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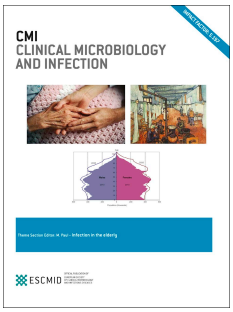

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ORIGINAL ARTICLE

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

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Running title: *B. burgdorferi* s.l. in patients with undefined disorder

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Abstract

Lyme borreliosis is a multisystem disorder with diverse spectrum of clinical manifestations, caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex. It is an infectious disease that can be successfully cured by antibiotic therapy on early stages; however, the possibility of appearance of persistent signs and symptoms of disease following antibiotic treatment is recognized today. It is known that Lyme borreliosis is mimicking multiple diseases that were never proven to have a spirochete etiology. Using complete modified Kelly-Pettenkofer medium we succeeded in cultivation of live *B. burgdorferi* sensu lato spirochetes from samples of humans who suffered from undefined disorders, had symptoms not typical for Lyme borreliosis, but undergone antibiotic treatment due to suspicion of having Lyme disease even though they were seronegative. We report the first recovery of live *Borrelia burgdorferi* sensu stricto from residents of southeastern United States and first successful cultivation of live *Borrelia bissettii*-like strain from resident of North America. Our results support the fact that *B. bissettii* is responsible for human Lyme borreliosis worldwide along with *B. burgdorferi* s.s. Involvement of new spirochete species in Lyme borreliosis changes the understanding and recognition of clinical manifestations of this disease.

Keywords: Lyme borreliosis, recovery of live spirochetes, antibiotic treatment, live *Borrelia burgdorferi*, live *Borrelia bissettii*

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 spirochete etiology [1]. Involvement of new or unknown *Borrelia* species in LB changes drastically the understanding and recognition of clinical manifestation 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 today.

Spirochetes from *Borrelia burgdorferi* sensu lato (s.l.) complex have developed sophisticated mechanisms for evasion of host innate and adaptive immune responses. Spirochetes are transmitted to vertebrates, including humans, via a tick bite together with tick saliva which contains a cocktail of immunomodulatory molecules [4]. Spirochetes themselves possess an array of outer surface proteins which 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]. Spirochetes 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 spirochetes into dormant forms, capable of withstanding unfavorable environmental conditions including antibiotic treatment [8, 9]. A major question is whether *B. burgdorferi* s. l. spirochetes are able to resist antibiotic challenge in transmissible form. Published studies support the fact that LB spirochetes establish persistent infection in different immunocompetent vertebrate hosts, extending from laboratory mice to nonhuman primates and humans [10]. However, until now,

66 persisting *Borrelia* were considered to be noninfectious and non-cultivable [10, 11]. Here we
67 present the first successful cultivation of live *Borrelia bissettii*-like strain in complete modified
68 Kelly-Pettenkofer (MKP) medium and successful recovery of *B. burgdorferi* sensu stricto (s.s.)
69 from residents of southeastern United States who suffered from undefined disorders. This study
70 would never happen if all the sample(s) have been evaluated by authors according to CDC
71 guidelines. All samples were "inappropriate" because: a) the region of sample origin, the
72 southeastern United States, is considered by CDC as non-endemic LB area; b) all patients
73 included in this study were seronegative according to the current CDC surveillance definition of
74 LB; c) all patients underwent extended treatment with doxycycline before the samples collection;
75 d) patients samples were not taken for cultivation purpose specifically. However, taking into
76 consideration the high value of any sample of human origin we decided to analyze them,
77 especially because the tick bite was recalled in majority of cases.

