Diagnostic Microbiology and Infectious Disease 93 (2019) 24–29

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

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

Novel real-time PCR assays for genomic group identification of tick-borne relapsing fever species *Borrelia hermsii*

Joseph J. Modarelli [a], Julie Piccione [b], Pamela J. Ferro [b], Maria D. Esteve-Gasent [a,*]

[a] Department of Veterinary Pathobiology, Texas A&M University College of Veterinary Medicine and Biomedical Sciences, 4467 TAMUx, College Station, TX 77843, United States
[b] Texas A&M Veterinary Medical Diagnostic Laboratory, 483 Agronomy Rd, College Station, TX 77840, United States

ARTICLE INFO

Article history:
Received 24 May 2018
Received in revised form 2 August 2018
Accepted 5 August 2018
Available online 8 August 2018

Keywords:
Borrelia hermsii
Genomic group
Molecular identification
Pathogenicity
Tick-borne relapsing fever
Nucleic acid amplification test

ABSTRACT

Borrelia hermsii is a non-Lyme borreliosis pathogen that is responsible for causing tick-borne relapsing fever in humans and animals in the western United States. *B. hermsii* has been described to encompass two divergent genomic groups, GGI and GGII, which have been suggested to maintain a unique geographical distribution and potential range of pathogenicity. Though the genomic groups have been extensively documented in the literature, a real-time PCR tool for identifying these genomic groups is lacking. This study describes the development and validation of two *flaB*-based quantitative real-time PCR assays for differentiating between the two genomic groups of *B. hermsii* while also maintaining specificity against other closely related *Borrelia* species. The diagnostic specificity of the assays were evaluated using a large panel of various *Borrelia* species, including a collection of 22 *B. hermsii* culture isolates purified from various hosts. The high sensitivity and specificity of the assays provide a useful tool for supporting future studies aimed at evaluating the geographical distribution as well as potential intraspecies pathogenicity within arthropod vectors and mammalian hosts.

1. Introduction

Tick-borne relapsing fever (TBRF) is a zoonotic blood-borne disease endemic throughout many regions of the world (Lopez et al., 2016; Talagrand-Reboul et al., 2018). Species responsible for causing TBRF in North America include *B. hermsii, B. turicatae,* and *B. parkeri*, though the later has not been found to play a major role in reported infections (Dworkin et al., 2008). Each of these three *Borrelia* species are transmitted through a soft tick vector including *Ornithodoros hermsi, O. turicata,* and *O. parkeri,* respectively. Human infections with TBRF are caused by *B. hermsii* and *B. turicatae*, with the majority of cases reported in the western United States and associated with *B. hermsii*. Although there is limited documentation of TBRF in dogs, most reports indicate infection with *B. turicatae* (Esteve-Gasent et al., 2017; Piccione et al., 2016; Whitney et al., 2007), with a more recent case study identifying *B. hermsii* (Kelly et al., 2014). TBRF infections are hypothesized to be underreported for several reasons, one of which is current diagnostics are confounded by genetically similar organisms such as *Borrelia burgdorferi* sensu lato, the causative agent of Lyme disease (LD) (Esteve-Gasent et al., 2017; Kelly et al., 2014; Piccione et al., 2016). Furthermore, signs and symptoms associated with TBRF during febrile states can be similar to LD and most other tick-borne diseases during initial stages of infection (i.e. flu-like illness) or when a readily identifiable rash is overlooked (Dworkin et al., 2008; Little et al., 2010; Stanek et al., 2012). However, TBRF is only detectable during limited stages, denoted as "relapses", which may further complicate an accurate diagnosis by limiting a window in which a presentation can be evaluated (Talagrand-Reboul et al., 2018). Currently, the most reliable method of TBRF diagnosis is the observation of spirochetemia through blood smear examination (Esteve-Gasent et al., 2017). While this diagnostic method is highly specific to TBRF *Borrelia* species, there is currently limited documentation to support microscopic detection during nonspirochetmic phases of infection (Piccione et al., 2016).

