Prevalence of Tick-borne Diseases in Questing Blacklegged Ticks (*Ixodes scapularis*) From Pike County, PA

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ABTRACT

The prevalence of tick-borne diseases (TBDs) have been on a steady rise throughout the United States. Since 2011, Pennsylvania has reported the highest number of Lyme disease cases annually. It has become a priority for local and state agencies to determine the prevalence of TBDs in local questing tick populations. This study represents the largest county-wide study surveying 1,000 *Ixodes scapularis* adult and nymph ticks collected from locations throughout Pike County, PA during spring and fall of 2018 and 2019. The overall two-year prevalence of *Borrelia burgdorferi* was 38.77% (95CI 35.71-41.88), *Anaplasma phagocytophilum* was 13.36% (95CI 11.30-15.64), *Babesia microti* was 5.06% (95CI 3.78-6.62), *Bartonella* species was 18.52% (95CI 16.15-21.09), *Mycoplasma* species was 3.24% (95CI 2.23-4.54), *Borrelia miyamotoi* was 1.52% (95CI 0.85-2.49), and Powassan virus lineage II was 1.72% (95CI 1.01-2.74). A coinfection prevalence of *B. burgdorferi* and *A. phagocytophilum* (p=0.0000), or *B. burgdorferi* and *B. microti* (p=0.0000) was significantly higher than the independent infection rate expected naturally. This large county-wide study emphasizes the urgency for diverse tick-borne disease surveillance studies, which include screening for more than *B. burgdorferi*.

Key words: Ixodes scapularis, tick, surveillance, Borrelia burgdorferi, Borrelia miyamotoi, Anaplasma phagocytophilum, Babesia microti, Bartonella spp., Mycoplasma spp., Powassan virus lineage II, deer tick virus, Pennsylvania, Coinfection.

INTRODUCTION

Tick-borne diseases (TBDs) in the United States have become a burden to public health, tripling within the last three years. Approximately 75% to 95% of vector-borne diseases reported in the U.S. are transmitted by ticks[1–3]. The geographic expansion of tick vectors and the etiologic agents they transmit has increased across the U.S. Changes of certain environmental conditions, such as host availability for vectors, forest fragmentation, invasive flora, global temperature, and humidity, are accountable for movement and expansion of tick populations[3–6]. New species, strains, and genetic variants of tick-borne illnesses are increasing, leading to a growth of pathogens capable of causing human and animal disease[2,7]. Each year, the CDC receives approximately 30,000 reported cases of Lyme diseases (LD). However, the CDC estimates an annual approximation of 300,000 U.S. citizens are at risk for LD. In 2018 alone, the U.S. reported a total of 33,666 confirmed and probable cases. In the same year, Pennsylvania reported 10,208 cases, approximately 30% of LD reports in the country[8]. The severity of TBDs in the state of PA is varied between the central, western and eastern regions. A statewide study of 1,855 adult blacklegged (*Ixodes scapularis*) ticks showed there were lower infection rates of the etiologic agent of LD, *Borrelia burgdorferi,* in the western region of PA between 2012 to 2014[9]. Decreasing TBDs is a challenge due to the highly invasive preventative measures involved with disrupting enzootic transmission to vectors[10]. Thus, the necessity for localized surveys, such as within a single county, can attribute to the surveillance and awareness of TBDs.

Numerous bacterial, viral, and protozoan agents can be passed from a tick vector to a mammalian host[3,10]. While any tick species can contribute to the spread of a TBD, *I. scapularis* is the main contributor to the increasing numbers of TBDs in PA and thus the most medically revelant[2]. The number of counties with an established *I. scapularis* population has doubled between 1996 and 2016, now pervasive in 44.7% of U.S. state counties. These geographical expansions of *I. scapularis* coincides with LD incidences[2,10]. Additionally, the presence of competent mammalian hosts for TBDs is essential due to the facilitation of horizontal transmission. This type of transmission in a three-host vector is ideal for pathogen survival. In the *I. scapularis* life cycle of 2 to 4 years, each life stage (larval, nymph, and adult) completes a blood meal in order to molt. Thus, there is greater acquisition of pathogens in older life stages such as the nymph and the adults[10]. The rate of transmission of a given TBD is dependent on the etiologic agent, anywhere from hours to days[3]. There are a few TBDs capable of transmitting via vertical transmission or transovarially, such as *Borrelia miyamotoi* and Powassan Virus Lineage II[10–12], of which the larval life stage becomes a concern. However, acquisition and maintenance of the other pathogens is primarily through blood meals and thus only life stages that have fed previously, nymphs and adults, are competent vectors[10].

As with other enzootic diseases, TBDs require competent hosts for pathogens that rely on horizontal transmission to pass to the vector. Humans, who acquire TBDs from ticks, are considered incidental hosts of both the vector and the pathogens. Human hosts are not necessary for survival of either ticks or their etiologic agents[10]. The first two life stages, larvae and nymphs, primarily acquire blood meals from small mammals like rodents. The largest life stage, the adult, prefers medium to large mammals such as white-tailed deer and humans. Competent hosts for TBDs are characterized by the ability to acquire, host, and transmit the disease to a vector. The primary competent host has been widely attributed to the white-footed mouse (*Peromyscus leucopus*). While white-tailed deer (*Odocoileus virginianus*) are a primary host for adult ticks, however; they lack competency to host certain TBDs like *B. burgdorferi*[2,7,10].

Coinciding with the geographic expansion of *I. scapularis* and the associated TBDs, the number of etiologic agents transmitted by *I. scapularis* has grown[10]. A protozoan agent, *Babesia microti*, is an intraerythrocytic parasite that invades red blood cells causing them to lyse[3,13]. The spirochete, *B. burgdorferi*, is the causative agent of LD. Infection with this spirochete may cause an erythema migrans (EM), a characteristic skin lesion of LD. However, about 30% of patients will not present or recall the presence of the EM[3]. There are three-stages of a clinical manifestation of LD, including an early localized, early disseminated, and then late disseminated[14]. Known late manifestations of LD include Lyme arthritis, Lyme carditis, neuropathy, and facial nerve palsy[3]. The vague flu-like symptoms related to LD is a concern for under and over diagnosis. Clinical suspicion has been shown to be minimally accurate when diagnosing LD in children, strengthening a requirement for laboratory confirmation[15]. Others mention studies that indicate LD is vastly underreported and LD cases are higher by 8 to 12 times. If this estimation is correct, LD rises to the top three reportable infectious diseases in the

U.S[3]. The Centers for Disease Control and Prevention (CDC) recognizes LD as the most prevalent vector-bone disease in the US[16]. Human granulocytic anaplasmosis is caused by an intraleukocytic bacterium called *Anaplasmosis phagocytophilum*[3,10]. These gram-negative bacteria infect host membrane-bound vacuoles, eliciting nonspecific illness that includes severe headache, fever, and general myalgia[3]. The flavivirus, Powassan virus lineage II, is transmitted primarily by *I. scapularis*. This vector was shown to bridge Powassan virus to human disease due to their high affinity for human host over that of other known enzootic vectors of the virus such as *Ixodes marxi* and *Ixodes cookei*[10,17,18]. Powassan virus lineage II, also known as the Deer Tick Virus, is neurovirulent, having known to cause meningoencephalitis. In the past 18 years, the number of Powassan virus cases has increased exponentially, initially 27 cases between 1958 and 1998 to 98 cases between 1999 and 2016[19]. The relapsing fever spirochete, *B. miyamotoi*, is another emerging enzootic disease only recently discovered to cause disease in humans[10,20,21]. The disease caused by *B. miyamotoi* is characteristic of recurrent fevers[3]. The danger of coinfections can not only lead to under or misdiagnosis of less prevalent diseases but may also increase disease severity in humans.

