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Co-circulating microorganisms in questing *Ixodes scapularis* nymphs in Maryland

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Abstract

Ixodes scapularis can be infected with *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, *Bartonella* spp., *Babesia microti*, and *Rickettsia* spp., including spotted-fever group *Rickettsia*. As all of these microorganisms have been reported in Maryland, the potential for these ticks to have concurrent infections exists in this region. To assess the frequency of these complex infections, 348 *I. scapularis* nymphs collected in 2003 were screened for these microorganisms by PCR with positives being confirmed by DNA sequencing. *Borrelia burgdorferi* was detected in 14.7% of nymphs. *Anaplasma phagocytophilum* (0.3%), *Rickettsia* spp. (19.5%), and an uncategorized agent (0.9%) was also detected. Dual infections were detected with *B. burgdorferi* and *Rickettsia* spp. as well as a triple infection with *B. burgdorferi*, *Rickettsia* spp., and an uncategorized agent. Infections with *B. burgdorferi* and *Rickettsia* spp. were statistically independent of one another. However, infection with *B. burgdorferi* and any one of these other microorganisms appears to occur more frequently than by chance alone, probably as a result of shared enzootic cycles. This study confirms that multiple microorganisms co-circulate with *B. burgdorferi* in *I. scapularis* in Maryland and demonstrates that *Rickettsia* spp. and *B. burgdorferi* circulate independently and at nearly equal frequencies, while *A. phagocytophilum* and other unrecognized organisms are less common.

Keyword Index

Ixodes scapularis; *Borrelia burgdorferi*; *Anaplasma phagocytophilum*; *Rickettsia* spp.; co-circulating

Introduction

The blacklegged tick *Ixodes scapularis* is an established vector of several human pathogens including bacteria, protozoa, and viruses (Burgdorfer and Gage 1986, Ebel et al. 2000, Adelson et al. 2004). *Borrelia burgdorferi*, the causative spirochete of Lyme disease, has historically been the most notorious pathogen associated with this tick due to the number of human cases. However, other microorganisms can also be present in *I. scapularis*. Although the frequency of transmission and rate of infection with other pathogens is considerably

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lower than that of *B. burgdorferi* in most geographic areas, these organisms are often maintained in an enzootic cycle involving *I. scapularis* and rodent hosts such as *Peromyscus leucopus*. Due to the similarities in the maintenance cycles of these organisms, concurrent infections with *B. burgdorferi* have been reported in ticks, but the effects of these complex infections on the transmission and pathogen maintenance of these agents are not completely understood (Halos et al. 2005). Other microorganisms with similar maintenance cycles are *Anaplasma phagocytophilum* (causing human granulocytic anaplasmosis [HGA]), *Babesia microti* (causing babesiosis), *Bartonella* spp., and *Rickettsia* spp. (Spielman 1976, Pancholi et al. 1995, Richter et al. 1996, Magnarelli et al. 1997, Raoult and Roux 1997, Schouls et al. 1999, Dumler et al. 2001, Sanogo et al. 2003). Study of these complex infections in *I. scapularis* will assist in determining whether infection with one agent affects the acquisition of other organisms and aid in understanding the effect that concurrent infection has on the transmission of individual agents.