TBRF species *B. hermsii* has been noted to diverge into two genetically distinct genomic groups (GG), denoted as GGI and GGII (Porcella et al., 2005; Schwan et al., 2007). This sub-speciation-like divergence has not yet been found to affect their subsequent human/animal host uniquely, but a recent study has suggested potentially distinctive modes of virulence associated with the two genomic groups in a pine squirrel model (Johnson et al., 2016). Further characterization of these genomic groups within affected hosts will provide insight into the plausibility of genomic group-dependent virulence and pathogenicity. The two genomic groups of *B. hermsii* have also indicated a potentially unique geographic distribution in a study limited to infected humans, chipmunks, and *Ornithodoros hermsi* ticks, sampled in the Western United States (Schwan et al., 2007). While preliminary, the isolates belonging to GGII were found to mostly cluster in northwestern regions while GGI isolates depicted a grouping pattern in both northwestern

* Corresponding author. Tel.: +1-979-845-1117.
E-mail address: MEsteve-Gassent@cvm.tamu.edu (M.D. Esteve-Gasent).

https://doi.org/10.1016/j.diagmicrobio.2018.08.001
0732-8893/

and southwestern regions. It is hypothesized that infected migratory birds may be responsible, at least in part, for the geographic distribution of *B. hermsii* genomic groups (Schwan et al., 2007).

Because of the genetic similarities between *B. hermsii* GGI and GGII, molecular detection in research laboratories has been primarily limited to multiple gene specific conventional PCR assays with GG identification via sequencing (Policastro et al., 2013). While this approach has proven to be mostly effective, it is time consuming, expensive, and heavily dependent on sample availability. Reliance on the multilocus sequence typing (MLST) method to identify GGs may inhibit future studies aiming to rapidly screen a collection of samples, or perform further downstream applications. As TBRF causing species are closely related to agents of LD borreliosis, PCR assays that target conserved genes, such as the *Borrelia flaB* gene, may cross amplify between the species in a similar fashion to the cross-reactivity seen on serologic platforms (Esteve-Gasent et al., 2017; Piccione et al., 2016). While alternative genes limited to TBRF species, such as the *Borrelia* immunogenic protein A (*bipA*) (Lopez et al., 2010) and the glycerophosphodiester phosphodiesterase gene (*glpQ*) (Schwan et al., 1996), might be more apt targets for real-time PCR (qPCR) development, *B. hermsii* GG discrimination may prove unsuccessful, though limited analysis of a hypothetical protein within the *bh0260* open reading frame conducted in a previous study has shown potential (Policastro et al., 2013). The availability of qPCR assays that can detect and identify the GG identities of *B. hermsii*, and exclude LD, could provide a beneficial diagnostic tool in both continued prevalence studies for *B. hermsii* within the U.S., as well as aid in further analysis of the geographic distribution and virulence of the two GGs. Therefore, the objective of this study was to develop qPCR assays that could differentiate *Borrelia* species and characterize *B. hermsii* GG identities.

2. Materials and methods

2.1. Borrelia species isolates and cultures

All TBRF *Borrelia* species and louse-borne relapsing fever *Borrelia* species isolates were cultured, purified, and provided by Dr. Tom Schwann at Rocky Mountain Laboratories in Montana. Species of the *Borrelia burgdorferi* sensu lato complex were cultured by Dr. Maria Esteve-Gasent at Texas A&M University College of Veterinary Medicine and Biomedical Sciences using conventional procedures (Barbour, 1984).

2.2. Design of qPCR primers and probe

All *B. hermsii flaB, bipA,* and *glpQ* gene sequences in GenBank® (n = 76, 40, and 82, respectively; queries were performed in January 2018) were evaluated between the two genomic groups (GGI and GGII). The *flaB* gene was selected as a candidate for further analyses based on relatively high sequence availability within the National Center for Biotechnology Information (NCBI) database, as well as gene sequence conservation within the species (Coordinators, 2017). Representative *B. hermsii* GGI and GGII *flaB* sequences (n = 38 and 38, respectively) were acquired, aligned, and evaluated, along with homologous and heterologous gene sequences from closely related *Borrelia* species (n = 192) (Tables S1 and S2). All gathered *Borrelia* DNA sequences were greater than 500 bp in length except for a single *B. coriaceae* sequence (315 bp). CLC Main Workbench 7.7 (CLCbio, Aarhus, Denmark) was utilized for gene sequence alignments and nucleotide mismatch identification. Additional analyses were performed with Primer Express 3.0 software (ThermoFisher, Waltham, MA) to verify primer/probe conformance to an annealing temperature of 60 °C and thermoprofile protocol developed for this project. Oligonucleotide sequences were then BLAST® searched against the NCBI database and respective pathogen genome to confirm specificity (Altschul et al., 1990). For GGI, primers Bh-1.flaB.512F and Bh-1.flaB.646R; and for GGII, primers Bh-2.flaB.511F and Bh-2.flaB.646R were designed to specifically anneal the gene fragment with the 3′ most position of the oligonucleotides binding over discovered single nucleotide polymorphism (SNP) sites (Table 1). The 27 bp probe Bh.flaB.615P was then designed to bind the amplicon of both genomic groups utilizing a degenerative oligonucleotide (R), and labeled with a Dual-Labeled BHQ® Probe (Biosearch Technologies, Petaluma, CA).