Bartonella henselae is the etiologic agent of Cat-scratch fever (CSD). The main route of transmission is through a scratch or bite from the main reservoir, cats. Cats acquire the disease from ectoparasites (such as fleas), thus CSD cases arise where cats and fleas are prevelant[22]. Cat fleas, *Ctenocephalides felis*, are the main vector of transmission from cat to cat. The viability of *Ixodes* tick's involvement in *Bartonella* infection is highly debated. In Europe, *Ixodes ricinus* successfully transferred *Bartonella birtlesii* to rodents in vivo. Contrary, this was not observed in *I. scapularis* and *B. henselae*. However, *Bartonella*-infected *Ixodes* ticks excrete the organism through saliva[23]. The Centers for Disease Control and Prevention (CDC) acknowledges that ticks are capable of carrying species of *Bartonella*, but lack transmission evidence between tick vector and *Bartonella*-infected humans[24]. *Mycoplasma fermentans* is another controversial tick microbe. In 1970, presence of *M. fermenatns* was found in the joints from rheumatoid arthritis patients and bone marrow from children with leukemia. Although this initially raised pathogenic concerns, no further verification was reported and frequent cell culture contamination have negatively impacted the pathogenic status of *M. fermentans*[25]. A retrospective study of 200 patients treated for Lyme disease found high prevalence of co-infection with species of *Bartonella* (46.50%) and *Mycoplasma* (82.00%). Additionally, presence of *M. fermentans* in ticks has been confirmed in California, New York, and New Jersey. However, there is no published scientific literature regarding the tick competency as a vector for *M. fermentans*[26]. Thus, for the purpose of this study *Bartonella* and *Mycoplasma* species found in the *I. scapularis* population will be discussed as a tick microorganism, not a TBD pathogen. These microorganisms may provide an insight of the interactions within the *I. scapularis* microbiome. Studies of polymicrobial infections indicate positive, negative, and neutral effects on each other's fitness influenced by access to nutrients, activation of tick immunity, and production of specific tick proteins[27]. The genus *Bartonella* belongs to the order Rickettsiales of which *Rickettsia* species are known tick symbionts within *I. scapularis*.

Specifically, *Rickettsia buchneri* may have a role in tick homeostasis due to its high abundance[27]. Thus, prevalence of other nonpathogenic tick organisms, such as *Bartonella* species and *Mycoplasma* species, should be incorporated in polymicrobial studies to analyze the potential of possible interactions.

Prevalence of coinfections have been previously reported from 1 to 28% of ticks, however 5 to 10% of ticks is more common[10]. Reported studies have shown that 40% of past LD patients are co-infected with *B. microti* and 13% are co-infected with *A. phagocytophilum*[28]. Other studies have indicated the survival advantage of coinfections of certain pathogens, such as the interaction between *B. burgdorferi* and *B. microti.* Coinfections may also enhance the severity of the illness. The first three reported human cases of LD and Babesiosis were hospitalized; one needed a blood transfusion with joint aspiration of a reoccurring swollen knee, the second received antibiotic therapy and still developed pulmonary edema, and the third had fatal pancarditis. This coinfection has exhibited an illness with more symptoms at a higher severity, however a suppressive effect was also observed[28]. Whether there is an increase of illness severity or not, coinfection of LD and Babesiosis remains a high threat to the public health due to different treatment regimens. While the antibiotic given for LD (doxycycline) is effective against other bacterial coinfections like Anaplasmosis, it is ineffective to treat Babesiosis[28]. Additionally, early intervention or removal of ticks combined with prompt diagnosis is the most effective way to decrease TBDs in humans[10].

Surveillance of TBDs is vital to monitor and detect emerging pathogenic agents. Such was the case of *B. miyamotoi*, that was first found in *I. scapularis*. Only twelve years after this discovery, it was shown to cause disease in humans[1]. The importance of known pathogens and their prevalence in specific geographic regions is necessary to provide spatial variation in the changing patterns of TBD risk. Decreasing TBDs is a challenge due to the highly invasive preventative measures involved with disrupting enzootic transmission to vectors[7]. Thus, the necessity for localized surveys, such as within a single county, can attribute to the surveillance and awareness of TBD. Data from this study will be utilized by local and state officials in their effort to combat the spread of ticks and TBDs throughout Pike County, PA as well as health-care providers in the diagnosis of TBDs through evaluating risk of exposure.

MATERIALS AND METHODS

Site Selection

Pike County, PA was divided into 9 grid sections where the longitudinal axis was labelled one through four and the latitudinal axis was labelled A, B, and C. Grid sections were labelled A2, A3, A4, B2, B3, B4, C2, C3 and C4 (**Figure 1**). A total of 100 *I. scapularis* ticks were collected from eight collection zones, 75 from one (B4), and 125 from another (B3). The borough of Milford PA is found in grid C2 but was treated as an individual collection zone and labelled MB making a total of 10 collection zones. Collection sites within each collection zone were labelled with the grid section followed by a number (ie: A2-1) in numerical order with the exception of the Milford borough sites which were labelled with letters based on the location of the collection site (**Figure 1**). Collection sites within the zones were chosen based on use by community members and presence of favorable tick habitat which bordered edges of forests with vegetation, wood and brush piles, shrubs and leaf litter. These sites included, state parks, state game lands, township buildings, schools, township parks, communities and hiking trails.

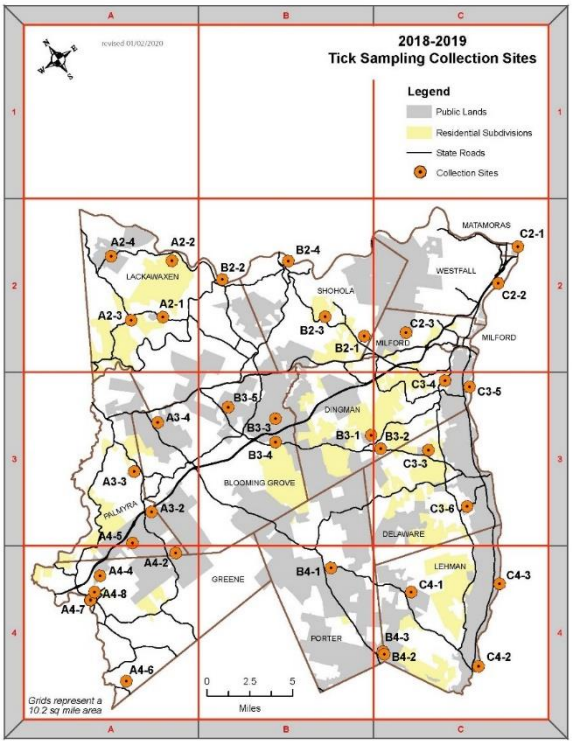

Figure 1. Grid and site locations of tick collections throughout Pike County, PA during 2018 and 2019.

Tick Collections

Ticks were collected from May through July (Spring) and October through November (Fall) in 2018 and 2019. Tick sampling was conducted by dragging corduroy cloths along the trails on nearby vegetation and leaf litter for several meters before being examined for ticks. Collected ticks were stored in microcentrifuge tubes labelled with collection zone and collection site. All *I. scapularis* life stages were collected, however only the nymph or adult life stages were tested for disease. Any tick species other than *I. scapularis* ticks found were collected and counted, but were not tested for diseases. Upon returning to the lab, ticks were sorted by location, species and life stage and then stored at -85°C until pathogen testing.

