Ticks and Tick-borne Diseases xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Microbiome analysis of *Ixodes scapularis* ticks from New York and Connecticut

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ARTICLE INFO

Keywords:
Ticks
Metagenomics
High-throughput sequencing
Borrelia
Bartonella
Ixodes scapularis

ABSTRACT

We employed high throughput sequencing to survey the microbiomes of *Ixodes scapularis* collected in New York and Connecticut. We examined 197 individual *I. scapularis* adults and pools from 132 adults and 197 nymphs. We detected *Borrelia burgdorferi* sensu stricto in 56.3% of individual ticks, *Anaplasma phagocytophilum* in 10.6%, *Borrelia miyamotoi* in 5%, *Babesia microti* in 7.6%, and Powassan virus in 3.6%. We did not detect *Borrelia mayonii*, *Ehrlichia muris eauclairensis, Bartonella* spp. or pathogenic *Babesia* species other than *B. microti*. The most abundant bacterium (65%), and only rickettsial species identified, was the endosymbiont *Rickettsia buchneri*. A filarial nematode was found in 13.7% of adult ticks. Fourteen viruses were detected including South Bay virus (22%) and blacklegged tick phlebovirus 1 and 2 (73%). This study provides insight into the microbial diversity of *I. scapularis* in New York State and Connecticut.

1. Introduction

The blacklegged tick, *Ixodes scapularis*, is among the most clinically important tick species in the United States (US). *I. scapularis* transmits the greatest diversity of pathogens of any tick within the US, including agents of Lyme disease (*Borrelia burgdorferi* sensu stricto and *Borrelia mayonii*), babesiosis (*Babesia microti*), anaplasmosis (*Anaplasma phagocytophilum*), *Borrelia miyamotoi* disease (*B. miyamotoi*), ehrlichiosis (*Ehrlichia muris eauclairensis*) and tick-borne encephalitis (Powassan virus) (Spielman et al., 1979; Burgdorfer et al., 1982; Pancholi et al., 1995; Telford et al., 1997; Tokarz et al., 2010; Pritt et al., 2011, 2016a, 2016b) Combined, these agents account for > 90% of all reported tick-borne diseases (Nelson et al., 2015; Connally et al., 2016). The promiscuous host selection of *I. scapularis*, coupled with the recent discovery and characterization of *B. mayonii* and *E. muris eauclairensis,* suggests that *I. scapularis* may harbor other yet-undiscovered human pathogens. The fact that *I. scapularis* is among the most frequent ticks encountered by humans advocates the need to catalogue all *I. scapularis*-associated microbes in order to identify the full spectrum of agents that can be transmitted by this tick.

Over the past decade, the employment of high-throughput sequencing (HTS) has substantially enhanced the detection of tick-borne agents. The use of HTS for tick microbiome analyses has also facilitated the discovery of novel tick borne-agents (Tokarz et al., 2014; Bouquet et al., 2017; Cross et al., 2018; Tokarz et al., 2018). In our previous work, we employed HTS to examine the virome of *I. scapularis* (Tokarz et al., 2014, 2018). In this study, we employed a metagenomic approach to survey the complete *I. scapularis* microbiome to gain insight into the capacity of *I. scapularis* to serve as a vector for additional agents of clinical significance.

2. Materials and methods

2.1. Tick extractions

All ticks were collected by dragging from sites in New York and Connecticut (Fig. 1). Ticks were extracted individually or in pools. All individual ticks were collected in 2016 and 2017 for this study. The homogenates from pooled ticks were obtained from ticks collected in 2015 and 2016 that were also previously used for virome analyses (Tokarz et al., 2018). To remove environmental contaminants, prior to extraction each individual tick was washed with 1 ml of 3% hydrogen peroxide, followed by 3 washes with 1 ml of nuclease free water. Ticks were homogenized in 100 µl of viral transport media, and the entire

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https://doi.org/10.1016/j.ttbdis.2019.04.011
Received 18 January 2019; Received in revised form 21 March 2019; Accepted 10 April 2019

