DETECTION OF THE BACTERIUM, *BORRELIA BURGDORFERI* IN *IXODES SCAPULARIS* AND *DERMACENTOR VARIABILIS* TICK SPECIES IN FREDERICK AND MONTGOMERY COUNTY, MARYLAND

by
Lisa Brown
B.S. Marine Science, Coastal Carolina University, 2012

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Accepted:

_____
Drew Ferrier, PhD, Faculty Adviser, Project Adviser

_____
Eric Annis, Ph.D. Director, Environmental Biology Program

_____
April M. Boulton, Ph.D. Dean of the Graduate School

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ABSTRACT:

Ixodes scapularis and *Dermacentor variabilis* are two common human-biting tick species in Maryland, and can both carry the bacterium, *Borrelia burgdorferi*, which causes Lyme disease in humans. However, *I. scapularis* ticks are the only species that can transmit the disease, due to complexities in their gene regulation, and a more robust immune system in *D. variabilis* ticks. There is a lack of scientific studies on tick pathogen pervasiveness in Frederick and Montgomery County, MD, so this study was conducted to obtain preliminary data on the presence of *B. burgdorferi* among several locations in these counties. Ticks were grouped based on species, life stage, and location of collection, and DNA extraction was performed on each pool. PCR and gel electrophoresis were then performed to detect *B. burgdorferi* in tick pool groups. Out of 9 pools of *I. scapularis* ticks, 6 were positive for *B. burgdorferi*, and out of 5 pools of *D. variabilis* ticks, 3 were positive for *B. burgdorferi*.

INTRODUCTION:

Ticks have served as causative agents for disease in humans for decades, and with changing climatic conditions and continued fragmentation of land use, ticks are increasing in their range and population densities. As a result, they are more frequently in contact with humans. In Maryland, the most prevalent human tick bites are from *Ixodes scapularis* (blacklegged tick/deer tick), *Dermacentor variabilis* (American dog tick/wood tick), and *Amblyomma americanum* (lone star tick). [1,2] The deer tick is a vector of several bacterial pathogens including *Borrelia burgdorferi* (Lyme disease), *Borrelia mayonii* (similar disease as Lyme), *Borrelia miyamotoi* (tick borne relapsing fever), *Anaplasma phagocytophilum* (anaplasmosis), *Ehrlichia muris eauclairensis* (ehrlichiosis), as well as Powassan virus, and a protozoan parasite, *Babesia microti* (babesiosis). [3] The American dog tick is also a vector for several bacteria; *Anaplasma phagocytophilum* (Anaplasmosis), *Ehrlichia muris eauclairensis* (ehrlichiosis), *Rickettsia rickettsii* (rocky mountain spotted fever), *Francisella tularensis* (tularemia), and while there is no evidence that Lyme disease is transmitted by *Dermacentor variabilis* tick bites, these ticks can carry *Borrelia burgdorferi.* Similar to *Ixodes scapularis*, *Dermacentor variabilis* can also carry Powassan virus.[3] The lone star tick, like the American dog tick, can carry bacteria including *Francisella tularensis* (Tularemia) and *Ehrlichia muris eauclairensis* (Ehrlichiosis) and can also carry and transmit heartland virus and bourbon virus.[3]

Ixodes scapularis is of high interest because they often carry and transmit *B. burgdorferi*, which is a parasitic spirochete bacterium that causes Lyme disease in humans. During the summer months when many children and adults are outside for recreation, deer ticks are in their larval and nymph life stages. Nymphs can transmit disease and are only the size of a poppy seed, making them extremely effective at remaining undetected and staying attached long enough to transmit *B. burgdorferi* and/or other pathogens.[1]

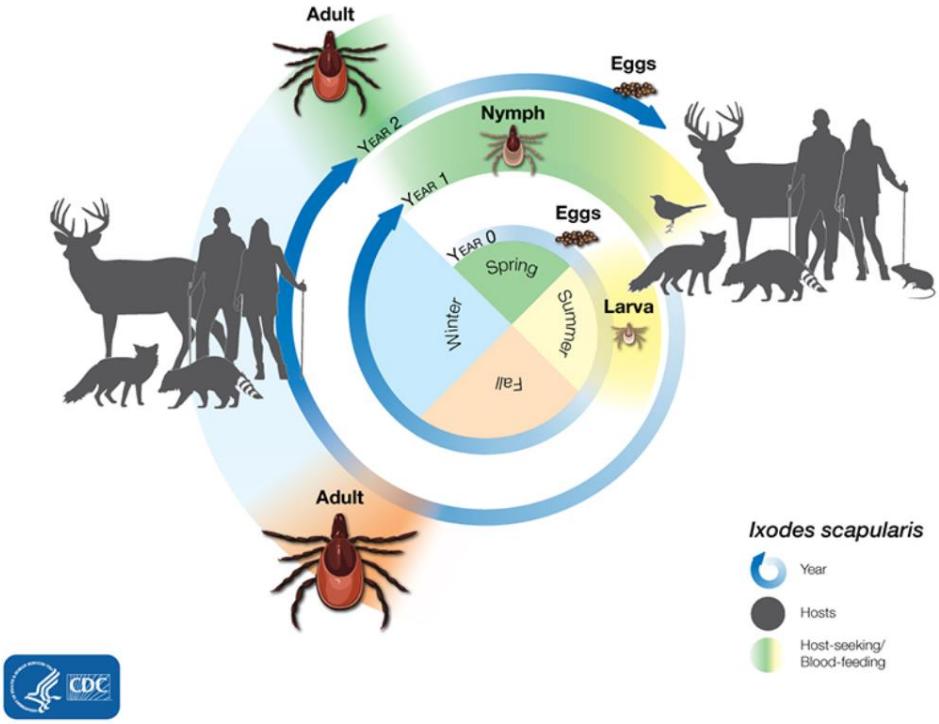

FIGURE 1. *I. scapularis* 4 stage life cycle beginning in the Spring of year 0 when eggs are developing and then hatching into larvae, and later molting into nymphs in the Spring of year 1. Their life cycle subsequently ends in the Spring of year 2 when they are adults and are mating, laying eggs, and then die.[3]

Ixodes scapularis ticks have a 4-stage life cycle over a period of two years in which they transform from egg to larvae to nymph to adult (Figure 1). In the spring of year two, adult females lay eggs typically on the ground near where they dropped off from their host. In the summer, eggs hatch into six legged larvae which find their first blood meal from a small mammal such as a mouse, chipmunk, or bird. The larvae fall off that host and remain in the soil

through the winter, going through developmental diapause[4], and then molt into nymphs the following spring. *Borrelia burgdorferi* cannot be passed from the adult female tick to its offspring via transovarial heredity, so the first opportunity for the deer tick to become infected with this pathogen is during the first blood meal when they are in their larval stage.[5] However, since larvae remain dormant during the winter, they do not have a chance to infect new hosts such as humans until they molt into eight-legged nymphs in the spring. At this stage, they may already be infected with *B. burgdorferi* from the blood meal during the larval stage, or they may become infected or reinfected from their blood meal as nymphs. The nymphs are active in seeking hosts from May through July and this is when they begin to transmit any pathogens they carry. The preferred hosts are white- footed mice, chipmunks, and some species of birds, which are all reservoirs for *B. burgdorferi*; humans and pets are incidental hosts.[6] The nymph life stage is more commonly the culprit in spreading disease to humans since their small size of less than 2 mm is extremely hard to detect. During the fall, nymphs molt into adults who seek larger mammal hosts such as white-tailed deer, and after male and females' mate, females lay their eggs and die. Deer ticks do not die in frost conditions, so some females will live through the winter until a blood meal in the Spring allows them to lay eggs.[7,8]

