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Isolation of live *Borrelia burgdorferi* sensu lato spirochaetes from patients with undefined disorders and symptoms not typical for Lyme borreliosis

N. Rudenko¹,², M. Golovchenko¹,², M. Vancova¹, K. Clark³, L. Grubhoffer¹,⁴ and J. H. Oliver, Jr.²

¹) Biology Centre Czech Academy of Sciences, Institute of Parasitology, Ceske Budejovice, Czech Republic, ²) Georgia Southern University, The James H. Oliver Jr Institute for Coastal Plain Sciences, Statesboro, GA, ³) Department of Public Health, University of North Florida, Jacksonville, FL, USA and ⁴) University of South Bohemia, Ceske Budejovice, Czech Republic

**Abstract**

Lyme borreliosis is a multisystem disorder with a diverse spectrum of clinical manifestations, caused by spirochaetes of the *Borrelia burgdorferi* sensu lato complex. It is an infectious disease that can be successfully cured by antibiotic therapy in the early stages; however, the possibility of the appearance of persistent signs and symptoms of disease following antibiotic treatment is recognized. It is known that Lyme borreliosis mimics multiple diseases that were never proven to have a spirochaete aetiology [1]. Involvement of new or unknown *Borrelia* species in Lyme borreliosis changes drastically the understanding and recognition of clinical manifestations of this disease. Our results support the fact that *B. bissetti* is responsible for human Lyme borreliosis worldwide along with *B. burgdorferi* s.s. The involvement of new spirochaete species in Lyme borreliosis changes the understanding and recognition of clinical manifestations of this disease.

**Keywords:** Antibiotic treatment, live *Borrelia burgdorferi*, live *Borrelia bissetti*, Lyme borreliosis, recovery of live spirochaetes

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**Corresponding author:** N. Rudenko, Biology Centre Czech Academy of Sciences, Institute of Parasitology, Branisovska 31, 37005, Ceske Budejovice, Czech Republic

E-mail: natasha@paru.cas.cz

**Introduction**

Lyme borreliosis (LB) is the most frequent tick-borne human disease in North America and in Europe. It has long been known that LB is mimicking a wider spectrum of diseases that were never proven to have a spirochaete aetiology [1]. Involvement of new or unknown *Borrelia* species in LB changes drastically the understanding and recognition of clinical manifestations of the disease. Even though antibiotic treatment resolves the majority of clinical manifestations of LB [2,3], the appearance of persistent signs and re-emerging symptoms of disease is recognized.

Spirochaetes from *Borrelia burgdorferi* sensu lato (s.l.) complex have developed sophisticated mechanisms for the evasion of host innate and adaptive immune responses. Spirochaetes are transmitted to vertebrates, including humans, through a tick bite together with tick saliva, which contains a cocktail of immunomodulatory molecules [4]. Spirochaetes themselves possess an array of outer surface proteins that are able to bind vector or host molecules and this protects them against the innate immune response during the initial phase of host colonization/invasion. Following successful host invasion, *Borrelia* has a tendency to reside in immune privileged sites in the host, which provides protection against the developing adaptive immunity of the host [5,6]. Spirochaetes seek protection from the
host immune system in host tissues [7]. It has also been suggested that *Borrelia* may protect itself by morphological changes of motile infective spirochaetes into dormant forms, capable of withstanding unfavourable environmental conditions including antibiotic treatment [8,9]. A major question is whether *B. burgdorferi* s.l. spirochaetes are able to resist antibiotic challenge in transmissible form. Published studies support the fact that LB spirochaetes establish persistent infection in different immunocompetent vertebrate hosts, extending from laboratory mice to non-human primates and humans [10]. However, until now, persisting *Borrelia* were considered to be non-infectious and non-cultivable [10,11]. Here we present the first successful cultivation of live *Borrelia bissettii*-like strain in complete modified Kelly–Pettenkofer (MKP) medium and successful recovery of *B. burgdorferi sensu stricto* (s.s.) from residents of the southeastern USA who suffered from undefined disorders. This study would not have happened if all the sample(s) had been evaluated by the authors according to CDC guidelines. All samples were ‘inappropriate’ because: a) the region of sample origin, the southeastern USA, is considered by the CDC as a non-endemic LB area; b) all patients included in this study were seronegative according to the current CDC surveillance definition of LB; c) all patients underwent extended treatment with doxycycline before the sample collection; and d) patient samples were not taken specifically for the purpose of cultivation. However, taking into consideration the high value of any sample of human origin we decided to analyse them, especially because the tick bite was recalled in most cases.

