

Adult *Ixodes scapularis* Ticks in a Lyme Disease Hot Spot are Vectors for Emerging Pathogens

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Abstract

A 2015 Center for Disease Control and Prevention study concluded that for northeastern United States, Lyme, a debilitating tick-borne disease is expanding westward with counties in western Pennsylvania identified as high-incidence areas. Lyme's primary vector, the black-legged tick (*Ixodes scapularis*), is also the host vector to at least four additional human pathogens. Thus, we tested adult *I. scapularis* ticks for co-infection in nine recreational areas located in a western Pennsylvania county categorized as high-incidence for Lyme. Specifically, we tested ticks for the Lyme disease pathogen, *Borrelia burgdorferi*, and two other emerging blood-borne pathogens, *Babesia microti* and *Anaplasma phagocytophilum*. Our analyses suggest that the three pathogens were independent in their distribution with *B. burgdorferi* present in 30%, *B. microti* in 27% and *A. phagocytophilum* in 4% of the tested *I. scapularis* samples. Forty-two percent (n = 106/252) of all *I. scapularis* ticks tested carried only one pathogen, of which only half (51%) of those carried *B. burgdorferi* and the rest carried either *B. microti* or *A. phagocytophilum*. Fifty-one percent of *I. scapularis* ticks tested carried at least one of the above three pathogens, with 9% of the ticks carrying more than one pathogen, of which 6% were co-infected with *B. burgdorferi* and *B. microti*. In recreational areas we sampled, a single bite from *I. scapularis* has the potential to transmit multiple pathogens which could result in concurrent infections and contribute to a variety of clinical symptoms not typically seen in Lyme disease patients. Our results considered in combination with previously published information on underreporting of human Lyme disease warrants the need for public health officials to be vigilant in the surveillance for tick-borne coinfections especially in areas endemic for Lyme.

Introduction

Vector-borne diseases are a major cause of human morbidity and mortality [1,2]. The black-legged tick, *Ixodes scapularis* (formerly known in the northeastern United States as the deer tick, *Ixodes dammini*) is the host vector to at least five human pathogens—*Anaplasma phagocytophilum*, *Babesia microti*, *Borrelia burgdorferi*, *Ehrlichia* species and Powassan virus [3], acquired by the tick after a blood meal from a co-infected wildlife host/ reservoir or as a result of sequentially feeding on diverse infected wildlife reservoirs. In addition to sharing the *I. scapularis* tick as a common vector, these pathogens can also share a common animal reservoir host, the white-footed mouse

(*Peromyscus leucopus*) [4-6]. Although Lyme disease is the leading vector-borne zoonotic disease caused by *B. burgdorferi* in the U.S., human co-infection with *B. microti* (Babesiosis) and *A. phagocytophilum* (Anaplasmosis) can occur in patients infected with *B. burgdorferi* [7-9]. Also, humans co-infected with more than one of the above mentioned zoonotic pathogens residing in an *I. scapularis* tick may be at risk of a harsher, more debilitating form of Lyme, Babesiosis or Anaplasmosis [7,10].

In recent years, Pennsylvania has led the United States in number of Lyme disease cases [1,11]. Because of human population density and proximity to the heavily populated areas of the northeastern U.S., there is a continuing misconception that Lyme disease cases are on the rise only in northeast Pennsylvania [12]. But over the past decade, many counties in western Pennsylvania have experienced an increase in incidence of Lyme Borreliosis in human adults and children [11] which is corroborated by the latest CDC finding that "The center of the high-incidence focus in the northeastern United States generally moved westward and northward, away from the coast of northern New Jersey and into east-central Pennsylvania" [13].

A combination of varying land use practices, diverse and abundant wildlife reservoirs (white-footed mouse, white-tailed deer, ground birds, eastern chipmunks, shrews), and recreational activities such as hiking, camping, fishing and hunting have increased the potential exposure of humans to *I. scapularis* ticks in western Pennsylvania. It is estimated that Pennsylvania's public recreational areas attract over 34 million visitors annually [14] and Pennsylvania ranks sixth in yearly sales of fishing licenses and second in yearly sales of hunting licenses [15]. Thus, the Commonwealth of Pennsylvania hosts a large human demographic that is at risk for exposure to multiple tick-borne pathogens.

The purpose of this study was to determine (i) the infection rates for *A. phagocytophilum*, *B. microti* and *B. burgdorferi* in *I. scapularis* collected in recreational areas of a western Pennsylvania county known to have a high-incidence for human Lyme disease; and (ii) the frequency of co-infections in these ticks which could translate to concurrent infections in humans via a single tick bite. Our results have added to the knowledge of

the microbial community residing in *I. scapularis* ticks in hot spot areas, which is important for increasing human vigilance and for public health professionals to make more informed prevention, diagnoses, and treatment decisions.