The impacts of climate change are playing a role in the ability of female ticks to survive throughout the winter, due to an increase in available moisture in the environment. As stated previously, ticks can survive frost, but in previous conditions of cold, dry winters, ticks would face mortality when moisture levels dropped. Ticks are quite sensitive to desiccation and death if they become dehydrated, however, now as air and ground temperatures do not get as cold in the winter, there is enough moisture for ticks to survive in large populations, seek hosts earlier than

usual in the early Spring, and lay eggs. The eggs are hatching sooner as well due to increased temperatures, impacting phenology of ticks and many other organisms as well. Maryland is going to continue to experience warmer, wetter winters, so this is another factor for why tick-borne diseases in humans will continue to increase. When there are more ticks in the environment, increased human contact is unavoidable.[31]

Lyme disease is the most common vector-borne disease in the United States and is of high concern due to the increase in cases reported, with an average of 467,000 diagnoses each year from 2010-2018.[9] Lyme disease in humans is often undiagnosed or misdiagnosed so this number of cases is likely much larger. Symptoms typically begin 7 to 14 days after the deer tick bite, and symptoms can include erythema migrans (a bullseye rash around the site of the bite) fever, malaise, headache, stiff neck, muscle and joint aches, and swollen lymph nodes. In many patients, the only symptom is the erythema migrans rash, but if it's in a location such as their scalp, where they cannot see it, then it is not diagnosed quickly and can disseminate to other tissues including nervous tissue in the body. Dissemination of *B. burgdorferi* can wreak havoc on the body, leading to cognitive slowing, headaches, facial palsy, lymphocytic meningoradiculitis, carditis, arthritis, and encephalitis.[10] *Borrelia burgdorferi* have a corkscrew-like shape and use internal periplasmic flagella to “swim” and migrate to dense tissues in the human body, such as the heart and joints. Due to these flagella, they can increase their velocity in viscous materials, allowing them to invade tissues that many other bacterial species cannot enter which results in disseminated Lyme disease.[11]

Long-term health issues from disseminated Lyme disease are often referred to as "chronic" Lyme disease and continue to be studied in epidemiological contexts. In recent years, celebrities and media figures have capitalized on chronic Lyme, and have coined the term "Lyme literate" to convince many people in the general public that their non-specific symptoms could be chronic Lyme disease. There is a high need for better scientific communication about this, and, specifically, data to provide evidence about the actual prevalence of *B. burgdorferi* in *I. scapularis* ticks. Many people don't understand the simple fact that Lyme disease is caused by bacteria that can be killed with antibiotics, and that disseminated Lyme disease is very rare.[12]

According to TickCheck,[13] a tick testing company which tests ticks for 30 pathogens, Maryland has had 25,725 confirmed cases of Lyme disease from 2000 to 2018. Out of that, 1,796 cases or 7% are from residents in Frederick County, and 3,432 or 13.4% are from residents in Montgomery County. Their data is from the CDC surveillance program which states that under-reporting is a limitation to the accuracy in these numbers, and Tick Check estimates that the cases could be approximately ten times higher with 257,250 in Maryland; 17,960 in Frederick County and 34,320 in Montgomery County. From confirmed cases in Frederick County, the data show 26 cases in 2000 compared to 110 in 2018, showing a 323% increase in confirmed Lyme disease cases. From confirmed cases in Montgomery County, the data show 80 cases in 2000 compared to 135 in 2018, indicating a 69% increase in confirmed Lyme disease cases. [13] The difference in confirmed cases was determined using percent difference between the two years.

Out of 430 deer ticks from Maryland tested by Tick Check, 21% tested positive for *B. burgdorferi,* and out of 109 dog ticks, 3% tested positive for *B. burgdorferi.* [13] Tick Check did

not specify which county these ticks were from, and there is currently little to no specific data on tick species and their pathogen loads in Frederick and Montgomery County, MD in the scientific literature. This study aims to be a starting point in data collection on tick borne pathogens in Frederick and Montgomery County, MD. Methods for tick collection and molecular biology assays used here can be applied in future studies so this research can continue and become more robust. The increase in Lyme disease cases among humans in Maryland places a demand for obtaining data on *I. scapularis* pathogen load, as they are the species of tick that transmits *B. burgdorferi,* the bacteria that causes Lyme disease. *Dermacentor variabilis* can also carry *B. burgdorferi;* however, there are no confirmed cases of Lyme disease from *D. variabilis,* so this tick will be assayed for *B. burgdorferi* for data collection purposes, but not for a connection to human Lyme disease.

There are factors to consider for tick collection in this study including the varying densities of nymphs and adults of *I. scapularis* (deer ticks) and *D. variabilis* (dog ticks) depending on the season/time of year. In June and July, adult dog ticks are much more active and higher in numbers than nymphs, compared to deer ticks which have a much higher number of larvae and nymphs in these months, but exhibit much lower adult activity. In August and September, dog ticks in both adult and nymph stages are much less active, and by October, they are not commonly found. Conversely, deer tick activity changes to become adult dominant toward the end of October, with less nymphs still active in August and little to no nymph activity in September and October.[14]

Dog ticks and deer ticks have unique habitats that impact their densities and host preference. *Ixodes scapularis* ticks are found primarily in leaf litter under tree canopies while *D. variabilis* ticks are more commonly found in open fields or grasslands. Many hosts such as white-tailed deer create and travel through paths in the woods[15], so these habitats were investigated as well. When determining collection sites, these variables were considered and played a large role in the total collection of ticks by species.

The objective of this study was to determine if *B. burgdorferi* is present in *I. scapularis* and *D. variabilis* tick species collected in Frederick and Montgomery County, MD. This study was designed to be a starting point for future students at Hood College to do similar research and expand on data collection, and other tick-borne pathogen testing. The methods used here are repeatable so that the next person taking on this research can start right away and will not have to spend time developing molecular biology assays.

Null Hypothesis: *Ixodes scapularis* ticks will have the same *Borrelia burgdorferi* positive PCR results when compared to *Dermacentor variabilis* ticks, regardless of life stage.

Alternate Hypothesis: *Ixodes scapularis* ticks will have a greater amount of *Borrelia burgdorferi* positive PCR results when compared to *Dermacentor variabilis* ticks, regardless of life stage.

MATERIALS AND METHODS:

Study Timeline:

Tick collection began in June 2022 and continued through August 2022. Several attempts to collect ticks in September were made with no success. Molecular biology assays to determine the presence or absence of *B. burgdorferi* began in August 2022 and continued through October 2022.

Tick Collection:

Questing ticks were collected at various sites in Frederick and Montgomery County, MD by primarily using the drag-sampling method. Questing refers to when ticks hold onto leaves and blades of grass by using their legs, and when hosts brush against the leaves or grass, they attach to the host. White flannel cloths 1 m^2 in size were attached to PVC pipe and were slowly dragged along vegetation in various areas where ticks could be including forest edges, interior forest, and highly disturbed areas such as walking paths[15] (Figure 2). Flannel cloths were also placed on top of vegetation for 5 minutes at a time. After dragging or placing the flannel over vegetation, the cloths were turned over and checked for ticks (Figure 2). Ticks adhere to the flannel and this method was the most consistent in obtaining ticks.[16] When ticks were found, they were carefully removed with tweezers and placed securely in a 10 ml conical tube. Each tube was used to collect several ticks at a time.

Another collection method employed was using a homemade tick trap made with cardboard and two-sided duct tape. A small bucket of dry ice was placed in the middle of the cardboard to

attract ticks with carbon dioxide, and the traps were checked after sitting over vegetated areas for 12-16 hours.

Some ticks were donated from a student at Hood College who removed them on him and his dog, and there were also donated ticks pulled off a deer found in Frederick.

