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MAJOR ARTICLE

Xenodiagnosis to search for *Borrelia burgdorferi* after antibiotic treatment of Lyme disease: a prospective cohort study

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Background: Some patients report non-specific symptoms after antibiotic therapy for Lyme disease (LD), raising questions about ongoing infection, despite no compelling evidence. We investigated whether xenodiagnosis could detect *Borrelia burgdorferi* in such patients, and if positive results correlated with symptoms.

Methods: Participants were adults who completed antibiotic treatment for LD 3-12 months earlier (post-therapy, n=40) or had persistent symptoms for ≥ 12 months after treatment, (post-treatment

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LD symptoms [PTLDS], n=20). Controls included one patient with erythema migrans (EM), one patient with untreated Lyme arthritis (LA), and 9 healthy volunteers (HV). Participants had 25-30 larval *Ixodes scapularis* ticks placed; ticks were collected 3-6 days later and tested for *B. burgdorferi.* The primary analysis evaluated if *B. burgdorferi* detection by xenodiagnosis was associated with persistence of symptoms in patients during the first year after treatment. This trial is registered with ClinicalTrials.gov, NCT02446626.

Results: Recovered ticks included 402 from post-therapy, 314 from PTLDS, 30 from the EM patient, 11 from the LA patient, and 80 from HV. All ticks tested negative for *B. burgdorferi* except for 1 tick from a recovered patient. An unplanned interim analysis led to the early termination of the study for futility.

Conclusion: Xenodiagnosis with larval *I. scapularis* ticks showed no evidence of *B. burgdorferi* in most patients after treatment, irrespective of symptoms. This may be due to absence of bacteria or to the low sensitivity of the technique in humans. This method is unlikely to detect persistent *B. burgdorferi* infection in humans and further research on the use of xenodiagnosis is unwarranted.

Keywords: Xenodiagnosis, *Ixodes scapularis*, Lyme disease, Post-Treatment Lyme Disease Symptoms, *Borrelia burgdorferi*

INTRODUCTION

Lyme disease (LD) starts at the tick bite site, with the erythema migrans (EM) skin lesion, followed by dissemination to other locations [1]. Most patients are successfully treated with recommended antibiotic therapy [2], but some have unexplained non-specific complaints lasting at least 6 months, named post-treatment Lyme disease symptoms (PTLDS) [3]. The cause of PTLDS is unknown [3, 4]. Whether *Borrelia burgdorferi* could persist after treatment remains a contentious issue. *B. burgdorferi* DNA and mRNA were detected in tissues from antibiotic-treated animals [5-13] and in *Ixodes scapularis* ticks feeding on these animals [7-12], but the implications of these findings for human disease remain unknown [14]. The first study using *I. scapularis* larva for xenodiagnosis of *B. burgdorferi* infection in humans [15] showed the procedure to be well tolerated, and ticks from a patient with PTLDS tested positive for *B. burgdorferi* DNA. In this study, we assessed whether detection of *B. burgdorferi* by xenodiagnosis was associated with symptoms after treatment.

METHODS

Study design and participants

The study was performed at five US centers, approved by the institutional review boards and conducted under an investigational device exemption approved by the US Food and Drug

Administration. All participants provided written informed consent. This study is registered with ClinicalTrials.gov NCT02446626.

All participants were ≥18 years of age. Post-therapy participants were diagnosed and treated for LD ≥3 but ≤12 months before the xenodiagnostic procedure. PTLDS participants were diagnosed and treated for LD > 12 months before the procedure and had persistent or relapsing symptoms that began or worsened within 6 months of LD diagnosis and treatment. All patients fulfilled the case definition of confirmed or probable LD [16] and antibiotic treatment followed guidelines [17]. One patient with EM and one patient with untreated Lyme arthritis (LA) were recruited as possible positive controls. Healthy volunteers (HV) were seronegative for *B. burgdorferi* antibodies and had no history of LD (Supplementary Figure S1).

Study procedures

Larval *I. scapularis* ticks were obtained from a laboratory-maintained colony at Tufts Veterinary School, as described [15]. Participants had 25 to 30 ticks placed under a dressing[18], with ticks collected three to six days later. If fewer than 14 engorged ticks were recovered, placement could be repeated. Participants completed a diary card for the first month and were contacted 7-10 days post-tick removal for adverse event assessment. Clinical evaluations were done at one- and three-months post-tick removal. Patients in the post-therapy group were also evaluated at 12 months after LD treatment. During evaluations, clinicians reviewed a symptom questionnaire covering the prior week and considered symptom relatedness to LD. Participants with at least one symptom possibly related to LD were classified as symptomatic. Quality of life and fatigue were assessed using the Short Form-36 version2 (SF-36v2), the Quality of Life in Neurological Disorders Fatigue scale short-form (Neuro-QoL-Fatigue), and the Fatigue Severity Scale (FSS).

The SF-36v2, Neuro-QoL-Fatigue and FSS data from the 12-month timepoint of 21 individuals, and from the tick placement visit of four individuals were used to develop models to quantitate symptom severity in PTLDS and previously published [19]. The logistic regression (LR) model uses Neuro-QoL-Fatigue, SF-36v2 Physical Functioning scale and Mental Health component (MCS) scores, and the decision tree (DT) model uses the Neuro-QoL-Fatigue score to predict membership in recovered versus symptomatic groups.

Testing of xenodiagnostic ticks

Initially, recovered ticks were divided between Tufts University (TU) and Ibis Biosciences (IB). At TU, ticks were tested by culture, outer surface protein A (OspA) PCR, and injection of lysates into severe combined immunodeficiency (SCID) mice with subsequent culture and PCR [15]. At IB, ticks were tested using an isothermal amplification reaction followed by PCR electrospray ionization mass spectrometry (IA/PCR/ESI-MS) for eight loci targeting seven *B. burgdorferi* genes [15]. With IB closure, testing was transferred to the Center for Infection and Immunity (CII) at Columbia University, where ticks were tested using a multiplex PCR targeting the same eight loci, and/or OspA qPCR. The Tick-Borne Disease Capture Sequencing assay (TBDCapSeq) [20]

was used to re-test DNA extracts from ticks, as described in the results. Details are available in Supplementary Materials.

Statistical analysis

A sample size of 86 individuals was planned to detect a between-group difference in the positivity rate if *B. burgdorferi* detection by xenodiagnosis was ≤ 5% in recovered and ≥30% in symptomatic individuals in the post-therapy group, with a Type I error rate of 0.05 and 80% power. Wilcoxon rank sum tests were used to compare various outcomes between groups. Fisher's exact tests were used to compare proportions. Exact 95% confidence intervals were calculated using the Clopper-Pearson method. When pairwise testing was performed, the Holm procedure was used to adjust for multiple comparisons. All analyses were done in R version 4.3.2, and p values ≤0.05 were considered statistically significant.

RESULTS

Subject characteristics

Between July 2015 and February 2020, 72 participants enrolled into the study, with 71 undergoing the xenodiagnostic procedure. These included 40 patients in the post-therapy group, and 20 in the PTLDS group, one patient with EM and one untreated patient with LA, and nine HV. The patient with EM had multiple skin lesions; ticks were placed at the primary lesion and collected at days six and seven after start of antibiotic treatment. Ten patients (seven post-therapy and three PTLDS) had a second procedure.

At tick placement, 25 post-therapy patients had recovered to their pre-LD health status (post-therapy-recovered), and 15 had symptoms possibly related to LD (post-therapy-symptoms). The most common manifestation was a single EM (N=21, 52.5%) (Table 1). The interval from start of symptoms to antibiotic treatment was longer for symptomatic compared to recovered patients (28 vs 5 days, p= .014), while the interval between treatment to xenodiagnostic procedure was similar (248 vs 304 days, p=NS) (Table 1).

The 20 PTLDS patients were enrolled a median of 2.6 years after LD diagnosis. The most common manifestation was early Lyme neuroborreliosis (N=7, 35%) (Table 1). PTLDS patients received a median of 97.5 days of antibiotic therapy (range=13-318 days). Eleven patients received more than 14 days of intravenous ceftriaxone (up to 84 days). PTLDS patients had longer interval from initial symptoms to treatment compared to recovered patients (40.5 vs 5 days p= .004) (Table 1).

Symptoms and Quality of Life Assessments

Post-therapy-recovered and HV had fewer symptoms (n=1) during the previous week (irrespective of LD relationship) compared to post-therapy-symptoms (n=4, p=.019) and PTLDS (n=6 symptoms; p= <0.05). Fatigue, sleep difficulties, concentration and memory complaints were more

common in the post-therapy-symptoms and PTLDS patients compared with post-therapy-recovered and HV (Table 2 and Supplementary Table S1). The median number of symptoms and the prevalence of each symptom was similar between post-therapy-symptoms and PTLDS. SF-36v2 Physical Health component (PCS), SF-36v2 MCS, Neuro-QOL-Fatigue and FSS scores were significantly different between post-therapy-recovered compared with post-therapy-symptoms ($p \leq 0.01$for all) and PTLDS ($p < 0.001$ for all), while similar between post-therapy-symptoms and PTLDS (Figure 1).

