Infection and Co-infection Rates of *Anaplasma phagocytophilum* Variants, *Babesia* spp., *Borrelia burgdorferi*, and the Rickettsial Endosymbiont in *Ixodes scapularis* (Acari: Ixodidae) from Sites in Indiana, Maine, Pennsylvania, and Wisconsin

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ABSTRACT In total, 394 questing adult blacklegged ticks, *Ixodes scapularis* Say (Acari: Ixodidae), collected at four sites were analyzed by polymerase chain reaction (PCR) for five microbial species: *Anaplasma phagocytophilum*, *Babesia microti*, *Babesia odocoilei*, *Borrelia burgdorferi*, and the rickettsial *I. scapularis* endosymbiont. Identities of genetic variants of *A. phagocytophilum* were determined by sequencing a portion of the 16S DNA. In 55% of infected ticks (193/351), a single agent was detected. In 45% (158/351), two or more agents were detected; 37% harbored two agents and 8% harbored three agents. One male tick, collected from Ft. McCoy, WI, harbored all four microbial genera. The highest rates of co-infection were by the *Ixodes* endosymbiont and *B. burgdorferi* (95/351). Two species of *Babesia* co-occurred within a single tick population in Wells National Estuarine Research Reserve, Wells, ME, whereas only *B. odocoilei* was found in other tick populations. Only *A. phagocytophilum* human anaplasmosis variant was detected in questing ticks from Tippecanoe River State Park, IN; from Wells; and Ft. McCoy, whereas a single infected tick from Presque Isle, PA, was infected by AP-Variant 1. Partially engorged ticks from deer in Tippecanoe River State Park were all infected with AP-Variant 1. Frequency of infections with each agent varied among populations. Rates and types of co-infections were not significantly different from random except for the *Ixodes* endosymbiont and *B. burgdorferi* in male ticks, which co-occurred less frequently than expected. Thus, *I. scapularis* hosts an array of pathogenic and symbiotic agents and potential evidence of interactions among microbial species was observed.

KEY WORDS *Ixodes scapularis*, microbial pathogens, polymerase chain reaction

The blacklegged tick, *Ixodes scapularis* Say (Acari: Ixodidae), is widely distributed in the northeastern United States and in much of the Midwest (Pinger et al. 1996, Dennis et al. 1998, Guerra et al. 2002). By 1996, *I. scapularis* had been detected in at least one county in all states east of the Great Plains (Dennis et al. 1998). In the Midwest, significant populations now occur in eastern Minnesota, western and central Wisconsin, northeastern Iowa, northern and eastern Illinois, northwestern Indiana, and the Upper Peninsula of Michigan. Most cases of Lyme disease are reported where high populations of *I. scapularis* and humans overlap, with >95% of the Lyme disease cases in the United States reported from the Northeast, Mid-Atlantic, and Upper Midwest (CDC 2004).

Cases of other tick-borne diseases also are related to the presence of *I. scapularis.* Pathogens transmitted by *I. scapularis* include *Borrelia burgdorferi,* the Lyme disease spirochete (Burgdorfer et al. 1982, Johnson et al. 1984); *Anaplasma phagocytophilum,* the agent of human granulocytic anaplasmosis (Chen et al. 1994, Dumler et al. 2001); and *Babesia microti*, the agent of human babesiosis in the United States (Spielman et al. 1985, Herwaldt et al. 1995). *I. scapularis* also has been found to transmit *Babesia odocoilei*, a tick-borne hemoprotozoan parasite that causes babesiosis in deer and other cervids (Waldrup et al. 1990, Holman et al. 2000). In addition to human and animal pathogens, *I. scapularis* harbors a symbiotic bacterium belonging to the alpha subclass of Proteobacteria, a member of the Spotted Fever Group (SFG) rickettsiae (Noda et al. 1997, Benson et al. 2004, Moreno et al. 2005). Because this symbiotic bacterium is so prevalent in *Ixodes* ticks, its co-occurrence with the other microorganisms may influence pathogen transmission (Moreno et al. 2006) as has been found in *Dermacentor* ticks (Burgdorfer et al. 1981, Macaluso et al. 2002).

Molecular evidence of co-infection with multiple human pathogens has been demonstrated for *I. ricinus* complex ticks collected in California, Wisconsin, and the northeastern United States (Piesman et al. 1987, Varde et al. 1998, Adelson et al. 2004, Holman et al.