**Methods**

**Patients study group**

In July–August, 2013, 34 samples were collected from 24 residents of the southeastern USA for microbiological testing as part of ongoing studies of tick-borne diseases in the southern USA (University of North Florida IRB approval #468310-3). Written informed consent was obtained from each patient before enrolment in the study. The group consisted of three samples from North Carolina, eleven samples from Georgia and ten from Florida. Five residents provided serum and plasma samples, the rest contributed either serum (eight samples) or plasma (15 samples). The major symptoms observed in patients included severe headache, nausea, muscle and joint pain, numbness and tingling sensations in extremities, neck pain, back pain, panic attacks, depression, dizziness, vision problems, sleep problems and shortness of breath. Tick bite was recalled by 17 patients (71%), six patients were uncertain and one reported no tick bite. Lesions at the tick bite sites that resembled erythema migrans were observed in 12 patients (50%) and were highly variable in size, from a few centimetres in diameter to 9–12 cm in some cases. Six patients did not report a history of erythema migrans and the last six patients were uncertain. All 12 patients that had a history of erythema migrans were suspected of having LB and were treated to some degree with antibiotics. All patients were tested for multiple tick-borne pathogens, including *Babesia microti*, *Rickettsia rickettsii*, *Ehrlichia chaffeensis* and *Borrelia burgdorferi*, using commercially available kits: tests for *Babesia microti*, *R. rickettsii* and *E. chaffeensis* (Focus Diagnostics, Cypress, CA, USA), Lyme IgM/IgG antibody ELISA and Lyme IgM and IgG Western blot (Quest Diagnostics, Madison, NJ, USA; Labcorp, Burlington, NC, USA, respectively). All patients had already undergone antibiotic treatments, some of extended duration, up to 9 months, that involved oral treatments with 100 mg doxycycline twice a day, as they were suspected of having LB.

**Cultivation media and culture seeding**

Our previous research confirmed the advantage of MKP medium over Barbour–Stoenner–Kelly–H (BSK-H) medium in the establishment of initial spirochaete culture from environmental or clinical samples. All clinical samples were provided by trained medical professionals. Blood was collected into the sterile closed vacutainers (Becton Dickinson, Franklin Lakes, NJ, USA), either clot tubes (for serum) or citrate-treated tubes (for plasma). After preparation, all serum and plasma samples were transferred under aseptic conditions into sterile polypropylene tubes and kept at +4°C. Samples from 11 patients were kept for 48 days before the culture seeding. The remaining samples, from 13 patients, were kept for 5 to 9 days before the culture seeding. The recipe for MKP medium that we used was published earlier [12]. The basic protocol that is routinely used in some European laboratories [13] was adjusted slightly according to our goals. Antibiotics were not included in the medium at any cultivation step. Briefly, 1 mL of each sample was inoculated into freshly prepared complete MKP medium, resulting in 6 mL of newly seeded culture that was grown at 34°C [14]. On day 30, all cultures were centrifuged at 2800 g for 20 min at room temperature. Media were replaced with fresh MKP and cultivation was continued at 34°C until the end of the 9th week, when the same procedure was repeated. Bacterial growth was determined by dark-field microscopy starting day 21 after culture initiation, and samples were examined thereafter on a weekly basis. Developed cultures were analysed by transmission electron microscopy starting day 21 after culture initiation, and samples were examined thereafter on a weekly basis. Developed cultures (14 weeks after seeding) were further re-cultivated in fresh MKP medium by dilution of 2 mL of established culture with 8 mL of fresh MKP (passage 2). To confirm spirochaete multiplication, the re-cultivation step was repeated once more (passage 3). After 11 weeks of cultivation, noticeably developing cultures were analysed by transmission electron microscopy.
microscopy and scanning electron microscopy. As a negative control, two tubes of clean MKP medium were used in each step of this protocol. As a positive control, two tubes of MKP were seeded with $10^5$ spirochaetes of *B. burgdorferi* s.s., strain B31.