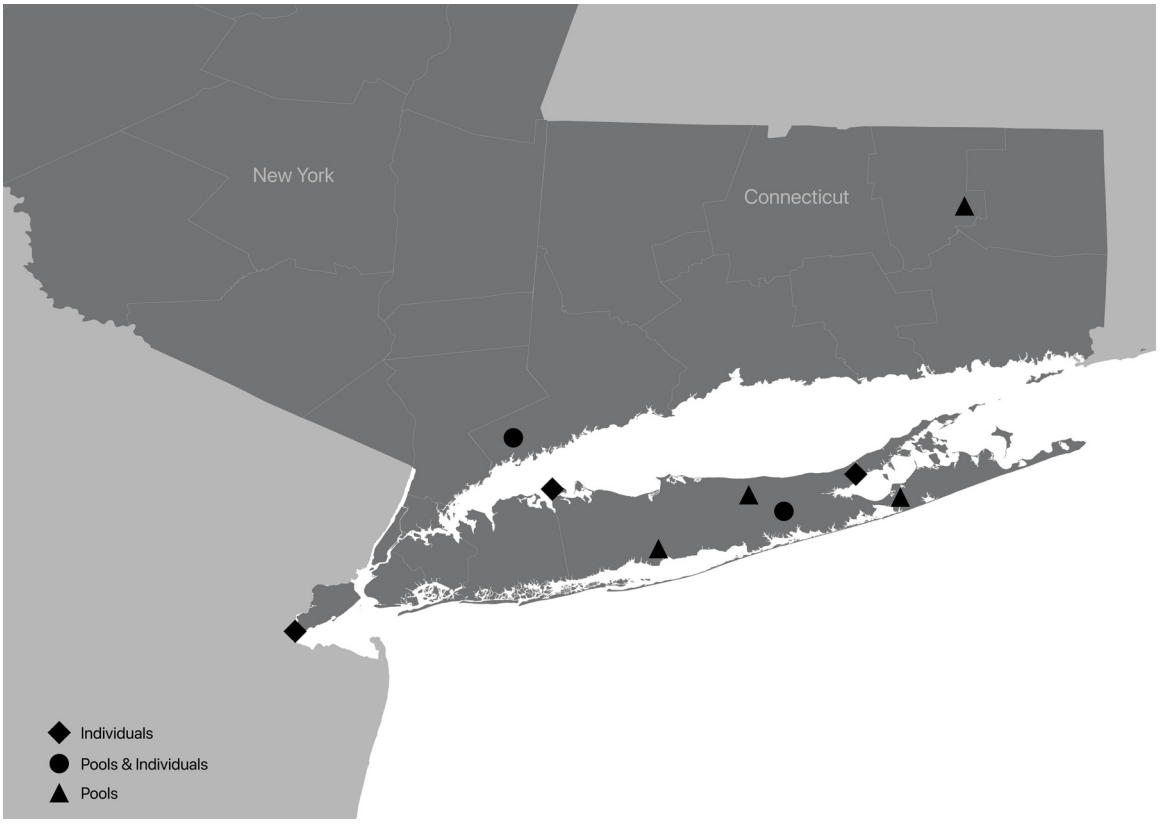

Fig. 1. Map of tick collection sites. Icons indicate the sites and the specimen types analyzed by high throughput sequencing (individual, pools or both).

volume was used for extraction of total nucleic acids (TNA) on the EasyMag platform (Biomerieux). TNA were eluted in 40 µl.

