

Prevalence of Tick borne diseases in questing adult *Ixodes scapularis* (Blacklegged ticks) collected from Milford Borough, Pennsylvania

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Introduction

In the United States, humans and animals are at increasing risk for acquiring tick borne diseases (TBDs), specifically in the Northeast and Mid-west. Ticks and the infectious organisms that they vector have always affected humans and domestic animals, and they continue to be a global health threat. Numerous bacterial, viral, and protozoan agents can be passed from a tick vector to a human or animal host, causing disease and even death (Jongejan and Uilenberg 2004). Screening of ticks for pathogens using molecular epidemiological tools can be used to determine the prevalence of tick borne pathogens in a particular geographic area.

Tick borne diseases are underreported and have continued to expand throughout the U.S. There are over 30,000 cases of Lyme disease reported yearly in the U.S., but recent studies indicate that the actual number of people infected may be upwards of 300,000 (Hinckley et. al. 2014; Nelson et. al. 2015). Since 2011, PA has reported the highest number of Lyme disease cases in the U.S., with over 11,443 cases in 2016 (CDC, 2017). Pike County in particular has had an increase in confirmed cases of Lyme disease from 13 in 2012 to 114 in 2016 (CDC, 2017).

There are three common hard bodied ticks in the northeast; the blacklegged tick (*Ixodes scapularis*), American dog tick (*Dermacentor variabilis*) and Lone star tick (*Amblyomma americanum*). Ticks acquire and transmit pathogens to and from a host through blood meals. Hard bodied ticks feed as larvae and nymphs with an additional meal taken by adult females. Any pathogen acquired during the three blood meals taken by females or the two blood meals taken by males, may be passed to the host through feeding. The most common tick transmitting TBDs in the U.S. is the blacklegged tick (*I. scapularis*). The blacklegged tick can transmit Lyme disease, babesiosis, anaplasmosis and *Borrelia miyamotoi*. Furthermore, some studies have identified the blacklegged tick as a potential vector for *Bartonella* and *Mycoplasma* species (Eskow et al. 2003; Angelakis et al. 2010).

In areas where TBDs are common, such as the northeast, pathogens co-circulate in the environment and within host, increasing the likelihood of co-infections. A co-infection can occur when a vector (tick) is carrying 2 or more pathogens and transmits multiple pathogens into the host. Co-infections can result in disease complications,

prognosis and treatment. In the northeast, co-infections within the blacklegged tick have become increasingly important, specifically co-infections of *Borrelia burgdorferi* and *Babesia microti* (Tokarz et. al. 2010; Harsh et. al. 2014).

Understanding prevalence rates of tick borne pathogens directly contributes to increased awareness of high-risk areas and the need for human and animal protection against tick bites. The purpose of this project is to determine pathogen prevalence within sampled locations of Milford Borough, Pike County PA. This project utilized molecular techniques as a means of exposure diagnostics for emerging and established tick borne pathogens.

Materials and Methods

Site Selection

Three residential sites in Milford Borough of Pike County Pennsylvania (Figure 1) were selected for tick collection. Site selection was based upon a number of parameters including; accessibility, prevalence of human foot traffic, and proximity to wildlife habitats such as rivers and forests. Sites that possessed abundant transitional zones with tall grass were selected. Site one was located off East Harford Street on Mott Street boarding Sawkill Creek (Figure 2). Site two was located on East Peach Alley directly off East Catherine Street. This site had direct access to the Delaware River and was located behind the ball park on Third Street (Figure 3). Site three was located behind residential homes where James Street intersects Pine Alley and 9th Street (Figure 4).

Each site had ideal tick habitat boarding edges of forest with vegetation which included Japanese barberry, wood and brush piles, shrubs and leaf litter. In addition, white-tailed deer tracks were identified at site three. Other wildlife host were noted during site selection which included chipmunks, ground hogs, birds and squirrels.