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2004, Swanson et al. 2006). Moreno et al. (2006) found evidence for the presence of *Rickettsia*, *Pseudomonas*, *Borrelia*, *Ralstonia*, *Anaplasma*, and other, less well-known bacterial genera in *I. scapularis* ticks collected in Westchester and Dutchess counties, NY. The risk for human co-infection with multiple pathogens differs by geographic location and depends on the prevalence of pathogens within the vertebrate reservoir hosts and *Ixodes* tick vectors (Swanson et al. 2006), and the frequency of tick-human interactions and possible microbial interactions within vectors.

Tick-borne pathogens must survive in the divergent environments of mammalian hosts and tick vectors, a phenomenon that could give rise to DNA sequence variation. For example, all of the samples of *A. phagocytophilum* obtained from human patients have identical 16S rRNA gene sequences. This strain has been designated *A. phagocytophilum* human anaplasmosis (AP-ha). A different strain, the AP-Variant 1 found in deer, differs by two base pairs from the human variant. Both the AP-ha and AP-Variant 1 types are transmitted by *I. scapularis* (Chen et al. 1994, Massung et al. 1998). Massung et al. (2002) described additional genetic variants of *A. phagocytophilum* detected in *I. scapularis* ticks and various mammals collected in Rhode Island and Connecticut. Besides AP-ha and AP-Variant 1, they described Variants 2, 3, and 4, which have not been detected in humans.

The purpose of this study was to examine the composition and dynamics of microbial communities in *I. scapularis* ticks by polymerase chain reaction (PCR) amplification of specific DNA target sequences. Mounting evidence suggests that *I. scapularis* can become infected with multiple pathogens after a bloodmeal from a co-infected host or as a result of sequentially feeding on differentially infected hosts. Moreover, co-infected *I. scapularis* can potentially lead to human simultaneous co-infection which may complicate diagnosis and treatment. The specific goals of this study were to determine 1) the infection rates for *A. phagocytophilum*, *B. microti*, *B. odocoilei*, *B. burgdorferi*, and the *I. scapularis* rickettsial endosymbiont in *I. scapularis* ticks over a broad geographic area where the ticks are endemic; 2) the frequency of co-infections in these ticks and their potential deviation from random expectations; and 3) the identity of *A. phagocytophilum* variants in these ticks. The results add to our understanding of microbial community composition in *I. scapularis* ticks.

Materials and Methods

Tick Collections. Questing adult ticks were collected at four sites in Indiana, Maine, Pennsylvania, and Wisconsin where populations of *I. scapularis* were known to occur (Fig. 1). The Indiana ticks were collected in October 2004 in Tippecanoe River State Park (SP), Pulaski County (41° 6′ 24.8″ N, 086° 34′ 59″ W). Maine ticks were collected in October 2003 in Wells National Estuarine Research Reserve, York County (43° 12′ 24″ N, 070° 19′ 54″ W) (provided by Dr. Robert Smith). Pennsylvania ticks were collected in October 2005 in Presque Isle SP, Erie County (42° 10′ 17″ N, 80° 05′ 40″ W). Wisconsin ticks were collected in April 2006 at Fort McCoy, Monroe County (44° 05′ 55″ N, 094° 37′ 51″ W).

One hundred questing adult ticks (42 females and 58 males) were collected in Tippecanoe River SP (42 females and 58 males) and Ft. McCoy (50 females and 50 male); 94 ticks (51 females and 43 males) were collected in Presque Isle SP. All ticks were collected by dragging a 1-m^2 white corduroy cloth attached to 1-m wooden dowel along deer trails. Ticks were transported to the laboratory, surface rinsed with 95% ethanol, and stored individually at −80°C.

DNA Extraction and Polymerase Chain Reaction Analysis. Ticks were ground with Teflon pestles in 1.7-ml microcentrifuge tubes in the presence of liquid nitrogen. Total DNA was extracted using a DNeasy tissue kit (QIAGEN, Valencia, CA). For *A. phagocytophilum*, a nested PCR assay specifically targeting a 546-bp fragment of the 16S rRNA gene was performed on the DNA samples according to Massung et al. (1998). The cycling conditions were described in Steiner et al. (2006). Additionally, three *A. phagocytophilum*-positive DNA samples obtained from previously collected, partially engorged ticks (from deer) (Steiner et al. 2006) were used for a comparison in this study because sequence variations in the 16S rRNA gene were anticipated (Massung et al. 2005).

