Identification of a novel pathogenic *Borrelia* species causing Lyme borreliosis with unusually high spirochaetaemia: a descriptive study

Bobbi S Pritt, **Paul S Mead**, **Diep K Hoang Johnson**, **David F Neitzel**, **Laurel B Respicio-Kingry**, **Jeffrey P Davis**, **Elizabeth Schiffman**, **Lynne M Sloan**, **Martin E Schriefer**, **Adam J Replogle**, **Susan M Paskewitz**, **Julie A Ray**, **Jenna Bjork**, **Christopher R Steward**, **Alecia Deedon**, **Xia Lee**, **Luke C Kingry**, **Tracy K Miller**, **Michelle A Feist**, **Elitza S Theel**, **Robin Patel**, **Cole L Irish**, and **Jeannine M Petersen**

Mayo Clinic, Rochester, MN, USA (B S Pritt MD, L M Sloan MT, E S Theel PhD, Prof R Patel MD, C L Irish BS, J A Ray MPH); Division of Vector Borne Diseases, US Centers for Disease Control and Prevention, Fort Collins, CO, USA (P S Mead MD, L B Respicio-Kingry MS, L C Kingry PhD, M E Schriefer PhD, A J Replogle BS, J M Petersen PhD); Wisconsin Department of Health Services, Madison, WI, USA (D K Hoang Johnson BS, Prof J P Davis MD, C R Steward MPH, A Deedon BS); Minnesota Department of Health, St Paul, MN, USA (D F Neitzel MS, E Schiffman MPH, J A Ray, J Bjork DVM); University of Wisconsin–Madison, Madison, WI, USA (Prof S M Paskewitz PhD, X Lee MS); and North Dakota Department of Health, Bismarck, ND, USA (T K Miller PhD, M A Feist BS)

Summary

Background— Lyme borreliosis is the most common tick-borne disease in the northern hemisphere. It is a multisystem disease caused by *Borrelia burgdorferi* sensu lato genospecies and characterised by tissue localisation and low spirochaetaemia. In this study we aimed to describe a novel *Borrelia* species causing Lyme borreliosis in the USA.

Methods— At the Mayo clinic, from 2003 to 2014, we tested routine clinical diagnostic specimens from patients in the USA with PCR targeting the *oppA1* gene of *B burgdorferi* sensu lato. We identified positive specimens with an atypical PCR result (melting temperature outside of the expected range) by sequencing, microscopy, or culture. We collected *Ixodes scapularis* ticks from regions of suspected patient tick exposure and tested them by *oppA1* PCR.

Findings— 100 545 specimens were submitted by physicians for routine PCR from Jan 1, 2003 to Sept 30, 2014. From these samples, six clinical specimens (five blood, one synovial fluid)

yielded an atypical *oppA1* PCR product, but no atypical results were detected before 2012. Five of the six patients with atypical PCR results had presented with fever, four had diffuse or focal rash, three had symptoms suggestive of neurological inclusion, and two were admitted to hospital. The sixth patient presented with knee pain and swelling. Motile spirochaetes were seen in blood samples from one patient and cultured from blood samples from two patients. Among the five blood specimens, the median *oppA1* copy number was 180 times higher than that in 13 specimens that tested positive for *B burgdorferi* sensu stricto during the same time period. Multigene sequencing identified the spirochaete as a novel *B burgdorferi* sensu lato genospecies. This same genospecies was detected in ticks collected at a probable patient exposure site.

Interpretation—We describe a new pathogenic *Borrelia burgdorferi* sensu lato genospecies (candidatus *Borrelia mayonii*) in the upper midwestern USA, which causes Lyme borreliosis with unusually high spirochaetaemia. Clinicians should be aware of this new *B burgdorferi* sensu lato genospecies, its distinct clinical features, and the usefulness of *oppA1* PCR for diagnosis.

Introduction

Lyme borreliosis is a spirochaetal tick-borne disease caused by some genospecies of the *Borrelia burgdorferi* sensu lato complex.[1–4] With 85 000 cases estimated annually in Europe and 300 000 cases estimated annually in the USA, it is the most common tick-borne disease in the northern hemi sphere.[5,6] Nearly all human infections are caused by three *B burgdorferi* sensu lato genospecies: *Borrelia garinii, Borrelia afzelii*, and *B burgdorferi* sensu stricto.[1] All three species cause Lyme borreliosis in Europe, whereas only *B burgdorferi* sensu stricto causes Lyme borreliosis in the USA.[7]

The clinical features of Lyme borreliosis are broad and seem to be associated with distinct tissue tropisms of specific *B burgdorferi* sensu lato genospecies.[8] Early localised infection typically results in erythema migrans rash, after which spirochaetes can disseminate to the nervous system, joints, and other organs. *B burgdorferi* sensu stricto is often associated with arthritis, *B garinii* with neurological effects, and *B afzelii* with acrodermatitis chronicum atrophicans.[8]

Lyme borreliosis is characterised by a low level of spirochaetaemia.[9] Spirochaetes are detectable by PCR in the peripheral blood of less than 50% of patients with erythema migrans, with average estimation of about 2330 genome copies per mL,[9,10] whereas the mean number of spirochaetes detected by culture of peripheral blood is only 0·1 spirochaetes per mL.[9] As expected, microscopic detection of *B burgdorferi* sensu lato spirochaetes has never been reported in peripheral blood, by marked contrast with relapsing fever borreliae, which have loads ranging from 10^5 spirochaetes per mL to more than 10^6 spirochaetes per mL, and are readily seen in peripheral blood.[11] We describe a new *B burgdorferi* sensu lato genospecies causing Lyme borreliosis with substantially elevated spirochaetaemia in acutely ill patients.

Methods

Patients

Mayo Medical Laboratories provides diagnostic PCR testing for Lyme borreliosis.[12] From Nov 1, 2003, to Sept 30, 2014, physicians throughout the USA submitted 100 545 specimens (synovial fluid, cerebrospinal fluid, EDTA [edetic acid]-anticoagulated whole blood, or fresh tissue) to our laboratory for routine clinical PCR testing. We interviewed patients with specimens yielding a PCR result that differed from that expected for *B burgdorferi* sensu stricto*, B garinii*, or *B afzelii*, to obtain clinical and epidemiological information. We reviewed medical records and requested additional samples. Patient follow-up and DNA sequencing of clinical specimens was approved by the Mayo Clinic institutional review board. Patients were interviewed by state public health officials as part of routine surveillance for a reportable condition.

Real-time PCR and DNA sequencing

We extracted DNA from diagnostic specimens using the MagNA Pure Instrument (Roche) and tested for *B burgdorferi* sensu stricto, *B afzelii*, and *B garinii* with a diagnostic real-time PCR assay that uses hybridisation probes and targets the chromosomal *oppA1* gene.[12,13] This assay is specific for *B burgdorferi* sensu lato and does not detect relapsing fever borreliae. We subjected PCR products to melting temperature analysis to differentiate *B burgdorferi* sensu lato genospecies (appendix). We established the number of *oppA1* copies with standard curves that were prepared with genomic DNA from *B burgdorferi* sensu stricto B31 and the MN14-1420 isolate; *oppA1* is present on the chromosome in a single copy in both genospecies. We used the Wilcoxon rank-sum test (two-sided) for *oppA1* PCR crossing point comparison.