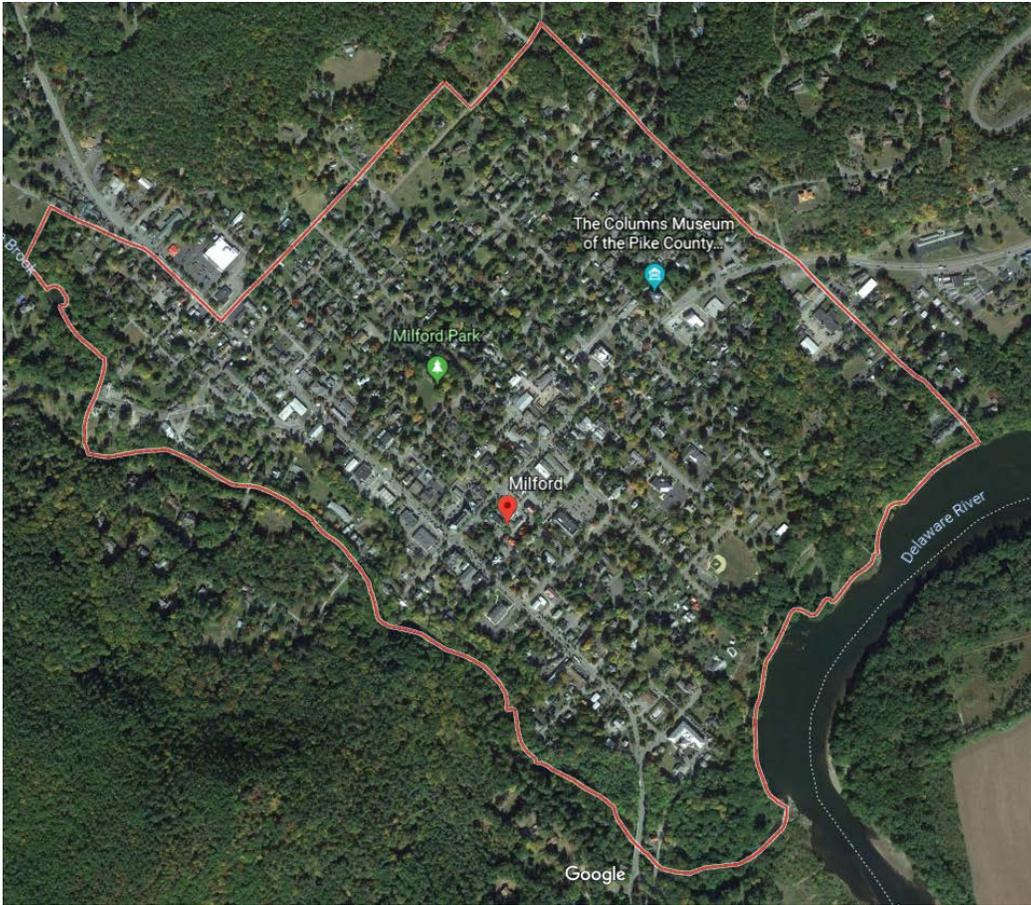


Figure 1: Milford Borough, Pennsylvania - The red outline depicts the boundary line of Milford Borough, Pike County PA.