2.3. qPCR optimization and amplification

DNA originating from *B. hermsii* was used throughout the optimization process as template DNA. The qPCR reactions were conducted with a reaction volume of 25.0 μL, using the Path-ID™ qPCR master mix kit (ThermoFisher, Waltham, MA) in an Applied Biosystems® 7500 Fast Real-Time PCR System (ThermoFisher, Waltham, MA). Optimal reaction conditions consisted of 12.5 μL 2× Path-ID™ qPCR buffer, 1.0 μL 25× primer/probe mix (450 nM primers and 125 nM probe), 8.0 μL template DNA, and 3.5 μL RNase-free water. The GGI assay consisted of a 25× primer/probe mix of Bh-1.flB.512F, Bh-1.flaB.646R, and Bh.flaB.615P. The GGII assay consisted of a 25× primer/probe mix of Bh-2.flaB.511F, Bh-2.flaB.646R, and Bh.flaB.615P. The oligonucleotide probe utilized for this study was labeled with FAM and BHQ-1 on the 5′ and 3′ ends, respectively. A temperature of 60 °C was selected as the optimal annealing temperature for both assays. Cycling conditions were as follows: initial denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 1 s and 60 °C for 30 s, for a total run time of 59 minutes. Fluorescence data was acquired at the conclusion of each annealing step.

2.4. Cloning positive amplification control

A plasmid control featuring both *B. hermsii* GGI and GGII target regions was utilized as a positive amplification control (PAC), and for determination of linear dynamic range, analytical sensitivity, and limit of detection (LOD). The plasmid PAC was synthetically generated commercially by inserting the select gene fragments into pUC57 plasmid DNA (GenScript, Piscataway, NJ) and cloned into TOP10 chemically competent *Escherichia coli* cells (ThermoFisher, Waltham, MA).

2.5. Specificity and sensitivity

To evaluate the diagnostic specificity of the qPCR assays, the respective primers and probe were used to screen 22 representative DNA samples purified from cell culture isolates for each *B. hermsii* genomic group (Table 1), as well as single DNA representatives from other TBRF species, louse-borne relapsing fever *Borrelia* species, and genospecies of the *Borrelia burgdorferi* sensu lato complex (Fig. 2). Specificity was

Table 1
Primer and probe sequences, Tm, and amplicon sizes of *Borrelia hermsii* genomic group real-time PCR assays.

Target	Primers/probe	Sequences (5′-3′)	Melting temperature (°C)	Product size
GGI	Bh-1.flaB.512F	GGACATTGAGAGTACATGTGGGC	59.4	135
	Bh-1.flaB.646R	CCTCTTGCTGTCCTATCTCTTGCA	60.8	
GGII	Bh-2.flaB.511F	TGGACATTGAGAGTACATGTGGGA	60.2	136
	Bh-2.flaB.646R	CCTCTTGCTGTCCTATCTCTTGCT	59.2	
GGI/GGII	Bh.flaB.615P	AGCCTGAGCRCCTTCACCTGCAAAAAGA	70.0	

