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Prevalence and Geographic Distribution of *Borrelia miyamotoi* in Host-Seeking *Ixodes pacificus* (Acari: Ixodidae) Nymphs in Mendocino County, California

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Abstract

Borrelia miyamotoi is an increasingly recognized human pathogen transmitted by *Ixodes* ticks in the Northern Hemisphere. In North America, infection prevalences of *B. miyamotoi* are characteristically low (<10%) in *Ixodes scapularis* (Say; Acari: Ixodidae) and Ixodes *pacificus* (Cooley & Kohls; Acari: Ixodidae), both of which readily bite humans. We tested 3,255 host-seeking *I. pacificus* nymphs collected in 2004 from 79 sites throughout Mendocino County in north-coastal California for presence of *B. miyamotoi*. The collection sites represented a variety of forest types ranging from hot, dry oak woodlands in the southeast, to coastal redwoods in the west, and Ponderosa pine and Douglas fir-dominated areas in the northern part of the county. We found that *B. miyamotoi* was geographically widespread, but infected *I. pacificus* nymphs infrequently (cumulative prevalence of 1.4%). Infection prevalence was not significantly associated with geographic region or woodland type, and neither density of host-seeking nymphs, nor infection with *Borrelia burgdorferi* sensu stricto was associated with *B. miyamotoi* infection status in individual ticks. Because *B. burgdorferi* prevalence at the same sites was previously associated with woodland type and nymphal density, our results suggest that despite sharing a common vector, the primary modes of enzootic maintenance for the two pathogens are likely different.

Keywords

Borrelia miyamotoi; *Ixodes pacificus*; relapsing fever spirochetes; tick-borne disease

Borrelia miyamotoi is a hard tick-borne relapsing fever spirochete first isolated from *Ixodes persulcatus* Schulze in Japan in 1994 (Fukunaga et al. 1995) that has recently gained recognition as an emerging human pathogen (Platonov et al. 2011, Krause et al. 2015, Molloy et al. 2015, Wagemakers et al. 2015). Human cases of *B. miyamotoi* disease have now been reported in temperate regions of Asia, Europe, and eastern North America where *I. persulcatus* Schultz, *Ixodes ricinus* L., and *Ixodes scapularis* (Say; Acari: Ixodidae), the ticks

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that likely serve as primary tick vectors of this pathogen to humans, are present (Platonov et al. 2011, Chowdri et al. 2013, Hovius et al. 2013, Sato et al. 2014, Molloy et al. 2015).

Natural infection with *B. miyamotoi* occurs in a variety of *Ixodes* spp., including the closely related group responsible for transmission of *Borrelia burgdorferi* sensulato (s.l.), the spirochetes that cause Lyme disease. In North America, vector competence for *B. miyamotoi* has been demonstrated for *I. scapularis* (Scoles et al. 2001, Breuner et al. 2017) and is suspected for Ixodes *pacificus* (Cooley & Kohls; Acari: Ixodidae). Though both the nymphal and adult stages of these ticks are frequently observed biting humans, nymphs are more commonly associated with pathogen transmission, and therefore, recognized as having greater epidemiological significance (Merten and Durden 2000, Eisen et al. 2017). For that reason, many surveys, including this one focus specifically on the nymphal stage. However, there is also evidence suggesting that transmission by larval ticks may account for some human exposure to *B. miyamotoi* (Barbour et al. 2009, Molloy et al. 2015, van Duijvendijk et al. 2016).

Globally, prevalence of *B. miyamotoi* in host-seeking *Ixodes* ticks ranges from 1 to 10%, with most local estimates falling closer to the low end (Scoles et al. 2001, Barbour et al. 2009, Rollend et al. 2013, Sinski et al. 2016, Rar et al. 2017). In California, *B. miyamotoi* infection prevalences of 1.6 and 1.7% were reported in *I. pacificus* nymphs from Mendocino and Sonoma counties (Mun et al. 2006, Crowder et al. 2014), and 1.4% statewide (Padgett et al. 2014). Collectively, these studies reveal that *B. miyamotoi* is widespread but usually at low prevalence in host-seeking tick populations. Current genotypic evidence supports categorizing the species into three types: Asian (primarily vectored by *I. persulcatus* and *I. ricinus*), European (*I. ricinus*), and American (*I. scapularis* and *I. pacificus*), with isolates showing little or no genetic variability within type (Geller et al. 2012, Barbour 2014, Crowder et al. 2014, Takano et al. 2014, Mukhacheva et al. 2015, Cook et al. 2016).