Assessment of Symptoms at 12 Months after Antibiotic Treatment

Of the 40 patients enrolled in the post-therapy group, 39 participants were evaluated at one year after treatment. One patient (recovered at eight months post-therapy) did not return for the 12-month assessment. Thirty patients were assessed as recovered, and nine had symptoms related to LD. For eight participants (six recovered and two symptomatic patients), the tick placement and the 12-month visits were combined. For the other 31 participants, the median interval between the two visits was 105 days (range=28-246 days). Symptoms related to LD resolved during this interval for seven participants. One participant developed symptoms possibly related to LD but did not complete the quality-of-life questionnaires at 12 months. Symptomatic patients had higher level of fatigue, as reflected by Neuro-QOL-Fatigue and FSS scores ($p=0.0011$ and $p=0.0029$, respectively) (Figure 2). Using the LR and DT models to assign status [19], 15 and 14 individuals were symptomatic, respectively. For the 17 individuals not included in the development of the models, the agreement rate with clinical categorization was 47% for the LR model and 65% for the DT model. The agreement between the models was 82% (Supplementary Table S2).

Testing of xenodiagnostic ticks

A total of 837 ticks from 68 participants were tested for *B. burgdorferi.* These included 402 ticks from 38 individuals in the post-therapy group, 314 ticks from 20 PTLDS individuals, 30 ticks from the EM patient, 11 ticks from the LA patient, and 80 ticks from eight HV. Three participants (one HV and two post-therapy patients) had no ticks recovered. Of these 837 ticks, 390 were tested by OspA PCR (385 also tested by culture), 245 by IA/PCR/ESI-MS and 202 by multiplex PCR/OspA qPCR. Lysates from ticks recovered from 60 participants were injected into SCID mice. The number of ticks tested by different assays are shown in Figures 3, Supplementary Figure S2 and Table S3. No samples were positive using these methods. The upper limit of the 95% confidence intervals for the different molecular assays for post-therapy and PTLDS groups combined varied from 1.1 to 2% (Table 3).

After the development of the TBDCapSeq assay [20], this assay was used to test DNA extracts from 198 ticks prepared at CII and 232 ticks prepared at IB (total=430 samples). Seventeen samples produced low quality of sequencing libraries or generated insufficient sequencing read counts and were excluded. Seventy-four DNA extracts processed and subsequently aliquoted at TU for analysis at CII were found to contain trace contamination with *B. burgdorferi*, as sequence

analysis demonstrated close homology to known laboratory strains. Consequently, the remainder of the samples processed at TU were not analyzed using TBDCapSeq.

The 430 samples tested by TBDCapSeq included 211 ticks from 34 individuals in the post-therapy group, 151 ticks from 19 PTLDS patients, 17 ticks from the EM patient, 5 ticks from the LA patient, and 46 ticks from seven HV (Figure 3 and Supplementary Table S3). Only one tick sample was positive for *B. burgdorferi*. This result was from a post-therapy-recovered Lyme arthritis patient, who had 21 ticks recovered, with 11 ticks tested using TBDCapSeq. TBDCapSeq analysis revealed one sequencing library positive for *B. burgdorferi*. The library generated 14,238 reads that mapped to *B. burgdorferi* . None of the other 23 samples sequenced on the same flow cell (including the 10 ticks from the patient) generated *B. burgdorferi* reads. To confirm this result, we re-sequenced the original positive library and made new sequencing libraries using the DNA of the positive sample and two negative samples. Each new library was constructed with alternative barcodes relative to the initial test. This analysis generated 62,597 reads from the original library and 75,166 from the new library (Supplementary Table S4). The sequencing reads were mapped to the *B. burgdorferi* B31, 297 and N40 genomes. Analysis of the sequences showed that the genome obtained from the tick did not correspond to any of these strains. A partial sequence from the *ospC* gene showed the highest similarity to a strain with *ospC* type N.

Therefore, xenodiagnosis using larval *I. scapularis* showed no evidence of *B. burgdorferi* in almost all recovered ticks from post-therapy patients and in none from PTLDS patients. However, ticks recovered from the patient with EM receiving antibiotic treatment, and the untreated LA patient were also negative for *B. burgdorferi* (Table 3).

Protocol completion futility analysis

Due to challenges in study enrollment, along with approaching 50% accrual and results indicating no evidence of *B. burgdorferi* in most samples, an unplanned interim analysis was initiated by the investigators to determine the likelihood of achieving the study goals. The analysis included data from 37 participants in the post-therapy cohort (28 recovered and nine with symptoms), excluding two individuals without recovered ticks and one lacking 12-month follow-up data. There were zero positives in the symptomatic group and one positive result in the recovered group (Supplementary Methods). The conditional power was 0.027%. Using the models to assign status, the conditional power was 0.654% for the LR model and 0.398% for the DT model. These low conditional power values led to early halting of the study, as stopping a study for futility is recommended if the conditional power is below 10%.

Adverse events

The xenodiagnostic procedure was well-tolerated, with no severe adverse events related to the procedure. Mild itching at the tick bite sites occurred in 43/71 individuals (61%) or 47/81 procedures (58%) (Table 4). For the ten individuals who had a second procedure, the incidence of

itching was similar between the procedures, with four experiencing mild itching and six having no itching at both procedures.

DISCUSSION

There is debate whether unexplained symptoms after LD treatment could signal persistent infection. The absence of sensitive direct diagnostic tests plays a significant role in this controversy. Xenodiagnosis using *I. scapularis* ticks, which mimics the natural transmission, is a minimally invasive method to detect *B. burgdorferi*. The first human study using this approach demonstrated the procedure to be feasible, but further research was needed to determine sensitivity and the significance of a positive result [15]. In this study, we investigated the connection between detection of *B. burgdorferi* through xenodiagnosis and persistence of symptoms in patients diagnosed and treated for LD within the prior year, and the detection rate in patients with PTLDS.

The main finding of this study is that xenodiagnosis using larval *I. scapularis* ticks shows no evidence of *B. burgdorferi* in almost all patients after antibiotic therapy for LD, irrespective of symptoms. As results were overwhelming negative, an unplanned futility analysis was performed. The very low conditional power (<1%) led to the decision to stop the study. Early stopping, though a limitation, is justified by the low chance of achieving the planned endpoint. This protects participants from entering a study with little chance of success and saves resources that can be allocated to more promising research.

The interpretation of negative results in the post-therapy and PTLDS subjects continues to be unclear [14]. We were unable to ascertain if negative results are due to absence of bacteria or low sensitivity of the technique in humans, as xenodiagnosis was negative in the two possible positive controls. However, both controls had factors potentially impacting the results. The patient with multiple EM had ticks collected at days 6 and 7 of antibiotic therapy, 48-72 hours longer than the EM patient enrolled in the initial study [15]. In the untreated LA case, it is possible that high levels of anti-*B. burgdorferi* antibodies, particularly against OspA, could negatively impact *B. burgdorferi* acquisition by ticks. Anti-OspA antibodies are known to decrease *B. burgdorferi* acquisition by ticks from infected hosts [21], and anti-OspA antibodies occur in almost 80% of patients with Lyme arthritis [22]; however, OspA antibodies were not assessed in this patient.

The interpretation of the single positive result using the TBDCapSeq assay is complex. Since there was only one positive sample, this result could represent an artifact, generated through accidental introduction of outside-source *B. burgdorferi* DNA. However, several aspects of the TBDCapSeq workflow make this unlikely. The CII laboratory has never cultured *B. burgdorferi*. Mouse tissues infected with B31 or N40 strains have been analyzed by TBDCapSeq, but the sequence obtained from the xenodiagnostic tick was dissimilar to both strains. The laboratory has examined whole blood samples from LD patients by TBDCapSeq [23]. Of these, <10% were positive, and only at a very low read count (<1000 reads), making these samples an unlikely source of contamination.

Finally, the positive tick was tested along with 23 other ticks that tested negative for *B. burgdorferi*, making extraction and sequencing reagents unlikely contamination sources.

The partial sequence from the *ospC* gene showed the highest similarity to a strain with *ospC* type N. While OspC type A and K predominates in disseminated disease in the northeastern US, OspC type N is also rarely found in invasive disease [24] but in only one of 49 synovial fluids from LA patients [25]. While we obtained reads from the chromosome and plasmids, only partial genome fragments were recovered and may not represent viable *B. burgdorferi* [14].

It is possible that xenodiagnosis using *I. scapularis* nymphs, instead of larvae, might increase the sensitivity of the procedure as nymphs take a larger amount of blood and feed for a longer period than larval ticks. However, *B. burgdorferi* prevalence in xenodiagnostic ticks was comparable across mouse studies using larvae [7, 9, 10, 13], and nymphs [5, 11]. Two non-human primate (NHP) studies used nymphs [8, 12]. For both studies, it is mentioned that *B. burgdorferi* was detected in xenodiagnostic ticks, but the number of positive ticks was not provided. Of note, *B. burgdorferi* is seldom found at late time-points even in untreated immunocompetent animals in the NHP model and culture is almost always negative [8, 12, 26].

This brings us to the role of host reservoir competence and the use of xenodiagnosis to ascertain infection status. A main requirement for a reservoir host is to be capable of transmitting spirochetes to feeding ticks [27]. However, hosts can acquire infection and not be able to transmit to ticks. Host infectivity to a permissive vector depends on many factors, including innate and adaptive immunological responses and duration of infection [28, 29]. Even with reservoir hosts, there is decrease ability to infect ticks with time after infection [30, 31]. Humans are considered non-reservoir or "dead-end" hosts, as are macaques and deer [32, 33], and may not have sufficient bacterial burden to be a source of infection to feeding ticks due to control of the infection, particularly at later timepoints.

This study reinforces the importance of early diagnosis and treatment of LD. Delayed antibiotic treatment was associated with prolonged symptoms and lower quality of life. Reassuringly, the number of recovered patients increased over time, indicating ongoing improvement. The discrepancy between the statistical models for PTLDS [19] and clinical categorization indicates that further refinements are necessary and highlights the need for an objective biomarker for more accurate PTLDS diagnosis.