Pathogen Detection

DNA and RNA extractions were performed on all *I. scapularis* adults and nymphs using a QIAamp Viral RNA kit (Qiagen, Redwood City, CA) following manufactures protocol. The measures taken to prevent contamination included small extraction groups of nineteen samples plus a RNA/DNA extraction blank control. A TaqMan primer-probe assay was used to target the 16S-23S intergenic spacer region specific for *Borrelia burgdorferi* and *Borrelia miyamotoi*, the Msp2 gene for *Anaplasma phagocytophilum*, and the 18S rRNA gene for *Babesia microti*. A SYBR green reverse transcriptase real-time PCR assay and melt curve analysis was used to determine presence of Powassan Virus Lineage II by targeting the NS5 gene. All amplification was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems) and an Applied Biosystems QuantStudio5 (Thermo Fisher-Scientific) following standard cycling conditions. Samples were only considered positive when amplification reached designated thresholds and Ct calls per assay with the typical sigmoid-shaped curves. Traditional PCR protocols were used to determine prevalence of *Bartonella* spp. and *Mycoplasma* spp. using primers specific for the ftsZ gene and 16s rRNA gene, respectively. A 1% agarose gel stained with ethidium bromide was used to confirm presence of *Bartonella* spp. and *Mycoplasma* spp. Each assay included a negative control where nuclease-free water was substituted for the DNA volume. All assays were validated using standards and guidelines developed by the Dr. Jane Huffman Wildlife Genetics Institute.

Statistical Analysis

A general linear model was used to determine significance of infection prevalence in *I. scapularis* ticks across years, life stage, canopy cover, season, and collection zones. Significance in the general linear model was evaluated using a two-way analysis of variance (ANOVA) type III. Pairwise differences of the binomial general linear model was used to determine differences of each collection zone. Canopy cover percentage was calculated at 1-km resolution for each site within the collection zones. The calculation only included Pennsylvania, any overlap with other states such as New Jersey was not included into the percentage. A binomial test was utilized for the confidence intervals of the proportion of infected ticks with 95% confidence intervals.

This same analysis was used to report the prevalence data of other published articles, such as Livengood et. al. 2020, Sanchez-Vicente et. al. 2019, and Tokarz et. al. 2010.

Analysis of co-infection rates were completed only with species-specific assays, including *B. burgdorferi*, *A. phagocytophilum*, *B. microti*, *B. miyamotoi*, and Powassan Virus Lineage II. The prevalence of *Bartonella* spp. and *Mycoplasma* spp. were reported separately, not integrated into co-infection analysis. Probabilistic model of species co-occurrence was used to determine if the observed co-infections occurred more frequently than what is distributed independently of one another based on their individual prevalence. The observed and expected frequencies were calculated between the different pathogens. The expected frequency is obtained through a distribution of each pathogen as random and independent of the others. Through analysis of these two frequencies, a probability that the co-infection occurs more frequently than what is observed independently is calculated. The statistical software package R 3.0.1 was used for statistical computing (The R Foundation for Statistical Computing, www.R-project.org). An alpha of 0.05 was used to determine statistical significance.

RESULTS

Tick Collection Data

In the years 2018 and 2019, tick collections from Pike County, PA yielded a total of 1,051 adult and nymph *I. scapularis* in the spring and fall season (**Table 1**). Of the 1,000 ticks tested, only 988 ticks were included in analysis due to extraction failure. The 988 *I. scapularis* were collected and tested from the designated collection zones: 264 adult females, 378 adult males, and 346 nymphs. During collections, a single adult female *Amblyomma americanum* was collected in zone C2, site 1. One *Haemaphysalis longicornis* nymph was identified from zone A3, site 2. One *Rhipicephalus sanguineus* larval was identified from zone A4, site 8. A total of 1,003 *Dermacentor variabilis* ticks were collected, identified, and stored, but not tested (**Table 1**). All *I. scapularis* life stages were observed at each zone location. Across all of the collection zones there was a disproportion of the life stage densities.

Table 1. Total ticks collected using drag clothes in Pike County, Pennsylvania from 2018 to 2019.

Species	Spring 2018	Fall 2018	Spring 2019	Fall 2019	Total
I. scapularis (adult female)	127	104	61	88	380
I. scapularis (adult male)	136	131	58	82	407
I. scapularis (nymph)	115	5	140	4	264
I. scapularis (larval)	152	0	8	0	160
I. scapularis (total)	378	240	259	174	1,211
D. variabilis (adult female)	278	0	202	0	480
D. variabilis (adult male)	230	0	292	0	522
D. variabilis (nymph)	1	0	0	0	1
D. variabilis (total)	509	0	494	0	1,003
A. americanum (adult female)	1	0	0	0	1
H. longicornis (nymph)	1	0	0	0	1
R. sanguineus (larval)	0	0	1	0	1

Pathogen Testing

Of the 988 *I. scapularis* ticks tested individually, molecular presence of all seven microorganisms (five pathogens and 2 microorganisms) were detected. Of the five pathogenic agents detected (excluding *Bartonella* and *Mycoplasma*), 46.26% (95CI 43.11-49.42) of *I. scapularis* ticks were infected with at least one TBD county-wide. Detection of a single pathogen occurred in 33.81% (95CI 30.86-36.85), while more than one pathogen was detected in 12.45% (95CI 10.45-14.67) of *I. scapularis* ticks.

A significantly higher prevalence of TBDs within adults was observed than nymphs due to overlapping 95% confidence intervals. Likewise, nymphs had a significantly lower coinfection rate than adults (Table 2). Additionally, there was only 1 nymph with molecular presence of three different TBDs compared to the 14 detected in adults, however this difference was not significant. This nymph had molecular presence of *B. burgdorferi*, *B. microti*, and Powassan Virus Lineage II from collection zone A2. The highest number TBDs detected within an individual tick was four pathogens. This adult female collected from zone B3 had molecular presence of *B. burgdorferi*, *A. phagocytophilum*, *B. microti*, and *B. miyamotoi* (**Table 2**). The highest total prevalence was *B. burgdorferi* at 38.77% (95CI 35.71-41.88), proceeded by *Bartonella* spp. at 18.52% (95CI 16.15-21.09), *A. phagocytophilum* at 13.36% (95CI 11.30-15.64), *B. microti* at 5.06% (95CI 3.78-6.62), *Mycoplasma* spp. at 3.24% (95CI 2.23-4.54), Powassan Lineage II Virus at 1.72% (95CI 1.01-2.74), and lastly *B. miyamotoi* at 1.52% (95CI 0.85-2.49). A similar order of microbial prevalence was observed in the adult life stage. Contrary, the nymph's highest molecular presence was *Bartonella* spp. (21.59%, 95CI 16.78-27.05). Only two nymphs were infected with Powassan Virus Lineage II (0.76%, 95CI 0.09-2.71), however *B. miyamotoi* nymphal prevalence (1.89%, 95CI 0.62-4.36) was similar to the adults and the total prevalence (**Table 3**). A binomial general linear model was used to evaluate if the following factors were determinants of individual pathogen prevalence: life stage, year, collection zone, season, and canopy cover. The significant determinants for the overall TBD prevalence (excluding *Bartonella* spp. and *Mycoplasma* spp.) included life stage (p=0.0000), year (p=0.0183), collection zone (p=0.0002), and season (p=0.0000). The pairwise comparison of the five pathogen prevalence among collection zones indicated zone MB's pathogen prevalence was statistically different than A2 (p=0.0188), A3 (p=0.0096), B3 (p=0.0003), B4 (p=0.0045), C3 (p=0.0009), and C4 (p=0.0086), however it was not significantly different than zone C2 of which it resides in (p=0.5577). When evaluating the determinants for *B. burgdorferi*, life stage (p=0.0000), zones (p=0.0007), and season (p=0.0297) were statistically significant. The prevalence of *B. burgdorferi* in collection zone MB (11.00%) was significantly different than prevalence of 52.00% in B3 (p=0.0039), 58.67% in B4 (p=0.0001), and 45.00% in C4 (p=0.0055). Additionally, the *B. burgdorferi* prevalence of zone B4 of 58.67% was significantly different from zone C2 prevalence of 32.69% (p=0.0233). No statistical significance among year, life stage, collection zones, or canopy cover were present in analysis of *A. phagocytophilum* prevalence. However, collection season was a strong determinant on *A. phagocytophilum* prevalence (p=0.0001). *A. phagocytophilum* prevalence was only significantly different between zone B2 and B4, respectively 16.00% and 6.67% (p=0.0512). The only significant determinant of *B. microti* prevalence was collection season (p=0.0274). The remaining two pathogens, *B. miyamotoi* and Powassan Virus Lineage II, did not have any significant determinants. The only insignificant determinant for *Bartonalla* spp. was canopy cover (p=0.2250), while *Mycoplasma* spp. prevalence was significantly influenced by zone (p=0.0000) and season (p=0.0375).