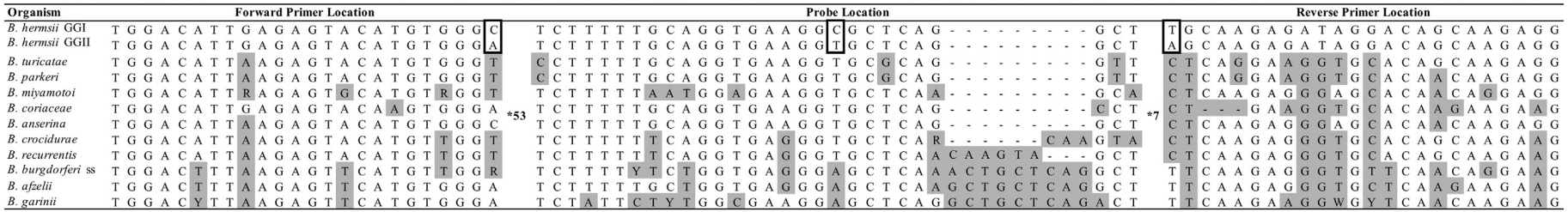

Fig. 1. Selected gene fragment alignments of the flaB gene with emphasis on regions containing forward, probe, and reverse oligonucleotide locations in linear orientation. Representative sequences of each Borrelia species is depicted for analysis with subsequent GenBank accession codes: New World relapsing fever Borrelia [*B. hermsii* GGI (AY597777), B. hermsii GGII (AY597806), *B. turicatae* (AY934629), *B. parkeri* (NZ_CP005851), *B. miyamotoi* (CP004217), *B. coriaceae* (NZ_CP005745), *B. anserina* (CP005829), Old World relapsing fever Borrelia [*B. crocidurae* (JX292925), *B. recurrentis* (DQ346814)], and Lyme disease related Borrelia [*B. burgdorferi* sensu stricto (ss) (AE000783), *B. afzelii* (CP018262), and *B. garinii* (CP007564)]. SNPs targeted for differentiation are represented by boxes. Base pair positions that are not conserved against the *B. hermsii* GGI and GGII sequences are highlighted in gray, where a position denoted with a dash "-" represents an in/del. Asterisks between oligonucleotide locations represent excluded base pairs in the interest of space.

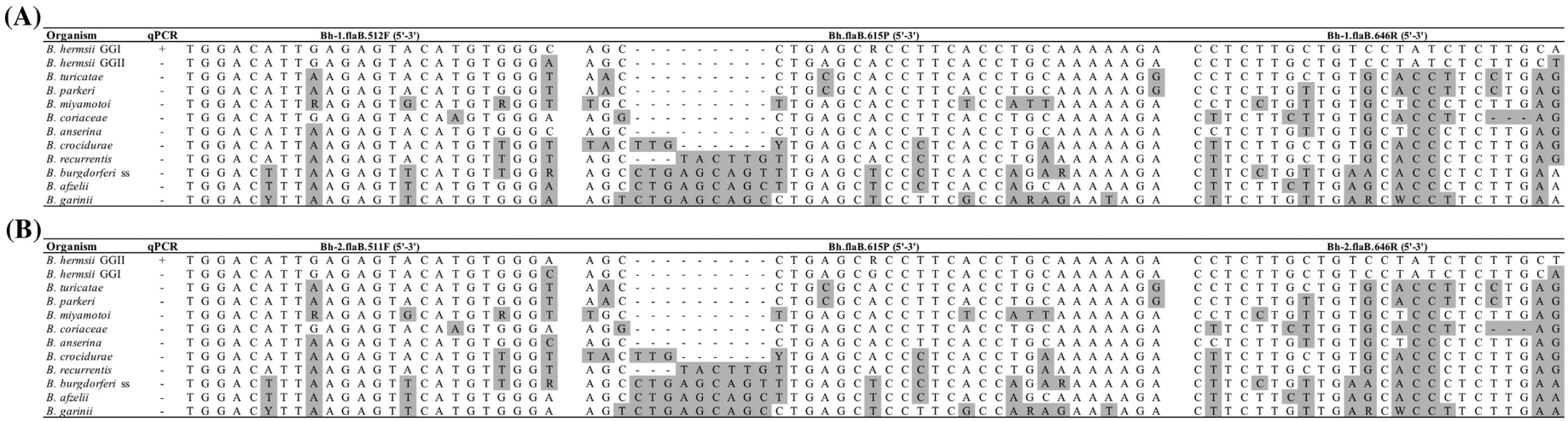

Fig. 2. Oligonucleotide alignments for the forward primer, reverse primer, and probe sequences of (A) *B. hermsii* GGI and (B) *B. hermsii* GGII assays in 5′ to 3′ orientation. Assays were evaluated for diagnostic specificity against closely related *Borrelia* species with the same sequences and accession codes as Fig. 1. Base pair positions that are not conserved against each assays primers/probe are highlighted in gray, where a position denoted with a dash "–" represents an in/del. The second column indicates qualitative specificity (positive or negative).