In conclusion, our study showed no evidence of *B. burgdorferi* in almost all xenodiagnostic ticks from LD patients after antibiotic therapy. While the interpretation of these negative results remains unclear, further research using xenodiagnosis to detect *B. burgdorferi* infection in humans is unlikely to show different results, and therefore unwarranted.

NOTES

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Data sharing: The relevant data are presented in the manuscript and the supplementary material. Additional de-identified data can be made available upon request to the corresponding author by qualified researchers whose proposed use of the data has been approved by the National Institute of Health Institutional Review Board and subject to the establishment of a data sharing agreement.

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Table 1. Baseline participant characteristics

Group	**Post-Therapy Recovered**	**Post-Therapy Symptoms**	**PTLDS**	**HV**
Number of participants	25	15	20	9
Median age, years [range]	59 [27-74]	61 [23-70]	59.5 [26-79]	47 [24-59]
Female sex (n)	9 (36%)	8	9	4
Race (n)				
Multiple	1 (4%)	0 (0%)	0 (0%)	2
White	24 (96%)	15	20	7
Ethnicity				
Hispanic or Latino	2 (8%)	0 (0%)	0 (0%)	3
Non-Hispanic or Latino	23 (92%)	14	20	6
Unknown or Not Reported	0 (0%)	1	0 (0%)	0 (0%)
Lyme disease presentation				
Single erythema migrans	15	6	3	NA
Multiple erythema migrans	3	0 (0%)	1	NA
Flu-like illness with seroconversion	2	3	3	NA
Early Lyme neuroborreliosis	1	2	7	NA
Carditis	2	0 (0%)	0 (0%)	NA
Lyme arthritis	2	3	4	NA
Late Lyme neuroborreliosis	0 (0%)	1	2	NA
Days from Symptoms Onset to Start of Therapy	5 [0 - 79]	28 [1 - 1041]	40.5 [2 - 1387]	NA
Days from Start of Therapy to Procedure	304 [114-373]	248 [139-380]	947 [450-6636]	NA

Data are n (%) or median [range]. Table does not include the patient with acute erythema migrans and the patient with untreated Lyme arthritis. PTLDS=post-treatment Lyme disease symptoms. HV=healthy volunteers. N=number

Table 2. Symptoms at tick placement

Placement Visit	PTR	PTS	PTLDS	HV	P value (PTR vs PTS)	P value (PTR vs PTLDS)	P value (PTS vs PTLDS)	P value (PTR vs. HV)	P value (PTS vs. HV)	P value (PTLDS vs. HV)
Joint Pain	12 (48 %)	13 (87 %)	14 (70%)	2 (22 %)	0.099	0.673	0.673	0.673	0.018	0.162
Muscle Pain	3 (12 %)	3 (20 %)	8 (40%)	1 (11%)	1	0.245	1	1	1	1
Dizziness	1 (4%)	2 (13 %)	6 (30%)	0 (0%)	1	0.202	1	1	1	0.687
Fatigue & Malaise	6 (24 %)	12 (80 %)	18 (90%)	1 (11%)	0.004	<0.001	1	1	0.007	<0.001
Feverish	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1	1	1	1	1	1
Headaches	5 (20 %)	3 (20 %)	10 (50%)	2 (22 %)	1	0.335	0.446	1	1	0.936
Paresthesias	6 (24 %)	4 (27 %)	9 (45%)	1 (11%)	1	1	1	1	1	0.643
Stiff neck	2 (8%)	4 (27 %)	8 (40%)	0 (0%)	0.695	0.085	0.977	1	0.777	0.166
Concentration and memory complaints	1 (4%)	8 (53 %)	13 (65%)	1 (11%)	0.003	<0.001	0.93	0.93	0.241	0.057
Difficulties with finding and	1 (4%)	4 (27 %)	9 (45%)	0 (0%)	0.226	0.014	0.777	1	0.777	0.135

recalling words										
Mood complaints	1 (4%)	2 (13 %)	6 (30%)	0 (0%)	1	0.202	1	1	1	0.687
Sleep problems	4 (16 %)	9 (60 %)	12 (60%)	0 (0%)	0.025	0.021	1	1	0.025	0.02
Tinnitus	3 (12 %)	4 (27 %)	4 (20%)	2 (22 %)	1	1	1	1	1	1
Number of symptoms	1 (0-6]	4 [1-9]	6 [1-11]	1 [0-3]	0.019	0.007	0.165	0.815	0.019	0.012

Data are n (%) or median [range] of participants presenting with the symptom in the week prior to the tick placement visit, unless otherwise stated. Comparisons between the 4 groups were performed using pairwise Wilcoxon rank sum test and adjusting the p-values for 6 comparisons using Holm's method. PTR= Post-Therapy Recovered. PTS= Post-Therapy Symptoms. PTLDS=post-treatment Lyme disease symptoms. HV=healthy volunteers.

Table 3. Testing of xenodiagnostic ticks

Presentation	**Culture**	**OspA PCR**	**SCID mouse assay***	**IA/PCR/ ESI-MS**	**Multiplex PCR and/or OspA qPCR**	**TBDCapSeq**	**All Tests**
Acute EM	0/13 = 0 (0, 0.247)	0/13 = 0 (0, 0.247)	0/1 = 0 (0, 0.975)	0/17 = 0 (0, 0.195)	ND	0/17 = 0 (0, 0.195)	0/30 = 0 (0, 0.116)
Lyme Arthritis	0/6 = 0 (0, 0.459)	0/6 = 0 (0, 0.459)	0/1 = 0 (0, 0.975)	ND	0/5 = 0 (0, 0.522)	0/5 = 0 (0, 0.522)	0/11 = 0 (0, 0.285)
Post Therapy	0/185 = 0 (0, 0.020)	0/188 = 0 (0, 0.019)	0/32 = 0 (0, 0.109)	0/121 = 0 (0, 0.030)	0/93 = 0 (0, 0.039)	1/211 = 0.005 (0.0001, 0.026)	1/402 = 0.002 (0.00006, 0.014)
PTLDS	0/147 = 0 (0, 0.025)	0/149 = 0 (0, 0.024)	0/18 = 0 (0, 0.185)	0/67 = 0 (0, 0.054)	0/98 = 0 (0, 0.037)	0/151 = 0 (0,0.024)	0/314 = 0 (0, 0.012)
Post Therapy plus PTLDS	0/332 = 0 (0,0.011)	0/337 = 0 (0,0.011)	0/50 = 0 (0,0.071)	0/188 = 0 (0,0.019)	0/191 = 0 (0,0.019)	1/332 = 0.003 (0.00008, 0.02)	1/716 = 0.001 (0.00004, 0.008)

Healthy Volunteer	0/34 = 0 (0,0.103)	0/34 = 0 (0,0.103)	0/8 = 0 (0,0.369)	0/40 = 0 (0,0.088)	0/6 = 0 (0,0.459)	0/46 = 0 (0,0.077)	0/80 = 0 (0,0.045)

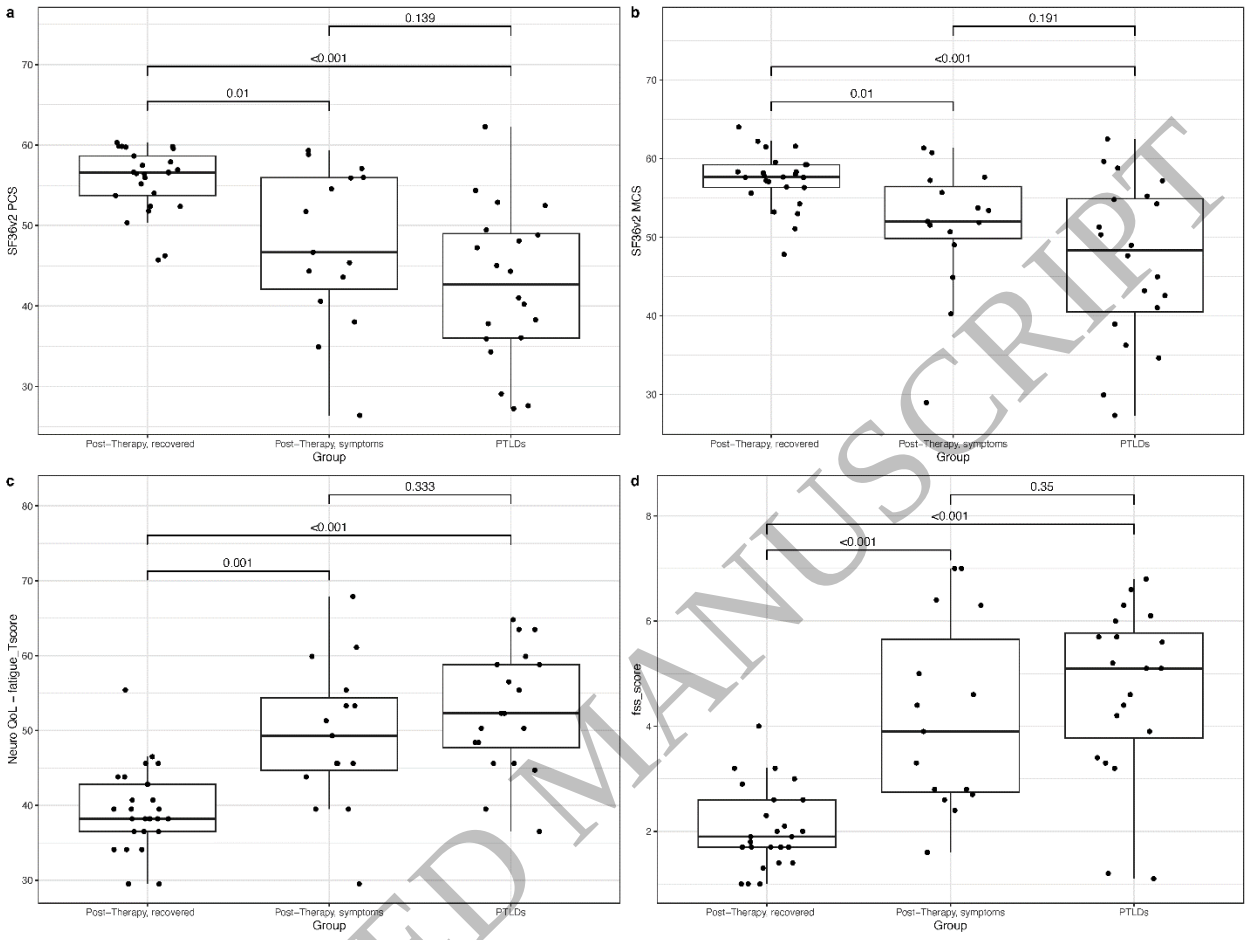
Data are number of positive results/number of ticks tested or *assay performed (exact Clopper-Pearson 95% confidence interval). OspA = outer surface protein A. PCR = polymerase chain reaction. qPCR= quantitative PCR. SCID: severe combined immunodeficiency. IA/PCR/ESI-MS = isothermal amplification PCR electrospray ionization mass spectrometry. TBDCapSeq = Tick-Borne Disease Capture Sequencing assay. EM = erythema migrans. PTLDS = post-treatment Lyme disease symptoms. ND = not done.

