

Persistence of Non-Cultivable *Borrelia burgdorferi* Following Antibiotic Treatment:

Critical Need for Further Research

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Lyme disease, caused by a number of closely related members of the *Borrelia burgdorferi* sensu lato family (*B. burgdorferi* sensu stricto in the United States) that are transmitted by closely related members of the *Ixodes persulcatus* family (*I. scapularis* and *I. pacificus* in the United States) is endemic in many parts of the world, with particularly high prevalence in the United States and Europe. Prevalence of human disease continues to rise, as does the geographic distribution of endemic areas. These events are enhanced by perturbation of the environment by humans, as well as global climate change, which favor habitation of the environment by *Ixodes spp.* vector ticks and suitable reservoir hosts. Interest in Lyme disease is rising globally, as Lyme disease is increasing in southern Canada, where infected ticks and reservoir hosts are extending their range from the United States, as well as an increase in prevalence throughout Europe and Asia.

I have been engaged in Lyme disease research since its initial discovery in coastal Connecticut in the late 1970's/early 1980's. At that time, I was on the faculty of the Yale School of Medicine, and collaborated with Dr. Steere and others to develop an animal model for studying mechanisms of disease and vaccine development. I have continued Lyme disease research upon joining the faculty at the University of California at Davis in 1997. I have been actively funded by NIH in Lyme disease research for over 25 years.

During the course of my Lyme disease research career, I have become saddened by the negative discourse and division that exists among various factions of the Lyme disease community, including the lay community, the medical community, and the scientific community (the so-called "Lyme Wars"). In particular, debate has intensified regarding efficacy and appropriate regimens for antibiotic treatment. Central to this debate is the Infectious Disease Society of America (IDSA) position that this is a simple bacterial infection that is amenable to simple antibiotic treatment, while also recognizing that something is happening in patients after treatment, known as Post Lyme Disease Syndrome (PLDS).

Lyme disease is exceedingly complex in humans, and this poses major challenges to accurate diagnosis and measuring outcome of treatment. It has been known for years that the acute signs of Lyme disease (erythema migrans, cardiac conduction abnormalities, arthritis, etc.) spontaneously regress without benefit of antibiotics, but their resolution is accelerated by treatment. There is overwhelming evidence in a variety of animal species as well as humans that *B. burgdorferi* persists without treatment, but the crucial question is does it survive following treatment, and if so, do surviving spirochetes cause "chronic" Lyme Disease or PLDS? These questions cannot be answered by speculative and expensive human clinical trials motivated by firmly held dogmatism.

Something strange is happening with Lyme disease. *Borrelia burgdorferi* persistently infects a myriad of fully immunocompetent hosts as the rule, not the norm of its basic biology. When

such a situation occurs, antibiotics may fail, since it is generally accepted that antibiotics eliminate the majority of bacteria, and rely upon the host to “mop up” the rest. If the bacteria are able to evade host “mopping”, then the logic of the scenario falters. It is not surprising, therefore, that experimental studies, using a broad spectrum of animal species (mice, dogs, monkeys) and a variety of antibiotics (doxycycline, amoxicillin, ceftriaxone, tigecycline) have all shown a failure to completely cure the animals of *B. burgdorferi* infection. What is surprising is that the surviving spirochetes can no longer be cultivated from tissues (culture is considered by some to be the gold standard for detecting viable *B. burgdorferi*), but their presence can be readily detected with a number of methods, including *B. burgdorferi*-specific DNA amplification (PCR), xenodiagnosis (feeding ticks upon the host and testing the ticks by PCR), detection of *B. burgdorferi*-specific RNA (indicating live spirochetes), and demonstration of intact spirochetes in tissues and xenodiagnostic ticks by labeling them with antibody against *B. burgdorferi*-specific targets. These surviving spirochetes are not simply “DNA debris” as some contend, but are rather persisting, but non-cultivable spirochetes. It remains to be determined if their persistence following treatment is medically significant. For example, humans are known to be persistently infected with a number of opportunistic pathogens, including viruses, bacteria, and fungi, which are held in abeyance by the immune response, without clinical symptoms. Their significance varies with individual human patients and their ability to keep them in check. Lyme disease is likely to be similar.

