Published in final edited form as:

Diagn Microbiol Infect Dis. 2018 November ; 92(3): 183–188. doi:10.1016/j.diagmicrobio.2018.06.007.

Critical Analysis of a Doxycycline Treatment Trial of Rhesus Macaques Infected with *Borrelia burgdorferi*

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Abstract

A critical analysis was conducted of a doxycycline treatment trial of Indian rhesus macaques. In this treatment trial, the investigators attempted to infect the primates with *Borrelia burgdorferi* sensu stricto by at least 10 tick bites from artificially infected ticks. None of the primates became ill; nevertheless, five primates were treated with a 28-day course of oral doxycycline. In contrast to the conclusions of the authors, the data did not convincingly document the existence of viable *B. burgdorferi* in antibiotic treated primates. The investigators were unable to cultivate the spirochete from any animal post-treatment using highly sensitive in vitro methods. Like many prior animal studies, the current study also did not document that the doxycycline exposure in these animals was similar to that expected in humans. Numerous additional methodologic problems are discussed.

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Disclosures: Dr. Wormser reports receiving research grants from Immunetics, Inc., Institute for Systems Biology, Rarecyte, Inc., and Quidel Corporation. He owns equity in Abbott/AbbVie; has been an expert witness in malpractice cases involving Lyme disease; and is an unpaid board member of the American Lyme Disease Foundation. Dr. Shapiro has received royalty payments from UpToDate; has been an expert witness in malpractice cases involving Lyme disease; and is an unpaid board member of the American Lyme Disease Foundation. Dr. Gerold Stanek is an unpaid member of the steering committee of the ESCMID Study Group on Lyme Borreliosis/ESGBOR. Dr. Franc Strle is an unpaid member of the steering committee of the ESCMID Study Group on Lyme Borreliosis/ESGBOR. Other authors: none.

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Keywords

Lyme disease; *Borrelia burgdorferi*; Treatment; Persistence; Doxycycline

Introduction

A treatment trial of Indian rhesus macaques that were infected with *Borrelia burgdorferi* sensu stricto (hereafter referred to as *B. burgdorferi*) by the bite of at least 10 artificially infected *Ixodes scapularis* ticks (nymphal stage) was reported recently. Results of the study were presented in two separate publications [1,2]. The first, authored by Embers et al., focused on serology, xenodiagnosis and what was characterized as a novel *in vivo* culture system [1]. The second one reported the histologic findings [2]. In both reports, polymerase chain reaction (PCR) and immunofluorescent antibody techniques (IFA) were used to identify *B. burgdorferi* in the non-human primates (NHPs). There were several discrepancies between the two publications in the reported methods of the study (Table 1). Although these discrepancies do not appear to be as serious as the errata that were published concerning a prior treatment trial of *B. burgdorferi* infected NHPs by Embers et al. [3], nevertheless, they do raise concerns about the accuracy of the two current publications. Also, within the first publication there were several inconsistencies, such as on the number of *in vivo* cultures that had become contaminated [1].

The study failed to document persistence of viable cells of *B. burgdorferi* in doxycycline treated NHPs by standard *in vitro* culture techniques [1,2]. *B. burgdorferi* also could not be recovered by culture of tissues from immunocompromised SCID mice after multiple attempts were made to infect these mice using material from xenodiagnostic ticks that had fed on the NHPs. Nevertheless, based on other testing methods, the authors claimed that they observed persistent infection with viable cells of *B. burgdorferi* despite treatment with doxycycline [1,2]. The authors also concluded from the results of their study that seronegativity and a wide variety of serologic responses do not exclude the possibility of persistent infection [1].

The validity of these conclusions, however, is highly questionable. In this analytic review, the following topics will be discussed: 1. whether this particular NHP model of *B. burgdorferi* infection mimics human disease; 2. the validity of a novel *in vivo* culture method for the isolation of *B. burgdorferi*; 3. whether the testing methods utilized documented residual infection by *B. burgdorferi*; 4. whether the doxycycline dosage used for the NHPs was demonstrated to provide equivalent antibiotic exposure to that expected in humans; and 5. an evaluation of the validity of the concept of viable but not cultivable cells of *B. burgdorferi*.

Does this NHP model mimic human *B. burgdorferi* infection?

