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Vector-Borne Diseases, Surveillance, Prevention

Tick-Borne Pathogens in Questing Blacklegged Ticks (Acari: Ixodidae) From Pike County, Pennsylvania

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Abstract

Active surveillance was conducted by collecting questing ticks from vegetation through a 2-yr survey in Pike County, Pennsylvania. Over a thousand blacklegged ticks (*Ixodes scapularis* Say) and American dog ticks (*Dermacentor variabilis* Say) were collected. A single specimen of the following species was collected: lone star tick (*Amblyomma americanum* L.), rabbit tick (*Haemaphysalis leporispalustris* Packard), and an Asian longhorned tick (*Haemaphysalis longicornis* Neumann). This study represents the largest county-wide study in Pennsylvania, surveying 988 questing *I. scapularis* adult and nymphs. Molecular detection of five distinct tick-borne pathogens was screened through real-time PCR at a single tick resolution. Respectively, the overall 2-yr adult and nymph prevalence were highest with *Borrelia burgdorferi* (Spirochaetales: Spirochaetacceae) (45.99%, 18.94%), *Anaplasma phagocytophilum* (Rickettsiales: Anaplasmataceae) (12.29%, 7.95%) where the variant-ha (8.29%, 3.03%) was overall more prevalent than the variant-v1 (2.49%, 4.17%), *Babesia microti* (Piroplasmida: Babesiidae) (4.97%, 5.30%), *Borrelia miyamotoi* (Spirochaetales: Spirochaetaceae) (1.38%, 1.89%), and Powassan virus lineage II [POWV]/deer tick virus (DTV) (2.07%, 0.76%). Adult and nymph coinfection prevalence of *B. burgdorferi* and *B. microti* (3.03%, 4.97%) and adult coinfection of *B. burgdorferi* and *A. phagocytophilum* or *A. phagocytophilum* and *B. microti* were significantly higher than the independent infection rate expected naturally. This study highlights the urgency to conduct diverse surveillance studies with large sample sizes to better understand the human risk for tick-borne diseases within small geographical areas.

Key words: Lyme disease, Powassan virus, babesiosis, anaplasmosis, tick-borne pathogen

Tick-borne diseases (TBDs) in the United States are a burden to public health. Approximately 75% to 95% of vector-borne diseases reported in the United States are transmitted by ticks (Wikel 2018, Wisely and Glass 2019, Rodino et al. 2020). Changes of certain environmental conditions, such as host availability for vectors, forest fragmentation, invasive flora, global temperature, and humidity, are accountable for the expansion of tick populations and TBDs (Allan et al. 2003, Adalsteinsson et al. 2018, Sonenshine 2018, Rodino et al. 2020). While any tick species can be associated with the spread of TBDs, the blacklegged tick (*Ixodes scapularis* Say) is the main contributor to the increasing numbers of TBDs in Pennsylvania (PA) and thus the most medically relevant (Wikel 2018).

In PA, the blacklegged tick has been associated with human bacterial pathogens, *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Borrelia miyamotoi*; protozoal pathogens such as *Babesia microti*; and viral pathogens such as Powassan virus (Hersh et al. 2014, Hutchinson et al. 2015, Farone et al. 2018, Edwards et al. 2019). Both the nymph and adult life stages of *I. scapularis* have been associated with human illness; however, the nymph life stage has primarily contributed to human Lyme disease (LD) infection in the eastern United States (Eisen and Dolan 2016). Their small size, relative to adults, may decrease visual detection and therefore prevention of disease transmission. Early removal of ticks is helpful for LD, whose causative agent *Borrelia burgdorferi* (Spirochaetales: Spirochaetaceae) takes 24 to 72 h to transmit (Eisen 2018). Other TBDs such as Powassan Virus Lineage II [POWV]/deer tick virus (DTV) transmits within 15 min attributed to its residency within the tick salivary glands (Madison-Antenucci et al. 2020). There are a few tick-borne pathogens (TBPs)

capable of transovarian transmission, such as *Borrelia miyamotoi* (Spirochaetales: Spirochaetaceae) and DTV (Eisen and Eisen 2018, Han et al. 2019, Pokutnaya et al. 2020). Acquisition and maintenance of the other pathogens are primarily through blood meals and thus only life stages that have fed previously, nymphs and adults, are competent vectors (Eisen and Eisen 2018).

New species and genetic variants of TBPs in *I. scapularis* are increasing and may lead to emergence of human and animal disease (Eisen et al. 2017, Wikel 2018). For example, human granulocytic anaplasmosis is caused by an intraleukocytic bacterium called *Anaplasma phagocytophilum* (Rickettsiales: Anaplasmataceae), which interferes with host membrane-bound vacuoles, eliciting nonspecific illness and symptoms (Eisen and Eisen 2018, Rodino et al. 2020). A distinction between two variants has surfaced: one that causes human anaplasmosis (variant-ha or Ap-ha) and one that is associated with ruminants (variant-1 or Ap-v1) (Trost et al. 2018). Distinguishing between these two strains in tick surveillance is critical for evaluating and understanding the public health concern for human anaplasmosis. In addition, *I. scapularis* can maintain and transmit more than one TBP. These vectors are defined as a coinfection where more than one TBP is present. In human illnesses, the danger of a coinfection includes under or misdiagnosis of less prevalent diseases and an increase in disease severity in humans; however, contradictory suppressive effects have been reported (Diuk-Wasser, Vannier, and Krause 2016). Whether there is an increase of illness severity or not, coinfection of distinct pathogens (bacterial, viral, or protozoan) remains a high threat to public health due to different treatment regimens. Additionally, early intervention or removal of ticks combined with prompt diagnosis is the most effective way to decrease TBDs in humans (Eisen and Eisen 2018).

Surveillance of TBPs in *I. scapularis* is vital to monitor and detect emerging pathogenic agents. Such was the case of *B. miyamotoi*, that was first found in *I. scapularis* in 2001 (Wisely and Glass 2019). Twelve years after this discovery, it was shown to cause disease in humans (Gugliotta et al. 2013). The importance of pathogen prevalence in specific geographic regions is necessary to analyze spatial patterns and expansions of TBD risk.

PA has the third highest incidence rate of LD, confirmed cases per 100,000 residents, averaged over 3 yr across the country (CDC 2019a). The distribution of TBPs in PA varies among regions (Hutchinson et al. 2015), with incidents rates as low as 40.7 cases in southeastern PA and as high as 170.44 cases in northwestern PA (PA Department of Health 2019). The variation seen within a single state advocates the necessity for localized surveys, such as within a single county, to properly ascertain the risk of TBDs.