The following report is a bit technical, but provides a summary of documented evidence of published and yet to be published experimental studies that provide compelling evidence for *B. burgdorferi* persistence following antibiotic treatment in animal model systems. It remains to be determined if humans are different, but the wide range of animal species studied (including non-human primates) predicts commonality from which extrapolation to humans is logical. Because of firmly entrenched opinion within the medical scientific community, evidence of persisting viable but non-cultivable spirochetes is slow to be accepted, and research proposals submitted to NIH that feature persistence following treatment are likely to receive prejudicial peer review in the contentious environment of Lyme disease*. Negative comments by peer reviewers of grant applications in the current financially austere NIH climate result in unfindable scores, if they are scored at all (triaged). I have no personal stake in this issue any more, as I am retiring within a year.

In my opinion, for such important and controversial studies to go forward, NIH will need to publish a specific call for applications, known as a “Request for Applications” (RFA), that requests research on the biological significance of persisting spirochetes following antibiotic treatment.

* a major weakness cited by a peer reviewer in a recent unfunded R01 application:

“The lay public that has so far denied the validity of scientific data will misunderstand the significance of...[persisting non-cultivable *Borrelia burgdorferi*]...and use it as additional evidence to support the idea of treatment-resistant Lyme disease.”

Persistence of Non-Cultivable *Borrelia burgdorferi* Following Antibiotic Treatment:

Critical Need for Further Research

Background:

There is widespread consensus among the mainstream medical community that relatively short-term courses of antibiotics can eliminate objective signs of Lyme borreliosis in patients, with the assumption that patients have been cured of infection. This has been articulated in the *IDSA Guidelines* in 2006 [3] and reaffirmed by an expert Lyme disease review panel in 2010 [4]. The *IDSA Guidelines* are in agreement with position statements of other medical and scientific organizations, including the European Federation of Neurological Societies, The European Union of Concerted Action on Lyme Borreliosis, the American Academy of Neurology, the Canadian Public Health Network, the German Society for Hygiene and Microbiology, several expert panels in various different countries, the American Lyme Disease Foundation, the CDC and NIH. An *Ad Hoc International Lyme Disease Group* has also affirmed this position [5,6].

This consensus is based upon clinically objective criteria, in keeping with sound medical practice. However, it is well established that patients with objective criteria of Lyme borreliosis may also have widely varied and subjective manifestations that do not necessarily fit objective clinical criteria [7,8,9]. There is agreement that when objective clinical signs are persistent, a rare patient may have chronic Lyme disease, and when objective clinical signs return in a treated patient, a rare patient may have recurrent Lyme disease. Under both circumstances, repeated antibiotic treatment is advised. A principal area of continuing but unresolved debate involves patients who experience disabling subjective symptoms following completion of appropriate antibiotic therapy. This has been recognized by the term “post-Lyme disease syndrome” (PLDS). *IDSA Guidelines* state that there is “no well-accepted definition of the PLDS”, and that “there is no convincing biologic evidence for the existence of systemic chronic *B. burgdorferi* infection among patients after receipt of recommended treatment regimens for Lyme disease.” [3] In the absence of objective clinical and diagnostic criteria, PLDS can never be proven to be, or not to be, associated with persistent infection with *B. burgdorferi*. Nevertheless, the vagaries of PLDS have promulgated a culture of “Lyme-literate physicians” (some literate, others not), emotionally charged lay support groups (well-intentioned, but often ill-informed), and speculative treatments, including scientifically unfounded and medically ill-advised long-term antibiotic regimens with outcomes that cannot be objectively proven. Therein lies the basis of what has been euphemistically termed the “Lyme Wars” [10]: a contentious debate that can never be won simply on strongly held conviction. What is needed is research on the basic biology of *B. burgdorferi*, including outcome after antibiotic treatment under controlled conditions in animal models. Animals are indeed different from humans, but knowledge gained with animal models lends credence to valid hypotheses that can then be rationally approached in human trials.