In this study the NHPs were infected by the bites of at least 10 artificially infected ticks; consequently, the number of organisms introduced into these NHPs is likely to have been substantially greater than would be expected from a single bite from a naturally infected tick [4]. Nevertheless, the resulting *B. burgdorferi* infection in the NHPs was assumed to mimic

human infection [5]. The authors stated that NHP models “recapitulate the multi-organ nature and progression of human Lyme disease,” including “erythema migrans, carditis, arthritis, and neuropathy of the peripheral and central nervous systems ...” [1]. However, not all NHP models of *B. burgdorferi* infection are the same. In the current NHP study [1,2], none of the primates appeared to develop any objective clinical manifestation of Lyme disease. Indeed, it was stated that none of the NHPs became ill in any discernible way. Although one of the 10 NHPs (IK14) developed a skin rash at the tick bite site that the authors called “erythema migrans,” this rash did not resemble typical erythema migrans based on the picture provided. It is unclear why the investigators did not use naturally infected ticks to transmit the infection, as had been done successfully in a prior NHP study [6]. In that study, 5 of 6 NHPs developed erythema migrans at the site of a tick bite, a statistically significant difference from the results of the current study, irrespective of whether the frequency of erythema migrans in the current study was 0/10 ($p=0.0014$ using the Fisher’s exact test 2-tailed) or 1/10 ($p=0.0076$). Lyme disease in humans in the United States is also very different from the *B. burgdorferi* infection created in the recent NHP study [1,2]. Studies in humans provide an estimate that more than 90% of those who become infected with *B. burgdorferi* develop an overt clinical illness, most commonly erythema migrans at the tick bite site [7,8].

Not only did primate IK14 not have erythema migrans based on the appearance of the skin lesion, this was the only NHP out of the 10 that had both negative cultures and negative PCR testing on two skin biopsy samples taken at the tick bite site before antibiotic therapy [1]. The authors speculated that the negative skin biopsy cultures and PCRs at the tick bite site in NHP IK14 occurred because they biopsied the center of the skin lesion rather than the periphery. However, available human data do not support this hypothesis [9].

Furthermore, IK14 is the only NHP that failed to develop a detectable serologic response to *B. burgdorferi* when tested at multiple time points [1]. Absence of evidence of infection with *B. burgdorferi* based on three different test methods, namely: culture and PCR (both performed at the skin site where the ticks had fed), and serologic response, is unprecedented in other studies of NHPs that were infected by tick bites [3,6,10]. Thus, the IK14 primate did not have convincing evidence of having been successfully infected with *B. burgdorferi*. In addition, the absence of a detectable serologic response occurred despite the fact that this NHP had very low doxycycline blood levels, much lower than the levels found for the four other NHPs that were treated with doxycycline [1]. Ironically, it is fortunate that this NHP did not acquire *B. burgdorferi* infection, as this animal, therefore, becomes a valuable control to assess the validity of the testing procedures that were performed to document persistence of *B. burgdorferi* infection. For example, the likely absence of *B. burgdorferi* infection in primate IK14 raises serious concerns about the significance of the non-motile “bodies” found on in-vitro culture of xenodiagnostic tick midgut contents and of having a positive RT-PCR for a single targeted gene, because this primate had such test results.

Validity of a novel in vivo culture method for *B. burgdorferi*

The authors have provided data on a novel in vivo culture technique [1]. In their study, heart tissue was inoculated into a dialysis membrane chamber (DMC), and the DMC was then

placed into the peritoneal cavity of a rat. The in vivo culture method was based on the premise that host adaptation of *B. burgdorferi* in NHPs might result in an altered phenotype that prevents propagation in an in vitro culture system. However, the technique employed simply transfers the material from one in vivo system to another in vivo system making the rationale for why this should be superior to simply analyzing the original tissue sample unclear. The only apparent difference would appear to be that there would be no immunologic response to *B. burgdorferi* in the rat.

It has been shown that *B. burgdorferi* can be readily recovered from mice with long standing infection by standard in vitro cultures [11]. Moreover, host adaptation in humans does not preclude successful in vitro cultivation of *B. burgdorferi* from tissue samples of the late cutaneous manifestation of Lyme disease called acrodermatitis chronica atrophicans [12]. Although most of the positive cultures for Lyme borrelia in patients with acrodermatitis chronica atrophicans have grown *B. afzelii*, the leading cause of this manifestation, some cultures have successfully grown *B. garinii* or *B. burgdorferi* [12]. It should also be noted that this DMC approach has never before been utilized for primary culture isolation; rather, it has been employed solely for the purpose of obtaining “host-adapted” spirochetes in sufficient numbers to facilitate molecular analysis [13–15].