**Molecular analysis of spirochaetes cultured from patients**

General processing of spirochaete samples, including DNA purification, PCR amplification, sequencing, sequence analysis and multilocus sequence analysis, was conducted according to our previously published protocols [15–17] and included six genomic loci: 5S-23S intergenic spacer region, 16S-23S internal transcribed region and genes encoding *ospA*, *ospC*, *flagellin* and *p66*.

**Electron microscopy**

Cultures that showed the presence of spirochaete DNA in PCR analysis with *B. burgdorferi* s.l.-specific PCR primers [15] were analysed using electron microscopy techniques.

Transmission electron microscopy. Spirochaetes cultured in MKP medium were pelleted at 460 g, washed in PBS, and fixed in 2.5% glutaraldehyde/0.1 M phosphate buffer (PB) for 1 h at room temperature. The pellets were washed three times in PB with 4% glucose, and centrifuged as before. Drops of sample solution were adsorbed onto glow discharged carbon/formvar-coated copper grids, and incubated for 3 to 5 minutes at room temperature. Grids were washed with three drops of deionized water, stained in 1% aqueous solution of uranyl acetate for 1 min, and dried. Transmission electron microscopy was performed on a JEOL 1010 microscope at an accelerating voltage of 80 kV. Images were captured with a MegaView III camera (SIS GmbH). For statistical evaluation, the images were taken randomly from at least two different grids. Cell lengths and diameters were measured using ImageJ, and results were statistically evaluated.

Scanning electron microscopy. Cultured spirochaetes were pelleted at 460 g, washed in PBS, and fixed in 2.5% glutaraldehyde in PB as described above. Cells were washed three times in PB with 4% glucose, and placed on polyl-lysine-coated cover slides. Samples were post-fixed in 1% osmium tetroxide for 15 min, and washed three times. Dehydration was performed in a graded series of acetone for 10 min at each step. Samples were dried using the critical-point drying method (CPD2 Pelco™, Ted Pella, Redding, CA, USA), mounted on aluminium stubs, gold-coated (sputter-coater Bal-tec SCD 050; Leica, Wetzlar, Germany), and examined in a field emission scanning electron microscope JSM 7401-F (JEOL, Tokyo, Japan).

**Results**

After 11 weeks of cultivation, atypically shaped microorganisms were observed in five out of 34 initial MKP cultures, i.e. one serum sample from North Carolina, two plasma samples from Georgia and two plasma samples from Florida. However, after 14 weeks of cultivation one serum sample from North Carolina and one plasma sample from Florida did not develop into growing cultures and did not show the presence of viable cells. PCR analysis of residual culture with *B. burgdorferi* s.l.-specific PCR primers confirmed the presence of *Borrelia*-related DNA in both of them. The remaining three plasma samples, M6p and M11p from Georgia, and M7p from Florida, developed into well-established cultures that confirmed their viability in two subsequent passages in MKP medium. Initially, all samples were seeded in duplicate into homemade complete MKP and BSK-H media under identical conditions. We do not present any data regarding the BSK-H part of the project as all BSK-H seeded samples were culture negative after 14 weeks of cultivation.

M6p and M11p strains were isolated from a married couple, both 48-years old, currently living in Georgia, USA. The male patient (M6p) grew up in Tampa, Florida and was an airline pilot. Although travelling internationally for several years, he denied being exposed to tick habitat outside the USA. During the past few years he had flown only within the USA. The patient recalled tick bites in several areas of Georgia and Florida; however, the vast majority of bites were encountered at the site of his current residence. No information is available regarding the tick species. In 2010 he began experiencing muscle and joint pain, especially in his hips. He sought clinical evaluation in August 2011. Tests for *Babesia microti* and *R. rickettsii* (Focus Diagnostics, USA) were negative. Tests for *E. chaffeensis* IgM were positive at 1:40 (reference range <1:20) and IgG was positive at 1:256 (reference range <1:64). Lyme IgM/IgG antibody ELISA (Quest Diagnostics) was positive at 2.56 (diagnostic cut off is >1.19), whereas Lyme IgM and IgG Western blots were negative (Quest Diagnostics). The patient completed 9 continuous months of oral doxycycline treatment (100 mg twice per day) with resolution of most symptoms for the following year. However, by June 2013 his previous symptoms had re-emerged.