Due to the expansive endemic regions for Lyme disease in the northern hemisphere, many of these other tick-borne organisms have a high potential to co-circulate with *B. burgdorferi* in vector populations. A recent review of co-infection studies determined that dual infection in *Ixodes* ticks is highest in areas where Lyme disease is endemic (Swanson et al. 2006). In the United States, studies have reported between 1% and 28% of *Ixodes* ticks are dually infected with any two of *B. burgdorferi*, *B. microti*, or *A. phagocytophilum* (Swanson et al. 2006). In *I. ricinus* collected in France, two pathogens were detected in 7.6% of ticks (Halos et al. 2005). In that study, any of three co-circulating pathogens (*B. burgdorferi*, *Babesia* spp., *Bartonella* spp.) were detected in adult ticks at a significantly higher rate than nymphs. In northern New Jersey, more than one pathogen (*B. burgdorferi*, *B. microti*, *A. phagocytophilum*, or *Bartonella* spp.) was detected by PCR in 14% of *I. scapularis*; 8.4% of these were concurrently infected with *B. burgdorferi* and *Bartonella* spp. (Adelson et al. 2004). Although these previous two studies, as well as others, have detected *Bartonella* spp., including *B. henselae*, in *Ixodes* spp., transmission of *Bartonella* spp. has not been demonstrated (Chang et al. 2001, Sanogo et al. 2003, Adelson et al. 2004, Morozova et al. 2004, Halos et al. 2005, Holden et al. 2006). In Delaware, where the prevalence of *A. phagocytophilum* in ticks is 4%, the density of *I. scapularis* has increased by 34% in a 10-year time span (Curran et al. 2000), dramatically increasing chances for humans to have contact with ticks and potentially be exposed to these tick-borne pathogens. Concurrently infected ticks also pose a potential increased risk to humans. Ticks collected from mice that are concurrently infected with *B. burgdorferi* and *A. phagocytophilum* have a higher infection prevalence with *A. phagocytophilum* than those from *A. phagocytophilum*-only infected mice (Thomas et al. 2001). Interestingly, ticks can efficiently co-transmit these pathogens (Levin and Fish 2000).

Studies of concurrent infection in ticks, mice, and humans have primarily focused on *B. burgdorferi*, *A. phagocytophilum*, and *B. microti*. Given the biology of *Bartonella* transmission and the apparent increase in *Rickettsia* spp. and unidentified endosymbionts and other microorganisms detected in ticks, concurrent infection in *I. scapularis* with these agents and *B. burgdorferi* is likely to occur in Lyme disease endemic regions. Understanding the effect that concurrent infections have on the transmission and maintenance of *B.*

burgdorferi is important in understanding the role that these complex infections play in the epidemiology of Lyme disease and other potential human pathogens. The prevalence of these concurrent infections in the mid-Atlantic region of North America is unknown. However, *B. microti* is known to be endemic in regions immediately north of Maryland and human cases of HGA have been reported in Maryland. Therefore, a preliminary survey of *I. scapularis* nymphs collected on the Eastern Shore of Maryland was conducted to determine the prevalence and geographic distributions of *B. burgdorferi*, *A. phagocytophilum*, *B. microti*, *Bartonella* spp., and *Rickettsia* spp. in the region. We hypothesized that the presence of other pathogens (*A. phagocytophilum*, *Rickettsia* spp., *Bartonella* spp, *B. microti*), either as a competing infection or as a co-circulating infection, would affect the prevalence rate of *B. burgdorferi* in *I. scapularis* nymphs.

Materials and Methods

Tick collection

Questing nymphs were collected between June and August 2003 by flagging a 50 m × 200 m grid at 27 forested (deciduous, pine, or mixed) locations on the Eastern Shore of Maryland (Figure 1). At each site, flagging for questing ticks was conducted for approximately 45 min within the grid. Flags were examined for ticks every 15 m, and ticks were placed in collection vials. In the lab, ticks were morphologically identified and stored in 1.5 ml centrifuge tubes at -20º C until further processing.

DNA extraction

DNA was extracted from each *I. scapularis* nymph using either a modified hexadecyltrimethylammoniumbromide (CTAB) extraction (Black et al. 1997, Anderson et al. 2004) or a QIAamp DNA MiniKit (Qiagen, Inc., Valencia, CA). Due to concerns about the long-term DNA stability of the extractions, the QIAamp DNA MiniKit was utilized to produce more stable extractions. The manufacturer's protocol for tissue extraction was followed for the QIAamp kit with the addition of an extended proteinase K digestion of approximately 15 h (overnight) and a fnal elution volume of 30 μl.

