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A molecular algorithm to detect and differentiate human pathogens infecting *Ixodes scapularis* and *Ixodes pacificus* (Acari: Ixodidae)

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ABSTRACT

The incidence and geographic range of tick-borne illness associated with *Ixodes scapularis* and *Ixodes pacificus* have dramatically increased in recent decades. Anaplasmosis, babesiosis, and *Borrelia* spirochete infections, including Lyme borreliosis, account for tens of thousands of reported cases of tick-borne disease every year. Assays that reliably detect pathogens in ticks allow investigators and public health agencies to estimate the geographic distribution of human pathogens, assess geographic variation in their prevalence, and evaluate the effectiveness of prevention strategies. As investigators continue to describe new species within the *Borrelia burgdorferi* sensu lato complex and to recognize some *Ixodes*-borne *Borrelia* species as human pathogens, assays are needed to detect and differentiate these species. Here we describe an algorithm to detect and differentiate pathogens in unfed *I. scapularis* and *I. pacificus* nymphs including *Anaplasma phagocytophilum*, *Babesia microti*, *Borrelia burgdorferi* sensu stricto, *Borrelia mayonii*, and *Borrelia miyamotoi*. The algorithm comprises 5 TaqMan real-time polymerase chain reaction assays and 3 sequencing protocols. It employs multiple targets for each pathogen to optimize specificity, a gene target for *I. scapularis* and *I. pacificus* to verify tick-derived DNA quality, and a pan-*Borrelia* target to detect *Borrelia* species that may emerge as human disease agents in the future. We assess the algorithm's sensitivity, specificity, and performance on field-collected ticks.

1. Introduction

The incidence and geographic range of human tick-borne illnesses caused by pathogens transmitted by the blacklegged tick, *Ixodes scapularis*, in the eastern United States have dramatically increased in recent decades. The western blacklegged tick, *Ixodes pacificus*, transmits several of the same pathogens in the far western United States (Eisen et al., 2017). Moreover, new human pathogens associated with these and other tick species continue to emerge in the United States (Eisen et al., 2016, 2017). *Ixodes scapularis* is an experimentally-confirmed vector of, and is naturally infected with, at least 7 human pathogens: *Borrelia burgdorferi* sensu stricto (s.s.) and *Borrelia mayonii* (Lyme borreliosis spirochetes), *Borrelia miyamotoi* (agent of *Borrelia miyamotoi* disease, a relapsing fever-like illness), *Anaplasma phagocytophilum* (agent of anaplasmosis), *Babesia microti* (agent of babesiosis), *Ehrlichia muris* subsp. *eauclarensis* (agent of ehrlichiosis), and Powassan virus lineage 2 (formerly termed deer tick virus, agent of Powassan virus disease) (Bakken and Dumler, 2008; Dolan et al., 2016, 1998; Ebel, 2010; Eisen et al., 2016; Johnson et al., 2015; Karpathy et al., 2016; Krause et al., 2015; Merten and Durden, 2000; Pritt et al., 2017, 2016a, 2016b, 2011; Scoles et al., 2001; Spielman, 1976; Steere et al., 1983; Teglas and Foley, 2006; Vannier and Krause, 2012). *Ixodes pacificus* is a vector of a subset of the human pathogens that *I. scapularis* transmits, including *B. burgdorferi* s.s, *A. phagocytophilum*, and most likely also *B. miyamotoi* (Eisen et al., 2016; Krause et al., 2015; Lane et al., 1994; Merten and Durden, 2000; Teglas and Foley, 2006).

Lyme borreliosis is the most commonly reported vector-borne disease in the United States. In recent years, state and local health departments have reported more than 30,000 cases annually, which is 3 times the number of cases reported each year in the early 1990's (Mead, 2015). Anaplasmosis and babesiosis case counts have also increased over recent years, though these diseases are not as common as Lyme borreliosis (Adams et al., 2016; CDC, 2016a, 2016b, 2016c). In 2016, state and local health departments reported more than 4000 cases of anaplasmosis and 1910 cases of babesiosis (CDC, 2016b).