A basic feature of Lyme borreliosis (without antibiotics) is that persistent infection is the rule, not the norm. This occurs in *B. burgdorferi*'s many reservoir hosts, and has been proven experimentally in *Peromyscus* mice [11], laboratory mice [12], rats [13], hamsters [14], gerbils [15], guinea pigs [16], dogs [17], and non-human primates [18]. Humans appear to be no different, as there are a number of documented case reports of persistent infection based on culture [19,20,21,22,23,24,25] and PCR [26,27,28,29,30]. *Borrelia burgdorferi* has evolved to persist in immunologically competent hosts as a survival strategy for maintaining its natural host-vector life cycle. Natural reservoir hosts and small laboratory animals are generally rodents. In such hosts, infection is generalized and persistent, including in the skin, wherein spirochetes can most efficiently interface with the vector tick. Both *in vivo* animal model studies

and *in vitro* studies have shown that *B. burgdorferi* spirochetes utilize an array of adhesins that engage virtually every component of the extracellular matrix to facilitate their dissemination [31], and sequestration within collagen as their preferred site of persistence [2,32,33]. Dissemination is also facilitated by bacteremia during early infection, which is generally cleared during the immune persistent phase of infection, and intermittent thereafter. Because humans are much larger, they experience localized infections, as evidenced by erythema migrans (EM), and sometimes disseminated, but randomly multifocal infection through bacteremia, which may result in pauciarticular arthritis, secondary EM, carditis, peripheral neuropathy, meningitis, and other objective clinical signs. It should be emphasized that Lyme disease in untreated humans (and experimental animals) is ephemeral, with “spontaneous” resolution (without antibiotic treatment) of EM, carditis, arthritis, and other signs [9,34]. Studies in animal models have shown that resolution of arthritis and carditis is mediated by the acquired humoral immune response of the host. Under these conditions, anatomically defined inflammation resolves, but infection persists [32,35,36]. Indeed, even during the pre-immune phase of infection, spirochetes populate many tissues with no evidence of inflammation (thus inflammation or “disease” does not necessarily correlate with spirochete presence). The random, multifocal nature of human infection, the ephemeral clinical signs, the myriad subjective symptoms, the clinical impracticality of culture or PCR, the insensitivity of culture, and the retrospective nature of serology all make objective diagnostic criteria for testing outcome of treatment in humans simply impossible. That is not the case with animal models.

In a recent critical review of studies involving antibiotic treatment of *B. burgdorferi*-infected animal models, it was stated that “in the treatment of other infections it is probably unrealistic to expect that antimicrobial therapy *per se* will eliminate every single microorganism from an infected host, and moreover, such an action would rarely if ever be required for a successful outcome...the role of antimicrobial therapy *in vivo* can be thought of in terms of “tipping the balance” in favor of the host’s own defenses against a particular pathogen”[37]. This may be true for “other infections” but when treating for *B. burgdorferi*, which persists in fully immunocompetent hosts as the rule of its natural behavior, “tipping the balance” in favor of the host poses a challenge.

In that regard, different laboratories, using various classes of antimicrobial drugs in different animal models, including mice [1,2,38,39,40], dogs [17,41,42], and non-human primates [43], have all demonstrated survival of *B. burgdorferi* following antibiotic treatment. What is unique about all of these studies is that spirochetes can be detected by PCR for *B. burgdorferi*-specific DNA (BbDNA), but not by culture. In mouse studies performed in this laboratory (see below), mice were treated with ceftriaxone, doxycycline, or tigecycline at various intervals of infection, and tissues were tested at intervals after treatment. Tissues remained BbDNA PCR-positive up to 12 months, but were consistently culture-negative. Morphologically-intact spirochetes could be visualized by immunohistochemistry in tissues from treated mice; ticks could acquire morphologically-intact *B. burgdorferi* and BbDNA from treated mice; ticks remained BbDNA-positive through molting into nymphs and adults; nymphs transmitted BbDNA to recipient immunodeficient (SCID) mice; allografts from treated mice transplanted into recipient SCID mice transferred BbDNA to recipient mice; and both tick- and transplant-inoculated mice had disseminated BbDNA. BbDNA-positive tissues were also positive for *B. burgdorferi*-specific RNA transcription. Furthermore, quantitative PCR indicated low-levels of replication during these various stages. The *IDSA Guidelines* have stated “the significance of continued PCR positivity needs to be better understood, but this phenomenon should not necessarily be construed to indicate persistence of viable *B. burgdorferi*” [3]. The above summarized behavior of PCR-positivity, RNA transcription, BbDNA transmission, BbDNA amplification, BbDNA dissemination, and morphologically intact spirochetes in both tissues and ticks strongly indicate the presence of persistent, viable, but uncultivable spirochetes.