Materials and Methods

Tick collection

Our nine study areas for questing *I. scapularis* adult tick collection (April–November 2013) are public conservation parks and lands in southwestern county of Indiana, Pennsylvania (Figure 1 inset) that are similarly forested and abundant with many mast producing trees such as oaks that provide abundant food resources for many mammalian vectors. Additionally, our study areas and the surrounding landscape contained an intermix of other land use types such as scattered shrub lands, natural gas wells, small managed grass openings, and park infrastructure such as roads and utility corridors which could result in the creation of edge habitats that contribute to *I. scapularis* tick survival and abundance by supporting the habitat preferred by many animal host reservoir species.

All ticks were collected by dragging a white cloth attached to a rope through vegetation and leaf litter along hiking trails and in adjacent forests. All ticks collected were immediately transferred to 70% ethanol and stored in a laboratory at the Indiana University of Pennsylvania (IUP, Indiana, Pennsylvania) until experimentation. Prior to pathogen testing, adult *I.*

scapularis ticks were identified as either male or female based on morphological differences [16].

DNA extraction and Polymerase Chain Reaction Analyses

Due to susceptibility of all *I. scapularis* genomic DNA (gDNA) to quick degradation (unpublished results), we modified our DNA extraction protocols to isolate gDNA from *I. scapularis* ticks. The Dilution and Storage protocol using dilution buffer and DNA release additive of the Thermo Scientific Phire Tissue Direct PCR master mix kit (Thermo Scientific, Waltham, MA) extracted gDNA for PCR directly from the tick without proteinase K digestion, and purification steps (Thermo Fisher Scientific, Inc). Each tick was placed into 20.5 μ l of dilution buffer/ DNA release additive mix. The reaction was incubated at 25°C for 5 min and then placed in a 98°C water bath for 2 min. The tubes were spun in a centrifuge and 1 μ l of the supernatant was used to measure DNA concentration using the NanoDrop (Thermo Fisher Scientific, Inc). All DNA samples were then stored at -20°C till further analyses. Following DNA extraction, we performed DNA amplification using conventional PCR technique with the T100 thermal cycler (Bio-Rad, Hercules, CA) on adult *I. scapularis* tick gDNA sample. Forward and Reverse primers specific for the *I. scapularis* tick and each of the three pathogens were custom made from Integrated DNA Technologies, Inc. (Coralville, IA). Positive controls were provided by past collaborators (see Acknowledgments), and two sets of negative controls (distilled water and a solution of dilution

