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1

2 **The generality of post-antimicrobial treatment persistence of**

3 **replicatively-attenuated *Borrelia burgdorferi* in a mouse model**

4

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13 **Short Title**: *Borrelia burgdorferi* Persistence

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23 **ABSTRACT**

24 A basic feature of infection caused by *Borrelia burgdorferi*, the etiological agent
25 of Lyme borreliosis, is that persistent infection is the rule, not the norm, in its many hosts.
26 The ability to persist and evade host immune clearance poses a challenge to effective
27 antimicrobial treatment. A link between therapy failure and the presence of persister cells
28 has started to emerge. There is growing experimental evidence that viable, but non-
29 cultivable spirochetes persist following treatment with several different antimicrobial
30 agents, then resurge after 12 months. The current study utilized the mouse model to
31 evaluate if persistence and resurgence occur following antimicrobial treatment in a
32 disease-susceptible (C3H/HeN) and disease-resistant (C57BL/6) mouse strain infected
33 with *B. burgdorferi* strains N40 and B31, to confirm the generality of these phenomena.
34 The status of infection was evaluated at 12 and 18-months after treatment. The results
35 demonstrated that persistent spirochetes remain viable for up to 18 months following
36 treatment, but divide slowly, thereby being tolerant to the effects of antimicrobial agents,
37 as well as being non-cultivable. The phenomenon of persistence and resurgence in
38 disease-susceptible C3H mice is equally evident in disease-resistant B6 mice, and not
39 unique to any particular *B. burgdorferi* strain. The results also demonstrate that following
40 antimicrobial treatment, both strains of *B. burgdorferi*, N40 and B31, lose one or more
41 small plasmids, resulting in attenuation. The biological relevance of attenuated *B.*
42 *burgdorferi* spirochetes is probably inconsequential. The study demonstrated that non-
43 cultivable spirochetes can persist in a host following antimicrobial treatment for a long
44 time but did not demonstrate their clinical relevance in a mouse model of chronic
45 infection.

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46 **INTRODUCTION**

47 *Borrelia burgdorferi,* the etiological agent of Lyme borreliosis has become an
48 important public health problem and the most prevalent tick-borne disease in the United
49 States (1, 2). It occurs across much of the northern hemisphere, causing considerable
50 morbidity and in some cases mortality in humans, domestic animals, and occasionally
51 wildlife. It is the most frequently diagnosed tick-borne disease in the United States.
52 According to the Centers for Disease Control and Prevention (CDC), it is estimated there
53 are more than 300,000 human cases of Lyme borreliosis annually (3), and is expecting to
54 rise (4).

55 Untreated human Lyme borreliosis results in disease with a wide range of clinical
56 symptoms and protean manifestation, depending on the stage of infection (5-9). The
57 treatment with antimicrobial agents of patients with diagnosed Lyme borreliosis mainly
58 resolves the objective clinical manifestations. Associated subjective symptoms often may
59 persist for many weeks, even months (10). However, a proportion of patients remain ill
60 (11, 12) and delayed treatment is associated with negative clinical outcomes (13, 14).
61 There are many reports that despite resolution of the clinical signs of infection after
62 treatment with antimicrobial agents, a minority of patients experience chronic Lyme
63 disease (15-22).

64 A hallmark of Lyme borreliosis is persistent infection. This has been
65 experimentally proven in *Peromyscus* mice (23), laboratory mice (24), rats (25), hamsters
66 (26), gerbils (27), guinea pigs (28), dogs (29), and non-human primates (30), and in
67 reported confirmed spontaneous cases in humans based on culture (31-37) and PCR (38-
68 41). Chronic cases require prolonged treatment, and treatment is often less effective (15,

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69 42-44). Several reports have provided evidence of *B. burgdorferi* presence in collagenous
70 tissues, following antimicrobial therapy during chronic infection in different animal
71 models, including mice (45-50), dogs (29, 51, 52), and non-human primates (48, 53, 54),
72 and in spontaneous cases in humans (17-21). What is unique about all of these studies is
73 that spirochetes can be detected by PCR for *B. burgdorferi*-specific DNA (BbDNA), but
74 not by culture. There is also strong scientific evidence that uninfected ticks were able to
75 acquire *B. burgdorferi* that survive various antimicrobial treatments and transmit them to
76 naïve hosts following the molt to the next stage (45-47, 50, 54).

77 The nature of the formation of persistent spirochetes is still unknown and is
78 matter of speculation. Several mechanisms have been proposed that involve a suppression
79 of the innate and adaptive immune systems, complement inhibition, induction of anti-
80 inflammatory cytokines, formation of immune complexes, antigenic variation, and
81 physical seclusion (55-59). *B. burgdorferi* has evolved to persist in immunologically
82 competent hosts as a survival strategy for maintaining its natural host-vector life cycle
83 (27, 53, 55, 60). Persistence of non-cultivable spirochetes has been shown to occur
84 following treatment with several different classes of antimicrobial agents, and the
85 phenomenon is likely explained by antimicrobial tolerance (in contrast to antimicrobial
86 resistance or inadequate antimicrobial treatment), in which all classes of antimicrobials
87 fail to completely eliminate non-dividing or slowly-dividing subpopulations of a broad
88 array of bacteria and fungi (61, 62). It has been known for decades that during in vitro
89 passage, *B. burgdorferi* is highly prone to plasmid loss (63-65), and therefore plasmid
90 loss is likely to also occur during the course of infection and increase over time.

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91 In mouse studies performed in this laboratory, mice were treated with ceftriaxone,
92 doxycycline, or tigecycline at various intervals of infection, and tissues were tested at
93 intervals after treatment. Tissues remained BbDNA PCR-positive up to 12 months but
94 were consistently culture-negative. Morphologically intact spirochetes could be
95 visualized by immunohistochemistry in tissues from treated mice; ticks could acquire
96 morphologically intact *B. burgdorferi* and BbDNA from treated mice; ticks remained
97 BbDNA-positive through molting into nymphs and adults; nymphs transmitted BbDNA
98 to recipient immunocompromised mice; allografts from treated mice transplanted into
99 recipient immunocompromised mice transferred BbDNA to recipient mice; and both tick-
100 and transplant-inoculated mice had disseminated BbDNA. BbDNA-positive tissues were
101 also positive for *B. burgdorferi*-specific RNA transcription. Furthermore, quantitative
102 PCR indicated low-levels of replication during these various stages (46, 47, 50). In the
103 most recent study we observed the resurgence of non-cultivable spirochetes in assessed
104 tissues of antimicrobial treated mice after 12 months, and the overall tissue spirochete
105 burden reached the levels detected in sham-treated mice at the same time point. Despite
106 the continued non-cultivable state, RNA transcription of multiple *B. burgdorferi* genes
107 was detected in host tissues, BbDNA was acquired by xenodiagnostic ticks, and
108 spirochetal forms could be visualized within ticks and mouse tissues by
109 immunofluorescence and immunohistochemistry, respectively. A number of host
110 cytokines were up- or down-regulated in tissues of both saline- and antimicrobial-treated
111 mice in the absence of histopathology, indicating host response to the presence of non-
112 cultivable spirochetes, despite the lack of inflammation in tissues (47).

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113 We hypothesize that during the course of infection, *B. burgdorferi* proliferates
114 and incidentally generates an increasingly heterogeneous population of replicatively-
115 attenuated spirochetes that have lost one or more small plasmids. These "attenuated"
116 spirochetes remain viable, but because of their plasmid loss, they divide slowly, thereby
117 being tolerant to the effects of antimicrobials, as well as being non-cultivable.
118 Results obtained from this study demonstrated the generality of spirochete persistence
119 and in particular, resurgence, following antimicrobial treatment by demonstrating the
120 phenomena in genetically susceptible C3H/HeN (C3H) and resistant C57BL/6 (B6)
121 mouse strains infected with *B. burgdorferi* strain N40 compared to strain B31 for up to 18
122 months after treatment.
123
124 **MATERIAL AND METHODS**
125 **Mouse infections.** All experiments involving vertebrate animals were approved
126 by the Institutional Animal Care and Use Committee (IACUC) at University of California
127 Davis (UCD). All animals were purchased from The Jackson Laboratory, Bar Harbor,
128 Maine, and were cared for by staff from Teaching and Research Animal Care Services
129 (TRACS), at UCD. Mice were maintained in an isolated room within filter-top cages and
130 were provided food and water *ad libitum*. The animals used in this study were C3H and
131 B6 specific-pathogen-free 5-week-old female mice and were maintained in cohorts of 5
132 mice per cage under identical husbandry conditions in the same animal room. Euthanasia
133 was performed by rapid carbon dioxide narcosis in accordance with guidelines of the
134 American Veterinary Medical Association (AVMA) (66). C3H and B6 mice were
135 randomly divided into two equal groups. One group of C3H and B6 mice was infected

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136 with *B. burgdorferi* N40 and another one with *B. burgdorferi* B31. Each mouse was
137 infected by syringe inoculation of 10^4 *B. burgdorferi* spirochetes at the mid-log phase in
138 0.1 ml of Barbour-Stoenner-Kelly-II (BSK-II) medium intradermally at the dorsal
139 thoracic midline.