We amplified and sequenced portions of the 16S rDNA, *ospC, flaB, rrf-rrl, oppA1, uvrA, rplB, recG, pyrG, pepX, clpX, nifS*, and *clpA* genes using previously described primers.[12,14–17] We analysed, assembled, and trimmed sequences in Lasergene v9·0 (DNASTAR). Using BLAST, 16S rDNA, *ospC, flaB*, and *rrf-rrl* sequences were compared with *B burgdorferi* sensu lato and relapsing fever borreliae sequences in GenBank. For construction of phylogenetic trees, we obtained homologous *B burgdorferi* sensu lato and relapsing fever borreliae sequences from GenBank and PubMLST (appendix). We used MEGA 5 (ClustalW) to align sequences and trees constructed by maximum likelihood analysis using the generalised time-reversible nucleotide substitution model with gamma distribution (four categories) followed by bootstrap analysis (1000 replicates).[18] Housekeeping genes were concatenated in frame in the order *uvrA, rplB, recG, pyrG, pepX, clpX*, and *clpA*, with or without *nifS* and exported into MEGA 5 to calculate pairwise genetic distances using the Kimura-2 model.

Microscopy and culture

Two clinical blood specimens with an atypical *oppA1* PCR melting temperature were available for microscopy and culture after storage at 4°C for either 5 days (one sample) or 39 days (one sample) (appendix). Other specimens were unavailable or previously frozen and

not amenable to microscopy and culture. We examined wet mounts from patient samples and cultures with dark-field microscopy at 400 times magnification (appendix).

Serological testing

We tested serum samples and plasma samples for antibodies reacting to *B burgdorferi* sensu stricto using FDA-cleared commercially available kits, following the recommended two-tiered algorithm (appendix).[19]

Tick collection and processing

We collected *Ixodes scapularis* ticks at approximate sites of possible patient exposure in Barron County, WI, USA, during 2013 and 2014 (appendix), and processed the ticks for PCR using a modification of a published protocol.[20] We also tested archived DNA from *I scapularis* collected in Eau Claire County, WI, USA, during 2009–10.

Role of the funding source

The funders of the study, Mayo Clinic and the US Centers for Disease Control and Prevention, had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

From Jan 1, 2012, to Sept 30, 2014, 9197 clinical specimens from residents of Minnesota, Wisconsin, and North Dakota were submitted to Mayo Clinic, Rochester, MN, USA, for routine diagnostic *B burgdorferi* sensu lato *oppA1* PCR testing. 3127 (34%) of 9197 specimens tested were blood samples, 1196 (13%) were synovial fluid, 4782 (52%) were cerebrospinal fluid, and 92 (1%) were tissue. 102 specimens were positive for *B burgdorferi*, including 13 blood, 81 synovial fluid, three cerebrospinal fluid, and five tissue samples. Six specimens (five blood, one synovial fluid) had positive *oppA1* PCR results with an atypical melting temperature (60·38–61·24°C), falling between the expected melting temperature for *B burgdorferi* sensu stricto (61·7–66·7°C) and *B afzelii* or *B garinii* (51·7–56·7°C) (table 1, figure 1A, figure 2, appendix).[12] No atypical melting temperatures were identified among 24 786 clinical specimens tested from 44 other states during the same time period (Fisher-Exact, p=0·00039), or among more than 66 562 clinical specimens from all states tested by the same method during 2003–11 (0 of 66 562 *vs* six of 33 983, p=0·00149). The five positive blood specimens with atypical melting temperatures were collected 1–4 days after onset of illness; the synovial fluid specimen was obtained 34 days after onset of illness (table 1). The median *oppA1* PCR crossing point was significantly lower (median 29·82, IQR 28·89–30·75) for the five blood specimens with atypical *oppA1* melting temperature compared with the 13 blood specimens that tested positive for *B burgdorferi* sensu stricto (median 34·51, IQR 33·73–35·55; p=0·0016; figure 3).

Sequence analysis of the atypical *oppA1* PCR products that were directly amplified from three patient specimens identified a *Borrelia* species with 89–95% similarity to *B burgdorferi* sensu lato genospecies (figure 4). Motile spirochaetes (two per 70 fields of

diluted blood) of the *B burgdorferi* sensu lato genospecies were microscopically recorded in blood from patient 6, obtained 1 day after illness onset and analysed 6 days later (figure 1B). The number of spirochaetes was estimated at around $8\cdot5 \times 10^4$/mL (appendix). No spirochaetes were seen in the haemolysed blood specimen from patient 5. Cultures of the *B burgdorferi* sensu lato genospecies (MN14-1420 and MN14-1539) were established from both available blood specimens (patients 5 and 6) after incubation for about 16 days. Spirochaetes were seen in primary and blind passaged cultures; sustained growth was achieved after cryopreservation and additional passage.

Based on the high quantity of spirochaetes that were detected microscopically, we estimated the number of *oppA1* copies per mL of blood for all 18 specimens that tested positive for *oppA1* PCR identified in Minnesota, Wisconsin, and North Dakota during 2012–14 by comparison of *oppA1* crossing point values to standard curves prepared with *B burgdorferi* sensu stricto B31 or MN14-1420 (appendix). For the five atypical *oppA1* positives, the median *oppA1* copy number was 180 times higher (median $8\cdot1 \times 10^5$, IQR $4\cdot6 \times 10^5$–$3\cdot6 \times 10^6$) when compared with the 13 *B burgdorferi* sensu stricto positives (median $4\cdot5 \times 10^3$, IQR $2\cdot3 \times 10^3$–$7\cdot5 \times 10^3$; figure 3).

Sequence analysis of 16S rRNA (1327 nucleotides), *ospC* (561 base pairs), *flaB* (435 base pairs), and *rrf-rrl* (253 base pairs) amplified from the two blood isolates substantiated that the *Borrelia* species was not identical to any other *Borrelia* species in GenBank (appendix). The closest sequence identity was to *B burgdorferi* sensu lato genospecies at 99% for 16S RNA, 85% for *ospC*, 97% for *flaB*, and 95% for *rrf-rrl*. Multilocus sequence analysis of seven genes, *uvrA, rplB, recG, pyrG, pepX, clpX*, and *clpA* genes (3774 nucleotides), showed that the spirochaetes isolated from patients 5 and 6 fell within the *B burgdorferi* sensu lato genospecies complex and were the same *B burgdorferi* sensu lato genospecies amplified from the blood of patients 1, 2, and 3. The blood isolates were further compared with 18 *B burgdorferi* sensu lato genospecies and three relapsing fever borreliae using an eight-gene multilocus sequence analysis (*uvrA, rplB, recG, pyrG, pepX, clpX, clpA*, and *nifS*; 4335 nucleotides) previously described for defining *B burgdorferi* sensu lato genospecies[21] (figure 4B). The highest pairwise similarity was to *B burgdorferi* sensu stricto (94·9–95·2% similarity, genetic distance 0·051–0·048), well above the threshold defined for separating genospecies (98·3% similarity, genetic distance 0·017),[21] substantiating that the organism detected in the six patients is a novel *B burgdorferi* sensu lato genospecies, and not a relapsing fever borrelia (eg, *B miyamotoi*). For comparison, the genetic distance recorded between the novel *B burgdorferi* sensu lato genospecies and *B burgdorferi* sensu stricto B31/Z41293 is greater than that seen between other formally recognised *B burgdorferi* sensu lato genospecies, including *B bissetti* and *B kurtenbachii* (0·035) and *B garinii* and *B bavariensis* (0·018).[14,21]

All patients were residents of the upper midwest (Minnesota, North Dakota, or Wisconsin) (figure 5). Median patient age was 36 years (range 10–67 years); four patients were male (table 1). Five presented with acute febrile illnesses, including four with rash. The sixth patient was afebrile but had a 1-month history of unilateral knee pain and swelling. Two patients were admitted to hospital, but none had a known immunocompromising disorder. Testing showed lymphopenia (four of five tested), mild thrombocytopenia (two of five), and

high hepatic transaminases (two of three). All patients reported onset of illness between May and July. Exposure to tick habitats in Minnesota or Wisconsin was reported by all patients and two recalled a tick bite less than 30 days before onset of illness. For one patient, the timing between tick bite and PCR sample acquisition was known to be 13 days.

