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1 # *Borrelia burgdorferi*, the causative agent of Lyme disease, forms
2 drug-tolerant persister cells.

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4
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8 **Running title: Persisters of *Borrelia burgdorferi***

9

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21 **ABSTRACT**

22 *Borrelia burgdorferi* is the causative agent of Lyme disease, which affects an
23 estimated 300,000 people annually in the US. When treated early, the disease usually
24 resolves, but left untreated, can result in symptoms such as arthritis and
25 encephalopathy. Treatment of the late stage disease may require multiple courses of
26 antibiotic therapy. Given that antibiotic resistance has not been observed for *B.*
27 *burgdorferi*, the reason for the recalcitrance of late stage disease to antibiotics is
28 unclear. In other chronic infections, the presence of drug-tolerant persisters has been
29 linked to recalcitrance of the disease. In this study, we examined the ability of *B.*
30 *burgdorferi* to form persisters. Killing of growing cultures of *B. burgdorferi* with antibiotics
31 used to treat the disease was distinctly biphasic, with a small subpopulation of surviving
32 cells. Upon regrowth, these cells formed a new subpopulation of antibiotic-tolerant cells,
33 indicating that these are persisters rather than resistant mutants. The level of persisters
34 increased sharply as the culture transitioned from exponential to stationary phase.
35 Combinations of antibiotics did not improve killing. Daptomycin, a membrane-active
36 bactericidal antibiotic, killed stationary phase cells, but not persisters. Mitomycin C, an
37 anti-cancer agent that forms adducts with DNA, killed persisters and eradicated both
38 growing and stationary cultures of *B. burgdorferi.* Finally, we examined the ability of
39 pulse-dosing an antibiotic to eliminate persisters. After addition of ceftriaxone, the
40 antibiotic was washed away, surviving persisters were allowed to resuscitate, and
41 antibiotic was added again. Four pulse-doses of ceftriaxone killed persisters, eradicating
42 all live bacteria in the culture.

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44 **Introduction**

45 All pathogens studied to date form persisters, dormant variants of regular cells
46 which are tolerant to killing by antibiotics. The ability to produce persisters explains the
47 puzzling recalcitrance of chronic infections to antibiotics that are effective against the
48 same pathogen *in vitro*. Indeed, many chronic infections are caused by drug-susceptible
49 pathogens [1, 2]. The immune system can effectively remove sessile cells from the
50 blood and many of the tissues, and this accounts for the efficacy of antibiotics, including
51 bacteriostatic compounds, in treating uncomplicated infections. When the immune
52 response is limited, the result is often a chronic infection [2]. Biofilms are a well-studied
53 case of immune evasion and serve as a paradigm for understanding chronic infections.
54 In biofilms, cells are protected from the large components of the immune system by a
55 surface exopolymer [3-5]. Antibiotics kill the regular cells, but dormant persisters
56 survive, and when the concentration of antibiotic drops, they resuscitate and repopulate
57 the biofilm [2]. This scenario is supported by our finding of high-persister (hip)
58 *Pseudomonas aeruginosa* selected in the course of prolonged antibiotic treatment [6].
59 Isolated from patients with late-stage cystic fibrosis, hip mutants can produce 1000
60 times more persisters than the parent strain; this indicates that selection for increased
61 tolerance (rather than resistance) provided the pathogen with a survival advantage.
62 Similarly, hip mutants are selected during treatment of oral thrush caused by *Candida*
63 *albicans* [7]. In *Salmonella typhimurium*, entrance of pathogens into human cells where
64 they are protected from the immune system is accompanied by a sharp increase in
65 persister formation and tolerance to killing by antibiotics [8]. In tuberculosis, dormant
66 cells are likely responsible for the need of a lengthy treatment of the acute stage and for

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67 the latent form of the disease. *Mycobacterium tuberculosis* hides from the immune
68 system in macrophages or in granulomas [9].
69 *B. burgdorferi* causes Lyme disease with 300,000 estimated cases annually in
70 the United States alone [10]. When treated early with antibiotics, the disease usually
71 resolves [11, 12]. If treatment is delayed, the pathogen spreads throughout the body
72 and can cause meningitis, arthritis, and carditis. Meningitis and carditis are mostly self-
73 limited, but Lyme arthritis can persist for years [13, 14]. A substantial proportion of
74 patients receiving their first course of antibiotics for Lyme arthritis do not respond fully to
75 a 28 day course of treatment. In such cases, retreatment with additional courses of
76 antibiotics is recommended [13, 15, 16]. *B. burgdorferi* avoids immune attack by
77 antigenic variation of surface components and by decreasing exposure of antigens [17-
78 19]. In this regard, Lyme disease resembles other chronic infections where the
79 pathogen is protected from the immune system, and persister cells may enable it to
80 survive treatment with antibiotics. In *Escherichia coli*, the model organism for the study
81 of persisters, dormant cells are formed primarily through expression of toxin/antitoxin
82 (TA) modules. Toxins confer dormancy by either inhibiting protein synthesis or by
83 decreasing the energy level of cells [20-22]. TA modules are widely spread among
84 bacteria, and are copiously present in some pathogens. *E. coli* has more than 30 TA
85 modules and *M. tuberculosis* over 75 [23, 24]. Interestingly, there are apparently no TA
86 modules in the genome of *B. burgdorferi* [25]. Virtually nothing is known about
87 persisters in this species. In this study, we report formation of drug-tolerant persisters in
88 *B. burgdorferi* and describe possible approaches to their elimination.
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Materials and methods

Bacterial strains and growth conditions: *Borrelia burgdorferi* B31 5A19 that had been passaged five times *in vitro* was kindly provided by Dr. Monica Embers [26]. *B. burgdorferi* was grown in BSK-II liquid media in a microaerophilic chamber (34°C, 3% O_2, 5% CO_2). Cultures were started by thawing -80°C glycerol stocks of *B. burgdorferi* (titer approximately 10^7 cfu/mL) and diluting 1:20 into fresh BSK-II media.