140 ***B. burgdorferi* culturing.** Two low-passage clonal strains of *B. burgdorferi* sensu
141 stricto were used. *B. burgdorferi* strains N40 and B31 were cloned by threefold limiting
142 dilution *in vitro* and passage in mice to prove infectivity and pathogenicity as described
143 previously (24). Frozen aliquots of clonal strains N40 and B31 were thawed and cultured
144 in modified BSK-II medium (67) at 33°C. Spirochetes were assessed for viability and
145 enumerated by dark-field microscopy using a Petroff-Hausser bacterial counting chamber
146 (Hausser Scientific, Horsham, PA) immediately prior to use, and diluted to appropriate
147 concentrations in BSKII medium. Mice were inoculated subdermally on the dorsal
148 thoracic midline with 10^4 mid-log phase spirochetes in 0.1 ml of BSK-II medium.
149 Infection status with cultivable *B. burgdorferi* was determined by culture of urinary
150 bladder and sub-inoculation site (deep dermis). Tissues were collected from mice
151 aseptically at necropsy, and then cultured in medium without antibiotic, as described
152 (68).

153 To determine if resurgent spirochetes regain cultivability at 18 months after
154 treatment and to increase their detectability, multiple tissue sites were collected and
155 cultured in four different media. In addition to urinary bladder and inoculation site we
156 also collected front joint, ear, quadriceps muscle, and spleen. Heart tissue was not
157 available for culture as was used for PCR and histology. To facilitate cultivation, several
158 media have been introduced, such as BSK-II medium, modified Kelly-Pettenkofer (MKP)

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159 medium, BSK-II + agarose, and BSK-II + carbohydrates medium (cordially provided by
160 Monica E. Embers of Tulane National Primate Research Center, Covington, Louisiana).
161 However, these media differ in their potential to support the growth of borreliae.

162 **Xenodiagnosis.** Laboratory-reared, pathogen-free *Ixodes scapularis* larvae were
163 provided by Melissa J. Caimano of Department of Molecular Biology and Biophysics,
164 UConn Health, Farmington, CT. All larvae were derived from single gravid ticks for
165 each study. Around 40 larval ticks were placed on each mouse 1 week prior to necropsy,
166 allowed to feed to repletion, collected, and then allowed to molt and harden into nymphs.
167 Cohorts of ticks collected from each mouse were maintained separately, so that ticks
168 within each cohort could be tested individually or in groups by qPCR. Nymphal ticks
169 were frozen in liquid nitrogen, ground with a mortar and pestle, and DNA was extracted
170 for PCR analysis.

171 **Antimicrobial treatment and monitoring.** The treatment with ceftriaxone was
172 initiated during the early immune/dissemination stage at 4 weeks post infection of *B.*
173 *burgdorferi* in immunocompetent C3H and B6 mice to mimic the stages found in human
174 disease. Previous studies in this laboratory have shown that the minimal inhibitory
175 concentration (MIC) and minimal bactericidal concentration (MBC) of ceftriaxone is
176 0.015 μg/ml and 0.06 μg/ml, respectively. The experimental design for this experiment
177 involved eight treatment groups, as depicted in Table 1. A ceftriaxone dosage of 16
178 mg/kg in 500 μl 0.9% normal saline was administrated intraperitoneally in mice twice
179 daily for 5 days, followed by once daily for 23 days. Over a 4-h period, serum
180 concentrations of ceftriaxone in mice were measured on the day of treatment by bleeding
181 mice at 0, 2 and 4 hours after treatment. Average serum concentrations of ceftriaxone

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182 were 18.625 μg/ml, and 1.795 μg/ml at 2 and 4 hours, respectively. As expected,
183 intraperitoneally administrated ceftriaxone accumulated rapidly in the circulation and
184 then decreased gradually during the following 2 hours. Studies with ceftriaxone in mice
185 have been challenged because the pharmacodynamics (T>MIC) of ceftriaxone do not
186 parallel those in humans. However, the ceftriaxone treatment regimen used in our studies
187 transiently achieves more than adequate serum concentrations. No differences were
188 observed in serum ceftriaxone concentrations between different mouse strains (C3H and
189 B6).

190 **Table 1**. The experimental design for the executed experiment involved eight treatment
191 groups.

Group	*B. burgdorferi*	Mouse strain	Treatment	12 months	18 months	n=
1	N40	C3H	ceftriaxone	5	5	10
2	B31	C3H	ceftriaxone	5	5	10
3	N40	B6	ceftriaxone	5	5	10
4	B31	B6	ceftriaxone	5	5	10
5	N40	C3H	saline	5	5	10
6	B31	C3H	saline	5	5	10
7	N40	B6	saline	5	5	10
8	B31	B6	saline	5	5	10

192

193 **Tissue processing for molecular analysis.** Mouse tissue samples for molecular
194 analysis were processed as previously described (47, 69), with slight modifications.
195 Briefly, samples of the heart base, ventricular myocardium (the heart was bisected
196 through the atria and ventricles, with one-half of the heart base and ventricles used for
197 nucleic acid extraction and the other half processed for histology), and the left tibiotarsal
198 joint were collected from each mouse. The samples were collected aseptically,
199 immediately weighed, snap-frozen in liquid nitrogen, and stored at −80°C before nucleic

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200 acid extraction. Total nucleic acid was extracted with QIAcube HT system (Qiagen,
201 Valencia, Calif.), according to the manufacturer's instructions for tissue or insects. The
202 system enables semi-automated high-throughput nucleic acid purification, yielding high-
203 quality pathogen nucleic acids free of contaminants and inhibitors. The copy number of
204 each *B. burgdorferi* target gene was expressed per milligram of tissue weight.

205 **Molecular quantitative analysis.** Because low DNA/RNA copy numbers were
206 expected in tissues from antimicrobial-treated mice, each sample was subjected to pre-
207 amplification. We used the same *B. burgdorferi* outer surface protein (*ospA*) and flagellin
208 (*flaB*), and a mouse glyceraldehyde-3-phosphate dehydrogenase (*gapDH*) assay primers,
209 standardized and optimized for real-time quantitative PCR (qPCR), as described (70).
210 Purified DNA/buffer was heated for 5 minutes at 95°C then placed on ice. Each reaction
211 contained Advantage buffer, Advantage 2 Polymerase (Takara Bio, Mountain View, CA),
212 0.2 mM each dNTP, 10 µM of each primer, and DNA template (100 ng/µl). Standard
213 amplification conditions were as follows: 1 min at 94°C, 25 cycles of 15 s at 94°C, 15 s
214 at 55°C, and 45 s at 70°C, 5 min at 70°C, and then 10 min at 4°C. The pre-amplified
215 products were diluted at a ratio of 1:10 and used as templates for qPCR analysis. To
216 assess transcriptional activity of *B. burgdorferi flaB* and *16S rRNA* genes, first-strand
217 cDNA was synthesized from total RNA using the QuantiTect Reverse Transcription Kit
218 (Qiagen) in 50-µL reactions, as described (70). To increase fidelity, efficiency, and
219 greater yield of cDNA, the Advantage® 2 Polymerase Mix (Clontech Laboratories) was
220 used, according to the manufacturer's instructions.

221 Two specific primers and one internal, fluorescence-labeled probe were designed
222 with Primer Express™ software (ThermoFisher Scientific), as described (71). The

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223 amplification efficiency (E) of all assays was calculated from the slope of a standard
224 curve generated on a 10-fold dilution in triplicate for every DNA sample, using the
225 formula $E=10^{(-1/\text{slope})}-1$. In order to obtain accurate and reproducible results, all assays
226 were determined to have an efficiency of >95%. To quantify the copy numbers of DNA
227 target genes, plasmid standards were prepared in order to create absolute standard curves,
228 as described (71). Based on the amplification efficiencies, detection limits were
229 approximately 10 copies of DNA per reaction, and the analytical sensitivity for each
230 target gene was in the range from <10 to 10^9 copies. All samples were assayed in
231 duplicate with positive and negative controls by the qPCR optimized assays and analyzed
232 for the presence of mouse *gapDH* in order to determine the efficiency of the nucleic acid
233 extraction, amplification, and as an indicator of inhibition. Amplification, data
234 acquisition, and data analysis were performed on a 7900HT Fast Real-Time PCR System
235 (ThermoFisher, Scientific). The thermal cycling conditions were as follow: 2 min at 50°C
236 to 10 min at 94.5°C, followed by 40 cycles of denaturation at 97°C for 30 sec, and
237 annealing and extension at 59.7°C for 1 min.
238 **Histology.** Formalin-fixed rear legs (demineralized in 15% formic acid) and
239 hearts were routinely processed for histology, paraffin-embedded and sectioned at 5 μm.
240 Sections were stained with hematoxylin and eosin. All tissues were evaluated by a board-
241 certified veterinary anatomic pathologist. Legs were examined for evidence of arthritis
242 and synovitis involving the knee and tibiotarsal joints as characterized by examination of
243 infiltration of neutrophils, synovial proliferation, and exudation of fibrin into joint or
244 tendon sheath lumina as well as neutrophil and macrophage infiltration of tissues at the
245 base of the heart (myocarditis). All determinations were made in a blinded fashion.