Descriptions of illness-associated rash varied from diffuse macular rashes involving the face, trunk, and upper extremities (figure 1A, patients 1 and 5) to a single 2 cm diameter erythematous leg lesion at the tick bite location (patient 6; table 1). Patient 3 presented with a single annular erythematous leg lesion with central punctum consistent with erythema migrans, and developed fever, leg and arm pain, and diffuse macular rash on the trunk, upper and lower extremities, and face within 8 h of receiving doxycycline. Differential diagnosis included Jarisch-Herxheimer reaction, drug eruption, and erythema multiforme. Doxycycline was discontinued after one dose. The patient improved without additional treatment, but 3 weeks later developed three erythema migrans lesions on the back and leg, which resolved after treatment with cefuroxime.

All six patients were given antibiotics (table 1). All five patients with febrile illnesses recovered; one with preexisting anaemia reported continuing fatigue. The patient with arthritis improved but reported persistent joint pain 6 months after treatment. Serum or plasma was available for five patients and was tested for reactivity to *B burgdorferi* sensu stricto antigens using the recommended two–tiered algorithm[19] (table 2). Patients 1, 3, and 4 were seropositive to *B burgdorferi* sensu stricto using this algorithm, including the 30 day cutoff for use of IgM immunoblot. Patient 5 had a positive EIA and IgM immunoblot in a sample obtained 32 days after onset of illness. All four seropositive patients had one or more samples positive using the first-tier C6 EIA; patients 3, 4, and 5 were positive using whole cell EIA. Two seropositive patients (3 and 5) had serial samples and sero converted from a negative to positive IgM immunoblot. The only specimen from patient 6 was obtained 1 day after illness onset and was seronegative.

Among archived and prospectively collected ticks *I scapularis*, 19 (2·9%, range 0–5·2%) of 658 were *oppA1* PCR positive for the novel *B burgdorferi* sensu lato genospecies and 195 (29·6%, range 9·8–33·3%) of 658 were positive for *B burgdorferi* sensu stricto; two were positive for both. Sequence analysis of *oppA1* for two ticks (EC10N1 and CP12150) and seven-gene multilocus sequence analysis for one tick (EC10N1) substantiated that the *B burgdorferi* sensu lato genospecies detected in *I scapularis* was the same identified in patients (figure 4A, appendix).

Discussion

We have identified a new *B burgdorferi* sensu lato genospecies (candidatus *Borrelia mayonii*) among patients and *I scapularis* ticks from the upper midwestern USA. A causal role in the patients' illnesses was suggested by the detection of DNA from this genospecies in patient specimens during acute illness, detection of motile spirochaetes in one blood specimen, culture of the novel *B burgdorferi* sensu lato genospecies from two patient specimens, development of a patient antibody response after illness onset, and clinical improvement after antimicrobial therapy active against other *B burgdorferi* sensu lato

genospecies. Failure to identify the organism in more than 90 000 clinical samples tested in previous years and from other states might suggest that this new species recently emerged in the upper midwestern USA.

Using an eight-gene multilocus sequence analysis and a published threshold for delineation of *B burgdorferi* sensu lato genospecies, we showed that the *Borrelia* species is a member of the *B burgdorferi* sensu lato group.

Spirochaetes were seen in the diluted blood of a patient who presented with a single erythema migrans lesion, estimated by microscopy at 10^5 genome copies per mL. The number of genomes in this specimen, based on the single-copy chromosomal gene *oppA1*, was estimated independently at 5×10^5 per mL. Importantly, the median *oppA1* copy number measured for the samples positive for *B burgdorferi* sensu stricto ($4{\cdot}5 \times 10^2$ per mL) agrees with that ($2{\cdot}3 \times 10^2$/mL) recorded previously using quantitative *flaB* PCR, thus supporting the use of the *oppA1* gene for *B burgdorferi* sensu lato quantitation.[10] For all five blood specimens from patients infected with the novel *B burgdorferi* sensu lato genospecies, the number of genomes was estimated to be 10^5–10^6 genome copies per mL. This number is similar to what has been reported for patients infected with relapsing fever borreliae and 50–8000 times higher than the blood specimens that tested positive for *B burgdorferi* sensu stricto during the same time period. The number of spirochaetes as estimated by both microscopy and PCR in blood from patients infected with the novel *B burgdorferi* sensu lato genospecies is greater than previously estimated for *Borrelia miyamotoi* (10^3–10^4 spirochaetes per mL of blood), a relapsing fever borreliae reported to cause human illness in the USA, Europe, and Russia.[22–25] Whether this high spirochaetaemia suggests a different tissue tropism for the new *B burgdorferi* sensu lato genospecies is an important question that needs to be further addressed; five of six novel *B burgdorferi* sensu lato genospecies PCR positives were blood specimens, whereas only 13 (13%) of the 102 *B burgdorferi* sensu stricto PCR positives detected during the same time period were blood, and 81 (79%) of 102 samples were synovial fluids.

Patients infected with the novel *B burgdorferi* sensu lato genospecies presented with differing clinical presentations when compared with patients infected with *B burgdorferi* sensu stricto. At least two patients presented with diffuse macular rash not typical of erythema migrans, including one rash that might have involved the palms and soles. Four patients presented with nausea or vomiting and two with fever over 39°C, symptoms not usually reported for Lyme borreliosis[7,26–28] but often reported among patients infected with relapsing fever borreliae.[29] Similarly, three patients had symptoms potentially consistent with neurological effects (confused speech, profound somnolence, visual difficulties) and two were admitted to hospital.

An important issue raised by identification of the novel *B burgdorferi* sensu lato genospecies is whether existing Lyme borreliosis diagnostic tests can detect infection with this organism. The six patients described here were fortuitously detected during routine clinical testing, because the diagnostic *oppA1* PCR used at Mayo Clinic detects and differentiates *B burgdorferi* sensu lato genospecies by melting temperature analysis. However, it is unknown if diagnostic PCR assays specific for *B burgdorferi* sensu stricto will detect the novel

genospecies. Regarding serology, the *B burgdorferi* sensu stricto C6 EIA was positive in all four patients with specimens obtained 3 days or more after onset of illness and *B burgdorferi* sensu stricto IgM immunoblots of specimens obtained 6 to 32 days after onset were positive for three patients. The *B burgdorferi* sensu stricto IgG immunoblot, however, was positive only for the patient with more than 30 days of untreated illness. *B burgdorferi* sensu stricto serology was negative for three specimens drawn 1–3 days after onset of illness.