BSK-II liquid medium was prepared according to protocol received from Monica Embers' lab by adding the following ingredients to 400 ml of deionized water and mixing thoroughly: 20 g bovine serum albumin (Sigma), 2 g neopeptone (Fluka), 0.8 g yeastolate (BD), 4 g HEPES sodium salt (Sigma), 2.4 g 10X CMRL (US Biologicals), 0.28 g sodium citrate (Fisher), 0.32 g sodium pyruvate (Sigma), 2 g glucose (Fisher), 0.16 g N-acetyl-glucoasmine (Sigma), 0.88 g sodium pyruvate (Sigma). The pH of the medium was adjusted to 7.6 and 24 ml of rabbit serum (Sigma) was added to the media. The medium was then filtered through a 0.22 µm filter.

Semi-solid plating was used to obtain cfu counts [27]. First, BSK 1.5X medium for semi-solid plating was prepared as in Samuels 1995 [27]. The following ingredients were added to 1 L of deionized water (LabChem, Inc) and mixed thoroughly: 8.33 g neopeptone (Fluka), 4.22 g yeastolate (BD), 9.99 g HEPES acid (Fisher), 8.33 g glucose (Fisher), 1.22 g sodium citrate (Fisher), 1.33 g sodium pyruvate (Sigma), 0.670 g N-acetyl-glucosamine (Sigma), 7.66 g sodium bicarbonate (Sigma). The pH of the media was adjusted to 7.5 and then 83.25 g of bovine serum albumin (Sigma) was added. The medium was stirred for one hour then filtered using a 0.22 µm filter. 1.5X BSK-II was stored at 4°C and used within 7 days of preparation. On the day of plating,

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113 125 ml of 1.5X BSK was mixed with 6 ml rabbit serum and 19 ml 1X CMRL (97.89
114 mg/mL) and equilibrated to 55°C. 1.7% agarose (Lonza) was melted and equilibrated to
115 55°C. When all ingredients had equilibrated to 55°C, 1.7% agarose was added to 1.5X
116 BSK at a ratio of 2:1 (BSK:agarose) to create BSK agarose. 8 ml of BSK agarose was
117 dispensed into 60mm Petri dishes as bottom agar and allowed to solidify. For top agar,
118 100 µl of the given dilution of *B. burgdorferi* was mixed with 5 ml of 55°C BSK agarose
119 and poured onto the bottom agar plates and allowed to solidify. The plates were
120 incubated in zip lock bags in microaerophilic chamber (34°C, 3% O_2, 5% CO_2) for at
121 least 21 days to obtain visible colonies.
122
123 **Antimicrobial agents:** Amoxicillin (Sigma), doxycycline hydrochloride (MP
124 Biomedicals), ceftriaxone disodium salt hemi (heptahydrate) (Sigma), and vancomycin
125 hydrochloride (Sigma) were dissolved in water. Mitomycin C (Sigma), gemifloxacin
126 mesylate (Tecoland Corporation), and spectinomycin dihydrochloride pentahydrate
127 (RPI) were dissolved in DMSO. Daptomycin cyclic lipopeptide (Sigma) was dissolved in
128 a 5 µg/ml solution of calcium chloride. Stock solutions of antibiotics were aliquotted and
129 stored at -20°C until use. Antibiotics did not undergo freeze-thaw cycles.
130
131 **Killing experiments:** *B. burgdorferi* was cultured in liquid BSK-II media for 3 days to
132 late-exponential growth phase or for 5 days to stationary phase. Antibiotics were then
133 added to the culture. The cultures were incubated in the microaerophilic chamber (34°C,
134 3% O_2, 5% CO_2). At a given time point, an aliquot of the culture was washed twice by
135 centrifuging the culture at 13.2k rpm for 5 minutes and resuspending the pellet in an

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136 equal volume of fresh BSK-II medium. The cultures were then serially diluted in fresh
137 BSK-II media. 100 µl of the appropriate dilution was mixed with 5 ml of BSK agarose
138 and poured as top agar. Plates were incubated in the microaerophilic chamber until
139 visible colonies appeared (at least 21 days).
140
141 **Growth-persister experiments:** Cultures of *B. burgdorferi* were started as described
142 above. At each time point, an aliquot of a growing culture was removed, diluted, and
143 plated for cfu counts to generate the growth curve. A second aliquot (1 mL or 3 mL) was
144 removed at the same time and challenged for five days with the indicated antibiotic.
145 After five days, an aliquot of challenged culture was removed, washed twice, diluted,
146 and plated for cfu counts to generate the persister curve.
147
148 **Minimum Inhibitory Concentration (MIC) testing:** A slightly modified version of the
149 broth microdilution [28] was used. *B. burgdorferi* was grown in liquid culture for three
150 days to reach exponential phase and then back diluted 1:10 into fresh BSK-II media to
151 make the inoculum solution. All antibiotics were prepared as stock solutions in solvent
152 (water or DMSO) based on the concentration to be tested and diluted in two fold
153 increments in a 96 well stock plate. 2 µl per well of the antibiotic stock solution was
154 transferred to the 96 well MIC plate to which 198 µl of the *B. burgdorferi* inoculum
155 solution was added (final inoculum of approximately 10^6 cells/well). Media, growth, and
156 vehicle controls were included on each plate. The MIC plate was covered with
157 Breatheasy Film (Diversified Biotech) and incubated in the microaerophilic chamber
158 (34°C, 3% O_2, 5% CO_2) for 72 hours. The lowest concentration of antibiotics that