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246 **Statistics.** Statistical analysis of qPCR data between antimicrobial treated and
247 sham treated mice at different time points were performed using independent samples *t*-
248 test or one-way analysis of variance, followed by multiple pairwise comparisons by
249 Tukey's honestly significant difference (HSD) test (SPSS 16.0 for Mac; SPSS Inc.,
250 Chicago, IL). Differences were considered significant with $p \leq 0.5$.
251 To statistically evaluate the severity of inflammation in collected tissue between
252 antimicrobial treated and sham treated mice Kruskal-Wallis test was performed using
253 GraphPad Prism, 7.03.
254
255 **RESULTS**
256 **Persistence and resurgence of *B. burgdorferi* N40 and B31 following**
257 **antimicrobial treatment in C3H and B6 mice.** Mouse tissue samples**,** heart base,
258 ventricular muscle, and tibiotarsal joint, were collected aseptically, immediately weighed,
259 snap-frozen in liquid nitrogen, and stored at −80°C before total nucleic acid extraction.
260 Quality control of qPCR was assessed by targeting mouse *gapDH* gene. Spirochetal DNA
261 was *flaB* and *ospA***.** Quantitative data were expressed as the number of DNA copies per
262 milligram of tissue weight. Mouse *gapDH* qPCR was positive from all assessed samples
263 (average Cq ± SD, 19.36 ± 4.69), confirming successful extraction of total nucleic acid.
264 Based upon *flaB*/*ospA* DNA qPCR on assessed tissue samples, all C3H and B6
265 mice inoculated with either *B. burgdorferi* strains N40 or B31 treated with saline were
266 qPCR-positive at 12 and 18 months after treatment in most tissues. The data in Table 2
267 and Table 3 summarize positive or negative *flaB/ospA* DNA qPCR results. Among
268 ceftriaxone-treated C3H and B6 mice infected with N40 or B31, 100% and 74% tested

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269 positive for *flab/ospA* DNA qPCR at tibiotarsal joints at 12 and 18 months after
270 treatment, respectively. Sporadically positive heart base and ventricular muscle samples
271 were detected in mice at 12 months after antimicrobial treatment (Table 2), but not in
272 mice at 18 months (Table 3).

273 **Table 2.** Analysis of *flaB/ospA* DNA in heart base, ventricular muscle, and tibiotarsus,
274 from *B. burgdorferi* infected mice treated with ceftriaxone and saline solution, then
275 necropsied **12 months** after completion of treatment

Mouse Strain	*Borrelia* Strain	Treatment	Heart base	Ventricular muscle	Tibiotarsus
C3H	N40	Cefriaxone	+	-	+
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	+	-	+
"	"	Cefriaxone	+	-	+
"	"	Cefriaxone	-	+	+
C3H	B31	Cefriaxone	-	-	+
"	"	Cefriaxone	+	-	+
"	"	Cefriaxone	+	-	+
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	+
B6	N40	Cefriaxone	-	+	+
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	+	-	+
B6	B31	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	+	-	+
C3H	N40	Saline	+	+	+
"	"	Saline	+	+	-
"	"	Saline	-	+	+
"	"	Saline	+	+	+
"	"	Saline	+	+	-
C3H	B31	Saline	+	+	+
"	"	Saline	+	+	-
"	"	Saline	+	+	-
"	"	Saline	+	+	+
"	"	Saline	+	+	+
B6	N40	Saline	-	+	-
"	"	Saline	+	+	+
"	"	Saline	+	+	-
"	"	Saline	+	+	+
"	"	Saline	+	+	-
B6	B31	Saline	-	+	+
"	"	Saline	-	+	+
"	"	Saline	+	+	-
"	"	Saline	N/A	N/A	N/A

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"	"	Saline	N/A	N/A	N/A

276

277 **Table 3**. Analysis of *flaB/ospA* DNA in heart base, ventricular muscle, and tibiotarsus,

278 from *B. burgdorferi* infected mice treated with ceftriaxone and saline solution, then

279 necropsied **18 months** after completion of treatment

Mouse Strain	*Borrelia* Strain	Treatment	Heart base	Ventricular muscle	Tibiotarsus
C3H	N40	Cefriaxone	-	-	-
"	"	Cefriaxone	-	-	-
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	-
"	"	Cefriaxone	N/A	N/A	N/A
C3H	B31	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	+
B6	N40	Cefriaxone	-	-	-
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	+
B6	B31	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	+
"	"	Cefriaxone	-	-	+
C3H	N40	Saline	+	+	+
"	"	Saline	+	+	+
"	"	Saline	-	+	+
"	"	Saline	+	+	+
"	"	Saline	N/A	N/A	N/A
C3H	B31	Saline	+	+	+
"	"	Saline	+	+	+
"	"	Saline	+	+	+
"	"	Saline	N/A	N/A	N/A
"	"	Saline	N/A	N/A	N/A
B6	N40	Saline	-	+	+
"	"	Saline	+	+	+
"	"	Saline	+	+	+
"	"	Saline	+	+	+
"	"	Saline	+	+	+
B6	B31	Saline	+	+	+
"	"	Saline	+	+	+
"	"	Saline	+	+	+
"	"	Saline	+	+	+
"	"	Saline	+	+	+

280

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281 Quantification of each target gene in assessed samples was accomplished by
282 preparing plasmid standards in order to create absolute standard curves to determine
283 starting copy number. Mean DNA copy numbers of *flaB/ospA* genes in collected tissue
284 samples among different mouse strains (C3H and B6) and different *B. burgdorferi* stra
285 ins (N40 and B31) of ceftriaxone-treated groups at 12 months after treatment were nearly
286 equivalent (mean ± SD, $2.97 \times 10^1 \pm 1.72 \times 10^1$). Among saline-treated groups
287 significantly higher DNA copy numbers of *flaB/ospA* were determined in C3H mice
288 infected with N40 and B31 in comparison to B6 mice infected with N40 and B31
289 ($P<0.05$) (Fig 1). Significantly higher copy numbers of *flaB/ospA* were determined in
290 tissue samples of saline-treated mice in comparison to the ceftriaxone-treated mice
291 ($P<0.05$) (Fig 1). Thus, these results suggested resurgence of spirochetes at 12 months
292 following antimicrobial treatment in both C3H and B6 mice infected with both *B.*
293 *burgdorferi* strains, N40 and B31, although all mice remained culture-negative. qPCR
294 results also revealed that a population of resurged spirochetes that survived antimicrobial
295 treatment and detected at 12 months, remained in mice even after 18 months. They were
296 exclusively detected in tibiotarsal joints of all treatment groups, with mean copy numbers
297 of *flaB/ospA* per milligram tissue that were nearly equivalent (mean ± SD, $1.52 \times 10^1 \pm$
298 9.74×10^0) (Fig 2). qPCR results of saline treated groups at 18 months were similar to the
299 results of the same treated groups at 12 months. *flaB/ospA* genes copies among saline
300 treated groups at 18 months were lower in B6 mice infected with N40 and B31 in
301 comparison to C3H mice infected with N40 and B31, but not significantly. Mean
302 *flaB/ospA* copy numbers in tibiotarsal joints at 18 months were significantly higher

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303 (*P*<0.05) in saline treated C3H mice infected with N40 and B31 in comparison to the
304 same groups of antimicrobial treated mice (Fig 2).
305 **Fig 1** and **Fig 2**
306 Analysis of *B. burgdorferi flaB* and *16S rRNA* transcriptional activity revealed
307 that most of the saline treated C3H and B6 mice infected with N40 or B31 strains were
308 positive. Transcriptional activity of *flaB* in samples from antimicrobial treated mice (C3H
309 and B6) necropsied at 12 and 18 months after treatment were 52% and 35%, respectively,
310 while *16S rRNA* was constitutively detected in all samples. The results indicate viability
311 among the persisting spirochetes.
312 **Confirmation of spirochetes non-cultivability at 12 and 18 months following**
313 **antimicrobial treatment.** The infection status of mice at 12 months after antimicrobial
314 treatment was assessed by culture of inoculation site and urinary bladder. For culture, we
315 used a modified BSK-II medium. The sensitivity of BSK-II medium for detection of
316 viable spirochetes was verified by serial 10-fold dilutions of a *B. burgdorferi* N40 and
317 B31 culture, as described (46). The culture results revealed that all saline treated mice
318 were positive, with significant numbers of spirochetes detected (Table 4). When mice
319 treated with ceftriaxone evaluated at 12 months after completion of treatment, none of the
320 tissues were culture positive. In single culture two non-motile spirochetes were observed
321 (Table 4). Resurgence at 12 months after treatment suggests that spirochetes have
322 regained some semblance of replicative activity and/or are disseminating once again
323 within mouse tissues.

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324 **Table 4**. Culture results for individual mice treated with ceftriaxone and saline solution
325 commencing at 4 weeks after inoculation, then subjected to necropsy at 12 months after
326 completion of treatment

Mouse Strain	*Borrelia* Strain	Treatment	Inoculation site	Urinary bladder
C3H	N40	Cefriaxone	-	-
"	"	Cefriaxone	-	-
"	"	Cefriaxone	-	-
"	"	Cefriaxone	-	-
"	"	Cefriaxone	-	-
C3H	B31	Cefriaxone	N/A	N/A
"	"	Cefriaxone	-	-
"	"	Cefriaxone	-	-
"	"	Cefriaxone	-	-
"	"	Cefriaxone	-	-
B6	N40	Cefriaxone	-	-
"	"	Cefriaxone	-	-
"	"	Cefriaxone	-	-
"	"	Cefriaxone	-	-
"	"	Cefriaxone	-	+
B6	B31	Cefriaxone	-	-
"	"	Cefriaxone	-	-
"	"	Cefriaxone	-	-
"	"	Cefriaxone	-	-
"	"	Cefriaxone	-	-
C3H	N40	Saline	++	+++
"	"	Saline	++	+++
"	"	Saline	++	+++
"	"	Saline	+	+++
"	"	Saline	+	++
C3H	B31	Saline	++	+++
"	"	Saline	+	+
"	"	Saline	+	++
"	"	Saline	++	++
"	"	Saline	++	++
B6	N40	Saline	++	+
"	"	Saline	+	+
"	"	Saline	+++	+
"	"	Saline	++	+
"	"	Saline	++	+++
B6	B31	Saline	+++	+++
"	"	Saline	+	+
"	"	Saline	++	++
"	"	Saline	N/A	N/A
"	"	Saline	N/A	N/A