Intriguingly, Lyme disease spirochetes typically infect *Ixodes* nymphs with greater frequency (prevalence 3–49%) than *B. miyamotoi* in areas where the two pathogens are sympatric (Barbour et al. 2009, Wagemakers et al. 2015, Rar et al. 2017) and there is considerably more genetic diversity within the *B. burgdorferi* s.l. species complex than *B. miyamotoi* (Girard et al. 2009, Margos et al. 2011, Mechai et al. 2015, Rar et al. 2017). In California, prevalence of *B. burgdorferi* s.l. in host-seeking *I. pacificus* nymphs is typically 2–3 times (3.2–4.9%) that of *B. miyamotoi* (Padgett et al. 2014).

Here we 1) describe the distribution and prevalence of *B. miyamotoi* in host-seeking *I. pacificus* nymphs collected throughout Mendocino County, CA, a county where both *B. miyamotoi* and *B. burgdorferi* s.s. are maintained in enzootic cycles and the ecology of the latter has been well-studied, 2) compare infection prevalence in nymphs across woodland types, and 3) compare these findings with those previously described for *B. burgdorferi* sensu stricto (s.s.) in the same sample population of ticks.

Materials and Methods

Study Sites and Tick Collection

The *I. pacificus* nymphs tested in this study were collected from 79 dense woodland sites throughout Mendocino County, CA, in 2004 (Eisen et al. 2006, Eisen et al. 2010). Locations were sampled twice within the period of peak nymphal activity in the spring (late April to early June). The collection sites were selected to reflect the variability of climate across the range of *I. pacificus* habitat in Mendocino County as well as to account for the ecological diversity associated with the different forest types where these ticks are present. Woodland categories were designated based on the predominant tree species at the sampling sites, including redwood ($n = 11$), coastal pine ($n = 3$), inland pine ($n = 8$), hardwood ($n = 19$), hardwood/conifer ($n = 22$), mixed tan oak/madrone or Douglas fir with a redwood influence ($n = 4$), or tanoak ($n = 12$).

Tree species found at sites categorized as redwood included coastal redwood (*Sequoia sempervirens* D. Don), Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco), tanoak (*Lithocarpus densiflorus* [Hook. & Arn.] Manos, Cannon & S.H. Oh), and California bay (*Umbellularia californica* [Hook & Arn.] Nutt.). Coastal pine sites included mainly Bishop pine (*Pinus muricata* D. Don) with coastal redwood or Douglas fir. Inland pine woodlands consisted of Ponderosa pine (*Pinus ponderosa* Douglas ex C. Lawson) and Douglas fir. Hardwood sites were comprised of *Quercus* spp. oaks, Pacific madrone (*Arbutus menziesii* Pursh) and California bay. Hardwood/conifer sites included mixed oaks, Pacific madrone, California bay, and Douglas fir or Ponderosa pine. Sites designated as mixed tanoak/madrone or Douglas fir with a redwood influence included mixed tanoak, Pacific madrone, or Douglas fir with redwood influence in or adjacent to the sampling site. Tanoak consisted primarily of tanoak with coastal redwood, Douglas fir, or Pacific madrone. More detailed climatic data and tick sampling methodology can be found in Eisen et al. (2006) and Eisen et al. (2010).

PCR-Based Detection of *B. miyamotoi* in Tick Samples

For most sites ($n = 59$), DNA extracts from at least 45 individual *I. pacificus* nymphs were tested. Another eight sites contributed between 11 and 32 nymphs, leaving 12 sites with 10 or fewer ticks tested. DNA had been previously extracted using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocol for animal tissue (Girard et al. 2009), eluted in 50 µl deionized sterile water, and stored at −20°C. We used a pair of real-time TaqMan PCR assays (Graham et al. 2016) to detect *B. miyamotoi*: a singleplex assay targeting the glycerophosphodiester phosphodiesterase (*glpQ*) gene of *B. miyamotoi*, and a duplex assay targeting the adenylosuccinate lyase (*purB*) gene of *B. miyamotoi* and the actin gene in *I. scapularis* and *I. pacificus*. The actin target allowed us to verify the presence of amplifiable DNA in each sample. Each reaction included 4.8 µl DNA extract and 5.2 µl master mix as described in Graham et al. (2016).