IDSA Guidelines also state that “unless proven otherwise, culture should be regarded as the gold-standard to address viability of *B. burgdorferi*” [3]. Culture may indeed be a gold standard when it is positive, but it is often not. Having worked with *B. burgdorferi* for over 25 years, it is apparent that not all isolates or strains can be easily cultured, and this is especially apparent during long-term infection. Thus, culture cannot be relied upon as a gold standard of viability. As noted above, our studies and those of others in mice, dogs and non-human primates have all reached similar conclusions: spirochetes are persisting, but are paradoxically non-cultivable. In ongoing studies (see below), we have found resurgence of non-cultivable spirochetes in tissues of mice (and by xenodiagnosis) at 12 months after antibiotic treatment.

Because persistence of non-cultivable spirochetes has been shown to occur following treatment with several different classes of antibiotics, the phenomenon is likely explained by antimicrobial tolerance (in contrast to antibiotic resistance or inadequate antibiotic treatment), in which all classes of antibiotics fail to completely eliminate non-dividing or slowly-dividing subpopulations of a broad array of bacteria and fungi [44,45]. A possible explanation for these attenuated antibiotic-tolerant spirochetes may be because of plasmid loss, in which spirochetes have lost critical genetic material that favors robust growth. It has been known for decades that during *in vitro* passage, *B. burgdorferi* is highly prone to plasmid loss [46,47,48], and therefore plasmid loss is likely to also occur during the course of infection and increase over time. This may explain why treatment success in humans [3,8] and laboratory mice [2,38] appears to be most effective during early infection. Treatment success is inversely correlated with spirochete populations, since spirochete burdens in mouse (and human) tissues are highest during early infection [49], when antibiotics work best. The biological (in contrast to medical) significance of attenuated spirochetes is probably insignificant, in that robustly dividing-, genetically-intact spirochetes would be selectively favored upon tick acquisition, transmission, and survival in reservoir hosts. The medical significance of attenuated persisting spirochetes is another matter, and compels further investigation.

Animal Studies. Various studies have shown efficacy of antibiotics in curing laboratory rodents of *B. burgdorferi* infection, based upon culture as the read out [50,51,52,53] [54,55] [56,57]. With the advent of increasingly sensitive PCR analyses, we and others have repeatedly demonstrated in dogs [17,41,42], mice [1,2,38,39,40] and rhesus macaques [43] that non-cultivable spirochetes persist following antibiotic treatment. Straubinger, et al. [17,41,42] found that despite treatment of infected dogs for 1 month with ceftriaxone, doxycycline, or azithromycin, BbDNA continued to be detected as late as 12 months after therapy, but tissues were consistently culture-negative. This seminal observation prompted Bockenstedt’s group in collaboration with our group to study mice infected with *B. burgdorferi* N40 and treated with ceftriaxone or doxycycline [1]. In that study, spirochetes could not be cultured from tissues of treated mice, but were detected by PCR in tissues for up to 9 months after treatment, and in ticks that fed upon treated mice at 3 months after treatment. Efforts to transmit spirochetes to naïve mice from infected ticks that fed upon treated mice were unsuccessful. However, spirochetes could be visualized in midguts of ticks that fed upon treated mice (Fig. 1). PCR analysis of spirochetes within the ticks suggested that they had lost one or more plasmids, based upon limited survey of gene targets on B31 lp25 and lp28-1, which are associated with potentially important virulence factors. It was concluded that spirochetes in antibiotic-treated mice were viable, but non-infectious and genetically attenuated.

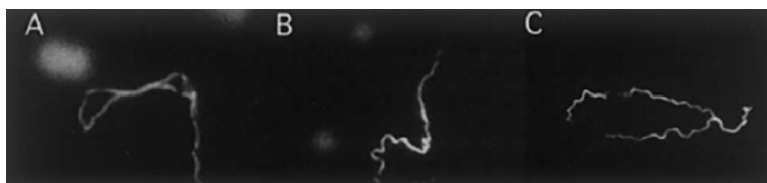
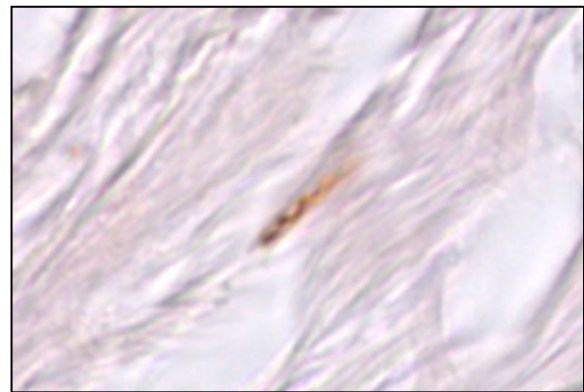


Fig. 1. Direct fluorescence antibody labeling of *B. burgdorferi* within midguts of xenodiagnosis ticks that fed upon mice treated with saline (A), ceftriaxone (B) or doxycycline (C) [1].

