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1 **Persistent *Borrelia* Infection in Patients with Ongoing Symptoms of Lyme Disease**

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Abstract

Introduction

Lyme disease is a tickborne illness that generates controversy among medical providers and researchers. One of the key topics of debate is the existence of persistent infection with the Lyme spirochete, *Borrelia burgdorferi*, in patients who have been treated with recommended doses of antibiotics yet remain symptomatic. Persistent spirochetal infection despite antibiotic therapy has recently been demonstrated in non-human primates. We present evidence of persistent *Borrelia* infection despite antibiotic therapy in patients with ongoing Lyme disease symptoms.

Materials & Methods

In this pilot study, culture of body fluids and tissues was performed in a randomly selected group of 12 patients with persistent Lyme disease symptoms who had been treated or who were being treated with antibiotics. Cultures were also performed on a group of 10 control subjects without Lyme disease. The cultures were subjected to corroborative microscopic, histopathological and molecular testing for *Borrelia* organisms in four independent laboratories in a blinded manner.

Results

Motile spirochetes identified histopathologically as *Borrelia* were detected in culture specimens, and these spirochetes were genetically identified as *Borrelia burgdorferi* by three distinct polymerase chain reaction (PCR) methods. Spirochetes identified as *Borrelia burgdorferi* were cultured from the blood of seven subjects, from the genital secretions of ten subjects, and from a skin lesion of one subject. Cultures from control subjects without Lyme disease were negative for *Borrelia* using these methods.

Conclusions

Using multiple corroborative detection methods, we showed that patients with persistent Lyme disease symptoms may have ongoing spirochetal infection despite antibiotic treatment, similar to findings in non-human primates. The optimal treatment for persistent *Borrelia* infection remains to be determined.

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53 **Introduction**

54

55 Lyme disease (LD) and similar Lyme-like borrelial infections are caused by members of the
56 *Borrelia burgdorferi* (Bb) sensu lato complex or by members of the *Borrelia* relapsing fever
57 complex such as *B. miyamotoi*, respectively.[1-4] Following initial infection, *Borrelia* spirochetes
58 can evade host defenses, sequester in immune privileged sites such as joints or the central
59 nervous system, and persist in pleomorphic forms.[5-8] Tickborne coinfections including *Babesia,*
60 *Anaplasma, Ehrlichia, Bartonella and Rickettsia* may complicate the clinical picture.[6,9,10] If LD is
61 not treated early in the course of infection, chronic illness may result and a variety of symptoms
62 may develop. These symptoms include fatigue, musculoskeletal pain, arthritis, cardiac disease
63 and neurological involvement with peripheral neuropathy, meningitis, encephalitis, cranial
64 neuritis and cognitive dysfunction.[6,8,11,12]

65

66 Although LD was first recognized in 1975, it remains a controversial illness and the topic of
67 polemic debate.[6,10,13-15] One viewpoint claims that persistent Lyme disease symptoms are
68 related to ongoing spirochetal infection despite antibiotic therapy. This scenario has been
69 demonstrated in animal models including rodents, dogs and horses using various detection
70 methods,[16-36] and a recent study in non-human primates showing "persistent, intact,
71 metabolically-active *B. burgdorferi* after antibiotic treatment of disseminated infection" offers
72 the strongest support for this pathogenesis.[37] Furthermore, comparable studies have suggested
73 persistent infection after antibiotic therapy as a cause of chronic symptoms in humans.[38-60] The
74 opposing viewpoint claims that persistent Lyme disease symptoms may be due to spirochetal
75 "debris" without active infection. While a number of studies from Europe and the USA have
76 demonstrated persistence of Bb DNA or antigens in human bodily tissues or fluids, very few
77 studies have demonstrated culture of live *Borrelia* spirochetes, the highest form of evidence for
78 persistent infection in chronic Lyme disease patients.[4,51,53,59]

79

80 In this pilot study, we present detailed evidence of persistent *Borrelia* infection despite
81 antibiotic therapy in 12 randomly-selected North American patients with ongoing LD

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82 symptoms. Spirochetal infection was demonstrated by corroborative microscopic,
83 histopathological and molecular detection of live *Borrelia* organisms in cultures of body fluids
84 and tissues from these patients.

85 **Methods**

86 **Subject selection**

87 Subjects included in the study were chosen at random from our North American patient
88 population. All of the LD patients in the study were either clinically diagnosed with LD or had
89 positive Bb serological testing prior to study participation. Serological testing for LD was
90 performed by a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory
91 (IGeneX Laboratory in Palo Alto, CA), as described in detail elsewhere.[60] Subjects with
92 Morgellons disease (MD) who were seropositive for LD were included in the study (see
93 below).[61] All subjects had been treated with antibiotics prior to the study, and symptomatic
94 patients who remained on antibiotic treatment were included in the study.
95
96 **Control selection**
97
98 Ten healthy subjects were recruited as controls after informed consent was obtained. These
99 subjects were then tested serologically for LD and those who were negative were accepted as
100 controls. Vaginal or seminal fluids were collected from negative controls and cultured for
101 *Borrelia*, as described below. Culture pellets underwent PCR testing for *Borrelia* in a blinded
102 manner at the University of New Haven and Australian Biologics, as described below. All
103 subjects were adults who gave informed consent to participate in the study. Signed informed
104 consent to collect specimens was obtained in accordance with the ethics approval
105 requirements for sample collection of the Western Institutional Review Board, Puyallup, WA.
106 Approval for anonymous sample testing was also obtained from the Institutional Review Board
107 of the University of New Haven, West Haven, CT. Additional signed informed consent to publish
108 the results was obtained from each subject.

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109 **Cultures**

110

111 To avoid contamination, all cultures were performed under strict aseptic conditions in a
112 laboratory that was free of *Borrelia* reference strains, and cultures of control and patient
113 samples were processed in an identical manner. Inocula were placed in Barbour–Stoner–Kelly H
114 (BSK) complete medium with 6% rabbit serum (Sigma Aldrich, #B8291) containing the following
115 antibiotics: phosphomycin (0.02 mg/ml) (Sigma Aldrich), rifampicin (0.05 mg/ml) (Sigma
116 Aldrich), and amphotericin B (2.5 µg/ml) (Sigma-Aldrich), as described previously.[62] Inocula
117 were prepared as follows:
118 A. Blood – whole blood (10 ml) was collected by venipuncture and left at room temperature to
119 clot, then centrifuged at low speed to separate red blood cells from sera. The serum
120 supernatants with a small amount of blood cells below the serum layer were collected and
121 were inoculated into the BSK medium.
122 B. Skin – whole calluses or skin from lesions were removed from MD subjects by scraping with a
123 scalpel blade.
124 C. Vaginal – vaginal secretions were collected by swabbing inside the vagina with sterile cotton-
125 tipped swabs that were then introduced into the BSK medium.
126 D. Seminal – semen was self-collected into a sterile vial, then was pipetted into the BSK
127 medium.

128

129 Eight ml tubes of inoculated medium were filled to minimize the airspace present, thus
130 providing a microaerobic environment, and incubated at 32°C. Culture fluid was examined by
131 dark-field microscopy for visible spirochetes weekly for up to 4 weeks. Cultures were
132 concentrated by centrifuging the fluid at 15,000 g for 20 minutes, retaining the pellet and
133 discarding the supernatant. For imaging, a small amount of culture pellet was resuspended in
134 50 µl 0.85% saline solution, washed and centrifuged again. The pellet was mixed with gelatin
135 and then fixed with formalin.

136

137 **Dieterle and anti-Bb immunostaining**

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138
139 Dermatological specimens and/or culture pellets from patients were fixed, sectioned and
140 processed for specialized staining at either McClain Laboratories LLC, Smithtown, NY, or the
141 Department of Biology and Environmental Science, University of New Haven, West Haven, CT,
142 as previously described.[59] Dieterle silver-nitrate staining was performed at McClain
143 Laboratories. Anti-Bb immunostaining was performed at McClain Laboratories or the University
144 of New Haven. In brief, immunostaining was performed using an unconjugated rabbit anti-Bb
145 polyclonal antibody (Abcam ab20950), incubated with an alkaline phosphatase probe (Biocare
146 Medical #UP536L), followed by a chromogen substrate (Biocare Medical #FR805CHC), and
147 counterstained with
148 hematoxylin. Positive and negative controls were prepared for comparison purposes using liver
149 sections from Bb-inoculated and uninfected C3H/HeJ mice followed by Dieterle and
150 immunostaining. Culture pellets from mixed Gram-positive bacteria (*Streptococcus* and
151 *Staphylococcus*) and Gram-negative bacteria (*Escherichia coli* and *Klebsiella*) were also prepared
152 for comparison purposes as negative controls to exclude cross-reactivity with commonly
153 encountered microorganisms.
154
155 **Molecular testing**
156
157 Patient and negative control samples were submitted in a blinded manner to the laboratories
158 performing polymerase chain reaction (PCR) amplification of DNA, as described below.
159
160 **PCR - University of New Haven**
161
162 DNA was extracted from culture pellets as previously described.[59] Reactions of blinded samples
163 were performed in triplicate.
164
165 *Borrelia* DNA in extracted samples was detected using a published TaqMan assay targeting a
166 139-bp fragment of the gene encoding the *Borrelia* 16S rRNA, as described previously.[59, 64]