For *Babesia* species, a PCR assay using the PIRO-A/PIRO-B primers targeting either a 408-bp (*B. odocoilei*) or 437-bp (*B. microti*) fragment of the 18S rRNA gene from *Babesia* species (Armstrong et al. 1998) was performed on DNA samples. Cycling conditions were described in Steiner et al. (2006).

For *B. burgdorferi*, a nested PCR assay targeting a specific 277-bp region of the *flagellin* gene of *B. burgdorferi* was performed on the DNA samples according to Schmidt et al. (1996). The cycling conditions were modified to a touchdown program for the primary reactions. Thirty-five cycles of the following were performed: denaturation at 94°C for 15 s, annealing for 15 s at 66°C (cycles 1–4), 62°C (cycles 5–8) at 58°C (cycles 9–35), and extension at 72°C for 5 min. The nested reaction was performed in the same manner as the primary reaction, but the initial annealing temperature was 60°C, followed by 56°C, and the final cycles at 52°C. Finally, a PCR assay targeting a 540-bp outer surface protein of SFG rickettsia was used to detect the *I. scapularis* endosymbiont (Regnery et al. 1991). Table 1 summarizes the sequences of primers used in this study. PCR products were fractionated in a 1.5% agarose gel in the presence of ethidium bromide and visualized by a UV transilluminator.

DNA Sequencing. After electrophoresis, PCR products were sliced from the gel and purified using the QIAquick gel extraction kit (QIAGEN). For some samples, aliquots of the purified products were sequenced on both strands using the PCR primers and dye terminator according to protocol by BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The sequencing was performed by the Indiana University Molecular Biol-