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159 showed inhibition of growth was interpreted as the MIC. All MIC assays were repeated
160 at least twice in triplicate.
161
162 **Results**
163 **Characterization of *B. burgdorferi* Persisters**
164 The presence of persisters is indicated by a biphasic killing pattern in a time-
165 dependent killing experiment. The bulk of the population is rapidly killed, followed by a
166 slower rate of death in a subpopulation of tolerant cells [29, 30]. In order to determine
167 whether *B. burgdorferi* forms persisters, time-dependent killing experiments were
168 performed with antibiotics commonly prescribed to patients with Lyme disease.
169 Doxycycline is a bacteriostatic protein synthesis inhibitor; amoxicillin and ceftriaxone
170 inhibit bacterial cell wall synthesis and are bactericidal for many bacteria. Minimum
171 inhibitory concentrations (MICs) of doxycycline, amoxicillin, and ceftriaxone were
172 determined (Table 1). Levels of antibiotics close to what is achievable with standard
173 clinically prescribed treatment dosing were chosen to evaluate persister formation in *B.*
174 *burgdorferi*, and we used colony forming unit (cfu) count to determine viability.
175 Amoxicillin (6 µg/ml, 100X MIC) and ceftriaxone (3 µg/ml, 300X MIC) at clinically
176 achievable levels killed the majority of cells in the first day, after which a slow phase of
177 death followed for the next 6 days (Figure 1a). This characteristic biphasic pattern of
178 killing is consistent with the presence of drug-tolerant persister cells.
179 Previous studies have shown that the persister fraction in other bacteria remains
180 relatively unchanged even as the antibiotic level increases. We sought to determine if *B.*
181 *burgdorferi* persisters behaved similarly in a dose-dependent killing experiment. As the

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182 concentration of amoxicillin and ceftriaxone increased, the fraction of surviving cells
183 remained largely unchanged (Figures 1b-c). Doxycycline is a bacteriostatic antibiotic,
184 but at higher concentrations appeared to effectively kill *B. burgdorferi* (Figure 1d).
185 Again, the fraction of surviving cells did not change significantly with increasing levels of
186 the compound. Thus, *B. burgdorferi* forms persisters capable of surviving very high
187 concentrations of antibiotics, which exceed what is clinically achievable.

188 Density-dependent formation is a common feature of persisters reported for all
189 pathogens examined so far, including *E. coli, Staphylococcus aureus, P. aeruginosa,*
190 and *M. tuberculosis* [6, 31-33]. In order to test this property in *B. burgdorferi*, samples
191 from a growing culture were removed over time, exposed to a lethal dose of antibiotic
192 for 5 days, and then plated for cfu. There was a characteristic dip in persister levels in
193 the early log phase, which is probably due to the resuscitation of dormant cells carried
194 over from the inoculum (Figure 2). At mid-log phase, there is a sharp increase in
195 persister levels, which continues as the density of the culture rises. In *E. coli*, once the
196 culture reaches stationary state, complete tolerance is achieved for β-lactams that only
197 kill growing cells [34]. In *B. burgdorferi*, we observe a very different picture – both
198 amoxicillin and ceftriaxone kill stationary cells fairly well, yet the fraction of persisters
199 continues to increase. One possibility is that this “stationary” culture actually represents
200 a steady state where some cells die and others grow.

201 Next, we tested whether the *B. burgdorferi* cells surviving antibiotic treatment are
202 drug-tolerant persisters or resistant mutants. For this, colonies produced by the
203 surviving cells were regrown and tested for MIC. The amoxicillin and ceftriaxone MIC
204 remained unchanged, showing that surviving cells had not acquired or developed a

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205 genetic mechanism for antibiotic resistance. The population grown from the surviving
206 cells produced the same level of persisters as the original population (Figure 3). These
207 experiments show that *B. burgdorferi* forms typical persister cells.
208

209 **Eradication of *B. burgdorferi* Persisters**

210 Drug combinations. Some antibiotics act synergistically, such as sulfonamide and
211 trimethoprim, polymixin and gentamicin, aminoglycosides and β-lactams [35], and we
212 wanted to see if a combination of compounds known to be active against *B. burgdorferi*
213 will increase efficiency of killing both regular and persister cells.

214 All possible two-drug combinations of amoxicillin, ceftriaxone, and doxycycline
215 were tested with a late-exponential phase culture in a time-dependent killing experiment
216 and found to be no more effective than the drugs used individually in killing of *B.*
217 *burgdorferi* (Figure 4a). Doxycycline actually inhibited the action of amoxicillin. We have
218 shown previously that fluoroquinolones and aminoglycosides can kill non-growing cells
219 [36, 37], and we next tested these compounds against *B. burgdorferi*. The pathogen is
220 generally poorly susceptible to compounds from these classes. However, the *B.*
221 *burgdorferi* MICs for gemifloxacin (fluoroquinolone) and spectinomycin (aminoglycoside)
222 are within achievable human dosing levels so we chose to test them [38-41] (Table 1).
223 Both gemifloxacin and spectinomycin were ineffective in killing *B. burgdorferi* at tested
224 concentrations (Figure 4b). Combining these compounds also did not improve killing
225 (Figure 4b).
226

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227 Experimental compounds. Having shown that combinations of clinically prescribed
228 antibiotics for Lyme disease are unable to effectively kill persister bacteria, we sought to
229 examine some novel potential antimicrobial agents. We recently showed that
230 acyldepsipeptide (ADEP4), an activator of the ClpP protease, effectively kills persisters
231 in *S. aureus* [42]. In the presence of ADEP4, the protease cleaves mature proteins,
232 forcing the cell to self-digest. However, ADEP4 did not have significant activity against
233 *B. burgdorferi* (not shown), which may be due to poor penetration.

234 We then considered whether knowledge of *B. burgdorferi* biology might be
235 exploited to predict vulnerability to existing approved compounds. *B. burgdorferi* lives
236 under microaerophilic conditions, where the capacity for energy generation is limited by
237 comparison to aerobic organisms. Daptomycin is the only approved membrane-acting
238 antibiotic that disrupts the proton motive force. The *B. burgdorferi* MIC to daptomycin
239 was fairly high, 12-25 μg/ml (Table 1), in accordance with published data [43].
240 Daptomycin was highly bactericidal against *B. burgdorferi*, but a remaining
241 subpopulation of persisters survived (Figure 5), suggesting that *B. burgdorferi* persisters
242 can tolerate a drop in the energy level. Next, we tested vancomycin. This large
243 glycopeptide antibiotic binds to lipid II, precursor of peptidoglycan, on the outside of the
244 cytoplasmic membrane. Vancomycin is highly effective against Gram-positive bacteria,
245 but does not penetrate across the outer membrane of Gram-negative species.
246 Surprisingly, the vancomycin MIC with *B. burgdorferi* is low, 0.25 μg/ml, similarly to
247 Gram-positive species. *B. burgdorferi* has an outer membrane; the basis for this
248 anomaly is unclear. Vancomycin effectively killed growing cells of *B. burgdorferi*, but not
249 persisters, and was comparable to ceftriaxone (not shown). We also tested teixobactin,