327

328 For the first time point of this study (12 months), a limited number of sample sites
329 (urinary bladder and inoculation site) were cultured. These are not optimal sites for
330 culture after antimicrobial treatment, since persisting spirochetes tend to be localized to

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331 heart base and joint tissue. To determine if resurgent spirochetes regain cultivability at 18
332 months after treatment, multiple sample types were collected such as joint, ear,
333 quadriceps muscle, spleen, urinary bladder, and inoculation site. Heart tissue was not
334 available for culture as was used for PCR and histology. To facilitate cultivation, several
335 media have been introduced, such as BSK-II medium, modified Kelly-Pettenkofer (MKP)
336 medium, BSK-II + agarose, and BSK-II + carbohydrates medium. The inoculated
337 cultures were incubated for 50 days at 33°C and examined weekly. Culture tubes were
338 gently but thoroughly stirred, and a drop of culture was examined by dark-field
339 microscopy. The culture results revealed that all saline treated mice were positive, with
340 significant numbers of motile spirochetes detected (Table 5, Fig 3A) in the majority of
341 tissue samples cultured. When mice treated with ceftriaxone were evaluated, one ear
342 tissues sample from B6 mouse infected with B31 (Fig 3B) and one front joint tissue
343 sample from B6 mouse infected with N40 (Fig 3C) were culture positive (Table 5).
344 Tissues were cultured in MKP and BSK-II + carb medium, respectively. After 4 weeks of
345 incubation spirochetes in cultured medium of ceftriaxone treated mice appeared non-
346 motile with irregular curves (dead), so we were unable to subculture them. These results
347 confirm our previous findings that antimicrobial-treated spirochetes are not cultivable,
348 probably as a result of being genetically impaired (plasmid loss) after treatment with
349 antimicrobials.

350 **Table 5.** Culture results for individual mice treated with ceftriaxone and saline solution
351 commencing at 4 weeks after inoculation, then subjected to necropsy at 18 months after
352 completion of treatment

Mouse Strain	*Borrelia* Strain	Treatment	IS[a]	Ear	UB[b]	QM[c]	FJ[d]	Sp[e]

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C3H	N40	Ceftriaxone	-	-	-	-	-	-
"	"	Ceftriaxone	-	-	-	-	-	-
"	"	Ceftriaxone	-	-	-	-	-	-
"	"	Ceftriaxone	-	-	-	-	-	-
C3H	B31	Ceftriaxone	-	-	-	-	-	-
"	"	Ceftriaxone	-	-	-	-	-	-
"	"	Ceftriaxone	-	-	-	-	-	-
"	"	Ceftriaxone	-	-	-	-	-	-
"	"	Ceftriaxone	-	-	-	-	-	-
B6	N40	Ceftriaxone	-	-	-	-	-	-
"	"	Ceftriaxone	-	-	-	-	-	-
"	"	Ceftriaxone	-	-	-	-	-	-
"	"	Ceftriaxone	-	-	-	-	-	-
"	"	Ceftriaxone	-	-	-	-	+	-
B6	B31	Ceftriaxone	-	-	-	-	-	-
"	"	Ceftriaxone	-	-	-	-	-	-
"	"	Ceftriaxone	-	-	-	-	-	-
"	"	Ceftriaxone	-	-	-	-	-	-
"	"	Ceftriaxone	-	+	-	-	-	-
C3H	N40	Saline	+	++	+	+++	+	+
"	"	Saline	+	+++	+	+++	+	+
"	"	Saline	+	+	++	+++	++	-
"	"	Saline	+	+	+	+	+	+
"	"	Saline	N/A	N/A	N/A	N/A	N/A	N/A
C3H	B31	Saline	+	+++	+	++	+	+
"	"	Saline	+	-	+	++	+	-
"	"	Saline	+	-	-	-	+	-
"	"	Saline	N/A	N/A	N/A	N/A	N/A	N/A
"	"	Saline	N/A	N/A	N/A	N/A	N/A	N/A
B6	N40	Saline	+	++	+	++	+	-
"	"	Saline	+	+	-	-	-	-
"	"	Saline	++	++	-	+	++	+
"	"	Saline	+	+	-	+	+	-
"	"	Saline	+	-	-	-	-	+
B6	B31	Saline	+++	+++	+++	+++	+++	+++
"	"	Saline	+	+	+	+	+	+
"	"	Saline	++	++	++	++	++	++
"	"	Saline	N/A	N/A	N/A	N/A	N/A	N/A
"	"	Saline	N/A	N/A	N/A	N/A	N/A	N/A

353 [a] Inoculation site; [b] Urinary bladder; [c] Quadriceps muscle; [d] Front joint; [e] Spleen

354

355 **Fig 3**

356 **Persistence of *B. burgdorferi* in xenodiagnostic ticks that fed upon**

357 **antimicrobial-treated mice.** Several days prior to necropsy, each mouse was infested by

358 placing approximately 40 larval *I. scapularis* ticks, derived from a single population of

359 laboratory-reared pathogen-free larvae. Ticks were allowed to feed to repletion. Engorged

360 larvae were collected and allowed to molt into nymphs and harden, thereby allowing

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361 post-feeding amplification of spirochete populations and minimization of inhibitory
362 products (blood) for PCR. Samples of 5 (saline treated) and 10 (ceftriaxone treated)
363 randomly selected nymphal ticks from each tick cohort from each mouse were collected.
364 Ticks were tested for the presence of *B. burgdorferi flaB/ospA* DNA by qPCR. Cohorts of
365 nymphs from each mouse treated with saline solution at 12 and 18 months were all
366 *flaB/ospA* PCR positive (Table 6). Mean copy numbers of *flaB/ospA* DNA ranged from
367 389 to 13,100 (mean ± SD, 3,120 ± 3,790) and from 97 to 11,950 (mean ± SD, 2,340 ±
368 2,820) copies per tick at 12 months and 18 months, respectively. In contrast, 9 of 20 and
369 3 of 19 ceftriaxone-treated mice had one *flaB/ospA* PCR positive tick each, among the ten
370 ticks tested from each mouse at 12 and 18 months, respectively (Table 6).
371 **Table 6**. *flaB/ospA* qPCR results of nymphal that ticks fed on mice treated with
372 ceftriaxone and saline solution prior to necropsy at 12 and 18 months after completion of
373 treatment

Mouse Strain	*Borrelia* Strain	Treatment	XenoDx 12 months	XenoDx 18 months
C3H	N40	Cefriaxone	1/10	0/10
"	"	Cefriaxone	0/10	0/10
"	"	Cefriaxone	1/10	1/10
"	"	Cefriaxone	1/10	0/10
"	"	Cefriaxone	0/10	N/A
C3H	B31	Cefriaxone	0/10	0/10
"	"	Cefriaxone	1/10	0/10
"	"	Cefriaxone	1/10	0/10
"	"	Cefriaxone	0/10	0/10
"	"	Cefriaxone	0/10	0/10
B6	N40	Cefriaxone	1/10	0/10
"	"	Cefriaxone	0/10	0/10
"	"	Cefriaxone	0/10	1/10
"	"	Cefriaxone	0/10	1/10
"	"	Cefriaxone	1/10	0/10
B6	B31	Cefriaxone	0/10	0/10
"	"	Cefriaxone	0/10	0/10
"	"	Cefriaxone	0/10	0/10
"	"	Cefriaxone	1/10	0/10
"	"	Cefriaxone	1/10	0/10
C3H	N40	Saline	5/5	5/5
"	"	Saline	5/5	3/5

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"	"	Saline	3/5	3/5
"	"	Saline	5/5	5/5
"	"	Saline	4/5	N/A
C3H	B31	Saline	5/5	3/5
"	"	Saline	4/5	5/5
"	"	Saline	5/5	3/5
"	"	Saline	5/5	N/A
"	"	Saline	3/5	N/A
B6	N40	Saline	2/5	3/5
"	"	Saline	4/5	4/5
"	"	Saline	3/5	2/5
"	"	Saline	5/5	5/5
"	"	Saline	4/5	4/5
B6	B31	Saline	3/5	5/5
"	"	Saline	2/5	2/5
"	"	Saline	4/5	4/5
"	"	Saline	N/A	3/5
"	"	Saline	N/A	5/5

374

375 **Histopathology of mouse tissues following antimicrobial treatment.** Here we
376 report on the general features of histopathology, legs were examined for evidence of
377 arthritis and synovitis, and hearts for neutrophil and macrophage infiltration. At 12
378 months after treatment inflammatory scores were greatest and more robust for
379 myocarditis in control C3H/N40 and C3H/B31 mice in comparison to antimicrobial
380 treatment groups (Fig 4). Tibiotarsal arthritis was mild, characterized predominantly by
381 synovial hyperplasia and hypertrophy with joint or tendon sheath effusion in both,
382 antimicrobial treated and control mice at 12 months. There are statistically significant
383 differences (Kruskal-Wallis test, $p = 0.0227$) between groups based on myocarditis
384 scores. Group of antimicrobial C3H/B31 mice has statistically significantly lower scores
385 than control C3H/B31 mice (Dunn's multiple comparisons test, $p = 0.043$). No
386 statistically significant differences (Kruskal-Wallis test, $p = 0.1187$) were observed
387 between groups based on tibiotarsal arthritis scores. Statistical analyses performed using
388 GraphPad Prism, 7.03 (Table 7). At 18 months inflammatory scores were greatest for
389 myocarditis in control groups C3H/N40, C3H/B31, and B6/N40 (Fig 4). The chronic