78 **Methods**

79 **Patients' study group**

80 In July-August, 2013, thirty four samples were collected from 24 residents of southeastern
81 United States for microbiologic testing as part of ongoing studies of tick-borne diseases in the
82 southern United States (University of North Florida IRB approval #468310-3). Written informed
83 consent was obtained from each patient prior enrolment into the study. The group consisted of 3
84 samples from North Carolina (NC), 11 samples from Georgia (GA) and 10 from Florida (FL).
85 Five residents provided serum and plasma samples, the rest contributed either serum (8 samples)
86 or plasma (15 samples). The major symptoms observed in patients represented severe headache,
87 nausea, muscle and joint pain, numbness and tingling sensations in extremities, neck pain, back
88 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 1 reported no tick bite. The lesions at the tick bite sites that resembled erythema migrans were observed in 12 patients (50%) and were highly variable in size, from few centimeters in diameter to 9-12 centimeters in some cases. Six patients did not report history of EM and the last 6 patients were uncertain. All twelve patients that had a history of EM 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, Rickettsia rickettsii* and *Ehrlichia chaffeensis* (Focus Diagnostics, USA), Lyme IgM/IgG antibody ELISA and Lyme IgM and IgG Western Blot (Quest Diagnostics, USA; Labcorp, USA, respectively). All patients had already undergone antibiotic treatments, some of extended duration, up to 9 months, that involved the 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 modified Kelly-Pettenkofer (MKP) medium over Barbour-Stoenner-Kelly-H (BSK-H) medium in establishment of initial spirochete 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, U.S.A), either the clot tubes (for serum) or citrate-treated tubes (for plasma). After the preparation all serum and plasma samples were transferred under aseptic conditions into a sterile polypropylene tubes and kept at +4^{o}C. Samples from 11 patients were kept for 48 days before the culture seeding. Remaining samples from 13 patients were kept from 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 into the medium at any cultivation step. Briefly, one milliliter of each sample was inoculated into freshly prepared complete MKP medium, resulting in 6 ml of newly seeded culture that was grown at 34^{o}C [14]. On day 30 all cultures were centrifuged at 2, 800 x g for 20 minutes at room temperature. Media was replaced by fresh MKP and cultivation was continued at 34^{o}C till 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 (14 weeks after seeding) were further re-cultivated in fresh MKP medium by dilution of 2 mls of established culture with 8 mls of fresh MKP (passage 2). To confirm spirochete multiplication, the re-cultivation step was repeated once again (passage 3). After 11 weeks of cultivation noticeably developing cultures were analyzed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). 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 10^2 spirochetes of *B. burgdorferi* s.s., strain B31.

Molecular analysis of spirochetes cultured from patients

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

Electron microscopy

Cultures that showed the presence of spirochete DNA in PCR analysis with *B. burgdorferi* s.l. specific PCR primers [15] were analyzed by electron microscopy techniques.

Transmission Electron Microscopy (TEM)

Spirochetes cultured in MKP media were pelleted at 460 × g, washed in PBS, and fixed in 2.5% glutaraldehyde/0.1 M phosphate buffer (PB) for 1 hr at room temperature (RT). The pellets were washed 3x 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 RT. 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 (TEM) was done 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 at least from two different grids. Cell lengths and diameters were measured using ImageJ, and results were statistically evaluated.

Scanning electron microscopy (SEM)

Cultured spirochetes 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 poly-L-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 aluminum stubs, gold coated (sputter coater Bal-tec SCD 050, Leica), and examined in a field emission SEM 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 1 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 3 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-II media under identical conditions. We do not present any data regarding the BSK-II part of the project as all BSK-II 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 United States. During the past few years he had flown only within the US. The patient recalled tick bites in several areas of Georgia and Florida, however, the vast majority of bites was encountered at 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 *B. microti* and *R. rickettsii* (Focus Diagnostics, USA) were negative. Tests for *E. chaffeensis* IgM was 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, USA) was positive at 2.56 (diagnostic cut off is >1.19), while Lyme IgM and IgG Western Blot was negative (Quest Diagnostics, USA). The patient completed 9

continuous months of oral doxycycline treatment (100 mg 2 x per day) with resolution of most symptoms for the following year. However, by June 2013 his previous symptoms 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 neighborhood. 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 patient 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 WB. 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 one year after the treatment.

The patient from Florida (sample M7p) was a 58 years 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 traveled from the site of her residence in the days prior to the tick bite. No information is available concerning the tick species. Patient's primary care physician suspected Lyme borreliosis. However, a Lyme IgM/IgG ELISA test was negative. Patient was prescribed 10 days of oral doxycycline (100 mg, 2 x per day), with following additional 30 days of doxycycline after that. After 40 days of continuous antibiotic treatment, the patient's blood was collected for analysis. At that time, 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, USA) tests conducted on the same blood samples were negative. Three presented patients 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 spirochete isolates M6p, M11p and M7p cultured from patients were measured and evaluated using negatively-stained cells and scanning electron microscopy (Table 1, Figure 1). Average lengths of M6p and M11p spirochetes from 1st and 3rd passages differed, contrary to measurements of M7p that did not change (Table 1).