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250 a compound we recently discovered, which also binds lipid II [44]. At 1.2 kDa,
251 teixobactin is considerably smaller than vancomycin (1.8 kDa), but it did not exhibit
252 good activity in killing *B. burgdorferi* (not shown).
253
254 Prodrugs. Growth under microaerophilic conditions suggests vulnerability to compounds
255 whose action depends specifically on a low oxygen environment. Nitroaromatic
256 compounds such as metronidazole are prodrugs that are converted into reactive drugs
257 by bacterial nitroreductases. These enzymes are expressed under anaerobic or
258 microaerophilic conditions, and target pathogens living in these environments (i.e.
259 *Helicobacter pylori, Clostridium difficile, E. coli*). We found that some nitroaromatic
260 compounds like nitrofurantoin are effective in killing *E. coli* persisters [45]. However, we
261 did not detect homologs of nitroreductases in the genome of *B. burgdorferi*. The MIC for
262 nitroaromatic compounds (nitrofurantoin, nitrofurazone, and metronidazole) was too
263 high to make them useful agents for killing *B. burgdorferi* persisters (data not shown).
264 Another compound that depends on a reductive environment for action is the
265 prodrug mitomycin C. Upon entering the cell, mitomycin C is reduced into an active drug
266 which then forms covalent adducts with DNA [46]. Originally discovered in a screen for
267 antibiotics, mitomycin C is now used as an anticancer agent. Cancers often create a
268 microaerophilic environment, which, together with rapid cell division, accounts for the
269 relatively selective action of mitomycin C against them. Functional RecBC and RecFOR
270 pathways are required to repair DNA damaged by mitomycin C in *E. coli* [46].
271 Interestingly, according to genomic data, *B. burgdorferi* lacks the genes of the RecFOR
272 pathway [47], further suggesting vulnerability to this compound.

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273 Mitomycin C eradicated a late exponential culture of *B. burgdorferi* within 24
274 hours, with no detectable persisters remaining (Figure 6a). This was observed with a
275 low, clinically achievable dose of the compound - 1.6 μg/ml, or 8X MIC. In a dose-
276 dependent experiment, eradication of a late exponential culture was achieved within 5
277 days with a 0.8 μg/ml (4X MIC) dose of the compounds (Figure 6b). Finally, mitomycin
278 C was tested against a stationary culture of *B. burgdorferi*. Surprisingly, eradication was
279 achieved with a low dose of 4X MIC within 24 hours (Figure 6c). It appears that a
280 stationary population is more susceptible to this compound than an exponentially
281 growing one.
282
283 Pulse-dosing. Apart from identifying compounds capable of killing persisters, it may also
284 be possible to eliminate them with conventional bactericidal antibiotics using pulse-
285 dosing. Based on our results, the level of persisters is lowest during early exponential
286 growth (Figure 2). We reasoned that allowing growth to resume and then re-treating
287 them as they enter exponential phase could kill persisters surviving an antibiotic
288 challenge. Eradication of the culture could then be achieved after several rounds of
289 killing and regrowth. To test this, a culture of *B. burgdorferi* was exposed to amoxicillin
290 or ceftriaxone. The surviving persisters were allowed to resuscitate for a short period of
291 time in fresh media, and then exposed to antibiotic again for a second round of killing.
292 Persisters were substantially diminished after four rounds of killing with amoxicillin, and
293 were eradicated below the limit of detection after four rounds of killing with ceftriaxone
294 (Figure 7). Additionally, we found that a ceftriaxone solution stored under experimental
295 conditions (in BSK-II media at 34°C, 3% O_2, 5% CO_2) does not lose activity, as

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296 measured by MIC against *B. burgdorferi,* for up to 20 days. The activity of amoxicillin
297 measured similarly, however, dropped 20-fold over 20 days which suggests degradation
298 over time. The resulting MIC was still lower than the concentration used in killing
299 experiments. This pulse-dosing experiment shows that a population of the pathogen can
300 be eradicated with conventional antibiotics commonly used to treat the disease.
301

302 **Discussion**

303 The presence of drug-tolerant persisters can explain the recalcitrance of chronic
304 infections to antimicrobial therapy, especially in cases when the disease is caused by a
305 susceptible pathogen. While some chronic infections are ancient – leprosy, syphilis,
306 tuberculosis – many cases in developed countries are consequences of otherwise
307 successful medical intervention. Various indwelling devices (catheters, prostheses,
308 heart valves) provide a substratum for biofilms that protect persisters from the immune
309 system [2]. Even in bacterial infections that are routinely successfully treated with
310 antibiotics, there is dependence upon the host immune system to control persisting
311 bacteria that are not eradicated by antibiotics. The role of the immune system becomes
312 evident when these same infections involve immunocompromised hosts and antibiotic
313 eradication of the infection becomes much more difficult.