The patients' infections were probably acquired by the bite of *I scapularis*, which transmits *B burgdorferi* sensu stricto in the USA. Two patients recalled a tick bite before illness and *I scapularis* that tested positive for the new *B burgdorferi* sensu lato genospecies were collected at two Wisconsin locations, including one visited by two patients. Prevalence of the novel species in tested *I scapularis* ranged from 0·6–4·9%. Non-detection of the new *B burgdorferi* sensu lato genospecies in *I scapularis* collected in the midwestern USA during 2004–07 further suggests that it might have recently emerged in this region.[30,31]

The identification of a novel *B burgdorferi* sensu lato genospecies causing Lyme borreliosis with substantially elevated spirochaetaemia and clinical features distinct from other recognised *B burgdorferi* sensu lato genospecies has important implications for accurate diagnosis, treatment and disease reporting. In view of the differing clinical manifestations for patients infected with the novel *B burgdorferi* sensu lato genospecies, it is likely that Lyme borreliosis is not being considered—and therefore not diagnosed—in some patients with this infection. The clinical range of illness must be better defined in additional patients to ensure that physicians can recognise the infection and distinguish it from other tick-borne infections. Many tick-borne pathogens have global distribution, therefore studies are needed to establish the geographic distribution of human beings and ticks infected with the novel *B burgdorferi* sensu lato genopecies. Finally, clinicians should be aware of the potential role of *oppA1* PCR for diagnosing infection with this novel pathogen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Baranton G, Postic D, Saint Girons I, et al. Delineation of *Borrelia burgdorferi* sensu stricto *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. Int J Syst Bacteriol. 1992; 42:378–383. [PubMed: 1380285]

2. Steere AC, Bartenhagen NH, Craft JE, et al. The early clinical manifestations of Lyme disease. Ann Intern Med. 1983; 99:76–82. [PubMed: 6859726]

3. Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwaldt E, Davis JP. Lyme disease-a tick-borne spirochetosis? Science. 1982; 216:1317–1319. [PubMed: 7043737]

4. Johnson RC, Schmid GP, Hyde FW, Steigerwalt AG, Brenner DJ. *Borrelia burgdorferi* sp. nov.: etiologic agent of lyme disease. Int J Syst Bacteriol. 1984; 34:496–497.

5. Lindgren, E.; Jaenson, TGT. Influences of climate and climate change, epidemiology, ecology and adaptation measures. Copenhagen, Denmark: World Health Organization; 2006. Lyme borreliosis in Europe.

6. Hinckley AF, Connally NP, Meek JI, et al. Lyme disease testing by large commercial laboratories in the United States. Clin Infect Dis. 2014; 59:676–681. [PubMed: 24879782]

7. Stanek G, Wormser GP, Gray J, Strle F. Lyme borreliosis. Lancet. 2012; 379:461–473. [PubMed: 21903253]

8. van Dam AP, Kuiper H, Vos K, et al. Different genospecies of *Borrelia burgdorferi* are associated with distinct clinical manifestations of Lyme borreliosis. Clin Infect Dis. 1993; 17:708–717. [PubMed: 7903558]

9. Aguero-Rosenfeld ME, Wang G, Schwartz I, Wormser GP. Diagnosis of Lyme borreliosis. Clin Microbiol Rev. 2005; 18:484–509. [PubMed: 16020686]

10. Liveris D, Schwartz I, McKenna D, et al. Quantitation of cell-associated borrelial DNA in the blood of Lyme disease patients with erythema migrans. Eur J Clin Microbiol Infect Dis. 2012; 31:791–795. [PubMed: 21842448]

11. Dworkin MS, Schwan TG, Anderson DE Jr, Borchardt SM. Tick-borne relapsing fever. Infect Dis Clin North Am. 2008; 22:449–468. viii. [PubMed: 18755384]

12. Babady NE, Sloan LM, Vetter EA, Patel R, Binnicker MJ. Percent positive rate of Lyme real-time polymerase chain reaction in blood, cerebrospinal fluid, synovial fluid, and tissue. Diagn Microbiol Infect Dis. 2008; 62:464–466. [PubMed: 18947959]

13. Hu LT, Pratt SD, Perides G, Katz L, Rogers RA, Klempner MS. Isolation, cloning, and expression of a 70-kilodalton plasminogen binding protein of *Borrelia burgdorferi*. Infect Immun. 1997; 65:4989–4995. [PubMed: 9393787]

14. Margos G, Hojgaard A, Lane RS, et al. Multilocus sequence analysis of *Borrelia bissettii* strains from North America reveals a new *Borrelia* species, *Borrelia kurtenbachii*. Ticks Tick Borne Dis. 2010; 1:151–158. [PubMed: 21157575]

15. Kugeler KJ, Gurfield N, Creek JG, Mahoney KS, Versage JL, Petersen JM. Discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts when screening ticks by PCR. Appl Environ Microbiol. 2005; 71:7594–7597. [PubMed: 16269811]

16. Rudenko N, Golovchenko M, Lin T, Gao L, Grubhoffer L, Oliver JH Jr. Delineation of a new species of the *Borrelia burgdorferi* sensu lato complex *Borrelia americana* sp. nov. J Clin Microbiol. 2009; 47:3875–3880. [PubMed: 19846628]

17. Wang G, Liveris D, Mukherjee P, Jungnick S, Margos G, Schwartz I. Molecular typing of *Borrelia burgdorferi*. Curr Protoc Microbiol. 2014; 34:12C.5.1–12C.5.31.

18. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011; 28:2731–2739. [PubMed: 21546353]

19. Centers for Disease Control and Prevention. Recommendations for test performance and interpretation from the Second National Conference on Serologic Diagnosis of Lyme Disease. MMWR Mor B Mortal Wkly Rep. 1995; 44:590–591.

20. Cao WC, Gao YM, Zhang PH, et al. Identification of *Ehrlichia chaffeensis* by nested PCR in ticks from southern China. J Clin Microbiol. 2000; 38:2778–2780. [PubMed: 10878087]

21. Margos G, Vollmer SA, Cornet M, et al. A new *Borrelia* species defined by multilocus sequence analysis of housekeeping genes. Appl Environ Microbiol. 2009; 75:5410–5416. [PubMed: 19542332]

22. Molloy PJ, Telford SR III, Chowdri HR, et al. *Borrelia miyamotoi* disease in the northeastern United States: a case series. Ann Intern Med. 2015; 163:91–98. [PubMed: 26053877]

23. Krause PJ, Fish D, Narasimhan S, Barbour AG. *Borrelia miyamotoi* infection in nature and in humans. Clin Microbiol Infect. 2015; 21:631–639. [PubMed: 25700888]

24. Hovius JW, de Wever B, Sohne M, et al. A case of meningoencephalitis by the relapsing fever spirochaete *Borrelia miyamotoi* in Europe. Lancet. 2013; 382:658. [PubMed: 23953389]

25. Platonov AE, Karan LS, Kolyasnikova NM, et al. Humans infected with relapsing fever spirochete *Borrelia miyamotoi*, Russia. Emerg Infect Dis. 2011; 17:1816–1823. [PubMed: 22000350]

26. Strle F, Ruzic-Sabljic E, Cimperman J, Lotric-Furlan S, Maraspin V. Comparison of findings for patients with *Borrelia garinii* and *Borrelia afzelii* isolated from cerebrospinal fluid. Clin Infect Dis. 2006; 43:704–710. [PubMed: 16912943]

27. Strle F, Ruzic-Sabljic E, Logar M, et al. Comparison of erythema migrans caused by *Borrelia burgdorferi* and *Borrelia garinii*. Vector Borne Zoonotic Dis. 2011; 11:1253–1258. [PubMed: 21612533]