Microorganism detection by PCR

The presence of *B. burgdorferi* was determined by PCR for two target gene fragments: flagellin (*flaB*) and outer surface protein C (*ospC*). The nested PCR for *flaB* followed the protocol of Johnson et al. (1992). The exterior reaction was conducted in a 25 μl volume. The interior reaction was conducted in 50 μl, using 1 μl of the exterior product as template for the second reaction. The *ospC* PCR amplified the 3′ end of the *ospC* fragment (Wang et al. 1999, Alghaferi et al. 2005, Anderson and Norris 2006). This semi-nested protocol used both exterior primers for the first round PCR and the interior forward and exterior reverse primers for the second round PCR. Only 1.5 μl (˜ 22 ng) of template DNA was used for the first round and 0.5 μl of exterior product was used as the template for the second round. Both the first and second rounds of PCR were conducted in 50 μl reactions. The positive control used was a pure *B. burgdorferi* culture provided by J.S. Dumler, Johns Hopkins University School of Medicine.

Anaplasma phagocytophilum 16S rRNA was detected using primers ge9f and ge10r described by Chen et al. (1994). Each 50 μl reaction contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% w/v gelatin, 400 μM each dNTP, 100 pmol each primer, 1 U *Taq* polymerase, and 1.0 μl (˜ 15 ng) template DNA. Cycling conditions involved an initial denaturation for 3 min at 95º C followed by 35 cycles of denaturation for 30 s at 94º C, annealing for 30 s at 55º C, and extension for 1 min at 72º C. The expected product size was 919 bp. The positive control was extracted genomic DNA provided by J.S. Dumler, The Johns Hopkins University School of Medicine.

Detection of a 238-bp fragment of the small subunit rRNA of *Babesia microti* was performed following the protocol of Persing et al. (1992) using primers Bab1 and Bab4. Modifications to the reaction mixture were 2.0 μl (˜ 30 ng) template DNA and 1 U *Taq* polymerase in each 20 μl reaction. Positive control material was extracted genomic DNA provided by E. Hofmeister, United States Geological Survey.

Bartonella spp. were identified through PCR amplification of a fragment of the citrate synthase (*gltA*) gene using primers BhCS.781p and BhCS.1137n as described by Norman et al. (1995). Each 50 μl reaction mixture was modified to contain 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% w/v gelatin, 400 μM each dNTP, 50 pmol each primer, 1 U *Taq* polymerase, and 1 μl (˜ 15 ng) template DNA. Cycling conditions were an initial denaturation for 5 min at 95º C followed by one cycle of denaturation for 20 s at 95º C, annealing for 30 s at 37º C, and extension for 2 min at 72º C followed by 35 cycles of denaturation for 20 s at 95º C, annealing for 30 s at 42º C, and extension for 2 min at 72º C. *Bartonella elizabethae* culture (ATCC 49927) obtained from ATCC (Manassas, VA) was used as the positive control.

Detection of a citrate synthase gene (*gltA*) fragment from *Rickettsia* spp. was used to determine the presence of *Rickettsia* spp. The primers (*Rp*CS.877p, *Rp*CS.1258n) and cycling conditions were previously described by Regnery et al. (1991). Each 50 μl reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% w/v gelatin, 400 μM each dNTP, 50 pmol each primer, ˜ 2.5 U *Taq* polymerase, and 1.5 μl (˜ 22 ng) template DNA. Samples which were positive for *Rickettsia* spp. were further tested for the presence of the *rOmpA* gene of spotted fever group (SFG) rickettsiae as described by Regnery et al. (1991), using primers *Rr*190.70p and *Rr*190.602n in a 50 μl reaction. Extracted genomic DNA for positive controls for both *Rickettsia* spp. and SFG- rickettsiae were provided by W. Nicholson, Centers for Disease Control and Prevention.

All PCR amplifications were completed on a PTC-200 thermal cycler (MJ Research, Inc., Watertown, MA). Positive and negative controls (no template) were performed for all PCR reactions. Products were visualized on 2% agarose gels using ethidium bromide. A sample was considered positive if a band of the expected size was visualized on agarose. Positive PCR results for *A. phagocytophilum*, *Rickettsia* spp., and *Bartonella* spp. were confirmed through direct sequencing and comparison to sequences available on GenBank. For samples positive for the *Rickettsia* spp. and *Bartonella* spp. *gltA*, sample sequences and sequences available on GenBank were aligned using BioEdit (Hall 1999) and ClustalX (Thompson et

al. 1997). Phylogenetic trees were constructed in ClustalX using the Neighbor Joining method and 1000 bootstrap replicates to evaluate the strength of the clustering analysis.