314 *B. burgdorferi* is a pathogen that can affect immunocompetent hosts. It
315 establishes long term infections of years to lifelong in both its natural (i.e. mice) and
316 incidental (i.e. humans) hosts in the absence of antibiotic therapy [14, 48]. Treatment in
317 the early stages of disease results in good outcomes. Delays in diagnosis and treatment
318 lead to sequelae that may require additional treatment. For example, patients who

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319 develop arthritis, which typically begins after 1 month of untreated infection, often do not
320 respond fully to a first course of 28 days of antibiotics [49]. The majority of these
321 patients have evidence of *B. burgdorferi* DNA in their synovial fluid and will respond to
322 additional one or two month courses of antibiotics [13, 16]. A smaller minority of patients
323 referred to as “antibiotic resistant Lyme arthritis” will continue to have arthritis with
324 synovial fluid that is PCR negative for *B. burgdorferi* DNA. These patients typically
325 respond to anti-inflammatory agents such as methotrexate or TNF-inhibitors. Both these
326 groups of patients should be distinguished from the highly controversial group of
327 patients with “chronic Lyme disease” that exhibit fatigue, myalgias and arthralgias
328 without objective evidence of disease. For the first group of Lyme arthritis patients
329 responsive to antibiotics, given that there is no reported resistance to clinically used
330 tetracyclines, β-lactams, and cephalosporins in the pathogen, the need for lengthy
331 courses of therapy is unclear. The presence of persister cells is one possible
332 explanation and this is a pattern that is seen in other infections where persister cells are
333 thought to be relevant for disease *in vivo*.
334 We found that similar to other pathogens, the pattern of killing of *B. burgdorferi*
335 by bactericidal antibiotics is biphasic, with a small subpopulation of surviving persisters.
336 These surviving clones are not resistant mutants; upon regrowth they form a new
337 persister subpopulation. Also similar to *E. coli, S. aureus*, and other pathogens, the
338 density of persisters increases as the culture deviates from strictly exponential growth,
339 reaching a maximum at stationary state. This is probably due to a deterioration of
340 growth conditions resulting in increasing numbers of dormant cells. However, of note,
341 the stationary state in *B. burgdorferi* is atypical, as amoxicillin and ceftriaxone continue

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342 to kill the majority of cells despite an increase in the level of persisters in the population.
343 Cell-wall acting antibiotics do not normally kill non-growing cells; one possibility is that
344 stationary state *B. burgdorferi* cultures represent a steady state of growing and dying
345 cells. The ability of β lactams to kill non-growing cells has also been observed in *M.*
346 *tuberculosis* where a combination of meropenem and a β lactamase inhibitor was able
347 to kill viable but non-replicative cells [50]. The authors speculate that peptidoglycan
348 remodeling continues in these non-replicating cells allowing for the activity of the β
349 lactam. This is another possible explanation of the killing we observe of stationary
350 phase *B. burgdorferi* with amoxicillin and ceftriaxone.
351 In a recently published study, Iyer, et al. [51] treated two different strains of *B.*
352 *burgdorferi* with ceftriaxone and were unable to detect live *B. burgdorferi* by subculture
353 in liquid medium. However, the cell density in that study was 10^7 cells/ml, and according
354 to our data, persister levels in this early exponential culture are low. In some of the
355 biological replicates treated with ceftriaxone, we have not been able to recover live
356 cells. At higher cell densities, the presence of persisters is unambiguous.
357 One common strategy for improving elimination of infective agents is to combine
358 existing compounds. For example, β lactams and aminoglycosides are known to
359 synergize with each other to achieve effective killing of *Enterococci* [52]. We tested
360 combinations of standard antibiotics used in treatment of Lyme disease as well as a
361 combination of a fluoroquinolone and an aminoglycoside, compounds that often
362 synergize and are capable of killing non-growing cells. However, there was no synergy
363 in killing *B. burgdorferi* with any of the tested combinations.

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364 We recently described efficient killing of persisters in *S. aureus* [42] and in *E. coli*
365 [45], and tested these compounds against *B. burgdorferi*. ADEP4, an activator of the
366 Clp protease, causes massive protein degradation in *S. aureus*, killing regular cells and
367 persisters. However, ADEP4 was not active against *B. burgdorferi*. We also reported
368 that nitrofuran prodrugs are effective in killing *E. coli* persisters. Nitrofurans are reduced
369 by bacterial nitroreductases into generally reactive compounds, explaining their activity
370 against persisters. Nitroreductases are expressed under anaerobic or microaerophilic
371 conditions. *B. burgdorferi* is a microaerophilic organism, but does not have obvious
372 homologs of a nitroreductase, and nitrofurans we tested were fairly inactive.

373 We also tested daptomycin, a lipopeptide that acts by increasing K^+ permeability
374 of the membrane. Being in a low-energy (microaerophilic) environment, the pathogen
375 may be vulnerable to membrane-acting compounds. Daptomycin killed the majority of
376 cells in a stationary culture, but the level of surviving persisters was comparable to that
377 of a stationary culture treated with ceftriaxone. In a recent publication daptomycin was
378 reported to kill *B. burgdorferi* persisters more effectively than regular cells [43]. This
379 conclusion was based on equating stationary cells with persisters. As follows from our
380 experiments, a stationary culture harbors a small subpopulation of persisters. The
381 actual level of stationary cells apparently surviving treatment by daptomycin in that
382 study was very high, 28%, as determined by live/dead staining. Under similar
383 conditions, we detect about 10^3 (0.002%) surviving persisters by cfu count. It appears
384 that live/dead staining may be over reporting the level of live *B. burgdorferi* cells.

385 Another weakness of the pathogen is its apparently limited ability for DNA repair.
386 Based on the genome, *B. burgdorferi* lacks *recFOR*. In *E. coli,* both RecBC and

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387 RecFOR are required for repair of DNA damage caused by mitomycin C, an anticancer
388 drug. Mitomycin C at a low, clinically achievable dose (8X MIC), eradicated *B.*
389 *burgdorferi* persisters in both exponential and stationary cultures within 24 hours. A
390 highly reduced environment activates mitomycin C, and this contributes to its selective
391 action in microaerophilic tumors. While the killing of persisters by mitomycin C is
392 impressive, given the toxicity of this drug, this is more of a proof-of-principle for a
393 compound exploiting the weaknesses of this pathogen rather than a clinically useful
394 agent. Treatment with mitomycin C can result in serious negative side effects and it
395 should not be used for treatment of Lyme Disease. This agent will be useful to examine
396 the possible contribution of persisters to the disease in an animal model of infection.