2.2. Library preparation

TNA (11 µl) was reverse transcribed using SuperScript III (ThermoFisher Scientific), treated with RNase H, followed by second-strand synthesis with Klenow fragment. Double-stranded DNA was sheared to a 200-bp average fragment length. Sheared DNA was purified and used for Illumina library construction using the KAPA Hyper Prep kit (KK8504, Kapa Biosystems). Libraries were quantified using an Agilent Bioanalyzer 2100 and sequencing was carried out on the Illumina HiSeq 4000 platform (Illumina, San Diego, CA, USA). Libraries were sequenced on 9 lanes (Table 1). Seven lanes included individual tick samples (16 to 32 tick samples per lane). Two lanes contained tick pools, including a lane with 8 pools of nymphs (20–25 nymphs per pool) and another lane that consisted of 10 pools of adult ticks. Six pools were from I. *scapularis*; we also sequenced 3 pools of *A. americanum* and 1 *D. variabilis* for microbiome comparison of the three tick species.

2.3. Bioinformatic and statistical analysis

The demultiplexed fastq files were trimmed using cutadapt software (v 1.8.3) (Martin, 2011) followed by generation of quality reports using FastQC software (v 0.11.5) (Andrews, 2010). The quality reports were used to determine filtering criteria based on the average quality scores of the reads, presence of indeterminate nucleotides and homopolymeric content of the reads. The reads were quality filtered and end-trimmed with PRINSEQ software (v 0.20.3) (Schmieder and Edwards, 2011). Host background levels were determined by mapping filtered reads against tick reference database using Bowtie2 mapper (v 2.2.9) (Langmead and Salzberg, 2012). The host-subtracted reads were de-novo assembled using MIRA (4.0) (Cheverux et al., 1999) and

Table 1
Pooling strategy for high throughput sequencing.

Sample type [#]	Site	[#] of ticks	Number of Raw Reads	Read Range
1. Tick Pools (N = 8)	Fifty Foot Cliff Preserve, CT	197[^]	368,789,299	5,8762,799 – 28,104,634
2. Tick Pools (N = 10)	Suffolk County[*], NY	220[&]	341,244,749	16,404,599 – 63,921,410
3. Individual ticks	NYC, NY	16	330,404,206	15,696,114 – 26,332,782
4. Individual ticks	Mianus State Park, CT	21	359,207,128	5,703,401 – 29,673,420
5. Individual ticks	Manorville, NY	32	331,736,276	3,958,492 – 21,425,762
6. Individual ticks	Manorville, Laurel Lake, NY	32	319,471,256	5,863,717 – 19,963,019
7. Individual ticks	Caumsett State Park, NY	32	240,880,684	4,691,670 – 13,987,925
8. Individual ticks	Caumsett State Park, NY	32	328,089,508	6,228,648 – 19,948,223
9. Individual ticks	Caumsett State Park, NY	32	327,787,247	7,027,998 – 20,798,708

[*] ticks originated from multiple sites.
[#] each row represents one lane on Illumina 4000.
[^] nymphs.
[&] only 132 were *I. scapularis*, in 6 pools.

MEGAHIT (v 1.0.4) (Li et al., 2015) assemblers. The contigs and unique singletons were annotated by homology search with Megablast against the GenBank nucleotide database. The sequences that showed poor or no homology at the nucleotide level were screened with BLASTX against the viral GenBank protein database. Sequences from viral BLASTX analysis were subjected to second round of BLASTX homology search against the complete GenBank protein database to correct for biased e-values and taxonomic mis-assignments. The abundance of the bacterial and invertebrate reads was determined from the top Megablast hit sequences. The read abundance was further normalized as counts per ten thousand filtered reads. Prevalence data for each agent was determined from the normalized reads obtained from individual ticks. In order to exclude non-tick borne bacterial sequences that can originate from environmental contaminants, we compared and subtracted reads that were also detected in negative control samples. Negative controls consisted of non-tick samples that went through the entire sequencing protocol, including extraction, library preparation and HTS.

The association of each identified agent with either male or female ticks was determined by comparing the prevalence of each agent in male ticks versus female ticks using Fisher's exact test, deriving a two-tailed p-value. Multiple comparisons were adjusted using Hochberg's step-up procedure controlling the family-wise error rate (FWER) at $\alpha = 0.05$ level (Hochberg, 1988). We also examined the association for every pair of agents using PAIRS (PAIRS, 2008) in which the observed C-score (Stone and Roberts, 1990) was compared to the expected C-score of the null model that represented complete randomness.