The female patient (sample M11p), the spouse of the male patient described above, grew up in Alabama, and also lives at their residence in Georgia, USA. Over the years she experienced multiple tick bites (tick species is unknown) in her current neighbourhood. Only bites in June 2010 resulted in a noticeable rash on her abdomen and thigh. The patient experienced headache, nausea, muscle and joint pain, predominantly in her hands and arms. In August 2011 she was tested for the
presence of multiple tick-borne pathogens. The Lyme IgM/IgG ELISA test gave borderline results at 1:18. Lyme IgM Western blot was negative. The presence of a single p41 band was observed on the Lyme IgG Western blot. Test results for all other tick-borne infections were negative. This patient also completed 9 months of continuous oral doxycycline treatment, with improvement of symptoms. Yet, her symptoms also returned approximately 1 year after the treatment.

The patient from Florida (sample M7p) was a 58-year-old female who grew up in New Jersey, moved to Georgia in 1983 and then to Tallahassee, Florida in 2006. In May 2013, she detected an attached tick, and experienced a rash at the tick bite site. She had not travelled from the site of her residence in the days before the tick bite. No information is available concerning the tick species. The patient’s primary-care physician suspected Lyme borreliosis. However, a Lyme IgM/IgG ELISA test was negative. She was prescribed 10 days of oral doxycycline after that. After 40 days of continuous antibiotic treatment, the patient’s blood was collected for analysis. At that time, the patient experienced headache, neck pain, back pain, panic attacks, depression, dizziness, vision problems and numbness or tingling in the extremities, sleep problems, tinnitus and shortness of breath. Lyme IgM and IgG Western blot (Labcorp) tests conducted on the same blood samples were negative. The three patients presented were all seronegative according to the current CDC surveillance definition of LB, yet B. burgdorferi s.l. was isolated from each of them.

Major morphological features of spirochaete isolates M6p, M11p and M7p cultured from patients were measured and evaluated using negatively stained cells and scanning electron microscopy (Table 1, Fig. 1). Average lengths of M6p and M11p spirochaetes from first and third passages differed, in contrast to measurements of M7p, which did not change (Table 1).

PCR analysis following sequencing of cultured strains with B. burgdorferi s.l. primers defined strains M6p and M11p (married couple from Georgia, USA) as B. burgdorferi s.s. Multilocus sequence analysis of M6p and M11p confirmed their close relatedness to B. burgdorferi s.s. strains originating from Ixodes scapularis ticks and LB patients from New York, Connecticut and Massachusetts, all regions where LB is endemic in the northeastern USA [18]. M6p and M11p strains carry the ospC allele, which has been associated with invasive B. burgdorferi s.s. strains isolated from human blood and cerebral spinal fluid in regions where LB is endemic [20]. The M6p and M11p carried the same ospC allele as earlier described for 15 B. burgdorferi s.s. strains isolated from the rodent host Peromyscus gossypinus and the non-human biting tick species Ixodes affinis, both specific to the southeastern USA. [16]. The multilocus sequence analysis showed that the M7p strain is closely related to B. bissettii. The six loci analysed revealed the highest levels of similarity to B. bissettii strain DN127 and B. bissettii strains isolated from Ixodes minor, I. scapularis and I. affinis ticks, and rodent hosts including Neotoma floridana and P. gossypinus, all collected in South Carolina and Georgia in 1994–95: 16S-23S intergenic spacer region (99–100%), 5S-23S intergenic spacer region (98%), and genes encoding ospC (99%), p66 (99%), flagellin (99%) and ospA (96%) (sample collection: JHO Jr, KC; sample analysis: NR, MG, unpublished data).

**Nucleotide sequence accession numbers**

Sequences determined in this study have been deposited into GenBank and given the indicated accession numbers (below). Numbers for each isolate are given for sequenced genomic loci in the following order: ospA, ospC, flagellin, p66, 5S-23S intergenic spacer region, 16S-23S internal transcribed region: KM269419, KM269449, KM269446, KM269452, KM269458, KM269455 for M6p; KM269420, KM269450, KM269447, KM269453, KM269459, KM269456 for M11p; KM269421, KM269451, KM269448, KM269454, KM269460, KM269457 for M7p.