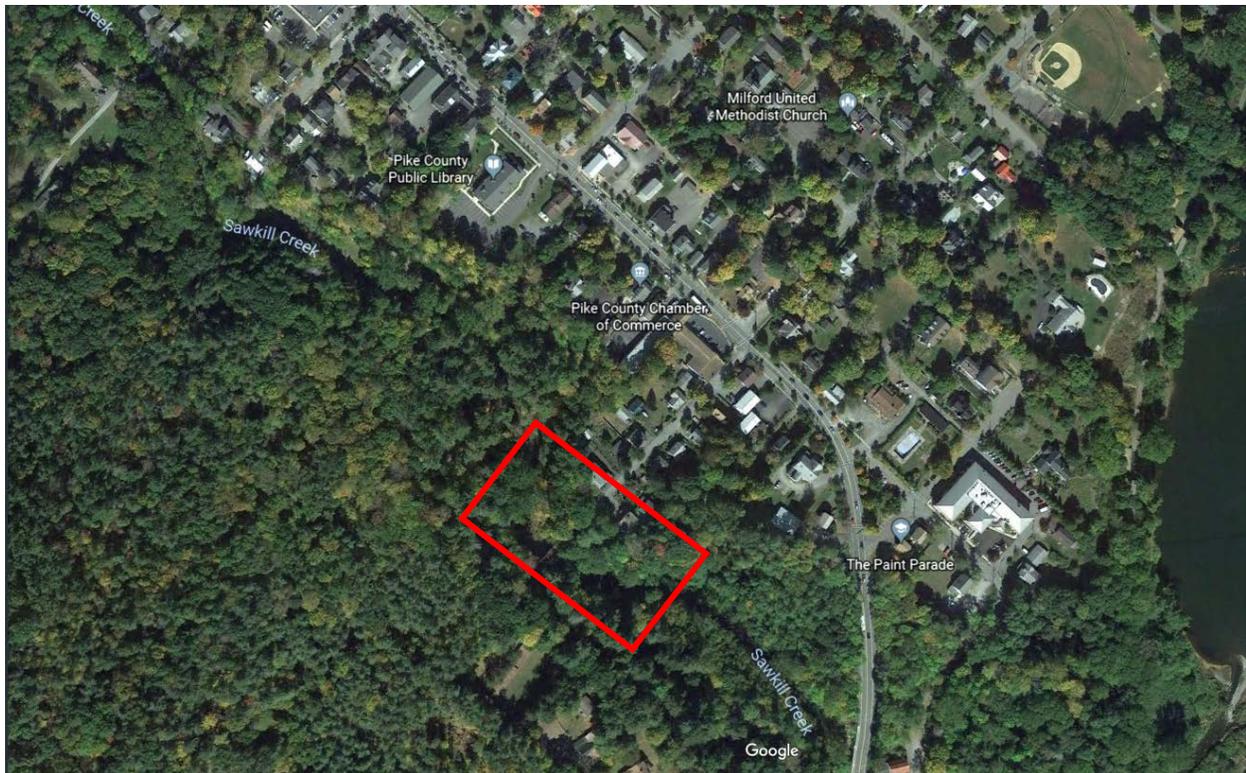


Figure 2: Site 1 – Located off East Harford Street at the end of Motts Street. This location has a bridge which overlooks Sawkill Creek connecting Milford Borough to the Milford Knob. The Milford Knob is a hiking and recreation area located in Pike County.



Figure 3: Site 2 – Located on East Peach Alley along the access to the Delaware River from East Catherine Street. This location was located directly behind the 3rd Street ball park.