These studies prompted further investigation of *B. burgdorferi* persistence following antibiotic therapy by examining mice treated with ceftriaxone during the early (3 weeks) or late stage (4 months) of *B. burgdorferi* N40 infection [2], since we had found that there are significant shifts into, or preferential survival of spirochetes in collagen during chronic infection [32]. Commencing at 3 weeks or 4 months of infection, mice were treated with ceftriaxone or saline for 1 month (16 mg/kg b.i.d. for 5 days, s.i.d. for 25 days), and then necropsied at 1 or 3 months after treatment. Tissues of mice were tested by culture, PCR, and allograft (ear skin) transplantation into naïve mice, and mice were tested by xenodiagnosis, using larval ticks. As before, spirochetes could not be cultured, but low copy numbers of BbDNA were detected by real-time quantitative PCR (qPCR) in tissues of treated mice. Treatment commencing at 3 weeks of infection was more effective at curing mice of infection (including BbDNA) than commencing treatment at 4 months. Allograft (ear tissue) transmission could not be demonstrated, but a low percentage of xenodiagnosis ticks were BbDNA-positive, and nymphal ticks from those tick cohorts transmitted BbDNA to naïve SCID mice, in which multiple tissues became BbDNA PCR-positive, but were culture-negative. Thus, in contrast to the previous study [1], our study found that ticks could both acquire and transmit infectious, non-cultivable spirochetes. This study also detected lp25 and lp28-1 gene targets in BbDNA-positive ticks, thereby challenging the hypothesis that spirochetes were genetically attenuated. Furthermore, morphologically-intact *B. burgdorferi* were found by immunohistochemistry in collagenous tissues of antibiotic-treated, culture-negative mice (Fig. 2):

Fig. 2. Immunohistochemical labeling of antigen-positive, morphologically-intact *B. burgdorferi* in a ligament of a mouse infected for 4 months, treated with ceftriaxone for 1 month, and then necropsied 1 month after completion of antibiotic treatment [2]



Studies with tigecycline. A valid criticism of mouse studies utilizing ceftriaxone is that the serum half-life of ceftriaxone is extremely short in the mouse, compared to humans. This is not the case with tigecycline. Tigecycline is a new first-in-class antibiotic that is highly active and bactericidal *in vitro* against multiple strains of *B. burgdorferi* compared to ceftriaxone, reaches serum concentrations well above (70 times) therapeutic levels with a half life of 12 hours [58]. We evaluated high and low doses of tigecycline, ceftriaxone (comparison group), and saline (control group) treatment in mice, commencing at 1 week, 3 weeks, or 4 months of infection [38]. Infection status was evaluated at 3 months after completion of treatment by culture, qPCR of multiple tissues, xenodiagnosis, tick-borne transmission, and allograft transplantation of joint and heart tissue into SCID mice. Previous studies revealed no allograft transmission using ear punch tissue (which has been found to be usually BbDNA-negative following treatment), so this experiment utilized tissues that were the most consistently BbDNA-positive (joint and heart) for persisting spirochetes. Results found no difference in effectiveness between ceftriaxone and tigecycline. Results also confirmed previous studies, demonstrating persistence of non-cultivable spirochetes, based upon qPCR, particularly in the heart base and joint tissues. As previously found, antibiotic treatment during the early stage of infection was more effective than treatment during the later stages of infection. The viability of non-cultivable spirochetes in antibiotic-treated mice was confirmed by transmission to SCID mice by allograft transplantation, with dissemination to multiple tissues in the recipient mice, and by xenodiagnosis, including acquisition of BbDNA by ticks, transmission by ticks to SCID mice, and survival through molting

of larval ticks to nymphs, and then to adults. As before, gene target copy numbers were consistently low, but increased slightly, rather than being diluted, during each transmission stage, indicating low levels of replication. In addition, BbDNA-positive heart base tissue from antibiotic-treated mice revealed RNA transcription of several *B. burgdorferi* genes. Results extended previous ceftriaxone and doxycycline studies, indicating that antibiotic treatment is unable to clear persisting spirochetes, which remain viable and infectious, but are slowly dividing.