FIGURE 2. Left: Use of the drag-sampling method to collect ticks at Homewood at Crumland Farms in Frederick County on the side of a walking trail. Right: Removal of ticks off the flannel cloth with tweezers and collection tubes.

Tick collection sites include the following in Frederick County: Carroll Creek Wildlife Park (39° 25' 36.4506", -77° 24' 3.87") and Homewood at Crumland Farms, The Lodge (39° 28' 36.9114", -77° 24' 37.3644") and in a residential area near Hood College (39° 25' 8.688", -77° 25' 1.0164"). In Montgomery County, ticks were collected behind a house in Damascus (39° 14' 1.413", -77° 12' 39.8082") (Figure 3).

Ticks were taken back to Hood College and frozen for at least 24 hours at -18°C, and species identity was determined using a stereoscope and taxonomic keys.[17] Ticks were tested for

pathogen presence as described below. Prior to DNA extraction, the date, location, location characteristics, collection method, common name, scientific name, sex, and life stage were

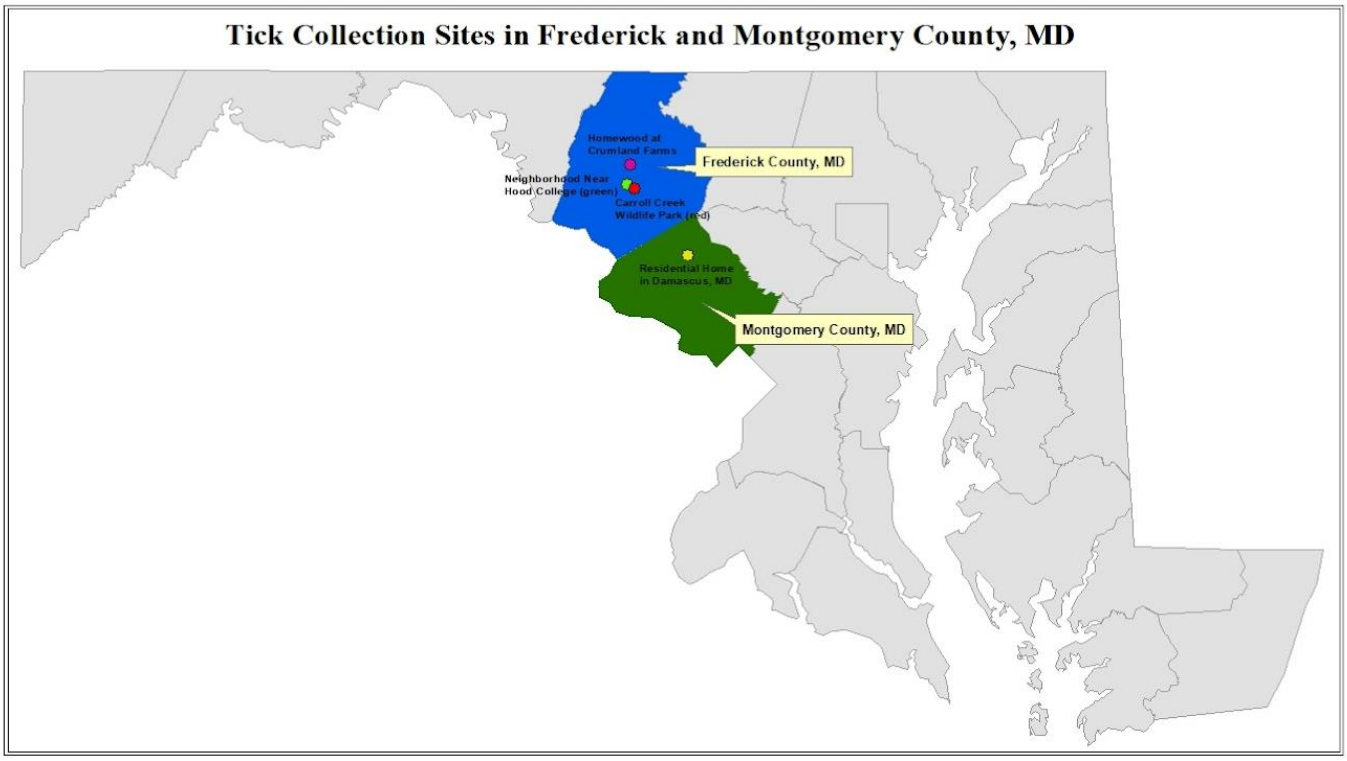

FIGURE 3. Map of Maryland with tick collection sites shown; in Frederick County, Homewood at Crumland Farms, a Neighborhood near Hood College, and in Montgomery County, a residential home in Damascus.

Tick Identification:

Correct identification of ticks by species and life stage was critical to this study, although it was a tedious and time-consuming process. It was imperative that ticks not be thawed and refrozen many times as this can degrade DNA before it is extracted. After trial and error, the method mostly used to identify ticks and store them for DNA extraction was to investigate them and cut them on the same day. Ticks were held in the freezer, removed, and looked at one by one for observation. The top and bottom of their bodies were investigated while looking at the field guide from tickencounter.com, a University of Rhode Island tick research program, to make

comparisons and determine the species, sex if applicable, and life stage of each tick.[14] After positive identification, the tick was placed in a 1.5 ml microcentrifuge tube, cut down the sagittal and oblique planes, and placed back in the freezer for future DNA extraction. Tick identification was completed over the course of several days, mostly in September and October. Female and male adult *D. variabilis* ticks have distinct differences in their appearance, making them easy to identify. All hard ticks have a scutum, which is a shield on the top side of the body. Both male and female *D. variabilis* ticks have a scutum with pale yellow and black markings, but the scutum on females covers about ⅓ of the body, while the scutum on males covers the entire top of the body (Figure 4). *Dermacentor variabilis* ticks have shorter mouthparts (including the hypostome and palps) than *I. scapularis*, and this was another identifying feature (Figure 4).

Ixodes scapularis ticks in larval and nymph life stages do not have features differentiated by sex, so it was not possible to determine if they were male or female. Nymphs were identified by their black scutum, long straight mouthparts, and the presence of eight legs. Larvae were identified by the brown features on the top of the body, the long, straight mouthparts, and the presence of six legs.[14] Nymphs and larvae were easily distinguished just by size as well, with nymphs being about 1.6 mm and larvae about 0.8 mm.

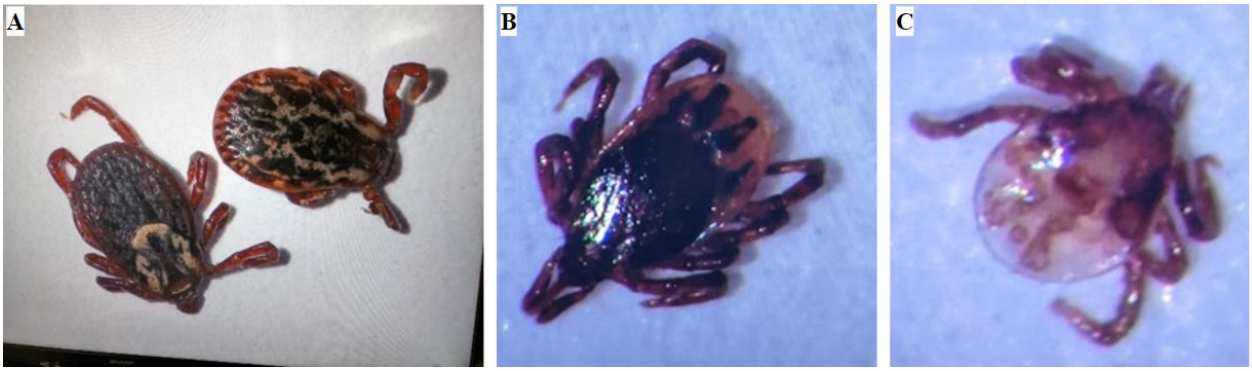

FIGURE 4. Collected ticks under a stereoscope to determine species and life stage. **A:** female and male adult stage *D. variabilis*, **B:** nymph stage *I. scapularis*, and **C:** larvae stage *I. scapularis*.