**Discussion**

Successful treatment of acrodermatitis chronica atrophicans and erythema migrans with penicillin [21,22] was reported long before the description of Lyme borreliosis in Connecticut and isolation of its causative agent, B. burgdorferi [2]. Years of research have extended the list of antimicrobial drugs used to treat LB [23]. Because evaluation of antibiotic treatment was based on success in obtaining positive spirochaete cultures after the treatment, different classes of antibiotics were considered to be effective [10], as all attempts to culture Borrelia using the established protocols failed, i.e. cultures were negative. Nevertheless, sceptical opinions about the effectiveness of antibiotic therapy in LB treatment circulated among the medical community for years due to the recurrent symptoms that some patients experienced in spite of the extended antibiotic challenge [11,24–26]. Laboratory studies on multiple immunocompetent hosts involving highly sensitive and specific molecular tools for pathogen detection revealed the presence of persistent spirochaetes in host tissues months after antibiotic treatments [10,11]. Neither detection of Borrelia-specific DNA by PCR and quantitative PCR in hosts, nor the detection of intact spirochaetes by immunochemistry or xenodiagnosis in culture-negative mice and the ticks that fed on them [11,24] provided sufficient evidence to verify the presence of live Borrelia. Recently, xenodiagnosis using I. scapularis nymphs was applied to patients to investigate whether live spirochaetes can
persist in humans after recommended courses of antibiotic treatment [27]. The majority of antibiotic-treated patients in that study were negative by xenodiagnosis. *Borrelia* specific DNA was detected in ticks removed from a patient with erythema migrans at an early stage of antibiotic treatment and from one post-treatment Lyme disease syndrome patient, the presence of persistent spirochaetes in whom was confirmed twice by two xenodiagnosis procedures conducted within an 8-month interval. Live spirochaetes were not recovered from either one of the samples available [27]. That study triggered an intensive discussion about the usefulness of applied xenodiagnosis [28] as the method and results of the study ‘provide the evidence against the biologic plausibility of a hypothesis’ of the persistence of *Borrelia* in patients after antibiotic treatment [28].

The lack of success in spirochaete cultivation from patients reported by multiple groups might have several explanations. One might be the structural alteration in spirochaete forms as a result of antibiotic challenge and induction of morphological changes that lead to the formation of dormant forms that are resistant to antibiotics. A recent study by Sapi et al. [29] on the analysis of the effect of different antibiotics on different morphological forms of two strains of *B. burgdorferi*, B31 and S297, using direct cell counting and dark-field microscopy, showed that doxycycline treatment reduced motile spirochaete forms by approximately 90%, but increased the number of cystic round body forms about twofold. *In vitro* evaluation of the same treatment with the use of fluorescence microscopy showed the reduction of spiral forms of *B. burgdorferi* by 94%, but confirmed that 5% of the remaining spirochaetes were alive. Approximately 5% of the round bodies population were dead, although the rest represented live cells [29]. One may speculate that atypical dormant spirochaete forms might persist in LB patients for years, regardless of antibiotic treatment and, probably, transform back into spiral motile forms under favourable conditions [8,9], which might coincide with discontinuous antibiotic treatment in some patients. All three culture-positive patients in our study underwent extended but interrupted treatment with doxycycline, which might explain the recurrent symptoms that they experienced. Recurrence of LB occurs because of either a relapse or a re-infection and this issue is as controversial as LB itself. Although in our study the possibility of re-infection of the patients cannot be completely ruled out, we suggest that it is highly improbable for the following reasons. The blood sample that resulted in culture of the M7p *B. bissettii*-like strain was taken after 40 days of oral doxycycline treatment. Taking into consideration the pharmacokinetics of doxycycline, we can speculate that it is possible that the antibiotic was still present in a patient’s body in a concentration sufficient to kill the majority of bacteria at the time-point of blood sample collection. The *B. burgdorferi* M6p and M11p strains originated from patients that underwent 9 months of oral doxycycline treatment and did not recall a tick bite after that. Doxycycline does not accumulate in human tissues at a scale that might keep the person bacteria-free during the year following treatment and its favourable pharmacokinetics might have rather low impact also. However, the molecular techniques that we used for amplification of the six loci from the *Borrelia* genome have previously proved to be highly sensitive in detection of multiple species or different spirochaete strains present in a single sample [16,17,30]. The uniformity of spirochaete species/strain genetic material was analysed three times after each re-cultivation and revealed identical sequences every time.

