**Supplemental Materials**

**Supplemental Table 1:** Clinical specimens tested in this study shown by specimen type and originating state

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| **Originating U.S. State or Territory** | **Specimen Type** |
| **Blood** | **CSF** | **Synovial Fluid** | **Tissue** |  |
| Alaska | 2 |  0 |  0 |  |
| Alabama | 54 |  0 | 1 |  |
| Arkansas | 260 |  0 |  0 |  |
| Arizona | 40 | 2 | 1 |  |
| California | 17 | 12 |  0 |  |
| Colorado | 20 | 5 | 2 | 1 |
| Connecticut | 9 |  0 |  0 |  |
| District of Columbia | 9 | 4 | 1 |  |
| Delaware | 72 | 49 | 16 |  |
| Florida | 50 | 48 | 2 |  |
| Georgia | 30 | 6 |  0 |  |
| Iowa | 68 | 38 | 6 |  |
| Idaho | 1 |  0 |  0 |  |
| Illinois | 24 | 49 | 3 |  |
| Indiana | 74 | 13 | 1 |  |
| Kansas | 170 | 18 | 1 |  |
| Kentucky | 25 |  0 |  0 |  |
| Louisiana | 24 | 2 |  0 |  |
| Massachusetts | 1 |  0 | 1 | 1 |
| Maryland | 57 | 5 | 6 | 1 |
| Maine | 5 |  0 |  0 |  |
| Michigan | 22 | 7 |  0 |  |
| Minnesota | 2497 | 239 | 75 | 3 |
| Missouri | 414 | 7 | 1 |  |
| Mississippi | 35 | 3 | 1 |  |
| Montana | 12 | 1 | 1 |  |
| North Carolina | 73 | 16 |  0 |  |
| North Dakota | 446 | 12 | 6 |  |
| Nebraska | 5 | 1 |  0 |  |
| New Jersey | 375 | 70 | 54 |  |
| New Mexico | 2 | 2 |  0 |  |
| Nevada | 1 |  0 |  0 |  |
| New York | 160 | 70 | 35 | 2 |
| Ohio | 92 | 26 | 3 |  |
| Oklahoma | 78 |  0 |  0 |  |
| Oregon | 2 | 5 |   |  |
| Pennsylvania | 28 | 67 | 26 |  |
| Rhode Island | 1 |  0 |  0 |  |
| South Carolina | 10 |  0 |  0 |  |
| South Dakota | 15 | 5 |  0 |  |
| Tennessee | 62 | 1 |  0 |  |
| Texas | 20 | 2 |  0 |  |
| Virginia | 80 | 49 | 32 |  |
| Vermont | 3 |  0 | 1 |  |
| Washington | 1 | 1 |  0 |  |
| Wisconsin | 592 | 18 | 24 | 1 |
| West Virginia | 84 | 6 |   |  |
| Wyoming | 2 |  0 |  0 |  |
| **Total (% of specimens)** | **6124 (84%)** | **859 (11.8%)** | **300 (4.1%)** | **9 (0.1%)** |

**Supplemental Table 2:** GenBank Accession numbers for genes sequenced in this study

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| Strain/gene | GenBank Accession # |
| *Candidatus B. johnsonii clpA* | MF062075 |
| *Candidatus B. johnsonii clpX* | MF062076 |
| *Candidatus B. johnsonii nifS* | MF062077 |
| *Candidatus B. johnsonii pepX* | MF062078 |
| *Candidatus B. johnsonii pyrG* | MF062079 |
| *Candidatus B. johnsonii recG* | MF062080 |
| *Candidatus B. johnsonii rplB* | MF062081 |
| *Candidatus B. johnsonii uvrA* | MF062082 |
| *Candidatus B. johnsonii* 16S rDNA | MF062083 |
| *Candidatus B. johnsonii flab* | MF062084 |
| *Candidatus B. johnsonii glpQ* | MF062085 |
| *B. hermsii* *clpA* | MF066941 |
| *B. hermsii clpX* | MF066942 |
| *B. hermsii nifS* | MF066943 |
| *B. hermsii pepX* | MF066944 |
| *B. hermsii pyrG* | MF066945 |
| *B. hermsii recG* | MF066946 |
| *B. hermsii rplB* | MF066947 |
| *B. hermsii uvrA* | MF066948 |
| *B. hermsii flab* | MF066949 |
| *B. hermsii glpQ* | MF066950 |
| *B. hermsii* 16S rDNA | MF066892 |