Prior to running individual samples through the assay, we confirmed a limit of detection equivalent to ≤5 genomes per reaction in the presence of background tick DNA from the Mendocino collection extracts. Specifically, we pooled equal volumes of DNA from

randomly selected specimens collected at 12 different sites. The pooled DNA tested positive for the tick actin target and negative for both *B. miyamotoi* targets. We then spiked aliquots of the *B. miyamotoi*- free tick DNA with genomic *B. miyamotoi* DNA. Using an estimated genome size of 1.3×10^6 nucleotides (Kingry et al. 2017), we calculated the approximate number of *B. miyamotoi* genome copies per ng of DNA. We determined the double-stranded concentration of our *B. miyamotoi* DNA stock using a Qubit 2.0 fluorometer and the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific Inc., Waltman, MA) immediately before preparing serial dilutions to achieve the equivalent of 10, 5, or 1 genome per reaction. Six replicates of each concentration were tested and all replicates containing the equivalent of five or more genomes were positive for both *B. miyamotoi* targets (*purB* and *glpQ*). Assuming at least one genome per spirochete, we would thus expect to consistently detect *B. miyamotoi* DNA in these samples down to five or fewer spirochetes per reaction.

PCR results were analyzed using CFX Manager 3.1 software (Bio-Rad, Hercules, CA) with the regression option selected for quantitation cycle (Cq) determination. Controls on each plate included a no-template control (elution buffer), DNA extracted from uninfected, colony-reared *I. scapularis* nymphs (Centers for Disease Control and Prevention, Fort Collins, CO), and recombinant plasmid DNA containing the *purB* and *glpQ* target sequences as previously described (Graham et al. 2016). Samples were considered positive if Cq values for both *B. miyamotoi* targets were ≤40. If we initially detected only one *B. miyamotoi* target in a sample, we repeated the PCR. This occurred only three times, and upon repeat, all three samples yielded corroborating results between the two targets.

Statistical Analyses and Mapping

Point estimates and confidence limits of infection prevalence were calculated based on maximum likelihood estimates (MLE) and score confidence intervals (Newcombe, 2012). Global Moran's Index was used in spatial clustering analysis based on point estimates of the density of infected nymphs and was performed using ArcMap software (ArcGIS v. 10.4, Redlands, CA). Logistic regression was used to assess the relationship between nymphal infection status and density of host-seeking nymphs among sites. A chi-squared test was used to determine whether co-infection of nymphs with *B. miyamotoi* and *B. burgdorferi* s.s. occurred as frequently as would be expected based on the probability that either agent by itself was infected. Logistic regression and chi-squared tests were performed using JMP 11.1.1 (SAS Institute, Cary, NC). A homogeneous stratum effect (HSE) test (Zhao 2014) and individual McNemar's tests, stratified by site, were used to evaluate any association of *B. miyamotoi* infection status with *B. burgdorferi* s.s. infections in ticks. Logistic regression was also used to analyze the relationship between *B. miyamotoi* prevalence and woodland type using Firth's correction (Firth 1993). The HSE test, McNemar's tests, and second logistic regression analysis were carried out in R (R Core Team 2017), with the latter using the 'brglm' package. Maps were developed using ArcMap projected to TealAlbers zone 10 NAD 1927.

Results and Discussion

Consistent with previous studies assessing a large number of ticks collected across multiple geographic locations (Barbour et al. 2009, Crowder et al. 2014, Padgett et al. 2014), we found that *B. miyamotoi* is geographically widespread in Mendocino County, but occurs at low prevalence in host-seeking *I. pacificus* nymphs. Of the 3,255 nymphs tested, 44 (1.4%; 95% CI: 1.0–1.8%) were PCR positive for *B. miyamotoi*. This overall infection prevalence of 1.4% for *B. miyamotoi* in *I. pacificus* nymphs is in remarkable agreement with previous studies testing ticks collected in southeastern Mendocino County (1.7%) (Mun et al. 2006) and statewide (1.4%) (Padgett et al. 2014), and is considerably lower than the prevalence of *B. burgdorferi* s.s. (average of 4.9%, range among sites 0–22%) previously reported for these host-seeking nymphal ticks (Eisen et al. 2010). We identified at least one *B. miyamotoi*-infected nymph from each of 24 locations (30.4% of sites sampled) extending from the hot, dry oak-woodland dominated southeastern corner of the county, to cooler and more humid conifer-dominated woodlands in the northeastern and northwestern parts of the county. Given the low infection prevalences observed, it is likely that *B. miyamotoi* is present at additional sites from which few ticks were available for testing. Notably, densities of host-seeking nymphs were low in coastal redwood, inland pine and Ponderosa pine dominated sites in the far western, and northern portions of the county resulting in wide confidence intervals (Fig. 1). Irrespective of tick abundance, risk for human exposure to *B. miyamotoi* is limited by the low prevalence of infection in ticks in these locations.