2.4. PCR

We designed a quantitative PCR assay for detection of *R. buchneri* targeting the *ompB* gene. For primer design, we used sequence data generated from this study, aligned with the *ompB* sequence of the reference strain (accession number EF433951.2). The assay consisted of fwd primer GATTACGGCAATCGAAGCTA, reverse primer, AGCATCGC CAATAAGAACAG and probe GCCGACGGTACAGTTATCACCGG.

We also designed an assay targeting the 16S rRNA gene of the genus *Bartonella*. To identify optimal sequences for primer design, we aligned sequences from 7 *Bartonella* species (*B. henselae* (accession number AJ223778), *B. vinsonii* (NR_104902), *B. phoceensis* (NR_115254), *B. queenslandensis* (EU111755), *B. senegalensis* (NR_125574), *B. acomydis* (NR_113288), *B. grahamii* (AB529507)) to agents with high homology to *Bartonella* (*Rhizobium petrolearium* (NR_116790)), agents identified in our HTS data (*Burkholderia* and *Mycobacteriacae* sp.), and known *I. scapularis*-transmitted pathogens (*B. burgdorferi* (L39081), *A. phagocytophilum* (AB196720)). Primer sites were chosen that displayed the highest specificity to *Bartonella*.

3. Results and discussion

We sequenced TNA from 197 individual adult *I. scapularis* (105 males and 92 females) on 7 lanes of Illumina HiSeq 4000 platform. Sequencing yielded between 240 and 359 million reads per lane, with a minimum of 3.96 million and a maximum of 29.7 million reads per individual tick sample (Table 1). For confirmation of the presence (or absence) of agents identified in individual ticks, we examined 17 tick pools on two lanes that generated 368 million and 341 million reads, respectively, with a range of 5.8 and 63.9 million reads per single pool.

3.1. Borrelia

We identified two species of *Borrelia* in the HTS data. *Borrelia burgdorferi* s.s. was detected in 111 (56.3%) individual ticks, and *Borrelia miyamotoi* in 10 (5.07%) (Table 2). Although several other *Borrelia* species have been detected in ticks throughout the US, we did not find evidence of any of these species in our samples (Marconi et al., 1995; Postic et al., 2007; Rudenko et al., 2009a, 2009b, Margos et al., 2010; Pritt et al., 2016a, 2016b, Margos et al., 2017). There was no significant difference in the prevalence of *Borrelia* in male or female ticks. Our results are consistent with published surveillance studies of *I. scapularis* by our group and others that reported significantly higher infection rates of *B. burgdorferi* s.s. relative to *B. miyamotoi* (Tokarz et al., 2017; Wroblewski et al., 2017; Cross et al., 2018). At the five sites analyzed in our study, the prevalence of *B. burgdorferi* s.s. ranged from 40% to 62.5%.

Table 2
Prevalence of *I. scapularis*-associated microbes.

Agent	Prevalence (# of positive ticks)
Bacteria	
Anaplasma phagocytophilum	10.6% (21)
Borrelia burgdorferi s.s.	56.3% (111)
Borrelia miyamotoi	5.07% (10)
Rickettsia buchnerii	65.0% (128)
Invertebrate	
Babesia microti[*]	8.6% (17)
Babesia odocoilei[*]	8.6% (17)
Ixodes scapularis nematode	13.7% (27)
Virus	
Blacklegged tick phlebovirus	73.10% (144)
BLTV-associated virus 1	5.10% (10)
BLTV-associated virus 2	1% (2)
Powassan virus	3.60% (7)
South Bay virus	21.80% (43)
Suffolk virus	9.60% (19)

[*] 2 ticks co-infected with *Babesia microti* and *Babesia odecolei*.

