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Regional prevalences of *Borrelia burgdorferi*, *Borrelia bissettiae*, and *Bartonella henselae* in *Ixodes affinis, Ixodes pacificus* and *Ixodes scapularis* in the USA

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ABSTRACT

The objective of this work was to determine the prevalence of *Borrelia* and *Bartonella* species in *Ixodes* spp. ticks collected from 16 USA states. Genus PCR amplification and sequence analysis of *Bartonella* and *Borrelia* 16SsRNA-23SsRNA intergenic regions were performed on DNA extracted from 929 questing adult ticks (671 *Ixodes scapularis*, 155 *Ixodes affinis*, and 103 *Ixodes pacificus*). Overall, 129/929 (13.9%) *Ixodes* ticks were PCR positive for *Borrelia burgdorferi* sensu stricto, 48/929 for *B. bissettiae* whereas 23/929 (2.5%) were PCR positive for a *Bartonella henselae. Borrelia bissettiae* or *B. burgdorferi* s.s. and *B. henselae* co-infections were found in *I. affinis* from North Carolina at a rate of 4.5%; in a single *I. scapularis* from Minnesota, but not in *I. pacificus*. For both bacterial genera, PCR positive rates were highly variable depending on geographic location and tick species, with *Ixodes affinis* (n = 155) collected from North Carolina, being the tick species with the highest prevalence's for both *Borrelia* spp. (63.2%) and *B. henselae* (10.3%). Based on the results of this and other published studies, improved understanding of the enzootic cycle, transmission dynamics, and vector competence of *Ixodes* species (especially *I. affinis*) for transmission of *Borrelia* spp. and *B. henselae* should be a public health research priority.

1. Introduction

Blacklegged ticks (Acari: Ixodidae) are obligate vertebrate blood feeders and known vectors for a variety of zoonotic viruses, bacteria, and protozoan pathogens worldwide. In the United States, some species, including *I. affinis*, *I. pacificus* and *I. scapularis* are generalist host feeders and are established or potential vectors for 16 zoonotic pathogens (Nelder et al., 2016), including *Borrelia burgdorferi* (causative agent of Lyme disease) (Clark, 2004; Clark et al., 2002; Harrison et al., 2010; Holden et al., 2006; Johnson et al., 2017; Maggi et al., 2010; Nadolny et al., 2011; Nelder et al., 2016; Oliver et al., 2003; Rudenko et al., 2013; Tokarz et al., 2017; Wroblewski et al., 2017), *Bartonella henselae* (bartonellosis/cat scratch disease) (Holden et al., 2006), *B. quintana* (bartonellosis/trench fever) (Chang et al., 2001, 2002), *Anaplasma phagocytophilum* (anaplasmosis) (Adelson et al., 2004; Belongia, 2002; Holden et al., 2006; Johnson et al., 2017; Murphy et al., 2017; Swanson and Norris, 2007; Tokarz et al., 2017; Wroblewski et al., 2017), *Babesia microti* (babesiosis) (Adelson et al., 2004; Belongia, 2002; Swanson and Norris, 2007), and *B. miyamotoi* (relapsing fever borreliosis/ *B. miyamotoi* disease) (Barbour, 2014; Breuner et al., 2017; Han et al., 2016; Johnson et al., 2017; King et al., 2017; Nelder et al., 2016; Tokarz et al., 2017; Wroblewski et al., 2017), and Ehrlichia muris-like (EMLA) (Murphy et al., 2017).

Lyme disease, caused by *B. burgdorferi* sensu stricto, is estimated to affect 300,000 people a year in the United States (Lyme-CDC, 2018). Lyme disease accounts for almost 75% of VBD cases reported from the highly endemic region of the northeastern United States (Paddock et al., 2016). As vectors for disease transmission, ticks are associated with approximately 95% of human vector-borne disease (VBD) cases reported to the Centers for Disease Control and Prevention (CDC) (Paddock et al., 2016). In recent years, bartonellosis has been recognized as an emerging/re-emerging zoonotic infectious disease

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caused by numerous mammalian reservoir-adapted *Bartonella* species. At least 18 *Bartonella* spp. have been implicated as a cause of disease pathology in animals or humans (Breitschwerdt, 2017). Several arthropod vectors, including sand flies, lice, fleas, biting flies and ticks are known to transmit various *Bartonella* spp. to animals and humans in the USA and abroad (Breitschwerdt, 2014, 2017; Breitschwerdt et al., 2010; Chomel and Kasten, 2010; Guptill, 2010; Mada and Joel Chandranesan, 2017; Mayne, 2011). *Bartonella* spp. infections have been associated with a wide range of diseases such as Carrión´s disease, trench fever, cat-scratch disease, bacillary angiomatosis, peliosis hepatis, endocarditis, myocarditis, chronic lymphadenopathy, and neurological and rheumatologic disorders. It is estimated that *B. henselae* infections affect between 13,000 to 45,000 people yearly in the USA alone (Nelson et al., 2016).