The implanted heart tissue became necrotic or disintegrated, potentially releasing tissue bound spirochetal remnants [1]. In prior studies of cells of *B. burgdorferi* placed into a DMC, the spirochetes multiplied rapidly and become highly motile [13]; and other data have demonstrated that inoculation of these organisms into cultures in vitro is typically successful [Ira Schwartz, unpublished data]. In the NHP study of Embers et al. [1], there is no mention of motility and no mention of successful subculture based on transfer to standard in vitro cultures of this or any other material obtained from primates or from xenodiagnostic ticks that had fed on them post-treatment.

Of the 10 samples obtained from the in vivo culture system and tested by RT-PCR, three were positive for a single gene target (from two treated NHPs and from one untreated NHP), but none was positive for both of the targets tested [1]. Transcript for *ospA* was detected by RT-PCR in the two treated NHPs. In previous studies using the DMC methodology, *ospA* transcript was not detected, consistent with its function as a tick phase gene [14,16]. Indeed, a reduction in *ospA* expression is considered the hallmark for the host-adapted state [14,17]. Standard PCR was not reported, but IFA was positive for four of the eight evaluable specimens (two were unevaluable due to contamination). So, of the four treated and infected NHPs (excluding IK14), none was positive based on detection of two RT-PCR gene targets in material obtained from the in vivo culture system. (A similar result of a positive RT-PCR result for only a single gene was found for material obtained from the xenodiagnostic ticks removed from the treated NHPs, whereas several of the untreated NHPs had a positive result for two RT-PCR gene targets on this material). There has been a justifiable precedent in other studies of NHPs for a PCR test result to be considered positive only when at least two separate gene targets were detected [3,18]. However, even if we presume that all three were true positives, and assume that the four control primates that were never tested in this way because they were only included in the histology part of the research project [2], would have tested negative, the p-value would still not have indicated a statistically significant difference

between the infected and uninfected groups (3/10 vs 0/4, $p = 0.51$). Both the absence of uninfected controls in the first part of the study and the small sample size of infected primates are serious methodological issues (Table 2) [1,2].

Comments on the results of other testing methods utilized to document residual infection by *B. burgdorferi*

The observations in this study [1,2] and those of a prior study on NHPs [3] are consistent with spontaneous resolution of *B. burgdorferi* infection over time in some, if not all, of the infected primates, indicated by the decline in the C6 antibody titer and failure to culture motile *B. burgdorferi* in the standard in vitro culture system after untreated infection (except at 1 or 2 weeks after tick feeding at the skin site where the ticks had fed and before antibiotic treatment) (Tables 3 and 4) [10]. This particular observation would seem to be consistent with the natural history of untreated *B. burgdorferi* infection in humans in the United States [19].

None of the tissues obtained post mortem from any of the NHPs grew *B. burgdorferi* in in vitro cultures [1,2]. In vitro cultures of ear, skin, heart, bladder, spleen and tibia tarsal joints of SCID mice that had been injected with the contents of xenodiagnostic ticks that had fed on *B. burgdorferi* infected NHP were also uniformly negative for *B. burgdorferi*. The authors speculated that the material obtained from xenodiagnostic ticks that had fed on infected NHPs failed to infect SCID mice because of a change in phenotype prompted by being in the tick midgut [1]. However, the authors do not try to establish this hypothesis by a control experiment using xenodiagnosis on infected (non SCID) mice and then attempting to infect SCID mice in the same manner as described for ticks that had fed on the infected NHPs.

Tissues of SCID mice that had been inoculated with the contents of xenodiagnostic ticks that had fed on *B. burgdorferi* infected NHPs were tested by RT-PCR using *ospA* and *flaB* primers [1]. No samples were positive by *ospA* RT-PCR and only one sample originating from an untreated NHP was positive by *flaB* RT-PCR; these negative findings apparently prompted the investigators to conduct a second round of xenodiagnosis for the NHPs followed by another attempt to infect SCID mice. In the second round “RNA from several mouse tissues was amplified by *ospA* primers,” although all in vitro cultures were again negative [1]. It is important to emphasize that in vitro cultures are highly sensitive and sometimes may be successful even if only a single *B. burgdorferi* cell is inoculated [20,21]. In other studies, in vitro cultures have been successful irrespective of whether the cells of *B. burgdorferi* originated in a human [5], in an animal [20,22,23], or in a tick [20,24]. Of course, like with PCR (especially nested PCRs) and IFA testing, false positive in vitro cultures have been reported [25,26]. The results of the study reviewed here, and that of other studies, are, however, consistent with persistence of spirochetal remnants [27,28].