Data analysis

To determine whether the infections with *B. burgdorferi* and the other pathogens were statistically independent, the odds ratio and 95% confidence intervals were calculated using Epi Info™ Version 3.3.2 (Dean et al. 2002).

Results

A total of 348 questing *I. scapularis* nymphs were collected from the Eastern Shore of Maryland during the summer months of 2003. Nymphal *I. scapularis* were collected at only 66.7% (18/27) of the sites. The majority of the ticks (n=333, 95.7%) were collected from 14 sites located in counties of the Upper Eastern Shore (Talbot, Caroline, Queen Anne's, and Kent counties) (Table 1). Although substantial numbers were collected, none of the adult, nymphal, or larval metastriate ticks was included in this analysis. The 27 forested sites were composed of deciduous trees (n=10, 37%), pine trees (n=2, 7.4%), or a mixture of deciduous and pine trees (n=15, 55.6%) (Table 1). Ticks, including nymphal *I. scapularis*, were collected at all three habitat types.

Extractions of *I. scapularis* nymphs were considered to be positive for *B. burgdorferi* if the PCR results were positive for either *flaB*, *ospC*, or both. The *B. burgdorferi* infection prevalence was 14.7% (51/348) in *I. scapularis*. Of the 51 positive samples, 10 (19.6%) were positive for *flaB* only, 18 (35.3%) were positive for *ospC* only, and 23 (45.1%) were positive for both. The infection prevalence was not significantly associated with the county of collection (χ^2=4.97, p=0.55), even when the counties were clustered as Upper or Lower Eastern Shore (Fisher's exact=0.21). Infected *I. scapularis* were present in all three forest types. Infection prevalence with each organism is shown in Table 1.

Anaplasma phagocytophilum 16S rRNA genes were detected in only 0.3% (1/348) of the nymphs. The presence of *A. phagocytophilum* was confirmed through sequencing of the amplicon and comparison to sequences available on GenBank using BLAST (Altschul et al. 1990) and revealed 98% identity with the 16S rRNA gene of *A. phagocytophilum*.

Using the *Bartonella* spp. primers, a PCR product of the expected size (356 bp) was obtained from three (0.9%) of the nymphs. These samples were sequenced directly using the primers described by Norman et al. (1995), but the recovered sequences did not cluster with those of *Bartonella* species available on GenBank (Figure 2). The sequences have been deposited into GenBank (Accession numbers EF662053-EF662055). Of these three nymphs, one was positive for both *Borrelia burgdorferi* and a non-SFG *Rickettsia* spp. DNA. Another tested positive for both *Rickettsia* spp. *gltA* and *ompA*.

Amplicons were obtained by PCR for *Rickettsia* spp. *gltA* in 19.5% (68/348) of the nymphs. These positive samples were subsequently analyzed for SFG-rickettsiae. The infection prevalence of SFG-rickettsiae was 63.2% (43/68) of the rickettsia-positive nymphs. Of the 68 nymphs that were positive for *Rickettsia* spp., 22.1% (15/68) were also positive for *B.*

burgdorferi. The odds ratio for concurrent infection with *B. burgdorferi* and *Rickettsia* spp. was 1.92 (95% CI: 0.93, 3.94) and not statistically significant, indicating that the infections are independent of each other. Simultaneous infection with SFG-rickettsiae and *B. burgdorferi* was detected in 11/15 (73.3%) of the nymphs, a relationship that was also not statistically significant (OR: 1.80; 95% CI: 0.51, 6.43). Representatives of *Rickettsia* spp. *gltA*-positive samples were sequenced to confirm the PCR results. Two had no full-length GenBank matches, with all matches being fragments less than 65 nucleotides. The sequences were similar to two derived using the *Bartonella*-specific primers. The sequence from the third sample had 99% identity with *Rickettsia* spp. *gltA* available on GenBank. This third sequence clustered with *Rickettsia* "*midichlorii*" (AY348295) and *R. helvetica* (AJ427878) (Figure 2). These sequences have been deposited into GenBank (Accession numbers EF662056-EF662058). None of the 348 nymphs were positive for *Babesia microti*.