397 Another peculiar feature of *B. burgdorferi* and a weakness of the pathogen is the
398 lack of development of resistance to any antibiotic used to treat Lyme disease. Even
399 attempts to raise mutants resistant to amoxicillin and ceftriaxone *in vitro* have been
400 unsuccessful. Joseph Bigger proposed an interesting strategy for elimination of
401 persisters in 1944, in the first publication describing these cells [53]. The rationale is to
402 add antibiotic to kill off regular cells; wash it away; allow the culture to start regrowing, at
403 which point persisters will resuscitate. Reintroducing antibiotics will kill the regrowing
404 bacteria. The argument against pulse dosing is that this protocol invites resistance
405 development. Given that this is not a concern for *B. burgdorferi*, pulse dosing may be an
406 effective strategy and we performed pulse dosing with amoxicillin and ceftriaxone.
407 Persisters were eradicated with ceftriaxone in four pulses. These experiments form the
408 basis for testing pulse dosing in an animal model, and if successful, in humans.

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409 While we have identified the presence of *B. burgdorferi* persisters in cultures of
410 the organism, the mechanisms by which they are able to survive remain unknown.
411 There are multiple pathways of persister formation in other bacteria. The study of
412 persisters so far identified redundant TA modules as a main component responsible for
413 persister formation in *E. coli* and *S. typhimurium* [8, 20, 22]. TA modules are widely
414 spread among bacteria, but are surprisingly absent from the genome of *B. burgdorferi*.
415 Other components leading to persister formation in *E. coli* have been detected as well -
416 the stringent response [54], various metabolic processes [55, 56], global regulators, and
417 protein stabilizing chaperones [56] . Future work will determine if these or other
418 processes are involved in persister formation in *B. burgdorferi* and if persisters play a
419 role in the pathogenesis of Lyme disease in humans.
420

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425 *burgdorferi* B31 5A19 strain and Dr. Yi-Pin Lin from Dr. John Leong's lab at Tufts
426 University for his help with the semi-solid plating method.

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603
604 **Figure Legends**
605 **FIG 1**

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606 Killing of *B. burgdorferi* by antibiotics. (a) Time-dependent killing. Antibiotics were
607 added to an exponentially growing culture, samples were taken over time, washed,
608 diluted, and plated in semi-solid BSK-II media for cfu counts. The culture was treated
609 with amoxicillin (Amox) (6 µg/ml) or ceftriaxone (Cef) (3 µg/ml) (n=9). (b-d) Dose-
610 dependent killing. A late-exponential culture of *B. burgdorferi* culture was exposed to
611 antibiotics for 5 days, and surviving cells were determined by cfu count. The culture was
612 treated with amoxicillin (b), ceftriaxone (c), or doxycycline (Dox) (d) (n=6). Error bars
613 represent standard error.
614
615 **FIG 2** Growth dependent persister formation in *B. burgdorferi*. Growth in BSK-II medium
616 was determined by cfu count. Persister levels were determined by taking samples from
617 the growing culture, exposing to antibiotic for 5 days, and counting cfu. (a), amoxicillin
618 (Amox) (6 µg/ml); (b), ceftriaxone (Cef) (3 µg/ml). N=6. Error bars represent standard
619 error.
620
621 **FIG 3** Persister formation is not heritable. Colonies recovered from a persister
622 experiment before and after antibiotic treatment were used to inoculate fresh BSK-II
623 media. The colonies were allowed to grow for 3 days and treated with the same
624 antibiotic used in the original persister experiment for 5 days. Persister levels of the
625 colonies recovered after antibiotic treatment (Persisters) were not significantly different
626 than the colonies recovered before antibiotic treatment (Control). N=5. Error bars
627 represent standard error. Amox = amoxicillin, Cef = ceftriaxone.
628

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629 **FIG 4** Killing of *B. burgdorferi* with drug combinations. (a) Time-dependent killing of late
630 exponential *B. burgdorferi* cultures exposed to the indicated antibiotics in combination.
631 Amoxicillin (Amox) (6 µg/ml), ceftriaxone (Cef) (3 µg/ml), and doxycycline (Dox) (2.5
632 µg/ml) (n=6). (b) Killing of late exponential *B. burgdorferi* exposed to gemifloxacin
633 (Gemi) (1.5 µg/ml) and/or spectinomycin (Spec) (160 µg/ml) singly or in combination
634 (n=6). An aliquot was taken at indicated time points, washed, diluted, and plated on
635 semi-solid BSK-II media for cfu counts. Error bars represent standard error.
636
637 **FIG 5** Killing of *B. burgdorferi* by daptomycin. Time dependent killing of stationary phase
638 *B. burgdorferi* exposed to daptomycin (81 µg/mL) (n=3). Error bars represent standard
639 error.
640
641 **FIG 6** Killing of *B. burgdorferi* by mitomycin C (MMC). (a, c) Time-dependent killing of *B.*
642 *burgdorferi*. Three independent cultures of *B. burgdorferi* either at late exponential
643 phase (a) or stationary phase (c) of growth were treated with MMC: 0.8 µg/ml (4X MIC)
644 or 1.6 µg/ml (8X MIC). (b) Dose-dependent killing of late exponential cultures of *B.*
645 *burgdorferi* culture after 5-day exposure to increasing concentrations of MMC. An
646 aliquot was taken at indicated time points, washed, diluted, and plated on semi-solid
647 BSK-II media for cfu counts. N=6. The x-axis is the limit of detection. An asterisk
648 represents eradication to the limit of detection.
649
650 **FIG 7** Pulse dosing results in effective killing of *B. burgdorferi* persisters. Late
651 exponential cultures of *B. burgdorferi* were treated with (a) ceftriaxone (Cef) (3 µg/ml) or

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652 (b) amoxicillin (Amox) (6 µg/ml) for 5 days. This represents the first round of killing. The
653 cultures were then washed and allowed to recover in fresh BSK-II media for 24 hours.
654 They were then treated again with amoxicillin (6 µg/ml) or ceftriaxone (3 µg/ml) for a
655 further 5 days to give the second round of killing. This was repeated for a total of four
656 rounds of killing with a 24 hours period of growth in fresh media between each round.
657 Bars represent the average of at least three independent cultures (n=3-6) and error bars
658 represent standard error. The x-axis is the limit of detection. An asterisk represents
659 eradication to the limit of detection.

