Diplorickettsia Bacteria in an *Ixodes scapularis* Tick, Vermont, USA

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An unexpected *Diplorickettsia* species closely related to the tickborne pathogen *D. massieliensis* was found in the microbiome of an *Ixodes scapularis* tick in Vermont, USA. This evidence of *Diplorickettsia* in North American ticks suggests a need for disease surveillance using molecular screening of ticks and serologic studies of humans.

The blacklegged tick, *Ixodes scapularis*, is a generalist arthropod ectoparasite that serves as a vector for an array of common human pathogens; novel disease-causing microbes have been discovered consistently in the tick for the past several decades. Tickborne bacterial infections causing illnesses such as anaplasmosis, *Borrelia miyamotoi* disease, and ehrlichiosis have all emerged in the United States in recent years (*1*–*3*), and it has been estimated that as many as half of all tickborne illnesses are caused by unknown pathogens (*4*).

We used 16S rRNA sequencing to survey for bacterial pathogens in *I. scapularis* ticks in western Vermont, USA. We collected ticks by drag sampling along 100 m transects using a 1 m^2 square of white denim. We collected ticks from 6 deciduous forest sites in Addison and Chittenden counties, Vermont (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/26/5/19-1135-App1.pdf), during May–July 2015. We extracted DNA from ticks using a phenol-chloroform extraction (*5*). In total, we extracted DNA from 97 ticks, 20 of which we selected based on DNA quality and quantity for 16S rDNA sequencing at Hudson Alpha Genomic Services Laboratory (Huntsville, AL, USA). We PCR amplified the V3 and V4 regions of the 16S rRNA gene from these ticks and 1 blank using 341F and 875R primers (*6*) and sequenced them on a MiSeq platform (Illumina, https://www.illumina.com), yielding a total of 15,302,568 reads. We used the DADA2 R package to identify amplicon sequence variants (ASVs) and assign taxonomy (*7*) (Appendix Figure 1). We used default settings in the DADA2 pipeline; however, we estimated error rates using the first 10 billion base pairs.

In a single adult male tick, an ASV assigned to the genus *Diplorickettsia* comprised 82% of the microbiome sequencing reads. The genus *Diplorickettsia* was originally defined by the species *D. massiliensis*, discovered in *I. ricinus* ticks in Europe (*8*). The ASV we identified shared 425 of 427 nt in the sequenced V3–V4 region of the 16S rRNA gene with the reference