Table 4. Site reactions

Tick Bite Site Reaction	**Number of Participants with reaction**	**Number of Procedures with reaction**
Pruritus	43 (61%)	47 (58%)
Pain/ Tenderness	21 (30%)	22 (27%)
Erythema	3 (4%)	3 (4%)
Vesicles	3 (4%)	3 (4%)

Data are n (%)

Figure 1. Health and fatigue scores at tick placement visit.



Corresponding data are in Supplementary Table S1. Computed pairwise Wilcoxon rank sum test p-values to test differences between the 3 different pairs of groups and adjusted for 3 comparisons using Holm's method. PTLDS= Post treatment Lyme disease symptoms. SF-36v2= Short Form-36 version 2. PCS= Physical Component Summary. MCS=Mental Component Summary. Neuro-QoL-Fatigue= Quality of Life in Neurological Disorders Fatigue scale short-form. FSS= Fatigue Severity Scale.

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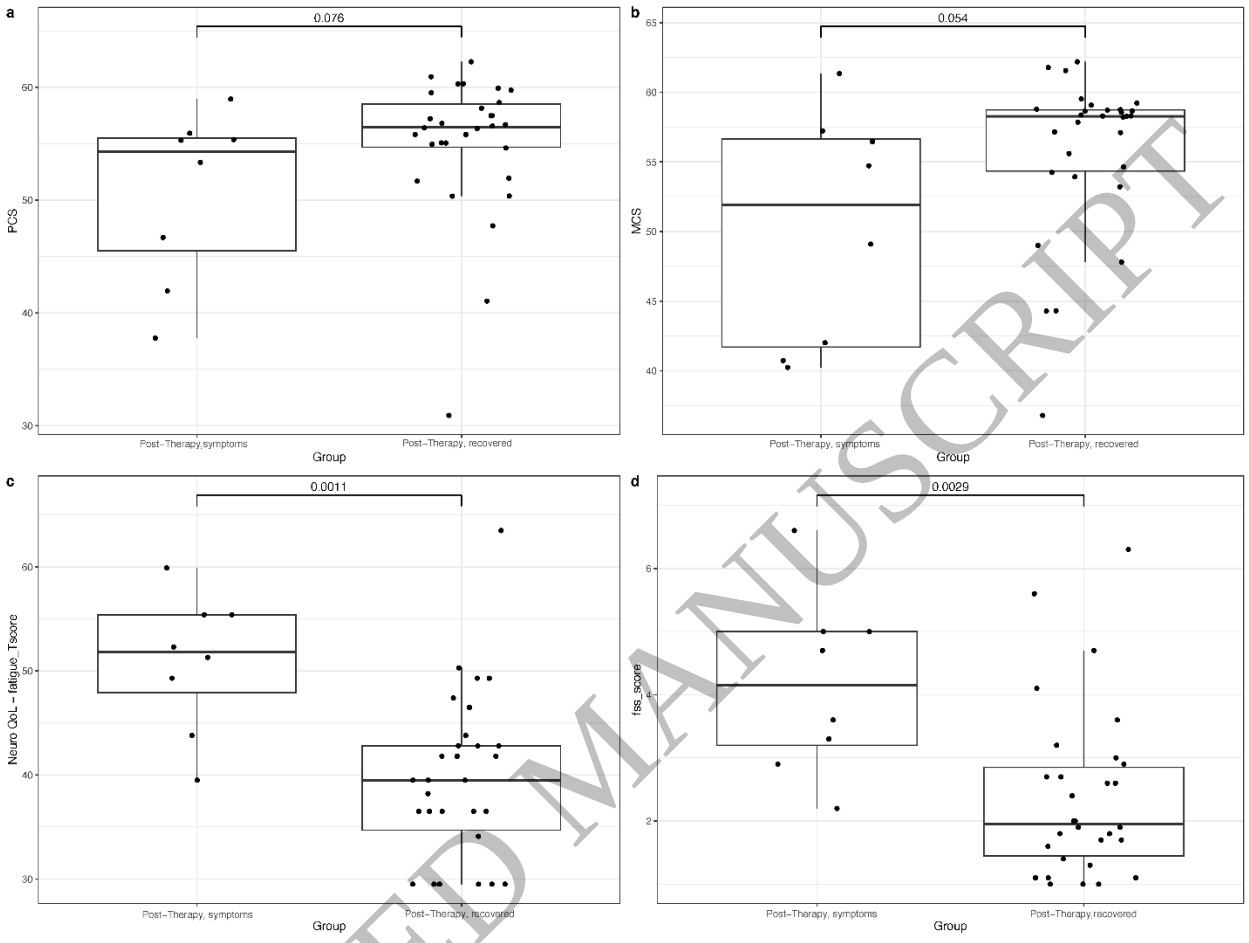
Figure 2. Health and fatigue scores at 12 months after antibiotic treatment



Corresponding data are in Supplementary Table S1. p values were calculated using the Wilcoxon rank sum test. PTLDS= Post treatment Lyme disease symptoms. SF-36v2= Short Form-36 version 2. PCS= Physical Component Summary. MCS=Mental Component Summary. Neuro-QoL-Fatigue= Quality of Life in Neurological Disorders Fatigue scale short-form. FSS= Fatigue Severity Scale.

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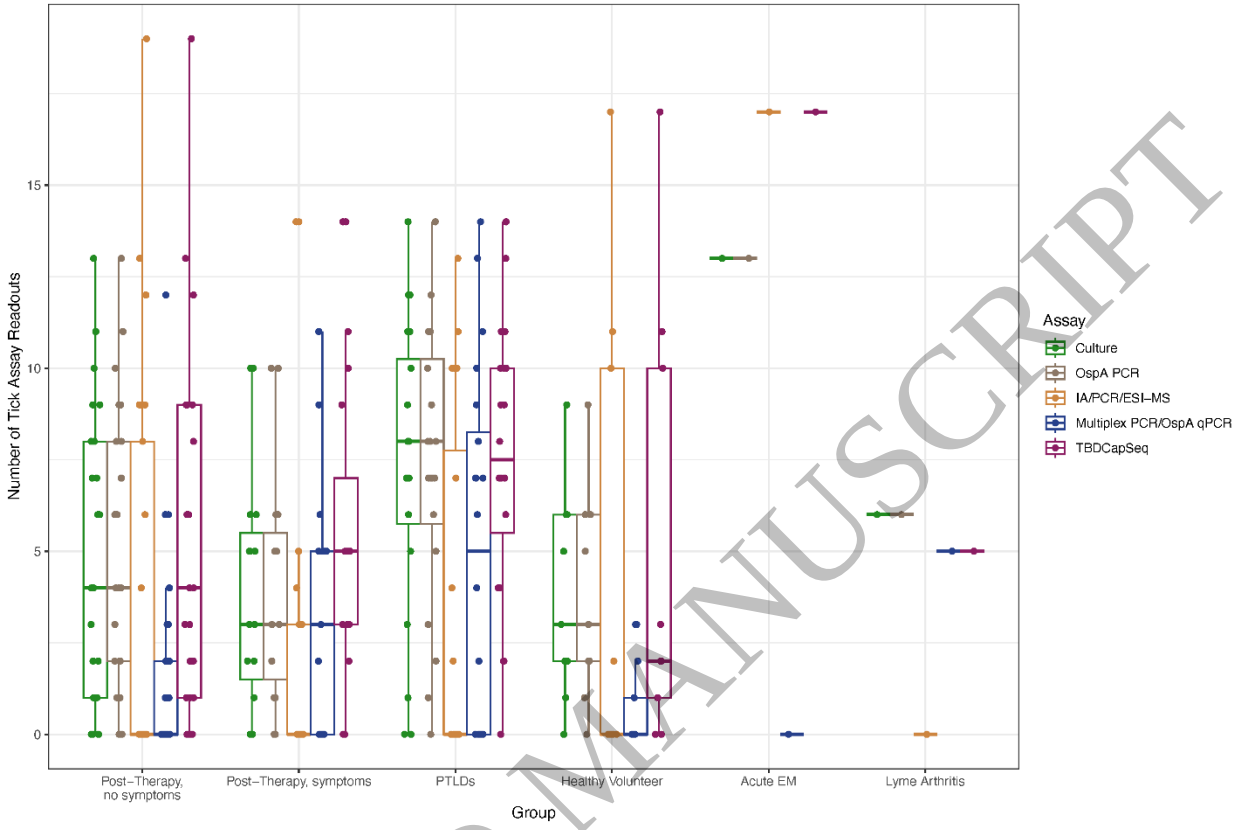
Figure 3. Number of xenodiagnostic ticks tested by assay and group



Corresponding data are in Supplementary Table S3. PTLDS= post-treatment Lyme disease symptoms. EM= erythema migrans. IA/PCR/ESI-MS= isothermal amplification PCR electrospray ionization mass spectrometry. PCR= polymerase chain reaction. qPCR= quantitative PCR. OspA= outer surface protein A. TBDCapseq= Tick-Borne Disease Capture Sequencing assay.