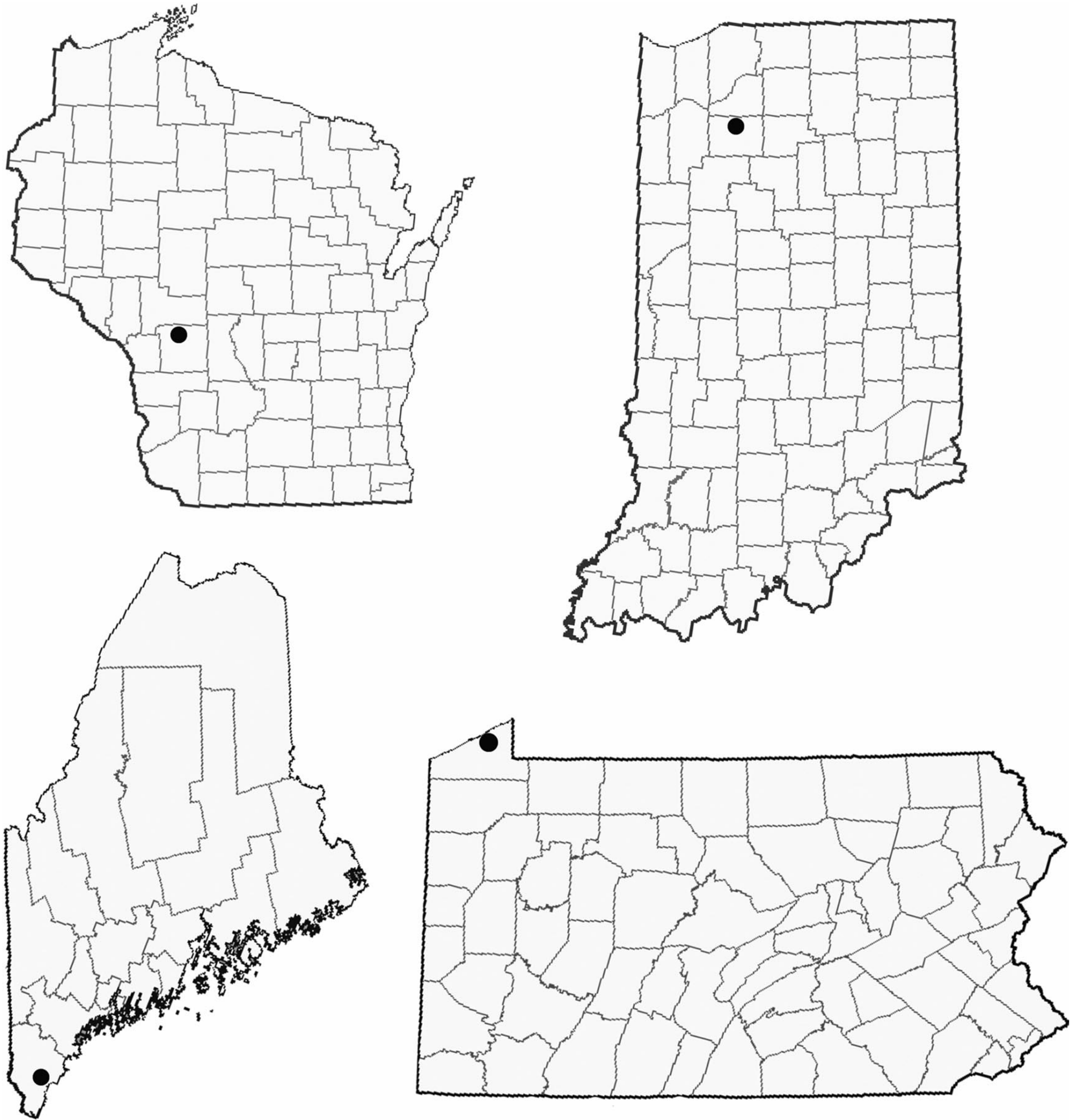

Fig. 1. Maps show the approximate location of tick collection sites in Monroe County, WI; Pulaski County, IN; Wells County, ME; and Erie County, PA.

ogy Institute on an ABI 3730 Analyzer from Applied Biosystems (http://imbi.bio.indiana.edu/).

The chromatograms representing the sequencing results were analyzed for confidence in nucleotide assignments and flanking sequences were cropped with Chromas 2.23 shareware (Cornell University, Ithaca, NY). A Basic Local Alignment Search Tool (BLAST) analysis (National Center for Biotechnology Information, Bethesda, MD) was performed to match the PCR product sequences with known sequences from the GenBank database (Altschul et al. 1990).

Cloning of PCR Products from *A. phagocytophilum*. The terminal sequences of most of the *A. phagocytophilum* PCR products could not be determined within the initial 76–84 nucleotide consensus region of the 16S rRNA gene, similar to the findings of Dugan et al. (2006). Complete sequences were obtained using the reverse primer. Some of the PCR products were cloned using the pGEM-T-Easy vector system (Promega, Madison, WI) according to the manufacturer instructions. Ten recombinant colonies from each cloning experiment were cultured, plasmids extracted using the QIAprep Spin Miniprep kit (QIAGEN), and inserts were sequenced using plasmid primers T7 and SP6 to confirm 5′ sequences of the 16S rRNA gene.

Ticks testing positive by PCR analyses (see below) were classified as being infected following standard terminology, as opposed to containing remnant DNA

Table 1. PCR oligonucleotide primers

Primer name	Species	Gene	Nucleotide sequence (5′–3′)	Product size (bp)	Reference
ge3a	*A. phagocytophilum*	16S rRNA	CACATGCAAGTCGAACGGATTATTC	932	Massung et al. 1998
ge10r			TTCCGTTAAGAAGGATCTAATCTCC		
nested					
ge9f			AACGGATTATTCTTTATAGCTTGCT	546	
ge2			GGCAGTATTAAAAGCAGCTCCAGG		
BBSCH31	*B. burgdorferi*	Flagellin	CACACCAGCATCACTTTCAGGGTCT	436	Schmidt et al. 1996
BBSCH42			CAACCTCATCTGTCATTGTAGCATCTTTTATTT		
FL-59			TTTCAGGGTCTCAGGCGTCTT	277	
FL-7			GCATTTTCAATTTTAGCAAGTGATG		
PIRO-A	*Babesia* spp	18S rRNA	AATACCCAATCCTGACACAGGG	408/437	Armstrong et al. 1998
PIRO-B			TTAAATACGAATGCCCCCAAC		
Rr190.70p	*R. rickettsii*	190-kDa atigen (Omp A)	ATGGCGAATATTTCTCCAAAA	532	Regnery et al. 1991
Rr190.602n			AGTGCAGCATTCGCTCCCCCT		

from microorganisms in prior bloodmeals, because the questing ticks collected were flat and several months removed from their previous bloodmeal.