Table 2. Detection of Tick-borne Diseases (TBDs) in Adults and Nymphs *Ixodes scapularis* collected from Pike County, Pennsylvania between 2018 – 2019.

	Nymph	%	95 CI		Adult	%	95 CI		Overall	%	95 CI	
			lower	upper			lower	upper			lower	upper
Ticks not carrying TBDs	186	70.45	64.55	75.89	345	47.65	43.96	51.36	531	53.74	50.58	56.89
Ticks carrying TBDs	78	29.55	24.11	35.45	379	52.35	48.64	56.04	457	46.26	43.11	49.42
Ticks carrying a single TBD	58	21.97	17.13	27.45	276	38.12	34.57	41.77	334	33.81	30.86	36.85
Ticks carrying more than one TBD	20	7.58	4.69	11.46	103	14.23	11.76	16.99	123	12.45	10.45	14.67
… two TBDs	19	7.2	4.39	11.01	88	12.15	9.86	14.76	107	10.83	8.96	12.94
… three TBDs	1	0.38	0.01	2.09	14	1.93	1.06	3.22	15	1.52	0.85	2.49
… four TBDs	0	0	0.00	1.39	1	0.14	0.00	0.77	1	0.10	0.00	0.56
Total ticks tested	264				724				988			

*includes molecular presence of five pathogens (*B. burgdorferi*, *A. phagocytophilum*, *B. microti*, *B. miyamotoi*, and Powassan Virus Lineage II).

Canopy cover was only a significant determinant in evaluation of overall TBD prevalence, excluding *Bartonella* spp. and *Mycoplasma* spp, and not on the individual microbial prevalence. The mean canopy cover of the collection zone sites was 80.58% (95CI 76.95-84.20). Site 1 in collection zone C2 had the lowest canopy cover (31.35%), while site 3 in collection zone B4 had the highest canopy cover (99.02%). The remaining sites were all above 62.05% canopy cover. There was a disproportion of ticks collected at sites within grids, thus molecular prevalence on the site level was not investigated. Collection zone C2 canopy cover ranged from 31.35% to 91.82%, while zone B4 ranged from 95.96% to 99.02% canopy cover. Zone C2 had the lowest *B. burgdorferi* prevalence (32.69%), while B4 had the highest *B. burgdorferi* prevalence (58.67%) amongst the grids. The prevalence between these zones are significant as previously stated through the generalized linear model (p=0.0233). Additionally, 99.04% of the ticks collected in zone C2 were adults (103/104) and 78.67% of the ticks collected in zone B4 were adults (59/75) (**Supplemental figure 1**).

The significance of co-infections of two microorganisms within the overall *I. scapularis* collected (n=988) was analyzed in 16 pairs, excluding 5 pairs that were removed from analysis due to an expected co-infection less than one. Of the 16 pairs analyzed, 7 pairs were found to have a significant positive association, suggesting that the co-infection occurred more often in their observed frequency than independently. The results indicate that co-infection with *B. burgdorferi* paired with *A. phagocytophilum* (p=0.0000) and *B. burgdorferi* paired with *B. microti* (p=0.0000) are more-likely to occur than what is expected randomly. The remaining significant co-infections included those co-infected with tick microorganisms (*Bartonella* spp. and *Mycoplasma* spp.). Of the pathogenic organisms detected 4 out of the 5 were significantly co-infected with *Bartonella* spp. The *I. scapularis* nymphs (n=264) were analyzed in 9 pairs, excluding 12 pairs due to an expected co-infection less than one. The only significant co-infection was between *B. burgdorferi* and *B. microti* (p=0.0011). The *I. scapularis* adults (n=724) were analyzed in 16 pairs, excluding 5 pairs due to an expected co-infection less than one. These results were similar to that yielded from the total *I. scapularis* analysis, however *A. phagocytophilum* and *B. microti* co-infection was only significant in the adult population (p=0.0237). Additionally, 3 out of the 5 pathogens were co-infected with *Bartonella* spp. (p<0.0459) (**Table 4**).

Table 3. Tick-borne disease prevalence *in Ixodes scapularis* by life stage (nymph and adult) and overall (nymph + adult).

Organism	*Ixodes scapularis* life stage											
	Nymph				Adults				Overall			
Total tested	264	%	95 CI		724	%	95 CI		988	%	95 CI	
			lower	upper			lower	upper			lower	upper
B. burgdorferi	50	18.94	14.40	24.20	333	45.99	42.32	49.70	383	38.77	35.71	41.88
A. phagocytophilum	28	10.61	7.16	14.96	104	14.36	11.89	17.13	132	13.36	11.30	15.64
B. microti	14	5.30	2.93	8.74	36	4.97	3.51	6.82	50	5.06	3.78	6.62
Bartonella spp.	57	21.59	16.78	27.05	126	17.40	14.71	20.37	183	18.52	16.15	21.09
Mycoplasma spp.	8	3.03	1.32	5.88	24	3.31	2.14	4.89	32	3.24	2.23	4.54
B. miyamotoi	5	1.89	0.62	4.36	10	1.38	0.66	2.53	15	1.52	0.85	2.49
Powassan Virus Lineage II	2	0.76	0.09	2.71	15	2.07	1.16	3.39	17	1.72	1.01	2.74

*These microorganisms are tick microoorganisms

Table 4. Probability of pathogens in questing *Ixodes scapularis* ticks using analysis of expected co-infection from individual prevalence and the observed co-infection.

I. scapularis	Pathogens & Microorganisms	Probability Co-infection	Expected Co-infection	Observed Co-infection	p-pos
Nymph (n=264)	*B. burgdorferi* + *B. microti*	0.010	2.6	8	0.0011
Adult (n=724)	*B. burgdorferi* + *A. phagocytophilum*	0.066	47.8	69	0.0000
	B. burgdorferi + *B. microti*	0.023	16.6	36	0.0000
	B. burgdorferi + *Bartonella* spp.	0.080	58.0	80	0.0000
	A. phagocytophilum + *B. microti*	0.007	5.2	10	0.0237
	A. phagocytophilum + *Bartonella* spp.	0.025	18.1	30	0.0012
	B. microti + *Bartonella* spp.	0.009	6.3	13	0.0046
	Bartonella spp. + *Mycoplasma* spp.	0.006	4.2	10	0.0040
Overall (n=988)	*B. burgdorferi* + *A. phagocytophilum*	0.052	51.1	77	0.0000
	B. burgdorferi + *B. microti*	0.020	19.4	44	0.0000
	B. burgdorferi + *Bartonella* spp.	0.072	70.8	90	0.0009
	A. phagocytophilum + *Bartonella* spp.	0.025	24.5	34	0.0172
	A. phagocytophilum + *Mycoplasma* spp.	0.004	4.3	9	0.0194
	B. microti + *Bartonella* spp.	0.009	9.3	16	0.0135
	Bartonella spp. + *B. miyamotoi*	0.003	2.8	6	0.0428