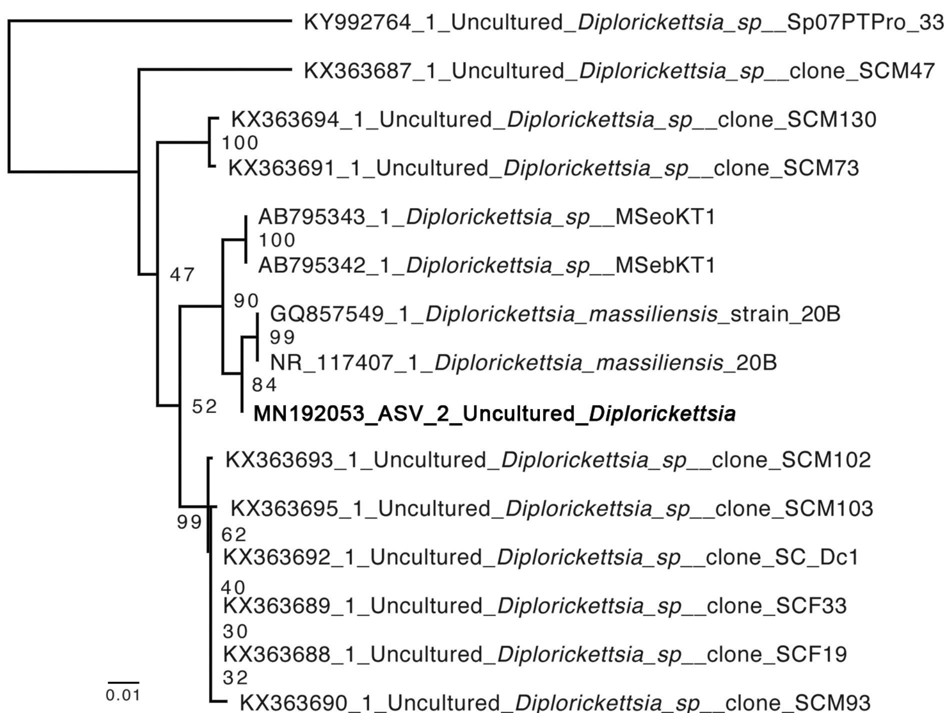

Figure. Neighbor-joining phylogenetic tree of a MAFFT alignment (https://mafft.cbrc.jp/alignment/server) of the V3–V4 region of the *Diplorickettsia* 16S rRNA gene, including the novel amplicon sequence variant identified in Vermont, USA (bold). A total of 427 bases were aligned and 363 conserved sites were used for neighbor-joining phylogeny, with 100 bootstrap iterations. The 341F and 875R primers were used to amplify these regions (*6*). Default alignment parameters were used for alignment and generation of phylogenetic tree. Numbers at nodes indicate bootstrap values after 1,000 bootstrapping iterations. GenBank accession numbers are indicated. Scale bar represents average number of substitutions per site.

sequence of *D. massiliensis* strain 20B (GenBank accession no. NR_117407.1) and was more closely related to this strain than any other previously sequenced *Diplorickettsia* in the National Center for Biotechnology Information (NCBI) nucleotide database (Figure). This tick was collected at the Sunny Hollow Colchester site (coordinates 44.518353°, -73.17112°) (Appendix Table 1).

D. massiliensis has been identified as a possible human pathogen; of patients in a hospital in France, 3 were found seropositive, and 1 found positive by quantitative PCR for the *D. massiliensis rpoB* gene (*9*). Our findings represent evidence of a *Diplorickettsia* bacteria in ticks in North America.

To confirm the presence of *Diplorickittsia* in the positive tick, we designed PCR primers to regions of the *D. massiliensis parC* and *ftsY* genes (Appendix Table 2) using primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast). We aligned primers against the NCBI nr database to ensure specific binding to *Diplorickettsia*. We also used DNA from a *Diplorickettsia*-negative tick (as determined by 16S sequencing) as PCR template to serve as a negative control. Successful amplification of regions of both genes confirmed the presence of *Diplorickettsia* DNA in the positive tick (Appendix Figure 2).

We Sanger sequenced amplicons from these PCR tests. We combined forward and reverse reads and trimmed them using the PEAR utility (https://cme.h-its.org/exelixis/web/software/pear). Each sequence showed high identity with previously sequenced *D. massiliensis* reference sequences via gapped alignment (247/252 bp *parC*, 298/310 bp *ftsY*). These results further suggest a close relationship between *Diplorickettsia* species we identified and *D. massiliensis*, but a lack of reference sequences for these genes from other species of *Diplorickettsia* makes it impossible to definitively assign this uncultured specimen to a particular species.

The high sequence similarity between the *Diplorickettsia* we identified and the previously identified pathogenic variety suggests the need for further study of the pathogenicity of this variant. Many genera of tickborne bacteria contain both pathogenic and nonpathogenic strains, and genetic similarity alone cannot confirm pathogenicity. Future work is needed to isolate this strain of *Diplorickettsia* and determine its ability to infect mammalian hosts and its transmissibility via tick bite. Experiments to test its ability to induce febrile illness in mammals would also help determine if *Diplorickettsia* spp. could cause a clinically significant infection in humans. Furthermore, serologic studies of patients with suspected tickborne diseases in the area surrounding the collection site are necessary to determine if this bacterium has infected persons in Vermont.