In 1926, Noguchi reported *Dermacentor andersoni* transmission of *B. bacilliformis* (Noguchi et al., 1926). Subsequently, historical evidence supporting ticks as competent vectors for transmission of *Bartonella* organisms was mostly circumstantial and primarily based on clinical case reports, epidemiological associations, or the detection of *Bartonella* spp. DNA in ticks of the genus *Ixodes* (Adelson et al., 2004; Chang et al., 2002; Dietrich et al., 2010; Halos et al., 2005; Nelder et al., 2016; Vilcins et al., 2009), *Amblyomma* (Billeter et al., 2008), *Dermacentor* (Billeter et al., 2011; Chang et al., 2002; Ebani et al., 2015), *Rhipicephalus* (Billeter et al., 2011; Ereqat et al., 2016), and *Hyalomma* (Ereqat et al., 2016) among others. The role of ticks as competent vectors for *Bartonella* transmission remains controversial (Harrison et al., 2012; Telford and Wormser, 2010; Tijsse-Klasen et al., 2011). Recent laboratory studies have provided evidence supporting *Ixodes ricinus* transmission of *B. henselae to* cats using an artificial tick feeding model (Cotte et al., 2008; Reis et al., 2011). *In vivo* laboratory transmission of *B. birtlesii* by *I. ricinus* nymphs was confirmed using a rodent model (Cotte et al., 2008; Reis et al., 2011). We are not aware of laboratory confirmation of *B. henselae* by an *Ixodes* sp.

Since a relatively high prevalence of *Bartonella* or *Borrelia* spp. have been reported in *Ixodes* spp. (Adelson et al., 2004; Chang et al., 2001; Dietrich et al., 2010) from the USA and Europe, the present study surveyed the presence of both bacterial genera in three *Ixodes* species: *I. affinis*, *I. pacificus,* and *I. scapularis*.

2. Methods

2.1. Tick collection, processing, and DNA extraction

In total, 816 individual adult questing ticks were tested, including 671 *I. scapularis*, 155 *I. affinis* (from North Carolina), and 103 *I. pacificus* from southern California (Table 1). Questing ticks were collected by dragging. No tick was visibly engorged. Each tick was individually cleaned using three washes of 75% ethanol and processed for DNA extraction using Qiagen DNeasy Blood & Tissue Kits® (Qiagen, Germantown, MD USA 20874). DNA quality and quantity was assessed using NanoDrop® (Thermo Fisher Scientific, Waltham, MA USA 02451) measurement at 260/280 ratio. House keeping DNA testing, targeting mitochondrial 16SrRNA DNA using primers Tick16S1s: 5' CTG CTC AAT GAT TTT TTA AAT TGC TGT GGT 3' and Tick16S460as: 5' CCG GTC TGA ACT CAG ATC AAG TAG GA 3' (developed and validated in our laboratory), was used to assess DNA quality for DNA amplification.

2.2. Molecular testing

Polymerase chain reaction, targeting DNA of *Bartonella* spp. and *Borrelia* spp., was performed as previously reported (Maggi et al., 2011, 2008, 2010; Mascarelli et al., 2016) with minor modifications. Briefly, screening of *Bartonella* ITS region was performed using oligonucleotides 325s: 5′ CTT CAG ATG ATG ATC CCA AGC CTT CTG GCG 3′ and 1100as: 5′ GAA CCG ACG ACC CCC TGC TTG CAA AGC A 3′ as forward and reverse primers, respectively. Amplification was performed in a 25-µl final volume reaction containing 12.5 µl of MyTaq™ Red masterMix Mix (Bioline) 0.2 µl of 100 µM of each forward and reverse primer (IDT® DNA Technology, Coralville, IA USA 52241), 7.5 µl of molecular–grade water, and 5 µl of DNA from each sample tested. PCR negative controls were prepared using 5 µl of molecular grade water. Positive controls for PCR were prepared by using 5 µl of *B. henselae* genomic DNA at a concentration of 0.001 pg/µl (0.5 genome copies per microliter). Conventional PCR was performed in an Eppendorf Mastercycler EP-gradient® (Eppendorf Inc., Westbury, NY USA 11590) under the following conditions: a single hot-start cycle at 95 °C for 2 min followed by 55 cycles of denaturing at 94 °C for 15 s, annealing at 68 °C for 15 s, and extension at 72 °C for 18 s. Amplification was completed by an additional cycle at 72 °C for 1 min, and products were analyzed by 2% agarose gel electrophoresis with detection using ethidium bromide under ultraviolet light. Amplicon products were sequenced (Genewiz©, Research Triangle Park, NC USA 27709) to establish species strain identification.