28. Logar M, Ruzic-Sabljic E, Maraspin V, et al. Comparison of erythema migrans caused by *Borrelia afzelii* and *Borrelia garinii*. Infection. 2004; 32:15–19. [PubMed: 15007737]

29. Dworkin MS, Anderson DE Jr, Schwan TG, et al. Tick-borne relapsing fever in the northwestern United States and southwestern Canada. Clin Infect Dis. 1998; 26:122–131. [PubMed: 9455520]

30. Hamer SA, Hickling GJ, Walker ED, Tsao JI. Increased diversity of zoonotic pathogens and *Borrelia burgdorferi* strains in established versus incipient *Ixodes scapularis* populations across the midwestern United States. Infect Genet Evol. 2014; 27:531–542. [PubMed: 24953506]

31. Hoen AG, Margos G, Bent SJ, et al. Phylogeography of *Borrelia burgdorferi* in the eastern United States reflects multiple independent Lyme disease emergence events. Proc Natl Acad Sci USA. 2009; 106:15013–15038. [PubMed: 19706476]

Research in context

Evidence before this study

Lyme borreliosis is a multisystem tick-borne disease of wide public health significance. It is the most frequently reported vector-borne disease in the temperate northern hemisphere and is caused by spirochaetes in the *Borrelia burgdorferi* sensu lato genospecies complex. There have been no previous descriptions of the pathogenic *Borrelia* species reported in this study in either ticks or human beings, or reports of Lyme borreliosis with high spirochaetaemia.

Added value of this study

The identification of a new *B burgdorferi* sensu lato genospecies causing Lyme borreliosis with substantially elevated spirochaetaemia and clinical features distinct from other *B burgdorferi* sensu lato genospecies has important implications for accurate diagnosis and treatment. In view of the differing clinical manifestations for patients infected with the novel *B burgdorferi* sensu lato genospecies, it is likely that Lyme borreliosis is not being considered in some patients with this infection. The medical and health-care community need to be aware of this new pathogen to recognise the infection and to treat patients appropriately.

Implications of all the available evidence

The discovery of a novel *B burgdorferi* sensu lato genospecies was attributable to the use of a diagnostic real-time PCR assay that detects and differentiates *B burgdorferi* sensu lato genospecies by melting temperature analysis. Those PCR assays designed specifically for detection of a single *B burgdorferi* sensu lato genospecies do not have the same ability to identify new or different genospecies. Since many tick-borne human pathogens have a global distribution (eg, *B burgdorferi, Babesia microti, Anaplasma phagocytophilum, Ehrlichia muris*, and *Borrelia miyamotoi*), the emergence of this pathogen highlights the need for widespread surveillance to look for emergence of this organism or related species in other parts of the world.
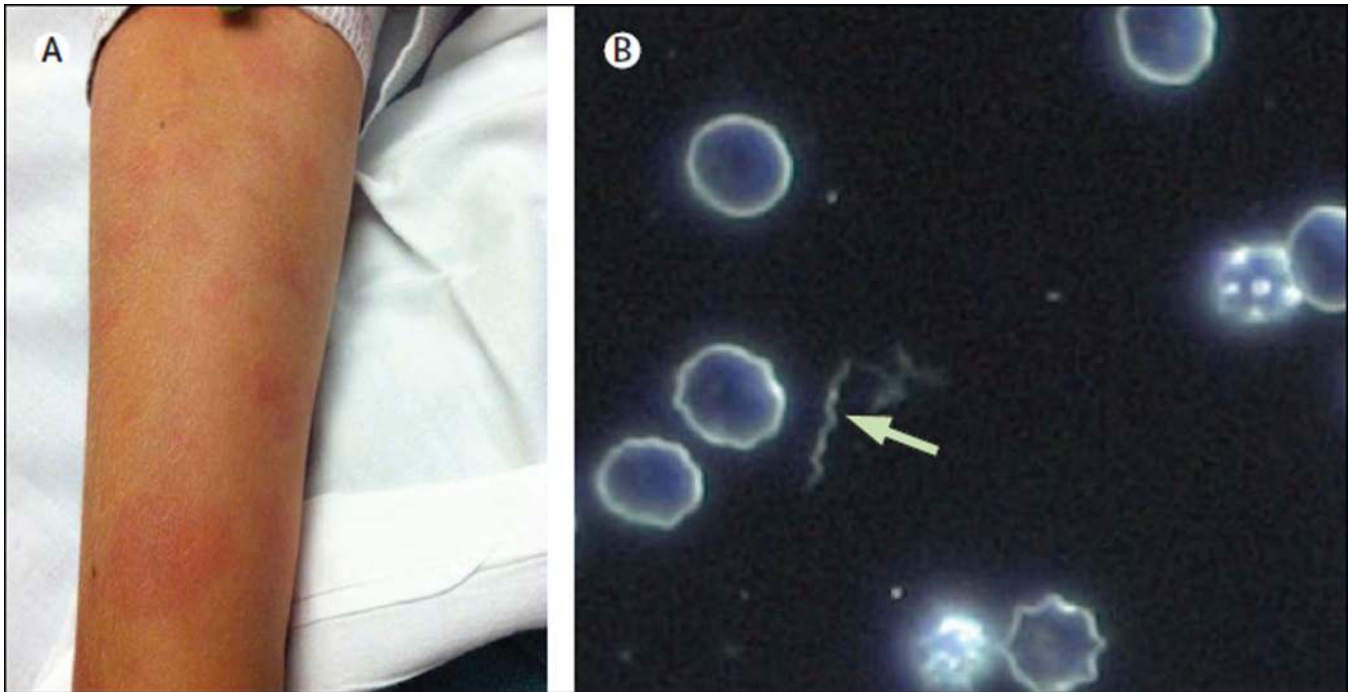
**Figure 1. Diffuse macular rash in patient 1 and dark-field microscopic visualisation of a spirochaete in patient 6**