Our attempts to cultivate live *Borrelia* from residents of southeastern US states was a logical step after extended analysis of a population of *B. burgdorferi* s.s. strains distributed in North and South Carolina, Georgia and Florida [16]. We found earlier that 30% of the southeastern *B. burgdorferi* s.s. strains analysed represent an ospC B type, the one most often detected in LB patients from endemic areas of the northeastern and Midwestern US states [16,20]. For LB to occur, humans must be exposed to invasive strains via a tick bite. However, the aforementioned southeastern ospC type B strains were cultured either from rodent hosts or tick vectors that rarely bite humans. So, at present we can only speculate whether patients in our study acquired the *B. burgdorferi* s.s.

### TABLE 1. Structural parameters of *Borrelia burgdorferi* s.l. strains M6p, M11p and M7p, measured on passage 1 and 3

<table>
<thead>
<tr>
<th>Parameter</th>
<th>First passage</th>
<th></th>
<th>Third passage</th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>M6p</td>
<td>M11p</td>
<td>M7p</td>
<td>M6p</td>
<td>M11p</td>
</tr>
<tr>
<td>Length ± SD (μm)</td>
<td>15.8 ± 5.2</td>
<td>15.1 ± 3.5</td>
<td>14.5 ± 3.9</td>
<td>14.2 ± 3.7</td>
<td>14.6 ± 3.8</td>
</tr>
<tr>
<td>Minimum–maximum (μm)</td>
<td>2.57–27</td>
<td>6–28</td>
<td>6–21</td>
<td>6–23</td>
<td>6–23</td>
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<tr>
<td>Diameter ± SD (μm)</td>
<td>0.244 ± 0.04</td>
<td>0.216 ± 0.04</td>
<td>0.276 ± 0.04</td>
<td>0.225 ± 0.04</td>
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</tr>
<tr>
<td>Wavelength (μm)</td>
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<td>ND</td>
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<td>Number of wavelengths</td>
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<td>ND</td>
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<td>5.7 (n = 41)</td>
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<td>59</td>
<td>59</td>
<td>52</td>
<td>53</td>
</tr>
</tbody>
</table>

Spirochaetes were prepared using a negative staining method. Parameters were measured using transmission electron microscope JEOL 1010. Abbreviations: ND, not determined; SD, standard deviation.

*Fragments.*
strains outside the southeastern USA or the strains were transmitted to patients through the bites of tick species other than *I. scapularis*, the traditionally recognized bridge vector in the eastern USA.

The recognition of *B. bissettii* as a causative agent of Lyme borreliosis in Europe was supported by the detection of spirochaetes in serum samples [31] and aortic valve tissue of LB patients from the Czech Republic [32]. In 2011, Girard et al. presented the first molecular evidence of *B. bissettii*-like DNA detected in residents of Mendocino County (CA, USA) [33]. Although one patient from that study had a history of erythema migrans and joint pain, the others had no history of LB signs, symptoms or diagnosis. Interestingly, all individuals with evidence of single infections or co-infections with a *B. bissettii*-like spirochaete were seronegative in previous tests conducted in 1988–89 [33].

Another noteworthy detail is that *B. bissettii*-like DNA sequences detected in serum samples of California residents formed a polytomy along with *B. bissettii* and *B. carolinensis* isolates in phylogenetic analysis [33], the same results as we obtained on MLST analysis of eight housekeeping genes from live *B. bissettii*-like strain M7p, isolated from residents of Florida (submitted for publication) [34]. Here, we present the first recovery of live *B. bissettii*-like spirochaetes (strain M7p) from North American residents. The taxonomic status of this strain requires further investigations.

*Borrelia bissettii* occurs in the western and southern USA, but rarely in the northeastern region. Records in the southeastern USA of naturally occurring *B. bissettii* infections in rodents, birds and *Ixodes* ticks [35] indicate that it is widely distributed and is not narrowly confined to particular vertebrate hosts or tick species in this region. Isolation of a live *B. bissettii*-like strain from a patient is strong evidence that this species, in addition to *B. burgdorferi* s.s., may cause human LB in North America. The successful cultivation of two *B. burgdorferi* s.s. strains and one *B. bissettii*-like strain from antibiotic-treated patients might suggest that active infection with persistent *Borrelia* may be the cause of recurrent symptoms and persistent disease. In addition, we cannot rule out the possibility that successful isolation of live spirochaetes from patients after extended antibiotic treatment might be the result of developed spirochaete resistance to doxycycline.

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Transparency Declaration

The authors have no conflicts of interest to declare.

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