PCR screening for *Borrelia* spp. DNA targeting the ITS region was performed using IGSA: 5' CACGA CTT TCT TCG CCT TAA AGC 3' and IGSB: 5' GTT AAG CTC TTA TTC GCT GAT GGT A 3' as forward and reverse primers, respectively. Amplification was performed in a 25 µl final volume reaction as described above for *Bartonella* DNA amplification. Positive PCR controls were prepared using 5 µl of DNA of genomic *B. burgdorferi* strain B31 at concentration of 0.005 pg/µl. Conventional PCR was performed in an Eppendorf Mastercycler EPgradient® under the following conditions: a single hot-start cycle at 95 °C for 2 min followed by 55 cycles of denaturing at 94 °C for 15 s, annealing at 64 °C for 15 s, and extension at 72 °C for 18 s. Amplification was completed by an additional cycle at 72 °C for 30 s. As described for *Bartonella* testing, products were analyzed by 2% agarose gel electrophoresis with detection using ethidium bromide under ultraviolet light, and positive samples were sequenced for species and strain identification.

Table 1
Borrelia burgdorferi s.s. and *Bartonella henselae* PCR results derived from *Ixodes* spp. ticks collected from different regions of the US.

Location	Number of ticks	*B. burgdorferi s.s.* positive ticks (%)	*B. henselae* positive ticks (%)	*B. burgdorferi*/*B. henselae* Co-infected ticks (%)
California	1[a]	0 (0)	0 (0)	0 (0)
California	103[b]	0 (0)	1 (1.0)	0 (0)
Connecticut	13[1]	8 (62)	0 (0)	0 (0)
Florida	2a[1]	0 (0)	0 (0)	0 (0)
Louisiana	1[a]	0 (0)	0 (0)	0 (0)
Maine	55[a]	0 (0)	0 (0)	0 (0)
Maryland	17[a]	7 (41)	0 (0)	0 (0)
Massachusetts	3[a]	2 (67)	0 (0)	0 (0)
Minnesota	62[a]	21 (34)	1 (2)	1 (2)
New Jersey	9[a]	4 (44)	0 (0)	0 (0)
New York	8[a]	6 (75)	0 (0)	0 (0)
North Carolina	348[a]	1 (0.3)	5 (1.4)	0 (0)
North Carolina	155[c]	55 (35.5)	16 (10.3)	3 (1.9)[d]
Pennsylvania	21[a]	15 (71)	0 (0)	0 (0)
Rhode Island	120[a]	8 (6.7)	0 (0)	0 (0)
Virginia	8[a]	0 (0)	0 (0)	0 (0)
Washington DC	2[a]	1 (50)	0 (0)	0 (0)
Wisconsin	1[a]	1 (100)	0 (0)	0 (0)

[a] *Ixodes* scapularis.
[b] *Ixodes* pacificus.
[c] *Ixodes affinis*.
[d] Four additional *I. affinis* ticks were co-infected with *B. bissettiae* and *B. henselae*.

3. Results

Based upon house keeping gene amplification, PCR inhibition was not detected for any tick extracted DNA sample (results not shown). The

Table 2
Prevalence of *Borrelia burgdorferi, Borrelia bissettiae, Bartonella henselae* and *Borrelia/B. henselae* co-infections in *Ixodes affinis*, *Ixodes pacificus*, and *Ixodes scapularis* ticks from the United States.