The association between *Borrelia burgdorferi* infection and detection of any other agent (*A. phagocytophilum*, *Rickettsia* spp., or *Bartonella*-like) produced an odds ratio of 2.94 (95% CI: 1.41, 6.09). This is statistically significant, indicating a trend towards concurrent infection.

Discussion

Several microorganisms are known to infect *I. scapularis*. This study confirms that at least three found in *I. scapularis* on the Eastern Shore in Maryland can co-circulate with *B. burgdorferi*. The most commonly detected microorganisms in *I. scapularis* nymphs were *B. burgdorferi* and *Rickettsia* spp., both individually and as co-circulating microorganisms. An unidentified agent or agents detected with *Bartonella* primers and *A. phagocytophilum* were rarely observed, and *Babesia microti* was not detected in any nymphs.

Our study relied on data from nymphal *I. scapularis* since we collected ticks during the summer months (June, July, and August), the times when nymphal ticks are most active (Wilson and Spielman 1985). Adults become active in the early spring and late summer and were therefore not collected at a high frequency. In addition, nymphs are considered to be more important epidemiologically in terms of transmission than are larvae or adults (Orloski et al. 2000).

The overall prevalence of *B. burgdorferi* in nymphal *I. scapularis* was 14.7%. In the northern counties of Queen Anne's, Kent, and Caroline, where 94% of the nymphs were collected, the prevalence was geographically stable, ranging from 13.3% to 15%. In the other fve counties of the Eastern Shore (Talbot, Wicomico, Worcester, Somerset, and Dorchester), the sample sizes were quite small, with fewer than ten nymphs collected per county. The reasons for the low density of *I. scapularis* in the southern Eastern Shore need to be determined to fully understand *B. burgdorferi* ecology in this region. Potential explanations include the lack of adequate tick habitat, the lack of suitable tick hosts or pathogen reservoirs, soil composition, or other abiotic factors.

The regional infection rate of *B. burgdorferi* in nymphal *I. scapularis* (14.7%) is low compared to nymphal infection rates from the western coastal plain of Maryland (40% to

98%) (Anderson 2004). These infection rates seem further distorted considering that the human incidence of Lyme disease in the northern part of this collection region can be as high as 80 cases per 100,000 persons while the incidence in the western coastal plains is usually less than 50 cases per 100,000 persons (Frank et al. 2002). This may be due to the small sample size; however, the prevalence of *B. burgdorferi* infection is consistent with the previously reported prevalence rate of 15% in *I. scapularis* collected from deer on the Eastern Shore (Amerasinghe et al. 1992, Amerasinghe et al. 1993).

Other researchers have examined the presence of *Rickettsia* spp. in *Dermacentor variabilis* in Maryland and found the prevalence of SFG-rickettsia to range from 3.8% to 8.6% (Schriefer and Azad 1994, Ammerman et al. 2004). *Rickettsia* spp. have been reported in *I. scapularis* from both laboratory colonies and from field specimens collected in Connecticut (Magnarelli et al. 1991, Magnarelli and Swihart 1991, Magnarelli et al. 1995, Noda et al. 1997). Our results not only establish the presence of *Rickettsia* spp. in field specimens of *I. scapularis* in Maryland, but also establish that the prevalence of SFG-rickettsiae in *I. scapularis* is higher than reported in *D. variabilis* (Schriefer and Azad 1994, Ammerman et al. 2004). The high prevalence of *Rickettsia* spp.-positive ticks among our samples (19.5%) and lack of epidemiologically associated human disease on the Eastern Shore of Maryland supports the hypothesis put forth by Noda et al. (1997) and Weller et al. (1998) that the rickettsial organisms detected are endosymbionts of *I. scapularis* rather than human pathogens.