Our study's purpose is to assess the pathogen prevalence within questing adult and nymphal *I. scapularis* in a large-scale survey (n = 1,000) of a localized region (Pike County, PA). LD case reports for Pike County were first reported in 1989 as 30.82 cases and since has escalated to 159.12 cases in 2019 (PA Department of Health 2019). Comparatively, the 2019 incidence rate of the entire state was less than half of the incidence rates found in Pike County (70.34 cases). Therefore, this county provides an ideal case study to demonstrate the importance of surveying localized regions to quantify regional variation in TBP.

Materials and Methods

Site Selection

Pike County, PA was divided into a grid of 9 primary zones for collection (Fig. 1). Variations in total ticks collected were based on the 2010 reported census for Pike County where the longitudinal axis C had the highest reported census for human population (U.S. Census Bureau 2022). Thus, we focused three additional tick collections within the borough of Milford which is found in grid C1 bordering C2. Collection sites within each grid were labeled with the grid section followed by a number (i.e., A1-1) in numerical order. The Milford borough sites (C1-4,5,6) were evaluated together and reported as C1-MB. Collection sites 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.

Tick Collections

Ticks were collected from May through July (Spring) and October through November (Fall) in 2018 and 2019 by dragging corduroy cloths along the trails of nearby vegetation and leaf litter for several meters before thoroughly examining the cloths for ticks. A total of 100 *I. scapularis* ticks were collected from six collection zones, 200 from one (C1), 75 from one (B3), and 125 from another (B2). Collected ticks were stored in microcentrifuge tubes labeled with collection zone and collection site. 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 via midsagittal cuts with a disposable scalpel and use of QIAamp Viral RNA kit (Qiagen, Redwood City, CA) following the manufacturer's protocol. The measures taken to prevent contamination included small extraction groups of nineteen samples plus an extraction blank control. Blank controls from the extraction process, including reagents and excluding a tick sample, were tested on the *B. burgdorferi* and *A. phagocytophilum* assays, being the most common tick pathogens, and the DTV assay for possible extraction contaminants. Validated TaqMan assays were used for specific pathogen detection (Table 1). A novel SYBR green reverse transcriptase real-time PCR assay and melt curve analysis was used to determine the presence of DTV by targeting the NS5 gene. The DTV primers used in this study are accredited to Coppe Healthcare Solutions (Knox et al. 2017). Samples that were positive on the Msp2 *A. phagocytophilum* were screened using a nested protocol (Massung et al. 1998) and sequenced with a published optimized primer (Edwards et al. 2019). All *A. phagocytophilum* positive samples were additionally screened with an SNP Genotyping assay and analyzed using an allelic discrimination plot (Krakowetz et al. 2014). The SNP Genotyping assay was validated with analysis of sequences with the two nucleotide variations seen amongst the variants of nine *A. phagocytophilum* positive samples (Massung et al. 1998, Edwards et al. 2019). All amplification was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems) and an Applied Biosystems QuantStudio5 (Thermo Fisher-Scientific) following the manufacturer's recommended cycling conditions for TaqMan Universal PCR Master Mix and primer concentration optimization. Samples were only considered positive when amplification reached designated thresholds and Ct calls per assay, not to exceed 40 cycles. Additionally, samples that were outside the melting temperature (Tm = 80–83) range in the melt curve analysis were determined negative. Each assay included a positive and negative control (nuclease-free water) that substituted for the DNA volume. The *B. burgdorferi* positive was a cell culture acquired from the ATCC (Manassas, VA) and *B. miyamotoi* positive from blood extraction

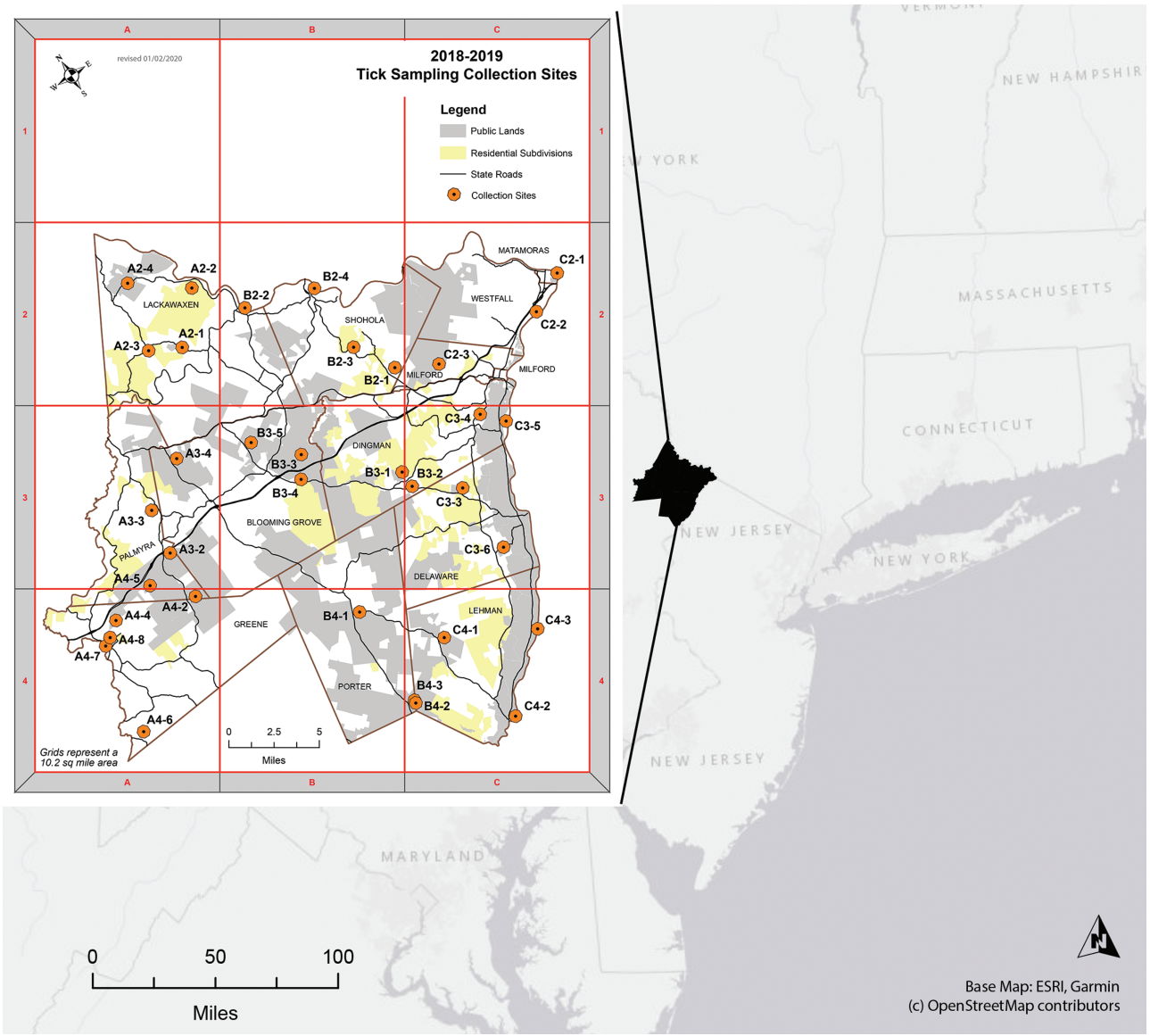

Fig. 1. Grid and site locations of tick collections throughout Pike County, PA during 2018 and 2019.