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167 Amplifications were conducted on a CFX96 Real-Time System (Bio-Rad) with cycling of 50°C for
168 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for
169 60 seconds, and fluorescent signals were recorded using CFX96 Real-Time software with the Cq
170 threshold set automatically.
171
172 Nested PCR primers for the 16S rRNA, flagellin (Fla), OspC, uvrA and pyrG genes were used as
173 previously described,[59, 64-66] with a final volume of 50 µl using 10 µl template DNA and final
174 concentrations of 20 mM Tris-HCl (pH 8.4), 50 mM KCl (1X Buffer B, Promega), 2 mM
175 MgCl2, 0.4 mM dNTP mix, 2 µM of each primer, and 2.5 U Taq polymerase (Invitrogen). The first
176 reaction used "outer" primers and the second reaction used "inner" primers, and 1 µl of PCR
177 product from the first reaction was used as template for the second. Cycling was programmed
178 as follows: 94°C for 5 minutes followed by 40 cycles of denaturation at 94°C for 1 minute,
179 annealing for 1 minute, and extension at 72°C for 1 minute, with a final extension step at 72°C
180 for 5 minutes. DNA products were visualized in 1-2% agarose gels.
181
182 PCR amplification was followed by Sanger sequencing. PCR products were extracted using the
183 QIAquick Gel Extraction kit (Qiagen) in accordance with the manufacturer's instructions. Eluates
184 were sequenced in both directions, then were compared by BLAST analysis using the GenBank
185 database (National Center for Biotechnology Information).
186
187 **PCR - Australian biologics**
188
189 DNA was extracted from culture pellets using the DNeasy Blood and Tissue kit ® (Qiagen) in
190 accordance with the manufacturer's instructions. Samples were forwarded to Australian
191 Biologics for *Borrelia* DNA and *Treponema denticola*/*Treponema pallidum* DNA testing. Blinded
192 samples were run in duplicate with positive and negative controls using primers for
193 the *Borrelia* 16S rRNA and rpoC gene targets, as previously described.[59,67,68] *Borrelia* DNA was
194 detected by real-time PCR targeting the 16S rRNA gene and/or by endpoint PCR targeting the
195 rpoC gene, as previously described,[59,67,68] using the Eco™ Real-Time PCR system with software

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196 version 3.0.16.0. Thermal profiles were performed with incubation for 2 minutes at 50°C,
197 polymerase activation for 10 minutes at 95°C then PCR cycling for 40 cycles of 10 secs at 95°C
198 dropping to 60°C sustained for 45 secs. The PCR signal magnitude generated (ΔR) was
199 interpreted as either positive or negative as compared to positive and negative controls.
200
201 For endpoint PCR, amplicons were visualized on 1-2% agarose gels and extracted from the gels
202 using the QIAquick Gel Extraction kit (Qiagen) in accordance with the manufacturer's
203 instructions. Sanger sequencing was used for gene analysis, as described previously.[59, 67, 68]
204
205 **PCR – University California Irvine**
206
207 The presence of Bb sensu stricto DNA in a set of blinded samples was confirmed by the
208 laboratory of Dr. Alan Barbour (University of California Irvine) by first quantitative PCR,[69] and
209 then by sequence of the PCR-amplified 16S-23S intergenic spacer.[70] The samples studied
210 included specimens from two of the subjects in this paper, Case 2 and Case 10, as described
211 below.
212
213 **Results**
214
215 **Subject Histories**
216
217 The clinical histories of the 12 study subjects with persistent Lyme disease symptoms (Cases 1-
218 12) are provided below, and the clinical characteristics of the subjects are summarized in Table
219 1. All subjects had received treatment with 2-4 weeks of antibiotics as recommended by Lyme
220 treatment guidelines endorsed by the Centers for Disease Control and Prevention (CDC).[14] Six
221 patients were taking antibiotics at the time of study sampling, as noted in Table 1. None of the
222 controls had received antibiotic therapy.
223
224 Case 1.

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225 The subject is a 50-year-old native Canadian woman who resided in an area endemic for LD in
226 eastern Canada. She did not recall an erythema migrans (EM) rash. She developed extreme
227 fatigue and musculoskeletal pain as well as ulcerative skin lesions along with symptoms of
228 formication. Magnification demonstrated filamentous inclusions within the lesions. The subject
229 was seronegative for anti-Bb antibodies excepting two indeterminate IgM bands showing
230 reactivity to the 41 kDa and the 93 kDA proteins, and a weakly positive IgG band showing
231 reactivity to the 41 kDa protein. She was clinically diagnosed with LD by a health care provider
232 in Canada and treated with antibiotics. The subject had discontinued antibiotics three weeks
233 prior to the sampling period, but continued treatment with naturopathic remedies. Despite
234 ongoing treatment with amoxicillin, the subject continues to have persistent symptoms of Lyme
235 disease.

236 Case 2.

237 The subject is a 54-year-old Caucasian woman who had a history of outdoor recreational
238 activity in Western Canada including areas in British Columbia that are endemic for LD. She
239 recalled an EM-like rash several years previously, and she did not receive treatment. She
240 developed significant joint pains, muscle aches, headaches, memory loss, fatigue and skin
241 lesions, and she initially tested negative for Lyme disease. She was clinically diagnosed by a
242 Canadian health care provider, and the diagnosis was confirmed later by an American health
243 care provider. She also had positive serological tests for *Babesia* and *Bartonella*. She did not
244 have prior knowledge of Morgellons disease, but she did have ulcerative lesions on her face and
245 torso consistent in appearance with the condition. Upon examination with a 50X handheld
246 microscope, filamentous inclusions were observed in her lesions. She has been aggressively
247 treated over the last few years with antibiotic combinations including intravenous ceftriaxone,
248 metronidazole, telithromycin, doxycycline, amoxicillin, ciprofloxacin, tinidazole and atovaquone
249 with little benefit.

250 Case 3.

251 The subject is a 63-year-old Caucasian man who had a history of outdoor recreational activity in

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252 endemic areas for Lyme disease, including Europe, Western Canada, and the USA (Connecticut
253 and Rhode Island). Although he recalls tick bites, he did not recall an EM rash. The subject
254 developed musculoskeletal pain and extreme fatigue. His wife (Case 4) had an EM rash and a LD
255 diagnosis that prompted him to get tested for LD. He was seroreactive for anti-Bb antibodies,
256 and DNA was detected in serum using PCR technology. He tested serologically positive for
257 *Babesia microti* and *Anaplasma phagocytophylum*. At the time of sampling he had ongoing
258 treatment with antibiotics, including doxycycline, clarithromycin, cefdinir, clindamycin,
259 tinadazole, atovaquone, clindamycin and hydroxychloroquine. He was symptomatic and taking
260 doxycycline at the time of sampling. His condition has since improved, but he still suffers from
261 musculoskeletal pain.

262 Case 4.

263 The subject is a 53-year-old Caucasian woman and the wife of Case 3. She had a history of
264 outdoor recreational activity in Lyme endemic areas of the USA and Canada. She has a history
265 of tick bites and recalled an EM rash after visiting both Connecticut and Rhode Island. Her
266 symptoms included seizures, neuropathy, palpitations and musculoskeletal pain. She had
267 serological testing for Bb and was initially negative, but she became seropositive after taking
268 antibiotics. She also had positive serological testing for *Babesia microti* and *Anaplasma*
269 *phagocytophylum*. She was symptomatic and taking antibiotics during the time of sample
270 collection. Antibiotics taken included doxycycline, telithromycin, minocycline, clindamycin,
271 clarithromycin, metronidazole, tinidazole, rifampicin, atovaquone, hydroxychloroquine and
272 mefloquine. The subject was taking clarithromycin and cefdinir at the time of sample collection.
273 She is currently asymptomatic.

274 Case 5.

275 The subject is a 40-year-old Caucasian woman living in Calgary, Canada, and the partner of case
276 6. She is a veterinarian and had a history of work exposure to ticks, and she had also travelled
277 to areas endemic for LD in Europe. She did not recall an EM rash. Her symptoms were primarily
278 musculoskeletal and severe headaches. She was seropositive for Bb and *Babesia*, and she had

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279 been treated with the following antibiotics: doxycycline, clarithromycin, metronidazole and
280 atovaquone. She had been taking doxycycline for one month at the time of sample collection.