Statistical Analyses. Variation in infection frequency among sites for each microbe was tested with chi-square tests with 3 degrees of freedom. The frequency of endosymbiont infection was compared between male and female ticks in each population with chi-square tests with one degree of freedom. Similarly, significant deviations from random co-occurrence were tested for each pair of microorganisms in each population with chi-square tests with 1 degree of freedom. For example, if microorganisms A and B each occurred at 50% frequency in a tick population, the expectation would be that 25% of ticks would be coinfected by A and B. Because this approach is limited to single pairs of microorganisms, we also calculated the C-score for each tick population, which quantifies the average co-occurrence of all unique pairs of microbial species within individual ticks (Gotelli 2000). The observed C-score was compared with the expected C-score from a random microbial community using the resampling algorithm in the EcoSim computer package (Gotelli and Entsminger 2003). Significant deviations from expected rates of coinfection could provide evidence of competitive or facilitative interactions among microorganisms within ticks (Burgdorfer et al. 1981, Macaluso et al. 2002, Moreno et al. 2006). For all analyses, the two *Babesia* species were combined into a single genus.

Results

PCR Results for *A. phagocytophilum*, *B. burgdorferi*, *Babesia* spp., and the *I. scapularis* Endosymbiont. Of 394 adult *I. scapularis* ticks tested (100 from each population except 94 from Presque Isle SP), 89% (351/394) were infected with at least one of the five species of microorganisms (Table 2). Infection rates for *B. burgdorferi* (sensu stricto in the United States) ranged from 72% for Tippecanoe River SP ticks to 35% for Ft. McCoy ticks. For *A. phagocytophilum*, the highest infection rates were found in samples from the Wells (16%) and Ft. McCoy (14%), whereas the lowest in-

Table 2. PCR test results for questing adult *I. scapularis* ticks from Tippecanoe River State Park, Indiana, Wells, ME; Presque Isle, PA; and Ft. McCoy, WI, for *A. phagocytophilum*, *B. burgdorferi*, *Babesia* species (*B. microti* and *B. odecoilei*), and the *Ixodes* endosymbiont

Name of microbe	Site (no. positive [%]/no. sequenced)			
	Tippecanoe River State Park, IN	Wells National Estuarine Research Preserve, ME	Presque Isle, PA	Ft. McCoy, WI
A. phagocytophilum				
AP-ha[a]	5 (5)/5	16 (16)/9	1 (1)/0	14 (14)/13
AP-Variant 1[b]	0	0	1	0
B. burgdorferi[c]	72 (72)/9	58 (58)/11	52 (55)/12	35 (35)/7
Babesia spp.				
B. odecoilei[d]	6 (6)/6	15 (15)/8	2 (2)/2	11(11)/11
B. microti[e]	0	7	0	0
Ixodes endosymbiont[f]	63 (63)/11	46 (46)/nd	61 (65)/13	82 (82)/nd
Co-infections[g]	46%	34%	33%	45%

nd, not done.
[a] Accession no. U02521.
[b] Accession no. AY193887.
[c] Accession no. AY884355.
[d] Accession no. AY237638.1.
[e] Accession no. AY144696.
[f] Accession no. AB002268.1.
[g] Two, three, or four agents in a tick.

Table 3. Co-infections in questing adult *I. scapularis* ticks from four populations

Ixodes Pop		Co-infection combination						C-score
		Ap-Bb[a]	Bb-Bab	Bb-Ie	Ap-Bab	Ap-Ie	Bab-Ie	
Tippecanoe River	O	4	4	41	0	3	5	173.67
SP IN	E	3.60	4.32	45.36	0.30	3.15	3.78	175.87
	χ^2	1.46 NS[b]	0.09 NS	2.40 NS	1.20 NS	0.02 NS	1.13 NS	NS
Wells ME	O	9	11	21	2	8	10	344.30
	E	9.28	8.70	26.68	2.40	7.36	6.90	341.61
	χ^2	0.10 NS	1.91 NS	5.37[c]	0.09 NS	0.11 NS	3.04 NS	NS
Presque Isle SP PA	O	1	1	30	0	1	2	122.50
	E	0.55	1.11	33.74	0.02	0.65	1.30	123.58
	χ^2	0.82 NS	0.02 NS	2.65 NS	0.00 NS	0.54 NS	0.52 NS	NS
Ft. McCoy, WI	O	8	4	29	1	14	8	174.83
	E	4.90	3.85	28.70	1.54	11.48	9.02	171.10
	χ^2	3.51[d]	0.01 NS	0.03 NS	0.25 NS	3.57[d]	0.72 NS	NS

O indicates the observed number of co-infections, E indicates the expected number of co-infections based on the individual frequencies of each microorganism, and the associated χ^2 value and statistical significance for each combination. The C-score is a test of all two- and three-way co-infections simultaneously (see text).