of *Hyaena* species blood, both were validated through sequencing via a nested IGS protocol (Bunikis et al. 2004). The positive control for *A. phagocytophilum* was obtained from a white-tailed deer (*Odocoileus virginianus*) blood extraction and confirmed with sequencing via a nested GE protocol (Massung et al. 1998). The *B. microti* positive was a total DNA extraction from an *I. scapularis*-infected tick from the passive surveillance studies at Dr. Jane Huffman Wildlife Genetics Institute and confirmed with sequencing of the 18s gene (Armstrong et al. 1998). The DTV synthetic positive was purchased from GeneWiz (South Plainfield, NJ). All assays were validated using standards and guidelines developed internally by the Dr. Jane Huffman Wildlife Genetics Institute consistent with CDC guidelines (CDC 2019c).

Statistical Analysis

All statistical analyses were carried out in R statistical software version 3.6.3 (R Core Team 2020). A generalized linear model (GLM) was used to model infection prevalence in *I. scapularis* with a binomial error distribution and fixed effects of sampling year, sampling season, life stage, canopy cover, and collection zone (Quinn and Keough 2002). A separate GLM was fit for each individual pathogen. Overall significance of each predictor's effect on infection prevalence was assessed using ANOVA with Type III sums of squares. QGIS 3.6 (QGIS.org 2019) was used to calculate percent canopy cover in a 1 km buffer surrounding each sampling point. Tree canopy data were derived from the 1-m resolution Pennsylvania Spatial Data Access (University of Vermont Spatial Analysis Laboratory 2015). One-kilometer buffers only included land area within Pennsylvania; any area of the 1 km buffer zone that crossed state lines was not included in the percentage. Collection zones were tested for significant differences in infection prevalence by computing estimated marginal means using the lsmeans() function in the R package emmeans (Lenth 2020). The 95% confidence intervals of pathogen prevalence of nymphs and adults were calculated using the binom.test() function (Hollander and Wolfe 1973). We used the cooccur() function in the cooccur library to analyze rates of coinfection among pathogens (Griffith et al. 2016). A probabilistic model of species co-occurrence was used to determine if the observed coinfections occurred significantly more or less frequently than the rate of co-occurrence that

would be expected in the dataset based on the observed prevalence of each pathogen if each was distributed independently. Pathogens with a low prevalence and a cooccurrence less than 1 were removed from statistical analysis. An alpha of 0.05 was used to determine statistical significance.

Results

Tick Collection Data

In the years 2018 and 2019, a total 2,100 ticks were collected (Table 2). This included five different species, where *I. scapularis* and *D. variabilis* Say (Acari: Ixodidae) were the most prevalent. More ticks were collected in the spring (n = 1,803) compared to the fall (n = 414) and among years in 2018 (n = 1,281) compared to 2019 (n = 936). During collections, a single adult female *Amblyomma americanum* Linnaeus (Acari: Ixodidae) was collected and identified in C1-1, and one *Haemaphysalis leporispalustris* Packard (Acari: Ixodidae) nymph from A2-1. This tick was identified by the cornua at the posterolateral margins present on the ventral basis capituli and the blunt spur on the palpal segment three that does not reach the anterior margin of the second segment (Egizi et al. 2019). One *Haemaphysalis longicornis* Neumann (Acari: Ixodidae) larval from A3-8 identified by the absence of cornua on the ventral basis capituli, presence of straight lateral margins on the dorsal basis capituli, and a slight projection on the second palpal segment (Egizi et al. 2019). All *D. variabilis* collected were found during the spring season. All *I. scapularis* adults (males and females) and nymphs were observed at each location (Supp Table S1 [online only]). Across all collections there was an uneven distribution of *I. scapularis* life stage densities, the lowest number of a life stage was one nymph in C1 and three nymphs in C2. Individual collections of other species are reported; however, the focus of this paper is specific to *I. scapularis*, and thus all further analyses were completed only on *I. scapularis*.

Pathogen Testing

Of the 1,000 *I. scapularis* tested, only 988 (346 adult females, 378 adult males, and 264 nymphs) were included in analysis. A total of 12 samples were eliminated from analysis as they were deemed contaminated due to amplification being detected within the extraction blank. TBP burden is summarized in Table 3. The *A. phagocytophilum* positive ticks were screened for validation and variant distinction post freeze. Loss of DNA quality could be the reason why variants were identified in only 97 out of the 110 ticks. The remaining 13 ticks were identified positive for *A. phagocytophilum* Msp2 gene presence, but without strain identification. Our variant discrimination based on sequencing of the 16s rRNA gene (Massung et al. 1998) and the SNP Genotyping assay (Krakowetz et al. 2014) allowed for detection of variant coinfection. However, our study did not discover any tick coinfected with Ap-ha and Ap-v1. Our binomial GLM demonstrated that the significant

Table 1. Real-time primers and probes used in this study

Species	Gene	Primer sequence	Probe sequence	Source
Borrelia burgdorferi	16s-23S	F—GCTGTAAACGATGCACACTTGGT R—GGCGGCACACTTAACACGTTAG	6FAM—TTCGGTACTAACTTTTAGTTAA—MGBNFQ	(Barbour et al. 2009)
Borrelia miyamotoi	16s-23S	F—GCTGTAAACGATGCACACTTGGT R—GGCGGCACACTTAACACGTTAG	ABY—CGGTACTAACCTTTCGATTA—QSY	(Barbour et al. 2009)
Anaplasma phagocytophilum	Msp2	F—ATGGAAGGTAGTGTTGGTTATGGTATT R—TTGGTCTTGAAGCGCTCGTA	ABY—TGGTGCCAGGGTTGAGCTTGAGATTG—QSY	(Courtney et al. 2004)
Babesia microti	18s rRNA	F—CAGGGAGGTAGTGACAAGAAATAACA R—GGTTTAGATTCCCATCATTCCAAT	VIC—TACAGGGCTTAAAGTCT—MGBNFQ	(Teal et al. 2012)
Deer tick virus	NS5	Sense F—GAAGCTGGGTGAGTTTGGAG Anti-sense R—CCTGAGCAACCAACCAAGAT		Coppe Healthcare Solutions
Anaplasma phagocytophilum Variants	16s rRNA	F—ACATGCAAGTCGAACGGATTATTCT R—GCTATCCCATACTACTAGGTAGATTCCT	(Ap-ha) VIC—CTGCCACTAACTATTCT—MGB (Ap-v1) FAM—CTGCCACTAATTATTCT—MGB	(Krakowetz et al. 2014)