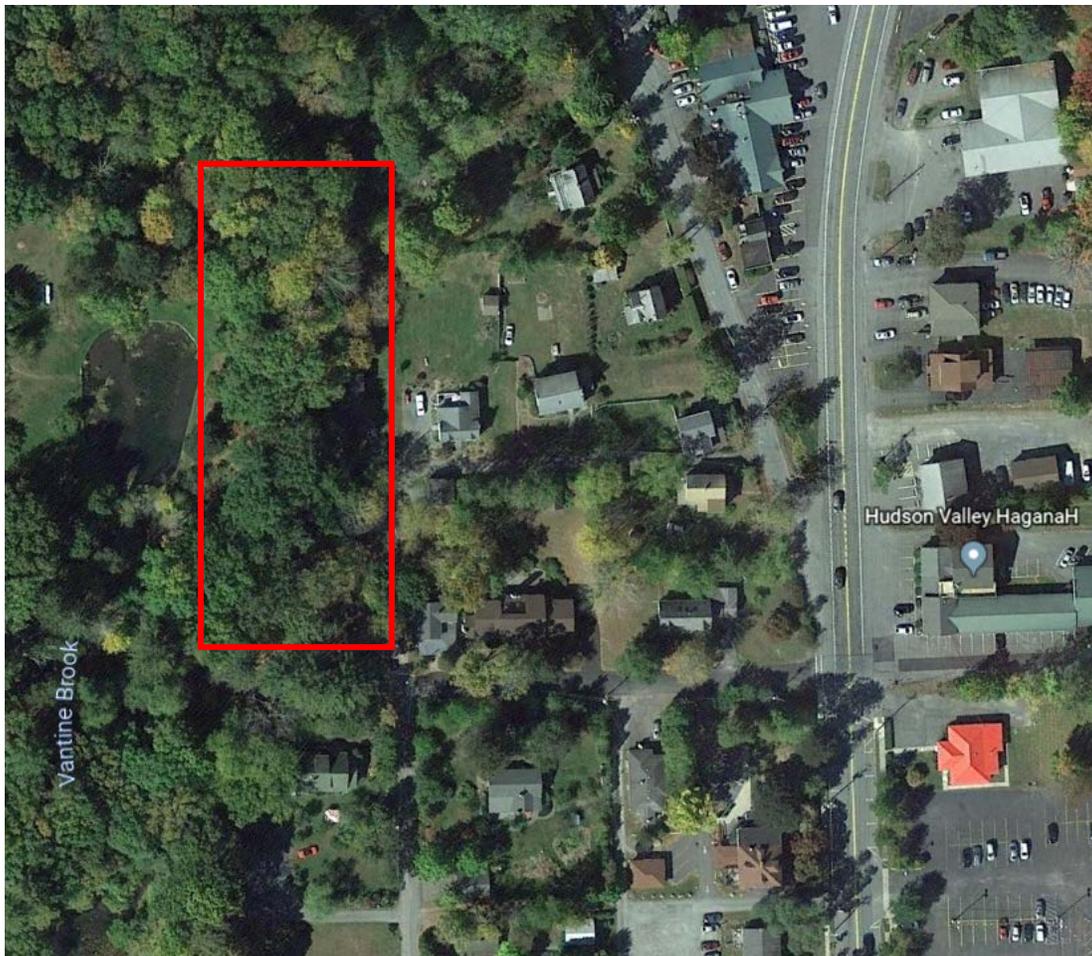


Figure 4: Site 3 – Located at the intersection of James and 9th Street. This location was directly behind residential homes boarding forest edge of the Vantine Brook.

Collection

Adult *Ixodes scapularis* ticks were collected between October and November of 2017. Ticks were collected using corduroy drag cloths which were drug along the ground and vegetation. Drags were completed for several meters and then examined for ticks. Ticks were placed into sterile 5mL falcon tubes, with each tube labeled with its perspective site and date. Ticks were stored at room temperature during collection. Upon returning to the lab ticks were sorted by collection site, and the identification of species and life cycle were documented using Ward's Guide to North American Ticks key (Ward's, Rochester, NY). Ticks were stored in sterile microcentrifuge tubes frozen at -20°C until DNA extraction.

DNA Extraction and PCR

DNA was extracted from ticks using a Qiagen DNeasy Blood and Tissue kit (Qiagen, Redwood City, CA) following manufacturers protocol.

To determine presence of pathogens, specific primer and probes were used for PCR following the Northeast Wildlife DNA Laboratory (NEWDL) standards. Real-time PCR was used to amplify the DNA for *Borrelia burgdorferi*, *Babesia microti*, *Anaplasma phagocytophilum* and *Borrelia miyamotoi*. All PCR was completed using a negative and positive control to validate results. To identify presence of *Bartonella* spp. and *Mycoplasma* spp., traditional PCR followed by gel electrophoresis to visualize the results. A positive and negative control was used to confirm positive samples. To determine *Bartonella henselae* and *Mycoplasma fermentans* positive samples, a SYBR green specific RT-PCR assay and traditional PCR assay was conducted on all *Bartonella* spp. and *Mycoplasma* spp. positive samples, respectively.