DNA Extraction:

To isolate genomic DNA from ticks, the Qiagen supplementary protocol, titled "Purification of total DNA from ticks using the DNeasy Blood & Tissue Kit for detection of *Borrelia* DNA" was followed.[30] Ticks were combined into pools according to collection location and life stage and they were tested together for the presence of *B. burgdorferi.* In pools 1,2,3,4 and 5 of adult *D. variabilis* ticks, each tick was cut in half down the sagittal plane, and then across the oblique plane, in accordance with the protocol, with an x-acto knife inside a 1.5 ml microcentrifuge tube. Additional ticks in each respective pool were also cut in the same tube and pushed to the bottom with the x-acto knife to make room at the top for cutting the next tick. The rationale to use whole bodies instead of half bodies in pools 1 and 2 was to be able to make comparisons with the concentration of DNA between whole-bodied and half-bodied pools. Adult ticks in pools 3, 4, and 5 were cut down the sagittal plane in a separate 1.5 ml tube and half the body of each tick was kept in that separate tube and placed in the freezer at -18°C, while the other half was placed in a pool tube in which all half bodies for all ticks in that group were placed. The adult half-bodies that were retained and put in the freezer were for the purpose of testing them individually for the presence of *B. burgdorferi* if the pool tested positive, but due to constraints on time and materials, they were not subjected to additional testing. Ticks in pools 6,7,8,9,10,11,12,13, and 14 consisted of nymph and larvae groups and ticks were cut down the sagittal plane with the most precision possible, although it was not possible to save half of the bodies for future analysis since they are so miniscule and difficult to handle.

Before the steps of DNA extraction could occur, the pooled ticks in the 1.5 ml tubes had to be transferred to 0.5 ml PCR tubes so the tube could fit in the thermal cycler for future incubations

during the extraction process. This occurred by following the first step in the protocol which was to add 180 µl of Buffer ATL to each 1.5 ml pool tube, followed by brief centrifugation to collect tissue debris at the bottom of the tube. A 200 µl size pipettor and tip were used to carefully transfer the buffer and pooled ticks into the 0.5 ml PCR tube. During aspiration of the buffer, the ticks adhered to the edge of the tip, and the liquid and ticks were transferred very quickly and carefully to the 0.5 ml PCR tube. Next, following the protocol, 20 µl of proteinase K was added to the tube and thoroughly vortexed for 15 s before incubation at 56°C. Incubation caused tissue and cell lysis, and after this, the exoskeleton was the only remaining debris. Incubation occurred for 45 minutes, and the Perkin Elmer Cetus DNA Thermal Cycler was used to hold the tubes at 56°C. After incubation, the tube was vortexed for 15 s and 200 µl of Buffer AL (without added ethanol) was added and thoroughly vortexed for 15 s. Each tube was then incubated at 70°C for 10 minutes, again using the same thermal cycler. Following incubation, 1 µl of carrier RNA (concentration of 10 mg/ml) was added and each tube was vortexed for 15 s. After the sample was vortexed, 230 µl of ethanol was added and the samples were vortexed again for 15 s. The mixture was then pipetted into a DNeasy mini spin column and placed in a 2 ml collection tube, and then centrifuged at 8000 rpm for 1 minute, using an Eppendorf model 5424 top loading centrifuge. The flow-through and collection tube was discarded, and the spin column was then placed in a new 2 ml collection tube. Then, 500 µl of Buffer AW1 was added and centrifuged at 8000 rpm for 1 minute, the flow-through and collection tube were discarded, and the spin column was placed in a new 2 ml collection tube. To wash away contaminants and further isolate the DNA, 500 µl of Buffer AW2 was added and centrifuged at 14,000 rpm for 3 minutes to dry the DNeasy membrane. The flow-through and collection tube were discarded, and the DNeasy spin column was placed in a clean 1.5 ml microcentrifuge tube (not provided by the Qiagen kit).

For adult tick pools, 105 µl of Buffer AE was added directly onto the DNeasy membrane, and for larvae and nymph pools, 35 µl of Buffer AE was added. This mixture was incubated at room temperature for 1 minute and was then centrifuged for 1 minute at 8000 rpm to elute. Lastly, another 30 µl of Buffer AE (for larvae/nymphs) and 100 µl (for adults) was added directly onto the membrane, and the sample was incubated for 1 minute and then centrifuged for 1 minute at 8000 rpm to elute the DNA. The DNeasy mini spin column was discarded and the extracted DNA with elution buffer was visibly seen at the bottom of the 1.5 ml microcentrifuge tube. The concentration of DNA extracted for each pool of ticks was immediately checked on a SPECTROstar Omega spectrophotometer to ensure adequacy for PCR amplification, and analysis with gel electrophoresis.

PCR and Gel Electrophoresis:

After DNA extraction, each pool of ticks was tested for *B. burgdorferi* through amplification of a region of the outer surface protein A gene (ospA) with PCR (polymerase chain reaction) using a BioRad C1000 Touch Thermal Cycler. Oligonucleotide primers (Forward – ATAGGTCTAATATTAGCCTTAATAGCAT, Reverse – AGATCGTACTTGCCGTCTT)[19] were purchased from Integrated DNA Technologies (IDT). Each PCR reaction was set up with a 20 µl final volume, of which the components are shown below (Table 1). To ensure no contamination, each reagent and sample was carefully pipetted into individual PCR tubes. The positive control of *B. burgdorferi* was at a much lower DNA concentration than the DNA of pooled tick groups, so 10 µl was used in each PCR reaction for the positive control, while only 1 µl of extracted DNA from tick pool groups was used in each PCR reaction (Table 1). Initially, 2 µl of DNA from tick pool groups was used in each PCR reaction but smearing on the agarose gel

indicated too much DNA was present, so this was reduced to 1 µl of DNA. The PCR reaction settings consisted of heating at 95 °C for 5 minutes for polymerase activation and initial DNA denaturation, full DNA denaturation at 95 °C for 15 s, 42 cycles of amplification including annealing at 60 °C for 20 s, extension at 74 °C for 30 s, the final step at 74°C for 10 min, and lastly, cooling to 12°C. [20]

TABLE 1. PCR Master Mixes

Negative Control	Cytiva PureTaq PCR Bead 16 µl DNAse, RNAse free water 2 µl forward primer 2 µl reverse primer
Positive Control	Cytiva PureTaq PCR Bead 6 µl DNAse, RNAse free water 2 µl forward primer 2 µl reverse primer 10 µl sample
Tick Pools	Cytiva PureTaq PCR Bead 15 µl DNAse, RNAse free water 2 µl forward primer 2 µl reverse primer 1 µl sample

Gel electrophoresis was then used to visualize which pooled groups of *I. scapularis* and *D. variabilis* ticks tested positive for *B. burgdorferi.* PCR products were loaded onto a 1.5% agarose gel stained with Gel Red Dye (Gel Red Nucleic Acid Gel Stain, 10,000x in Water). This was prepared by weighing 1.5 grams of dried agarose powder, mixing it with 100 ml of TAE (tris base, acetic acid and EDTA) buffer, adding 10 µl of gel red dye, and microwaving for 1 minute and 30 s to homogenize the solution. Halfway through microwaving, it was picked up and swirled for mixing, and to make sure it wasn't overboiling, which can affect the concentration of agarose once the gel cools. After taking it out of the microwave, it was set out to cool to 55°C

before carefully pouring 40 ml into the gel tray. The remaining 60 ml was kept for future use. A 10-well comb was then added to the gel tray and the gel was placed in a refrigerator at 4°C for 15 minutes to solidify. The gel was added to the gel electrophoresis system and 1X TAE buffer was added; just enough to cover the top of the gel.[21] Each PCR product had 4 µl of Gel Loading Dye Blue (6X) added to it, and in each gel well, 6 µl of each PCR product was carefully pipetted in. In the last well, 6 µl of Quick-Load® 100 bp DNA Ladder was added, and the machine was run at 96V for an hour, until the DNA had run about 60-70% down the gel. A Syn Gene Gel Box transilluminator was then used to visualize the results on the gel, and images were acquired for data collection and analysis.