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660 **Table 1:** Selected antibiotics tested against *B. burgdorferi.*

Drug	Class	MIC (µg/ml)	Max. serum concentration (µg/ml)
Amoxicillin	β-lactam	0.06	7.6 [57]
Ceftriaxone	Cephalosporin	0.01	256.9 [58]
Doxycycline	Tetracycline	0.25	2.6- 5.9 [59]
Gemifloxacin	Fluoroquinolone	0.125	2.33 [38]
Spectinomycin	Aminoglycoside	2	140-160 [41]
Daptomycin	Lipopeptide	12.5-25	55-133 [60]
Vancomycin	Glycopeptide	0.25	40 [61]
Mitomycin C	Antitumor antibiotic	0.2	3.2 [62]

The MIC was determined by broth microdilution method.

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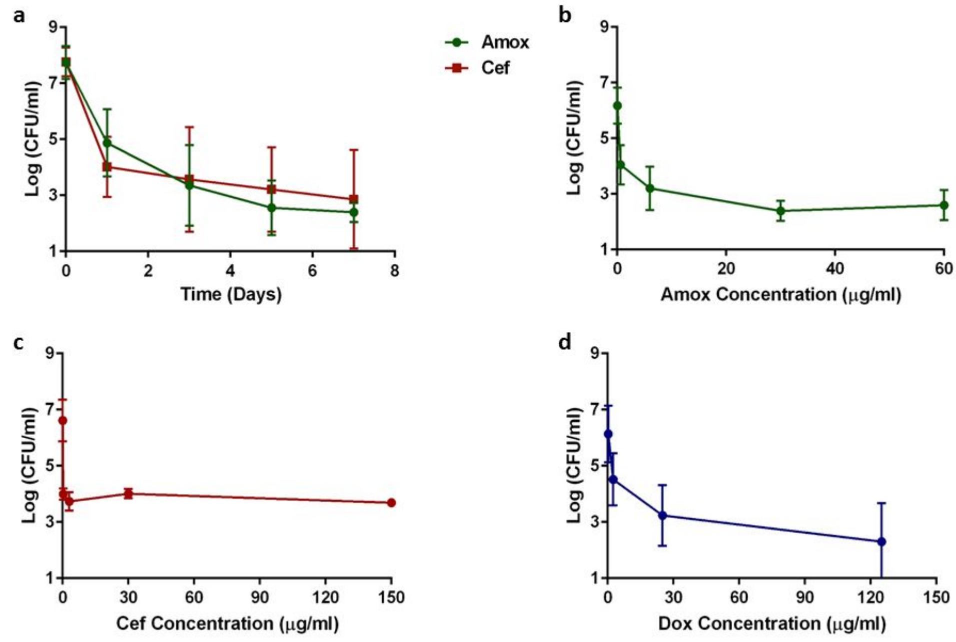

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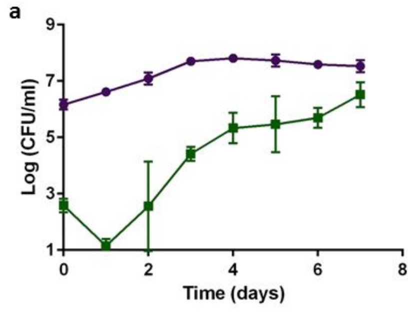
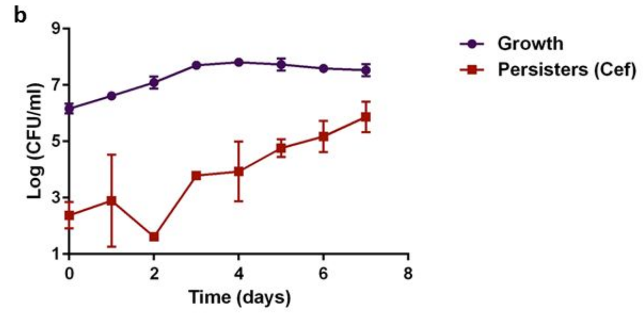


Growth
Persisters (Amox)



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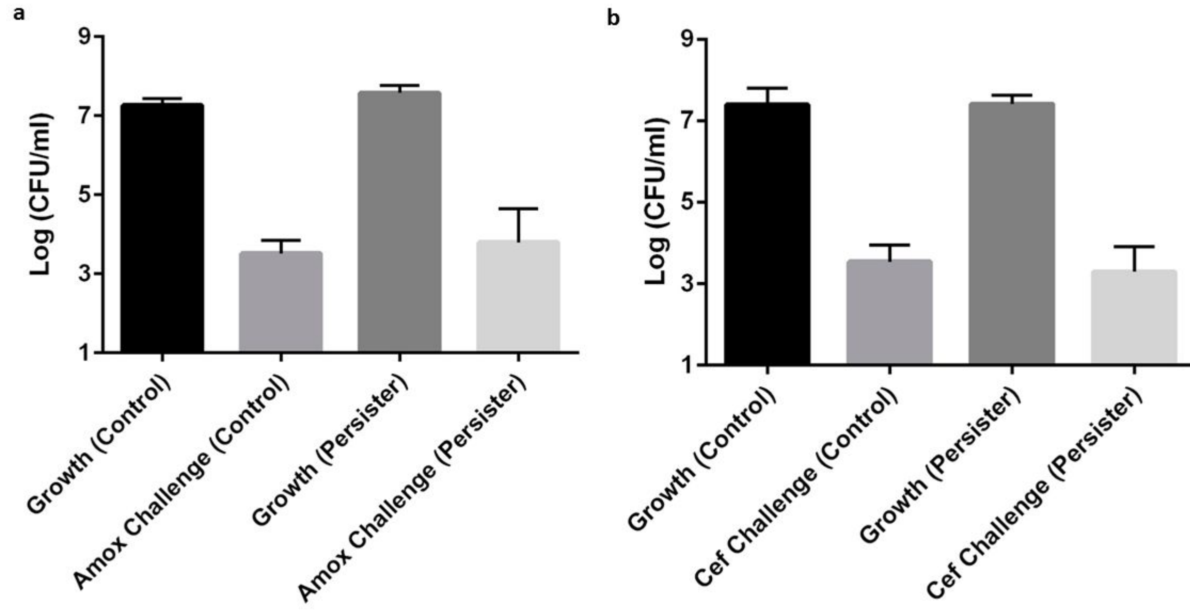