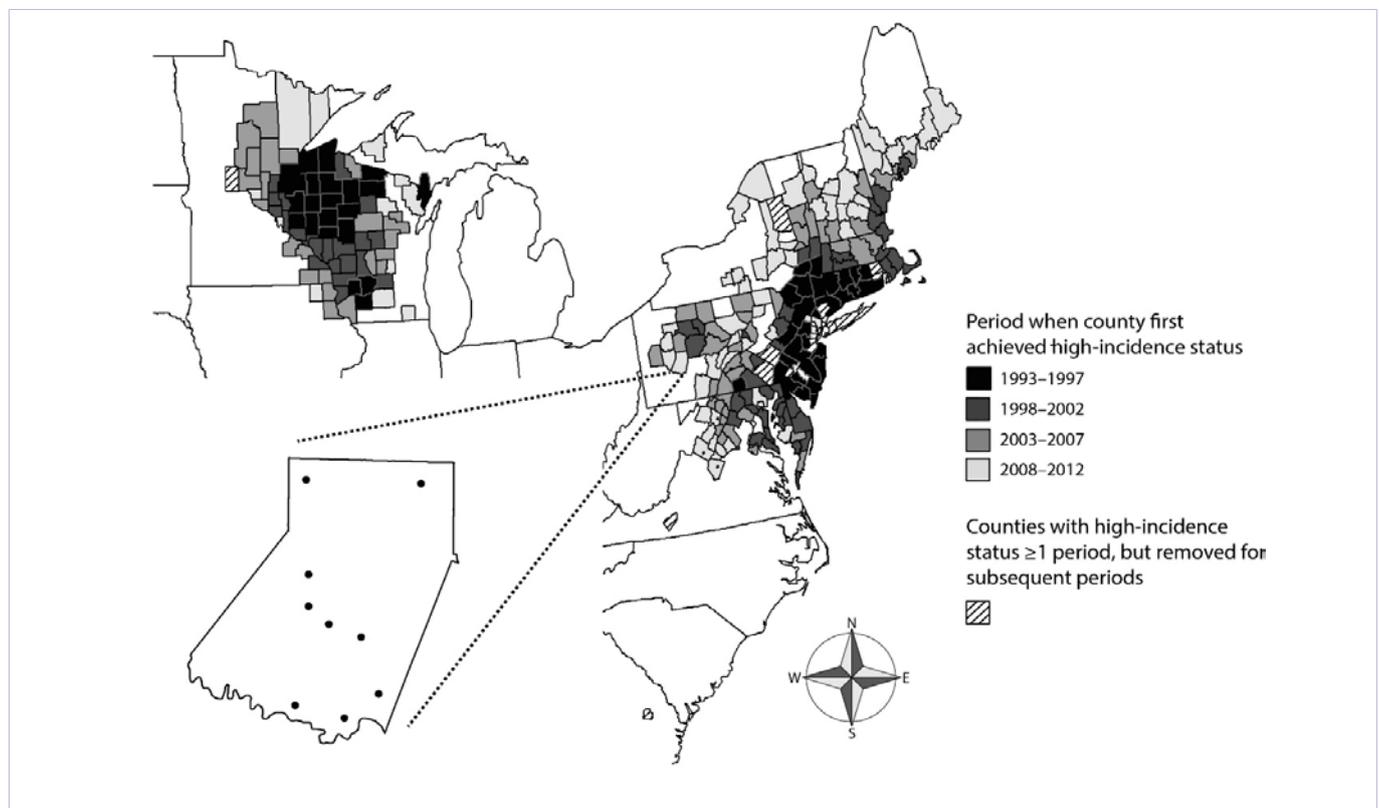


Figure 1: Adapted with permission from Kugeler, et al. [13] of United States counties with high incidence of Lyme disease with inset of southwestern Pennsylvania county, location of the nine high risk recreational areas (closed circles), our *Ixodes scapularis* sampling sites.

buffer/ DNA release additive mix) were used in place of extracted DNA to control for contamination.

To detect *A. phagocytophilum*, we used the ge3a-F/ ge10r-R primer set (GenBank accession number KJ942183.1). The forward primer was 5' CAC ATG CAA GTC GAA CGG ATT C 3' and the reverse primer were 5' TTC CGT TAA GAA GGA TCT AAT CTC C 3' amplifying a 932-base pair (bp) product specific for the *A. phagocytophilum* 16S rRNA gene. Amplification was performed as follows: 3 min at 98°C (one cycle) followed by 40 cycles for 5 sec at 98°C, 5 sec at 55°C, 90 sec at 72°C. *A. phagocytophilum* was not further differentiated between the human pathogenic (AP-Ha) and the nonpathogenic (AP-variant 1) strains [12].

We used the PIRO-AF/ PIRO-BR primer set to detect *B. microti* [17]. The forward primer was 5' ATT ACC CAA TCC TGA CAC AGG G 3' and the reverse primer was 5' TTA AAT ACG AAT GCC CCC ACC 3' amplifying a 437-bp product specific for the *B. microti* 18S rRNA gene. Amplification was performed as follows: 3 min at 98°C (one cycle) followed by 40 cycles for 5 sec at 98°C, 5 sec at 55°C, 30 sec at 72°C.

To detect *B. burgdorferi*, we used the FL6-F/ FL7-R primer set (GenBank accession number X63413.1). The forward primer was 5' TTC AGG GTC TCA AGC GTC TTG GAC T 3' and the reverse primer were 5' GCA TTT TCA ATT TTA GCA AGT GAT G 3' amplifying a 276-bp product specific for the *B. burgdorferi* flagellin gene [18]. Amplification was performed as follows: 3 min at 98°C (one cycle) followed by 40 cycles for 5 sec at 98°C, 5 sec at 55°C, 30 sec at 72°C.

To confirm that all PCR reactions had *I. scapularis* tick DNA, we amplified the 16S rRNA gene of *I. scapularis* tick using the *Iscap16SF/Iscap16SR* primer set (Genbank accession number KF146646). The forward primer was 5' CGG TCT GAA CTC AGA TCA AG 3' and the reverse primer was 5' GGG ACA AGA AGA CCC TAT G 3' amplifying a 320-bp product specific for the 16S rRNA gene of the *I. scapularis* tick.

All PCR products were separated by horizontal gel electrophoresis on a 1.2% Tris-acetate EDTA (TAE) ethidium bromide agarose gel followed by analyses using a Gel Doc XR

Imager (Bio-Rad, Hercules, CA).