Persistence of multiple *B. burgdorferi* isolates following antibiotic treatment. We tested efficacy of ceftriaxone (vs. saline) in mice infected with *B. burgdorferi* N40, B31, 72a, 118a, or Bo126. In saline-treated mice, we were unable to culture 72a, and rarely 118a, yet tissues contained BbDNA at infection-level copy numbers and distribution, and mice seroconverted at a titer indicating active infection. There was no difference in sensitivity of any isolate to antibiotic, with all treated mice being positive for BbDNA. Results support the generality of spirochete persistence.

Resurgence of spirochetes at 12 months after treatment. It has been speculated that non-cultivable spirochetes would eventually die out following treatment [1,37]. We obtained supplemental funds from the National Research Foundation for Tick-Borne Diseases and from NIH/NIAID to support a long-term study, in which mice were followed for up to one year after completion of treatment. Mice were infected with *B. burgdorferi* cN40 for 30 days, treated with ceftriaxone for 30 days, and then necropsied at 2, 4, 8 and 12 months after treatment. Spirochetes could be cultured from inoculation site and urinary bladder of saline-treated mice, but could not be cultured from any of the antibiotic-treated mice at any interval. qPCR results (*flaB*) for saline-treated mice indicated widespread persistent infection in multiple tissues for up to 1 year after treatment:

flaB real-time Q-PCR and Xenodiagnosis Results in Saline-Treated Mice

Interval	Inoculation Site	Ear	Heart Base	Ventricular Muscle	Tibiotarsus	Quadriceps Muscle	XenoDx
2 mos	+	+	+	+	+	+	2/2
	+	+	+	+	+	+	2/3
	+	+	+	+	+	+	2/3
	+	+	+	+	+	+	2/2
4 mos	+	+	+	+	-	+	0/1
	+	+	+	+	-	+	NA
	+	+	+	+	-	+	NA
	+	+	+	+	-	+	NA
8 mos	+	+	+	+	-	+	NA
	+	+	+	+	-	+	NA
	+	+	+	+	-	+	2/2
	+	-	+	+	-	+	3/3
12 mos	+	+	+	+	+	+	5/5
	+	+	+	+	+	+	5/5
	+	+	+	+	-	+	4/5
	+	+	+	+	+	+	5/5
	+	+	+	+	+	+	4/5
	+	-	+	+	+	+	4/5
	+	-	+	+	+	-	2/5
	+	-	+	+	+	+	2/5
+	+	+	+	+	+	4/5	

In contrast to saline-treated mice, remarkably different results were found in mice treated with antibiotic:

flaB real-time Q-PCR and Xenodiagnosis Results in **Antibiotic-Treated Mice**

Interval	Inoculation Site	Ear	Heart Base	Ventricular Muscle	Tibiotarsus	Quadriceps Muscle	XenoDx
2 mos	---	---	---	---	+++	---	0/3
	---	+--	++-	---	---	---	0/4
	---	---	---	+++	+--	---	0/2
	---	+--	---	---	+--	---	0/2
4 mos	---	---	---	---	---	---	NA
	---	---	---	---	---	---	NA
	---	---	+--	---	---	---	0/3
	---	---	---	---	++-	---	NA
8 mos	---	---	---	---	+--	---	NA
	---	---	---	---	---	---	3/4
	---	---	---	---	---	---	1/2
	---	---	---	---	---	---	NA
	---	---	---	+--	---	---	0/3
	---	---	---	---	+--	---	1/6
	---	---	---	---	---	---	1/3
	---	---	---	---	---	---	2/2
12 mos	+--	---	+++	+++	+--	+-	0/10
	+--	---	+++	+--	---	+++	1/10
	---	++-	+++	+++	+++	+-	1/10
	+++	+--	++-	+--	+--	+-	1/10
	+++	---	+++	+++	---	+++	1/10
	+++	+++	+++	+--	+++	+++	0/10
	+++	---	+++	+++	+++	+++	1/10
	+++	+++	+++	+++	+++	+++	1/10

Saline control tissues were tested as single samples, whereas antibiotic-treated mouse tissues were tested in triplicate, and expressed as such in the above table. Results revealed resurgence of spirochete distribution in tissues at 12 months. There was also an increase in *flaB* copy numbers in many samples from antibiotic-treated mice, with nearly 20% of the 12 month antibiotic samples containing 100 or more (up to 339) spirochetes/mg tissue (within range of saline-treated samples). Notably, although tissue samples were rarely positive at 8 months, xenodiagnosis at 8 months presaged resurgent activity prior to the 12 month interval.

