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CLINICAL THERAPEUTICS

Molecular Testing of Serial Blood Specimens from Patients with Early Lyme Disease during Treatment Reveals Changing Coinfection with Mixtures of *Borrelia burgdorferi* Genotypes

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ABSTRACT *Borrelia burgdorferi* is the etiological agent of Lyme disease. In the current study, we used direct-detection PCR and electrospray ionization mass spectrometry to monitor and genotype *B. burgdorferi* isolates from serially collected whole-blood specimens from patients clinically diagnosed with early Lyme disease before and during 21 days of antibiotic therapy. *B. burgdorferi* isolates were detected up to 3 weeks after the initiation of antibiotic treatment, with ratios of coinfecting *B. burgdorferi* genotypes changing over time.

KEYWORDS *Borrelia burgdorferi*, Lyme disease, antibiotic, genotype, time course

Lyme disease is caused by the bite of a tick infected with the spirochete *Borrelia burgdorferi*. The CDC estimates ~300,000 new cases of Lyme disease in the United States per year (1). The near-pathognomonic skin rash erythema migrans (EM) is the only specific clinical sign that uniquely points to Lyme disease. However, the EM lesion is not seen in ~30% of infected individuals (2), and even when present, it often does not have the hallmark bull's-eye appearance characteristic of infection with a *Borrelia* species (3, 4). The current approved serological two-tier test for Lyme disease detects the presence of host antibodies for *B. burgdorferi* antigens but cannot distinguish active infection from prior exposure (5, 6), so there is no way to test for cure or measure response to treatment. We previously used broad-range PCR electrospray ionization mass spectrometry (PCR/ESI-MS) to directly detect and genotype *B. burgdorferi* isolates from ticks and whole blood from patients with early Lyme disease (7–9). This assay can also distinguish *B. burgdorferi* genotypes, even when present in mixtures (7, 8), which is important because coinfecting genotypes are generally common in wild hosts (10) and *Ixodes scapularis* ticks (7). Using our multilocus typing method, we have a much higher genotype resolution than *ospC* typing alone (7), and we now have identified nearly 90 unique *B. burgdorferi* genotypes (unpublished data). Our aim in this first-of-its-kind study was to use our direct molecular assay for *B. burgdorferi* to monitor microbiological response to treatment and to determine how long after initiating antibiotic therapy *B. burgdorferi* isolates can be detected in blood from patients with early Lyme disease. Furthermore, the present study was designed to identify genotype(s) of the infecting strain(s) of *B. burgdorferi* isolates in these serially collected specimens.

The study was approved by the Johns Hopkins Medicine Institutional Review Board, and written informed consent was obtained from all participants before enrollment. Four patients from an area in Maryland where Lyme disease is endemic were enrolled during the summer/fall of 2015 and 2016. All participants had a physician-documented and diagnosed EM and were excluded for the presence of an immunosuppressive

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TABLE 1 PCR/ESI-MS primer sequences and gene targets

Primer pair ID	Primer code	Gene target	Primer sequence (5′→3′)
BCT3511	BCT8229F	*gyrB*	TGCATTTGAAAGCTTGGCATTGCC
	BCT8230R		TCATTTTAGCACTTCCTCCAGCAGAATC
BCT6092	BCT13037F	*rplB*	TCATCCACATGGTGGTGGTGAA
	BCT13040R		TGCGAGTCTTATAGCCTTTAGTAGGC
BCT6095	BCT13043F	*rpoC*	TACAAAGGAATGGGAATGTTATTGTGGT
	BCT8236R		TGCGAGCTCTATATGCCCCAT
BCT6101	BCT13044F	*leuS*	TCATGTTGGTCATCCGGAAGGATA
	BCT13049R		TGTATTGCATAACTTTCAGCAGGAAG
BCT3517	BCT8241F	*flaB*	TGCTGAAGAGCTTGGAATGCA
	BCT8242R		TACAGCAATTGCTTCATCTTGATTTGC
BCT3518	BCT8243F	*ospC*	TGACGGTATTTTTATTTATATCTTGTAATAATTCAGG
	BCT8244R		TTTGCTTATTTCTGTAAGATTAGGCCCTTT
BCT3519	BCT8245F	*hbb*	TCGAATAATGTTATTGAGTTTAGATCTTTTGGTAC
	BCT8246R		TGGACGAAAATACGCAACATGATGATC
BCT3520	BCT8247F	*hbb*	TGTCTTTTCCAAGAAGACCAAAGGTTACTAA
	BCT8248R		TACCCTTAAGCTCTTCAAAAAAAGCATC

illness or medication, pregnancy, or a history of receiving the Lyme vaccine. Of the four participants, two (patients A and D) self-reported a history of diagnosed and treated Lyme disease. All participants were antibiotic naive at the time of enrollment, and a day 0 specimen was collected before initiating a 21-day course of doxycycline. Three of the four participants said they missed no doses, and one of the four (patient A) missed one dose. A total of 20 ml of whole blood was collected at days 0, 1, 2, 4, 8, and 21 into two 10-ml EDTA purple-cap blood collection tubes and frozen for analysis by PCR/ESI-MS. Nucleic acids were extracted from four 5-ml aliquots of EDTA whole blood, and PCR/ESI-MS was performed as previously described (11). Three of the primer pairs (BCT6092, BCT6095, and BCT6101) were modified from our previous studies to improve their performance in the presence of a high background of human DNA without changing their targets (7, 8). The sequences of all primers used in this study are shown in Table 1; when a core six of eight primers produced a signature, we assigned a genotype to the detection as described previously (7).