Table 2. Total ticks collected using drag cloths 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)	530	240	267	174	1,211
D. variabilis (adult female)	301	0	166	0	467
D. variabilis (adult male)	244	0	175	0	419
D. variabilis (total)	545	0	341	0	886
A. americanum (adult female)	1	0	0	0	1
H. leporispalustris (nymph)	1	0	0	0	1
H. longicornis (larval)	0	0	1	0	1

Drags were used to collect questing ticks at the collection sites shown in Fig. 1.

determinants of overall TBP prevalence were pathogen type, season, life stage, and collection grid, but not year or canopy cover (Table 4). Both life stages had a higher prevalence of singly infected ticks than coinfected ticks, and the nymphal coinfection rate was lower than adults (Table 3). There was only 1 nymph with molecular presence of three different TBPs compared to the 13 detected in adults. This nymph had molecular presence of *B. burgdorferi*, *B. microti*, and DTV from collection grid A1. The highest number of TBPs detected within an individual tick was four pathogens. This adult female, collected from grid B2, had molecular presence of *B. burgdorferi*, *A. phagocytophilum (*variant Ap-ha), *B. microti*, and *B. miyamotoi*. Molecular presence of all five TBPs was detected in both life stages and sexes (nymph, adult male, and adult female) (Table 5). Specific collection grids were found to be significantly different in overall TBP prevalence via estimated marginal means, where results were averaged over levels of pathogen type, year, life stage, and season (Table 6). The total pathogen prevalence of C1-MB was statistically different than grids A1 ($P = 0.027$), B1 ($P = 0.032$), B2 ($P < 0.001$), B3 ($P = 0.016$), C2 ($P = 0.010$), and C3 ($P = 0.041$); however, it was not significantly different than grid C1 where C1-MB resides within ($P = 0.787$). The most common pathogens *(B. burgdorferi*, *A. phagocytophilum* including both variants, and *B. microti*) were detected in all collection grids and C1-MB in either adult or nymph ticks, but not both. The presence of *B. miyamotoi* or DTV was found in all grids and C1-MB, except A3 (Supp Table S1 [online only]). The pathogen with the greatest prevalence in adults was *B. burgdorferi,* proceeded by *A. phagocytophilum* (specifically Ap-ha), *B. microti*, DTV, and lastly *B. miyamotoi* (Table 5). The same prevalence patterns were found in nymphs, except *A. phagocytophilum* variants were separately lower than *B. microti* and *B. miyamotoi* prevalence was greater than DTV. The *A. phagocytophilum* variant with the highest adult prevalence was Ap-ha, surpassing *B. microti* prevalence. The nymphal *A. phagocytophilum* variant prevalence was slightly higher in Ap-v1. When evaluating the determinants for *B. burgdorferi* prevalence, life stage, collection grid, and season were statistically significant (Table 7). Collection year and canopy cover percent had no effect of *B. burgdorferi* prevalence. The collection grids that were significantly different in *B. burgdorferi* prevalence than C1-MB, based on estimated marginal means, was B2 ($P = 0.009$), B3 ($P = 0.001$), and C3 ($P = 0.006$) (Table 6). Statistical significance was found in the total *A. phagocytophilum* prevalence among year ($P = 0.030$) and season ($P = 0.001$), but not in life stage, collection grid, or canopy cover. The only significant determinant among the *A. phagocytophilum* variants was season ($P = 0.025$) in Ap-ha prevalence (Table 7). The only significant determinant of the overall *B. microti* prevalence was collection season. No significant determinants were identified for *B. miyamotoi* and DTV, possibly a result of low overall prevalence.

Coinfection rates were found to be significant with analysis of the expected rates, based on individual prevalence, compared with the observed rates (Table 8). Three TBPs (DTV, *B. miyamotoi*, and Ap-v1) were removed from coinfection analysis due to lower individual prevalence resulting in an expected co-occurrence of less than 1. The overall *I. scapularis* collected ($n = 988$) showed the most prevalent pathogens to have coinfection rates that occurred more than what is expected randomly included *B. burgdorferi*, *A. phagocytophilum,* and *B. microti*. There is a significant interaction between *B. burgdorferi* and Ap-ha and *B. microti* and Ap-ha in the overall *I. scapularis*, including both nymph and adult populations. The *I. scapularis* nymphal coinfections that showed significance was *B. burgdorferi* and *B. microti* ($P = 0.001$) and *B. burgdorferi* and Ap-ha ($P = 0.001$). Adult *I. scapularis* indicated infection with *B. burgdorferi* and *A. phagocytophilum* ($P < 0.001$), *B. burgdorferi and B. microti* ($P < 0.001$), and *A. phagocytophilum* and *B. microti* ($P = 0.024$) occurred more frequently than expected. Only the human active *A. phagocytophilum* variant was significant in these interactions: *B. burgdorferi* and Ap-ha ($P < 0.001$) and *B. microti* and Ap-ha ($P = 0.007$) where Ap-v1 coinfection significance could not be evaluated due to the low individual prevalence.

Discussion

As TBDs continue to rise, surveillance of ticks and their pathogenic agents are of upmost medical and veterinarian importance to monitor current and emerging TBPs. This study demonstrates the significance of developing scientific methods using large sample sizes over small geographical regions for better resolution of the burden associated with TBPs. In addition, this study has highlighted the importance in strain differentiation and coinfection exposure of TBPs to better understand the public health risk associated with surveillance efforts.