PCR analysis with following sequencing of cultured strains with *B. burgdorferi* s.l. primers defined strains M6p and M11p (married couple from Georgia, U.S.A.) as *B. burgdorferi* sensu stricto. Multilocus sequence analysis of M6p and M11p confirmed their close relatedness to *B. burgdorferi* s.s. strains originated from *Ixodes scapularis* ticks and LB patients from states New York, Connecticut and Massachusetts, LB endemic regions of Northeastern United States [18]. M6p and M11p strains carry the *ospC* B allele which has been associated with invasive *B. burgdorferi* s.s. [19] strains isolated from human blood and cerebral spinal fluid in LB endemic regions [20]. The M6p and M11p carried the same *ospC* allele as earlier described fifteen *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 United States. [16]. The MLSA analysis showed that M7p strain is closely related to *B. bissettii.* Six analyzed loci 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 1994-1995: 16S-23S IGS (99-100%), 5S-23S IGS (98%), and genes encoding *ospC* (99%), *p66* (99%), *flagellin*

(99%) and *ospA* (96%) (samples collection: J. H. Oliver Jr., K. Clark; samples analysis: Rudenko N., Golovchenko M., 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 IGS, 16S-23S ITR: 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 (ACA) and erythema migrans (EM) 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 extended the list of antimicrobial drugs used to treat LB [23]. Because evaluation of antibiotic treatment was based on success in obtaining of positive spirochete 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, skeptical opinions about 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, 25, 26]. Laboratory studies on multiple immunocompetent hosts involving highly sensitive and specific molecular tools for pathogen detection revealed the presence of persistent spirochetes in host tissues months after antibiotic treatments [10, 11]. Neither detection of *Borrelia*-specific DNA by PCR and qPCR in hosts, nor

248 the detection of intact spirochetes by immunochemistry or xenodiagnosis in culture-negative
249 mice and ticks that fed on them [11, 24] provided sufficient evidence to verify the presence of
250 live *Borrelia*. Recently, xenodiagnosis using *Ixodes scapularis* nymphs was applied to patients to
251 investigate whether live spirochetes can persist in humans after recommended courses of
252 antibiotic treatment [27]. The majority of antibiotic treated patients in that study were negative
253 by xenodiagnosis. *Borrelia* specific DNA was detected in ticks removed from a patient with EM
254 at an early stage of antibiotic treatment and from one post treatment Lyme disease syndrome
255 patient, the presence of persistent spirochetes in whom was confirmed twice by two
256 xenodiagnosis procedures conducted within an eight month interval. Live spirochetes were not
257 recovered from either one of the samples available [27]. That study triggered an intensive
258 discussion about the usefulness of applied xenodiagnosis [28] as the method and results of the
259 study "provide the evidence against the biologic plausibility of a hypothesis" of the persistence
260 of *Borrelia* in patients after antibiotic treatment [28].

261 The lack of success in spirochete cultivation from patients reported by multiple groups
262 might have several explanations. One might be the structural alteration in spirochete forms as a
263 result of antibiotic challenge and induction of morphological changes that lead to formation of
264 dormant forms resistant to antibiotics. A recent study by Sapi and colleagues [29] on analysis of
265 the effect of five different antibiotics on different morphological forms of two strains of *B.*
266 *burgdorferi*, B31 and S297, using direct cell counting and dark field microscopy, showed that
267 doxycycline treatment reduced motile spirochete forms approximately 90%, but increased the
268 number of cystic round body forms about twofold. *In vitro* evaluation of the same treatment with
269 the use of fluorescent microscopy showed the reduction of spiral forms of *B. burgdorferi* by
270 94%, yet confirming, however, that 5% of remaining spirochetes were alive. Approximately 5%