Two human pathogens transmitted by *I. scapularis*, *B. mayonii* and *Ehrlichia muris eauclairensis,* were not detected in individual ticks or tick pools. Although these agents have been detected in up to 3% of *I. scapularis* in the Upper Midwest, they have not yet been found outside of this region (Pritt et al., 2011; Johnson et al., 2015; Murphy et al., 2017). Our results confirm that *B. mayonii* and *E. muris eauclairensis* have not gained a significant foothold in the *I. scapularis* populations in the Northeast (Stromdahl et al., 2014).

3.2. Anaplasma

A. phagocytophilum, the agent of human granulocytic anaplasmosis, was present in 21 (10.6%) ticks. The majority (90%) of the *A. phagocytophilum*-positive ticks were infected with the Ap-ha strain. We did not detect other *Anaplasma* species.

3.3. Babesia

Babesia was the lone protozoan identified in our tick samples. We detected *Babesia* sequences in 32 (16.2%) ticks. Two species were present. Fifteen ticks (7.6%) were infected with *B. microti*, the primary species implicated in babesiosis in the United States (CDC, 2018). Another 15 ticks were infected with *B. odocoilei*, a species pathogenic in white-tailed deer and other animals, but not connected to human disease (Spindler et al., 1958; Gallatin et al., 2003; Holman et al., 2003). Two additional ticks were co-infected with both *B. microti* and *B. odecoilei*. Two other species, *B. duncani* and *B. divergens* have also been implicated in babesiosis in the United States (Persing et al., 1995; Herwaldt et al., 1996; Beattie et al., 2002; Yabsley and Shock, 2013; Herc et al., 2018; Scott and Scott, 2018). *B. duncani* has been found on the West Coast, although the vector(s), prevalence and true geographic distribution of this species are unknown. Similarly, limited data exists for *B. divergens,* although this species has been linked with *I. dentatus*, a tick that feeds primarily on rabbits and birds (Goethert and Telford, 2003). The absence of *B. duncani* and *B. divergens* sequences in our data suggests that *I. scapularis* is not a vector for these species, at least in the Northeastern part of the US.

Table 3
Comparison of *Bartonella* 16S rRNA assays.

Primer source	Primer sequence 5' to 3'	# of ticks tested	# of positive ticks	# of *Bartonella* positives PCR products*	BlastN* (% homology)
This study	Fwd- AATTTGTGCTAATACCGTATACGTCCT Rev- CACCTCTTCCACACTCAAGATA	45	0	0	N/A
Adelson et al	Fwd- GGAATTCCCTCCTTCAGTTAGGCTGG Rev- CGGGATCCCGAGATGGCTTTTGGAGATTA	45	10	0	*Phyllobacterium sp* (100%), *Ensifer sp.* (100%)

* bacteria were identified through BlastN homology searches.