PCR Controls:

The use of positive and negative controls for PCR are vital for obtaining accurate results. The negative control used was DNAse, RNAse free water. The positive control was DNA from *B. burgdorferi* strain B3, ordered from ATCC. The positive control concentration was determined using a 1:10 serial dilution to detect the final limit of detection. The original stock of *B. burgdorferi* DNA was a solution of 5 µg of dried *B. burgdorferi* DNA mixed with 350 ml of RNase, DNase free water. Nine 1 ml screw cap microcentrifuge tubes were set up next to the first tube, consisting of the stock, and labeled 2-10. To know the volume of stock to be pipetted out, the final volume for each tube was determined to be 500 µl, and a calculation of $x/500 = 0.1$, $x=50$ µl was used to establish the volume of the DNA being diluted. Each tube labeled 2-10 then had 450 µl of DNase, RNase free distilled water pipetted into them, and then 50 µl of the stock tube was pipetted into tube number 2, and that tube was vortexed for 15 s. Next, 50 µl was pipetted from tube 2 into tube 3, and vortexed for 15s. Following this, 50 µl was pipetted from

tube 3 into tube 4 and vortexed for 15s. This method was repeated for all subsequent tubes.[22] These samples were then amplified through PCR, with a total volume of 20 µl. Two negative controls consisting of water, and a DNA ladder were used to ensure accurate PCR results. The results were visualized using gel electrophoresis and showed strong positives from tubes 1(the stock), 2, and 3, and then the DNA diluted out and was faint from tubes 4 and 5 and showed up negative from tubes 6-10 (Figure 5). The dilution from tube 2 was chosen as the positive control, with a DNA concentration of 1.43ng/µl.

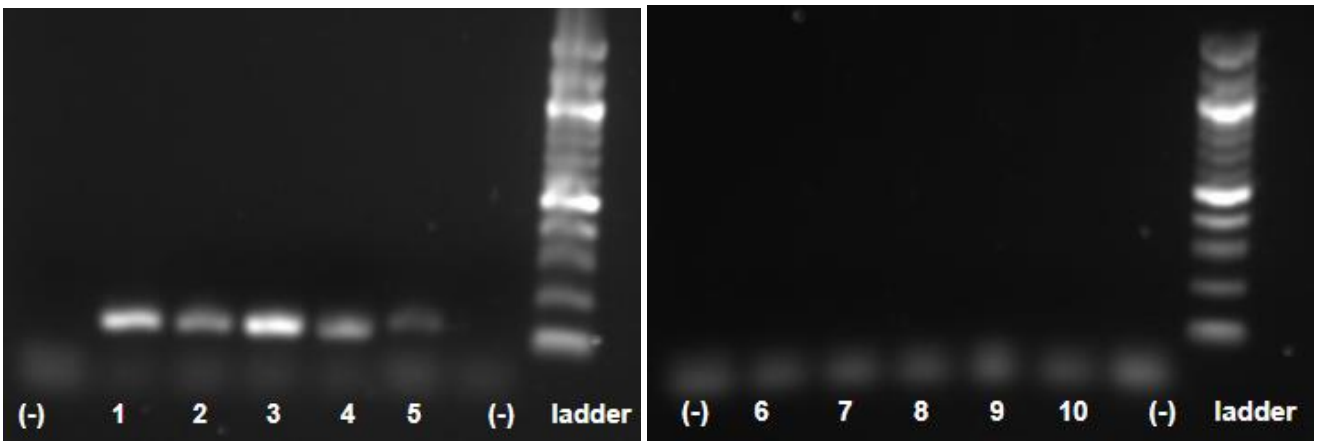

FIGURE 5. Gel electrophoresis results from a 1:10 serial dilution of 5 ug/350 µl (14.28ng/µl) of *B. burgdorferi* DNA, using primers for the ospA gene. Tube 1 was the stock solution, tube 2 had a concentration of 1.43ng/µl, tube 3 had a concentration of 0.142 ng/µl, tube 4 had a concentration of 0.0142 ng/µl, and tube 5 had a concentration of 0.00142 ng/µl. Tubes 6-10 showed no positive results.

RESULTS:

Following DNA extraction of each pool, the concentration of DNA was measured, and these data were important in determining how much volume of DNA from each pool to amplify through PCR and visualize via gel electrophoresis. The DNA concentration of the positive control containing *B. burgdorferi* was 1.42 ng/µl, and 10 µl of this sample was used in each PCR

reaction. The concentration of DNA extracted from each of the tick pools was significantly higher than the positive control, and DNA was smeared on the agarose gel after using 2 µl of each tick pool DNA sample after PCR. It was concluded that too much volume of each pool DNA sample was being used, due to the highly concentrated DNA (Table 2). In subsequent PCR reactions, 1 µl of each tick pool DNA was used, and that resulted in clear results on the gel (Figure 6). The ospA gene of *B. burgdorferi* was targeted with specific primers during PCR, and through gel electrophoresis, a 100 bp section of the gene was visualized (Figure 6). The high DNA concentrations from tick pools are due to the presence of all DNA from each tick, DNA from any pathogens (including *B. burgdorferi* or others), and any DNA from organisms that touched the outside of the ticks in the environment and left trace DNA. When comparing the concentration of the positive control to the tick pools, it's important to understand that the positive control was specifically clean DNA from just *B. burgdorferi*, and primers targeted a part of the ospA gene within that DNA.

TABLE 2. Results from tick collection & identification, DNA extraction, PCR, and gel electrophoresis.

	Collection Location	# of Females	# of Males	Life stage	Total # of Ticks/Pool	Species	Half Body or Whole Body	DNA Concentration [ng/ul]	Detection of *B. burgdorferi*
Pool 1	Carroll Creek Wildlife Park off Montevue	3	2	adult	5	*D. variabilis*	Whole	335.43	Negative
Pool 2	Homewood at Crumland Farms	4	6	adult	10		Whole	501.75	Positive
Pool 3	Homewood at Crumland Farms	3	6	adult	9		Half	176.03	Positive
Pool 4	Near Hood College	4	2	adult	6		Half	231.79	Negative
Pool 5	Homewood at Crumland Farms	4	4	adult	8		Half	205.61	Positive
Pool 6	Residential Home in Damascus	NA		larvae	7	*I. scapularis*	Whole	252.04	Positive
Pool 7	Residential Home in Damascus			larvae	11			245.66	Negative
Pool 8	Residential Home in Damascus			nymph	2			308.96	Positive
Pool 9	Residential Home in Damascus			larvae	7			161.42	Negative
Pool 10	Residential Home in Damascus			larvae	10			145.18	Positive
Pool 11	Residential Home in Damascus			larvae	7			264.69	Positive
Pool 12	Residential Home in Damascus			larvae	10			209.9	Positive
Pool 13	Residential Home in Damascus			larvae	11			267.26	Negative
Pool 14	Donated off a Deer			nymph	5			458.76	Positive
Total = 108									

A total of 108 ticks were collected and tested in pools, based on location and life stage, for the presence of *B. burgdorferi*. *Ixodes scapularis* ticks accounted for 70 of the total ticks collected;