of round bodies' population were dead, although the rest represented the live cells [29]. One may speculate that atypical dormant spirochete forms might persist in LB patients for years, regardless of antibiotic treatment and, probably, transform back into spiral motile forms under favorable 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 Lyme borreliosis 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 antibiotic was still present in a patients' body in concentration sufficient to kill the majority of bacteria at the time point of blood sample collection. *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 organism bacteria-free during the year following treatment and its favorable pharmacokinetics might have rather low impact in it as well. However, molecular techniques that we used for amplification of 6 loci from *Borrelia* genome proved to be highly sensitive in detection of multiple species or different spirochete strains present in a single sample earlier [16, 17, 30]. The uniformity of spirochete species/strain genetic material was analyzed three times after each re-cultivation and revealed identical sequences every time.

Our attempts to cultivate live borrelia from residents of southeastern United States was a logical step after extended analysis of a population of *B. burgdorferi* s.s. strains distributed in NC, SC, GA and FL [16]. We found earlier that 30% of analyzed southeastern *B. burgdorferi* s.s. strains represent an *ospC* B type, the one most often detected in LB patients from endemic areas of the Northeastern and Midwestern United States [16, 20]. In order for LB to occur, humans must be exposed to invasive strains via 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. strains outside the southeastern part of the United States 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 U.S.

The recognition of *B. bissettii* as a causative agent of Lyme borreliosis in Europe was supported by detections of spirochetes in serum samples [31] and aortic valve tissue of LB patients from the Czech Republic [32]. In 2011 Girard and colleagues presented the first molecular evidence of *B. bissettii*-like DNA detected in residents of Mendocino County (California, USA) [33]. While one patient from that study had a history of EM and joint pain, the other 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 spirochete were seronegative in previous tests conducted in 1988-1989 [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 8 housekeeping genes from live *B. bissettii*-like strain M7p, isolated from resident of Florida

316 [submitted for publication]. Here, we present the first recovery of live *B. bissettii*-like
317 spirochetes (strain M7p) from North American resident. The taxonomic status of this strain
318 requires further investigations.

319 *B. bissettii* occurs in the western and southern USA, but rarely in the northeastern region.
320 The records of naturally occurring in the southeastern US *B. bissettii* infections in rodents, birds,
321 and *Ixodes* ticks [34] indicate that it is widely distributed and is not narrowly confined to
322 particular vertebrate hosts or tick species in this region. Isolation of live *B. bissettii*-like strain
323 from a patient is strong evidence that this species, in addition to *B. burgdorferi* s.s., may cause
324 human Lyme borreliosis in North America. The successful cultivation of two *B. burgdorferi* s.s.
325 strains and one *B. bissettii*-like strain from antibiotic treated patients might suggest that active
326 infection with persistent *Borrelia* may be the cause of recurrent symptoms and persistent disease.
327 We also can not rule out the possibility that successful isolation of live spirochetes from patients
328 after extended antibiotic treatment might be the result of developed spirochete resistance to
329 doxycycline.

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Transparency Declaration

The authors have no conflicts of interest to declare.

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452 Figure Legend

453 Morphology of *Borrelia burgdorferi* M6p strain (passage 3) imaged in SEM JEOL 7400F at 4

454 kV, working distance 8 mm (A, B) and TEM JEOL 1010 80 kV (C). Bars - 1 μm.

455

Table 1. Structural parameters of *B. burgdorferi* s.l. strains M6p, M11p and M7p, measured on passage 1 and 3.

	1st passage			3rd passage		
	M6p	M11p	M7p	M6p	M11p	M7p
Length ± SD (µm)	15.9 ± 5.2	15.1 ± 3.5	14.5 ± 3.9	12.4 ± 3.7	13.6 ± 3.1	14.6 ± 3.8
Minimum - maximum (µm)	2.5* — 27	6 — 28	6 — 21	6 — 23	6 —23	6 — 28
Diameter ± SD (µm)	0.244 ± 0.04	0.216 ± 0.04	0.276 ± 0.04	0.225 ± 0.04	0.240 ± 0.04	0.323 ± 0.05
Wavelength (µm)	ND	ND	ND	3.16 ± 0.8 (n = 43)	1.79 ± 0.24 (n = 40)	2.48 ± 0.5 (n = 43)
Number of wavelengths	ND	ND	ND	3.7 ± 1.2 (n = 38)	5.7 (n = 41)	4.6 (n = 42)
Number of cells	67	59	59	52	53	53