281 Case 6.

282 The subject is a 42-year-old Caucasian man living in Calgary, Canada, and the partner of case 5.
283 He is a veterinarian and had a history of work exposure to ticks. He had also travelled to areas
284 endemic for LD in Europe. He did not recall an EM rash. His symptoms were primarily
285 musculoskeletal, severe headaches, memory loss, vision problems and extreme fatigue. He was
286 seropositive for Bb and *Babesia,* and he had been treated with the following antibiotics:
287 doxycycline, clarithromycin, metronidazole and atovaquone. He had been taking doxycycline
288 for one month at the time of sample collection.

289 Case 7.

290 The subject is a 36-year-old Caucasian woman living in Calgary, Canada. She was bitten by many
291 ticks while working as a tree planter in the mountains, but she does not recall an EM rash. In
292 September 1997 she developed profound fatigue, migratory joint pains, peripheral neuropathy
293 and personality changes consistent with depression. She was seropositive for Bb, and she was
294 eventually treated with intramuscular penicillin, amoxicillin, and minocycline over two years.
295 She remains symptomatic despite antibiotic treatment.

296 Case 8.

297 The subject is a 39-year-old Caucasian man residing in Calgary, Canada. He has a history of
298 hiking, camping and other outdoor activities in Alberta and Manitoba, Canada, but no known
299 tick bites or EM rash. He complains of joint pain, low back pain and headaches, and he has been
300 treated for sciatica, depression, insomnia, and anxiety. He also has an extensive history of
301 periodontal disease with recurrent gingival infections, and he has received multiple courses of
302 penicillin and amoxicillin over many years. He had positive serological testing for Lyme disease,
303 and he has not been tested for tickborne coinfections.

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304 Case 9.

305 The subject is a 71-year-old Caucasian woman living in Ontario, Canada and the partner of Case
306 10. She was 40 years old when she became ill in 1986 with severe flu-like symptoms, fatigue,
307 severe pelvic pain, blurred vision, rib soreness and night sweats. She did not recall a tick bite or
308 an EM rash. The patient had not knowingly visited a Lyme disease endemic area. She consulted
309 six different physicians over a period of four years before being treated with six weeks of
310 doxycycline for what was diagnosed as pelvic inflammatory disease in 1988, and her symptoms
311 transiently improved. She was clinically diagnosed with Lyme disease in 1990 by a physician in
312 Ontario, as the Ontario government's ELISA test was "negative" for Lyme disease. Over the next
313 20 years the subject was intermittently treated with doxycycline and her symptoms improved,
314 but never completely resolved, and other symptoms developed such as muscle aches, joint
315 pains, sleep disturbances, bladder and urethral pain, and cognitive impairment. These
316 symptoms waxed and waned over the years. She experienced multiple Jarisch-Herxheimer
317 reactions with repeated doxycycline treatment. The subject's two children were treated for
318 congenital Lyme disease between 1990 and 2004 and are asymptomatic today. In May 2011,
319 the subject was tested by a CLIA-approved laboratory in the USA and was found to be
320 serologically positive for Lyme disease.

321 Case 10.

322 The subject is a 72-year-old Caucasian man living in Ontario, Canada and the partner of Case 9.
323 He was 41 years old at the onset of symptoms in 1986 with flu-like muscle aches, joint pains
324 and unrelenting fatigue. He did not recall a tick bite or an EM rash. The subject had not
325 knowingly visited a Lyme disease endemic area. He had consulted 12 different doctors over a
326 period of four years before getting a confirmed diagnosis of Lyme disease, at which point he
327 had developed severe arthritic symptoms, significant neurological symptoms including
328 encephalopathy and dementia with brain magnetic resonance imaging (MRI) showing
329 hyperintense white matter lesions. His antibiotic regimens over 20 years included: tetracycline,
330 amoxicillin plus probenecid, doxycycline, clarithromycin, intravenous ceftriaxone, and

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331 intramuscular benzathine penicillin G. When the subject was on antibiotics he had relief of
332 many symptoms, but he was never completely free of symptoms associated with Lyme disease.
333 He had multiple Jarisch-Herxheimer reactions when new antibiotic regimens were initiated.
334 The two-tiered Lyme disease serology test performed in Canada failed to show positivity for
335 Lyme disease, but the subject subsequently sent blood to a CLIA-approved laboratory in the
336 United States and was found to be seropositive for Lyme disease.

337 Case 11.

338 The subject is a 57-year-old Caucasian woman living in Calgary, Canada. She had exposure to
339 ticks while hiking and camping in Canada, but she did not recall an EM rash. She developed
340 musculoskeletal and neuropsychiatric symptoms and was diagnosed with LD after testing
341 serologically positive. She also had positive testing for *Babesia, Ehrlichia* and *Bartonella*. She
342 received intermittent antibiotic therapy with multiple oral, intramuscular and intravenous
343 antibiotics, and her symptoms improved while on antibiotics but relapsed when the antibiotics
344 were discontinued. She remains symptomatic after five years of antibiotic treatment.

345 Case 12.

346 The subject is a 46-year-old Caucasian woman living in Alberta, Canada who did not have a
347 history of tick bite or EM rash. She has suffered with skin lesions consistent with Morgellons
348 disease for more than a decade and was diagnosed with Lyme disease in the USA after testing
349 serologically positive for Bb. She has had severe gastrointestinal problems that have
350 necessitated frequent hospitalizations. The gastrointestinal difficulties began after she had
351 gastric bypass surgery. Her intestines form lesions that fuse together, causing blockages. She
352 has been treated aggressively with antibiotics before and after this study by doctors both in the
353 USA and Canada with only minimal benefit. Antibiotic therapy included multiple treatments
354 with intravenous ceftriaxone, doxycycline, clarithromycin and amoxicillin.
355

356 **Microscopy and Histopathology**

357 Case 1. Whole calluses were submitted for sectioning, Dieterle and anti-Bb immunostaining.

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358 Spirochetes were visible in both Dieterle and anti-Bb immunostains. Blood culture was
359 performed and fluid from the culture demonstrated spherical bodies under darkfield
360 microscopy. Dieterle staining and anti-Bb immunostaining was not performed.

361 Case 2. Blood culture was performed and fluid from the culture demonstrated spherical bodies
362 under darkfield microscopy. Dieterle staining and anti-Bb immunostains demonstrated
363 spherical bodies. Anti-Bb immunostaining was positive. Vaginal culture was performed and fluid
364 from the culture demonstrated spirochetes and biofilm under darkfield microscopy. Dieterle
365 staining and anti-Bb immunostaining demonstrated spirochetes and biofilm. Skin specimens
366 were not submitted for staining or culture. Repeat blood and vaginal cultures were positive for
367 *Borrelia* by immunostaining and PCR (see Tables 2-4).

368 Case 3. Blood culture was performed and fluid from the culture demonstrated spherical bodies
369 and occasional spirochetes under darkfield microscopy. Dieterle staining and anti-Bb
370 immunostains demonstrated spherical bodies and occasional spirochetes. Anti-Bb
371 immunostaining was positive. Seminal culture was performed and fluid from the culture
372 demonstrated spirochetes under darkfield microscopy. Dieterle staining and anti-Bb
373 immunostaining demonstrated spirochetes. Repeat blood culture was positive for *Borrelia* by
374 immunostaining and PCR (see Tables 2-4).

375 Case 4. Blood culture was performed and fluid from the culture demonstrated spherical bodies
376 and occasional spirochetes under darkfield microscopy. Dieterle staining and anti-Bb
377 immunostains demonstrated spherical bodies and occasional spirochetes. Anti-Bb
378 immunostaining was positive. Vaginal culture was performed and fluid from the culture
379 demonstrated spirochetes under darkfield microscopy. Dieterle staining and anti-Bb
380 immunostaining demonstrated spirochetes. Repeat blood culture was positive for *Borrelia* by
381 immunostaining and PCR (see Tables 2-4).

382 Case 5. Blood culture was performed and fluid from the culture demonstrated spherical bodies
383 under darkfield microscopy. Dieterle staining and anti-Bb immunostaining demonstrated
384 spherical bodies. Vaginal culture was performed and fluid from the culture demonstrated

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385 spirochetes under darkfield microscopy. Dieterle staining and anti-Bb immunostaining
386 demonstrated spirochetes. Repeat blood culture was positive for *Borrelia* by immunostaining
387 and PCR (see Tables 2-4).

388 Case 6. Blood culture was performed and fluid from the culture demonstrated spherical bodies
389 under darkfield microscopy. Dieterle staining and anti-Bb immunostains demonstrated
390 spherical bodies. Anti-Bb immunostaining was positive. Seminal culture was performed and
391 fluid from the culture demonstrated spirochetes under darkfield microscopy. Dieterle staining
392 and anti-Bb immunostaining demonstrated spirochetes. Repeat blood culture was positive for
393 *Borrelia* by immunostaining and PCR (see Tables 2-4).