Alt text for figures

Figure 1. Boxplots with individual data points comparing the scores from health and fatigue scales between groups, with statistical values, at time of tick placement.

Figure 2. Boxplots with individual data points comparing the scores from health and fatigue scales between participants who recovered and those with symptoms, with statistical values, at 12 months after antibiotic treatment.

Figure 3. Boxplots with individual data points comparing the number of xenodiagnostic ticks tested by each assay and by study group.

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Supplementary Materials

Supplementary Tables and Figure

Table S1. Symptoms and health scores at tick placement visit and 12 months after antibiotic treatment

Code	All symptoms Tick Placement	All symptoms 12-month	Clinician Attribution Tick Placement	Clinician Attribution 12-month Assessment	Interval between Tick Placement and 12-month (days)	FSS at tick placement	Neuro QoL Fatigue at tick placement	SF-36v2 MCS at tick placement	SF-36v2 PCS at tick placement
A-001	none	none	Recovered	Recovered	220	1.4	39.5	58.02	53.73
A-002	A, D, F,I,J, L	A, D, F, I, J, L	Recovered	Recovered	140	3.2	45.6	57.61	46.25
A-003	A, M	A, M	Recovered	Recovered*	0	1.8	36.5	59.22	56.56
A-004	A,D,L	B	Recovered	Recovered	49	4	55.4	57.21	45.73
A-005	F,G	A, G,L	Recovered	Recovered	98	3	43.8	56.38	59.84
A-006	A	M	Symptoms	Recovered	28	1.6	29.5	57.64	58.83
A-007	A,F,M	M	Recovered	Recovered	46	1.7	38.2	61.49	59.82
A-008	none	none	Recovered	Recovered	170	2.1	34.1	58.31	56.62
A-009	A, D,L	D	Symptoms	Symptoms	246	2.8	45.6	52	57.09
A-010	A	none	Recovered	Recovered	233	1	29.5	62.21	59.55
A-011	A,D,F,H,I ,J,L	A,D,F,I,L	Symptoms	Symptoms	161	2.7	53.3	53.73	45.38
A-012	H	M	Recovered	Recovered*	0	2	29.5	61.56	58.64
A-013	A,B	A,F,G,H, M	Recovered	Symptoms	245	2.3	34.1	64.01	52.39
A-014	A,B,D,I	none	Symptoms	Recovered	97	4.6	49.3	60.75	44.35
A-015	A, D, L	none	Symptoms	Recovered	130	2.6	43.8	51.54	55.98
A-016	A	F,M	Symptoms	Recovered	105	2.8	39.5	55.67	59.32
A-017	A, D,F,G,M	A,G,M	Recovered	Recovered	208	1.3	38.2	57.07	51.81
A-018	A	A,H	Recovered	Recovered*	0	2	38.2	54.24	59.74
A-019	D	none	Recovered	Recovered	35	2.6	45.6	52.99	55.18
A-020	none	none	Recovered	Recovered*	0	1.9	39.5	59.52	60.31
A-021	A,C,D,F, H,I,K,L, M	D,F,I,K,L, M	Symptoms	Symptoms	81	6.3	61.1	28.97	40.6
A-022	A,D,M	A,D,M	Symptoms	Symptoms*	0	3.3	39.5	61.34	55.92
A-023	A,B,G,K	A,G	Recovered	Recovered	91	1	38.2	57.81	56.93
A-024	A,D,F,H,I ,K,L	A,D,F,H	Symptoms	Symptoms	144	4.4	53.3	44.89	43.6
A-025	A,G,L	A,L	Recovered	Recovered	193	1.7	40.7	51.08	52.38
A-026	none	A	Recovered	Recovered	38	1	36.5	58.31	56.62
A-027	B,D,G,I,J, L	B,D,G,I,J, K,L,M	Symptoms	Recovered	200	7	67.9	51.88	26.42
A-028	none	not done	Recovered	Not done	not done	1.4	40.7	57.65	55.94
A-029	A,C,D,G,	A	Symptoms	Recovered	104	6.4	51.3	50.68	34.93
A-030	none	C	Recovered	Recovered	42	2.9	43.8	55.6	54.01

A-031	D	none	Recovered	Recovered	245	1.7	36.5	56.32	57.91
A-032	A,G, L,M	A,B,D,G, L,M	Symptoms	Symptoms	89	3.9	45.6	53.39	54.56
A-033	A,B,D,F, G,H	A,B,D,F, G,H	Recovered	Recovered*	0	2.6	46.5	53.21	50.36
A-034	A,G,L	A,D	Recovered	Recovered*	0	3.2	42.8	47.81	57.48
A-035	D, I, J, L	A,B,D	Symptoms	Symptoms	232	7	59.9	49.02	38.03
A-036	none	B,F	Recovered	Recovered	36	1.7	39.5	59.21	56.45
A-037	none	none	Recovered	Recovered	33	1.7	34.1	57.61	59.86
A-038	A, D, H, I, L	G	Symptoms	Recovered	96	2.4	45.6	57.22	51.74
A-039	C	I	Recovered	Recovered	197	1.9	38.2	58.18	56.37
A-040	A,B,D,G, I,J	A,G,J	Symptoms	Symptoms*	0	5	55.4	40.24	46.68
B-001	A,B,C,D, F,G,H,I	N/A	PTLDS	N/A	N/A	3.9	45.6	54.79	38.29
B-002	D,L	N/A	PTLDS	N/A	N/A	5.1	52.3	59.63	36.07
B-003	A, D, H,I,K,L	N/A	PTLDS	N/A	N/A	5.2	55.4	36.26	37.82
B-004	A, D, F,H	N/A	PTLDS	N/A	N/A	3.3	48.4	54.24	48.09
B-005	A,D,I,L	N/A	PTLDS	N/A	N/A	6	55.4	34.65	49.46
B-006	A, B, C, D, F, G, H, I, J, K, L	N/A	PTLDS	N/A	N/A	5.6	58.8	27.33	47.25
B-007	B, D,G, I, J, K	N/A	PTLDS	N/A	N/A	5.7	56.5	51.29	34.31
B-008	I, J, L	N/A	PTLDS	N/A	N/A	3.2	44.7	50.32	52.48
B-009	A,D,G,J, M	N/A	PTLDS	N/A	N/A	5.7	50.3	41.03	62.28
B-010	B, D, F, G, I, J, K, L	N/A	PTLDS	N/A	N/A	6.3	63.5	38.94	27.62
B-011	A, B, D, F, H, I, J, L	N/A	PTLDS	N/A	N/A	1.2	39.5	55.22	52.89
B-012	C,D,F,G,I ,J	N/A	PTLDS	N/A	N/A	3.4	45.6	58.78	44.32
B-013	A, C,D,G,H, I,J,L,M	N/A	PTLDS	N/A	N/A	4.4	64.8	29.94	27.26
B-014	A,C,D,F, L	N/A	PTLDS	N/A	N/A	4.6	48.4	47.64	35.92
B-015	A,D,F,H, L, M	N/A	PTLDS	N/A	N/A	6.1	59.9	57.18	29.1
B-016	A,B,D	N/A	PTLDS	N/A	N/A	4.2	50.3	42.57	48.83
B-017	A, D, F, G, I, J, K, L	N/A	PTLDS	N/A	N/A	6.8	58.8	48.97	41.02
B-018	A,B,D,G, I,K	N/A	PTLDS	N/A	N/A	5.1	52.3	44.97	45.03
B-019	B, F, I	N/A	PTLDS	N/A	N/A	1.1	36.5	62.48	54.36

B-020	A,C,D,F, H,I,K,L, M	N/A	PTLDS	N/A	N/A	6.6	63.5	43.19	40.23
C-001	A,F	N/A	Acute EM	N/A	N/A	2.1	38.2	53.1	57.27
D-001	A,B,D,G, H,L	N/A	Lyme Arthritis	N/A	N/A	2.4	43.8	59.12	47.37
E-001	none	N/A	HV	N/A	N/A	1	29.5	62.19	59.91
E-002	D,J,M	N/A	HV	N/A	N/A	2.4	46.5	55.1	53.26
E-003	F,M	N/A	HV	N/A	N/A	1.1	29.5	59.27	57.56
E-004	none	N/A	HV	N/A	N/A	1.1	34.1	56.98	61.47
E-005	F	N/A	HV	N/A	N/A	1	43.8	49.62	62.66
E-006	A,B	N/A	HV	N/A	N/A	1.8	45.6	52.44	54.56
E-007	A,G	N/A	HV	N/A	N/A	1.6	39.5	53.48	58.76
E-008	none	N/A	HV	N/A	N/A	1	29.5	61.49	59.82

A=joint pain. B=muscle pain. C=dizziness. D=fatigue & malaise. E=feverish. F=headache. G= paresthesias. H=stiff neck. I=concentration and memory complaints. J=difficulties with finding and recalling words. K=mood complaints. L=sleep problems. M=tinnitus. N/A= not applicable. Recovered= post-therapy, no symptoms possibly related to Lyme disease. Symptoms= post-therapy, symptoms possibly related to Lyme disease. PTLDS= post-treatment Lyme disease symptoms. EM= erythema migrans. HV= healthy volunteer.*=12-month assessment performed together with tick placement visit. SF-36v2= Short Form-36 version 2. MCS=mental component summary score. PCS= physical component summary score. Neuro-QoL-Fatigue= Quality of Life in Neurological Disorders Fatigue scale short-form. FSS= Fatigue Severity Scale.