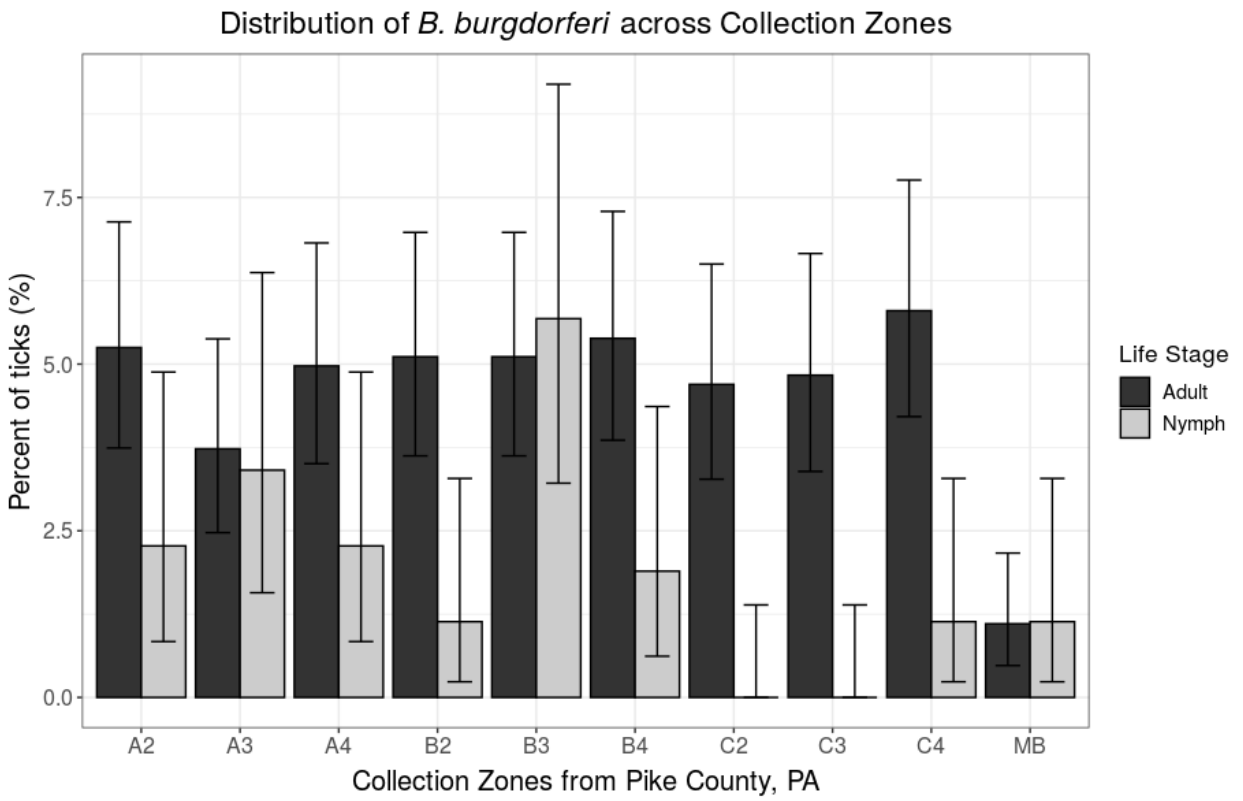

Figure 2. Distribution of positive *I. scapluaris* ticks infected with *B. burgdorferi* by life stage across collection zones in Pike County, PA from 2018 and 2019. The lower and upper bounds represent the 95 confidence interval. No nymphs collected from either zone C2 (n=1) and C3 (n=3) were positive. Percentages of ticks are derived from the total life stage, adults (n=724) and nymphs (n=264).

DISCUSSION

The aim of this study was to investigate the most common microorganisms present within the *I. scapularis* tick population of Pike County, PA. To the authors' knowledge, this study is the largest study conducted within a PA county that tests for five pathogens (three bacterial, one protozoan, and one viral) and two bacterial tick microorganisms. Other studies of disease prevalence in *I. scapularis* ticks of PA include a four-year study, where 1,721 nymphs were tested for *B. burgdorferi*, *A. phagocytophilum*, *B. microti*, and *B. miyamotoi* across three different counties within the Lehigh Valley region[29]; a previous study in Lehigh Valley tested 423 questing adult and nymphs for same four pathogens[30]; a statewide study, including 67 PA counties, tested 1,363 adults for *B. burgdorferi*, *A. phagocytophilum*, *B. microti*[9]; 115 ticks (74 nymphs and 41 adults) were tested in Gettysburg National Military Park for presence of *B. burgdorferi*[31]; across four counties in southwestern PA, 294 field-collected ticks (195 nymphs and 99 adults) and 31 *O. virginianus*-collected ticks were tested for *B. burgdorferi* and *A. phagocytophilum* [32]; in the northwestern and southeastern PA, 454 adults (372 questing and 84 engorged females from *O. virginianus*) were tested for *B. burgdorferi* and *A.*

phagocytophilum[33]; and a state-wide analysis of 299 adults tested for 20 different genomic targets[34]. Originally, 1,000 *I. scapularis* nymphs and adults total were tested, however only 988 were included in analysis due to failure to extract RNA. This marks the largest collection of ticks from one county in PA, comprising of a diverse disease testing panel of seven different microorganisms.

Tick species

The total ticks collected in our two-year study surmounted to 2,217 ticks, including five different tick species and all three life stages of *I. scapularis* (n=1,211). The vector tested for disease included the *I. scapularis* tick adult female, adult male, and nymph (n = 1,051) **(Table 1)**. Adult and nymph ticks have different potentials of acquiring and transmitting TBDs. The *I. scapularis* tick is a three-host tick, where each life stage feeds on a different host. Once a larval molts into a nymph it has completed one blood meal or one chance to acquire a TBD. Tick acquisition of TBDs from a host increases with the amount of blood meals, thus adults have a higher prevalence of TBDs[35]. However, adult ticks, due to their larger size, can be detected and removed earlier than nymphs[9]. In our survey, life stage was a significant determinant of overall TBDs, prevalence of *B. burgdorferi* and of a tick microorganism (*Bartonella* spp.). To accurately evaluate polymicrobial prevalence of the I. scapularis tick, it is important to include all host seeking life stages that have completed a blood meal.

In recent decades, ticks have expanded their geographic territories due to several factors such as climate change, host availability, humidity tolerance, freezing temperature durations, and human habitat[5]. The *D. variabilis* tick was prevalent in the spring collection, surmounting to 1,003 ticks (**Table 1**). The *D. variabilis* tick, distinguishable by its decorative shield, is a known vector for Rocky Mountain spotted fever (RMSF), Tularemia, and Ehrlichiosis[36,37]. One *A. americanum* tick adult female was found in zone A2, during the spring of 2018. The *A. americanum* tick is a vector for Erhlichiosis, Tularemia, Tick-borne relapsing fevers and Southern Tick Associated Rash Illness, a relative to LD. It may also have an association to an anaphylactic reaction with red meats known as Alpha-Gal[38]. A single *H. longicornis* tick nymph was found in zone A3, during the spring of 2018. The *H. longicornis* tick is an invasive species from Asia which was first documented in 2017 in New Jersey and is not currently known to carry or transmit TBDs in the United States[39]. A larval *R. sanguineus* was identified in zone A4, during the Spring of 2019. The *R. sanguineus* is the most widespread tick worldwide and is a known vector for several etiologic agents such as *Coxiella burnetti*, *Echrlichia canis*, and *Rickettsia* species[40]. This survey of Pike County ticks is more diverse than neighboring regions. A survey in the Lehigh Valley region found only two other species, *D. variabilis* and *I.* cookei, in addition to *I. scapularis*[29]. Documentation in local areas, such as a specific county, can aid to reinforce the data on expansion of ticks. Knowledge of these tick parameters can be used for the spatial and geographic distribution of health risks allotted to the different tick species.