assessed by demonstrating failure to amplify DNA, in duplicate testing, from each species other than the GGI or GGII targets. To determine the analytical sensitivity of the assay, a dilution series (spanning 6 dilutions) for each pathogen was conducted with the constructed plasmid PAC (working stock at 7.59×10^{-5} ng/μL) to determine a quantification cycle (Cq) cutoff for the qPCR assays, as well as provide the LOD of gene copies in genome copy equivalents per microliter (CGE/μL). Linear dynamic range analysis was then generated based on the dilution series for each assay to calculate qPCR efficiency as established in a previous publication (D'Haene et al., 2010). Finally, an R^2 value was assigned to each assay based on the generated data. The plasmid DNA concentration was quantified using a NanoDrop (ThermoFisher, Waltham, MA) device, and the associated Cq values given for each pathogen was used to determine the LOD copy value. Formula used to determine LOD:

$$\text{number of copies} = \left([\text{plasmid}]\ \text{ng} * 6.022 \times 10^{23}\right) / \left(\text{amplicon length bp} * 1 \times 10^{9} * 650\right).$$

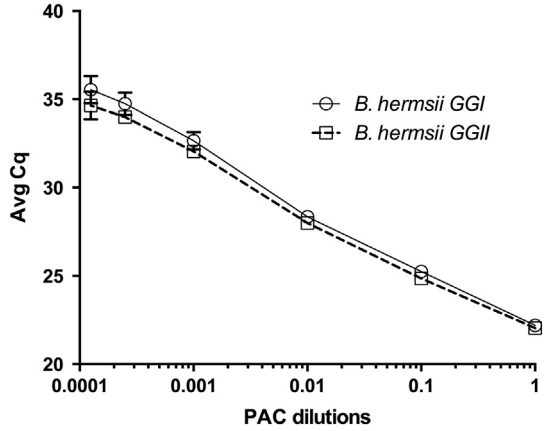

Fig. 3. Analytical sensitivity of real-time PCR (qPCR) for *Borrelia hermsii* genomic groups I and II. Plasmid positive amplification control (PAC) was serially diluted (6 steps) and analyzed by qPCR for each assay. Each dilution target was tested in duplicate.

3. Results

3.1. Sequence analysis

Sequence analysis of the *flaB* gene revealed two single-nucleotide polymorphisms (SNPs) separated by 90 base pairs that represent pyrimidine and purine nucleotide mutations conserved within all *B. hermsii* genomic group gene sequences (Fig. 1). The selected gene region also contained additional mismatch sites in respect to other *Borrelia* species that made the location ideal for qPCR species differentiation, as corroborated in a previous study that highlighted potential regions for *Borrelia* species discrimination (Pickens, 1992).

3.2. Heterologous Borrelia species analytical specificity

For all strain controls listed in Figs. 1 and 2, and Table 2, DNA amplification was only detected when a specific *B. hermsii* genomic group was used as template DNA with the respective genomic group assay. Although this study only utilized DNA originating from one representative strain for each *Borrelia* species other than *B. hermsii*, in silico sequence analysis revealed that all respective species share 100% similarity within target gene regions (Table S2).

Table 2
Borrelia hermsii isolates screened by genomic group specific qPCR assays.

Isolate*	Source	Locality	qPCR Results
GGI			
DAH	Human	Washington	I
MAN	Human	California	I
FRO	Human	Washington	I
FRE	Human	Washington	I
MIL	Human	Idaho	I
BRO	Human	Idaho	I
SWA	Human	Idaho	I
CAR	Human	Idaho	I
EST-7	Chipmunk	Colorado	I
BAK	Human	Washington	I
BYM	Human	Idaho	I
GGII			
MTW-4	Human	California	II
YOR	Human	California	II
HAN	Human	Idaho	II
REN	Human	Washington	II
OKA-1	Human	B.C. Canada	II
GMC	Human	Washington	II
CMC	Human	Washington	II
LAK-2	Human	Montana	II
LAK-3	Human	Montana	II
LAK-5	Human	Montana	II
YBT-10	Tick†	Montana	II

* GGI, genomic group I; GGII, genomic group II.
† Tick species represented as *Ornithodoros hermsi*.