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390 periarteritis and tenosynovitis were observed in few control C3H/B31 and B6/N40 mice,
391 respectively, a pathohistological findings frequently observed in borrelial chronically
392 infected mice (Fig 5). Osteoarthritis was very mild and considered a background age-
393 related finding. There are statistically significant differences (Kruskal-Wallis test, p =
394 0.0019) between groups based on myocarditis scores. Control groups C3H/N40 and
395 C3H/B31 have statistically significantly higher scores than antimicrobial treated group
396 C3H/B31 (Dunn's multiple comparisons test, p = 0.046, p = 0.007). No statistically
397 significant differences (Kruskal-Wallis test, p = 0.1431) were observed between groups
398 based on tibiotarsal arthritis scores. Statistical analyses were performed using GraphPad
399 Prism, 7.03 (Table 7).
400 **Table 7.** Histopathological results of heart base and tibiotarsal joint of *B. burgdorferi*
401 infected mice treated with either ceftriaxone or sham treated with saline solution

Mouse Strain	*Borrelia* Strains	Treatment	Heart base 12 months	Tibiotarsus 12 months	Heart base 18 months	Tibiotarsus 18 months
C3H	N40	Cefriaxone	0	1	1	0
"	"	Cefriaxone	0	0	1	0
"	"	Cefriaxone	1	1	0	1
"	"	Cefriaxone	1	1	0	1
"	"	Cefriaxone	0	1	N/A	N/A
C3H	B31	Cefriaxone	N/A	N/A	0	0
"	"	Cefriaxone	0	0	0	0
"	"	Cefriaxone	0	0	0	0
"	"	Cefriaxone	0	0	0	0
"	"	Cefriaxone	0	0	0	0
B6	N40	Cefriaxone	1	0	0	0
"	"	Cefriaxone	1	0	1	1
"	"	Cefriaxone	0	1	1	0
"	"	Cefriaxone	1	0	0	1
"	"	Cefriaxone	0	1	0	0
B6	B31	Cefriaxone	1	0	1	0
"	"	Cefriaxone	1	0	0	0
"	"	Cefriaxone	0	0	1	0
"	"	Cefriaxone	0	0	0	0
"	"	Cefriaxone	1	0	0	0
C3H	N40	Saline	1	0	1	1
"	"	Saline	2	1	1	1
"	"	Saline	1	1	2	1
"	"	Saline	3	1	2	1

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"	"	Saline	1	1	N/A	N/A
C3H	B31	Saline	1	0	2	0
"	"	Saline	1	1	2	0
"	"	Saline	2	1	2	1
"	"	Saline	3	1	N/A	N/A
"	"	Saline	3	1	N/A	N/A
B6	N40	Saline	0	1	1	0
"	"	Saline	0	1	1	0
"	"	Saline	1	0	1	1
"	"	Saline	1	1	2	1
"	"	Saline	1	1	1	1
B6	B31	Saline	2	1	1	0
"	"	Saline	1	1	0	0
"	"	Saline	1	0	1	1
"	"	Saline	N/A	N/A	1	0
"	"	Saline	N/A	N/A	1	0

402 Myocarditis scores by graduation (0-3): 0 = not present; 1 = minimal to mild, focal to
403 focally extensive, lymphoplasmacytic infiltrates; 2 = moderate, multifocal,
404 lymphoplasmacytic infiltrates; 3 = marked, coalescing to diffuse, lymphoplasmacytic
405 infiltrates. Arthritis scores by graduation (0-1): 0 = not present; 1 = synovial
406 hyperplasia/hypertrophy and effusion with or without minimal lymphoplasmacytic
407 infiltrates
408
409 **Fig 4** and **Fig 5**
410 **Plasmid content of attenuated spirochetes.** In our previous work we conducted
411 experiments utilizing primarily strain N40, so the designed qPCR assays were based on
412 its genome sequences. For analysis of plasmid presence in tissue samples from C3H and
413 B6 mice infected with *B. burgdorferi* B31 strain, fewer genes were targeted. A
414 comparison of plasmid profile, size, and GC content between N40 and B31 strains appear
415 to have major structural differences, so only four designed qPCR assays targeting *bptA,*
416 *arp, ospA,* and *erp23* are homologous.
417 Among 12-month and 18-month samples, all gene targets were detected in tissue
418 samples from saline-treated C3H and B6 mice infected with either *B. burgdorferi* N40

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419 and B31 strains (Table 8 and 9). *B. burgdorferi* genomes of N40 strain in tissue samples
420 with high Bb DNA copy numbers from 12 antimicrobial-treated mice were uniformly
421 missing *bptA* (lp25), *erp22* (cp32-9), and *erp23* (cp34-2), and variably missing *arp* (lp28-
422 5), *eppA* (cp9), and *ospC* (cp26), whereas *ospA* (lp54) was detected in all examined tissue
423 samples (Table 8). All assessed tissue samples of mice infected with strain B31 were
424 uniformly missing *bptA* (lp25) and *arp* (lp28-5), and variably missing *erp23* (cp34-7). As
425 in tissue samples from mice infected with strain N40, *ospA* (lp54) was detected in all
426 examined samples (Table 9). Data suggest loss of small linear and circular plasmids in
427 persisting spirochetes at 12 and 18 months after treatment with antimicrobial. No
428 difference was observed between different *B. burgdorferi* strains (B31 and N40) or in
429 genetically different strains of mice that are disease-susceptible (C3H) and disease-
430 resistant (B6). Most notable was the uniform absence of lp25 in both *B. burgdorferi*
431 strains, N40 and B31, loss of which significantly attenuates infectivity in mice, with
432 markedly reduced, but detectable levels of infection. Results also demonstrated
433 amplification of multiple gene targets, thereby verifying the specificity of residual
434 BbDNA results (Tables 8 and 9).

435 **Table 8**. Analysis of plasmid presence in heart base (HB), ventricular muscle (VM), and
436 tibiotarsus (TT) of mice infected with *B. burgdorferi* **N40** strain then treated with either
437 ceftriaxone or sham treated with saline solution

Mouse Strain	Treatment /Time	Sample Type	chrom *flaB*	lp25 *bptA*	lp28-5 *arp*	lp54 *ospA*	cp9 *eppA*	cp26 *ospC*	cp32-9 *erp22*	cp34-2 *erp23*
C3H	Saline/12	HB	+	+	+	+	+	+	+	+
B6	"	TT	+	+	+	+	+	+	+	+
C3H	Saline/18	HB	+	+	+	+	+	+	+	+
B6	"	VM	+	+	+	+	+	+	+	+
C3H	Ceftriaxone /12	HB	+	-	-	+	-	-	-	-
C3H	"	TT	+	-	-	+	-	+	-	-

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C3H	"	TT	+	-	-	+	-	-	-	-
B6	"	TT	+	-	+	+	-	-	-	-
B6	"	HB	+	-	-	+	-	-	-	-
B6	"	TT	+	-	-	+	-	-	-	-
C3H	Ceftriaxone /18	TT	+	-	-	+	-	-	-	-
C3H	"	TT	+	-	-	+	-	-	-	-
C3H	"	TT	+	-	+	+	+	+	-	-
B6	"	TT	+	-	-	+	-	-	-	-
B6	"	TT	+	-	-	+	-	-	-	-
B6	"	TT	+	-	-	+	-	-	-	-

438

439 **Table 9**. Analysis of plasmid presence in heart base (HB), ventricular muscle (VM), and
440 tibiotarsus (TT) of mice infected with *B. burgdorferi* **B31** strain then treated with
441 ceftriaxone and sham treated with saline solution

Mouse Strain	**Treatment/Time**	**Sample Type**	**chrom *flaB***	**lp25 *bptA***	**lp28-1 *arp***	**lp54 *ospA***	**cp34-7 *erp23***
C3H	Saline/12	VM	+	+	+	+	+
B6	"	VM	+	+	+	+	+
C3H	Saline/18	HB	+	+	+	+	+
B6	"	HB	+	+	+	+	+
C3H	Ceftriaxone/12	TT	+	-	-	+	-
C3H	"	HB	+	-	-	+	-
C3H	"	TT	+	-	-	+	-
B6	"	TT	+	-	-	+	+
B6	"	HB	+	-	-	+	-
B6	"	TT	+	-	-	+	-
C3H	Ceftriaxone/18	TT	+	-	-	+	-
C3H	"	TT	+	-	-	+	-
C3H	"	TT	+	-	-	+	-
B6	"	TT	+	-	-	+	-
B6	"	TT	+	-	-	+	-
B6	"	TT	+	-	-	+	-

442

443 **DISCUSSION**

444 The most commonly prescribed antimicrobial agents for the treatment of human
445 Lyme borreliosis, such as penicillin, amoxicillin, cefotaxime, cefuroxime, ceftriaxone,

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446 doxycycline, and erythromycin, have shown to be effective against *B. burgdorferi* (72,
447 73). It is important to mention that treatment during early infection is more effective in
448 clearing the pathogen than treatment during later infection in humans (74) and in the
449 mouse model (46). Over the past several years, there have been numerous well-supported
450 reports of *Borrelia* detection after completed antimicrobial therapy (46, 50, 53, 60, 75,
451 76). All of these studies have in common that spirochetes were detected by PCR for
452 BbDNA, but not by culture. Morphologically intact spirochetes were visualized by
453 immunohistochemistry in tissues from antimicrobial-treated mice; ticks could acquire
454 those spirochetes and transmit them to recipient mice. Mice tissues were positive for *B.*
455 *burgdorferi*-specific RNA transcripts.