Tick-borne disease Surveillance

Of the 988 *I. scapularis* ticks analyzed, about half of the ticks tested were positive for a TBD (46.26%, 95CI 43.11-49.42) (**Table 2**). A similar survey in Suffolk County, New York, screened for the same TBDs in *I. scapularis* adults and nymphs (n=678). They reported that 63.42% (95CI 59.67-67.06) of ticks were positive for at least one TBD, significantly higher than ours[27]. This study of a neighboring state was comparable due to the spring and fall collections, area of collection localized to a county, and inclusion of both life stages. However, their ratio of adults to nymphs was higher than ours, the adults (n=620) almost ten times the nymphs (n=58) compared to our ratio where adults (n=724) are about three times the nymphs (n=264). The higher probability of adult acquisition of TBDs may drive the higher infection rate seen in Suffolk County, NY. A PA statewide study screened for 15 microorganisms, of which only the following were found; *B. burgdorferi, A. phagoctyophilum, B. microti, B. miyamotoi*, and *R. parkeri*. They reported 60.2% (95CI 54.41-65.79) of *I. scapularis* adults collected in 2013 carried a microorganism[34]. Any differences could be influenced by the inclusion of a nonpathogenic organism (*Rickettsia* spp. endosymbionts). Our adult TBD prevalence was lower, however not significantly due to overlapping of the 95CI (52.35%, 95CI 48.64-56.04). Additionally, our adult infection rate was significantly lower than another New York state survey which found 71.33% (95CI 65.71-76.50) *I. scapularis* adults infected with the same five distinct TBDs (*B. burgdorferi*, *A. phagocytophilum*, *B. microti*, *B. miyamotoi*, and Powassan virus)[41]. This variation of small-scale studies indicates the necessity for larger scale studies within localized area. Our adult coinfection rate of TBDs was 14.23% (95CI 11.76-16.99) (**Table 2**). This was significantly lower than the PA statewide study that reported adults carrying more than one microorganism at 20.40% (95CI 15.99-25.42). The Suffolk County, NY study found an *I. scapularis* (adult and nymph) coinfection rate of 21.68% (95CI 18.63-24.98)[27]. This was significantly higher than our total *I. scapularis* co-infection rate of 12.45% (95CI 10.45-14.67) (**Table 2**). The known health issues of co-infection of TBDs is a public health concern[28]. These differences in prevalence amongst studies provides an insight on TBD diversity. Our study found a significant variation of positive ticks amongst collection zones across a single county. This may imply the statistical importance of large sample sizes combined with small geographical collection zones. In addition, the strength of an assay contributes to the accuracy of infection rate. Our study maintained extensive controls for tick extractions and PCR testing, including the sensitivity and specificity of our TaqMan assays through the combined power of primers and probes. In conclusion, better representation of the polymicrobial nature of ticks needs a high standard of experimental design and assay development.

Lyme Disease (*B. burgdorferi*)

Our prevalence of *B. burgdorferi* within *I. scapularis* ticks was 45.99% (95CI 42.32-49.70) in adult ticks and 18.94% (95CI 14.40-24.20) in nymph ticks for an overall prevalenceof 38.77% (95CI 35.71-41.88) (**Table 3**). Other PA studies that included both *I. scapularis* life stages reported lower *B. burgdorferi* infection from 21.00% (n=115) to 36.39% (107/294)[31,32]. The infection rate of the latter excludes the engorged adults removed from *O. virginianus* (n=7/31)[32]. The PA nymphal prevalence was reported from 24.8% (95CI 22.8-26.9) to 42.56%

(83/195), higher than our Pike County nymphal *B. burgdorferi* prevalence[29,31,32]. The *B. burgdorferi* infection rate in adults was found between 13.1% (25/191) in southeastern PA and 61.6% (162/263) in the northwestern PA[9,33,34]. Our adult infected prevalence lies within this extensive variety discovered across the state, however similar to more recent PA statewide studies that found 45.5% (136/299) and 47.4% (646/1363) adults positive with *B. burgdorferi*[9,34]. The latter survey found a higher prevalence within the northeastern region, consisting of Pike County (52%, n=277)[9]. A large localized study, such as this, highlights the comprehensive prevalence of TBDs within tick populations. When evaluating an overall infection rate across the state, infection rates can vary from 20-75% whereas this localized study is more specific to each region of Pike County. Even on a small county scale within Pike County there were differences of *B. burgdorferi* prevalence (**Figure 2**). Milford Borough, which exist in zone C2, was significantly different than a majority of the other zones (A2, A3, B3, B4, C3, and C4). This data can be used to assist physicians in understanding the potential risk for exposure to a TBD following the bite of a tick.

Anaplasmosis (*A. phagocytophilum*)

The occurrence of human Anaplasmosis is on the rise across the nation, conversely there have been low reports of human infections in PA[34]. Our overall prevalence of *A. phagocytophilum* detected in Pike County was 13.36% (95CI 11.30-15.64); nymphal infection of 10.61% (95CI 7.16-14.96) and 14.36% (95CI 11.89-17.13) infection rate in adults (**Table 3**). This is comparable to a study conducted in four southwestern counties of PA who reported an *A. phagocytophilum* prevalence of 14.29% (42/294) in questing adult and nymphs, excluding the engorged adults removed from *O. virginianus* (n=6/31). Conversely to our data, their nymphal infection of 17.44% (34/195) was higher than the adult infection of 8.08% (8/99)[32]. The *I. scapularis* study in Gettysburg National Military Park did not identify *A. phagocytophilum* in either adult (n=74) or nymph (n=41) populations[31]. The nymphal survey of the Lehigh Valley region in PA found a significantly lower *A. phagocytophilum* prevalence of 3.4% (95CI 2.4-3.9), consisting of both strains (Ap-v1 and Ap-ha)[29]. Lower adult incidences of *A. phagocytophilum* have also been reported from 1.7% to 3.3% in PA surveys[29,33,34]. A study of three PA counties discerned from the northwestern (1.9%) and the southeastern adult prevalence (39.8%), considerably higher than our adult prevalence [33]. However, a statewide survey found the lowest infection rate in the northcentral region (2%) to the highest infection rate in the northeastern region (4%)[9]. Discrepancies in TBD prevalence can be explained by distinction between strains of *A. phagocytophilum*. In our detection of *A. phagocytophilum*, we did not distinguish between the two known strains currently in PA circulation, human pathogenic strain (Ap-ha) and the variant that is not known to cause human infection (Ap-v1)[9,29]. The survey in Lehigh Valley, PA distinguished the two strains through sequencing, discovering a prevalence of 3.5% for Ap-v1 and 0.8% for Ap-ha. This is a three-fold difference in prevalence between the two variants. Nevertheless, the co-infection of *B. burgdorferi* and *A. phagocytophilum* was highest in Ap-ha (0.6%) than Ap-v1 (0.1%)[29]. Conversely, a study in the northeastern region of New York found a higher prevalence of Ap-ha (4%) than Ap-v1 (2.4%) in questing nymphs from 2011 to 2012.

Their average *A. phagocytophilum* prevalence across two years was 8.3% of which 1.9% were unable to be classified as either strain[43]. Our high prevalence of *A. phagocytophilum* found in Pike county may be augmented due to lack of strain differentiation, however the published literature of strain prevalence is contradictory. The combined strain prevalence of Lehigh Valley nymphs was still significantly lower than our Pike County nymphal prevalence. This reinforces the necessity for local studies in regard to public health awareness of human Anaplasmosis.

Babesiosis (*B. microti*)

Babesiosis occurs at a lower incidence in PA than what might be expected given the high prevalence of LD. Additionally, 94% of Babesiosis cases are in other regions of the U.S., not including PA[29]. However, Babesiosis infection is not mandatory to report in the state of PA. Healthcare providers can elect to report cases to the Pennsylvania Department of Health, who stated an increase of 20-fold from the past 12 years[44]. Our nymphal infection prevalence of *B. microti* was 5.30% (95CI 2.93-8.74) and adult infection prevalence was 4.97% (95CI 3.51-6.82) for an overall prevalence of 5.06% (95CI 3.78-6.62) (**Table 3**). The *I. scapularis* study in Gettysburg National Military Park did not identify *B. microti* in either adult (n=74) or nymph (n=41) populations[31]. The nymphal survey of the Lehigh Valley region in PA found a lower *B. microti* prevalence of 2.8% (95CI 2.1-3.7), however not significant based on overlapping 95CI. Our adult *B. microti* prevalence was slightly higher than other recorded adult studies in PA, ranging from 0.67% to 3.52% statewide[9,34]. In the same statewide study, their highest *B. microti* prevalence of 5.5% came from the northcentral region. Their northeastern infection rate, consisting of Pike County, was 3.5% (n=277)[9]. However, neighboring states such as New Jersey have recorded incidences of B*. microti* as high as 8.41% in *I. scapularis* ticks[45]. Pike County's relatively high occurrence of *B. microti* is a significant health risk.