[a] Ap, *A. phagocytophilum;* Bb, *B. burgdorferi;* Bab, *Babesia* spp.; and Ie, *I. endosymbiont.*

[b] Nonsignificant.

[c] $P < 0.05$.

[d] $P < 0.06$.

fection rate (1%) was found among the Presque Isle SP ticks. For *Babesia* species, the highest infection rates were in ticks from Wells (15%) and Ft. McCoy (11%). The frequency of infection by the *I. scapularis* endosymbiont ranged from 46% (Wells) to 82% (Ft. McCoy). There was significant variation in infection frequency among populations for all four groups of microorganisms (*B. burgdorferi:* $\chi^2 = 28.21, P < 0.0001$; *A. phagocytophilum:* $\chi^2 = 18.14$, $P = 0.0004$; *Babesia:* $\chi^2 = 11.78, P = 0.0082$; *I. scapularis endosymbiont:* $\chi^2 = 28.19$, $P < 0.0001$). There were significantly lower endosymbiont infection rates in male versus female ticks in every population, e.g., Tippecanoe River SP (females 98% infected, males 38% infected; $\chi^2 = 13.78$, $P = 0.0002$), Wells (females 86% infected, males 6% infected; $\chi^2 = 34.78$, $P < 0.0001$); Presque Isle SP (females 94% infected, males 30% infected; $\chi^2 = 7.85$, $P = 0.005$); and Ft. McCoy (females 100% infected, males 64% infected; $\chi^2 = 3.95$, $P = 0.0469$). No differences in infection frequencies were detected between sexes for the other microorganisms (data not shown).

Babesia isolates were sequenced to determine whether they were *B. microti* or *B. odocoilei.* Sequencing results revealed that the six positive ticks from Tippecanoe River SP, the two positive ticks from Presque Isle SP and the 11 positive ticks from Ft. McCoy were all infected with *B. odocoilei.* In contrast, eight of the *Babesia* isolates from Wells ticks were infected with *B. odocoilei* and seven with *B. microti,* indicating the co-occurrence of multiple *Babesia* species within single tick populations.

Overall, 55% of infected ticks (193/351) harbored a single agent, whereas 45% (158/351) harbored multiple infections. Of these, 37% (130/351) harbored two agents, and 8% (27/351) harbored three agents. A single male tick collected from Wisconsin harbored all four microbial genera. The double infections with *B. burgdorferi* and the *Ixodes* endosymbiont were the most prevalent type of multiple infections at all sites making up 61% (96/158) of dual infections. Double infections with *Babesia* spp. and the *I. scapularis* endosymbiont (11/158) and with *A. phagocytophilum* and the *I. scapularis* endosymbiont (10/158) were much less frequent. Fourteen triple infections with *A. phagocytophilum*, *B. burgdorferi* and the *I. scapularis* endosymbiont were observed; seven of these were in Ft. McCoy ticks. Ten triple infections with *B. burgdorferi*, *Babesia* sp., and the *I. scapularis* endosymbiont were found, including three in Wells ticks infected with *B. microti.* There was one triple infection from Maine with *A. phagocytophilum*, *B. odocoilei,* and the *I. scapularis* endosymbiont.

Considering pairwise co-infections, one of 24 combinations (six types of pairs × four populations) differed significantly from random expectations. There were significantly fewer *B. burgdorferi*/*Ixodes* endosymbiont co-infections than expected in the Wells population (Table 3). Two co-infections were significantly different from expected at $P < 0.06$ in the Ft. McCoy population. At $P < 0.05$, one in 20 comparisons would be expected to be significant by chance alone. To avoid the problem of multiple comparisons and to incorporate three-way co-infections into the analysis, a C-score analysis was conducted. Observed C-scores were similar to expected C-scores and in no case did observed C-scores differ significantly from random expectations (Table 3).

Given the differences in endosymbiont infection frequency in male and female ticks, and the significantly fewer *B. burgdorferi*/*Ixodes* endosymbiont co-infections than expected in the Wells population (Table 3), we further explored whether there were nonrandom co-infections by sex. Across sites, in male ticks, an association was found in infection rates between *B. burgdorferi* and the *Ixodes* endosymbiont. Infection rates with *B. burgdorferi* were higher when the *Ixodes* endosymbiont not detected ($\chi^2 = 11.81, P < 0.001$). Even when the Wells data (in which detected infections were lower than other sites) were excluded,

the association remained significant ($\chi^2 = 6.41$, $P < 0.011$).