Low Density Array (LDA) studies. LDA is a medium-throughput method based on a qPCR or a reverse transcription qPCR (RT-qPCR) platform. Forty three *B. burgdorferi* N40 genes were tested simultaneously, including genes associated with attachment, bacterial membrane, motility, metabolism, cellular processes, cell division, complement regulation, and metabolism (*General Methods*). Among 12 month samples, all gene targets were detected in 5 tissue samples from 4 saline-treated mice, whereas *B. burgdorferi* genomes in 16 tissue samples with high Bb DNA copy numbers from 6 antibiotic-treated mice were uniformly missing BBK32 (lp36), and variably missing *ospE*, various *erps* (cp32s), *vlsE* (located on N40 lp36), *arp* (located on N40 lp28-5), *bptA* (lp25), and *eppA* (cp9). Although preliminary, data suggest loss of small linear and circular plasmids in persisting spirochetes. Most notable was the uniform absence of lp36, loss of which significantly attenuates infectivity in mice, with markedly reduced, but detectable levels of infection [59]. Results also demonstrated amplification of multiple gene targets, thereby verifying the specificity of residual Bb DNA results. LDA was also utilized to detect RNA

transcription of various genes from cDNA-processed samples. Because of low copy numbers in samples, most gene transcripts were below detection levels in antibiotic-treated mice, but *dbpA* transcription was consistently found in most samples, and *bmpD*, *CRASP1*, *erp23* and *p23-T2* transcription were variably detected in samples at 12 months. Although preliminary, LDA analysis confirmed our previous findings [38] of RNA transcription by persisting spirochetes, indicating metabolic viability of non-cultivable spirochetes (in contrast to residual DNA debris).

This mouse study was repeated to confirm the findings of persistence and resurgence of non-cultivable spirochetes, with similar results of detection of non-cultivable spirochetes by PCR in tissues at 12 months following completion of ceftriaxone treatment. In addition, ticks were fed upon antibiotic-treated mice at 12 months after completion of antibiotic treatment, and found once again to be PCR-positive, as before. In addition, small numbers of spirochetes were visible by immunofluorescence within tick midgut preparations:

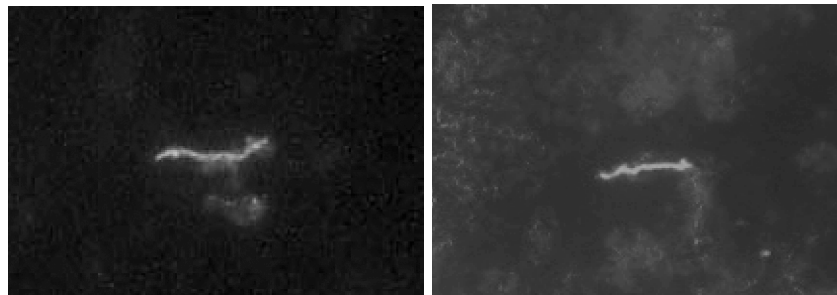


Fig. 4. Immunofluorescence staining of *B. burgdorferi* spirochetes in the midgut of ticks that fed upon a saline-treated infected control mouse at 12+ months of infection (left image) and on a mouse infected with non-cultivable spirochetes at 12 months following completion of antibiotic treatment (right image).

Tissues of mice infected with non-cultivable *B. burgdorferi* at 12 months following completion of antibiotic treatment were examined by immunohistochemistry for the presence of spirochetes. Rare, morphologically-intact spirochetes expressing immune-reactive antigen were found in heart tissue:

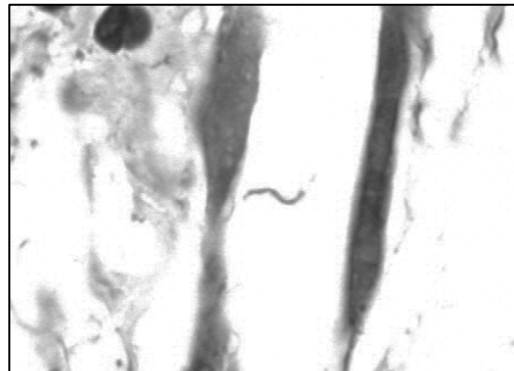


Fig. 5. Immunohistochemical staining of *B. burgdorferi* spirochete entering a lymphatic vessel in the heart base of a mouse infected with non-cultivable spirochetes at 12 months following completion of antibiotic treatment.