***B. miyamotoi* Disease (*B. miyamotoi*)**

Our overall prevalence of *B. miyamotoi* was 1.52% (95CI 1.52-2.49). Our nymphal prevalence (1.89%, 95CI 0.62-4.36) was slightly higher than the adults infected with *B. miyamotoi* (1.38%, 95CI 0.66-2.53), however not determined significant through the GLM (**Table 3**). This observation could be due to the transovarian transmission observed in *B. miyamotoi*[20,46,47]. Adult ticks collected from *O. virginianus* were pooled into 409 groups (n=1,990) and tested for *B. miyamotoi*. They reported a maximum likelihood estimation tick infection rate of 7.64/1000 (95CI 4.46-12.29). This pooled estimation of 0.76% *B. miyamotoi* infected ticks is similar to our *B. miyamotoi* adult prevalence.[46]. In contrast, a recent PA statewide study found 0.33% (1/299) adults positive with *B. miyamotoi*, lower than our adult prevalence[34]. Additionally, the nymphal survey of the Lehigh Valley region in PA found a lower *B. miyamotoi* prevalence of 0.3% (95CI 0.1-0.7), however not significantly due to overlapping 95CI[29]. *B. miyamotoi* is not as dominant as its sister spirochete, *B. burgdorferi*, but surveillance of this tick-borne relapsing fever remains imperative.

Deer Tick Virus (Powassan Virus Lineage II)

The prevalence of Powassan Virus lineage II within *I. scapularis* ticks collected was 2.07% (95CI 1.16-3.39) in adult ticks and 0.76% (0.09-2.71) in nymph ticks for a total prevalence of 1.72% (95CI 1.01-2.74) (**Table 3**). Our positive adults were higher than hunter-harvested white-tailed deer statewide (0.05%, 1/1990) and unengorged adult ticks statewide that did not find a single tick positive for Powassan Virus (0.00%, 0/299)[17,34]. However, the bordering state of NY found an equivalent prevalence of Powassan Virus lineage II in *I. scapularis* adults (2.45%, 7/286)[41]. Despite the rarity of human infection with Powassan virus, the reported cases have increased recently. The majority of cases reside in northeast of the U.S[48]. From 2010 to 2019, PA has had 6 confirmed cases of Powassan virus, while neighboring states report higher cases such as NY and NJ, respectively 20 and 12[49]. Although Powassan encephalitis is considered rare, only 40 cases diagnosed since 1958, its fatality rate of 10% to 15% is a clinical concern[41]. Additional analysis to differentiate between Powassan Lineage I and II is needed to determine the nature of this public health concern.

Canopy Cover

Canopy cover was a significant determinant in the total TBD prevalence, excluding the tick microorganisms *Bartonella* and *Mycoplasma* species. Pike County is a heavily wooded area of northeastern, PA. Although our collection sites were diverse (31.35% to 99.02%) compared to our mean canopy cover (80.58%, 95CI 76.95-84.20), it was not a significant determinant on the individual TBD prevalence, including *B. burgdorferi*. Only one site's canopy cover percent, zone C2 site 1 at 31.35%, was lower than 62.05% (zone MB site DR). Out of three sites, C2 collection zone's canopy cover percentage mean was 62.33%, ranging from 31.35% to 91.82% and the zone with the highest site canopy cover percent was zone B4, ranging from 95.96% to 99.02% for a mean of 97.98%. However, our pairwise comparison of zones found *B. burgdorferi* prevalence was significantly lower in zone C2 (32.69%) than in zone B4 (58.67%) (p=0.0233). Canopy cover percent was used as a measure of forest fragmentation. Previous studies have shown that forest fragmentation leads to a dilution of mammalian species and elevation of TBD reservoirs like *P. leucopus*, which may influence an increase in Lyme disease risk[4]. However, our GLM did not find canopy cover percent as a significant determinant of *B. burgdorferi* prevalence. In previous studies, increased infection prevalence was observed with increased forest fragmentation and decreased host diversity[4,50,51]. However, other studies have discredited this relationship between forest fragmentation and pathogen prevalence. These studies discuss discrepancies on this long held belief of LD dilution effect is contributed to large geographic areas, increasing other variables such as mammalian species density, and small sample size per area[52,53]. Our analysis of forest fragmentation supports the lack of a dilution effect, specifically with *B. burgdorferi* prevalence. Other influences might be the ratio of adults to nymphs. A higher concentration of adults, who have a greater chance of acquiring TBDs, may lead to a higher prevalence rate of specific collection zones. However, the zone with the highest adult concentration, zone C2 (99.04%), had the lowest *B. burgdorferi* prevalence. Other factors influencing collection zone prevalence differences could be host availability and seasonality.

Co-infections

Co-infections are identified by a tick carrying two or more diseases which can complicate medical diagnosis and treatment. The commonly reported prevalence of co-infections has been identified as less than 5 to 10%, but also have been reported to range from 1 to 28% within the *I. scapularis* tick population[29]. The results derived from our study indicate a co-infection rate of 12.45% (95CI 10.45-14.67) (**Table 2**). The highest co-infection observed was *B. burgdorferi* and *A. phagocytophilum* at 7.79% (95CI 6.20-9.65). Other PA surveys have found a *B. burgdorferi* and *A. phagocytophilum* co-infection rate ranging from less than 0.01% to 1.5%[9,17,29]. However, the neighboring state of NY found a higher co-prevalence of the pair at 15.73% (45/286)[41]. Co-infections of *B. burgdorferi* and *A. phagocytophilum* are primarily observed due to individual prevalence, not influenced by the interaction of the specific etiologic agent[28,29]. Contrary, we found that a co-infection with *B. burgdorferi* and *A. phagocytophilum* occurred more frequently in the observed frequency than independent occurrence in our total ticks (p=0.0000) and adults (p=0.0000) (**Figure 4**). The nymphal co-infection was not significant regarding *B. burgdorferi* and *A. phagocytophilum* prevalence. Conversely, the Lehigh Valley survey observed significance in coinfection frequencies of Ap-ha strain and *B. burgdorferi* in *I. scapularis* nymphs[29]. Despite the interference effect reported in tick acquisition of *A. phagocytophilum* and *B. burgdorferi* from infected *P. leucopus*, other studies have shown a higher co-infection prevalence of the pair than what is expected[54–56]. Studies reviewing host-collected ticks found a positive association between *B. burgdorferi* and *A. phagocytophilum* in meso-mammal hosts, such as Virginia opossums (*Diospyros virginiana*) and racoons (*Procyon lotor*). This positive association was not seen in the questing ticks nor the other host-collected ticks (small mammals, squirrels, and birds)[57]. This idiosyncratic pattern is a noteworthy public concern with the westward spread of *A. phagocytophilum* in questing ticks alongside an increase of HGA incidences.