	N=	*B. burgdorferi* Infected (%)	*B. bissettiae* Infected (%)	*B. henselae* Infected (%)	*Borrelia* spp. + *B. henselae* Co-Infected (%)
Ixodes scapularis	671	74 (11.0)	0 (0)	6 (0.9)	1 (0.1)
Ixodes affinis	155	55 (35.5)	48 (31)	16 (10.3)	7 (4.5)
Ixodes pacificus	103	0 (0)	0 (0)	1 (1.0)	0 (0)
Total *Ixodes* spp.	929	129 (13.9)	48 (5.2)	23 (2.5)	8 (0.9)

limit of detection for DNA amplification was 0.005 pg/μl and 0.001 pg/μl for *B. burgdorferi* and *B. henselae* DNA, respectively, as previously established (Lantos et al., 2014; Maggi et al., 2012, 2010). *Borrelia* and *Bartonella* DNA were amplified in all PCR positive controls (0.005 pg/μl and 0.001 pg/μl concentration, respectivelly), but not from any of the PCR negative controls used during this work.

3.1. Bartonella DNA screening

B. henselae, the only *Bartonella* species PCR amplified and sequenced from ticks in this study, was found in California (1/103 *I. pacificus*), Minnesota (1 of 62 *I. scapularis*), and North Carolina (5/348 *I. scapularis*, 16/155 *I. affinis*), for an overall prevalence of 2.6% (Table 2). The highest prevalence of *B. henselae* infection was found in *I. affinis* collected from NC, where *B. henselae* was amplified from 16 tick samples (10%).

3.2. Borrelia DNA screening

74 of 671 (11%) *I. scapularis* and 55/155 (36%) *I. affinis* were PCR positive for *B. burgdorferi*. *Borrelia* DNA was not amplified from 103 *I. pacificus* ticks from southern California or from *I. scapularis* collected in Florida, Louisiana or Virginia. The prevalence of *B. burgdorferi* in *I. scapularis* varied from 0.5–71.4% in the remaining states (Table 1). In addition to *B. burgdorferi*, *B. bissettiae* DNA, a member of the *B. burgdorferi* sensu lato complex (Maggi et al., 2010; Rudenko et al., 2014, 2011), was PCR amplified and sequenced from 48/155 (31%) *I. affinis* tick extractions. *Borrelia bissettiae* DNA was not amplified from ticks from other states. No other *Borrelia* spp. were amplified from the ticks in this study. Overall, *B. bissettiae* and/or *B. burgdorferi* DNA was amplified and sequenced from 98/155 (63.2%) *Ixodes affinis* (Table 2).

3.3. Co-infection

B. burgdorferi and *B. henselae* co-infections were infrequently documented among the 929 *Ixodes* ticks tested. In Minnesota, only 1/21 (5%) *B. burgdorferi* positive ticks was co-infected with *B. henselae*. Co-infection was more prevalent for *I. affinis* (Table 2). *B. burgdorferi* and *B. henselae* co-infection was found in 3/155 (2%) *I. affinis*, whereas *B. bissettiae* and *B. henselae* co-infection was found in 4/155 (2.6%) *I. affinis,* with an overall co-infection rate of 7/155 (4.5%) ticks tested.

4. Discussion and conclusions

In the United States, *Ixodes* ticks are the predominant vectors for *B. burgdorferi* transmission among animals and humans. Based upon PCR amplification of *Bartonella* DNA from questing ticks, *Ixodes* spp. are potential vectors for *B. henselae* transmission. In this study, we determined the prevelance of *Bartonella* and *Borrelia* spp. DNA in three *Ixodes* adult species (*I. scapularis*, *I. affinis*, and *I. pacificus*) collected from different regions of the US. As previously reported, our results indicate a higher prevalence for *B. burgdorferi* in the North and Northeast, than for other regions of the US (Table 1), with an average of 11.0% for *I. scapularis*, 35.5% for *I. affinis*, and 0% for *I. pacificus* in southern California (Table 2). Interestingly, other than *B. bissettiae,* no other *Borrelia* spp. DNA was amplified in this study. In *I. affinis*, *B. bisettiiae* or *B. burgdorferi* DNA was amplified from 63.2% of 155 *I. affinis* from North Carolina (Maggi et al., 2010) compared to 0.3% of 348 *I. scapularis* ticks from North Carolina tested in an identical manner. Although *I. affinis* is infrequently reported to attach to humans, even infrequent exposures to this tick species may carry a risk for transmission of *B. burgdorferi* and/or *B. bisettii.*