Table S2. Status assessment using the logistical and decision tree models at 12 months after antibiotic therapy

Code	FSS	Neuro QoL Fatigue	SF-36v2 Physical Functioning	SF-36v2 MCS	SF-36v2 PCS	Logistic Regression PTLDS Model Score	Logistic Regression Model PTLDS Probability	Decision Tree model PTLDS Probability	Clinician Attribution	Scores used to develop models
A-001	1.7	34.1	55.63	58.75	55.81	0.03	Recovered	Recovered	Recovered	YES
A-002	4.1	50.3	49.88	58.66	41.05	0.53	PTLDS	PTLDS	Recovered	YES
A-003	1.8	36.5	57.54	59.22	56.56	0.02	Recovered	Recovered	Recovered	YES
A-004	2.4	36.5	57.54	57.85	56.34	0.05	Recovered	Recovered	Recovered	YES
A-005	2.9	36.5	55.63	58.71	56.4	0.04	Recovered	Recovered	Recovered	YES
A-006	1	29.5	57.54	58.3	59.51	0.01	Recovered	Recovered	Recovered	YES
A-007	1.1	29.5	57.54	62.19	59.91	0.00	Recovered	Recovered	Recovered	YES
A-008	1.8	39.5	57.54	58.78	55.08	0.04	Recovered	Recovered	Recovered	YES
A-009	2.2	43.8	57.54	54.72	58.95	0.59	PTLDS	PTLDS	Symptoms	YES
A-010	2.7	36.5	51.8	61.77	51.94	0.01	Recovered	Recovered	Recovered	YES
A-011	2.9	51.3	55.63	56.46	53.34	0.63	PTLDS	PTLDS	Symptoms	YES
A-012	2	29.5	57.54	61.56	58.64	0.00	Recovered	Recovered	Recovered	YES
A-014	3	42.8	57.54	58.57	57.48	0.06	Recovered	Recovered	Recovered	YES
A-015	1	29.5	57.54	57.1	56.8	0.03	Recovered	Recovered	Recovered	No
A-016	1	47.4	57.54	49	55.81	0.99	PTLDS	PTLDS	Recovered	No
A-017	3.6	43.8	53.71	57.15	54.95	0.37	Recovered	PTLDS	Recovered	YES
A-018	2	38.2	57.54	54.24	59.74	0.49	Recovered	Recovered	Recovered	YES
A-019	2.6	41.8	57.54	55.6	60.94	0.36	Recovered	Recovered	Recovered	YES
A-020	1.9	39.5	57.54	59.52	60.31	0.02	Recovered	Recovered	Recovered	YES
A-021	5	55.4	51.8	42.01	41.94	1.00	PTLDS	PTLDS	Symptoms	YES

A-022	3.3	39.5	55.63	61.34	55.92	0.01	Recovered	Recovered	Symptoms	No
A-023	1.1	29.5	57.54	58.27	57.21	0.01	Recovered	Recovered	Recovered	YES
A-024	3.6	49.3	55.63	57.22	55.36	0.42	Recovered	PTLDS	Symptoms	YES
A-025	4.7	49.3	46.06	54.63	50.35	0.98	PTLDS	PTLDS	Recovered	No
A-026	1.9	29.5	55.63	59.07	51.68	0.01	Recovered	Recovered	Recovered	YES
A-027	6.3	63.5	47.97	44.28	30.91	1.00	PTLDS	PTLDS	Recovered	No
A-029	5.6	49.3	53.71	36.79	47.72	1.00	PTLDS	PTLDS	Recovered	No
A-030	2.7	42.8	57.54	53.93	56.69	0.70	PTLDS	Recovered	Recovered	No
A-031	1.1	36.5	55.63	58.64	58.14	0.04	Recovered	Recovered	Recovered	No
A-032	4.7	48.4	55.63	49.09	55.3	1.00	PTLDS	PTLDS	Symptoms	No
A-033	2.6	46.5	51.8	53.21	50.36	0.96	PTLDS	PTLDS	Recovered	No
A-034	3.2	42.8	57.54	47.81	57.48	1.00	PTLDS	Recovered	Recovered	No
A-035	6.6	59.9	46.06	40.73	37.76	1.00	PTLDS	PTLDS	Symptoms	No
A-036	1.3	41.8	57.54	58.21	54.61	0.07	Recovered	Recovered	Recovered	No
A-037	1.7	29.5	57.54	58.29	60.3	0.01	Recovered	Recovered	Recovered	No
A-038	1.4	41.8	53.71	58.36	55.06	0.15	Recovered	Recovered	Recovered	No
A-039	1.6	39.5	53.71	44.31	62.26	1.00	PTLDS	Recovered	Recovered	No
A-040	5	55.4	49.88	40.24	46.68	1.00	PTLDS	PTLDS	Symptoms	No

Logistic Regression Model categorize participants with probability < 0.5 as recovered and ≥ 0.5 as PTLDS. Decision Tree model categorizes Neuro-QoL Fatigue t-scores ≤ 42.8 as recovered and t-scores > 42.8 as PTLDS. A-013 and A-028 had no questionnaires data at 12 months. PTLDS= Post treatment Lyme disease symptoms. Neuro-QoL-Fatigue= Quality of Life in Neurological Disorders Fatigue scale short-form. FSS= Fatigue Severity Scale.SF-36v2= Short Form-36 version 2. MCS=Mental Component Summary. PCS= Physical Component Summary

Table S3. Testing of xenodiagnostic ticks

ID Code	Patient Group	Number of ticks recovered	Number of ticks tested by culture	Number of ticks tested by ospA PCR	SCID mouse assay performed*	Number of ticks tested by IA/PCR/ESI-MS	Number of ticks tested by multiplex PCR and/or OspA qPCR	Number of ticks tested by TBDCapSeq
A-001	Post Therapy	13	4	4	1	9	0	9
A-002	Post Therapy	9	0	0	0	9	0	9
A-003	Post Therapy	14	6	6	1	8	0	8
A-004	Post Therapy	22	9	9	1	13	0	13
A-005	Post Therapy	20	8	8	1	12	0	12
A-006	Post Therapy	8	3	3	1	5	0	5
A-007	Post Therapy	0	0	0	0	0	0	0
A-008	Post Therapy	19	10	10	1	9	0	9
A-009	Post Therapy	19	5	5	1	14	0	14
A-010	Post Therapy	27	8	8	1	19	0	19
A-011	Post Therapy	3	0	0	0	3	0	0
A-012	Post Therapy	0	0	0	0	0	0	0
A-013	Post Therapy	10	4	4	1	6	0	6
A-014	Post Therapy	5	2	2	1	3	0	3
A-015	Post Therapy	13	3	3	1	4	6	10
A-016	Post Therapy	4	1	1	1	3	0	3
A-017	Post Therapy	7	3	3	1	4	0	4
A-018	Post Therapy	1	1	1	0	0	0	0
A-019	Post Therapy	7	7	7	1	0	0	0
A-020	Post Therapy	7	4	4	1	0	3	3
A-021	Post Therapy	11	6	6	1	0	5	5
A-022	Post Therapy	11	6	6	1	0	5	5
A-023	Post Therapy	13	7	7	1	0	6	6
A-024	Post Therapy	10	5	5	1	0	5	5

A-025	Post Therapy	4	2	2	1	0	2	2
A-026	Post Therapy	3	0	2	0	0	1	1
A-027	Post Therapy	1	0	1	0	0	0	0
A-028	Post Therapy	23	11	11	1	0	12	12
A-029	Post Therapy	19	10	10	1	0	9	9
A-030	Post Therapy	7	6	6	1	0	1	1
A-031	Post Therapy	2	1	1	1	0	1	1
A-032	Post Therapy	4	2	2	1	0	2	2
A-033	Post Therapy	17	13	13	1	0	4	4
A-034	Post Therapy	2	1	1	1	0	1	1
A-035	Post Therapy	8	5	5	1	0	3	3
A-036	Post Therapy	12	9	9	1	0	3	3
A-037	Post Therapy	4	2	2	1	0	2^	2
A-038	Post Therapy	21	10	10	1	0	11^	11
A-039	Post Therapy	17	11	11	1	0	6^	6
A-040	Post Therapy	5	0	0	0	0	5^	5
B-001	PTLDS	7	0	0	0	7	0	7
B-002	PTLDS	15	5	5	1	10	0	0
B-003	PTLDS	19	8	8	1	11	0	11
B-004	PTLDS	17	7	7	1	10	0	10
B-005	PTLDS	7	3	3	1	4	0	4
B-006	PTLDS	21	8	8	1	13	0	13
B-007	PTLDS	21	11	11	1	10	0	10
B-008	PTLDS	3	1	1	1	2	0	2
B-009	PTLDS	22	12	12	1	0	10	10
B-010	PTLDS	14	7	7	1	0	7	7
B-011	PTLDS	15	8	8	1	0	7	7
B-012	PTLDS	19	10	10	1	0	9	9
B-013	PTLDS	4	0	2	0	0	2	2
B-014	PTLDS	28	14	14	1	0	14	14
B-015	PTLDS	14	7	7	1	0	7	7