3.3. Borrelia Hermsii genomic group specificity

Of the 22 different *B. hermsii* DNA isolates purified from various host samples represented by Table 2, the qPCR assays were able to successfully detect and identify the genomic group classification for 100% of the isolates. No cross amplification was observed for either qPCR assay against DNA isolated from a differing genomic group identity (e.g. GGI did not amplify GGII, and vice versa).

3.4. Linear dynamic range, analytical sensitivity, and efficiency

The linear dynamic ranges and efficiencies of the qPCR assays, using the listed primer and probes (Table 1), were determined using serial dilutions of the plasmid PAC (Fig. 3). The efficiency and R^2 calculations for each assay was 93% and 0.996, and 99% and 0.997 for GGI and GGII, respectively. The analytical sensitivity and LOD was determined to be 16 CGE/μL of plasmid DNA for each assay (Table S3). A reproducible Cq value at 16 CGE/μL of plasmid DNA was determined to be 36, therefore, a sample with a Cq ≤ 36 was considered positive. A Cq value between 36.0 and 38.0 should be considered suspect and retested for confirmation due to potentially detecting copies below the LOD.

4. Discussion

This report describes the development and validation of two *flaB* based real-time PCR (qPCR) assays for detection of both genomic groups of *B. hermsii*. The assays can be used independently to ascertain specific genomic group identities of *B. hermsii* positive samples. In comparison, past studies have utilized multiple genes for sequence analysis and subsequent genomic group identification of *B. hermsii* (Johnson et al., 2016; Policastro et al., 2013). Additionally, this study revealed that two SNPs found within the *flaB* gene was sufficient to indicate group type when targeted with a qPCR assay following the detailed optimized reaction conditions. As the assays differentiate between GGs' SNPs at the primer level, and therefore does not rely on an oligonucleotide probe to maintain specificity, SYBR® Green based assays may also be utilized per preliminary results (data not shown). In that respect,

analyses revealed a potential to multiplex the assays, as initial generated data revealed unique melting curves between GGI and GGII samples (data not shown). Further studies are needed to evaluate the use of a SYBR® Green assay for differentiation of the genotypes.

Borrelia species responsible for TBRF in susceptible hosts generally display a cyclic form of clinical manifestation, hence the name relapsing fever (Dworkin et al., 2008). In humans, the febrile stage of TBRF persists for roughly three to four days and is associated with spirochetemia (Lopez et al., 2016). The bacteria are then systematically targeted for elimination by the host immune system, and the host becomes asymptomatic for an afebrile stage that lasts roughly seven to ten days (Lopez et al., 2016). TBRF spirochetes utilize antigenic variation during this stage to alter outer surface proteins, allowing a population minority to escape from immune system detection and a subsequent relapse to occur within the host (Dworkin et al., 2008). However, studies have not reached a consensus on whether TBRF spirochetes can be regularly detected by qPCR methodology through all stages of disease (Lopez et al., 2014). As the assays presented in this study depict a limit of detection of 16 CGE/μL further studies are needed to confirm whether the assays are sufficient in detecting spirochetes from a host during the afebrile state. Further, diagnostic validation analyses conducted in this study utilized only genomic DNA purified from cultures originating from wildlife, tick, and human isolates. Blood samples from patients with active infection and ticks were not utilized in this study. Further investigations are warranted in order to verify the potential of the individual assays in a diagnostic setting.

In conclusion, as future studies continue to assess the geographic range, natural reservoir, and genomic group virulence of *B. hermsii*, more efficient and specific molecular detection methodologies for the TBRF pathogen are necessary. The sensitive and specific qPCR assays developed here may provide a beneficial molecular tool for use in ecological studies of *B. hermsii* as well as in the differential diagnosis of hosts infected with the pathogen.