Site-specific MLE for infection prevalence ranged from 0 to 10.5% (Fig. 1), and the MLE for density of infected nymphs collected by dragging leaf-litter or fir-needle areas was less than 1 per 100 m^2 at all sites. There was no significant evidence of spatial clustering of sites based on density of infected nymphs (Global Moran's I., z = 1.47, *P*-value = 0.14), indicating that the risk of encountering nymphs infected with *B. miyamotoi* does not appear to be concentrated within geographical foci. The nymphs included in this study represent a subset of those that were screened previously for *B. burgdorferi* s.s. (Eisen et al. 2010), enabling direct comparisons of infection prevalence and spatial patterns of infection between *B. burgdorferi* s.s. and *B. miyamotoi* in host-seeking *I. pacificus*. Woodland type was not a significant predictor of *B. miyamotoi* prevalence (*P*-value = 0.52), nor was density of questing nymphs associated with *B. miyamotoi* infection status at these locations (*P*-value = 1.0). As with estimates of infection prevalence per site, it is possible that relationships between *B. miyamotoi* and environmental factors could be obscured by the low infection prevalence in ticks. However, prevalence of *B. burgdorferi* s.s. in these nymphs was previously shown to vary significantly according to woodland type, and was also positively associated with density of host-seeking nymphs (Eisen et al. 2010). The results from Mendocino County align with those recently reported for *I. ricinus*, whereas in contrast to the Lyme disease spirochete *Borrelia afzelii*, *B. miyamotoi* prevalence in the same sample of nymphs was not associated with forest type (Ruyts et al. 2017).

Notably, only a single tick tested positive for both *B. miyamotoi* and *B. burgdorferi* s.s., which did not differ significantly (*P*-value = 0.43) from the number of co-infections expected based on prevalence of the two species independently. Individual tick infection status was significantly different statistically across sites, so separate McNemar's tests

analyzed the relationship of the two *Borrelia* species by site. Forty-five of 55 sites tested (23 sites did not have enough information for the test to run) failed to show a statistically significant relationship, suggesting that any potential interaction occurring between *B. burgdorferi* s.s. and *B. miyamotoi* is unlikely to influence infection status in ticks. These results correspond with a previous report where *B. miyamotoi* and *B. burgdorferi* s.s. occurred at frequencies independent of one another in *I. scapularis* (Barbour et al. 2009).

Such comparisons reiterate important questions previously raised regarding the transmission cycle of *B. miyamotoi* and in particular, why prevalence of this spirochete in nymphs is consistently lower than that of sympatric *B. burgdorferi* s.l. spirochetes, despite sharing common vectors and exposure to the same potential reservoir hosts. One possibility is that in contrast to rodents infected with *B. burgdorferi* s.l. group spirochetes, which frequently exhibit prolonged or chronic periods of infectiousness (Brown and Lane 1992, Richter et al. 2004, Hanincova et al. 2008, Salkeld et al. 2008), a brief duration of active *B. miyamotoi* infection in rodents (Taylor et al. 2013, van Duijvendijk et al. 2016) may limit the temporal window for naïve feeding ticks to acquire infection. Perhaps more importantly, the uniformly low prevalence of *B. miyamotoi* in ticks across different habitats and in the various vertebrate host communities they feed on in Mendocino County and elsewhere (Fukunaga et al. 1995, Bunikis and Barbour 2005, Scott et al. 2010, Hamer et al. 2012, Taylor et al. 2013, Burri et al. 2014, Szekeres et al. 2015, Wagemakers et al. 2017) suggests that the local composition of host species may not be a major factor influencing the prevalence of infection in ticks. It is possible that there may not be strong amplifying reservoir hosts for *B. miyamotoi*, and that enzootic transmission involving most, or all hosts is inefficient. Moreover, acquisition of *B. miyamotoi* by uninfected ticks feeding on naturally infected hosts may be inefficient. Instead, transovarial transmission may play a more significant role than horizontal transmission in the perpetuation of *B. miyamotoi*. The lack of genotypic diversity among *B. miyamotoi* isolates may be further evidence of this. While it is established that vertebrate hosts drive genotypic diversity of *B. burgdorferi* s.l. and the geographic distribution of these genotypes (Kurtenbach et al. 2002, Brisson et al. 2004, Vollmer et al. 2011, Rudenko et al. 2014), the relative similarity among *B. miyamotoi* isolates suggests that host-mediated selection associated with horizontal transmission is not occurring at a magnitude equal to that in *B. burgdorferi* s.l. Instead, vertical transmission of spirochetes from female ticks to their offspring, which holds little or no importance in natural maintenance of *B. burgdorferi* s.l. (Rollend et al. 2013), but is well-recognized in relapsing fever *Borrelia* species associated with soft ticks (Barbour 2005), is likely to be equally or more important than enzootic transmission to natural maintenance of *B. miyamotoi*. Among the studies where definitive confirmation of *B. miyamotoi* infection by PCR was available, as many as 73% (Scoles et al. 2001) of the progeny from a single *I. scapularis* female, and more than 90% (Richter et al. 2012) of larvae from an unspecified number of *I. ricinus* females acquired infection via transovarial transmission. Future research should define the frequency of transovarial passage at a population level over several generations, assess the efficiency of this route in comparison to horizontal transmission, and further explore why *B. miyamotoi* is surprisingly scarce in nature, despite its ability to utilize multiple routes of transmission.