Of note, in the histologic study, inflammatory tissue sites were found in all 10 NHPs that had been exposed to infected ticks; however, inflammatory tissue sites were also found in 3 of the 4 uninfected control NHPs that were utilized for this aspect of the study, a non-significant difference ($p=0.29$) [2]. In the NHPs with a positive IFA, there was no validation by a positive 23S rRNA probe directed at *B. burgdorferi* at the same tissue sites. In addition,

a nested quantitative RT-PCR targeting the *ospA* and *oppA-2* genes applied to tissues with evidence of inflammation failed to show any positive findings. By nested PCR testing targeting the 5S–23S ribosomal gene, positive results were obtained in 4 untreated primates and 1 treated primate, but not from the IK14 primate [1].

In the histologic study, inflammation in heart tissue was observed in primate IK14 with detection of spirochetes by the anti-OspA antibody but not with the polyclonal antiborrelial antibody, suggesting that the anti-OspA antibody finding is invalid [2]. Alternatively, there may be cross reactivity between OspA and an unidentified antigen naturally present in heart tissue of NHPs. No experimental data were provided that would explain why only a monoclonal antibody directed to OspA would be reactive with a putative *B. burgdorferi* cell, when a polyclonal antibody to multiple antigens of *B. burgdorferi* was not reactive. Also, the serologic response to OspA for all of the NHPs was non-existent throughout the experiment, suggesting minimal expression of this protein [1].

As we and others have stated before, neither the detection of DNA or mRNA establishes viability definitively, whereas a positive in vitro culture does [29–36]. IFA tends to be highly subjective and poorly reproducible [29,37]. What precautions were taken to safeguard against non-specific sticking of aggregates that might be present in the IFA reagents? Were the reagents routinely micro-centrifuged before use to remove such aggregates, the occurrence of which increases with repeated freezing and thawing of ligand reagents?

Was doxycycline exposure in this study demonstrated to be equivalent to that found in humans?

In many prior animal studies, no attempt was made to reproduce the antibiotic exposure that would be found in humans [11,38]. The doxycycline exposure in the current NHP study was not demonstrated to have been equivalent to that expected in humans. Only two trough levels were determined per primate in the current study over a 28-day period [1], and the results were based on bioassays, which can be problematic with wide discrepancies depending on the bacterium used to assess the drug levels [3,29]. Out of the few blood samples tested, it was shown that 1 of the 5 animals (20%; 95% C.I.: 1% to 72%) had much lower trough blood levels of doxycycline [1]. A number of factors can influence doxycycline blood levels in humans including whether fasting at the time the drug was taken [39,40]. Also, the urine pH can substantively affect doxycycline blood levels [41].

When considering the limitations of the study, it is clear that doxycycline blood levels should be measured by chemical assays (which have been available for many years) rather than biologic assays, and the area under the concentration time curve (AUC) determined for non-protein bound drug [29,39,40,42]. The investigators have not provided any information on the percentage of doxycycline in blood that was protein bound in this or any other species of NHP [1,3]. In humans, the $t_{1/2}$ of doxycycline ranges from 12 to 25 hours depending on the study [39]. Based on the prior study with Chinese NHPs infected with *B. burgdorferi*, it can be inferred that the $t_{1/2}$ in this animal was only approximately 5 hours [3]. A prior study that involved two Indian Rhesus macaques that received oral doxycycline without food found a $t_{1/2}$ of 6.76 hours [43]. In that study the doxycycline dosage was 5mg/kg per dose.

The 25 mg twice daily dosage chosen to treat the NHPs in the current study was actually somewhat lower than the 50 mg twice daily dosage used in a prior study from the same institution of NHPs infected with *B. burgdorferi*; in that study this higher dosage was regarded as ineffective (Table 3) [3]. Although the decision to use the 25 mg twice daily dosage was based on a separate study of Indian macaques, only 2 animals were studied, the animals were fasting and the doxycycline levels were not measured by a chemical assay [40,43].