Statistical analyses

Data were tabulated and analyzed in Microsoft Excel (Microsoft Corp; Redmond, WA) and SPSS (IBM Corp.; Armonk, NY; Ver. 23). Data summarizations included assessment of pathogen frequencies within and across tick genders. Primary analyses were conducted using Chi-square tests to assess tick gender differences in pathogen frequencies. Chi-square tests also were used to test for the non-independence of multiple pathogens within ticks. Predicted frequencies of tick pathogen combinations were estimated by the combinations of their independent assortments [18,19].

Results

Of the 252 adult *I. scapularis* ticks tested, fifty-one percent (128/ 252) were infected with at least one of the three microbial species with *B. burgdorferi* being present in 30% (76/ 252) of tick samples, followed by *B. microti* (27%, 69/ 252) and *A. phagocytophilum* (4%, 9/ 252). Forty two percent (105/ 252) ticks tested were infected with only one of the three pathogens with approximately half of those ticks (51%, 54/ 105) infected with *B. burgdorferi* and the rest carried either *B. microti* (47%, 49/ 105) or *A. phagocytophilum* (2%, 2/ 105). Nine percent (23/ 252) ticks tested were infected with at least two of the three pathogens of which 6% (16/ 252) were co-infected with *B. burgdorferi* + *B. microti*, 1% (3/ 252) with *B. burgdorferi* + *A. phagocytophilum*, < 1% (1/ 252) with *B. microti* + *A. phagocytophilum*. Forty-nine percent of ticks tested (124/ 252) were uninfected and 1% (3/ 252) infected with all three pathogens. Male and female ticks were marginally different in their *B. burgdorferi* ($\chi^2 = 3.71$, $df = 1$, $p = 0.054$) and *A. phagocytophilum* ($\chi^2 = 3.11$, $df = 1$, $p = 0.078$), but not in their *B. microti* ($\chi^2 = 0.30$, $df = 1$, $p = 0.584$; Table 1) prevalence rates. Males and females did not differ in the relative proportions of ticks carrying, 0, 1, or >1 pathogen ($\chi^2 = 2.36$, $df = 2$, $p = 0.307$) nor in the overall frequency of ticks carrying pathogens of any kind ($\chi^2 = 1.22$, $df = 1$, $p = 0.269$; Table 1).

The three pathogens were relatively independent in their distribution among ticks, both in the actual combinations of

Table 1: Comparative prevalence rate of *Borrelia burgdorferi*, *Babesia microti* and *Anaplasma phagocytophilum* in adult male and female *Ixodes scapularis* collected from nine public recreational areas in southwestern Pennsylvania.

Pathogens in <i>I. scapularis</i>	total		M		F		M vs F		
	n	rate	n	rate	n	rate	Chi square	df	p
<i>B. burgdorferi</i>	76	0.302	23	0.232	53	0.346	3.71	1	0.054
<i>B. microti</i>	69	0.274	29	0.293	40	0.261	0.30	1	0.584
<i>A. phagocytophilum</i>	9	0.036	1	0.010	8	0.052	3.11	1	0.078
no pathogens	124	0.492	53	0.535	71	0.464	2.36	2	0.307
only 1 pathogen	105	0.417	40	0.404	65	0.425			
more than 1 pathogen	23	0.091	6	0.061	17	0.111			
at least 1 pathogen	128	0.508	46	0.465	82	0.536	1.22	1	0.269

Table 2: Observed and expected prevalence rates of *Borrelia burgdorferi*, *Babesia microti* and *Anaplasma phagocytophilum* in adult *Ixodes scapularis* collected from nine public recreational areas in southwestern Pennsylvania.

Pathogens in <i>I. scapularis</i>	Observed		Expected		Observed vs. expected		
	(n)	rate	(n)	rate	Chi square	df	p
<i>B. burgdorferi</i> (Bb)	76	0.302	--	--	--	--	--
<i>B. microti</i> (Bm)	69	0.274	--	--			
<i>A. phagocytophilum</i> (Ap)	9	0.036	--	--			
Bb + Bm	16	0.063	20.81	0.083	2.36	3	0.501
Bb + Ap	3	0.012	2.71	0.011			
Bm + Ap	1	0.004	2.46	0.010			
Bb + Bm + Ap	3	0.012	0.74	0.003			
no pathogens	124	0.492	123.24	0.489	0.33	2	0.850
only 1 pathogen	105	0.417	102.02	0.405			
more than 1 pathogen	23	0.091	26.73	0.106			
at least 1 pathogen	128	0.508	128.76	0.511	--	--	--

pathogens present in the ticks sampled ($\chi^2 = 2.36, df = 3, p = 0.501$) as well as in the relative number of pathogens observed within tick samples ($\chi^2 = 0.33, df = 2, p = 0.850$; Table 2). *B. burgdorferi* was found unevenly among the nine recreational areas sampled ($\chi^2 = 48.19, df = 8, p < 0.001$) whereas *B. microti* was not different in prevalence among areas sampled ($\chi^2 = 5.89, df = 8, p = 0.660$).