Human granulocytic anaplasmosis was not a reportable disease in Maryland until 2002, so long term data regarding human cases is not available. However, the prevalence of infection in Maryland is low (0.4%-1.8%), based on samples from either blood donors or from patients with concurrent Lyme disease (personal communication, J.S. Dumler). Based on serological data from samples collected between 1987 and 1997, the estimated prevalence of HGA in Maryland was 1.6% (Comer et al. 1999). In 2001-2002, the incidence of HGA in Maryland was reported to be 0.46 to 1.62 cases per million people (Demma et al. 2005). Serological data supports the presence of *A. phagocytophilum* antibodies in the reservoir *P. leucopus* in Maryland with a prevalence of 6.7% (Nicholson et al. 1998). In mid-Atlantic and northeastern states, the prevalence of antibodies in *P. leucopus* ranges from 4.3% in New Jersey to 23.0% in New York (Nicholson et al. 1998). In the eastern United States, the prevalence of *A. phagocytophilum* in *I. scapularis* ranges from 1.6% in the southeast to between 4% and 90.9% in the northeast (Magnarelli et al. 1995, Telford et al. 1996, Schwartz et al. 1997, Varde et al. 1998, Curran et al. 2000, Fang et al. 2002). The *A. phagocytophilum* prevalence of 0.3% observed in *I. scapularis* nymphs in this study is much lower than reported from surrounding regions, but is consistent with the low frequency of clinical HGA observed in Maryland. It is also consistent with results from other surveys conducted in our laboratory, where *A. phagocytophilum* has not been detected in approximately 1,000 ticks screened (unpublished data). Our use of a single-step PCR protocol rather than a nested protocol may have resulted in a conservative prevalence of *A. phagocytophilum* since nested PCR has a greater sensitivity (Massung and Slater 2003). However, in *I. scapularis* collected in 1997 and 1998 throughout Maryland, Delaware, Pennsylvania, and New Jersey, only three of 979 (0.3%) ticks were positive for *A.*

phagocytophilum (Bunnell 1999). The consistency between our data and that of Bunnell indicates that infection with *A. phagocytophilum* in *I. scapularis* is not common but appears to be regionally stable.

Bartonella spp., including human pathogens, have been detected in *I. pacificus* (Chang et al. 2001); however, maintenance in the ticks and transmission to mammals has not been demonstrated. In our study, 0.9% of *I. scapularis* nymphs contained DNA for an organism using a PCR primer set designed for amplification of a *gltA* gene fragment from *Bartonella* spp. (Norman et al. 1995). However, sequencing results indicated that the amplicons did not originate from any known *Bartonella* spp. In fact, the sequences did not cluster with any citrate synthase sequences available from GenBank. These sequences did cluster with other unidentified citrate synthase amplicons recovered from *D. variabilis* by our laboratory. The sequence alignments and phylogenetic tree both confirm the presence of an unknown agent or agents in Maryland *I. scapularis*. These entities appear to be most closely related to *Bartonella* spp. The definitive identification of these organisms is pending in expanded studies in our laboratory.

Although *B. burgdorferi* and *Rickettsia* spp. were co-circulating in 4.3% of the nymphs, the presence of these organisms appears to be independent of each other. Different enzootic maintenance mechanisms could afect the rate at which each organism occurs in ticks. Unlike *B. burgdorferi*, SFG-rickettsiae can be maintained through transovarial transmission.

Concurrent infections with *Borrelia burgdorferi* and any of the microorganisms studied (*Rickettsia* spp., *Bartonella* spp., or *A. phagocytophilum*), when considered together, do not occur independently. However, concurrent infections may not necessarily occur as a result of concurrent *B. burgdorferi* infection. The chances of concurrent infections with *B. burgdorferi* and other tick-borne pathogens may increase due to their common transmission cycles.