Table footnotes

Spirochetes were prepared by negative staining method. Parameters were measured using TEM JEOL 1010.SD-standard deviation;

*-fragments; ND-not determined

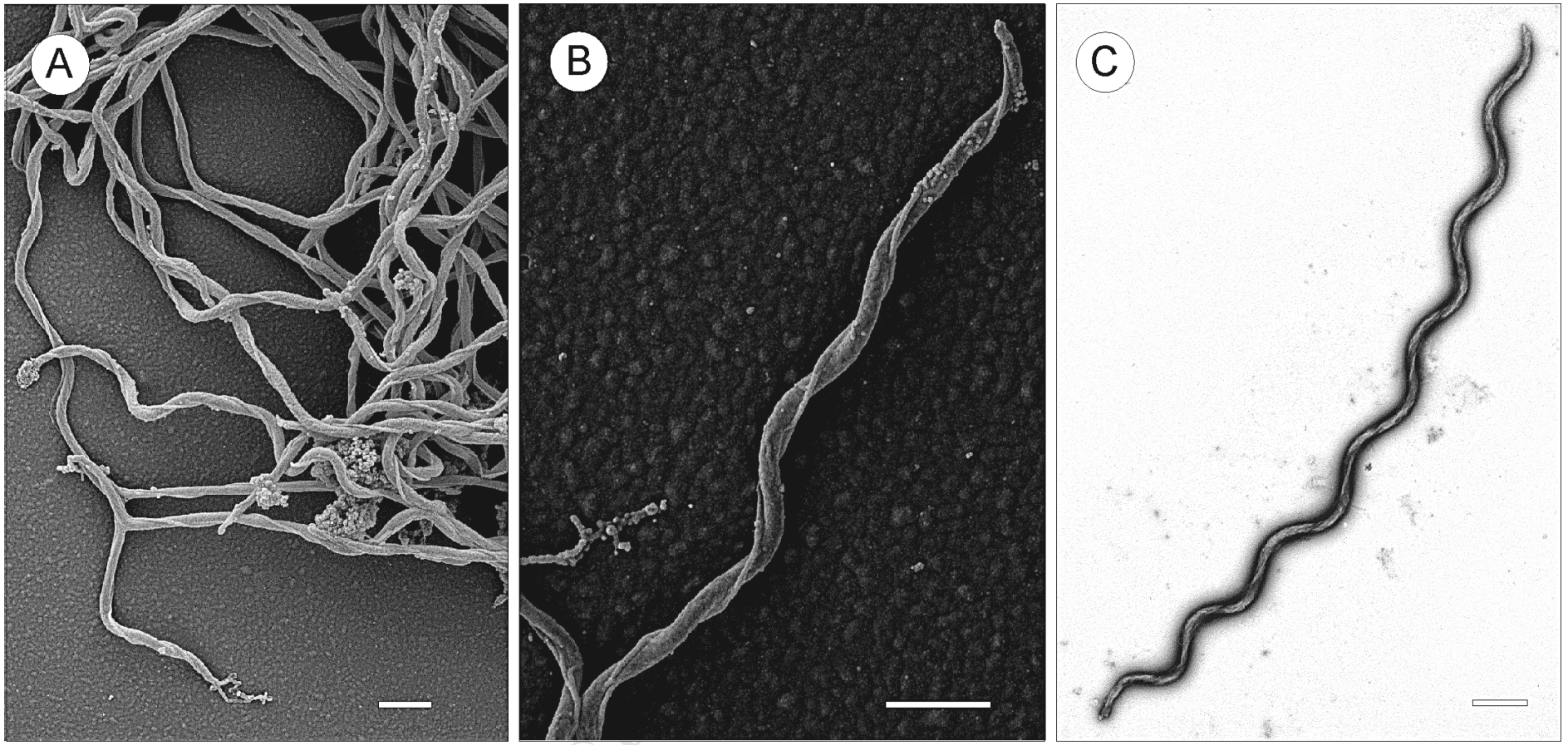

Figure 1