Tick Species

A total of 2,100 ticks were collected in our 2-yr study, including five different species and all three life stages of *I. scapularis* ($n = 1,211$). In recent decades, ticks have expanded their geographic ranges due to several factors such as climate change, host availability, humidity tolerance, freezing temperature durations, and human habitat (Sonenshine 2018). All *D. variabilis* were collected in the spring, congruent with other studies on their

Table 3. Detection of tick-borne pathogens in *Ixodes scapularis* collected from Pike County, Pennsylvania between 2018 and 2019

			95% CI				95% CI				95% CI	
Life stage, Percent	Nymph	%	Lower	Upper	Adult	%	Lower	Upper	Overall	%	Lower	Upper
Ticks not carrying TBPs	191	72.35	66.53	77.66	349	48.20	44.51	51.91	540	54.66	51.49	57.79
Ticks carrying TBPs	73	27.65	22.34	33.47	375	51.80	48.09	55.49	448	45.34	42.21	48.51
Ticks carrying a single TBP	55	20.83	16.10	26.24	282	38.95	35.38	42.61	337	34.11	31.15	37.16
Ticks carrying more than one TBP	18	6.82	4.09	10.56	93	12.85	10.49	15.50	111	11.23	9.33	13.37
two TBPs	17	6.44	3.80	10.11	79	10.91	8.73	13.41	96	9.72	7.94	11.74
three TBPs	1	0.38	0.01	2.09	13	1.80	0.96	3.05	14	1.42	0.78	2.37
four TBPs	0	0.00	0.00	1.39	1	0.14	0.00	0.77	1	0.10	0.00	0.56
Total ticks tested	264				724				988			

This includes the molecular prevalence of *B. burgdorferi*, *A. phagocytophilum* (Ap-v1, Ap-ha, and undetermined variants), *B. microti*, *B. miyamotoi*, and deer tick virus. The values are the individual infected counts. The percent values are presented with 95% confidence intervals (CI).

Table 4. ANOVA with Type III sums of squares parameter estimates for explanatory variables in binomial GLM model for overall pathogen prevalence

Parameter	LR Chisq	df	Pr(>Chisq)
Pathogen	844.77	4	<0.001
Year	0.18	1	0.668
Life stage	17.08	1	0.001
Collection grid	29.34	9	0.001
Season	20.24	1	<0.001
Canopy cover percent	0.05	1	0.816

propensity of spring activity (Burg 2001). The distribution of the *H. longicornis* tick is noteworthy due to its invasiveness, parthenogenetic reproduction, and ability to overwinter (Occi et al. 2019). *Haemaphysalis longicornis* was previously reported in five PA counties (Bucks, Centre, Chester, Montogomery, and Philadelphia) by an active tick surveillance across the state (Beard 2018, Price et al. 2020). Our active surveillance updates the range expansion of a single *H. longicornis* larva in PA as far north as Pike County and correlates with the temporal abundance of life stages in other studies, where nymph activity peaks in early summer or spring (Occi et al. 2019). *Haemaphysalis leporispalustris* is a competent vector of *Francisella tularensis* and *Rickettsia* species among wildlife populations (Roth et al. 2017, Zellner and Huntley 2019, Hamer et al. 2021). The immature stages are known to parasitize migratory birds (Hamer et al. 2021). Findings from the present tick survey of Pike County found greater diversity than neighboring regions. In the Lehigh Valley region only three species, *D. variabilis, I. scapularis,* and *I. cookei* were reported (Edwards et al. 2019). Continued active surveillance efforts in local areas will contribute to the documentation of tick expansion into new geographic regions (Sonenshine 2018).

Tick-Borne Pathogen Surveillance

The focus of this study was to determine the presence and prevalence of the pathogens within the *I. scapularis* population of Pike County, PA. Of the 988 *I. scapularis* analyzed, roughly half of the adults (51.80%) and one-quarter of the nymphs (27.65%) tested positive for a TBP (Table 3). When evaluating coinfections, nymphs (6.82%) were observed to be half of the overall adult coinfection (12.85%). This study highlights the importance of analyzing both adult and nymph life stages for tick surveillance. Adult ticks have completed two blood meals and thus exposed to two different reservoirs when compared to nymphs. Surveillance of coinfections in nymphs evaluates human health risk and better interpretation of local reservoirs. In comparison, adults provide a wider range of surveillance for the less prevalent pathogens. The two-fold difference between Pike County's adult and nymph population, and significance in life stage on pathogen prevalence ($P = 0.001$), mimics the potential double exposure of reservoirs (Table 4). The known health issues of coinfection with TBDs are a public health concern (Diuk-Wasser et al. 2016). Our study found a significant variation of positive ticks amongst collection grids across a single county (Table 4). This supports the importance of large sample sizes combined with small geographical collection sites.

Lyme Disease (*B. burgdorferi*)

The CDC estimates over 400,000 people are affected with LD annually in the United States, despite the significantly lower reported

Table 5. Tick-borne pathogen prevalence in *Ixodes scapularis* by life stage (nymph and adult) and overall (nymph + adult)

	Ixodes scapularis life stage											
Pathogen	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	21	7.95	4.99	11.90	89	12.29	9.99	14.91	110	11.13	9.24	13.26
Ap-ha	8	3.03	1.32	5.88	60	8.29	6.38	10.54	68	6.88	5.38	8.64
Ap-v1	11	4.17	2.10	7.33	18	2.49	1.48	3.90	29	2.94	1.97	4.19
Undetermined	2	0.76	0.09	2.71	11	1.52	0.76	2.70	13	1.32	0.70	2.24
B. microti	14	5.30	2.93	8.74	36	4.97	3.51	6.82	50	5.06	3.78	6.62
B. miyamotoi	5	1.89	0.62	4.36	10	1.38	0.66	2.53	15	1.52	0.85	2.49
Deer tick virus	2	0.76	0.09	2.71	15	2.07	1.16	3.39	17	1.72	1.01	2.74

Table 6. Estimated marginal means of collection grid overall pathogen and *B. burgdorferi* prevalence averaged over levels of pathogen type, year, life stage, and season

		Estimated marginal means of collection zones				
	Contrast	Estimate	SE	df	*z*-ratio	*P* value
Total pathogen prevalence	A1: C1-MB	1.001	0.301	Inf	3.357	0.027
	B1: C1-MB	1.016	0.307	Inf	3.311	0.032
	B2: C1-MB	1.325	0.284	Inf	4.675	<0.001
	B3: C1-MB	1.045	0.297	Inf	3.515	0.016
	C2: C1-MB	1.043	0.286	Inf	3.648	0.010
	C3: C1-MB	0.910	0.282	Inf	3.232	0.041
	B2: C1	0.832	0.240	Inf	3.461	0.019
B. burgdorferi prevalence	B2: C1-MB	1.570	0.426	Inf	3.688	0.009
	B3: C1-MB	1.979	0.454	Inf	4.357	0.001
	C3: C1-MB	1.487	0.391	Inf	3.803	0.006

Only collection grids that were significant ($P = 0.05$) are shown.