63 were larvae, and 7 were nymphs. All the 63 *I. scapularis* larvae and 2 of the *I. scapularis* nymphs were collected from a residential home in Damascus, Montgomery County using the drag-sampling method in leaf litter under tree canopy, in areas with dense Japanese stiltgrass, and across mowed grass. The other 5 *I. scapularis* nymphs were collected from a dead deer found in Frederick. Out of the 7 pools of *I. scapularis* larvae, 4 (57%) were positive for *B. burgdorferi.* This means that a range of 4 (6%) to 34 (54%) individual larvae could have been infected. Nymphs accounted for 2 out of 7 pools of *I. scapularis* ticks, and 100% were positive for *B. burgdorferi* (Figure 6). A range of 2 (28%) to 7 (100%) individual nymphs could have been infected. Nymphs can be infected either from the larval stage tick obtaining the pathogen from its first blood meal, and retaining it when molting into the nymph stage, or they can become infected upon molting into nymphs, after a blood meal from an infected host.[26]

There were 38 *D. variabilis* ticks collected and all were adults. Of the 5 pools of *D. variabilis* ticks, 3 were positive, all from one location; Homewood at Crumland Farms in Frederick County. A range between 3 and 27 individual *D. variabilis* ticks could have been infected with *B. burgdorferi.* Based on previous studies, it's likely that a smaller number of individuals carried *B. burgdorferi* as there is a lower positive detection rate for *B. burgdorferi* among *D. variabilis* ticks.[26] These ticks were collected using the drag-sampling method and were in grassy areas along a walking trail. There were reports of tick bites or finding ticks on their bodies from residents who use that trail early in the summer, and in an area where there is a bench to sit and look at the pond is where we found most of the ticks. The grass was open to the sun (i.e., not covered under the tree canopy) which is the preferred habitat of dog ticks.[26] There were 2 other pools of *D. variabilis* ticks; one consisted of 6 ticks found in a residential neighborhood near

Hood College, and the other consisted of 5 ticks collected from Carroll Creek Wildlife Park off Montevue Avenue in an open grassy area. Both locations are in Frederick County. Neither of these pools tested positive for the presence of *B. burgdorferi,* meaning that 11 out of 38 total *D. variabilis* ticks (29%) were negative for *B. burgdorferi (*Figure 6)**.**

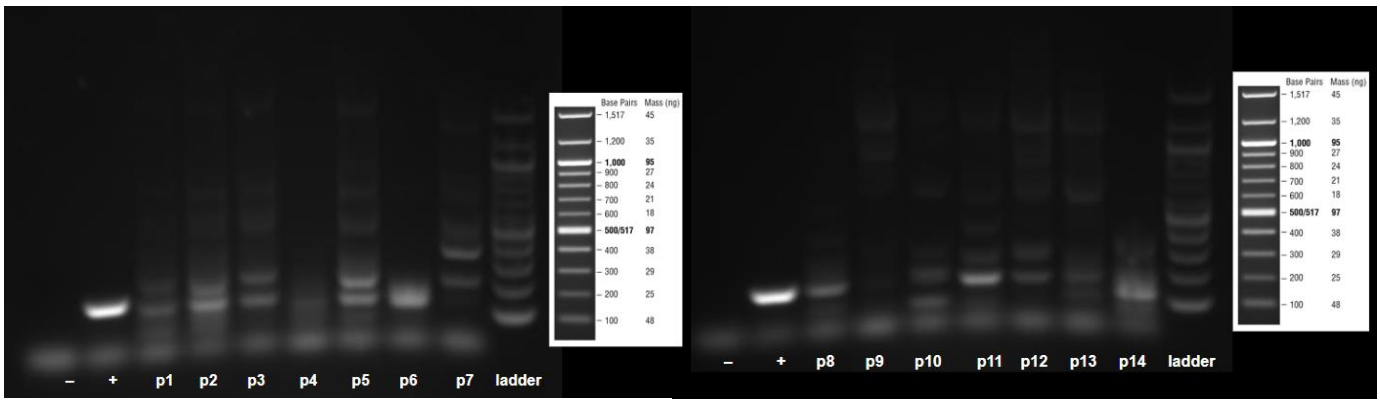

FIGURE 6. Gel electrophoresis results following amplification of DNA samples from pooled tick groups. Pools 2,3,5,6,8,10,11,12, and 14 have detection of *B. burgdorferi,* and pools 1,4,7,9, and 13 do not. Pool reference numbers are described in detail in Table 2.

The data collected were limited in scope due to lack of success in collecting more ticks. Another site in Montgomery County that was surveyed yet yielded no ticks was near College Gardens Park in Rockville, MD; the habitats surveyed included grass areas near a pond, wooded areas under tree canopy in leaf litter on a hiking trail, and along dense Japanese stiltgrass areas beside the trail. In Frederick County, Schifferstadt Architectural Museum, Waterford Park, and Frederick Municipal Forest were all surveyed, in open grass areas and under tree canopies in leaf litter, but no ticks were found. Of the 5 locations that successfully yielded ticks, 4 were in Frederick County, and 1 was in Montgomery County. Of these, 3 locations included tick pools that tested positive for *B. burgdorferi*, and 2 of these pools from Damascus and Homewood included questing ticks that were collected via the drag-sampling method. The residential home in Damascus had 5 out of 8 positive pools of *I. scapularis* larvae (out of 70 total ticks) and one

positive pool of nymphs, out of two total nymph pools (Table 3). This could be indicative of a large population of white-footed mice in this habitat as they are reservoir hosts for *B. burgdorferi* and have been found to be the most common host for larval *I. scapularis* ticks.[25] Homewood at Crumland Farms was the other site where questing ticks were collected using the drag-sampling method and 3/3 (100%) of adult *D. variabilis* pools tested positive for *B. burgdorferi* (Table 3).

TABLE 3. Detection of *B. burgdorferi* by Collection Location

Location	Tick Species	Number of Positive Pools	Number of Negative Pools	Grand Total
Residential Home in Damascus (Montgomery County)	*I. scapularis*	5	3	8
Animal Control - Picked off 1 Deer (Frederick County)	*I. scapularis*	1		1
Homewood at Crumland Farms (Frederick County)	*D. variabilis*	3		3
Carroll Creek Wildlife Park off Montevue (Frederick County)	*D. variabilis*		1	1
Near Hood College (Frederick County)	*D. variabilis*		1	1
Grand Total of Pool Groups		**9**	**5**	**14**

DISCUSSION:

It was surprising that 4 out of 7 larval *I. scapularis* tick pools tested positive for *B. burgdorferi,* considering the data from previous studies which show a low detection rate in larval deer ticks.[24,26,27] Since larvae were pooled together in groups ranging from 7 to 11 individuals, it's impossible to know how many individual larvae were infected, and that is a limitation of this study. However, these results can be used to make inferences on the population of reservoir hosts such as white-footed mice in the habitat where these ticks were collected. Previous studies that showed a lower incidence of larval deer ticks harboring *B. burgdorferi* ascribed this as being due

to lack of a first blood meal for the larvae, as they hatch out of eggs and may take weeks to months to finally attach to a host and obtain a blood meal.[24] The individual larval ticks that were part of the 3 pools of *I. scapularis* ticks that tested negative did not have a blood meal yet, or had a blood meal from an uninfected host. Pools 6 and 7 were collected on July 23, 2022, and pools 9,10,11,12, and 13 were collected on August 23, 2022; it's difficult to draw conclusions on the impact of this, because pool 6 tested positive while pool 7 tested negative, and pools 10,11, and 12 tested positive while pools 9 and 13 tested negative. There isn't a trend in these data to suggest a correlation with date. It's not surprising that almost all ticks collected were in the larval stage, because according to Tickencounter.com, larvae in the mid to southeast region of the United States are in greater numbers in July, August, and begin to decline in September.[24]