B-016	PTLDS	17	6	6	1	0	11	11
B-017	PTLDS	15	9	9	1	0	6	6
B-018	PTLDS	25	12	12	1	0	13	9
B-019	PTLDS	12	8	8	1	0	4	4
B-020	PTLDS	19	11	11	1	0	8^	8
C-001	Acute EM	30	13	13	1	17	0	17
D-001	Lyme Arthritis	11	6	6	1	0	5	5
E-001	HV	19	9	9	1	10	0	10
E-002	HV	23	6	6	1	17	0	17
E-003	HV	4	2	2	1	2	0	2
E-004	HV	17	6	6	1	11	0	11
E-005	HV	0	0	0	0	0	0	0
E-006	HV	5	2	2	1	0	3	3
E-007	HV	2	1	1	1	0	1	1
E-008	HV	5	3	3	1	0	2	2
E-009	HV	5	5	5	1	0	0	0
	Total:	837	385	390	60	245	202	430

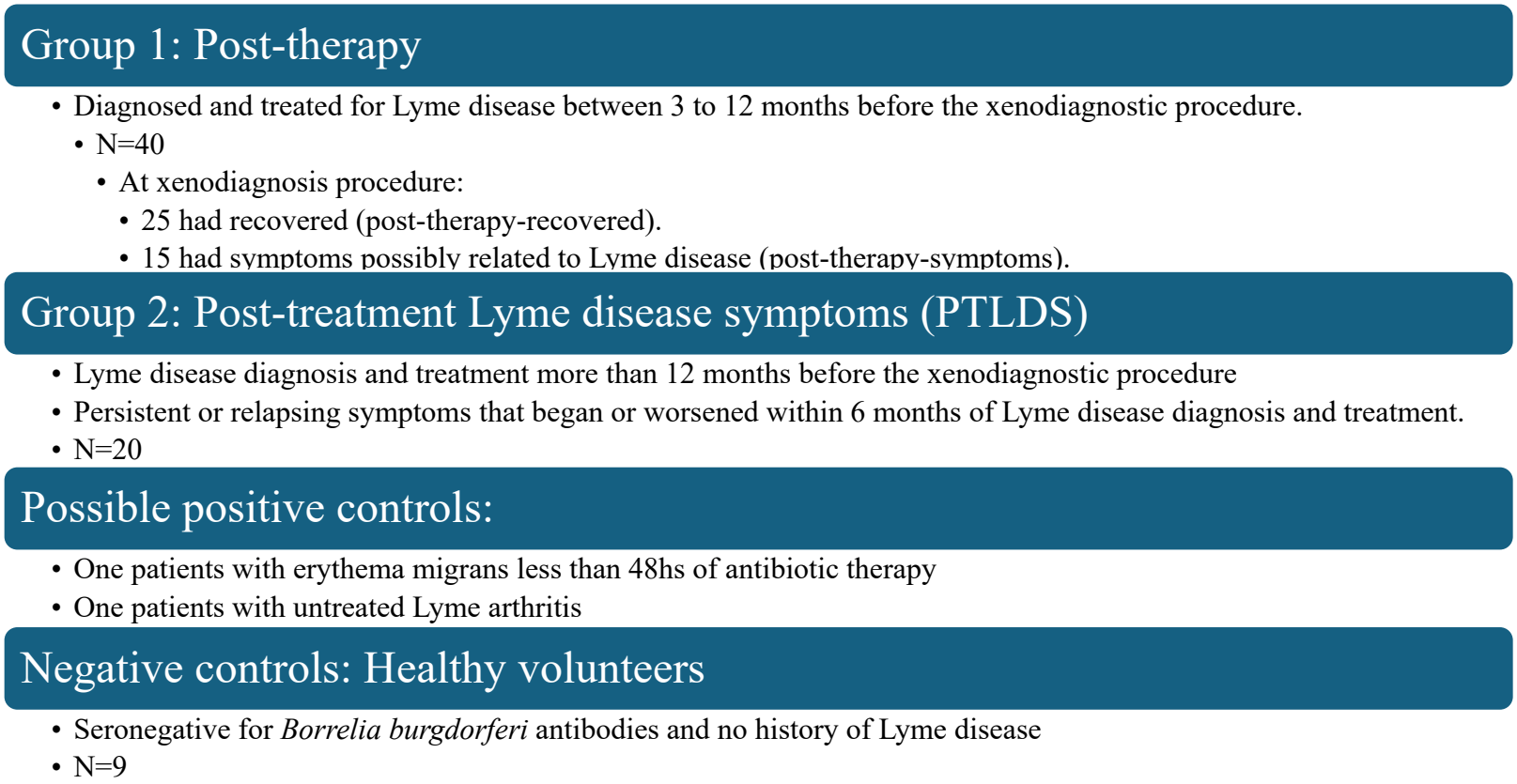
*1=performed; 0=not performed. ^Only OspA qPCR performed. #= One tick sample was positive for *B. burgdorferi*. EM= erythema migrans. PTLDS= post-treatment Lyme disease symptoms. HV= Healthy Volunteer. OspA= outer surface protein A. PCR = polymerase chain reaction. SCID= severe combined immunodeficiency. IA/PCR/ESI-MS= isothermal amplification PCR electrospray ionization mass spectrometry. qPCR= quantitative PCR. TBDCapseq= Tick-Borne Disease Capture Sequencing assay.

Table S4. TBDCapSeq detailed information for the positive tick sample.

Assay	1	2	3
Total number of reads	23,515,639	148,919,023	150,553,322
Total number of reads mapped to *Borrelia burgdorferi*	14238	62,597	75,166
Chromosome	5788	20,285	29,739
lp25	986	1,503	5,576
lp28-2	904	4,944	4,812
lp28-3	1,364	5,258	7,032
lp28-4	244	1,496	1,396
lp36	372	3,812	2,052
lp38	1,329	6,046	6,954
cp32	11	1,060	48
cp9	473	691	2,649
cp32-1	97	108	547
cp32-3	102	443	580
cp32-4	227	388	1,053
cp32-6	124	199	806
cp32-7	492	18	2,788
cp32-8	80	259	515
cp32-9	18	1,195	241
lp56	143	1,510	742
lp5	19	695	51
lp21	128	1	621
lp17	913	2,381	4,437
lp28-1	121	1,099	876
lp54	348	9,206	1,651

TBDCapSeq= Tick-Borne Disease Capture Sequencing assay. Lp=linear plasmid. Cp=circular plasmid.

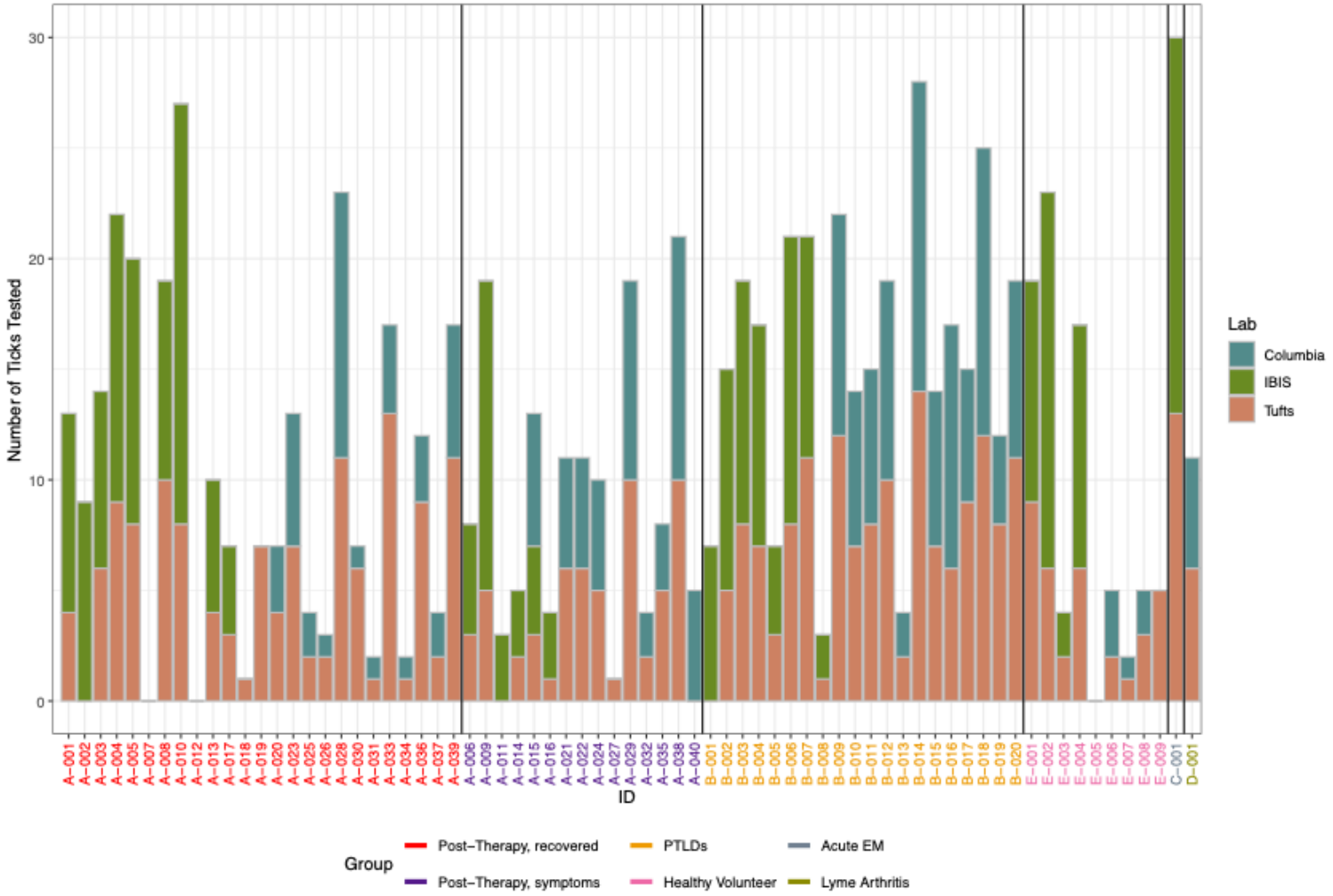
Figure S1. Study groups



Alt text for Figure S1. Chart describing the different study groups.