There is substantial overlap among the areas that report Lyme borreliosis, anaplasmosis, and babesiosis. Based on cases reported between 2008 and 2015, fourteen states in the Northeast, mid-Atlantic and upper Midwest regions have been classified as high Lyme borreliosis incidence states (Schwartz et al., 2017). In 2016, these 14 states

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reported more than 96% of Lyme disease, anaplasmosis and babesiosis cases (CDC, 2016b). Although it is not associated with a notifiable condition, *Borrelia miyamotoi* was recently recognized as another tick-borne human pathogen in the United States. The spirochete appears to have a broad geographic distribution, and clinicians have documented human cases in northeastern and midwestern states (Chowdri et al., 2013; Crowder et al., 2014; Gugliotta et al., 2013; Jobe et al., 2016; Krause et al., 2015, 2013; Molloy et al., 2015; Nelder et al., 2016). Given that the etiologic agents of Lyme disease, *Borrelia miyamotoi* disease, anaplasmosis, and babesiosis are sympatric in several regions, it is not surprising that researchers have observed evidence of co-infection with two or more of these agents in both ticks and humans (Barbour et al., 2009; Belongia et al., 1999; Fiorito et al., 2017; Johnson et al., 2017; Krause et al., 2002, 2014; Pritt et al., 2016b). Investigators and public health officials need assays that reliably detect a range of pathogens associated with *I. scapularis* and *I. pacificus* in field-collected ticks, including ticks infected with multiple pathogens, to estimate the geographic distribution of pathogens and to assess geographic variation in their prevalence.

Hojgaard et al. (2014b) described a testing algorithm employing paired TaqMan real-time polymerase chain reaction (PCR) assays to detect *B. burgdorferi* s.s., *A. phagocytophilum*, and *Ba. microti* in *I. scapularis*. The algorithm incorporated 2 different targets per pathogen as well as an *I. scapularis* actin target. We subsequently determined that the actin primer-probe set could be used to verify DNA integrity in both *I. scapularis* and *I. pacificus*-derived samples (Graham et al., 2016). The algorithm included 2 *Borrelia* targets: a non-coding segment of the chromosome, "gB31", which is present in *Borrelia* species including *B. burgdorferi* s.s. and *B. miyamotoi*, and a segment of the flagellin gene (*fliD*), which is present in *B. burgdorferi* sensu lato (s.l.) species including *burgdorferi* s.s., but not in *B. miyamotoi*. Without a *B. miyamotoi*-specific target, however, identification of *B. miyamotoi* in ticks required additional amplification and sequencing. Moreover, the algorithm could not differentiate ticks co-infected with *B. burgdorferi* s.s. and *B. miyamotoi* from ticks infected with *B. burgdorferi* s.s. alone (both tested positive for the gB31 and *fliD* targets) (Hojgaard et al., 2014b).

Following the recent discovery of *B. mayonii*, a new member of the *B. burgdorferi* s.l. complex that causes Lyme borreliosis (Pritt et al., 2016a, 2016b), we found that the 2 multiplex assays were not adequately specific. That is, we could not differentiate *B. burgdorferi* s.s. from *B. mayonii* using the gB31 and *fliD* targets. Additional testing suggested that the algorithm also detected both the gB31 and *fliD* targets in some, but not all, other *B. burgdorferi* s.l. species. There are 9 named species within the *B. burgdorferi* s.l. complex that are present in the United States: *Borrelia americana*, *Borrelia andersonii*, *Borrelia bissettiae*, *B. burgdorferi s.s.*, *Borrelia californiensis*, *Borrelia carolinensis*, *Borrelia kurtenbachii*, *Borrelia lanei* sp. nov., and *B. mayonii* (Margos et al., 2017; Pritt et al., 2016b; Schotthoefer and Frost, 2015). Investigators have detected *B. burgdorferi* s.s. and *B. mayonii* as well as *B. andersonii* and *B. kurtenbachii* in field-collected *I. scapularis* (Burgdorfer et al., 1982; Hamer et al., 2012; Johnson et al., 1984; Lin et al., 2001; Margos et al., 2010; Pritt et al., 2016a, 2016b). *Ixodes pacificus* is naturally associated with *B. burgdorferi* s.s. as well as *B. americana*, *B. bissettiae*, *B. californiensis*, and *B. lanei* sp. nov. (Fedorova et al., 2014; Lane et al., 2013; Margos et al., 2017, 2016; Postic et al., 2007, 1998; Rudenko et al., 2009). *Borrelia burgdorferi* s.s. and *B. mayonii* have been culture confirmed as human pathogens in the United States (Benach et al., 1983; Pritt et al., 2016a, 2016b; Steere et al., 1983). Golovchenko et al. (2016) recently isolated a *B. bissettiae* strain from a Florida resident, but they could not report specific clinical manifestations of the infection due to a lack of clinical evidence. *Borrelia bissettiae* and/or other species may emerge as human pathogens in the United States in the future. Therefore, it is advantageous to detect and differentiate the full range of *Borrelia* in field-collected ticks.