Statistics

To determine if there is an association between infection rates and location, and infection rate and tick gender, a Chi-Square using SPSS Statistics v24.0 was used (IBM, 2016). An alpha of 0.05 was used to determine statistical significance.

Results

In total 100 adult *Ixodes scapularis* (blacklegged/deer ticks) ticks were collected from Milford Borough, with 54 from site one, 26 from site two and 20 from site three (Table 1). During collection, one nymph *Ixodes scapularis* was collected but not tested or included in final tick count.

A total of 51/100 (51%) ticks were carriers of at least one tick borne disease. The highest infection rate identified was Lyme disease (*Borrelia burgdorferi*) with 37/100 (37%) positive ticks. Other pathogen prevalence's ranged from 4-8 percent (Table 2). A total of 7 ticks tested positive for *Bartonella* spp. A SYBR green RT assay specific for *Bartonella henselae* was conducted on 6 of the positive samples. A prevalence of 1 percent (1/100) of ticks tested positive for *B. henselae*. A total of 5 ticks tested positive for *Mycoplasma* spp. A *Mycoplasma fermentans* specific assay was conducted and found 0/5 (0%) of the ticks positive for *M. fermentans*.

An overall co-infection rate of 11 percent was identified with 4 percent of ticks carrying three tick borne pathogens (Table 3). Furthermore, 9/11 (81.8%) of co-infections were Lyme disease with another tick borne disease and 2/11 (18.2%) did not involve Lyme disease bacteria. There was no significance between infection prevalence and site location ($\chi^2 = 1.989$; $p=0.370$) or prevalence of co-infections and site locations ($\chi^2 = 3.015$; $p=0.222$). No relationship was identified in prevalence of Lyme disease and site locations ($\chi^2 = 5.861$; $P=0.056$) or between sex of adult tick and prevalence of pathogens ($\chi^2 = 0.034$; $P=0.854$).

Table 1: Total number of female and male ticks collected from sites 1, 2 and 3.

Site	Total collected adult <i>Ixodes scapularis</i> ticks	Females	Males
Site 1	54	25	29
Site 2	26	12	14
Site 3	20	9	11
Total	100	46	54

Table 2: Infection rate of *Borrelia burgdorferi*, *Babesia microti*, *Anaplasma phagocytophilum*, *Borrelia miyamotoi*, *Bartonella* spp. and *Mycoplasma* spp. in adult *Ixodes scapularis* ticks collected from Milford Borough, PA. (*) Asterisk indicates assays screened only on positive samples.

Site	<i>Borrelia burgdorferi</i>	<i>Babesia microti</i>	<i>Anaplasma phagocytophilum</i>	<i>Borrelia miyamotoi</i>	<i>Bartonella</i> spp.	<i>Mycoplasma</i> spp.	* <i>Bartonella henselae</i>	* <i>Mycoplasma fermentans</i>	Total Infection rate
Site 1	16/54 (29.6%)	2/54 (3.7%)	4/54 (7.1%)	4/54 (7.1%)	4/54 (7.4%)	3/54 (5.56%)	1/54 (1.85%)	0/54 (0.0%)	26/54 (48.1%)
Site 2	9/26 (34.6%)	0/26 (0.0%)	1/26 (3.85%)	0/26 (0.0%)	3/26 (11.5%)	1/26 (3.8%)	0/26 (0.0%)	0/26 (0.0%)	12/26 (46.1%)
Site 3	12/20 (60.0%)	2/20 (10.0%)	3/20 (15.0%)	1/20 (5.0%)	0/20 (0.0%)	1/20 (5.0%)	0/20 (0.0%)	0/20 (0.0%)	13/20 (65.0%)
Total	37/100 (37.0%)	4/100 (4.0%)	8/100 (8.0%)	5/100 (5.0%)	7/100 (7.0%)	5/100 (5.0%)	1/100 (1.0%)	0/100 (0.0%)	51/100 (51.0%)

Figure 5: Prevalence of tick borne diseases in adult *Ixodes scapularis* collected from Milford Borough, Pike County PA.