Discussion

To our knowledge, this study is the most comprehensive report of prevalence of three microbial pathogens, *B. burgdorferi*, *B. microti* and *A. phagocytophilum* in *I. scapularis* ticks tested from recreational areas in a region recently designated as “a center of high incidence focus” for human Lyme disease [13, Figure 1]. The three most striking patterns observed from our study are (i) 51% of the 252 total *I. scapularis* ticks tested were infected with at least one of the three microbial pathogens; (ii) 42% of the 252 total *I. scapularis* ticks tested were infected with only one pathogen of which only 51% (105/ 252) were identified as carrying the Lyme disease pathogen *B. burgdorferi*, while the other 49% of the ticks were infected with non-Borreliosis pathogens, causative agents of emerging blood-borne debilitating diseases; and (iii) 1% of the ticks sampled had the potential to transmit all three pathogens via a single bite to humans.

Nine percent of the ticks sampled were co-infected with more than one pathogen which is not surprising given that zoonotic pathogens share a common *I. scapularis* tick vector and small mammal reservoirs [4-6]. We saw no evidence that coinfection occurred more or less often than expected based upon the co-infections predicted by combinations of the pathogens independent frequencies. Our coinfection rates for *B. burgdorferi*

+ *A. phagocytophilum* and *B. microti*+ *A. phagocytophilum* were similar to a prior study conducted in southwestern Pennsylvania [18]. In spite of these three pathogens potentially sharing some common animal reservoirs, we found *B. burgdorferi* to have an uneven distribution (independent of sample size) among the nine recreational areas we sampled which could be due to differences in prevalence of hosts these ticks feed on during the larval or nymphal stages of their development [10]. Additionally, the number and/or source of blood meals, and thus the opportunity for infection, could possibly be the reason for marginal differences in *B. burgdorferi* ($p = 0.054$) and *A. phagocytophilum* ($p = 0.078$) prevalence between male and female ticks [10]. The 27% prevalence rate for *B. microti* in this study could be attributed to regional variation [20] and sampling of *I. scapularis* ticks for microbial pathogens in a high incidence southwestern Pennsylvania county compared to prior studies which sampled ticks from random locations in western Pennsylvania [18,19,21]. In support of our findings, a study by Piesman, et al. [22] demonstrated the wide prevalence of *B. microti* (2%-47%) in adult *I. scapularis* from four areas in eastern Massachusetts.

Although the biotic and abiotic factors influencing the distribution of *I. scapularis* ticks and their transmission of the zoonotic pathogens they carry are complex [23] and beyond the scope of this manuscript, it is obvious that Lyme disease in southwest Pennsylvania is on the rise [11,13]. During a five year period (2010-2014), Pennsylvania Department of Health [11] reported a seven fold increase in Lyme disease incidence rate in southwest Pennsylvania (75.72 in 2014 versus 11.5/100,000 persons in 2010) as compared to a two-fold increase in northeast Pennsylvania (54.4 in 2014 versus 30.51 in 2010), which the CDC

have corroborated in their recent findings [13].

It has been previously reported that human Lyme and Babesiosis are commonly diagnosed in overlapping geographic areas, especially in regions with high human densities of the northeast (New York, New Jersey) and Midwest (Minnesota, Wisconsin) United States [24]. To date, the human incidence rate for Anaplasmosis and Babesiosis for Pennsylvania is not reported by CDC. But, given our findings and the high incidence rates for Anaplasmosis and Babesiosis in states bordering Pennsylvania (New York and New Jersey) [1], human infection by one of these two emerging blood-borne pathogens is possible via a single *I. scapularis* tick bite [25] which could result in concurrent infections and contribute to a variety of clinical symptoms not typically seen in Lyme disease patients.

Due to current observed changes in the seasonal and environmental landscape of our planet, the upward trend in *I. scapularis* tick population size and co-infection rates with the zoonotic pathogens is likely to continue [2]. Although factors affecting tick coinfection and risks posed to humans need further study, it is warranted that the scientific community, public health officials, and health care providers, think beyond Lyme so as to be unbiased in their diagnosis of other emerging tick-borne diseases as well as co-infection complications that could result from a single *I. scapularis* tick bite. We also urge managers and public users of recreational areas to be aware of the risks of coinfections and become more familiar with tick prevention measures and symptoms of these emerging tick-borne diseases.

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