The concept of viable but not cultivable cells of *B. burgdorferi*

The authors of this study [1,2] and several studies performed in *B. burgdorferi* infected mice by an overlapping group of investigators have suggested that there is persistence of viable but uniformly not cultivable *B. burgdorferi* despite antibiotic therapy [11,20,44], and which fail to cause disease when introduced into SCID mice [20] (SCID mice would typically develop tissue inflammation when infected with *B. burgdorferi* [45]). Why this would be expected to occur is unclear and has not been validated in several animal studies conducted by other investigators [24,27,46]. This phenomenon has also not been observed in in vitro studies of *B. burgdorferi* persists after exposure to antibiotics [47,48].

Although the concept of viable but not cultivable is clearly controversial, the fact that no cultivable cells of *B. burgdorferi* remain after antibiotic therapy has been confirmed in a mouse system in which infected mice were treated with just a 5 day course of ceftriaxone, and following treatment extracts of the entire mouse devoid of the gastrointestinal tract were cultured in vitro, but viable *B. burgdorferi* could not be recovered [49]. Similarly, in human studies, there has been no convincing evidence for the persistence of cultivable *B. burgdorferi* cells in almost every treated patient in the United States who has been evaluated to date, when using established and reliable culture methods, including patients with antibiotic-refractory arthritis or with post-treatment Lyme disease syndrome (PTLDS) [5,21,50–53]. The term PTLDS refers to patients with residual and persistent, subjective symptoms despite treatment with antibiotics that effectively resolved an objective manifestation, such as the erythema migrans skin lesion [5].

Conclusions

In conclusion, the current primate study [1,2], unfortunately, does not clarify the many concerns and questions raised by a prior primate study by Embers et al. [3,29]. Numerous serious methodologic issues were identified in the current study [1,2]. Most significantly, this study did not establish the existence of viable *B. burgdorferi* in doxycycline-treated NHPs. The current study certainly does not invalidate the efficacy of antibiotic therapy for *B. burgdorferi* infection in patients or the utility of serologic testing for establishing a diagnosis of extracutaneous manifestations of Lyme disease. The study also does not clarify the etiology of PTLDS.

Acknowledgments

The authors thank Dr. Phil Baker, Julia Singer, Sophia Less, Artemio Zavalla and Lisa Giaratano for their assistance.

Funding: This publication was made possible in part by support from CTSA Grant Number UL1 TR001863 from the National Center for Advancing Translational Science (NCATS), a component of the National Institutes of Health (NIH), to EDS. The findings and conclusions of this paper are those of the authors and do not necessarily represent the official position of the NIH.

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Highlights

1. A doxycycline treatment trial of non-human primates (NHP) was critically reviewed.
2. NHP were infected with *Borrelia burgdorferi* by at least 10 tick bites.
3. Doxycycline exposure was not shown to be similar to humans receiving usual doses.
4. Multiple other methodologic concerns were identified.
5. The findings did not prove persistence of viable *B. burgdorferi* in treated NHP.

Table 1

Contradictions between Two Publications Describing the Same Treatment Trial

Variable	Report 1 [1]	Report 2 [2]
Age of primates	2–3 years	3–4 years *
Number of ticks initially placed on primate	20	10
Dose of doxycycline	4.2–5.7 mg/kg twice daily, i.e., 25 mg twice daily	5 mg/kg twice daily

* Likely due to the investigators using the age of the NHPs when they were sacrificed for this phase of the study, rather than the age at study entry.

Table 2

Methodological Problems (also see text)