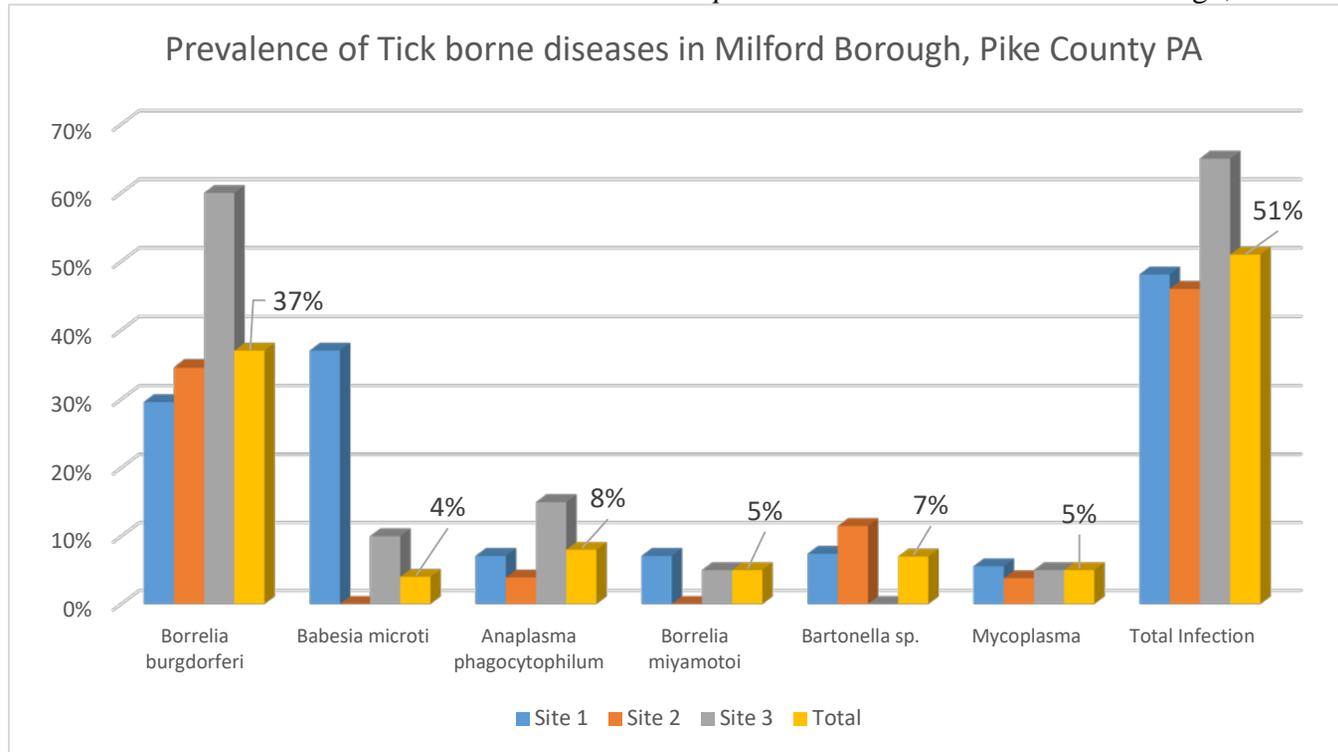


Table 3: Coinfection rate of *Borrelia burgdorferi*, *Babesia microti*, *Anaplasma phagocytophilum*, *Borrelia miyamotoi*, *Bartonella* spp. and *Mycoplasma* spp. in adult *Ixodes scapularis* ticks collected from Milford Borough, PA.