Concurrently infected nymphs have the potential to transmit more than one pathogen to a host during feeding which is important because altered immune responses can occur in mice and in humans with multiple infections (Belongia 2002). In human concurrent infections with *B. burgdorferi* and *B. microti*, the increased severity of clinical Lyme disease seen with the dual infection is thought to be the result of an increased *B. burgdorferi* spirochete load and the immune response elicited by *B. microti* (Persing and Conrad 1995, Krause et al. 1996). In mice infected with both *B. burgdorferi* and *A. phagocytophilum*, the immunological response elicited by each of the pathogens results in increased densities of spirochetes (Holden et al. 2005). However, recent *in vitro* data demonstrated that a greater proportion of *B. burgdorferi* were able to cross an endothelial cell barrier when *A. phagocytophilum*-infected cells were present, thereby possibly explaining the increased spirochete load in the both the blood and tissue (Nyarko et al. 2006).

Borrelia burgdorferi remains the primary microorganism of concern with *I. scapularis* on the Eastern Shore of Maryland; however, other microorganisms, some pathogenic for humans, can co-circulate with *B. burgdorferi* in these ticks. While concurrent infections with *B. burgdorferi* and any one of the other microorganisms in this study appears related,

probably due to the overlap between the common enzootic cycles, the sample size is relatively small. Therefore, the expanded examination with a larger sample size of the prevalence of these microorganisms and the association between concurrently infecting microorganisms in both adult *I. scapularis* and in *P. leucopus* would help to understand these enzootic cycles. The potential epidemiological risk associated with concurrent infections in the vector population and subsequent transmission of complex bacterial infections to humans is not yet fully understood. This is complicated by a paucity of epidemiological data for illness associated with many of these potential or confirmed pathogens. Further study is warranted to better delineate the distribution, identity, and risks associated with transmission of these microorganisms to humans.

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Figure 1.
Tick collection sites on the Eastern Shore of Maryland.

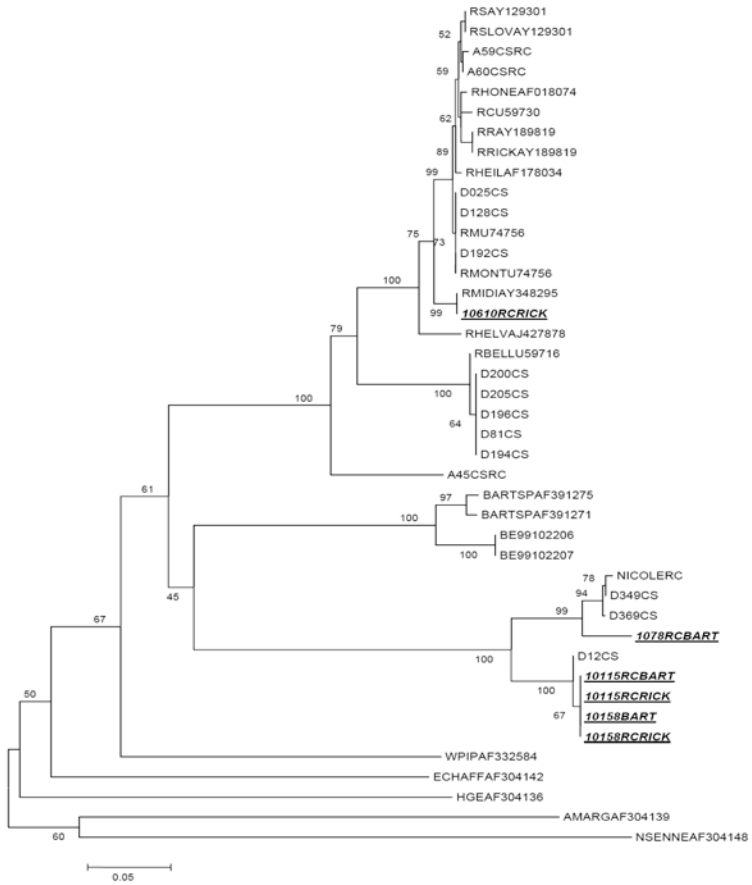

Figure 2.

Neighbor-joining phylogenetic tree based on alignment of citrate synthase gene fragments from *Rickettsia* spp. and *Bartonella* spp. Samples from this study are underlined and italicized. (GenBank Accession Numbers EF662053-EF662058).