Persistence of non-cultivable spirochetes in antibiotic-treated rhesus macaques. In collaboration with Mario Philipp and Monica Embers at Tulane National Primate Research Center, we blindly analyzed tissues by qPCR from rhesus macaques treated with ceftriaxone and doxycycline [72]. Several tissues were confirmed to be BbDNA-positive. As in dog and mouse studies, animals were culture-negative and xenodiagnosis-positive. Morphologically-

intact spirochetes were observed by immunofluorescence in the ticks that fed upon treated animals:

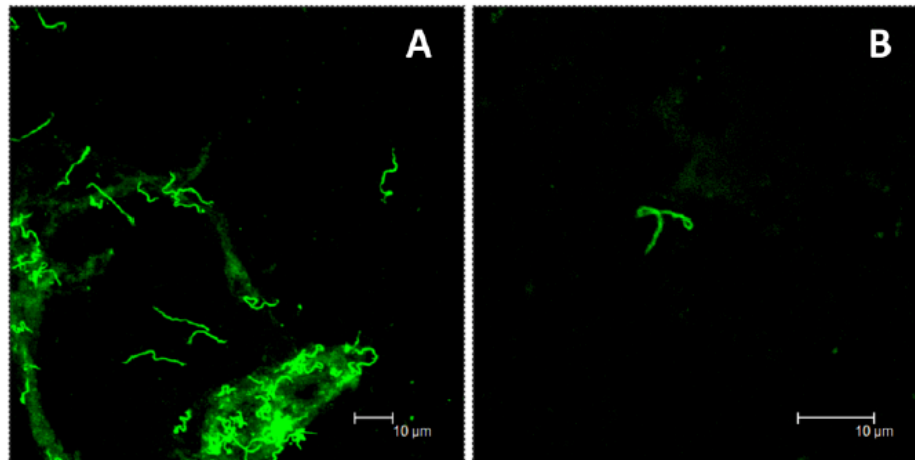


Fig 5. Spirochetes recovered by xenodiagnosis from macaques treated with antibiotic. Images represent immunofluorescent staining of *B. burgdorferi* in xenodiagnostic tick midgut culture (A) or tick midgut preparation (B) from treated animals [72].

Summary of Key Findings in Published and Preliminary (unpublished) Animal Studies

- Studies in mice, dogs and non-human primates have demonstrated persistence of non-cultivable spirochetes following treatment with several different bacteriostatic and bactericidal antibiotics.
- Non-cultivable spirochetes can be visualized as morphologically intact, antigen-positive spirochetes in ticks feeding upon antibiotic-treated mice and macaques, and in tissues of antibiotic-treated mice for 12 or more months after completion of antibiotic treatment.
- Non-cultivable spirochetes in antibiotic-treated mice can be acquired by ticks, transmitted by ticks, and survive molting of ticks from larvae to nymphs and to adults, confirming their viability.
- Non-cultivable spirochetes can be transmitted from antibiotic-treated mice to recipient SCID mice through tick-borne infection or transplantation of tissue allografts, and disseminate in recipient mice.
- Persisting non-cultivable spirochetes transcribe RNA, confirming their metabolic viability.
- Low copy numbers of target DNA of non-cultivable spirochetes are present in tissues of mice following antibiotic treatment, with evidence of very low but increasing levels of replication when acquired by ticks, transmitted by ticks, in different stages of ticks, and following transmission to recipient hosts.
- Non-cultivable spirochetes resurge at 12 months after antibiotic treatment, with increased BbDNA copy numbers and widespread dissemination in host tissues.
- Preliminary results suggest that resurgent non-cultivable spirochetes have lost small linear and circular plasmids, which may explain their attenuated, low-replicative behavior.

Conclusion:

“...the significance of continued PCR positivity needs to be better understood, but this phenomenon should not necessarily be construed to indicate persistence of viable *B. burgdorferi*.” *IDSA Guidelines*

Persisting viable but non-cultivable *B. burgdorferi* is now a convincing phenomenon based upon a number of animal-based (mouse, dog and primate) studies using a number of different antibiotics, and the significance of continued infection indeed needs to be better understood. It is time to recognize that Lyme disease is not a simple bacterial infection.

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