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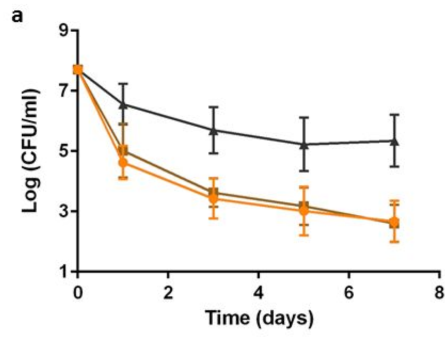

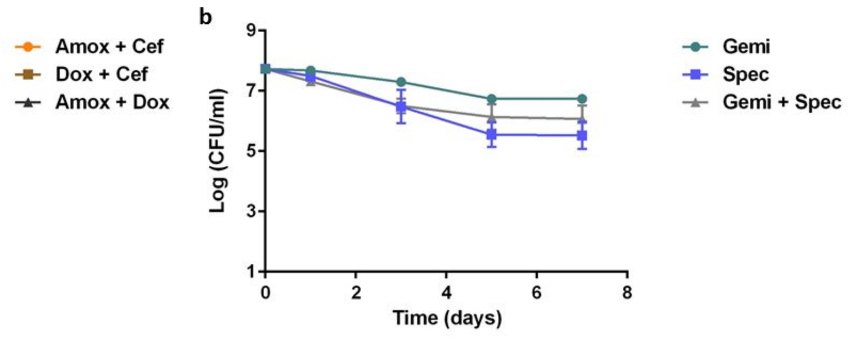

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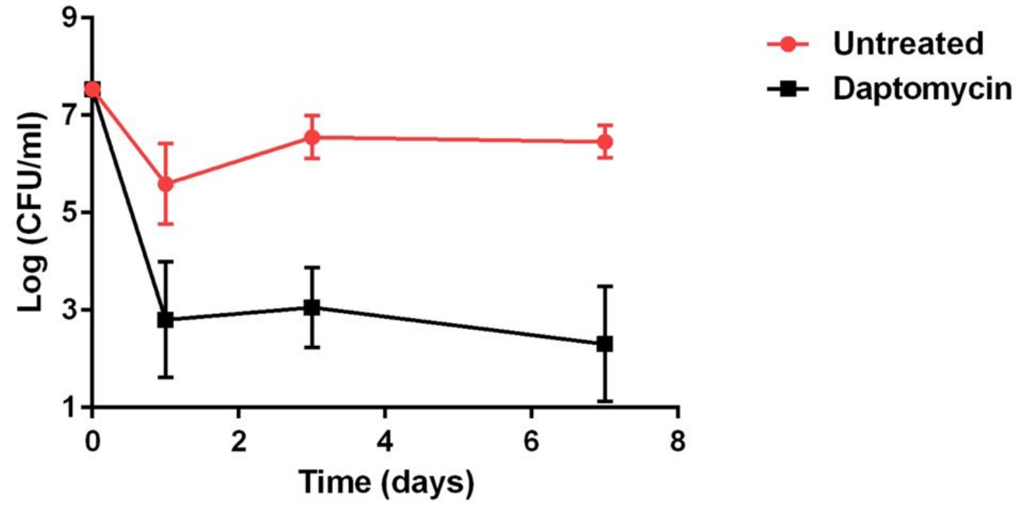

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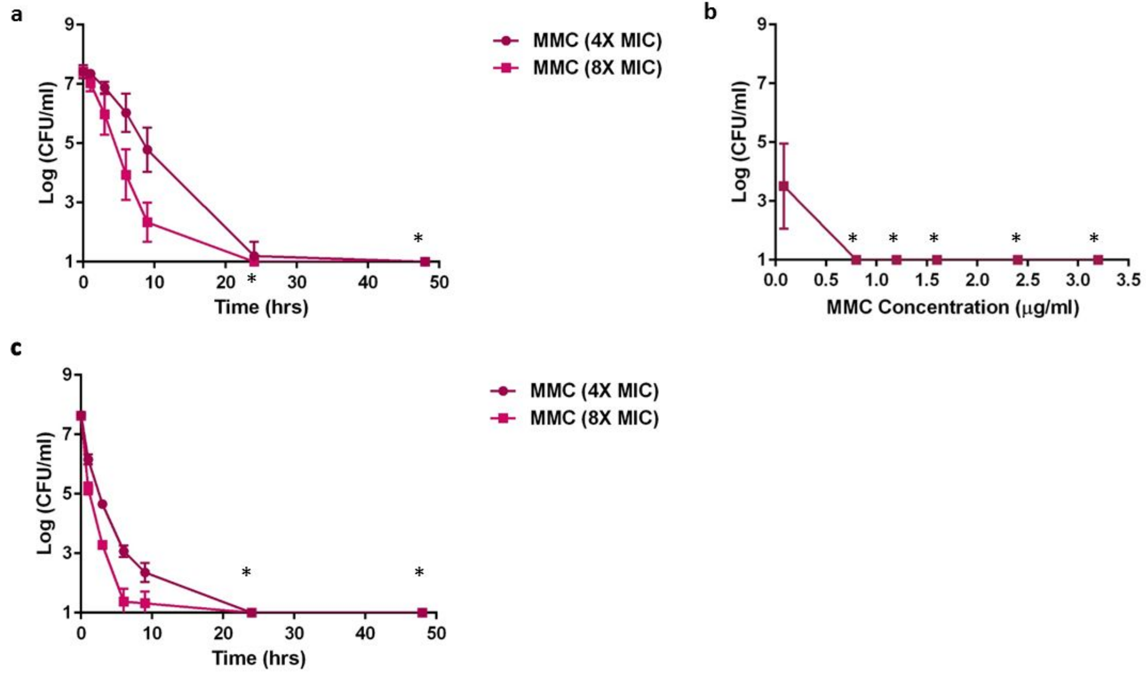

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