456 In the current study, we evaluated persistence at 12 and 18 months after
457 ceftriaxone treatment in two strains of mice that are disease-susceptible (C3H) and
458 disease-resistant (B6). We recognize that inbred mice do not represent genetically
459 heterogeneous humans but use of two genetically and phenotypically disparate mouse
460 strains will confirm the generality of persistence, and more importantly resurgence,
461 following antimicrobial treatment. Disease severity and tissue spirochete burdens vary
462 among mouse strains (77), although the ID50 among mice is identical (78). C3H/HeJ
463 mice are susceptible to infection by Gram-negative bacteria as a result of spontaneous
464 mutation in *Tlr4* gene (79), which may influence the persistence. B6 mice harbor
465 equivalent tissue spirochete burdens as C3H mice, become persistently infected, and have
466 normal macrophage function (79).

467 Our results have shown that, indeed, persistence and resurgence in fully
468 immunocompetent B6 mice is similar to those in disease-susceptible C3H mice, which

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469 confirm the generality of persistence in a mouse model. These results confirm our
470 previous finding (47) that a subpopulation of viable, antimicrobial-tolerant, but slowly
471 dividing, and persistent spirochetes of *B. burgdorferi* resurged in mice 12 months after
472 treatment and re-disseminated into multiple tissues. Importantly, copy numbers of
473 spirochetal DNA in tissue samples of antimicrobial treated disease-resistant B6 mice was
474 nearly equivalent to those of disease-susceptible C3H mice. However, significantly
475 higher DNA copy numbers of spirochetal DNA were determined in saline-treated groups
476 of C3H mice infected with either N40 or B31 in comparison to antimicrobial-treated mice
477 (C3H and B6). This finding is in concordance with the results published by Armstrong et
478 al. (80) that C3H mice develop more severe disease than B6 mice, with greater
479 spirochetes number and later clearance.

480 Despite the fact that tissues of treated mice remained BbDNA PCR-positive at 12
481 months with significant copy numbers of targeted genes, culture was consistently
482 negative. Actually, in a single cultured urinary bladder we observed two non-motile
483 spirochetes for up to three weeks of incubation. On the other hand, all saline-treated mice
484 were culture-positive. Our studies (46, 47, 50) and those of others (45, 49, 81) in mice,
485 dogs (29, 52) and non-human primates (53, 54) have all reached similar conclusions:
486 spirochetes are persisting, but are paradoxically non-cultivable. It was suggested that the
487 persisting remnants of *B. burgdorferi* in the tissues of infected mice after antimicrobial
488 treatment is DNA or DNA-containing structures rather than live bacteria (76, 82, 83). In
489 their *in vitro* study Iyer et al. (84) could not successfully subculture spirochetes after
490 exposure to ceftriaxone. However, BbDNA was detected by PCR for up to 56 days in
491 aliquots from both ceftriaxone-treated and untreated cultures. Pavia and Wormser (85)

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492 demonstrated that *B. burgdorferi* cannot be cultured from experimentally infected mice
493 after ceftriaxone treatment with only 5 daily doses. The treatment regimen used in the
494 study was judged based on the absence of a positive culture.

495 IDSA Guidelines state that "unless proven otherwise, culture should be regarded
496 as the gold-standard to address viability of *B. burgdorferi*"(86). Culture may indeed be a
497 gold standard when it is positive, but it is often not. It is apparent that not all isolates or
498 strains can be easily cultured, and this is especially apparent during long-term infection.
499 It is becoming increasingly clear that in *B. burgdorferi* infected and antimicrobial treated
500 animals, spirochetes were non-cultivable but viable, as have been demonstrated by
501 xenodiagnoses (46, 47, 50, 54), immunohistology (46, 47, 75), and transcriptional activity
502 of mRNA (47, 50, 54). Here we demonstrated that morphologically intact *B. burgdorferi*
503 that survived antimicrobial treatment in disease-susceptible C3H mice as well in disease-
504 resistant B6 mice could be acquired by larval ticks. Ticks remained BbDNA-positive
505 through molting into nymphs. In addition, transcription of the chromosomally-encoded *B.*
506 *burgdorferi 16S rRNA* and *flaB* genes were detected in treated mice, indicating that
507 spirochetes are metabolically active and alive at 12 months after treatment. The data
508 obtained in this study indicate that in a significant number of culture-negative tissue
509 samples following antimicrobial treatment, complementary methods in diagnostic
510 microbiology should be considered. The clinical significance and the prognostic value of
511 these findings have to be more deeply investigated. It was shown that dead bacterial
512 DNA can be detected for up to 4 to 5 months after antimicrobial treatment (87), as
513 extracellular DNA is very prone to degradation (88, 89). Bacterial mRNAs, on the other
514 hand, have very short half-lives as degraded by exonucleases very fast. The half-life

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515 ranges from a few minutes to several hours, depending on the bacterial strain (90-92).
516 These finding support the fact that non-cultivability of antimicrobial-tolerant and
517 persistent spirochetes does not negate their viability.
518 Our previous studies in mice (46, 47, 50) and those of others (49, 81) are all based
519 on *B. burgdorferi* N40 strain. Although N40 probably represents *B. burgdorferi*, the
520 generality of long-term spirochete persistence following antimicrobial treatment should
521 be evaluated with other *B. burgdorferi* strains. Therefore, we used *B. burgdorferi* strain
522 B31 and confirmed persistence and resurgence at 12 months after antimicrobial treatment
523 in both, C3H and B6, mice. Our results indicate that disseminated spirochetes of two
524 different *B. burgdorferi* strains can persist in mice at 12 and 18 months following
525 antimicrobial treatment. Non-cultivable spirochetes persist in mice (93), dogs (29), and
526 non-human primates (54) inoculated with alternate strains of *B. burgdorferi*, so we are
527 confirming that persistence and resurgence are not unique to N40.
528 An additional long-term interval, holding mice up to 18 months, facilitated
529 several observations regarding persistent antimicrobial-tolerant spirochetes. Within the
530 observation period, there was molecular evidence of BbDNA in 79% of all assessed mice
531 following antimicrobial treatment. Spirochetal DNA was exclusively detected in
532 tibiotarsal joint, but not in heart base and ventricular muscle. Copy numbers of BbDNA
533 are not significantly different between treated C3H and B6 mice infected with either N40
534 or B31. Interestingly, BbDNA copy numbers in tibiotarsal joints of all antimicrobial
535 treated mice at 18 months are equivalent to those at 12 months, suggesting that
536 antimicrobial-tolerant spirochetes remained persistent exclusively in connective-tissue.
537 One of the proposed mechanisms of *B. burgdorferi* immune evasion and persistence is

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538 sequestration in connective-tissue, that make them less accessible to cells and molecules
539 of the host's immune system (55). It was suggested that the joint or a tissue adjacent to
540 the joint is the niche of persisting *B. burgdorferi* in antimicrobial-treated mice (49). There
541 is scientific evidence that tissues with greater decorin expression levels such as joint
542 harbored most spirochete loads during chronic infection (94). The findings of our study
543 reinforce the notion of persistence of viable spirochetes following antimicrobial treatment
544 and further implicate the connective-tissue as a privileged site that may partake to its
545 failure. Although spirochetes resurged 12 months after antimicrobial treatment, overt
546 disease was not present. Also, despite spirochetes presence, postmortem gross signs of
547 disease were not observed in assessed tissues (heart, joint) of treated mice at 18 months.
548 Studies in animal models have shown that resolution of arthritis and carditis is mediated
549 by the acquired humoral immune response of the host. Under these conditions,
550 anatomically defined inflammation resolves, but infection persists (95-97). Indeed, even
551 during the pre-immune phase of infection, spirochetes populate many tissues with no
552 evidence of inflammation (thus inflammation does not necessarily correlate with
553 spirochete presence).

554 Spirochetal RNA was detected in the joint tissue of C3H and B6 mice infected
555 with either N40 or B6 following treatment at 18 months, suggesting their viability.
556 Interestingly, xenodiagnosis was positive in only three of nineteen treated animals. We
557 are speculating that the inability to retrieve spirochetes from all treated mice could be
558 ascribed to a clearance of spirochetes from the heart tissue. Migration of spirochetes from
559 joints to tick attachment sites, that is usually a head and/or a dorsal part of a mouse body,
560 is more distant than from a heart. It was also suggested that the efficiency of spirochetal

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561 acquisition from a host to a tick depend on the intensity and duration of infection, and it
562 is significantly less during chronic infection (98). However, we recovered a few slow-
563 growing spirochetes by culture from joint tissues of two mice, utilizing several specially
564 prepared media that were cordially provided by Monica E. Embers. We were unable to
565 propagate the recovered spirochetes as they lysed after 4 weeks. Obviously, the used
566 media were unable to support the prolonged growth of slow growing and impaired
567 spirochetes.

568 Here we have shown that following antimicrobial treatment, both strains of *B.*
569 *burgdorferi*, N40 and B31, generates an increasingly heterogeneous population of
570 replicatively-attenuated spirochetes that have lost one or more small plasmids. These
571 "attenuated" spirochetes remain viable, but because of their plasmid loss, they divide
572 slowly, thereby being tolerant to the effects of antimicrobials, as well as being non-
573 cultivable. The phenomenon of persistent, non-cultivable *B. burgdorferi* spirochetes after
574 antimicrobial treatment has been explained by antimicrobial tolerance. Unlike
575 antimicrobial-resistance, antimicrobial-tolerance to all classes of antimicrobials fail to
576 completely eliminate non-dividing or slowly-dividing subpopulations of a broad array of
577 bacteria and fungi (62, 99). It has been shown that *in vitro* passage of *B. burgdorferi* is
578 predisposed to plasmid loss (63-65). This phenomenon probably occurs throughout the
579 infection and elevates over time.