Pool 8 was a combination of 2 nymphal *I. scapularis* ticks from 2 separate locations; near Hood College, and in the same area the larval ticks were found in Damascus. These ticks were pooled together since they were the only 2 nymphs not pulled off the deer found in Frederick. Pool 8 was positive for *B. burgdorferi*, and it could have been one or both ticks that were infected. Both nymphs were collected in July 2022, when nymphs are actively seeking hosts for blood meals. It was quite unexpected to continue to collect larval ticks and not nymphs in July and August, as many studies show high activity of nymphs throughout the summer months, which is why so many humans become infected with Lyme disease.[1,9,14]

Pool 14 was composed of 5 nymphal *I. scapularis* ticks and results showed detection of *B. burgdorferi* for this pool. These ticks were plucked directly off a dead deer found in Frederick in early September from a colleague who saved them for analysis in this study. Deer are not

reservoir hosts for *B. burgdorferi,* so even though *I. scapularis* are called deer ticks, it's not because they become infected from deer, it's because adult deer ticks mate on deer.[6] Individual nymph tick(s) in pool 14 that harbored *B. burgdorferi* had to have been infected from a previous blood meal on a different host.

Based on the low detection rate of *B. burgdorferi* in *D. variabilis* ticks from the literature,[6,29] it was unexpected to have 3 pools (pool 2,3, and 5) test positive. It could have been just 1 tick per pool that had *B. burgdorferi* in its tissues, and in that case, it would only be 3/38 ticks or an 8% positivity rate, but unfortunately with the ticks not being tested individually, the rate is unknown. It was also interesting that 27 of 38 ticks were collected from one location: Homewood at Crumland Farms in Frederick County. Pool 2 and 3 were collected on July 21, 2022, and pool 5 was collected on July 22, 2022. Pool 2 consisted of 10 ticks, pool 3 included 9 ticks, and pool 5 consisted of 8 ticks (Table 2). The large population of *D. variabilis* ticks in this specific location has implications beyond this study; *D. variabilis* ticks are known to carry and transmit *R. rickettsii,* the bacteria that cause rocky mountain spotted fever in humans, in Maryland. [3] As stated previously, several residents that frequent the path/bench area found these ticks on them, and it would be beneficial to inform the residents about potential disease risk from *D. variabilis* tick bites.

The positive results for *D. variabilis* were less predictable than positive results for *I. scapularis* tick pools because previous studies demonstrate a much higher prevalence of *B. burgdorferi* in *I. scapularis* ticks. A vector competency meta-analysis of studies on ticks and *B. burgdorferi* infection showed evidence that larval *D. variabilis* ticks can be infected with *B. burgdorferi but*

acquiring the spirochete bacteria seemed to vary widely with a range of 10-61% of larval ticks becoming infected. [26] This study also showed evidence that larvae can pass *B. burgdorferi* across stages to their nymph form after molting, with a 3.3% rate of this happening; only 2 out of 61 larval ticks were found to pass it to their nymph stage. To see if *D. variabilis* could transmit the pathogen, 51 hosts were used for the ticks to feed on, and there was no definitive evidence that showed the transmission of *B. burgdorferi* to any of the hosts. With *I. scapularis* ticks, transstadial passage from larval to nymph stage was observed to be 40-100%, and most studies showed a rate of passage to be over 80%. Vector competency was shown to be significant, with a study finding 94% (50/53) of *I. scapularis* hosts to be infected with *B. burgdorferi,* demonstrating a definitive and successful transmission for nearly every blood meal.[26]

The ospA gene was chosen as the target gene for amplification via PCR, and primers for this gene were purchased. The decision to detect *B. burgdorferi* using the ospA gene was based on many studies indicating that ospA is an important gene for the tick-spirochete relationship, especially for *I. scapularis* ticks.[28] *Borrelia burgdorferi* can adapt to conditions inside many different hosts due to highly organized gene expression. When *I. scapularis* ticks initially feed on reservoir hosts which harbor *B. burgdorferi,* they attract *B. burgdorferi* by first producing a salivary protein, SALP25D, which reduces inflammation at the bite site to evade detection. The activity of this protein attracts *B. burgdorferi* to the ticks and they use their ability to move via their flagella to enter the tick through the mouth. The movement of *B. burgdorferi* into the tick impacts its gene expression in that it causes upregulation of the Hk1-Rr1 gene, and downregulation of the RpoS gene, which increases the quantity of c-di-GMP, a molecule involved in the regulation of outer surface protein expression, including OspA and OspB

(Figure 7). These proteins protect the pathogen against harmful components in the host's blood such as antibodies. The OspA and OspB genes also allow *B. burgdorferi* to adhere to the tick's midgut, increasing their population and subsequently allowing for passage to a host the next time the tick has a blood meal.[6,27]

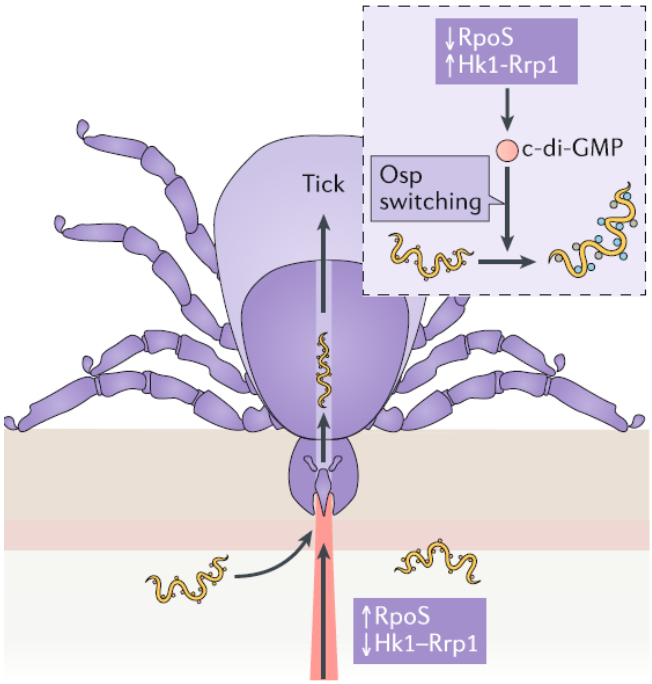

FIGURE 7. A representation of *Ixodes scapularis* ticks on the surface of a human skin, with the transmission of *B. burgdorferi* taking place due to complex gene expression. [6]

Dermacentor variabilis does not produce the SALP25D protein which sets off the chain reaction of other protein activity in *I. scapularis* and attracts *B. burgdorferi* to the tick. This could partially explain why *I. scapularis* ticks are vectors for *B. burgdorferi* and *D. variabilis* ticks are not, but there are other factors to consider. *Dermacentor variabilis* ticks have a more robust innate immune system as compared to *I. scapularis* and they produce antimicrobial proteins in their hemolymph plasma that are more effective at lysing *B. burgdorferi* cells. There is evidence that *B. burgdorferi* can reach tissues in *D. variabilis*, and, therefore, they sometimes test positive

for the presence of *B. burgdorferi;* however, more often, the hostile environment of the hemolymph plasma kills the bacteria within minutes, long before they can embed in tissues within the ticks. *Dermacentor variabilis* ticks infected with *B. burgdorferi* have little to none of these bacteria in their midguts, which is where the bacteria must be to move into the host when the tick is feeding. Instead, when *B. burgdorferi* is present in *D. variabilis,* it is embedded in their tissues, and therefore, cannot be transmitted to hosts. This is interesting to consider since 3 of 5 *D. variabilis* pools tested positive in the current study; the tick(s) that were infected had no chance of transmitting this pathogen to humans or other hosts since the spirochetes cannot survive in the midgut. This information is important to communicate with the public as Lyme disease is the most common tick-borne disease, and most people do not have the background knowledge on ticks to know that only *I. scapularis* ticks transmit *B. burgdorferi.*[29]