Literature regarding *B. burgdorferi* and *B. microti* indicate higher rates of co-infection than expected where pathogen prevalence was assorted independently. This positive interaction may indicate a host that is competent for both and thus vector acquisition of the pair could occur in one blood meal[29,55,57]. Similarly, we found our co-infection of *B. burgdorferi* and *B. microti* (4.45%, 95CI 3.25-5.93) occurred more frequently than what was expected independently (p = 0.0000). This significance was sustained in the nymph (p=0.0011) and adult populations (p=0.0000) (**Figure 4**). Our total *B. burgdorferi* and *B. microti* co-infection is higher than other PA studies that reported co-infection prevalence of 0.70% to 2.00%[9,29,34]. Our high coinfection rate might be influenced by host prevalence within Pike County. *I. scapularis* ticks collected from hosts, specifically from small mammals such as small rodents and shrews, share this elevated pattern of coinfection. These mammalian hosts, carriers of both etiologic agents either by low resistance or high tolerance of infection, could indicate dual vector acquisition of these pathogens from a single blood meal[57]. This may increase concern of acquiring two TBDs from a life stage that has only had one blood meal prior, the nymph. In our study, we found 8 nymphs (3.03%, 95CI 1.32-5.88) and 36 adults (4.97%, 95CI 3.51-6.82) that were positive for both *B. burgdorferi* and *B. microti*. In the Lehigh Valley region, 25 *I. scapularis* nymphs were

found co-infected with the pair (1.5%, n=1,721). The medical prominence is not only due to the increased disease severity and duration, but the distinct treatment regimens[15,16]. The antibiotics commonly used to eliminate the spirochete that causes LD, such a doxycycline, will not effectively treat Babesiosis, caused by a protozoan. PA medical practioners within Pike County should be alert of the high co-infection tendency of a single tick bite.

This study documents the co-infection rate of three pathogens (1.52%, 95CI 0.85-2.49) and four pathogens (0.10%, 0.00-0.56) (**Table 2**). One *I. scapularis* adult female co-infected with four different pathogens (*B. burgdorferi*, *A. phagocytophilum*, *B. microti*, and *B. miyamotoi)* and one nymph co-infected with three distinct TBDs; a bacteria, a protozoan, and a virus (*B. burgdorferi*, *B. microti*, and Powassan Virus Lineage II). The potential of co-infection transmission and viability in a human host is a concern for medical agencies, complicating diagnosis and treatment.

Co-infection of TBDs is of the highest clinical concern[9,34]. There is a variation of treatment regimens specific to certain TBD, specifically *B. microti*, a protozoan, and *B. burgdorferi*, a spirochete bacterium. The persistence of flu-like symptoms after treatment for TBDs may be an indication of a co-infection[5]. Co-infections are overlooked due to the similarity in symptoms and diagnostic methods that produce cross-reactivity[32]. There is evidence of an increased severity of disease when dealing with TBD coinfections[9]. Similar county-wide studies, encompassing a diverse TBD panel that includes more than *B. burgdorferi* alone, should continue due to the risk and danger of co-infections. Surveillance of these pathogens and their vectors should continue to provide information for medical diagnosis, to maintain the known geographic distribution and abundance of ticks and their associated diseases, and to aid the public health message to prevent TBD. Further testing of other ticks found in these geographical areas is needed to better understand the distribution and prevalence of other TBDs such as Rocky Mountain Spotted fever, Ehrlichiosis and Tularemia. Although these diseases are thought to be rare, these TBDs are detrimental to one's health and few studies in PA have documented their prevalence.

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SUPPLEMENTAL figure 1. *I. scapularis* microorganism prevalence amongst collection zones and separated by life stage (adult and nymph) and sex (female and male).

I. scapularis	A2	A3	A4	B2	B3	B4	C2	C3	C4	MB	Total
Female	30	26	39	42	35	27	49	43	34	21	346
Male	44	27	27	42	34	32	54	38	48	32	378
Adult	74	53	66	84	69	59	103	81	82	53	724
Nymph	26	47	34	16	56	16	1	3	18	47	264
Total	100	100	100	100	125	75	104	84	100	100	988

B. burgdorferi	A2	A3	A4	B2	B3	B4	C2	C3	C4	MB	Total
Female	18	13	21	22	20	18	17	22	19	5	175
Male	20	14	15	15	17	21	17	13	23	3	158
Adult	38	27	36	37	37	39	34	35	42	8	333
%	51.35%	50.94%	54.55%	44.05%	53.62%	66.10%	33.01%	43.21%	51.22%	15.09%	45.99%
Nymph	6	9	6	3	15	5	0	0	3	3	50
%	23.08%	19.15%	17.65%	18.75%	26.79%	31.25%	0.00%	0.00%	16.67%	6.38%	18.94%

A. phagocytophilum	A2	A3	A4	B2	B3	B4	C2	C3	C4	MB	Total
Female	4	2	12	4	4	2	6	12	6	1	53
Male	9	5	1	8	6	3	6	4	9	0	51
Adult	13	7	13	12	10	5	12	16	15	1	104
%	17.57%	13.21%	19.70%	14.29%	14.49%	8.47%	11.65%	19.75%	18.29%	1.89%	14.36%
Nymph	1	7	0	4	8	0	0	0	0	8	28
%	3.85%	14.89%	0.00%	25.00%	14.29%	0.00%	0.00%	0.00%	0.00%	17.02%	10.61%

B. microti		A2	A3	A4	B2	B3	B4	C2	C3	C4	MB	Total
	Female	4	4	1	1	4	3	1	3	1	1	23
	Male	4	1	2	1	2	1	1	0	1	0	13
	Adult	8	5	3	2	6	4	2	3	2	1	36
	%	10.81%	9.43%	4.55%	2.38%	8.70%	6.78%	1.94%	3.70%	2.44%	1.89%	4.97%
	Nymph	2	4	0	1	4	1	0	0	1	1	14
	%	7.69%	8.51%	0.00%	6.25%	7.14%	6.25%	0.00%	0.00%	5.56%	2.13%	5.30%

B. miyamotoi		A2	A3	A4	B2	B3	B4	C2	C3	C4	MB	Total
	Female	1	1	0	0	0	0	0	4	0	1	7
	Male	0	0	0	0	0	0	1	1	1	0	3
	Adult	1	1	0	0	0	0	1	5	1	1	10
	%	1.35%	1.89%	0.00%	0.00%	0.00%	0.00%	0.97%	6.17%	1.22%	1.89%	1.38%
	Nymph	0	2	0	1	2	0	0	0	0	0	5
	%	0.00%	4.26%	0.00%	6.25%	3.57%	0.00%	0.00%	0.00%	0.00%	0.00%	1.89%

Powassan Virus Lineage II		A2	A3	A4	B2	B3	B4	C2	C3	C4	MB	Total
	Female	1	1	0	1	1	0	0	2	1	0	7
	Male	1	0	0	0	1	1	3	2	0	0	8
	Adult	2	1	0	1	2	1	3	4	1	0	15
	%	2.70%	1.89%	0.00%	1.19%	2.90%	1.69%	2.91%	4.94%	1.22%	0.00%	2.07%
	Nymph	1	0	0	0	1	0	0	0	0	0	2
	%	3.85%	0.00%	0.00%	0.00%	1.79%	0.00%	0.00%	0.00%	0.00%	0.00%	0.76%

Bartonella spp.		A2	A3	A4	B2	B3	B4	C2	C3	C4	MB	Total
	Female	8	11	13	10	14	8	4	12	9	0	89
	Male	8	6	5	1	0	5	3	4	5	0	37
	Adult	16	17	18	11	14	13	7	16	14	0	126
	%	21.62%	32.08%	27.27%	13.10%	20.29%	22.03%	6.80%	19.75%	17.07%	0.00%	17.40%
	Nymph	6	17	11	0	11	3	0	3	1	5	57
	%	23.08%	36.17%	32.35%	0.00%	19.64%	18.75%	0.00%	100.00%	5.56%	10.64%	21.59%

Mycoplasma spp.		A2	A3	A4	B2	B3	B4	C2	C3	C4	MB	Total
	Female	0	1	0	0	6	0	0	6	0	1	14
	Male	0	0	1	2	0	1	1	4	0	1	10
	Adult	0	1	1	2	6	1	1	10	0	2	24
	%	0.00%	1.89%	1.52%	2.38%	8.70%	1.69%	0.97%	12.35%	0.00%	3.77%	3.31%
	Nymph	0	0	0	0	3	0	0	0	1	4	8
	%	0.00%	0.00%	0.00%	0.00%	5.36%	0.00%	0.00%	0.00%	5.56%	8.51%	3.03%