Figure S2. Number of ticks tested at each laboratory, by participant.

PTLDs= Post treatment Lyme disease symptoms. EM= erythema migrans.



Alt text for Figure S2. Chart showing number of ticks tested at each laboratory, by participant.

Supplementary methods

Testing by culture and PCR and SCID Mouse Assay (Tufts University)

Recovered live fed larval ticks were kept in a humidified chamber for 1 to 2 weeks before processing. Dead larval ticks were processed immediately as detailed below but not subjected to culture due to the high likelihood of contamination by mold.

Individual live fed larval ticks were brushed with 70% ethanol and then transferred to a 1.5mL Eppendorf tube containing 25µL of sterile PBS, where the larva was crushed with a sterile pipet tip. A portion of the lysate was placed in 1.3mL of BSK, containing rifampicin and phosphomycin, and amphotericin. The cultures were monitored weekly by darkfield microscopy for 6 weeks. At the end of 6 weeks, the cultures were centrifuged, and the pellet was processed for DNA (DNeasy). PCR was performed using primers for *ospA* as previously reported. A second portion of the tick lysate was used for direct PCR also using primers for ospA.

Approximately 5µL of each tick lysate from an individual participant was pooled together to inject into a SCID mouse. SCID mice were monitored for infection by culture and PCR of ear punch biopsies at two weeks after tick feeding, and at four weeks by culture and PCR of skin, ankle joint, heart, and bladder tissues. All cultures were monitored by darkfield microscopy. PCRs were performed as above using primers for *ospA*.

PCR

DNA was extracted using the Qiagen DNeasy as per the manufacturer's instructions. PCR was performed using primers for *Borrelia burgdorferi* gene outer surface protein (osp) A(1). In control testing using spiked samples, the sensitivity of this PCR was between 1-10 organisms. Positive results were confirmed by PCR testing for *Borrelia burgdorferi* flaB, recA and ospC (2) and by a second primer set for ospA (ospA-1F and ospA-1R) outside the original screening primers to distinguish potential amplicon contamination.

Primer name	Gene	Sequence
ospA-F	ospA	CTGCAGCTTGGAATTCAGGCACTTC
ospA-R	ospA	GTTTTGTAATTTCAACTGCTGACCCCTC
ospA-1F	ospA	ATGAAAAAATATTTATTGGGAATAGGTCTAATAT
ospA-1R	ospA	TTATTTTAAAGCGTTTTTAATTTCATCAAGTTT
recA-F	recA	GTGGATCTATTGTATTAGATGAGGCTCTCG
recA-R	recA	GCCAAAGTTCTGCAACATTAACACCTAAAG
flaB-F	flaB	GCAGCTAATGTTGCAAATCTTTTC
flaB-R	flaB	GCAGGTGCTGGCTGTTGA
ospC-F	ospC	ATGAAAAAGAATACATTAAGTGC

ospC-R	ospC	ATTAATCTTATAATATTGATTTTAATTAAGG

Culture

Samples were placed in Barbour-Stoenner-Kelly (BSK) II. Cultures were held for 6 weeks with weekly darkfield microscopy analysis; at 6 weeks, the medium was centrifuged for 5 minutes at 18,000-x g and the sediment analyzed by darkfield microscopy and PCR.

IA/PCR/ESI-MS

Ticks and skin biopsies were extracted as previously described (3) with the following modifications: Qiagen DNEasy columns from the DNEasy Blood and Tissue Kit (Qiagen, Valencia CA) were used and samples were eluted in 200uL of AVE elution buffer (Qiagen). For each set of extractions performed at least 1 extraction control was performed. Borrelia enrichment was performed as previously described (3). Borrelia DNA was selectively amplified with an isothermal amplification (IA) reaction for the 8 loci included in the PCR electrospray ionization mass spectrometry (PCR/ESI-MS) Borrelia genotyping assay. The IA enriched extracts were analyzed on the Borrelia genotyping assay as previously described (3, 4). The PCR/ESI-MS genotyping assay employs eight PCR primer pairs that target seven Borrelia genes. Two primer pairs (BCT3514 and BCT3515) are used to determine the species of Borrelia and the remaining primer pairs are used to genotype the organisms. A sample was considered positive if any of the 8 primer pairs from the assay produced Borrelia specific amplicons. (3) The sensitivity of this assay is 0.6 genomes. The unique combination of primer basecount signatures used in the assay can also be used to distinguish genotypic variation.(3, 4)

Tick-borne Disease Capture Sequencing (TBDCapSeq)

TBDCapSeq is a next generation sequencing (NGS) assay that incorporates a capture enrichment step prior to Illumina sequencing. Capture enrichment features a pool of agent-specific biotinylated DNA probes that are designed along the entire length of a genome to selectively bind and capture the template of interest prior to NGS. This approach enables >100-to-10,000-fold improvement in detection over unbiased NGS methods, with assay sensitivity that can surpass quantitative PCR (5). The TBDCapSeq probe set was designed to specifically target tick-borne pathogens and the utility of this assay for detection of tick-borne agents in clinical specimens and ticks has already been documented (6). For detection of *B. burgdorferi*, TBDCapSeq employs probes designed from strains B31, 297, and N40, as well as all representative OspC types (6). In addition, the capture probes have the capacity to detect sequences differing by up to 40% from known nucleotide sequences used for the probe design, ensuring optimal recognition of *B. burgdorferi* genetic heterogeneity. All TBDCapSeq assays were performed as outlined previously at the Center for Infection and Immunity (CII) at Columbia University (6). Briefly, sequencing libraries were generated and pooled, followed by capture with the TBDCapSeq probe set and NGS. To control for cross-contamination, no known positives for *B. burgdorferi* were assayed alongside the tested samples. Following

demultiplexing, sequencing reads obtained from every sample were directly mapped to the genome of the B31 strain of *B. burgdorferi*.

Sequencing Library preparation

Two types of samples were tested by TBDCapSeq. One set consisted of DNA previously extracted at IBIS or TUFTS University. A total of 253 tick DNA extracts from IBIS were analyzed. These were tested in pools of 3 to 5 samples per library, for a total of 63 sequencing libraries. An additional 30 samples (7 libraries) either produced low quality of sequencing libraries or generated insufficient sequencing read counts and were excluded. An additional 47 sequencing libraries were tested consisting of DNA from samples extracted at TUFTS University. These samples were composed of pre-pooled DNA from ticks, murine tissues, or culture.

In addition, whole ticks were shipped frozen to CII where total nucleic acids (TNA) from each individual tick were extracted using the EasyMag Extraction Platform (Biomerieux). TNA were split into two batches, one used for PCR assays, and the other for NGS. Each sample was first tested by a qPCR assay targeting the *ospA* gene (see below). Sequencing libraries were generated from *i)* NA from individual tick samples, or *ii)* NA from pools consisting of up to 5 tick samples, all obtained from the same patient. In total, 193 ticks were extracted and tested within 70 sequencing libraries by TBDCapSeq. Another 8 ticks were not examined due to limited NA available for testing (ticks were broken apart and insufficient material was available for NA extraction and/or low quality of sequencing libraries).

OspA qPCR

5ml of TNA was tested with the Invitrogen RNA UltraSense One-Step Quantitative RT-PCR (cat 11732927) master mix containing primers CCTTCAAGTACTCCAGATCCATTG (forward), and AACAAAGACGGCAAGTACGATC (reverse) and the probe FAM-CAACAGTAGACAAGCTTGA-MGB targeting the *ospA* gene. The reactions were run on a Bio-Rad CFX96 qPCR thermocycler at 95°C for 10 min followed by 45 cycles of 95°C 15s, 60°C for 30s.

Multiplex PCR

A two-step approach was used to test TNA from each individual tick. First, 5ml of TNA from each tick was used as template in a multiplex PCR, using primer sets from (4), with each primer at a concentration of 0.5mM. In a second step, 1 ml of the reaction from step 1 was used as template in individual, single-plex reactions for each primer pair. For positive controls, identical side by side PCR reactions were run, using a positive control standard that included the sequence of the PCR product for each primer pair. Each of these sequences contained an altered nucleotide composition that would distinguish it from an authentic *B. burgdorferi* sequence. PCR products for steps 1 and 2 were visualized by electrophoresis and sequenced.

Protocol Completion Futility Analysis

The planned sample size was 43 per group. Using Jeffreys prior, we determined the posterior distributions for the probability of a positive test as beta distributions for the groups. For the symptomatic group, we simulated positivity results for the remaining 34 subjects using a binomial distribution with a probability of 0.5/9.5. For the recovered group, we did the same for the remaining 15 subjects with a probability of 1.5/28.5. We generated a binomial random variable from these posterior distributions to estimate the data for each group and applied Fisher's exact test to assess the null hypothesis at the 0.05 level. This process was repeated 1,000,000 times to calculate the conditional power.

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