In summary, this study evaluated a large sample of *I. pacificus* nymphs collected from a single ecologically diverse Californian county for presence of *B. miyamotoi*. Our results support estimates from previous studies conducted at differing spatial scales in the region, and were presented in contrast to patterns of *B. burgdorferi* s.s. infection among the same group of ticks. Although *B. miyamotoi* is broadly distributed throughout Mendocino County across a variety of woodland types where *I. pacificus* is present, density of infected nymphs was consistently low due to low prevalence of infection in host-seeking nymphal ticks. Prevalence of *B. miyamotoi* in nymphs was not associated with any of the geographic or ecologic factors predictive of *B. burgdorferi* s.s. infection prevalence. Much about the natural history of *B. miyamotoi* remains to be explored, including the relative contributions of horizontal, transovarial, and co-feeding transmission to its natural maintenance.

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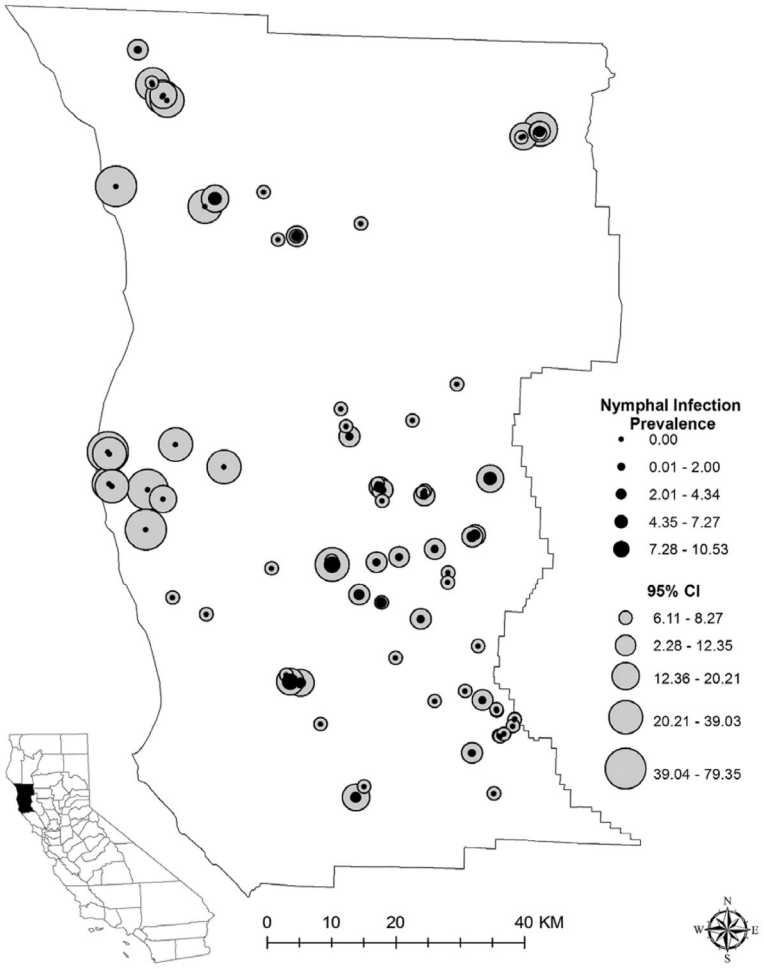

Fig. 1.

Infection prevalence estimates for *Borrelia miyamotoi* in *Ixodes pacificus* nymphs in Mendocino County, CA.