Bartonella henselae was the only *Bartonella* species detected in this study. A very low prevalence of *B. henselae* DNA was found in *I. scapularis* from all regions surveyed (Table 1). The highest *B. henselae* prevalence was found in *I. affinis* ticks, with 10% of the 155 surveyed ticks being *B. henselae* PCR/DNA sequence positive. Based upon DNA sequencing, two *B. henselae* 16S-23S ITS strain types (*B. henselae* Houston 1 or San Antonio 2 strain) were identified. Our prevalence results are in contrast to previous studies where *Bartonella* DNA prevalences as high as 34.5% and 37.7% to 38.2% have been reported in New Jersey (USA) for *I. scapularis* and in Europe for *I. ricinus* (Adelson et al., 2004; Corrain et al., 2012; Dietrich et al., 2010; Ebani et al., 2015). This discrepancy could be attributed to regional (even at small geographical scale) differences, as reported from France (Vayssier-Taussat et al., 2016), where *B. henselae* prevalence varied from 0 to 36% in *Ixodes ricinus* collected within relatively close proximities.

Two different (and perhaps co-related) facts may account for differences in *Bartonella* prevalence among Ixodid ticks from the same or from disparate geographic regions: spatial-temporal distribution (clustering) and host association differences between larvae, nymph, and adult ticks (Dietrich et al., 2010; Nelder et al., 2014; Paul et al., 2016). Dietrich et al. reported a 14-fold higher *B. henselae* DNA prevalence in *I. ricinus* nymphal ticks when compared with adult ticks from the same geographic regions (Dietrich et al., 2010). Similarly, PCR positive rates varied from 11.8% to 38.2% for nymphs and from 0% to 12% for adult ticks depending on the region surveyed (Dietrich et al., 2010). Host preference, regional and/or seasonal variances seem to be the most likely reasons for the un-even pathogen prevalences for *Bartonella* and *Borrelia* spp. reported in *I. ricinus* in Europe (Bogumila and Adamska, 2005; Corrain et al., 2012; Cotte et al., 2010; Dietrich et al., 2010; Egyed et al., 2012; Halos et al., 2005; Hercik et al., 2007; La Scola et al., 2004; Raileanu et al., 2017; Sytykiewicz et al., 2012; Tijsse-Klasen et al., 2011) and in *I. scapularis* in the US (Adelson et al., 2004; Becker et al., 2016; Belongia, 2002; Hinrichsen et al., 2001; Lukin and Grabarev, 1999; Nelder et al., 2016). The potential impact/s of environmental, geospatial and tick life stage were not assessed in this study.

In this study, co-detection of *B. henselae* and *B. burgdorferi* DNA within a tick was infrequent, with *I. affinis* ticks being the species with the highest percentage of co-infection (5%).

Our co-infection findings also differ somewhat from previous reports from the US and Europe. Co-infection with *Bartonella* spp. and *B. burgdorferi* was reported in 8.4% of 107 *I. scapularis* tested from New Jersey (Adelson et al., 2004). In contrast, co-infection with *B. henselae* and *B. burgdorferi* was not documented in 419 adults *I. ricinus* from Germany and France (Dietrich et al., 2010). It is important to note that, for most of the states surveyed in this study, a very low number of ticks were available for testing. Therefore, our results are not representative of optimal tick sample size or distributions necessary for accurate

assessment of pathogen prevalence's within the three *Ixodes* spp. throughout the US. However, we were able to confirm *B. henselae* DNA in all three tick species tested. In addition to vectorial differences among the three *Ixodes* spp., host preferences, regional, and/or seasonal variances may be responsible in part for differences in *Bartonella* and *Borrelia* spp. prevalence's.

Based upon the results of this and other studies (Clark et al., 2002; Harrison et al., 2010; Maggi et al., 2010; Rudenko et al., 2013), a thorough understanding of the enzootic cycle as well as the vector competence of *I. affinis* for potential transmission of both *B. bissettiae, B. burgdorferi* and *B. henselae* should be emphasized. With the advent of new and improved diagnostic technologies, its seems that bartonellosis and borreliosis disease awareness continues to increase (Nelder et al., 2016). Nevertheless, due to human population growth into suburban and rural areas, fluctuations in tick's host species densities, and the apparent climate changes that may favor *Ixodes* tick expansions and survival (Nelder et al., 2014; Paul et al., 2016), the full spectrum of animal and human tick-transmitted diseases remains incompletely characterized and funding to investigate the impacts will provide a more thorough risk assessment.

Authors' contribution

EBB and RGM supervised and conducted the study as presented here. MT and TM collected ticks for testing. TR assisted with PCR testing of extracted tick DNA. All authors contributed to, read and approved the final version of the manuscript.

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Competing interests

The authors declare that they have no competing interest.

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