Table 1

Summary of tick collections and PCR status of *I. scapularis* nymphs on the Eastern Shore of Maryland.

County	Site Number	Habitat Type	*Ixodes scapularis* nymphs	*Ixodes scapularis* adults	*Dermacentor variabilis* adults	*Amblyomma americanum* adults	Metastriate nymphs	Larvae (Y/N)	*B.b.*	*Rickettsia* spp. (SFG)	*A.p.*	*Bartonella* spp.	*B.m.*
All Sites Total			348	7	109	276	1973		51	68 (43)	1	3	0
Caroline	108	Deciduous	40	2	9	7	79	N	6	4 (2)	0	0	0
	109	Mixed forest	4	0	1	4	72	N	0	0	0	0	0
	Total		44	2	10	11	151		6	4 (2)	0	0	0
Dorchester	103	Deciduous	2	0	2	8	27	N	0	0	0	0	0
	104	Mixed forest	2	1	6	3	38	N	0	0	0	0	0
	126	Mixed forest	0	0	3	0	41	Y	0	0	0	0	0
	127	Mixed forest	0	0	0	0	139	Y	0	0	0	0	0
	Total		4	1	11	11	245		0	0	0	0	0
Kent	110	Pine	60	2	8	4	86	N	8	4 (4)	1	0	0
	111	Deciduous	0	0	2	0	0	N	0	0	0	0	0
	112	Deciduous	0	1	0	1	0	N	0	0	0	0	0
	Total		60	3	10	5	86		8	4 (4)	1	0	0
Queen Anne's	101	Mixed forest	69	1	48	156	394	N	17	29 (24)	0	2	0
	102	Mixed forest	62	0	3	9	33	N	5	3 (2)	0	0	0
	105	Mixed forest	15	0	1	3	4	N	1	0	0	0	0
	106	Mixed forest	33	0	13	13	125	N	9	3 (2)	0	0	0
	107	Deciduous	28	0	1	45	351	N	1	1 (0)	0	1	0
	114	Deciduous	17	0	3	0	19	Y	1	17 (5)	0	0	0
	124	Deciduous	0	0	0	0	3	N	0	0	0	0	0
	125	Deciduous	0	0	0	0	8	N	0	0	0	0	0
	Total		224	1	69	226	937		34	53 (33)	0	3	0
Somerset	120	Deciduous	1	0	0	1	280	Y	0	1 (0)	0	0	0
	121	Deciduous	3	0	0	2	223	Y	1	1 (0)	0	0	0
	Total		4	0	0	3	503		1	2 (0)	0	0	o

County	Site Number	Habitat Type	*Ixodes scapularis* nymphs	*Ixodes scapularis* adults	*Dermacentor variabilis* adults	*Amblyomma americanum* adults	Metastriate nymphs	Larvae (Y/N)	*B.b.*	*Rickettsia* spp. (SFG)	*A.p.*	*Bartonella* spp.	*B.m.*
Talbot	113	Mixed forest	5	0	1	8	39	Y	0	1 (0)	0	0	0
	Total		5	0	1	8	39		0	1 (0)	0	0	o
Wicomico	115	Mixed forest	2	0	2	1	28	N	1	2 (2)	0	0	0
	116	Mixed forest	2	0	1	0	33	N	0	1 (1)	0	0	0
	117	Mixed forest	1	0	2	9	91	N	1	0	0	0	0
	118	Pine	0	0	0	0	10	Y	0	0	0	0	0
	Total		5	0	5	10	162		2	3 (3)	0	0	0
Worcester	119	Mixed forest	0	0	1	0	36	Y	0	0	0	0	0
	122	Mixed forest	2	0	1	0	17	Y	0	1 (1)	0	0	0
	123	Mixed forest	0	0	1	2	20	Y	0	0	0	0	0
	Total		2	0	3	2	73		0	1 (1)	0	0	0

Abbreviations: *B.b. - Borrelia burgdorferi; A.p. - Anaplasma phagocytophilum ; B.m. - Babesia microti*