In addition, our findings suggest the need for further study of the prevalence of *Diplorickettsia* in North America ticks. We have developed PCR primers (Appendix Table 2) to facilitate future study of this bacterium and have demonstrated via sequencing that these primers accurately amplify their target *Diplorickettsia* genes. We have deposited the partial *Diplorickettsia* 16S rRNA, *ftsY*, and *parC* genes sequenced in this study into the NCBI GenBank database (accession nos. MN192053, MN640996, MN640997). The raw sequencing reads are available through the NCBI sequence read archive (accession no. PRJNA557440).

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Case of *Babesia crassa*–Like Infection, Slovenia, 2014

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We report a case of *Babesia crassa*–like infection in an asplenic patient in Slovenia in 2014. We diagnosed the infection using microscopy, 18S rRNA sequencing, and serology and monitored parasitemia using digital PCR. With its increasing occurrence, babesiosis should be included in differential diagnoses for immunocompromised patients displaying fever.

Babesia infections occur worldwide and cause disease mainly in animals, but disease occurs occasionally in humans. Infections in humans are mostly attributable to *B. microti*, *B. duncani*, and *Babesia* sp. MO1 in North America; *B. divergens*, *B. venatorum*, and *B. microti* in Europe; and *B. venatorum*, *B. crassa*–like parasite, *B. microti*, *Babesia* sp. XXB/HangZhou, and *Babesia* sp. KO-1 in Asia (*1*,*2*). Transmission occurs predominantly through tick bites, but humans have acquired infections via contaminated blood products and through the transplacental and perinatal routes (*1*). Most patients with *Babesia* infections in Europe were reported to be asplenic or immunocompromised. Typical clinical signs and symptoms include fever (up to 40°C), parasitemia (20%–80%), severe anemia, muscle weakness, fatigue, and late-onset jaundice with dark urine, and sometimes complications develop. Long-term clinical follow-up that includes blood smear examination and PCR analysis is necessary because relapse and persistence of parasitemia can occur in spite of treatment. The application of novel molecular methods has revealed that the host range of many *Babesia* species is less restricted than previously thought. New species or animal pathogens are increasingly being discovered as causing *Babesia* infections in humans. We report a *B. crassa*–like infection in a patient in Slovenia in 2014.

In 2014, a 55-year-old woman, living on the outskirts of Murska Sobota, Slovenia, sought medical treatment for a 6-day history of intermittent fever up to 39°C, myalgia, headache, poor appetite concomitant with weight loss, fatigue, sweating, and dark urine. She previously had a splenectomy and partial pancreatectomy 5 years previous because of cystic adenoma and adrenal incidentaloma without hormonal activity. She reported no history of travel, tick bite, animal contact, or blood transfusions.

Her blood pressure was 115/70 mm Hg, heart rate 83 beats/min, and body temperature 36.6°C, and a physical examination indicated no significant clinical findings. The first basic blood analysis revealed thrombocytopenia (platelets 85×10^9/L). A differential blood analysis indicated that the concentration of large unstained cells was elevated (0.41×10^9/L, reference range $0–0.4 \times 10^6$/L). Biochemical laboratory testing showed mild fluctuations in liver functioning: aspartate aminotransferase 1.22 (reference range 0.17–0.51) µkat/L, alanine aminotransferase 1.13 (reference range 0.17–0.68) µkat/L, γ-glutamyltransferase 1.08 (reference range 0.03–0.51 µkat/L) µkat/L, and alkaline phosphatase 1.88 (reference range 0.5–2.0) µkat/L. C-reactive protein was 51 mg/L (150 [reference range 0.76–28.5] nmol/L), and mild erythrocyturia was present. Giemsa-stained blood smears showed unusual inclusions in erythrocytes, Howell-Jolly bodies, mild anisocytosis, some atypical lymphocytes, and some large thrombocytes. We observed many ring forms and some paired piriform shapes of *Babesia* spp. in blood smears (Figure), and parasitemia was 1% (Appendix Table,