Coinfection	Number of Coinfections	Site (s)
Lyme disease and Babesiosis	2	1 and 3
Lyme disease and Anaplasmosis	1	3
Lyme disease and <i>Borrelia miyamotoi</i>	1	1
Lyme disease and <i>Mycoplasma</i>	1	1
Anaplasmosis and <i>Bartonella</i>	1	1
<i>Borrelia miyamotoi</i> and <i>Mycoplasma</i>	1	1
<i>Prevalence of 2 pathogens</i>	7/100 (7%)	
Lyme disease, <i>Mycoplasma</i> and <i>Borrelia miyamotoi</i>	1	1
Lyme disease, <i>Mycoplasma</i> and Anaplasmosis	2	2 and 3
Lyme disease, Babesiosis and Anaplasmosis	1	3
<i>Prevalence of 3 pathogens</i>	4/100 (4%)	
Total Co-infections	11/100 (11%)	

Table 4: Co-infection prevalence by collection sites. There was no relationship between collection site and co-infection prevalence ($\chi^2 = 3.015$; $p=0.222$).

Collection Location	Co-infection Prevalence
Site 1	6/54 (11.1 %)
Site 2	1/26 (3.8%)
Site 3	4/20 (20 %)

Discussion

This is the first study completed in Milford Borough, PA to determine the prevalence of six pathogens transmitted by blacklegged ticks. Ticks were collected from areas where humans and pets are frequent. Results of the study identified 51 percent of the ticks sampled were positive for a single organism and 11 percent of ticks were co-infected with two or three tick borne diseases (Table 3).

There was no relationship between infection rates and collection sites ($\chi^2 = 1.989$; $p=0.370$) or prevalence of co-infections and site locations ($\chi^2 = 3.015$; $p=0.222$). When evaluating the relationship between Lyme disease infections and collection sites, no significance was identified when using an alpha of 0.05 ($\chi^2 = 5.861$; $p=0.056$). In this study, sites 1 and 2 had a lower Lyme disease infection prevalence and overall infection rate in comparison to site 3. When evaluating the geographical landscape of collection sites, site 3 has more transitional zones with forest and grasslands than sites 1 and 2 (Figures 2-4). Fragmented forest have lower biodiversity with higher populations of white-footed mice and small mammals which result in higher infection rates (Allan et. al. 2003; Jackson et. al. 2006). Similar to studies conducted by Allan et. al. (2003) and Jackson et. al. (2006), although infection rates were greater in fragmented populations, there was no statistical significance.

A co-infection rate of 11 percent between all sites was identified with site 3 having the highest co-infection rate of 20 percent (Table 4). Other studies from surrounding regions have also identified high co-infection rates. A study conducted in New York identified co-infections of *B. burgdorferi* and *B. microti* at 6.68% and 16.8%, within blacklegged ticks (Tokarz et. al. 2010; Harsh et. al. 2014). A PA state wide surveillance study conducted in 2013 by Hutchinson et. al., found a co-infection rate of 2% for *B. burgdorferi* and *B. microti* which is less than the 3% identified within this study. To date, the clinical implications of polymicrobial infections are unknown. Due to the differences in medical treatment for bacterial (Lyme disease) and a protozoan (babesiosis) infection, medical practitioners should be aware of the risk for co-infections from a single tick bite. Furthermore, disease manifestations between pathogens are similar and testing solely for Lyme disease can result in misdiagnosis, difficulties with treatment and delayed recovery for the patient.

In conclusion, a high prevalence of adult blacklegged ticks from Milford Borough PA were carriers of a tick borne disease. A high co-infection rate of 11 percent was identified within the 100 sampled ticks with a 4 percent co-infected with 3 pathogens. Overall, this study confirms that Lyme disease is only one tick borne organism circulating in the environment. Other tick

borne diseases are becoming increasingly common with infection rates as high as 15 percent. For accurate diagnosis, it is recommended Physician's in endemic areas screen for all possible tick borne diseases.

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