394 Case 7. Vaginal culture was performed and fluid from the culture demonstrated spirochetes
395 under darkfield microscopy. Dieterle staining and anti-Bb immunostaining demonstrated
396 spirochetes.

397 Case 8. Seminal culture was performed and fluid from the culture demonstrated spirochetes
398 under darkfield microscopy. Dieterle staining and anti-Bb immunostaining demonstrated
399 spirochetes.

400 Case 9. Vaginal culture was performed and fluid from the culture demonstrated spirochetes,
401 including one that was quite actively motile, under darkfield microscopy. Dieterle staining and
402 anti-Bb immunostaining demonstrated spirochetes.

403 Case 10. Seminal culture was performed and fluid from the culture demonstrated spirochetes
404 under darkfield microscopy. Dieterle staining and anti-Bb immunostaining demonstrated
405 spirochetes. Repeat seminal culture was positive for *Borrelia* by immunostaining and PCR (see
406 Tables 2-4).

407 Case 11. Vaginal culture was performed and fluid from the culture demonstrated spirochetes,
408 including one that was quite actively motile, under darkfield microscopy. Dieterle staining and
409 anti-Bb immunostaining demonstrated spirochetes.

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410 Case 12. Blood culture was performed and fluid from the culture demonstrated spherical
411 bodies under darkfield microscopy. Dieterle staining and anti-Bb immunostains demonstrated
412 spherical bodies. Anti-Bb immunostaining was positive. Skin culture was performed and fluid
413 from the culture demonstrated spirochetes under darkfield microscopy. Dieterle staining and
414 anti-Bb immunostaining demonstrated spirochetes.

415 The results of darkfield microscopy, Dieterle silver stains and anti-Bb immunostaining from
416 Cases 1-12 are summarized in Table 2. Examples of these spirochete detection methods are
417 shown in Figure 1A-1C.

418 **Molecular testing**

419 **PCR Detection of *Borrelia***

420 PCR detection of *Borrelia* was performed for research purposes only. No data resulting from
421 this study was used diagnostically.

422 Samples (whole dermatological calluses, blood culture, vaginal cultures, and seminal cultures)
423 from the study patients were submitted for PCR detection and sequencing of *Borrelia* DNA at
424 both the University of New Haven, CT, and Australian Biologics, Sydney, Australia in a blinded
425 manner. *Borrelia* DNA was detected by at least one laboratory for all 12 patients, and amplicon
426 sequences consistent with Bb DNA were obtained for 10/12 patients. Blinded negative cultures
427 from healthy, seronegative subjects along with the blinded suspected positive cultures from LD
428 patients were sent to University of New Haven and Australian Biologics. All negative controls
429 were PCR-negative for *Borrelia* strains.

430 Australian Biologics performed PCR for the detection of *Treponema denticola* and *Treponema*
431 *pallidum* on all study samples and blinded controls. Treponemal DNA was not detected in any
432 samples. For Cases #2 and #10, additional PCR and sequencing on genital cultures was
433 performed at the University of California, Irvine, in the laboratory of Dr. Alan Barbour. In these
434 samples, *Borrelia* DNA was detected with qPCR and then confirmed as Bb sensu stricto by
435 sequence of the 16S-23S intergenic spacer.

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436 Positive PCR results are summarized in Table 3, and positive sequencing results are summarized
437 in Table 4. PCR sequences and BLAST analyses are shown in Supplemental Figure 1.

438 **Discussion**

439

440 In this pilot study, we cultured live *Borrelia* organisms from 12 antibiotic-treated subjects with
441 persistent Lyme disease symptoms, thus showing that viable spirochetes can be found in LD
442 patients despite antibiotic therapy. Half of these subjects were taking antibiotics at the time of
443 sampling. Patient cultures showed *Borrelia* spiral forms and spherical bodies, as described in
444 other publications.[7,59,66] We demonstrated the presence of *Borrelia* infection in cultures from
445 these patients using corroborative microscopy, histopathology and PCR techniques, and we
446 obtained sequences for amplicons from 10/12 patients. Repeat cultures of blood, semen and
447 vaginal secretions were positive for Bb by microscopy, histopathology and PCR in six patients
448 tested by four different laboratories. Cultures from healthy *Borrelia*-seronegative controls were
449 consistently negative, making the possibility of *Borrelia* contamination in LD patient samples
450 extremely unlikely.

451

452 Persistent *Borrelia* infection may result in part from the wide variety of tissues and fluids that
453 support spirochetal growth.[16-37] The tissues susceptible to *Borrelia* infection include fibroblasts,
454 skin, synovial tissue, ligaments, cardiac tissue, glial cells, neurons, endothelial cells, lymphoid
455 tissue and hepatic tissue.[5,48,59,71-81] The pleotropic nature of *Borrelia* infection may allow the
456 spirochete to evade the host immune system and antibiotic therapy, as outlined below.

457

458 The role of cysts and biofilms in persistent *Borrelia* infection is controversial.[10, 82, 83] Ongoing
459 Lyme disease symptoms may arise from spirochetes hidden in biofilms or surviving as cell wall-
460 deficient cysts (L-forms), by intracellular *Borrelia* sequestration or by sequestration within
461 privileged sites where antibiotics do not attain therapeutic levels.[13,37,84-86] Regardless of the
462 mechanism by which *Borrelia* spirochetes persist in tissues, persistent *Borrelia* infection
463 requires treatment, and options at present are limited and controversial.[10,13,83] The controversy
464 is fueled by disagreement over viability of the spirochetes, as described below.

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465

466 Although there is evidence of post-treatment *Borrelia* infection in animals and humans, some
467 researchers speculate that *Borrelia* antigens and DNA detected in studies are merely
468 spirochetal “debris”.[36, 87-89] Wormser et al. offered an “amber” hypothesis as a possible
469 explanation for persistent symptoms, namely that persistent Lyme arthritis is caused by non-
470 viable spirochetes enmeshed in joints within host-derived fibrinous or collagenous matrices.[88]
471 Bockenstedt et al. proposed that the inflammation seen in mice described in their study
472 following antibiotic treatment was caused by *Borrelia* DNA and proteins representing non-
473 infectious spirochetal “debris” deposited in tissues.[36]

474 In contrast, those who support the idea that active infection is responsible for persisting Lyme
475 disease symptoms propose that there are various protective mechanisms providing spirochetal
476 resistance or tolerance to antibiotics including intracellular invasion, biofilm formation, cell-wall
477 deficient L-forms, cysts, and the formation of persister cells.[78,84-86] Furthermore the “amber”
478 and “debris” hypotheses of symptom persistence are difficult to support because *Borrelia* DNA
479 is rapidly cleared from murine tissues after prompt antibiotic treatment,[21] and the DNA of non-
480 viable spirochetes is cleared from mouse tissue within several hours.[90] The present study
481 confirms the presence of live *Borrelia* spirochetes in patients who had been treated with
482 antibiotics for persistent Lyme disease symptoms.
483

484 Recent studies have focused on “persister cells” and “sleeper cells” as spirochetal agents of
485 persistence in Lyme disease.[91-93] The concept involves organisms that are tolerant to antibiotics
486 and can downregulate their metabolic needs via a “stringent response” to survive in a hostile
487 environment, only to reemerge when the environment becomes more favorable. A similar
488 mechanism of persistent infection has been described in *E. coli*, *Mycobacteria* and *Salmonella*.[93]
489 The survival of metabolically tolerant spirochetes in privileged sites would explain our findings
490 of viable *Borrelia* in antibiotic-treated patients once the antibiotics are withdrawn and culture
491 conditions are optimized. The factors that influence viability of “persister cells” and “sleeper
492 cells” in patients with persistent Lyme disease symptoms merit further study.
493

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494 Three of our study subjects had a controversial skin condition commonly called Morgellons
495 disease (MD). [61,94-98] The distinguishing feature of this skin condition is the presence of white,
496 black, or brightly colored filaments that lie under, are embedded in, or project from skin lesions
497 (see Figure 1D). While some medical practitioners erroneously consider MD to be a purely
498 delusional disorder, MD appears to be a *Borrelia* filamentous dermatitis.[94,95] MD patients
499 exhibit symptoms that resemble those of Lyme disease, such as fatigue, joint pain, and
500 neuropathy, and the skin condition has been shown to be associated with *Borrelia* infection.[94-98]
501 Spirochetes from different *Borrelia* species have been detected in MD patient
502 specimens.[61,94,99,100] We obtained positive *Borrelia* cultures from all three of our MD subjects.

503 The mechanism of MD filament evolution has not been resolved, but as collagen and keratin
504 filaments arise from proliferative keratinocytes and fibroblasts in human epithelial tissue, we
505 speculate that *Borrelia* infection alters keratin and collagen gene regulation.[99,100] *Borrelia*
506 bacteria can invade fibroblasts and keratinocytes where they survive and replicate
507 intracellularly.[74,76,101] As shown by in vitro studies, *Borrelia* spirochetes can be isolated from
508 keratinocyte and fibroblast monolayers despite treatment with antibiotics.[74,76] Persistent
509 refractory infection in MD patients may therefore result in part from sequestration of live
510 *Borrelia* spirochetes within keratinocytes and fibroblasts.