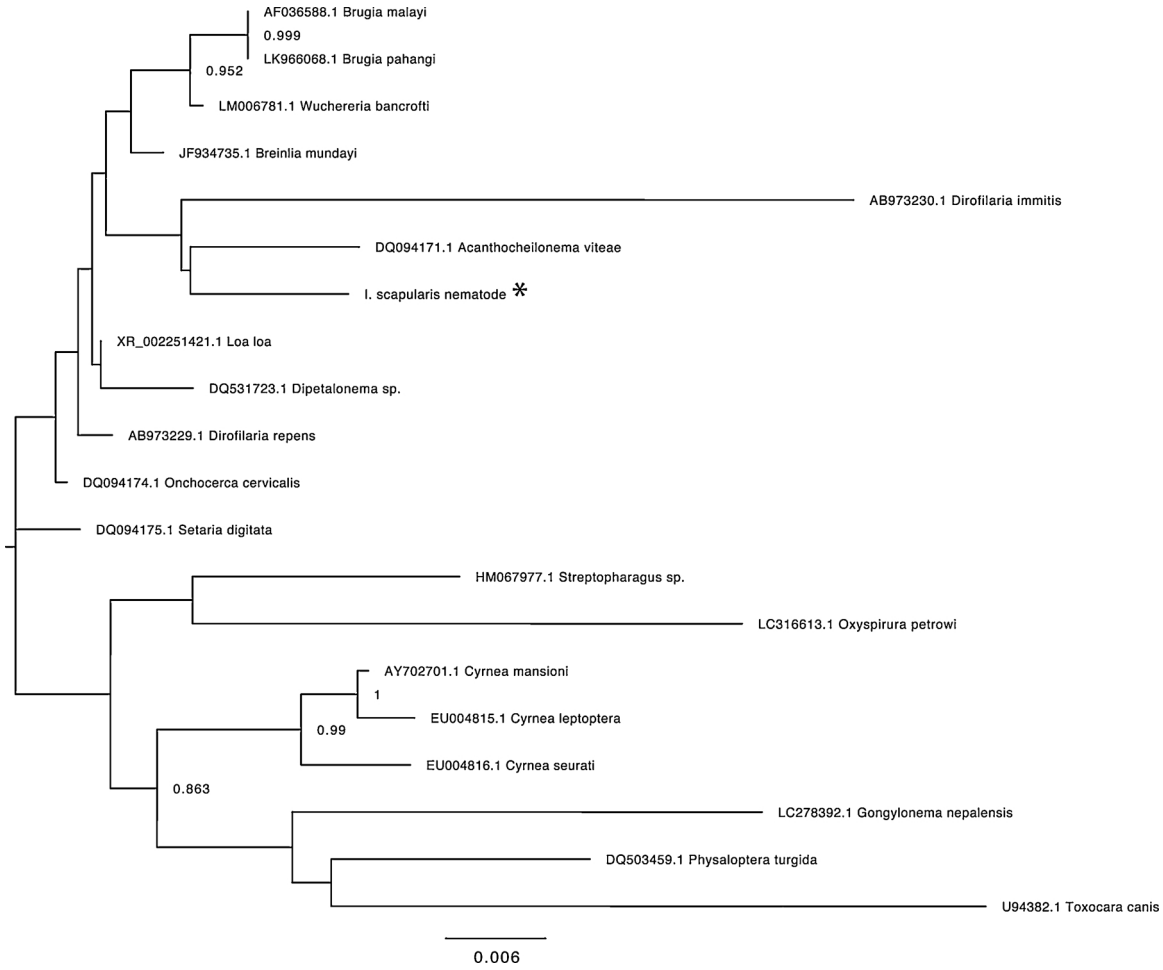

Fig. 2. Phylogenetic characterization of a putative nematode identified in this study. Relationships were inferred through the alignment of a 1562 nucleotide fragment of the 18 s rRNA gene of related filarial and non-filarial nematodes using CLUSTALW in Geneious 10.2.4. The sequence obtained in this study is indicated with an asterisk. The phylogenetic tree was constructed in MEGA 7.0.26 and the robustness of each node was determined using 1000 bootstrap replicates.

3.4. Rickettsia

A *Rickettsia* species was first identified in *I. scapularis* in 1991, and has subsequently been referred to as *Rickettsia cooleyi*, *Rickettsia midichlorii* and the recently adopted name, *Rickettsia buchneri* (Magnarelli et al., 1991),(Billings et al., 1998; Benson et al., 2004; Moreno et al., 2006; Troughton and Levin, 2007; Kurtti et al., 2015). Like many other tick-associated *Rickettsia*, *R. buchneri* is suspected to be an endosymbiont. The endosymbiotic *Rickettsia* of ticks are transovarially transmitted, and presumed not to establish infection in other invertebrate hosts (Cheng et al., 2013). *I. scapularis* has not been implicated as a host of other species of *Rickettsia*. *R. buchneri* was the lone *Rickettsia* identified in our study. We detected *R. buchneri* in 128 (65%) of adult ticks (Table 2). Congruent with reports that infection is more common in female ticks, we detected *R. buchneri* in 89% of females versus 44% of males (Cross et al., 2018). This female tick-specific association of *Rickettsia* was the only significant sex-specific association among all identified agents. When assessed by quantitative PCR, the *Rickettsia* burden was not significantly different between male and female ticks (data not shown). To determine the prevalence of *R. buchneri* in immature stages of *I. scapularis*, we tested TNA of 25 nymphs by quantitative PCR, all 25 were *Rickettsia*-positive, suggesting that the loss in *Rickettsia* occurs at the time of the molt from nymphs to adults.