Table 7. ANOVA with Type III sums of squares parameter estimates for explanatory variables in binomial GLM model for the most prevalent diseases: *B. burgdorferi*, *A. phagocytophilum*, and *B. microti*

	Borrelia burgdorferi			Anaplasma phagocytophilum			Babesia microti		
Parameter	LR Chisq	Df	Pr(>Chisq)	LR Chisq	Df	Pr(>Chisq)	LR Chisq	Df	Pr(>Chisq)
Year	2.83	1	0.093	4.70	1	0.030	0.375	1	0.541
Life stage	35.81	1	<0.001	0.02	1	0.881	0.597	9	0.440
Collection grid	28.82	9	0.001	15.89	9	0.069	8.48	1	0.486
Season	4.73	1	0.030	11.64	1	0.001	4.87	1	0.027
Canopy cover percent	0.00	1	0.974	0.41	1	0.522	0.00	1	0.982

Table 8. Probability of pathogens in questing *Ixodes scapularis* using analysis of expected coinfection from individual prevalence and the observed coinfection

I. scapularis	Pathogens & Microorganisms	Probability Co-infection	Expected Co-infection	Observed Co-infection	p-pos
Nymph	*B. burgdorferi* + *B. microti*	0.010	2.7	8	0.001
(n = 264)	*B. burgdorferi* + Ap-ha	0.006	1.5	6	0.001
Adult	*B. burgdorferi* + *A. phagocytophilum*	0.057	40.9	58	<0.001
(n = 724)	*B. burgdorferi* + Ap-ha	0.038	27.6	43	<0.001
	B. burgdorferi + *B. microti*	0.023	16.6	36	<0.001
	A. phagocytophilum + *B. microti*	0.006	4.4	9	0.024
	Ap-ha + *B. microti*	0.004	3.0	8	0.007
Overall	*B. burgdorferi* + *A. phagocytophilum*	0.043	42.6	65	<0.001
(n = 988)	*B. burgdorferi* + Ap-ha	0.027	26.4	49	<0.001
	B. burgdorferi + *B. microti*	0.020	19.4	44	<0.001
	A. phagocytophilum + *B. microti*	0.006	5.6	10	0.042
	Ap-ha + *B. microti*	0.003	3.4	9	0.005

The pathogens not listed (Deer tick virus, *B. miyamotoi*, and Ap-v1) were removed from analysis because the low expected occurrence was less than 1.

cases each year (CDC 2019). PA has reported the highest number of cases annually, accounting for 28 percent of total reported cases in the United States (CDC 2019a). Prevalence of *B. burgdorferi* within adult *I. scapularis* was 45.99% (95% CI: 42.32–49.70) and 18.94% (95% CI: 14.40–24.20) in nymphs for an overall prevalence of 38.77% (95% CI: 35.71–41.88) (Table 5). Other PA studies that included both *I. scapularis* life stages (adults and nymphs) reported lower overall *B. burgdorferi* infection from 21.00% ($n = 115$) to 36.39% ($n = 294$) (Brown et al. 2015, Han et al. 2019). Our adult-infected prevalence falls within the range observed across the state and is similar to more recent PA statewide studies that found 45.5% (136/299) and 47.4% (646/1363) adults positive with *B. burgdorferi* (Hutchinson et al. 2015, Livengood et al. 2020). Our localized study is highly specific to each region of Pike County and includes a larger sample size ($n = 988$) than that of the statewide study. Even on a small county scale, there were differences in *B. burgdorferi* prevalence across Pike County (Fig. 2). The fluctuations in *B. burgdorferi* prevalence in endemic areas highlight the need for continued surveillance with large sample sizes to better understand the public health impact. These data can be used to assist physicians in understanding the potentially higher risk for exposure to *B. burgdorferi* following the bite of a tick from Pike County, PA.

Observable differences between a small county scale provide insights into significant explanatory variables on *B. burgdorferi*

prevalence such as life stage, season, and collection grid (Table 7). Milford Borough's (C1-MB) *B. burgdorferi* prevalence was significantly different than three other grids (Table 6). The sites from C1-MB had lower *B. burgdorferi* prevalence (Fig. 2) and are characterized by fragmented forests dispersed across the small borough. GLM analysis found no effect of canopy cover percent on *B. burgdorferi* prevalence. Similarly, *Borrelia* species prevalence amongst natural and urban areas were not significantly different (Kowalec et al. 2017) and there is emerging evidence against the dilution effect hypothesis (Zolnik et al. 2015). Unlike canopy cover, collection grid was a significant explanatory variable on *B. burgdorferi* prevalence (Table 7). The lack of significance of canopy cover paired with the significance of collection grid on *B. burgdorferi* prevalence may warrant future investigation on the influence of fragmented forests.

Anaplasmosis (*A. phagocytophilum*)

The CDC reports increased human anaplasmosis across the nation and PA; however, there have been few reports of human infections in Pike County, PA (PA Department of Health 2020, CDC 2021). Our overall prevalence of *A. phagocytophilum* detected in Pike County was 11.13% (95% CI: 9.24–13.26), nymphal infection of 7.95% (95% CI: 4.99–11.90), and a 12.29% (95% CI: 9.99–14.91) infection rate in adults (Table 5). PA studies have reported higher *A. phagocytophilum* prevalence in southwestern counties, 14.29% (42/294) (Brown et al. 2015), and lower incidences from 1.7% to 3.4% in PA surveys (Courtney et al. 2003, Edwards et al. 2019, Livengood et al. 2020). The high prevalence rates in Pike County questing ticks may correlate to a newly identified *A. phagocytophilum* endemic area. Discrepancies in TBP prevalence might be explained by the distinction between strains of *A. phagocytophilum*.

In the present study, detection of *A. phagocytophilum* was further evaluated through analysis of two known strains currently in PA circulation. The human active variant (Ap-ha) is the agent for human anaplasmosis which shares a reservoir with *B. burgdorferi*, *Peromyscus leucopus* (Keesing et al. 2014). The *A. phagocytophilum* variant 1 (Ap-v1) strain that is not known to cause human infection is associated with ruminants (Massung et al. 2005). In the present study, the higher nymphal prevalence of Ap-v1 (4.17%, 95% CI: 2.10–7.33) may imply larval host preferences or higher densities of other mammals circulating Ap-v1, such as striped skunks (*Mephitis mephitis*) (Keesing et al. 2014). Nymphal life stages prefer small to medium mammals, such as raccoons and opossums (Ginsberg et al. 2021). Similarly, the Lehigh Valley nymphal survey found a higher prevalence of Ap-v1 (3.5%) compared to Ap-ha (0.8%), a three-fold difference between the variants. The higher Ap-ha prevalence (8.29%, 95% CI: 6.38–10.54) seen in our adult populations might indicate nymphal preferences of small or medium mammals, known for Ap-ha infection, such as the white-footed mouse (Keesing et al. 2014). The lack of *A. phagocytophilum* variant coinfection in this study is congruent with studies in Canada (Krakowetz et al. 2014) and other northeastern U.S. studies (Steiner et al. 2008, Edwards et al. 2019). Coinfection of the variants appears to be uncommon, with evidence of a single *Ixodes ricinus* (Acari: Ixodidae) to have a dual infection (Massung et al. 2002). The few coinfections between *A. phagocytophilum* variants warrant evaluation of host and vector residency dynamics.