1	Lack of adequate numbers and types of controls.
A.	Failure to include doxycycline treated but uninfected primates.
B.	Failure to include controls bitten by uninfected ticks.
C.	Failure to include any uninfected controls, except for the histopathologic section of the study.
2	Lack of data needed to evaluate antibiotic-exposure and to make comparisons with what would be expected in humans.
A.	Failure to frequently monitor doxycycline blood levels.
B.	Failure to measure peak blood level of doxycycline at any time point.
C.	Failure to use a non-biologic assay for measurement of doxycycline blood levels.
D.	Failure to measure protein binding of doxycycline in the NHPs studied.
E.	Failure to provide data on the area under the curve for unbound doxycycline, i.e., the drug level remaining after excluding the amount of drug bound to serum proteins.
F.	Failure to demonstrate that antibiotic exposure was similar to that expected in humans receiving doxycycline treatment, due to the above concerns.
3	Sample size very small precluding showing statistical significance of findings.
4	Assumption that a positive RT-PCR proves viability of <i>Borrelia burgdorferi</i> .
5	Use of a NHP with serologic evidence consistent with having had a prior undefined spirochetal infection.
6	Use of an “in-vivo culture” method without demonstrating either spirochetal motility or ability to subculture “isolates” that were “recovered” in the assay under standard in vitro culture conditions.
7	Failure to test for doxycycline resistance in “persisters.”
8	Failure to document that the ticks used to feed on the NHPs were free of other pathogens.
9	Failure to conduct immunofluorescence testing (IFA) in tissues without inflammation.
10	Failure to indicate how many of the xenodiagnostic specimens were positive on midgut smear using an IFA with polyclonal anti-Borrelia species antibody (what was stated was that there were “few positives”) and instead reporting on the results of an IFA with an anti-OspA antibody (that apparently was more likely to be positive).
11	Failure to explain in number 10 above why an IFA with the polyclonal antibody would be expected to be less sensitive than with the anti-OspA antibody.
12	Failure to document that the pathologists were blinded with regard to the tissue samples from the uninfected primates.
13	Failure to explain why in some of the IFA images the spirochetes have atypical configurations relative to the morphology expected for live and intact <i>B. burgdorferi</i> .
14	Inability to judge antibiotic efficacy based on resolution of an objective clinical manifestation of <i>Borrelia burgdorferi</i> infection, since none occurred.

Comparison of Two Treatment Studies of Primates Infected with *Borrelia burgdorferi* and Treated with a 28-day Course of Doxycycline

Table 3

Characteristic	2012 Study [3]	2017 Study [1,2]	Comment
Type of Primate	Chinese rhesus macaques 3–4 years of age N=5 (3 treated, 2 untreated)	Indian rhesus macaques 2–3 years of age N=10 (5 treated, 5 untreated)	In 2017 study, the origin of the uninfected control primates was not stated (e.g., were they Chinese, Indian or other?); uninfected controls used only for the histopathological analysis.
Route of Infection	3 needle inoculations: 2 subcutaneous, 1 intradermal, each containing 1×10^8 organisms	10 bites from infected ticks	In 2017 study, the ticks were infected by capillary tube of an in vitro cultivated strain of <i>Borrelia burgdorferi</i> .
Strain of <i>Borrelia burgdorferi</i>	B31.5A19	B31.5A19	
Timing, Dosage, and Duration of Doxycycline	4 months after needle inoculation; 50 mg twice daily po with food for 28 days or >12mg/kg/day	4 months after tick inoculation; 25 mg twice daily po with food for 28 days or 4.2–5.7 mg/kg twice daily	
Outcome Parameters	In vitro culture; standard PCR targeting <i>flaB</i> and <i>OspC</i> genes, histopathology/immunofluorescence to localize inflammation and spirochetes; C6 serology; xenodiagnosis; standard RT-PCR targeting <i>OspA</i> and <i>bbl26</i> genes.	In vitro culture; 5-antigen serologic assay; standard PCR targeting <i>flaB</i> , <i>OspA</i> , <i>OspC</i> ; xenodiagnosis; tick contents underwent individual staining and culture and pooled for RT-PCR and SCID mouse inoculation; novel in vivo culture system; immunostaining; in situ hybridization targeting the 23rRNA of <i>B. burgdorferi</i> .	In the 2017 study nested PCR was used for tissue DNA detection targeting the 5S–23S intergenic sequence, and a nested RT-PCR was used targeting the <i>ospA</i> and <i>oppA-2</i> genes.

Table 4

Study Findings That Suggest That No Viable Spirochetes Were Found

1	No positive in vitro culture of any material for which culture was attempted, aside from the skin cultures done within 1 month of tick inoculation and prior to antibiotic treatment.
2	Inability to convincingly infect SCID mice with material from “positive” xenodiagnostic ticks or to apparently cause disease in these mice.
3	No objective clinical manifestations of Lyme disease in the primates studied.
4	No demonstration of spirochetal motility.

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