A. *phagocytophilum* Variants. Sequence analysis of the PCR products by using primers ge9f and ge2 revealed the presence only of AP-ha in ticks collected in Tippecanoe River SP (5/5 sequenced), Wells (9/16 sequenced), and Ft. McCoy (13/14 sequenced). Sequencing analysis of the PCR products from a single positive tick collected in Presque Isle SP revealed the AP-Variant 1 genotype.

All the sequences were analyzed by BLAST and compared with the AP-ha 16S RNA gene sequence reported by Chen et al. (1994, GenBank accession number U02521). Representative sequences were checked for variation, according to Massung et al. (2002), at positions #76, 84, 157, 176, 284, and 299, corresponding to A,G,A,G,C and A respectively. All samples of questing ticks collected in Tippecanoe River SP, Wells, and Ft. McCoy matched the AP-ha sequence. In contrast, partially engorged ticks collected from deer in Tippecanoe River SP (samples 261, 263 and 278) were all infected with the AP-Variant 1 with G,A,A,G,C and A at the positions mentioned earlier (Courtney et al. 2003, GenBank accession number AY 193887). A single positive sample from Presque Isle SP was also infected by the AP-Variant 1.

Discussion

Results from a survey of nearly 400 ticks from four sites in broadly distributed states indicated that the *Ixodes* rickettsial endosymbiont occurred at the highest frequency (≈63%), followed by *B. burgdorferi* (≈55%), and *A. phagocytophilium* and *Babesia* spp. each occurred at an average prevalence of 9%. There were significant differences in prevalence among tick populations for each microorganism and a significant difference in endosymbiont frequency between male and female ticks. Approximately 40% of all ticks were simultaneously co-infected by at least two microbial agents, but there were no overall deviations from random co-occurrence.

Infection rates can vary from region to region and year to year (Paskewitz et al. 2001, Jackson et al. 2002, Holman et al. 2004, Caporale et al. 2005). However, *B. burgdorferi* infection rates (55%) described in this report for northwestern Pennsylvania are similar to those reported by Courtney et al. (2003). The values for Tippecanoe River SP, IN, are higher (70%) in this report than reported for a previous year (55%) (Steiner et al. 2006). The increasing incidence of infection may be due to conditions at this site where an abundance of vertebrate hosts with high levels of infection are capable of passing on the spirochetes to ticks, keeping the enzootic cycle going the following season with newly hatched larvae (unpublished data). Ticks collected from Ft. McCoy, WI had the lowest *B. burgdorferi* infection rate of the four populations tested (35%), but they had the highest *A. phagocytophilum* infection rate (14%) along with Wells, ME (16%). The *A. phagocytophilum* infection rate for the ticks from Presque Isle, PA, was the lowest of any of the sites we examined and is in agreement with the findings of Courtney et al. (2003) at the same site.

We detected the presence of both *B. microti* and *B. odocoilei* in Wells, ME, ticks, but we found only *B. odocoilei* in ticks from Ft. McCoy, Tippecanoe River SP, and Presque Isle SP. These findings, which indicate that *B. odocoilei* is more prevalent than *B. microti,* but that the two species can co-occur in the same tick population, are in agreement with the findings of Armstrong et al. (1998) who analyzed tick samples from Maine, Massachusetts, and Wisconsin. These authors suggested that the acquisition and transstadial passage of *B. microti* by the tick is relatively inefficient when compared with that of *B. odocoilei.* Our results, indicating nearly equal infection frequencies of *B. microti* and *B. odocoilei* for Maine ticks, suggest that the efficiency of passage is similar for both microorganisms. However, *B. odocoilei* is not known to infect humans (Homer and Persing 2005); therefore, it may be of little current concern to public health officials. Nevertheless, the possibility of interactions between the human pathogenic and nonpathogenic *Babesia* species deserves further investigation.

We found that 64% of questing ticks were infected with the *I. scapularis* endosymbiont. In contrast, Moreno et al. (2006) reported the presence of this endosymbiont in almost all ticks tested. Despite our differences in detection rates, clearly this microorganism is both widespread and common. As a consequence of the high rates of infection, the likelihood of co-detection with other microorganisms increased. Other tick-borne endosymbionts also occur at high frequencies and generate similarly high rates of co-infection (Niebylski et al. 1997, Jasinskas et al. 2007).