580 Clinical relevance of microbial persistence has been demonstrated for
581 *Burkholderia pseudomallei* (100), *Escherichia coli* (101), *Pseudomonas aeruginosa*
582 (102), *Candida albicans* (103), *Streptococcus pneumonia* (104), *Staphylococcus aureus*
583 (101, 105), *Mycobacterium tuberculosis* (106, 107), *Legionella* spp. (108, 109), and

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584 *Salmonella enterica* (110). The persistent cells have transient tolerance to antimicrobial
585 agents and their ability to revert back to a sensitive state, in which cells rapidly divide
586 make them important in chronic infections. There are two major consequences of the
587 presence of persister cells: 1) the continuous presence of viable cells during consecutive
588 rounds of antimicrobial treatment, which contribute to the emergence of antimicrobial
589 resistance and 2) experimental evidence indicates that prolonged exposure to
590 antimicrobial agents leads to the selection of high persistent mutants (111, 112). The
591 biological relevance of attenuated *B. burgdorferi* spirochetes is probably inconsequential,
592 while their clinical relevance was subject of this study. Our study demonstrated that non-
593 cultivable spirochetes can persist in a host following antimicrobial treatment for a long
594 time, but did not demonstrate their clinical relevance in a mouse model of chronic
595 infection. The clinical relevance of attenuated spirochetes following antimicrobial
596 treatment and their eventual fate in other animal models require further studies.
597

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911

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916 **AUTHORS CONTRIBUTION**

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917 Conceived and designed the experiments: EH. Performed the experiments: EH DI EE.
918 Analyzed the data: EH DI EE. Contributed reagents/materials/analysis tools: DI EE.
919 Wrote the paper: EH DI.
920

921 **FIGURE LEGEND**

922 **Figure 1.** Copy numbers of *B. burgdorferi* DNA of *flaB/ospA* genes in heart base (HB),
923 ventricular muscle (VM), and tibiotarsus (TT) of C3H and B6 mice infected with either
924 N40 or B31 strains at 12 months after ceftriaxone and saline solution treatment.
925 *Significantly higher copy number ($p<0.05$)

926 **Figure 2.** Copy numbers of *B. burgdorferi* DNA of *flaB/ospA* genes in heart base (HB),
927 ventricular muscle (VM), and tibiotarsus (TT) of C3H and B6 mice infected with either
928 N40 or B31 strains at 18 months after ceftriaxone and saline solution treatment.
929 *Significantly higher copy number ($p<0.05$)

930 **Figure 3.** Darkfield images of cultured *B. burgdorferi* from mouse tissues necropsied 18
931 months after completion of treatment. Magnification 400x. **A.** Note numerous
932 spirochetes from ear culture of saline solution treated C3H mouse infected with strain
933 N40. **B.** Ear culture of ceftriaxone treated B6 mouse infected with strain B31. **C.** Front
934 joint of ceftriaxone treated B6 mouse infected with strain N40

935 **Figure 4**. Histology of the heart base of ceftriaxone and saline solution treated mice
936 necropsied at 12 and 18 months after treatment

937 **Figure 5.** Chronic periarteritis observed in a C3H mouse infected with *B. burgdorferi*
938 B31 then treated saline solution and necropsied 18 months after treatment (**A** and **B**).

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939 Tenosynovitis observed in a B6 mouse infected with B. burgdorferi N40 then treated with
940 saline solution and necropsied at 18 months after treatment (**C**).
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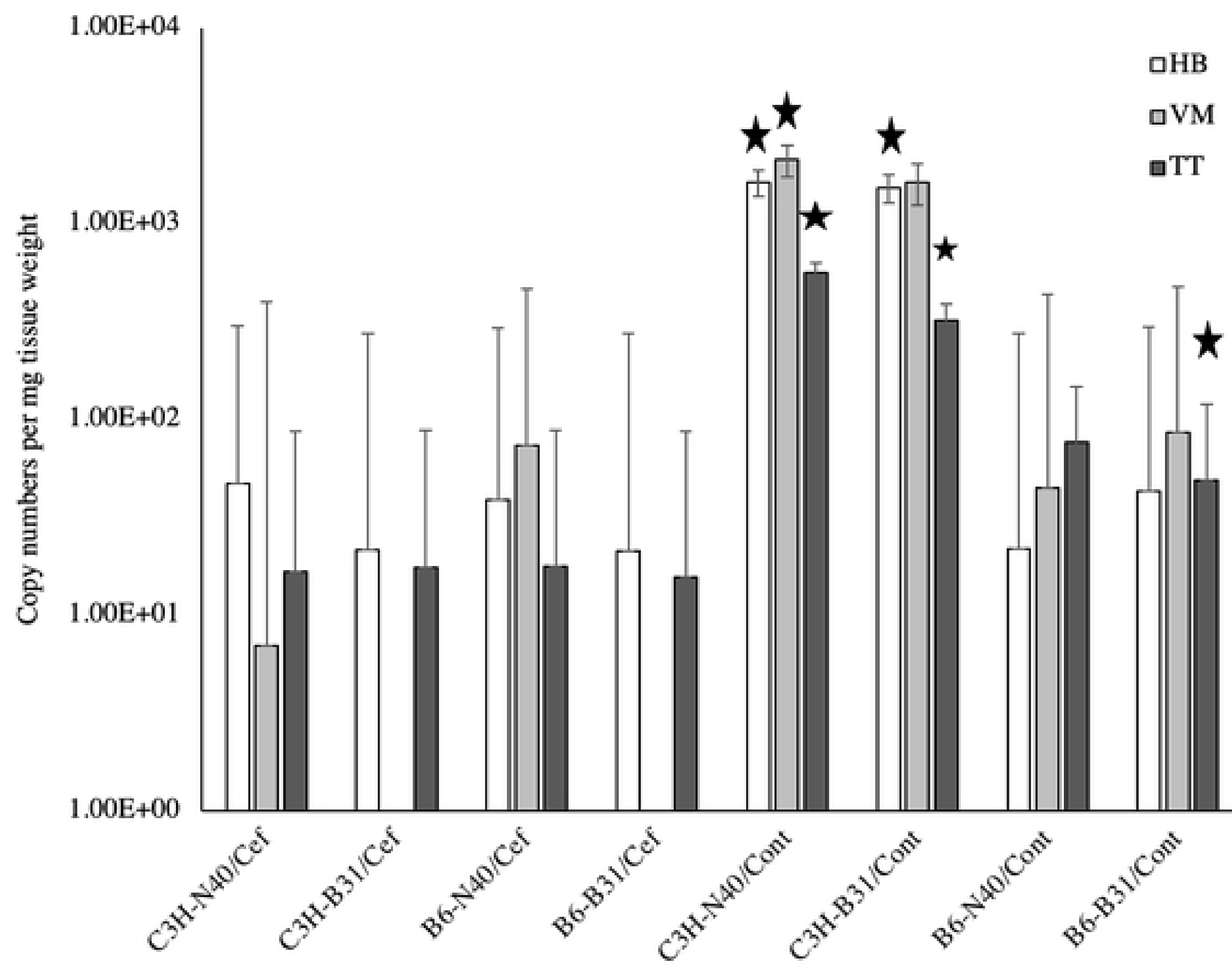
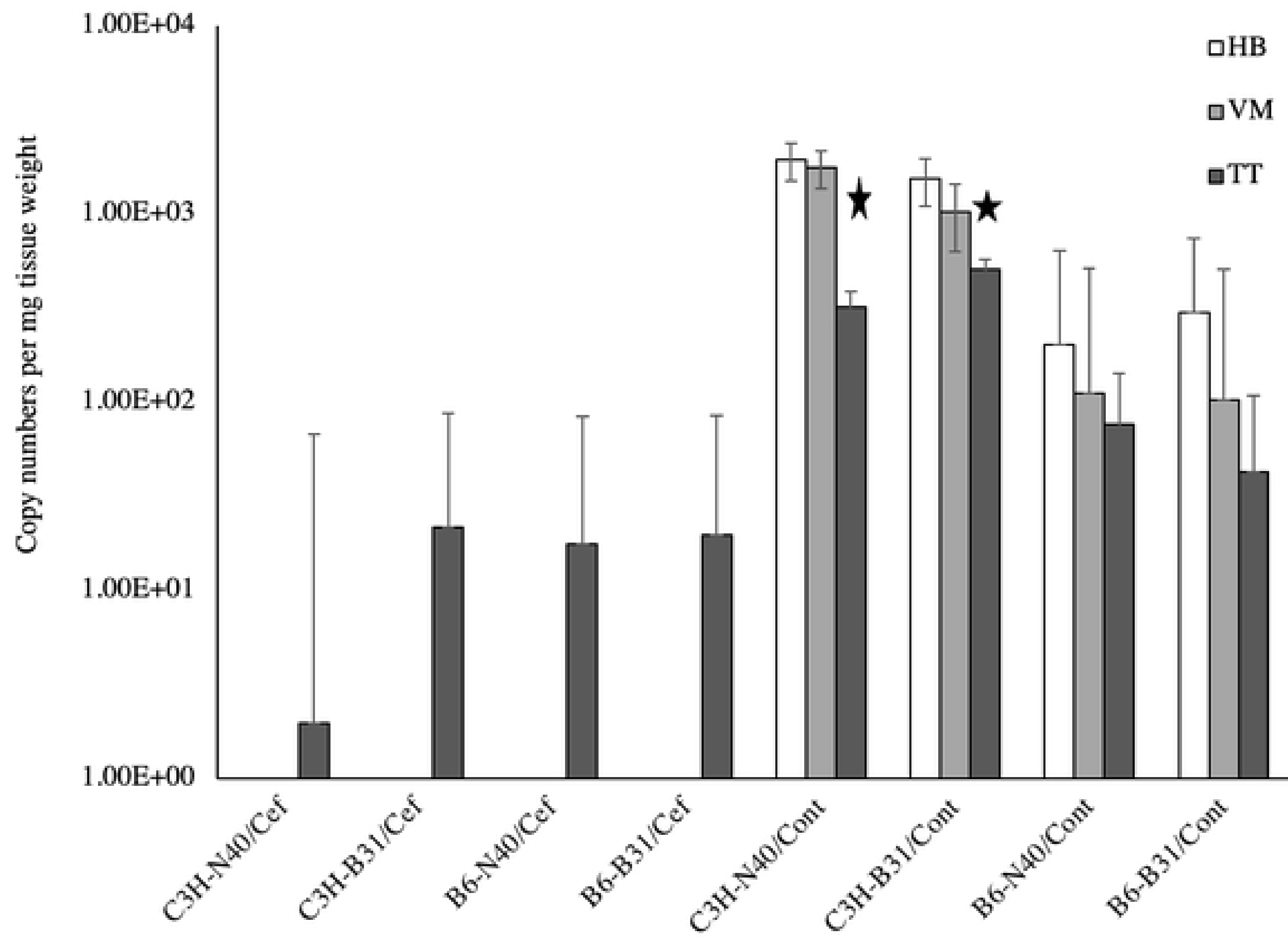