(A) Diffuse macular rash seen 4 days after onset of symptoms in patient 1. Rash was reported by patient's caregiver to involve the palms and soles, but this was not documented in the medical record. (B) Dark-field microscopic visualisation (400× magnification) of a single spirochaete in diluted blood from patient 6.
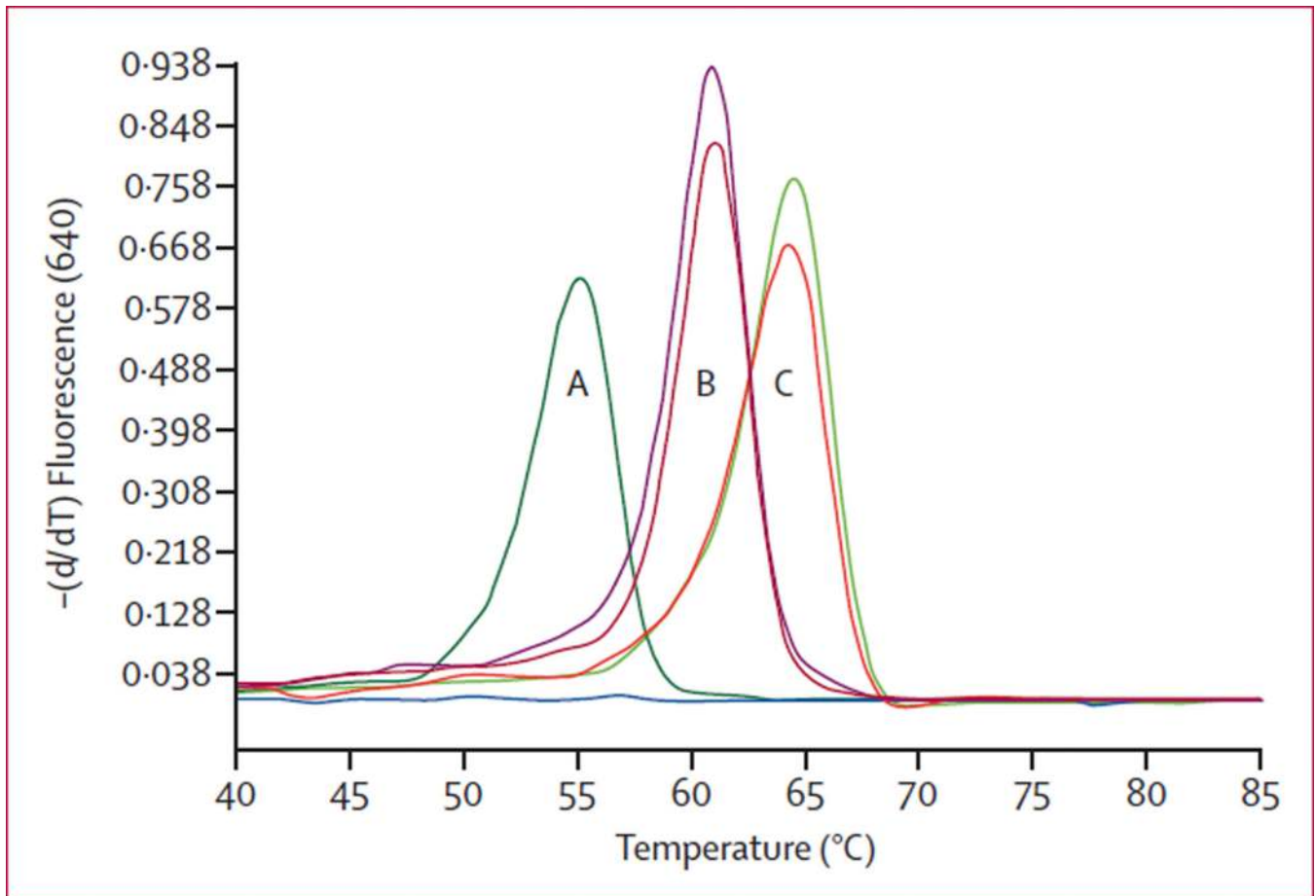
Figure 2. Representative *oppA1* PCR melting temperature peaks of *Borrelia* genospecies
Representative melting temperature peaks in °C for *B afzelii* (A peak; acceptable range
51·7–56·7°C), novel *B burgdorferi* sensu lato genospecies (B peaks; 60·38–61·24°C), and *B burgdorferi* sensu stricto (C peaks; 61·7–66·7°C). Y-axis represents the negative derivative of the ratio of the FRET signal (LC-Red640 flouresence) and background fluorescein fluorescence.
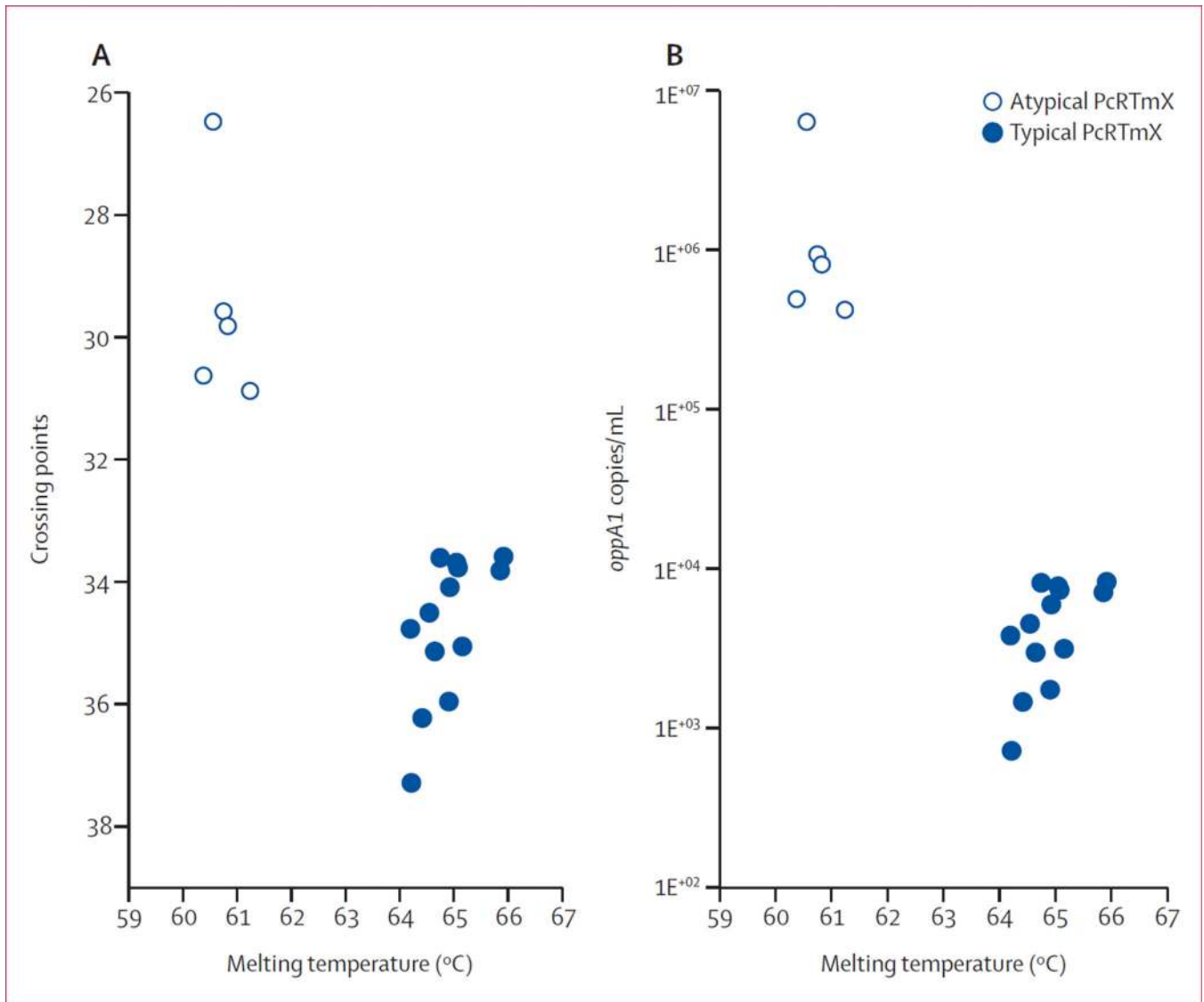
Figure 3. Comparison of *oppA1* PCR melting temperature, crossing points, and estimated *oppA1* copy number in *B burgdorferi*–positive blood specimens

Comparison of melting temperature and crossing point for the five atypical *oppA1* PCR positive blood specimens (open circles) and 13 *B burgdorferi* sensu stricto *oppA1* PCR positive blood specimens (closed circles).