511 *Borrelia* spirochetes have been detected in vaginal and seminal secretions.[13,100] We cultured
512 *Borrelia* spirochetes in genital secretions from 10 of our study subjects who had taken or were
513 currently taking antibiotic therapy. Bb is a complex organism that is related to the spirochetal
514 agent of syphilis, and therefore may have similar infectious capabilities.[13,100,102] As outlined
515 above, *Borrelia* spirochetes penetrate tissues, can form cystic structures, hide in biofilms,
516 become intracellular, and sequester in privileged sites (brain, eye and synovium).[9,10,13,83,103-105]
517 These specialized abilities of the *Borrelia* spirochete suggest that the genital tract could harbor
518 infection. The vagina and the seminal vesicles are privileged sites, and that may explain why the
519 organism can persist in the genital tract despite antimicrobial therapy in a manner similar to
520 syphilis, chlamydia, human immunodeficiency virus (HIV), Ebola and Zika virus.[102,106-110]

521 In summary, in this pilot study we demonstrated persistent infection despite antibiotic therapy

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522 in 12 North American patients with ongoing symptoms of LD. Cultures were positive in all 12
523 patients in our study, indicating that the *Borrelia* spirochetes were replicating and therefore
524 alive. The spirochetes were genetically identified as Bb in a blinded fashion using PCR and gene
525 sequencing in three separate laboratories. In contrast, cultures from control subjects without
526 Lyme disease were negative for *Borrelia* spirochetes. Our findings provide evidence that
527 persistent infection rather than spirochetal "debris" was at least in part responsible for ongoing
528 symptoms in these cases of Lyme disease, and the results mirror recent observations in a non-
529 human primate model of treated Lyme disease.[37] Larger clinical studies using corroborative
530 techniques are needed to confirm the findings in this pilot study.

531
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541
542 **Conflicts of Interest**:

543 The authors have no conflicts of interest to declare.

544

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942 **Supporting Information Legends**

943

944 Figure 1

945 A: Darkfield microscopy of culture fluid from Case 7 showing live spirochetes.
946 Magnification 1000x.
947 B: Dieterle silver stain of culture fluid from Case 10 showing live spirochetes.
948 Magnification 1000x.
949 C: Borrelia immunostain of culture fluid from Case 9 showing live spirochetes.
950 Magnification 1000x.
951 D. Typical dermal filaments from patient with Morgellons disease. Magnification 100x.

952

953 Table 1: Clinical Characteristics of Study Patients. EM, erythema migrans; MD,
954 Morgellons disease; MS, musculoskeletal; F, fatigue; N, neurological; *Bab*, *Babesia*
955 *microti* or *Babesia duncani*; *Bart*, *Bartonella henselae; Ana, Anaplasma*
956 *phagocytophilum*; *Ehr*, *Ehrlichia chafeensis*. Abx, on antibiotics at time of testing.
957 Table 2: Summary of Microscopy Results from Patient Culture Samples. N/A, not
958 available
959 Table 3: Summary of PCR Results from Patient Culture Samples. *Sequenced; RT, real
960 time PCR; N, nested PCR; E, endpoint PCR; N/A not available; (+/-) One specimen was
961 positive for Bb DNA, one specimen was negative (different collection dates).
962 Table 4: Summary of BLAST Sequence Analysis of Patient PCR Samples. F, forward
963 sequence; R, reverse sequence. UNH, University of New Haven; AB, Australian Biologics;
964 UCI, University of California Irvine.

965

966 Supplemental Figure 1: PCR Sequences and BLAST Analyses from Individual Patient
967 Cultures. UNH, University of New Haven; AB, Australian Biologics; UCI, University of
968 California Irvine.

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971 Table 1. Patient Clinical Characteristics

Case #	Age/ Gender	EM Rash	Sx	MD Lesions	LD Seroreactivity	Co-infections	Abx
Case 1	50F	No	MS, F	Yes	Negative	Unknown	Yes
Case 2	54F	Yes	MS, F	Yes	Negative	*Bab, Bart*	No
Case 3	63M	No	MS, F	No	Positive	*Bab, Ana*	Yes
Case 4	53F	Yes	MS, F	No	Negative, seroconverting to positive	*Bab, Ana*	Yes
Case 5	40F	No	MS, N	No	Positive	*Bab*	Yes
Case 6	42M	No	MS, N	No	Positive	None	Yes
Case 7	36F	No	MS, N	No	Positive	None	Yes
Case 8	39M	No	MS, N	No	Positive	Unknown	No
Case 9	71F	No	MS, F, N	No	Positive	None	No
Case 10	72M	No	MS, F, N	No	Positive	None	No
Case 11	57F	No	MS	No	Positive	*Bab, Ehr, Bart*	No
Case 12	46F	No	MS	Yes	Positive	*Bab*	No

972 EM, erythema migrans; MD, Morgellons disease; MS, musculoskeletal; F, fatigue; N,
973 neurological; *Bab*, *Babesia microti* or *Babesia duncani*; *Bart*, *Bartonella henselae; Ana,*
974 *Anaplasma phagocytophilum; Ehr, Ehrlichia chafeensis.* Abx, on antibiotics at time of testing.

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976 Table 2: Microscopy of Patient Samples

Case #	Sample type	Darkfield	Dieterle	Bb immunostain
Case 1	whole callus	N/A	spirochetes	positive, spirochetes
	blood culture	spirochetes	N/A	N/A
Case 2	blood culture	spherules	spherules	positive, spherules
	vaginal culture	spirochetes	spirochetes	positive, spirochetes, biofilm
Case 3	blood culture	spirochetes/ spherules	spirochetes/ spherules	positive spirochetes/ spherules
	seminal culture	spirochetes	spirochetes	positive, spirochetes
Case 4	blood culture	spirochetes/ spherules	spirochetes/ spherules	positive spirochetes/ spherules
	vaginal culture	spirochetes	spirochetes	positive, spirochetes
Case 5	blood culture	spherules	spherules	positive, spherules
	vaginal culture	spirochetes	spirochetes	positive, spirochetes
Case 6	blood culture	spherules	spherules	positive, spherules
	seminal culture	spirochetes	spirochetes	positive, spirochetes
Case 7	vaginal culture	spirochetes	spirochetes	positive, spirochetes
Case 8	seminal culture	spirochetes	spirochetes	positive, spirochetes
Case 9	vaginal culture	spirochetes	spirochetes	positive, spirochetes
Case 10	seminal culture	spirochetes	spirochetes	positive, spirochetes
Case 11	vaginal culture	spirochetes	spirochetes	positive, spirochetes
Case 12	blood culture	spherules	spherules	positive, spherules
	skin culture	spirochetes	spirochetes	positive, spirochetes

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Table 3: PCR Analysis of Patient Culture Samples

Case #	Sample type	University of New Haven	Australian Biologics	UC – Irvine
1	whole callus	16S rRNA (N), pyrG (N)*, fla (N)*	N/A	N/A
	blood culture	pyrG (N), fla (N)*	N/A	N/A
2	blood culture	16S rRNA (N)	16S rRNA (RT), rpoC (E)*	N/A
	vaginal culture	pyrG (N)*, fla (N)	16S rRNA(RT)*	qPCR 16S-23S intergenic spacer
3	blood culture	16S rRNA (N)*	16S rRNA(RT)	N/A
	seminal culture	16S rRNA (RT), 16S rRNA (N)*, fla (N)	16S rRNA(RT), rpoC (E)*	N/A
4	blood culture	16S rRNA (N), pyrG (N)	16S rRNA(RT), rpoC (E)*	N/A
	vaginal culture	16S rRNA (RT), 16S rRNA (N), pyrG (N), fla (N)	16S rRNA(RT), rpoC (E)*	N/A
5	blood culture	16S rRNA (RT), 16S rRNA (N), pyrG (N)	16S rRNA(RT)	N/A
	vaginal culture	16S rRNA (N)	16S rRNA(RT), rpoC (E)*	N/A
6	blood culture	16S rRNA (RT), 16S rRNA (N), pyrG (N)	16S rRNA(RT)	N/A
	seminal culture	16S rRNA (RT),16S rRNA (N)	16S rRNA(RT)	N/A
7	vaginal culture	16S rRNA (N)*	N/A	N/A
8	seminal culture	16S rRNA (N)*	N/A	N/A
9	vaginal culture	pyrG (N)	16S rRNA(RT)	N/A
10	seminal culture	pyrG (N)	16S rRNA(RT)	(+/-) qPCR16S-23S intergenic spacer
11	vaginal culture	16S rRNA (N)	16S rRNA(RT), rpoC (E)*	N/A
12	whole callus	uvrA (N)*	N/A	N/A
	blood culture	pyrG	16S rRNA(RT)	N/A