Acknowledgments

The authors would like to thank Dr. Tom Schwan from the Rocky Mountain Laboratories NIAID Facility in Montana for providing tick-borne and louse-borne relapsing fever DNA controls and strains used in this study. The research reported here was supported by Texas A&M AgriLife and Texas A&M Veterinary Medical Diagnostic Laboratory (TVMDL) seed grant, Molecular Diagnosis of Zoonotic Tick-Borne Diseases.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diagmicrobio.2018.08.001.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215(3):403–10.
Barbour AG. Isolation and cultivation of Lyme disease spirochetes. Yale J Biol Med 1984;57(4):521–5.
Coordinators NR. Database resources of the National Center for biotechnology information. Nucleic Acids Res 2017;45(D1):D12–7.
D'Haene B, Vandesompele J, Hellemans J. Accurate and objective copy number profiling using real-time quantitative PCR. Methods 2010;50(4):262–70.
Dworkin MS, Schwan TG, Anderson Jr DE, Borchardt SM. Tick-borne relapsing fever. Infect Dis Clin North Am 2008;22(3):449–68. [viii].
Esteve-Gasent MD, Snell CB, Adetunji SA, Piccione J. Serological detection of tick-borne relapsing fever in Texan domestic dogs. PLoS One 2017;12(12), e0189786.
Johnson TL, Fischer RJ, Raffel SJ, Schwan TG. Host associations and genomic diversity of *Borrelia hermsii* in an endemic focus of tick-borne relapsing fever in western North America. Parasit Vectors 2016;9(1):575. https://doi.org/10.1186/s13071-016-1863-0.
Kelly AL, Raffel SJ, Fischer RJ, Bellinghausen M, Stevenson C, Schwan TG. First isolation of the relapsing fever spirochete, *Borrelia hermsii*, from a domestic dog. Ticks Tick Borne Dis 2014;5(2):95–9.
Little SE, Heise SR, Blagburn BL, Callister SM, Mead PS. Lyme borreliosis in dogs and humans in the USA. Trends Parasitol 2010;26(4):213–8.
Lopez JE, Krishnavahjala A, Garcia MN, Bermudez S. Tick-borne relapsing fever spirochetes in the Americas. Vet Sci 2016;3(3):16. https://doi.org/10.3390/vetsci3030016.
Lopez JE, Schrumpf ME, Nagarajan V, Raffel SJ, McCoy BN, Schwan TG. A novel surface antigen of relapsing fever spirochetes can discriminate between relapsing fever and Lyme borreliosis. Clin Vaccine Immunol 2010;17(4):564–71.
Lopez JE, Vinet-Oliphant H, Wilder HK, Brooks CP, Grasperge BJ, Morgan TW, et al. Real-time monitoring of disease progression in rhesus macaques infected with *Borrelia turicatae* by tick bite. J Infect Dis 2014;210(10):1639–48.
Piccione J, Levine GJ, Duff CA, Kuhlman GM, Scott KD, Esteve-Gassent MD. Tick-borne relapsing fever in dogs. J Vet Intern Med 2016;30(4):1222–8.
Pickens S. The decade for natural disaster reduction: the role of health care workers. Nurs Health Care 1992;13(4):192–5.
Policastro PF, Raffel SJ, Schwan TG. *Borrelia hermsii* acquisition order in superinfected ticks determines transmission efficiency. Infect Immun 2013;81(8):2899–908.
Porcella SF, Raffel SJ, Anderson Jr DE, Gilk SD, Bono JL, Schrumpf ME, et al. Variable tick protein in two genomic groups of the relapsing fever spirochete *Borrelia hermsii* in western North America. Infect Immun 2005;73(10):6647–58.
Schwan TG, Raffel SJ, Schrumpf ME, Porcella SF. Diversity and distribution of *Borrelia hermsii*. Emerg Infect Dis 2007;13(3):436–42.
Schwan TG, Schrumpf ME, Hinnebusch BJ, Anderson D, Konkel ME. GlpQ: an antigen for serological discrimination between relapsing fever and Lyme borreliosis. J Clin Microbiol 1996;34(10):2483–92.
Stanek G, Wormser GP, Gray J, Strle F. Lyme borreliosis. Lancet 2012;379(9814):461–73.
Talagrand-Reboul E, Boyer PH, Bergstrom S, Vial L, Boulanger N. Relapsing fevers: neglected tick-borne diseases. Front Cell Infect Microbiol 2018;8:98. https://doi.org/10.3389/fcimb.2018.00098.
Whitney MS, Schwan TG, Sultemeier KB, McDonald PS, Brillhart MN. Spirochetemia caused by *Borrelia turicatae* infection in 3 dogs in Texas. Vet Clin Pathol 2007;36(2):212–6.