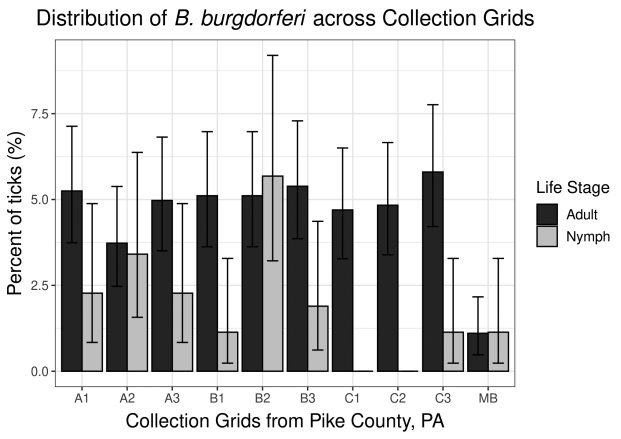

Fig. 2. Distribution of positive *I. scapularis* infected with *B. burgdorferi* by life stage across collection grids in Pike County, PA from 2018 and 2019. The lower and upper bounds represent the 95% confidence interval. No nymphs collected from either grid C1 ($n = 1$) or C2 ($n = 3$) were positive. Percentages of ticks are derived from the total life stage, adults ($n = 724$), and nymphs ($n = 264$).

Human cases of anaplasmosis are estimated at 4,151 annually, being primarily endemic in the Northeastern United States (Madison-Antenucci et al. 2020). Cases of anaplasmosis have tripled in Pike county in a single year, between 2018 and 2019 (PA Department of Health 2020). Due to the risk of human disease transmission from nymphal ticks, the higher Ap-ha prevalence in this study is integral to the Pike County public health awareness to prevent misdiagnosis or underrepresentation. Few studies have implemented *A. phagocytophilum* variant detection in surveillance studies, despite the higher human health risk associated with the human active anaplasmosis. The higher Ap-ha prevalence in this study warrants the inclusion of variant discrimination for future surveillance studies.

Babesiosis (*B. microti*)

Babesiosis occurs at a lower incidence in PA than what might be expected given the high prevalence of LD (Ingram and Crook 2020). Additionally, 94% of babesiosis cases are reported from seven states in the United States, not including PA (Gray 2019). However, babesiosis infection does not warrant mandatory reporting in the state of PA. Four states bordering PA had the highest reported cases, including NY (2,257 cases) and NJ (869 cases) (Gray 2019). Healthcare providers can elect to report cases to the Pennsylvania Department of Health, who stated an increase of 20-fold from the past 12 yr (Ingram and Crook 2020). Our nymphal infection prevalence of *B. microti* was 5.30% (95% CI: 2.93–8.74) and adult infection prevalence was 4.97% (95% CI: 3.51–6.82) for an overall prevalence of 5.06% (95% CI: 3.78–6.62) (Table 5). Life stage was not a significant determinant on *B. microti* prevalence, despite lack of documentation for transovarial transmission (Uilenberg 2006). A lack of two-fold amplification of *B. microti* prevalence in adults may reflect similar feeding preferences of larvae and nymphs. The white-footed mouse is a *B. microti* reservoir and a popular blood meal source for larvae and nymphs (Vannier et al. 2015). This phenomenon, a lack of life stage significance, was also seen in *A. phagocytophilum* prevalence. This may warrant future research in transstadial and transovarial transmission dynamics, as the possible drive of pathogen maintenance over host availability.

The nymphal survey of the Lehigh Valley region in PA found a lower *B. microti* prevalence of 2.8% (95% CI: 2.1–3.7). Our adult *B. microti* prevalence was higher than other recorded adult studies in PA, ranging from 0.67% to 3.52% statewide (Hutchinson et al.

2015, Livengood et al. 2020), where the northeastern infection rate, consisting of Pike County, was 3.5% ($n = 277$) (Hutchinson et al. 2015). Pike County's relatively high occurrence of *B. microti* poses a significant health risk. Disease severity and treatment of this protozoan ranges from moderate disease, with oral antimicrobials, to severe disease, with intravenous administration (Vannier et al. 2015). From 2011 to 2015, almost half of babesiosis patients were hospitalized (Gray 2019). The high nymphal *B. microti* prevalence found in our study should alert Pike County health providers of the risk of babesiosis exposure in patients.

B. miyamotoi Disease (*B. miyamotoi*)

Borrelia miyamotoi disease is not reportable in PA, despite concerns of transovarial transmission and the risk of cross reactivity with the C6 peptide ELISA LD test (Barbour et al. 2009, Molloy et al. 2018, Han et al. 2019). Overall prevalence of *B. miyamotoi* was 1.52% (95% CI: 1.52–2.49). Nymphal prevalence (1.89%, 95% CI: 0.62–4.36) was slightly higher than the adults infected with *B. miyamotoi* (1.38%, 95% CI: 0.66–2.53), however, not determined significantly through the GLM (Table 5). The higher nymphal prevalence might be driven by the transovarial transmission observed in *B. miyamotoi* and *I. scapularis* (Barbour et al. 2009, Dibernardo et al. 2014, Farone et al. 2018). A recent PA statewide study found 0.33% (1/299) adults positive with *B. miyamotoi*, lower than adult prevalence in this study (Livengood et al. 2020). Additionally, the nymphal survey of the Lehigh Valley region in PA found a lower *B. miyamotoi* prevalence of 0.3% (95% CI: 0.1–0.7) (Edwards et al. 2019). The variation in prevalence of less frequent TBPs, such as *B. miyamotoi*, continues to support the need of surveillance studies to include large sample sizes over small geographic regions for better resolution of TBPs and human risk. *Borrelia miyamotoi* disease is not reportable and therefore little is known about human cases in tick endemic areas. Recent retrospective studies, using PCR testing from acutely febrile patients in the northeastern United States, found 0.19% to 0.84% positive *B. miyamotoi* blood samples (Molloy et al. 2015, Marcos et al. 2020). These retrospective studies indicate low prevalence of *B. miyamotoi* relative to the other TBPs in the northeastern region. *Borrelia miyamotoi* is not as dominant as its sister spirochete, *B. burgdorferi*, but surveillance of this tick-borne relapsing fever remains imperative as a comparatively high risk for the Pike County, PA residents as documented here.