* Sequenced; RT, real time PCR; N, nested PCR; E, endpoint PCR; N/A not available; (+/-) One specimen was positive for Bb DNA, one specimen was negative (different collection dates)

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Table 4: BLAST Sequence Analysis of Patient PCR Samples

Case #	Culture specimen	Sequence	Length	E-value	BLAST match Bbss	LAB
1	callus	pyrG	680	0.0	100%	UNH
	callus	fla	367	2e-172	100%	UNH
	blood	fla F	364	2e-176	99%	UNH
	blood	fla R	367	2e-172	99%	UNH
2	vaginal	pyrG F	656	0.0	99%	UNH
	vaginal	pyrG R	659	0.0	99%	UNH
	vaginal	rpoC	79	4e-32	100%	AB
	vaginal	16S-23S Intergenic spacer	474	0.0	100%	UCI
3	blood	16S rRNA F	415	0.0	99%	UNH
	blood	16S rRNA R	415	0.0	99%	UNH
	seminal	16S rRNA	388	0.0	99%	UNH
	blood 1 month Abx	rpoC	103	0.11	96%	AB
	seminal 1 month Abx	rpoC	146	9e-57	100%	AB
	seminal 4 months Abx	rpoC	158	3e-52	98%	AB
4	vaginal	rpoC	118	1e-51	99%	AB
5	vaginal	rpoC	109	6e-47	99%	AB
7	vaginal	16S rRNA	396	0.0	99%	UNH
8	seminal	16S rRNA	221	7e-10	100%	UNH
10	seminal	16S-23S Intergenic spacer	474	0.0	99%	UCI
11	vaginal	rpoC	156	1e-25	100%	AB
12	callus	uvrA F	653	0.0	99%	UNH
	callus	uvrA R	651	0.0	99%	UNH

F, forward sequence; R, reverse sequence; Bbss, *B. burgdorferi* sensu stricto; Abx, antibiotics

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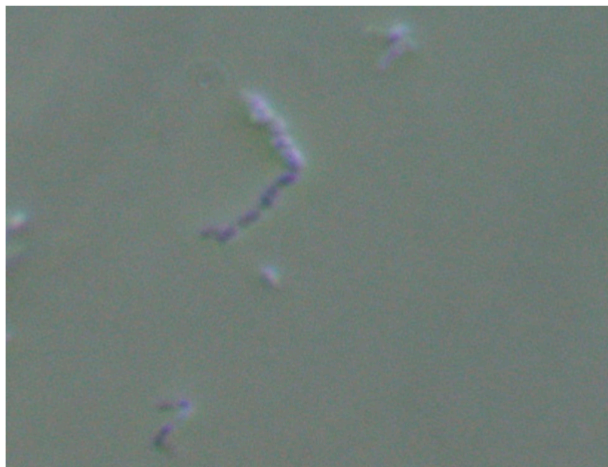
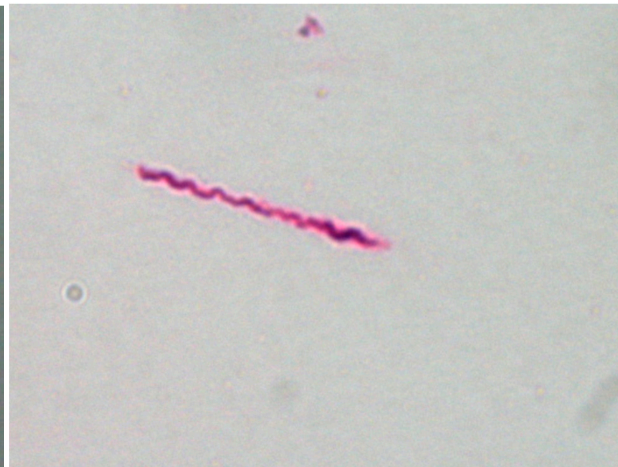
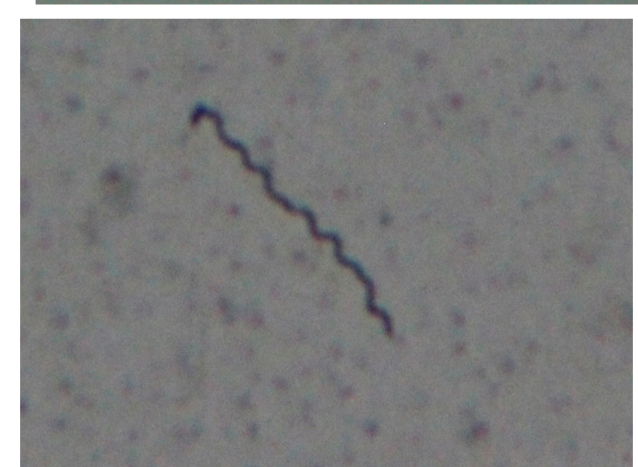
A

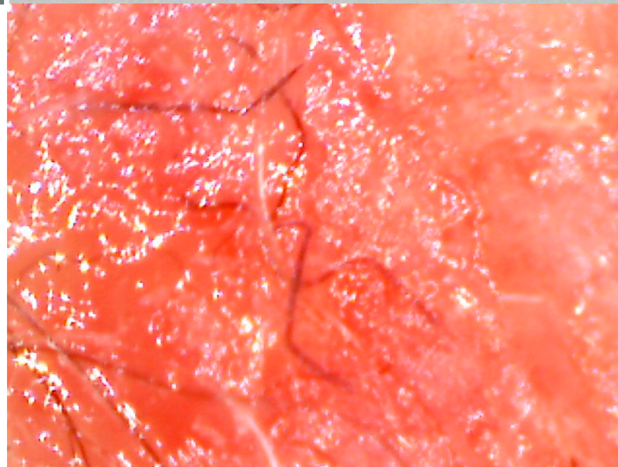


B





C



D

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Supplemental Figure 1

SEQUENCES FROM UNIVERSITY OF NEW HAVEN

Sequences for Case #1 UNH

Whole callus
pyrG

NNNNNNNNNNNANNNNNNNNNNNNNNNGGGAGTGGTAATATTTCTTTTATTCATTTAAC
ATATGTGCCAAGTCCAGCTGGAATTAATGAGCAAAAATCTAAACCTACTCAACAAAGTGT
TAAAACCTTAAATAAAGCAGGTATTTTCCCCGATTTAATTATTGCTAGAAGTTCACAAGTA
TTGACAGACCAAATCAGAAAAAAAGTGGCAATGTTTTGCAATGTTGAGAGCACTTCTATT
ATTGACAATGTTGATGTTTCTACTATTTATGAAATTCCTATATCTTTTTATAAGCAGGGTGT
ACATGAGATTTTAAGCTCTAAGTTAAATATTAAGGTTGATCCAAAAATAGAAGAGCTTTC
AAAGCTTGTAGGAGTTATAAAATCTAATTTTTTTGTGCCTAAAAAAATTATTAATATTGCT
ATTTGTGGTAAATATGCTGAACTTGATGATTCTTATGCATCAATTAGAGAGTCTTTGGTTC
ATGTTGCAGCCCATTTGGATTTGCTTATTAAAAGCACTTTAATTGATTCTAATGATTTAAA
TGAGAGCTGTTTAAAAGAGTTTGACGGCATTATTGTTCCTGGCGGCTTTGGAGGCAAAGG
ATATGAAGGTAAAATTATGGCTATTAAATATGCTCGTGAGAATAATATTCCCTTTCTTGGA
ATTTGNCCTTGNNN

Length 680 bp, BLAST 100% identity with Bbss, e value 0

Whole callus
Fla

NNNNNNNNNNNNNNNNNNNNANNACTNNNNNNNNNCGAAATTAATAGAATTGCTGATCAA
GCTCAATATAACCAAATGCACATGTTATCAAACAAATCTGCTTCTCAAAATGTAAGAACA
GCTGAAGAGCTTGGAATGCAGCCTGCAAAAATTAACACACCAGCATCACTTTCAGGGTCT
CAAGCGTCTTGGACTTTAAGAGTTCATGTTGGAGCAAACCAAGATGAAGCTATTGCTGTA
AATATTTATGCAGCTAATGTTGCAAATCTTTTCTCTGGTGAGGGAGCTCAAACTGCTCAGG
CTGCACCGGTTCAAGAGGGTGTTCAACAGGAAGGAGCTCAACAGCCAGCACCTGCTACAG
CACCTTCA