The hypothesis tested in this study: *Ixodes scapularis* ticks will have a greater amount of *Borrelia burgdorferi* positive PCR results when compared to *Dermacentor variabilis* ticks, regardless of life stage, was supported. There were 4 of 7 pools of *I. scapularis* ticks that tested positive, with a total of 34 potential individuals infected, and there were 3 of 5 positive pools of *D. variabilis* ticks, with a total of 27 potential individuals infected. Overall, *I. scapularis* pools showed a higher positivity; however, without knowing specifics on individual ticks, it's hard to draw firm conclusions. Knowing the background now about the strong immune system of *D. variabilis* and its quick response to fighting off *B. burgdorferi,* it is likely that many fewer *D. variabilis* ticks were infected than *I. scapularis* ticks. There are also previous studies which support this contention. Research conducted in three Canadian maritime provinces[23] from 2012 – 2020 investigated the presence of *B. burgdorferi* in *I. scapularis* and *D. variabilis.* For *D.*

variabilis, results showed a 1.9% positivity rate for *B. burgdorferi* (12/619 ticks) across all three locations over the course of 8 years. *I. scapularis* ticks had a much higher percent positivity rate at 15.8% (871/5507 ticks). Authors of another study[24] in Prince George's County, MD collected ticks from six sites both from questing ticks and ticks found on trapped mice. *Borrelia burgdorferi* was detected in 0% (0/12) *D. variabilis* ticks collected but was detected in 23.6% (21/89) of *I. scapularis* ticks collected, across all locations. These data are important to compare to in future studies that can investigate the presence of *B. burgdorferi* and/or other pathogens on an individual basis.

Future Studies and Limitations:

Students at Hood College who want to embark on tick pathogen research now have a starting point. An excellent next step would be to run the same assays described here, but for the purpose of detecting *R. rickettsii,* the bacteria that causes rocky mountain spotted fever, in *D. variabilis* ticks. In addition to this, for even more data on tick borne pathogens present in Maryland, future tick studies could include the use of multiplex PCR to detect *A. phagocytophilum, E. muris eauclairensis, B. burgdorferi,* and *Powassan virus,* which *I. scapularis* and *D. variabilis* can both carry. To make this research even more in-depth, DNA fragments in the agarose gel could be cut out and sent to a laboratory facility for genetic sequencing to determine the exact genotypes of pathogens present in each sample. This could be used to provide data on the populations of pathogens in the reservoir hosts, and to track mutations over time. Climate change could be driving beneficial mutations in the pathogens, allowing them to adapt and evolve over time and that could be the focus of a future study, making connections to human disease incidence and potential increase as climate change impacts worsen.

Binary splitting pooling is a method used when there are limitations in studies such as low pathogen detection, high cost of reagents and other lab materials, and time constraints. Binary splitting would have been utilized had more time and materials been available and would have provided specifics on which individual ticks in each pool were carrying *B. burgdorferi.* If a positive result came from a sample of pooled ticks, the next step would be to extract the DNA from each individual half-bodied tick, and to then do a pool of two smaller groups, adding 5 µl of extracted DNA from each individual tick into the new smaller pool. If there is one group that is positive and one that is negative, the next step is to proceed with smaller pools with the positive group (or both if needed) to determine which individual tick(s) are positive.[18] This method can be used in future studies to get more robust and detailed data on the presence of *B. burgdorferi* in each individual tick. This would be most suited for adult ticks, but it is extremely difficult to save half the bodies of nymph and larval ticks, so it would be best to do individual DNA extraction on those in the future to determine pathogen detection at the individual level.

Another recommendation for future studies is to use a water bath for incubations during the DNA extraction process. Using a thermal cycler was difficult because the only temperature resistant tubes are 0.5 ml and those small tubes were too difficult to maneuver while cutting the ticks, so that is why I transferred them from a 1.5 ml tube to a 0.5 ml tube. Future students who take on this research should procure a water bath with a flotation device for the tubes to sit in so that time is not wasted on transferring the material from one tube to another.

For tick collection, I suggest focusing on habitats in which ticks of specific species are often found, based on the scientific literature, which is leaf litter under tree canopies for deer ticks, and

open grassy areas for dog ticks. Results from this study indicate that these are the most preferred habitats for each species, so in the future is a researcher is looking at just Lyme disease in deer ticks, the focus should be collection in leaf litter habitats under trees. I advise prospective tick collectors to have many different locations within one site to look for ticks. For example, if you go to a park, use the drag-sampling method in at least 5 areas within the park for maximum success.

The Center for Coastal & Watershed Studies (CCWS) at Hood College started a program in the summer of 2022 for the Fredrick community to send ticks in for analysis, and if students and faculty can grow this program in addition to regular tick surveys using the drag-sampling method, the number of ticks collected and assayed would increase greatly and more spatial data could be analyzed to see if there are hotspots of disease in the county, and possibly surrounding counties. The CCWS department could also reach out to the Maryland Department of Natural Resources and inquire about a potential partnership so that ticks from roadkill or diseased animals could be tested for pathogens by students at Hood College. This would be an opportunity to collect more data on tick pathogens in Frederick and could propel a long-term study on tick-borne pathogen prevalence so that trends can be analyzed.

Previous studies have investigated tick pathogen load by trapping small mammals including mice and chipmunks, which are reservoir hosts for *B. burgdorferi.*[2] If time and resources permit, a student embarking on a thesis could take on a study that involves trapping animals and testing ticks found on them. This could give insight into the pathogen load in those hosts in the surrounding area to extrapolate on the threat of tick-borne disease to humans.

There were many limitations for this study including the imbalance in ticks collected. There were 108 total ticks collected and tested; the only adults collected were of *D. variabilis* species, and the only larvae and nymphs collected were of *I. scapularis* species, so it was very difficult to compare results between tick species and life stages. Due to a limited budget, more materials could not be purchased to provide a more sophisticated analysis.

Shortcomings in field collection and laboratory processing of ticks also hampered this study. There were not enough extraction, PCR, and gel electrophoresis reagents to do individual DNA extraction and subsequent assays to determine which individuals were infected with *B. burgdorferi.* Instead, the pool method was used, which worked well, but binary splitting would have been a fantastic next step to narrow down the positive pools to the individual level.

Another limitation in collecting ticks was that it was a labor-intensive, time-consuming process. Two undergraduate students and I were responsible for collecting ticks starting in June, and there were only 4 out of 16 days in which ticks were found and collected. These students could no longer help by mid-August, and I was going out on my own to collect ticks which included only 3 successful days out of 10 days trying to find them. There were many challenges to overcome during the tick collection process, including getting the ticks into the tubes, especially larval ticks because they are incredibly tiny. It was difficult to use tweezers since the tip of the tweezers is so much larger than their bodies, so in the future, obtaining a tool, or specialized small tweezers, that are more suited to larval tick collection would be helpful. The larval ticks often fell off the tweezers or off the conical tube and ended up on my hands, the cloth, or lost, which was anxiety

inducing as these could harbor *B. burgdorferi,* and cause Lyme disease. There have been confirmed cases of Lyme disease in residents of the home in Damascus, so the results from this study confirm there is an elevated risk of contracting Lyme disease from the ticks in the surrounding habitat.

Ticks are incredible in evolutionary terms, with their ability to evade detection and carry so many pathogens across distinct species. I truly hope another student can continue to grow this important research as it is likely that Lyme disease and other tick-borne illnesses will increase as climate change and land fragmentation continue over time.

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