Deer Tick Virus (Powassan Virus Lineage II)

Despite the rarity of human infection with DTV, the reported cases have increased recently, where a majority reside in the northeastern United States (CDC 2019b). From 2010 to 2019, PA has had 6 confirmed cases of DTV, while neighboring states report higher cases such as NY and NJ, respectively 20 and 12 (CDC 2020). Although Powassan encephalitis is considered rare with only 40 cases diagnosed since 1958, its fatality rate of 10–15% is a clinical concern (Tokarz et al. 2010). The prevalence of DTV within *I. scapularis* was 2.07% (95% CI: 1.16–3.39) in adults and 0.76% (0.09–2.71) in nymphs for a total prevalence of 1.72% (95% CI: 1.01–2.74) (Table 5). Higher adult prevalence agrees with the findings that more DTV isolates were obtained from adults than other life stages, regardless of transovarian observations (Madison-Antenucci et al. 2020). This may warrant investigation of transovarian maintenance of DTV against transstadial transmission. Only one other statewide study in PA of ticks collected from hunter-harvested white-tailed deer has documented molecular evidence of DTV in a single tick (0.05%, 1/1990) (Campagnolo et al. 2018, Livengood et al. 2020). However, the bordering state of NY found an equivalent prevalence of DTV in *I. scapularis* adults (2.45%, 7/286) (Tokarz et al. 2010). Adult females have a greater chance of acquiring DTV from a blood meal than nymphs and larvae from infected hosts, respectively, 57%, 40%, and 10% (Madison-Antenucci et al. 2020). Additionally, a model analysis found cofeeding of an uninfected tick alongside an infected one provided long-term maintenance of the virus in the environment (Madison-Antenucci et al. 2020). Although adult ticks are regarded to have lower human transmission risk than nymphs, the fast transmission time of DTV increases their significance to public health (Eisen 2018). The high adult infection rates found in Pike County still pose valid concern, despite the lower rates in the nymph population.

Coinfections of TBPs

Complications of medical diagnosis and treatment arise with ticks coinfected with TBPs. The commonly reported prevalence of coinfections is less than 10%, but may range from 1 to 28% within the *I. scapularis* population (Hutchinson et al. 2015, Diuk-Wasser et al. 2016, Edwards et al. 2019, Livengood et al. 2020). Our study indicates a coinfection rate of 11.23% (95% CI: 9.33–13.37) (Table 3). Coinfections of *B. burgdorferi* and *A. phagocytophilum* are primarily observed due to individual prevalence, not influenced by the interaction of the specific etiologic agent (Diuk-Wasser et al. 2016). On the contrary, we found a positive correlation between *B. burgdorferi* and *A. phagocytophilum* coinfection where the observed frequency was greater than independent occurrence in total ticks ($P < 0.001$) and adults alone ($P < 0.001$) (Table 8). The nymphal coinfection of *B. burgdorferi* and specifically Ap-ha variant was significant ($P = 0.001$). This significant interaction between *B. burgdorferi* and Ap-ha might be indicative of a high reservoir population in Pike County, specifically small mammals such as *P. leucopus* and *Tamias straitus*. Similarly, the Lehigh Valley survey observed significance in nymphal coinfection frequencies of Ap-ha strain and *B. burgdorferi* in *I. scapularis* nymphs (Edwards et al. 2019). Despite the interference effect reported in tick acquisition of *A. phagocytophilum* and *B. burgdorferi* from infected *P. leucopus* (Levin and Fish 2001), other studies have shown a higher coinfection prevalence of the pair than what is expected (Hamer et al. 2014, Stewart and Bloom 2020). This discrepancy in coinfection between *B. burgdorferi* and *A. phagocytophilum* warrants future investigation with strain distinction. The significant nymphal coinfection prevalence is a noteworthy public concern with the westward spread of *A. phagocytophilum* in questing ticks alongside an increase of anaplasmosis incidences.

Literature regarding *B. burgdorferi* and *B. microti* indicate higher rates of coinfection than expected where pathogen prevalence was assorted independently. This positive interaction may indicate a host that is competent for both. Thus, vector acquisition of the pair could occur in one blood meal (Hersh et al. 2014, Edwards et al. 2019, Stewart and Bloom 2020). Similarly, we found coinfection of *B. burgdorferi* and *B. microti* ($P < 0.001$) occurred more frequently than what was expected independently. This significance was sustained in the nymph ($P = 0.001$) and adult populations ($P < 0.001$) (Table 8). Total *B. burgdorferi* and *B. microti* coinfection (4.45%, 95% CI: 3.25–5.93) (Table 5) is higher than other PA studies that reported coinfection prevalence of 0.70% to 2.00% (Hutchinson et al. 2015, Edwards et al. 2019, Livengood et al. 2020). High coinfection rate might be influenced by host prevalence within Pike County. *Ixodes scapularis* 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 (Hersh et al. 2014). This may increase concern of acquiring two TBPs from a life stage that has only had one blood meal prior, the nymph. The increased disease severity, duration, and the distinct treatment regimens make coinfections of *B. burgdorferi* and *B. microti* medically prominent (Diuk-Wasser et al. 2016). The antibiotics commonly used to eliminate the spirochete that causes LD, such a doxycycline, will not effectively treat babesiosis, caused by a protozoan. Medical Practitioners within Pike County should be alerted of the high coinfection tendency of a single tick bite.

This study documents the coinfection rates of three pathogens (1.52%, 95% CI: 0.85–2.49) and four pathogens (0.10%, 0.00–0.56) (Table 3). One female coinfected with four different pathogens (*B. burgdorferi*, *A. phagocytophilum Ah-ha*, *B. microti*, and *B. miyamotoi)*. One nymph coinfected with three distinct TBPs: a bacterium, a protozoan, and a virus (*B. burgdorferi*, *B. microti*, and DTV). The potential of coinfection transmission and viability in a human host is a concern for medical agencies, complicating diagnosis and treatment.

Coinfection of TBPs is of the highest clinical concern (Hutchinson et al. 2015, Livengood et al. 2020). Similar county-wide studies, encompassing a diverse TBP panel that includes more than *B. burgdorferi* alone, should continue due to the risk associated with coinfections. 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 TBDs. Results highlighted in this study have indicated significant differences in TBP prevalence between adjacent collection grids. Inclusion of multiple life stages across collection grids benefits the detection of less prevalent TBPs, like *B. miyamotoi* and DTV, as seen in the absence of pathogen prevalence across grids (Supp Table S1 [online only]). Without this fine scale resolution of tick samples, pathogen detection may have been lost resulting in a misinterpretation of risk associated with TBPs. Continued surveillance efforts should include multiple collection sites throughout a county with larger sample sizes to truly emphasize the risk of TBPs. Lastly, further testing of other ticks found in these geographic areas is needed to better understand the distribution and prevalence of other TBPs 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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Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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