Figure1



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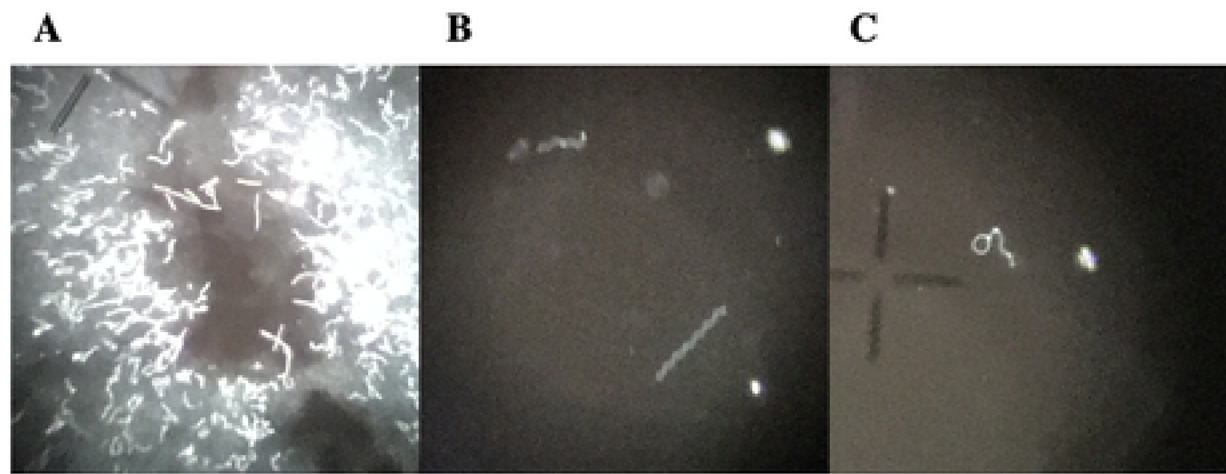

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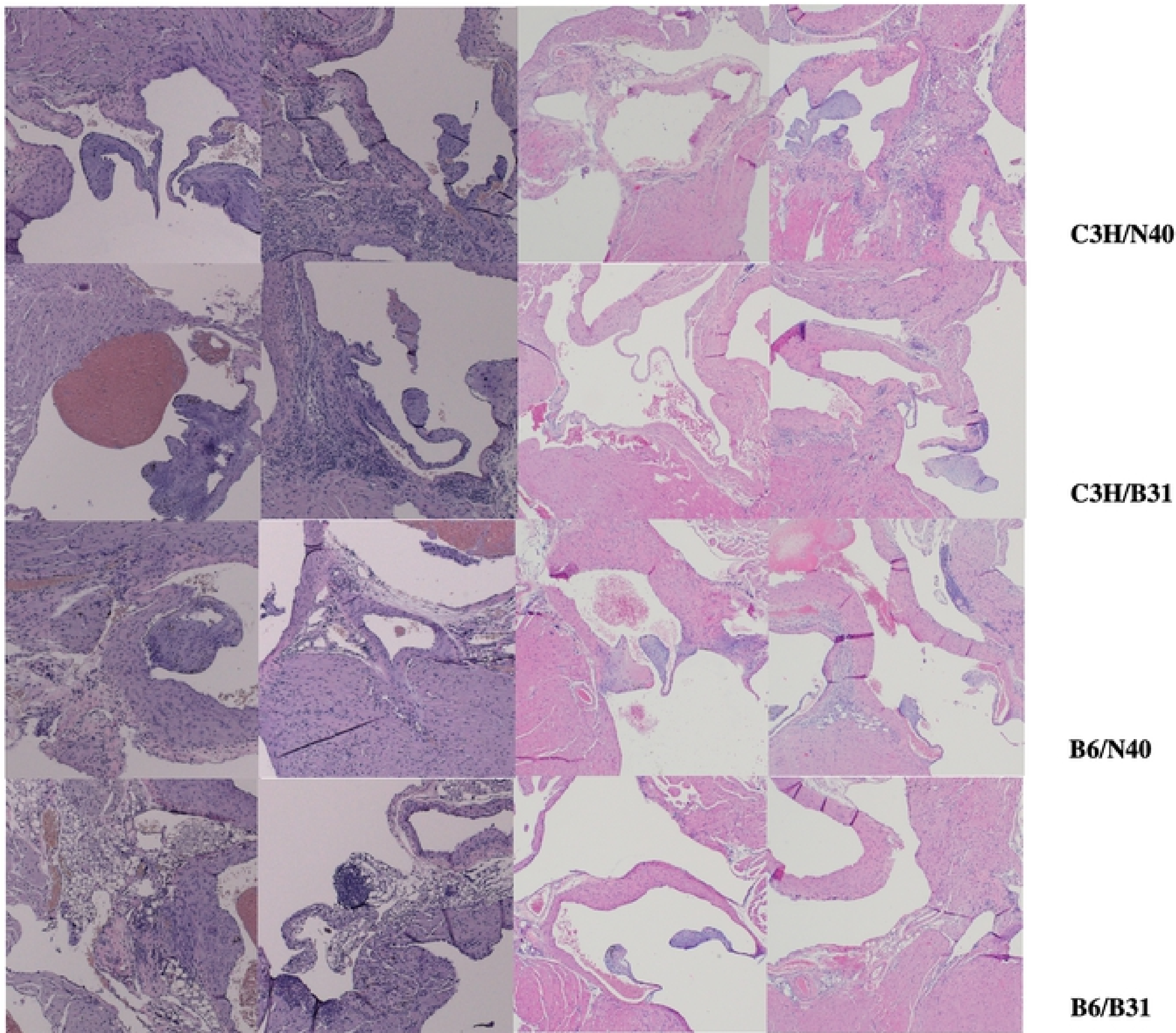

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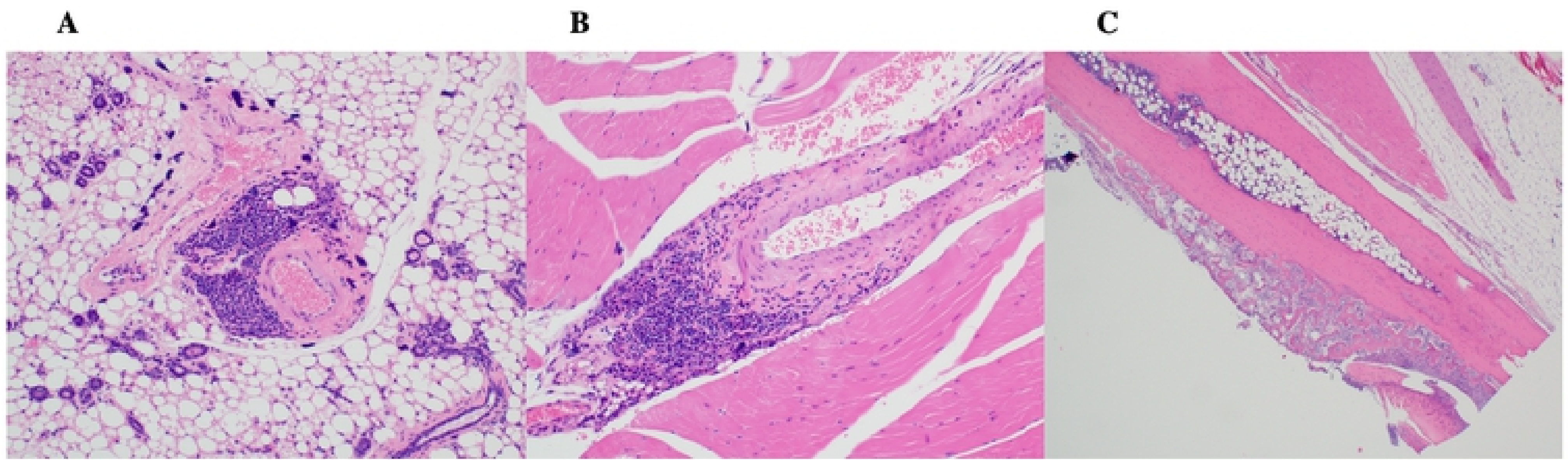

Figure5