3.5. Bartonella

Bartonella species have been promoted as agents of tick-borne disease (Eskow et al., 2001; Telford and Wormser, 2010). Reports of *Bartonella* DNA in ticks, coupled with detection of this bacteria in patients with Lyme disease, have led to the proposal that *Bartonella* is a tick-transmitted pathogen and an upsurge in *Bartonella* testing of patients with a suspected tick-borne illness (Eskow et al., 2001; Adelson

Table 4
Co-infections detected by high throughput sequencing.

Two agents	
Agents	Number of positive ticks
B. burgdorferi s.s. *B. miyamotoi*	7 (3.5%)
B. burgdorferi s.s. Powassan virus	5 (2.5%)
B. burgdorferi s.s. *B. microti*	9 (4.5%)
B. burgdorferi s.s. *A. phagocytophilum*	9 (4.5%)
A. phagocytophilum *B. microti*	1 (< 1.0%)
A. phagocytophilum *B. miyamotoi*	1 (< 1.0%)
Three agents	
Agents	Number of positive ticks
B. burgdorferi s.s. *A.phagocytophilum* *B. miyamotoi*	1 (< 1.0%)
B. burgdorferi s.s. *A. phagocytophilum* Powassan virus	1 (< 1.0%)
B. burgdorferi s.s. *A. phagocytophilum* *B. microti*	4 (2.0%)

et al., 2004; Holden et al., 2006).

We did not detect *Bartonella* sequences in individual ticks or in tick-pools. We speculate that differences between our findings and published reports implicating *I. scapularis* as potential vectors of *Bartonella* may reflect several factors. First, the presence of microbial DNA within a tick does not conclusively establish the tick's competency as a vector, or the viability of the microbe. The DNA may instead be a remnant of a previous blood meal, and its concentration sufficient to be amplified and detected by sensitive molecular assays such as PCR. Rodents, a typical source of blood meals for *I. scapularis*, are frequent hosts of *Bartonella* species (Heller et al., 1998; Bermond et al., 2000; Gundi et al., 2004, 2009; Tadin et al., 2016). Therefore, some ticks that have previously fed on a *Bartonella*-infected mouse may contain residual *Bartonella* DNA. Second, inappropriate assay design in tick surveillance studies can contribute to an inaccurate assessment of *Bartonella* prevalence in ticks. One such study, from 2004 by Adelson et al, reported *Bartonella* in 33% of *I. scapularis* nymphs in New Jersey. The forward and reverse primers employed for the tick screening targeted a conserved fragment of the *Bartonella* 16S rRNA gene. However, both primers were also homologous to the corresponding 16S rRNA sequence from a wide range of soil bacteria and may have amplified trace amounts of soil bacterial contaminants in tick homogenates. This has also been suggested previously (Telford and Wormser, 2010). In addition, we found that the resulting 240 nucleotide fragment is not sufficient to adequately discriminate between a PCR product obtained from *Bartonella* spp and soil bacteria. To address this confound, we designed alternative 16S rRNA *Bartonella*-specific assays that would exclude soil bacteria. We tested multiple primer pairs, with the highest degree of specificity achieved with the primer pair shown in Table 3. We then tested TNA from 45 ticks from our study and did not identify any *Bartonella*-positive ticks. However, when we tested the same samples with primers employed by Adelson et al, 10 (13%) were positive. When we sequenced these PCR products, all were 100% identical to soil bacteria and not *Bartonella* (Table 3). Our results do not exclude that a portion of Lyme disease patients may indeed have a *Bartonella* infection. Nonetheless, we postulate that these infections are not due to *I. scapularis* transmission but may be acquired through alternative means of exposure, such as contact with infected domestic cats, or flea bites (Klotz et al., 2011). We also acknowledge that our results are limited to *I. scapularis* within the New York metropolitan area and do not exclude a role for other tick species in *Bartonella* transmission (Holden et al., 2006).