(B) Comparison of melting temperature and estimated *oppA1* copy number (genomes per mL of blood) for five atypical *oppA1* PCR positive blood specimens (open circles) and 13 *B burgdorferi* sensu stricto *oppA1* PCR positive blood specimens (closed circles).
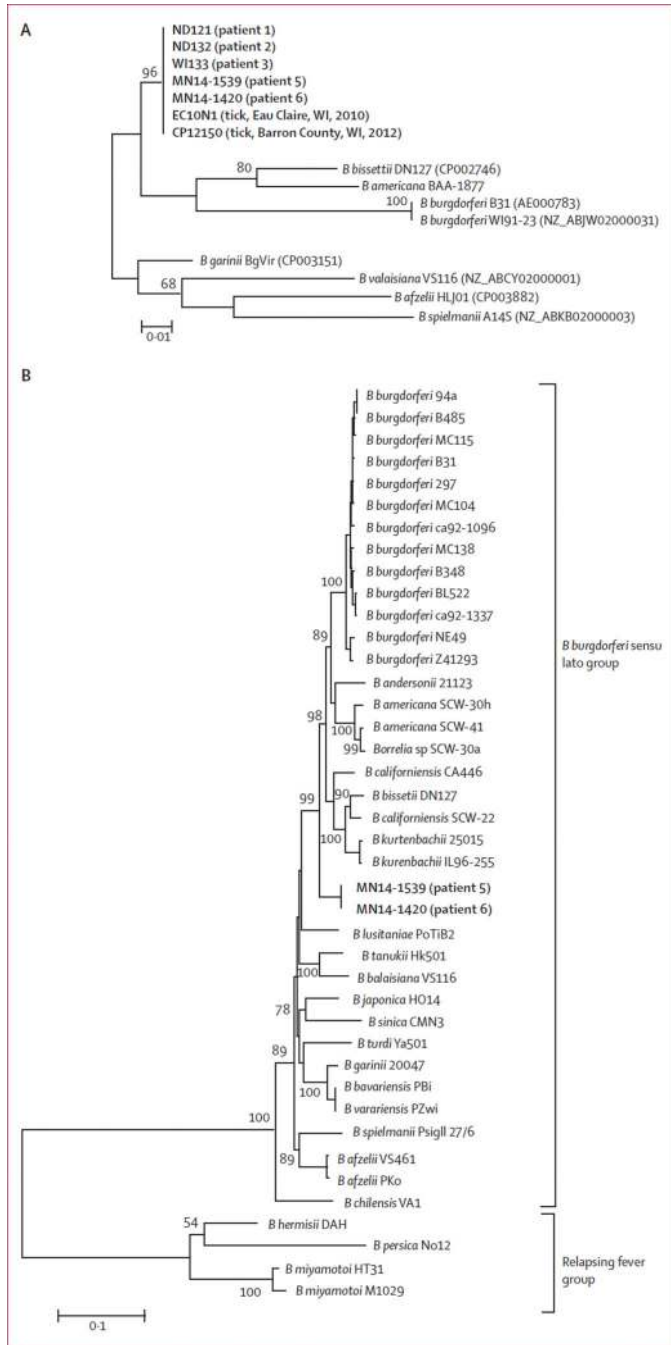
Figure 4. Phylogenetic analyses

(A) Phylogenetic analysis of a 149 base pair fragment of the *oppA1* gene amplified from patient specimens (MN14-1539, MN14-1420, WI133, ND132, and ND121) and tick specimens (CP12150 and EC10N1) compared with seven different species of the *B burgdorferi* sensu lato complex. There is no homologous sequence in relapsing fever borreliae. Bootstrap support values greater than 50% are shown. The scale bar corresponds to 0·01 substitutions per nucleotide position. Accession numbers are indicated for available *Borrelia* species *oppA1* sequences retrieved from GenBank. The *B americana* BAA-1877

oppA1 gene sequence was generated in this study. GenBank does not allow deposition of sequences shorter than 200 bp; *oppA1* sequences generated in this study are available by request. (B) Phylogenetic analysis of eight concatenated housekeeping genes: *uvrA, rplB, recG, pyrG, pepX, clpX, clpA, and nifS*, amplified from patient isolates (MN14-1539, MN14-1420) compared with 18 different *B burgdorferi* sensu lato genospecies and three relapsing fever species. Bootstrap support values greater than 50% are shown. The scale bar corresponds to 0·1 substitutions per nucleotide position. The source of other *Borrelia* species gene sequences is shown in the supplemental methods. Sequence nomenclature (eg, MN14-1539, WI133) represents the state from which the diagnostic specimens were submitted for testing and does not necessarily show the patient's state of residence.
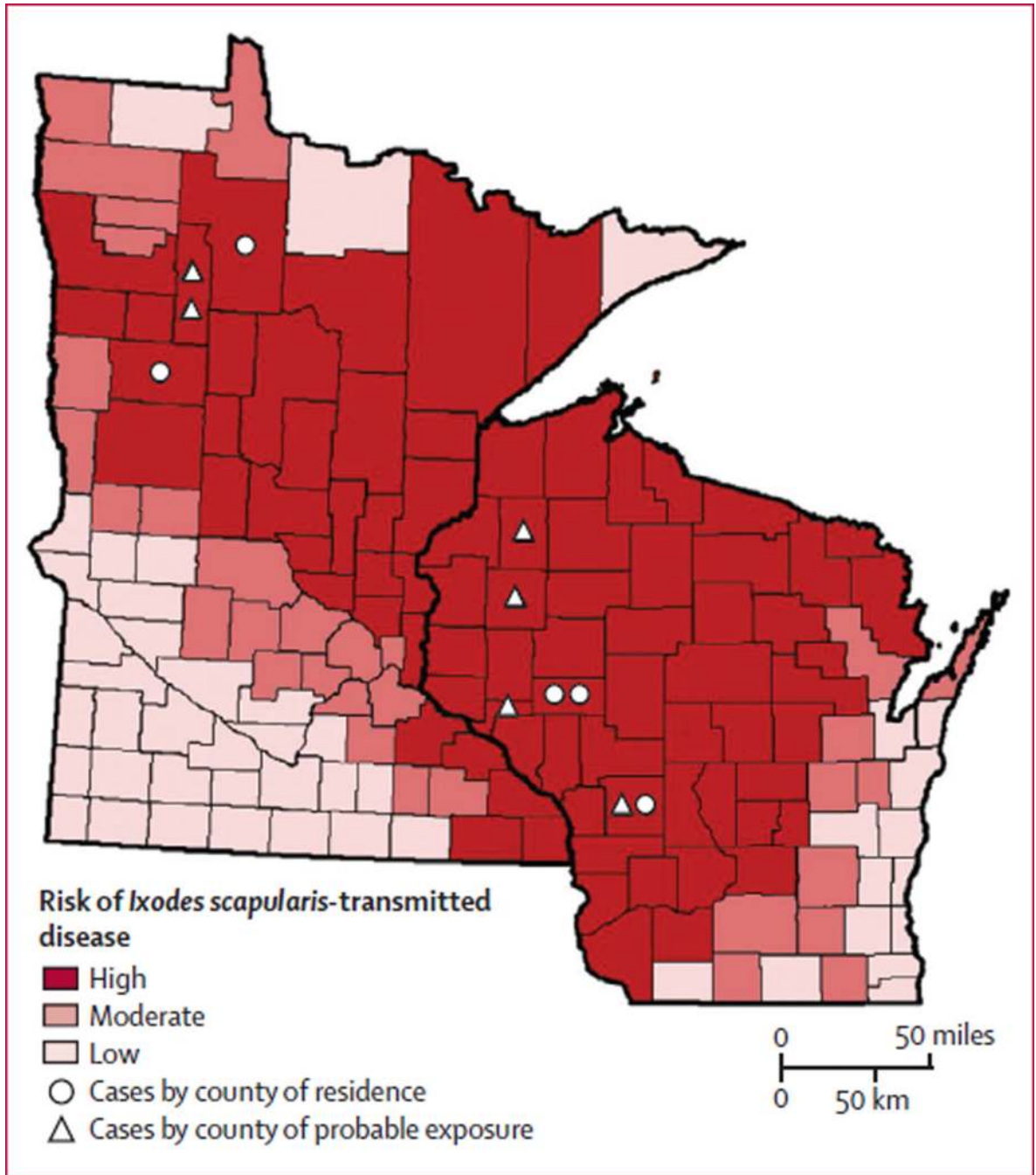
Figure 5. Probable counties of patient exposure to ticks in Minnesota and Wisconsin in relation to risk of diseases transmitted by *I scapularis*