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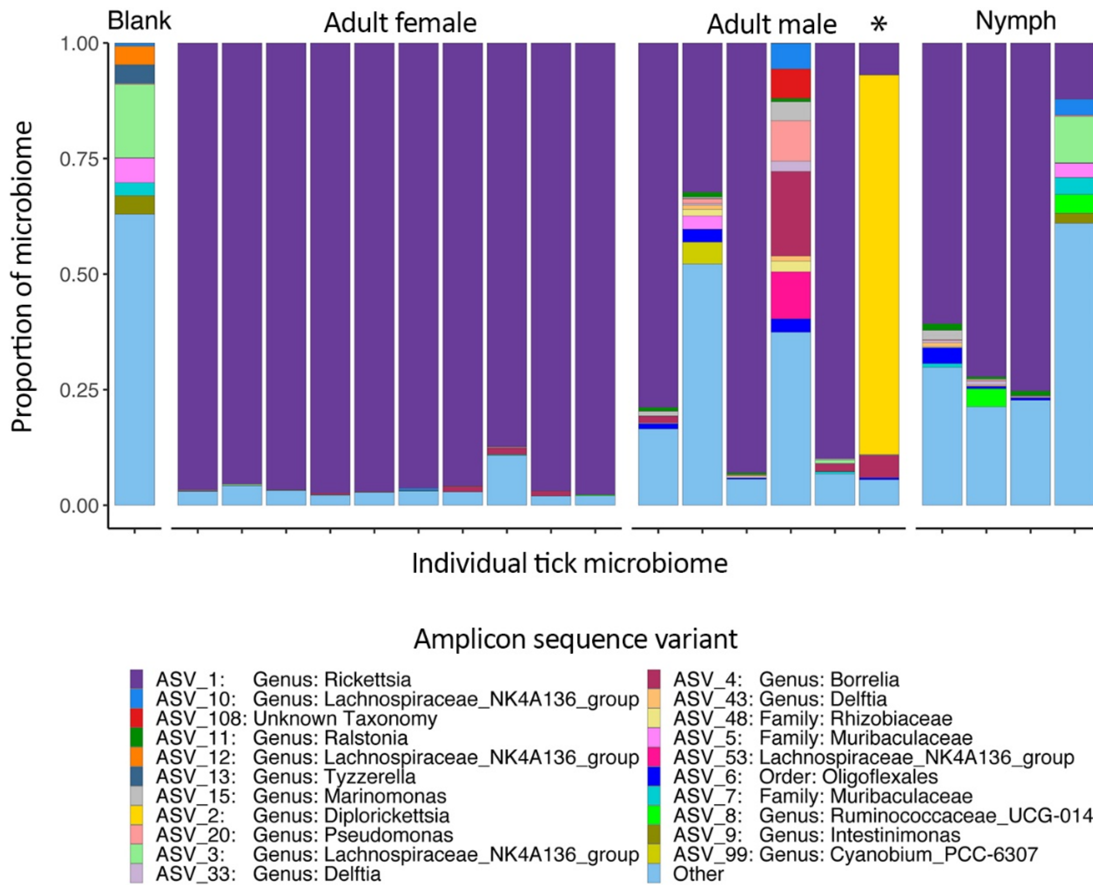
Appendix