3.6. Other bacteria

Previous studies have shown that the diversity of *I. scapularis* bacteriome is limited (Ross et al., 2018). In our samples, *R. buchneri, Borrelia*, or *Anaplasma* (when present) typically accounted for > 80% of all bacterial reads. We also identified reads for *Wolbachia, Mycobacteria,* and *Burkholderia*. With the exception of *Wolbachia*, the low number of reads from other genera suggest that these bacteria may represent environmental contaminants.

3.7. Virome

We previously identified sequences of 21 putative viruses in *I. scapularis* (Tokarz et al., 2018). Fourteen of these viruses were also present in the individual ticks from this study, along with sequences of a novel totivirus. Consistent with our previous data, the most prevalent viruses were the transovarially transmitted Blacklegged tick phleboviruses 1 and 2 (found in 73% of the ticks), South Bay virus (22%), and Suffolk virus (10%). The pathogenic Powassan virus (deer-tick lineage) was present in 7 (3.6%) ticks.

Three rare viruses, Laurel Lake virus, *Ixodes scapularis* associated virus-5 and *Ixodes scapularis* associated virus-6, were all present in a single tick (tick sample RTs-604). All three viruses have limited homology to viruses identified in metagenomic analyses of fungi. In addition, the HTS data for RTs-604 were unique as it contained > 2 million reads for *Cordyceps brongniartii*, an entomopathogenic fungus. We also identified a tick pool that was positive for these three viruses as well as *C. brongniartii*. These data suggest that all three viruses are likely of fungal origin.

3.8. I. scapularis filarial nematode

Filarial nematodes were first observed in *I. scapularis* homogenates

34 years ago (Beaver and Burgdorfer, 1984). Recently, filarial nematode sequences were identified in I. scapularis from Connecticut (by PCR) and Wisconsin (by HTS) (Namrata et al., 2014; Cross et al., 2018). We identified nematode sequences in 27 (13.7%) ticks. Phylogenetic analysis of the complete 18S rRNA sequence indicated that it belongs to a distinct filarial nematode species with the highest genetic similarity to *Acanthocheilonema viteae* (Fig. 2). In addition, all ticks positive for *Wolbachia* were also positive for the filarial nematode. Since *Wolbachia* are known endosymbionts of nematodes the *Wolbachia* reads presumably originated from the nematodes, as suggested previously (Cross et al., 2018).

3.9. Co-infections

Thirty-eight ticks (19%) were co-infected with known pathogens (*A. phagocytophilum, B. burgdorferi* s.s., *B. miyamotoi, B. microti*, and Powassan virus) (Table 4). Thirty-two ticks (16%) were co-infected with two agents, and 6 (3%) with 3 agents. We also examined associations between the bacterial, eukaryotic and viral agents identified in our study. Two positive associations were identified (*B. microti* with BLTV1 and BLTPV and B. *burgdorferi* s.s.). In the recently published study by Cross et al, a significant association between *B. burgdorferi* s.s. and South Bay virus was identified (Cross et al., 2018). However, we did not observe this association in our sample set.

4. Conclusion

Our study provides further insight into the microbiome of *I. scapularis*. In addition to identifying known pathogens, we surveyed *I. scapularis* for other microbial agents that have the potential to cause disease. In conjunction with recently published microbiome studies from other geographical sites, we now possess a better understanding of the spectrum of agents that can be harbored by *I. scapularis*, which can serve to focus research and clinical treatment.

Acknowledgments

We'd like to thank Nischay Mishra and Simon H. Williams for assistance with the manuscript.

This work was supported by a grant from the Steven & Alexandra Cohen Foundation(SACF CU15-4008).

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