I scapularis-transmitted diseases in the figure were Lyme borreliosis, babesiosis, and anaplasmosis. The county of residence for each patient (indicated with a circle) is deemed a county of potential exposure except for the patient from North Dakota, whose county of residence is not shown. Some patients had probable exposures in one or more county in addition to their county of residence (indicated with a triangle). The risk of disease transmitted by *I scapularis* is based on county-specific mean annual reported incidence of confirmed Lyme borreliosis and confirmed and probable human anaplasmosis and babesiosis

in Minnesota and Wisconsin in 2007–13. Counties with 10·0 or fewer cases per 100 000 people were classified as low risk, counties with 10·1–24·9 cases per 100 000 people were classified as moderate risk, and counties with 25·0 or more cases per 100 000 people were classified as high risk.

Table 1

Demographic, clinical, and laboratory features in patients infected with suspected novel *B burgdorferi* sensu lato genospecies

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Demographic features						
Age (years)	10	65	11	21	67	51
Sex	Male	Male	Male	Female	Female	Male
Tick exposure	Probable	Probable	Known bite	Probable	Probable	Known bite
Symptoms						
Fever	Yes	Yes	Yes	No	Yes	Yes
Headache	Yes	Yes	Yes	No	Yes	Yes
Neck pain	Yes	Yes	Yes	No	No	No
Fatigue	Yes	Yes	Yes	No	Yes	No
Myalgia	Yes	Yes	No	No	Yes	Yes
Nausea or vomiting	Yes	No	Yes	No	Yes	Yes
Arthralgia (site)	No	No	No	Yes (left knee)	No	No
Other	Profound somnolence	..	Confused speech	..	Chills, abdominal and lumbar back pain, flashing lights	..
Physical findings						
Measured temperature (°C)	40	NA	39·7	Afebrile	38·2	NA
Rash	Many erythematous macules on face, trunk, arms (figure 1A)*	NA	Initial macule, enlarged to erythema migrans; diffuse macular rash after single dose of doxycycline; many erythema migrans 28 days later	NA	Many erythematous macules on trunk and upper extremities	2 × 2 cm macule on leg at site of possible tick bite
Other	..	..	..	Swelling left knee	..	..
Laboratory results [normal range for adults and children aged 10–11 years combined]						
Days of illness before specimen collection for PCR	1	4	2	34	3	1
oppA1 PCR melting temperature (°C)	61·24	60·75	60·83	61·19	60·56	60·38
Crossing point	30·88	29·58	29·82	34·20	26·48	30·63
Estimated number of oppA1 copies per mL	$4·2×10^5$	$9·4×10^5$	$8·1×10^5$	Not determined	$6·4×10^6$	$4·9×10^5$

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
White blood cell count (×10⁻⁹/L) [3·4–10·5]	7·4	3·4	4·6	NA	12·4	5·3
Lymphocyte count (×10⁻⁹/L) [0·90–6·50]	0·74	0·31	0·44	NA	0·93	0·30
Platelet count (×10⁻⁹/L) [150–450]	184	113	122	NA	215	150
Haemoglobin (g/dL) [12·0–17·5]	14	NA	14·7	NA	9·6	15·5
Aspartate aminotransferase (U/L) [8–60]	46	NA	NA	NA	118	23
Alanine aminotransferase (U/L) [7–55]	33	NA	NA	NA	69	27
Treatment and outcome						
Antimicrobial therapy	Ceftriaxone (1 day), amoxicillin (21 days; dosage NA)	Doxycycline (dosage and duration NA)	Initial treatment: doxycycline (discontinued after 1 × 50 mg dose)[†]	Initial treatment: doxycycline (100 mg twice per day for 28 days)[‡]	Doxycycline (100 mg twice per day for 21 days)	Doxycycline (100 mg twice per day for 14 days)
Hospital admission	4 Days	No	No	No	1 day	No
Outcome	Recovered	Recovered	Recovered	Persistent joint pain	Improved, lingering fatigue (pre-existing anaemia)	Recovered

Clinical findings and symptoms were recorded by medical staff at time of initial patient presentation. NA=not available.

*
Rash was reported by patient's caregiver to involve the palms and soles, but this was not documented in the medical record.

†
Subsequent treatment for patient 3 was initiated 3 weeks after illness onset, and consisted of cefuroxime, 500 mg twice per day for 21 days.

‡
For patient 4, subsequent treatment consisted of amoxicillin, 500 mg three times per day for 21 days.

Table 2

Serological test results from patients infected with the novel *B burgdorferi* sensu lato genospecies

	Days from onset of illness to collection of specimen	B burgdorferi EIA—whole cell	B burgdorferi EIA-C6	B burgdorferi IgM immunoblot (number of bands detected/number of possible bands); specific antigens detected	B burgdorferi IgG immunoblot (number of bands detected/number of possible bands); specific antigens detected
Patient 1	6	Not available	Positive	Positive (2/3); 23, 41	Negative (1/10); 41
Patient 3	2	Not available	Equivocal	Negative (0/3)	Negative (0/10)
Patient 3	29	Not available	Positive	Positive (3/3); 23, 39, 41	Negative (2/10); 23, 41
Patient 3	104	Positive	Positive	Negative (0/3)	Negative (4/10); 18, 23, 39, 41
Patient 4	266	Positive	Positive	Negative (1/3); 23	Positive (5/10); 23, 39, 41, 45, 58
Patient 5 (plasma)	3	Negative	Positive	Negative (0/3)	Negative (0/10)
Patient 5	32	Positive	Positive	Positive (2/3); 23, 39	Negative (2/10); 23, 41
Patient 6 (plasma)	1	Negative	Negative	Negative (0/3)	Negative (1/10); 41

Specimens from patient 2 were not available for testing. IgM immunoblot was deemed second tier positive for *B burgdorferi* if two or more of a possible three bands (21–25 kDA[OspC], 39 kDA[BmpA], and 41 kDA[Fla]) are detected within 30 days of onset.[17] IgG immunoblot was deemed second tier positive for *B burgdorferi* if five or more of a possible ten bands (18 kDa, 21 kDa[OspC], 28 kDa, 30 kDa, 39 kDa[BmpA], 41 kDa[Fla], 45 kDa, 58 kDa[not GroEL], 66 kDa, and 93 kDa) were detected.