Appendix Table 1. Sampling locations for *Ixodes scaularis* tick, Vermont, USA

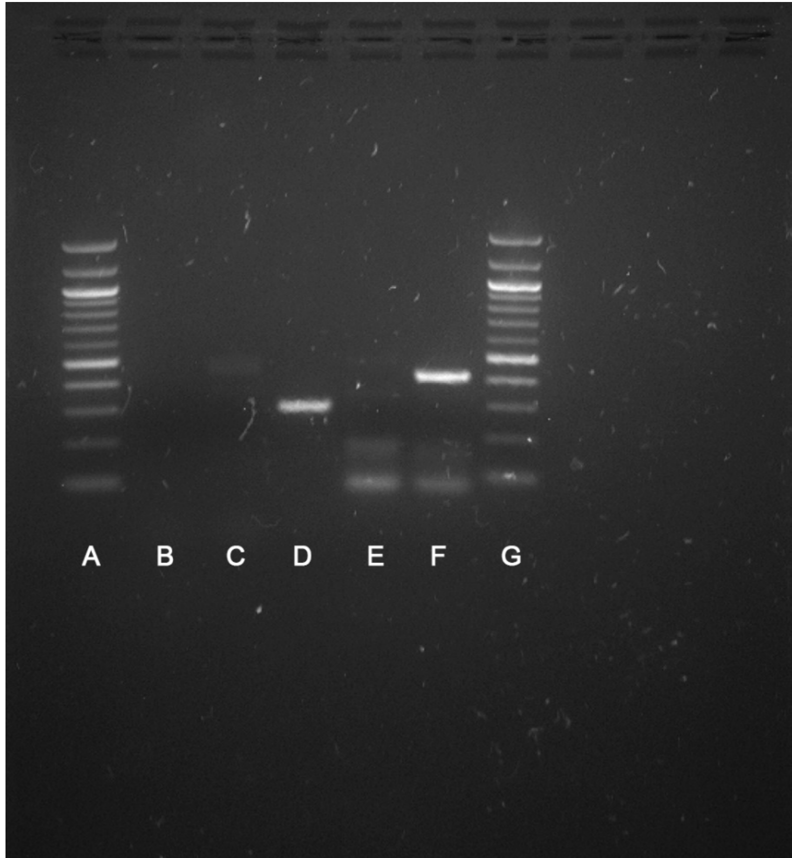
Site	Latitude, °	Longitude, °	Mean elevation, m
Sunny Hollow Colchester	44.518353	−73.17112	93
Shelbourne Town Forest	44.369066	−73.143851	122
New Haven	44.113699	−73.156602	119
Chipman Hill	44.023385	−73.162146	221
Chipman Hill (Backside)	44.026342	−73.159889	213
Orwell	43.818445	−73.264537	140

Appendix Table 2. Primers used for PCR of novel *Diplorickettsia* bacteria found in *Ixodes scaularis* tick, Vermont, USA

Primer	Melting temperature, °C	Primer sequence	Amplicon length, bt
ftsY forward	60	TCATCGATGGCCAAGCTGTT	386
ftsY reverse	60	TTTCACCTTCGCGAGCTCTT	
parC forward	60	ACTCGACCATCCAAAAGCGT	272
parC reverse	60	TCACCAAACGTGTCGGTTGT	



Appendix Figure 1. Microbiome of each tick via 16s RNA sequencing. Each vertical bar represents an individual tick, colored by the proportion of the microbiome made up of each amplicon sequence variant (ASV). For the top 22 ASVs, taxa information is shown as the lowest order to which the ASV can be identified. Asterisk indicates the tick in which *Diplorickettsia* was identified.



Appendix Figure 2. Presence of *Diplorickettsia* is confirmed by PCR for the *parC* and *ftsY* gene. A) New England Biolabs (https://www.neb.com) 100bp ladder; B) negative control, using the *parC* primers but no DNA; C) *Diplorickettsia*-negative tick with *parC*; D) *Diplorickettsia*-positive tick with *parC* (272 bp amplicon); E) *Diplorickettsia*-negative tick with *ftsY;* F) *Diplorickettsia*-positive tick with *ftsY* (386 bp amplicon); G) NEB 100 bp ladder. We used NEB Phusion 2X master mix for PCR, using 25 μL reactions, 1 μL of tick prep DNA (unquantified); final primer concentration for each sample was 0.4 μM. PCR temperatures were 98°C for 30 sec, followed by 35 cycles of 98°C for 10 sec, 61°C for 15 sec, 72°C for 20 sec, followed by 72°C for 10 min.