Length 367 bp, BLAST 100% identity with Bbss, e value 2e-172

Blood culture
Fla

Forward
NNNNNNNNNNNNANNNNANCAACNNACAGACGAAATTAATAGAATTGCTGATCAAGCTC
AATATAACCAAATGCACATGTTATCAAACAAATCTGCTTCTCAAAATGTAAGAACAGCTG
AAGAGCTTGGAATGCAGCCTGCAAAAATTAACACACCAGCATCACTTTCAGGGTCTCAAG
CGTCTTGGACTTTAAGAGTTCATGTTGGAGCAAACCAAGATGAAGCTATTGCTGTAAATA
TTTATGCAGCTAATGTTGCAAATCTTTTCTCTGGTGAGGGAGCTCAAACTGCTCAGGCTGC
ACCGGTTCAAGAGGGTGTTCAACAGGAAGGAGCTCAACAGCCAGCACCTGCTACAGCAC
CTTCA

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Length 364 bp, BLAST 99% identity with Bbss, e value 2e-176

Reverse
NNNNNNNNNNNNNNNNNNNANNACTNNNNNNNNCGAAATTAATAGAATTGCTGATCAA
GCTCAATATAACCAAATGCACATGTTATCAAACAAATCTGCTTCTCAAAATGTAAGAACA
GCTGAAGAGCTTGGAATGCAGCCTGCAAAAATTAACACACCAGCATCACTTTCAGGGTCT
CAAGCGTCTTGGACTTTAAGAGTTCATGTTGGAGCAAACCAAGATGAAGCTATTGCTGTA
AATATTTATGCAGCTAATGTTGCAAATCTTTTCTCTGGTGAGGGAGCTCAAACTGCTCAGG
CTGCACCGGTTCAAGAGGGTGTTCAACAGGAAGGAGCTCAACAGCCAGCACCTGCTACAG
CACCTTCA

Length 367 bp, BLAST100% identity with Bbss, e value 2e-172

Sequences for Case #2 UNH

Vaginal culture
pyrG

Forward
ATAGCCATAATTTTACCTTCNTATCCTTTGCCTCCAAAGCCGCCAGGAACAATAATGCCGT
CAAACTCTTTTAAACAGCTCTCATTTAAATCATTAGAATCAATTAAAGTGCTTTTAATAAG
CAAATCCAAATGGGCTGCAACATGAACCAAAGACTCTCTAATTGATGCATAAGAATCATC
AAGTTCAGCATATTTACCACAAATAGCAATATTAATAATTTTTTTAGGCACAAAAAAATTA
GATTTTATAACTCCTACAAGCTTTGAAAGCTCTTCTATTTTTGGATCAACCTTAATATTTAA
CTTAGAGCTTAAAATCTCATGTACACCCTGCTTATAAAAAGATATAGGAATTTCATAAATA
GTAGAAACATCAACATTGTCAATAATAGAAGTGCTCTCAACATTGCAAAACATTGCCACT
TTTTTTCTGATTTGGTCTGTCAATACTTGTGAACTTCTAGCAATAATTAAATCGGGGAAAA
TACCTGCTTTATTTAAGGTTTTAACACTTTGTTGAGTAGGTTTAGATTTTTGCTCATTAATT
CCAGCTGGACTTGGCACATATGTTAAATGAATAAAAGAAATATTACCACTCCCAATCTCC
TGTCTTATTTGTCTTACTGTCTCAATAAATAAAATATTTTTCCATAT 99% B31 sequence, it has one nucleotide difference

Length 656 bp, BLAST 99% identity with Bbss, e value 0

Reverse
GATTGGGAGTGGTAATATTTCTTTTATTCATTTAACATATGTGCCAAGTCCAGCTGGAATT
AATGAGCAAAAATCTAAACCTACTCAACAAAGTGTTAAAACCTTAAATAAAGCAGGTATT
TTCCCCGATTTAATTATTGCTAGAAGTTCACAAGTATTGACAGACCAAATCNGAAAAAAA
GTGGCAATGTTTTGCAATGTTGAGAGCACTTCTATTATTGACAATGTTGATGTTTCTACTA
TTTATGAAATTCCTATATCTTTTTATAAGCAGGGTGTACATGAGATTTTAAGCTCTAAGTT
AAATATTAAGGTTGATCCAAAAATAGAAGAGCTTTCAAAGCTTGTAGGAGTTATAAAATC
TAATTTTTTTGTGCCTAAAAAAATTATTAATATTGCTATTTGTGGTAAATATGCTGAACTTG
ATGATTCTTATGCATCAATTAGAGAGTCTTTGGTTCATGTTGCAGCCCATTTGGATTTGCTT
ATTAAAAGCACTTTAATTGATTCTAATGATTTAAATGAGAGCTGTTTAAAAGAGTTTGACG
GCATTATTGTTCCTGGCGGCTTTGGAGGCAAAGGATATGAAGGTAAAATTATGGCTATTA
AATATGCTCGTGAGAATAATATTCCCTTTCTTGGAATTTGTCTTGGTATAA

Length 659 bp, BLAST 99% identity with Bbss, e value 0

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Sequences for Case #3 UNH

Blood culture
16S rRNA

Forward
NNNNNNNNNNNNNNNNNNNNNNNGNNTGTAGCATACATTCAGTGGCGAACGGGTGAGT
AACGCGTGGATGATCTACCTATGAGATGGGGATAACTATTAGAAATAGTAGCTAATACCG
AATAAGGTCAGTTAATTTGTTAATTGATGAAAGGAAGCCTTTAAAGCTTCGCTTGTAGATG
AGTCTGCGTCTTATTAGCTAGTTGGTAGGGTAAATGCCTACCAAGGCAATGATAAGTAAC
CGGCCTGAGAGGGTGAACGGTCACACTGGAACTGAGATACGGTCCAGACTCCTACGGGA
GGCAGCAGCTAAGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCGACACTGCGTGAA
TGAAGAAGGTCGAAAGATTGTAAAATTCTTTTATAAATGAGGAATAAGCTTTGTAGGA

Length 415 bp, BLAST 99% identity with Bbss, e value 0

Reverse
NNNNNNNNNNNNNNNNNNNNNNTCTTCNNNCCNCNGNNGNGCGCTCCGTCAGGCTTTCGC
CCATTGCGGAAGATTCTTAGCTGCTGCCTCCCGTAGGAGTCTGGACCGTATCTCAGTTCCA
GTGTGACCGTTCACCCTCTCAGGCCGGTTACTTATCATTGCCTTGGTAGGCATTTACCCTA
CCAACTAGCTAATAAGACGCAGACTCATCTACAAGCGAAGCTTTAAAGGCTTCCTTTCAT
CAATTAACAAATTAACTGACCTTATTCGGTATTAGCTACTATTTCTAATAGTTATCCCCAT
CTCATAGGTAGATCATCCACGCGTTACTCACCCGTTCGCCACTGAATGTATTGCTACATCC
CGTTTGACTTGCATGCTTAAGACGCACTGCCAGCGTTAGTTCTAAGCCAGGA

Length 415 bp, BLAST 100% identity with Bbss, e value 0

Seminal culture
16S rRNA

GTAGCATACATTCAGTGGCGAACGGGTGAGTAACGCGTGGATGATCTACCTATGAGATGG
GGATAACTATTAGAAATAGTAGCTAATACCGAATAAGGTCAGTTAATTTGTTAATTGATG
AAAGGAAGCCTTTAAAGCTTCGCTTGTAGATGAGTCTGCGTCTTATTAGCTAGTTGGTAGG
GTAAATGCCTACCAAGGCAATGATAAGTAACCGGCCTGAGAGGGTGAACGGTCACACTG
GAACTGAGATACGGTCCAGACTCCTACGGGAGGCAGCAGCTAAGAATCTTCCGCAATGGG
CGAAAGCCTGACGGAGCGACACTGCGTGAATGAAGAAGGTCGAAAGATTGTAAAATTCT
TTTATAAATGAGGAATAAGCTTTGTAGGA

Length 388 bp, BLAST 99% identity with Bbss, e value 0

Sequences for Case #7 UNH

Vaginal culture
16S rRNA

NNNNNGNNNNNNGCNNNCTTCNGTGGCGACGGGTGAGTAACGCGTGGATGATCTACCTA
TGAGATGGGGATAACTATTAGAAATAGTAGCTAATACCGAATAAGGTCAGTTAATTTGTT
AATTGATGAAAGGAAGCCTTTAAAGCTTCGCTTGTAGATGAGTCTGCGTCTTATTAGCTAG

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TTGGTAGGGTAAATGCCTACCAAGGCAATGATAAGTAACCGGCCTGAGAGGGTGAACGG
TCACACTGGAACTGAGATACGGTCCAGACTCCTACGGGAGGCAGCAGCTAAGAATCTTCC
GCAATGGGCGAAAGCCTGACGGAGCGACACTGCGTGAATGAAGAAGGTCGAAAGATTGT
AAAATTCTTTTATAAATGAGGAATAGCCTTTTGTAGGA