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| **Supplemental Table 3: New alleles identified in this study** |
| Sample | Bbsl genospecies | Allele | Closest Allele Match | Position\* | Base |
| 15-0078 | *B. burgdorferi* | *pyrG* | *pyrG 1* | 313 | A |
|  | *B. burgdorferi* | *uvrA* | *uvrA 7* | 520 | T |
| 15-1840 | *B. burgdorferi* | *pepX* | *pepX 3* | 415 | T |
| 15-5375 | *B. burgdorferi* | *pyrG* | *pyrG 1* | 412 | A |
| 15-8607 | *B. burgdorferi* | *nifS* | *nifS 4* | 310 | T |
| 15-8808 | *B. burgdorferi* | *nifS* | *nifS 1* | 368 | G |
| 15-5922 | *B. mayonii* | *pepX* | *pepX 191* | 363 | G |
| \*Closest allele match indicates the most similar sequence in Borrelia pubMLST  |   |   |
| \*\*Nucleotide Position is based on comparison to allele size in the Borrelia pubMLST  |  |  |

**Supplemental Methods**

**PCR**

DNA extraction performed at the Mayo Clinic utilized the Roche MagNA Pure 2.0 instrument (Total Nucleic Acid Kit; Roche Diagnostics, Indianapolis, IN). DNA extraction performed at CDC utilized the Roche MagNA Pure 96 (DNA and Viral NA Small Volume Kit, Roche). To guide the use of Bbsl or RF primers for amplicon sequencing*, Borrelia* PCR positive DNA samples were characterized as *B. burgdorferi* or *B. mayonii* using a previously described duplex TaqMan PCR assay targeting the oligopeptide permease periplasmic A2 gene (*oppA2*) [1] or *B. miyamotoi* TaqMan assay targeting the 16S rRNA gene [2].

**Evaluation of pan-*Borrelia* TaqMan PCR**

Specificity of the 16S rRNA pan-*Borrelia* TaqMan PCR assay was verified with 1-100 ng of genomic DNA extracted from 14 *B. burgdorferi* *sensu lato* strains (9 genospecies; *B. burgdorferi sensu stricto, B. afzelii, B. americana, B. andersonii, B. bavariensis, B. carolinensis, B. garinii, B. japonica,* and *B. kurtenbachii*), 12 relapsing fever *Borrelia* strains (6 genospecies; *B. miyamotoi, B. coriaceae, B. parkeri, B. recurrentis, B. turicatae* and *B. hermsii*), 35 strains of 18 otherbacterialspecies (*Bartonella, Staphylococcus*, *Pasteurella, Enterobacter, Escherichia, Cedecea, Klebsiella, Serratia, Acinetobacter, Moraxella, Proteus, Pseudomonas, Ochrobactrum, Salmonella, Streptococcus, Legionella*, *Afipia* and *Leptospira)*. DNA was purified using the QIAamp DNA Minikit (Qiagen) and associated protocols, which included an RNase A treatment step. Purified DNA was suspended in Buffer AE and quantified using a NanoDrop 8000 UV Spectrophotometer. PCR sensitivity was evaluated using tenfold serial dilutions (10 ng/µl to 1 fg/µl) of *Borrelia burgdorferi* B31 DNA tested in triplicate. Detection to 10 fg (~10 genomic equivalents) was verified by testing tenfold serial dilutions (10 ng/µl to 1 fg/µl) of two other *Borrelia* species, *Borrelia carolinensis* SCW22 and *Borrelia hermsii* NE95-0544.

**Locus Amplification**

For the 8 housekeeping genes, 3-15 µl of purified template DNA was utilized. Resulting PCR amplicons were visualized on 2% agarose gels. For *B. burgdorferi*, the annealing temperature for PCR using the outer primers was modified to 52°C for *clpA, clpX, pyrG*, and *recG*, and 54°C for *nifS, pepX, rplB*, and *uvrA*. For amplification of RF *Borrelia,* degenerate *clpA* and *nifS* primers were designed: *clpA*-forward 5’AAAAACATCRAYYTTTTCATTTTTTAGTA3’, *clpA*-reverse 5’TTGACYTATTAGATGGTCTTGG3’; *nifS*-forward 5’GAAAMAKTMAAAATC MTAAGGAAAG3’, *nifS*-reverse 5’CAATAATTCCTGCAATGTTTGGTG3’. For amplification of 16S rDNA, *flaB,* and *glpQ,* 5-10 µl of template DNA was used with published PCR conditions [3].

**References**

1. Pritt BS, Respicio-Kingry LB, Sloan LM, et al. Borrelia mayonii sp. nov., a member of the Borrelia burgdorferi sensu lato complex, detected in patients and ticks in the upper midwestern United States. Int J Syst Evol Microbiol **2016**; 66(11): 4878-80.

2. Platonov AE, Karan LS, Kolyasnikova NM, et al. Humans infected with relapsing fever spirochete Borrelia miyamotoi, Russia. Emerg Infect Dis **2011**; 17(10): 1816-23.

3. Schwan TG, Raffel SJ, Schrumpf ME, et al. Phylogenetic analysis of the spirochetes Borrelia parkeri and Borrelia turicatae and the potential for tick-borne relapsing fever in Florida. J Clin Microbiol **2005**; 43(8): 3851-9.