Despite the shortcomings of the 2 multiplex assays described by Hojgaard et al. (2014b), we found that combining sensitive, multiplex, real-time PCR assays incorporating a tick DNA control and multiple targets for each pathogen was an efficient and reliable approach for high-throughput testing of field-collected ticks (Feldman et al., 2015; Johnson et al., 2017; Morshed et al., 2015). We therefore sought to refine and expand our pathogen testing scheme in accordance with the ever-growing complexity of *Ixodes*-borne *Borrelia*. Our goals included (1) incorporating targets to detect and differentiate the 3 *Ixodes*-borne *Borrelia* species known to cause human disease in the United States (*B. burgdorferi* s.s., *B. mayonii*, and *B. miyamotoi*) from each other and from other *Ixodes*-borne borreliae, (2) integrating a target that would allow us to detect other *Borrelia* species for putative identification by sequencing and/or to bank for future testing if new pathogenic *Borrelia* species emerge or known species are shown to cause human illness, (3) incorporating at least 2 targets per pathogen, and (4) maintaining the ability to verify DNA integrity. While others have developed molecular testing schemes to detect and differentiate multiple pathogens in *Ixodes* ticks (e.g., Courtney et al., 2004; Dibernardo et al., 2014; Eshoo et al., 2015; Tokarz et al., 2009, 2017; Ullmann et al., 2005; Wroblewski et al., 2017), we know of no published algorithm to detect and differentiate the etiologic agents of Lyme borreliosis (both *B. burgdorferi* s.s. and *B. mayonii*), *Borrelia miyamotoi* disease, anaplasmosis, and babesiosis, and certainly not one that achieves all 4 of our goals.

Here we describe an algorithm to detect and differentiate *A. phagocytophilum*, *Ba. microti*, *B. burgdorferi* s.s., *B. mayonii*, *B. miyamotoi*, and other *Borrelia* species in unfed *I. scapularis* and *I. pacificus*. The algorithm includes: (1) paired multiplex TaqMan real-time PCR assays for the detection of *A. phagocytophilum*, *Ba. microti*, and *Borrelia*, (2) a duplex TaqMan real-time PCR assay to detect and differentiate *B. burgdorferi* s.s. and *B. mayonii* in *Borrelia*-positive samples, (3) paired TaqMan real-time PCR assays to detect *B. miyamotoi* in *Borrelia*-positive samples, and (4) sequencing protocols to putatively identify other *Borrelia* species, verify *B. mayonii* positives, and resolve suspect real-time PCR results (Fig. 1). We assessed the algorithm's sensitivity to each target pathogen, its overall specificity, and its performance on field-collected *I. scapularis* nymphs from Minnesota.

2. Materials and methods

2.1. Real-time PCR

Our testing algorithm incorporated 5 real-time PCR assays (Fig. 1). All primer and probe sequences and per-reaction concentrations appear in Table 1. For the first step, in which we sought to detect *A. phagocytophilum*, *Ba. microti*, and *Borrelia*, we modified the paired multiplex TaqMan real-time PCR assays described by Hojgaard et al. (2014b). The modified assays incorporated a pan-*Borrelia* primer-probe set adapted from Parola et al. (2011) as well as adjustments to reaction volume, some primer concentrations, cycling conditions, and the quantitation cycle (Cq) determination mode. The first assay targeted segments of the genes encoding flagellin (*fliD*) in *B. burgdorferi* s.s. and *B. mayonii*, and the genes encoding *A. phagocytophilum* P44 outer membrane proteins (*p44*), and *Ba. microti* secreted antigen 1 (*sa1*). For simplicity, we hereafter refer to the *fliD* target as "*B. burgdorferi* s.l. *fliD*" because the assay detected this target in multiple *B. burgdorferi* s.l. species including both *B. burgdorferi* s.l. species known to cause human disease in the United States. The first assay also included a tick actin target to verify the integrity of DNA samples derived from *I. scapularis* and *I. pacificus*. Hereafter, we refer to this assay as "M1b," because it incorporates the same targets as the assay previously termed "M1" (Hojgaard et al., 2014b) with modified *fliD* primer concentrations. The second assay targeted *Borrelia* 16S rDNA, the genes encoding *A. phagocytophilum* major surface protein 4 (*msp4*), and *Ba. microti* 18S rDNA. We developed this assay to replace "M2" (Hojgaard et al., 2014b). As it incorporated a unique combination of targets, we named it "M3." For both M1b and M3, each 10-µl reaction included 1X iQ Multiplex Powermix (Bio-Rad Laboratories, Hercules, CA, USA), 200 nM each probe,