Length 396 bp, BLAST 99% identity with Bbss, e value 0

Sequences for Case #8 UNH

Seminal culture
16S rRNA

AGCTTTGTAGGACCTGGCTTAGAACTAACGCTGGCAGTGCGTCTTAAGCATGAGGAATAA
GCTTTGTAGGACCTGGCTTAGAACTAACGCTGGCAGTGCGTCTTAAGCATGAGGAATAAG
CTTTGTAGGACCTGGCTTAGAACTAACGCTGGCAGTGCGTCTTAAGCATGAGGAATAAGC
TTTGTAGGACCTGGCTTAGAACTAACGCTGGCAGTGCGTCT

Length 221 bp, BLAST 100% identity with Bbss, e value 7e-10

Sequences for Case #12 UNH

Whole callus
uvrA

Forward
CNNNNNNNNNNNNNNNNANNGNNTATCAGGTAGTCTATCTGGTGGCGAGGCTCAGCGTAT
TAGGCTTGCTACTCAAATAGGATCAGCACTTTCGGGTGTTATTTATGTTCTTGATGAGCCA
AGTATTGGTCTTCATCAAAGAGATAATGAAAAATTAATCTCTACTCTTGTTAATCTTAAAA
ATCTTGGTAATACTGTAATTGTTGTTGAACATGATGAGCAAACTTTGCGTACTGCGGACTA
TATTATTGATATGGGTCCTGGTGCTGGAATTCTTGGAGGGGAAATAGTTGCAAAGGGAGC
CTTGATTGATATTTTAAATAGCAAAAATAGTTTAACTGGTCAATATCTTAGCGGCAAGTTT
AAAATAGATGTTCCAAGCTCTAGAAGAAAGGCAGATAAGGGAGAAATTTTGCTTTTGGGC
TCTAATAAAAATAATCTTAAAAATATAGACTTAAGTATCCCTTTGGGAGTTTTTACCGTAA
TAACAGGTGTTTCTGGTAGCGGAAAAAGTACTTTACTTAACGAGGTGTTATATCCAGCTCT
TGATAGTAGATTAAAGCTTAATGAAAAGTATTGTGATGGCTTTAAAGATATTGTTGGGTAT
GAAAAAATCGATAAAATTATTCAAATAAATCAAAAACCCCAATAGGA

Length 653 bp, BLAST 99% identity with Bbss, e value 0

Reverse
NNNNNNNANTNNNNCNACNNNNNNATATCTTTAAAGCCATCACAATACTTTTCATTAAGC
TTTAATCTACTATCAAGAGCTGGATATAACACCTCGTTAAGTAAAGTACTTTTTCCGCTAC
CAGAAACACCTGTTATTACGGTAAAAACTCCCAAAGGGATACTTAAGTCTATATTTTTAA
GATTATTTTTATTAGAGCCCAAAAGCAAAATTTCTCCCTTATCTGCCTTTCTTCTAGAGCTT
GGAACATCTATTTTAAACTTGCCGCTAAGATATTGACCAGTTAAACTATTTTTGCTATTTA
AAATATCAATCAAGGCTCCCTTTGCAACTATTTCCCCTCCAAGAATTCCAGCACCAGGACC
CATATCAATAATATAGTCCGCAGTACGCAAAGTTTGCTCATCATGTTCAACAACAATTACA
GTATTACCAAGATTTTTAAGATTAACAAGAGTAGAGATTAATTTTTCATTATCTCTTTGAT
GAAGACCAATACTTGGCTCATCAAGAACATAAATAACACCCGAAAGTGCTGATCCTATTT

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GAGTAGCAAGCCTAATACGCTGAGCCTCGCCACCAGATAGACTACCTGATATTCTATTTA
AATATAAATAAGAAAGGCCAACATCAATTAANANTTTTNNNGCN

Length 651 bp, BLAST 99% identity with Bbss, e value 0

SEQUENCES FROM AUSTRALIAN BIOLOGICS

Sequences for Case #2 AB

Vaginal culture
rpoC

GGAGATGTTGTAAAAGCAGGAGATATGCTTTGTGATGGTAGAATTAATCCTCATGATGTG
CTTGAAATTTTAGGTGGGA

Length 79 bp, BLAST 100% identity with Bbss, e value 4e-032

Sequences for Case #3 AB

Blood culture
rpoC

TAAAGGGTACTCTCTAGCCTTTTACCTTTTTGATTAGACTCCCACCTAAAATTTCAAGCAG
GGGATTCTACATCTACTAAGCCTTTTACCTTTTTGAATTGAA

Length 103bp, BLAST 96% identity with Bbss, e value 0.11

Seminal culture, 1 month on abx
rpoC

ATTTCTGCAGTGACATCCATGCTATCTCCTGCTTTTACAACATCTCCATCTCTAACCAAAA
GATGTTTTCCAGCTGGAATATAATGCTTATGTTCAACCCCATACTCATCTAAAATATTAAT
AAGCCTTTTACCTTTTTGAATTGA

Length 146bp, BLAST 100% identity with Bbss, e value 9e-57

Seminal culture, 4 months on abx
rpoC

TTAAGAGGCAGCAGCACGTGTATGAGTGTGAATACATAAGTGTTATATTCCAGCTGGAAA
ACATCTTTTGGTTAGAGACGGAGATGTTGTAAAAGCAGGAGATATGCTTTGTGATGGTAG
AATTAATCCTCATGATGTGCTTGAAATTTTAGGTGGGA

Length 158bp, BLAST 98% identity with Bbss, e value 3e-52

Sequences for Case #4 AB

Vaginal culture

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rpoC

TGCTTTTACAACATCTCCGTCTCTAACCAAAAGATGTTTTCCAGCTGGAATATAATGCTTA
TGTTCAACCCCATACTCATCTAAAATATTAATAAGCCTTTTACCTTTTTGAATTGAA

Length 118bp, BLAST 99% identity with Bbss, e value 1e-51

Sequences for Case #5 AB

Vaginal culture
rpoC

TCCAGCTGGAAAACATCTTTTGGTTAGAGATGGAGATGTTGTAAAAGCAGGAG
ATATGCTTTGTGATGGTAGAATTAATCCTATGATGTGCTTGAAATTTTAGGTGG
GA

Length 109 bp, BLAST 99% identity with Bbss, e value 6e-47

Sequences for Case #11 AB

Vaginal culture
rpoC

TATGGTAATGATCATGTGTATGAGGTAGGACGGTGGCGATGATTGTCCTTTTAG
GTGGCAAGGTGGGATGCTTGATGTTTTTGGTGGAAAAGCAGGAGATATGCTTT
GTGATGGTAGAATTAATCCTCATGATGTGCTTGAAATTTTAGGTGGGAA

Length 156 Bp, BLAST 100% identity with Bbss, e value 1e-25

SEQUENCES FROM UNIVERSITY OF CALIFORNIA, IRVINE

Sequences for Case #2 UCI

16S-23S Intergenic spacer
Vaginal culture

ATGCGTTTTGCTTCACATCACTGTTTCGCTTCGCTTTGTACAGGCCATTGTAGCACGTGTGTAGCCCAGGACATA
AGGGCCATGATGATTTGACGTCATCCTCACCTTCCTCCGACTTATCACCGGCAGTCTTATCTGAGTCCCCACCAT
TACATGCTGGTAACAGATAACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACACCTCACAGCACGAGCTGA
CGACAACCATGCAGCACCTGTATATAGACCCCAAACGGGGAATAATTATCTCTAACTATATCCTATATATGTCAA
GCCCTGGTAAGGTTCCTCGCGTATCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCC
TTTGAGTTTCACTCTTGCGAGCATACTCCCCAGGCGGCACACTTAACACGTTAGCTTCGGTACTAACTTTTAGTT
AACACCAAGTGTGCATCGTTTACAGC

Length 474bp, BLAST 100% identity with Bbss, e value 0

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Sequences for Case #10 UCI

16S-23S Intergenic spacer
Seminal culture

ATGCGTTTTGCTTCACATCACTGTTTCGCTTCGCTTTGTACAGGCCATTGTAGCACGTGTGT
AGCCCAGGACATAAGGGCCATGATGATTTGACGTCATCCTCACCTTCCTCCGACTTATCAC
CGGCAGTCTTATCTGAGTCCCCACCATTACATGCTGGTAACAGATAACAAGGGTTGCGCT
CGTTGCGGGACTTAACCCAACACCTCACAGCACGAGCTGACGACAACCATGCAGCACCTG
TATATAGACCCCAAACGGGGGATAATTATCTCTAACTATATCCTATATATGTCAAGCCCTG
GTAAGGTTCCTCGCGTATCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCG
TCAATTCCTTTGAGTTTCACTCTTGCGGGCATACTCCCCAGGCGGCACACTTAACACGTTA
GCCTCGGTACTAACTTTTAGTTAACACCAAGTGTGCATCGTTTACAGC

Length 474bp, BLAST 99% identity with Bbss, e value 0