Fewer studies have quantified the prevalence of the *Ixodes* endosymbiont in part because it is not a known human pathogen. Nevertheless, the potential for interactions between rickettsial endosymbionts and human pathogens has been demonstrated (Macaluso et al. 2002). More generally, both pathogenic and symbiotic *Rickettsia* are associated with a wide range of arthropod and invertebrate taxa, and they can be transmitted contagiously and transovarially through eggs (Perlman et al. 2006). Rickettsial endosymbionts in *I. scapularis* have been described several times based on microscopic and DNA sequence analyses (Noda et al. 1997, Schabereiter-Gurtner et al. 2003, Benson et al. 2004). Moreno et al. (2006) used temporal temperature gradient gel electrophoresis to identify and quantify microbial communities in *I. scapularis.* They found that *Rickettsia* were the most abundant DNA sequences obtained, and were found in 100% of their pooled and individual tick samples. In this study, we found <100% infection, with significantly lower infection rates in male ticks. The lower infection rate in males may be a PCR artifact resulting from lower rickettsial cell number and/or density in male ticks. We have found that the ubiquitous *Coxiella* endosymbiont of *Amblyomma americanum* (L.) (Jasins-

kas et al. 2007, Klyachko et al. 2007) occurs at significantly lower cell density in males than females as determined by quantitative PCR (V. S. Silvanose, K. C., and C. F., unpublished data). Given transovarial transmission, male ticks play no role in endosymbiont reproduction and the absence or reduced density of endosymbionts in males in not surprising. Some *Rickettsia* manipulate the reproductive behavior of host arthropods and kill males, producing all-female populations (Perlman et al. 2006). However, there is no evidence of sex ratio alteration in ticks that exhibit 50:50 male:female ratios.

Peromyscus leucopus, the white-footed mouse, is the primary reservoir for AP-ha and *B. microti* (Spielman et al. 1979, Spielman et al. 1985, Chen et al. 1994). *Odocoileus virginianus,* the white-tailed deer, is the primary reservoir for *B. odocoilei* (Emerson and Wright 1968, 1970) and for AP-Variant 1 (Massung et al. 2005). Co-infection of questing adult ticks by AP-ha and *B. odocoilei,* or by AP-Variant 1 and *B. microti,* would require infection by one agent at the larval stage and the second agent at the nymphal stage. We did not detect such dual infections. *P. leucopus* also is the primary reservoir for *B. burgdorferi* and the host for larvae and nymphs of *I. scapularis. B. burgdorferi* has efficient transstadial persistence from larvae to adult in *I. scapularis,* so co-infections with this pathogen may occur simultaneously. Similarly, the *I. scapularis* endosymbiont is maintained transovarially; therefore, co-infections with this agent are not constrained by vertebrate host type.

Michalski et al. (2006) tested ticks and deer blood collected in western and eastern sites of the Wisconsin River in Wisconsin. They described the known AP-ha and AP-Variant 1 strains of *A. phagocytophilum* and the new AP-Variant WI-1 and AP-Variant WI-2 from deer blood. In examining questing ticks from Ft. McCoy, WI, this study detected the AP-ha variant in the 13 of 14 samples sequenced despite the reported abundance of nonpathogenic AP-Variants 1, 2, 3, and 4 (Massung et al. 2002). Massung et al. (2002) suggested that a lower incidence of human anaplasmosis could be expected in areas where these variants predominate due to a competitive advantage over AP-ha. The results of this study suggest that our collections from Indiana, Maine, and Wisconsin harbor the pathogenic variant of *A. phagocytophilum* (AP-ha); therefore, they represent a human health risk. The only tick positive for *A. phagocytophilum* collected in Presque Isle SP, PA, harbored the nonpathogenic genotype AP-Variant 1.

In conclusion, our results reveal complex microbial communities associated with *I. scapularis,* including the coexistence of *A. phagocytophilum* variants and *Babesia* species within single tick populations, and widespread co-infections of the *Ixodes* rickettsial endosymbiont with *B. burgdorferi* and other microbial agents. There was significant variation among populations in the frequency of infection by each microorganism, but the rates and types of co-infections were not significantly different from random except for the *Ixodes* endosymbiont and *B. burgdorferi* in male ticks, which co-occurred less frequently than expected. These results indicate that *I. scapularis* hosts a diverse array of pathogenic and symbiotic microorganisms and provide some potential evidence of interactions among microbial species.

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