B. burgdorferi isolates were detected and genotyped at day 0 and subsequent time points by PCR/ESI-MS testing of whole blood collected from two of the four participants (patients A and B); patients C and D tested negative in all specimens. For patient A, eight of eight specific primer pairs detected our *B. burgdorferi* genotype 25 at day 0 (Fig. 1A, B, and D; Fig. 2). *B. burgdorferi* isolates were not detected at days 1 and 4 (blood was not collected at day 2 for patient A) (Fig. 1A). However, *B. burgdorferi* isolates were detected by six and seven of eight primer pairs at days 8 and 21, respectively (Fig. 1A), and at each of these time points, two genotypes (6 and 25) were detected. At these later visits, genotype 6 was determined as the major genotype and genotype 25 as the minor genotype (Fig. 1C and E; Fig. 2). For patient B, coinfection with two *B. burgdorferi* genotypes (major genotype 7 and minor genotype 77) was detected by PCR/ESI-MS from blood collected at day 0 (Fig. 2; Fig. 3B and D). After the start of antibiotic treatment, there was a genotypic shift, with genotype 77 becoming the major genotype detected at day 1 (Fig. 2). Evidence of both genotypes was observed through day 4, with genotype 7 becoming undetectable by day 8 (Fig. 2). Specifically, primer pair BCT6101 generated base-count signatures corresponding to genotypes 7 and 77 at days 0 and 1, but genotype 77 was the only genotype detected by primer pair BCT6101 at day 2 (Fig. 2; Fig. 3C). Base-count signatures corresponding to genotype 77 were last detected at day 8 (Fig. 2; Fig. 3E). *B. burgdorferi* isolates were not detected (0 of 8 primer pairs) at day 21 (Fig. 3A).

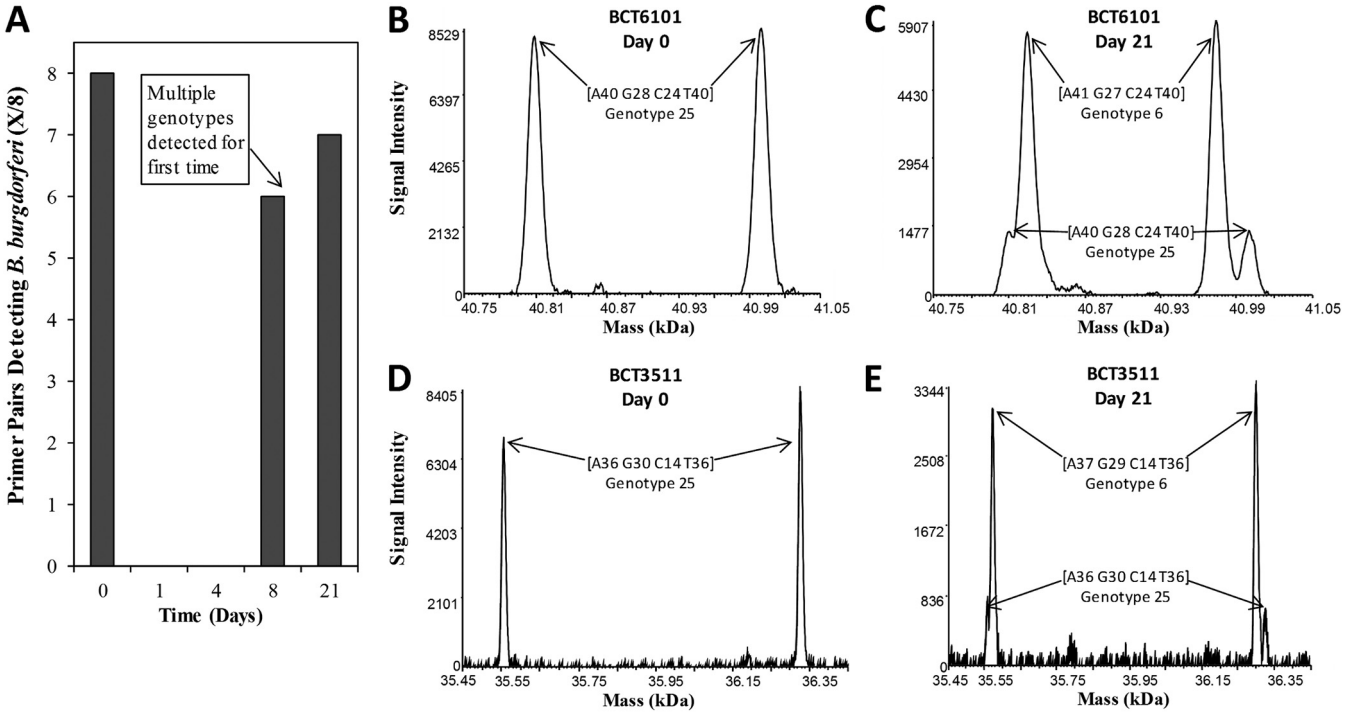

FIG 1 *Borrelia burgdorferi* primer pair detections and deconvolved spectral data of PCR amplicons derived from patient A. PCR/ESI-MS was performed on serial whole blood from a patient with clinically diagnosed early Lyme disease with EM. Specific primer pairs detecting *B. burgdorferi* isolates are plotted over time (A), displayed as cumulative detections from four 5-ml aliquots of 20 ml blood. Primer pairs BCT6101 (*leuS*) (B) and BCT3511 (*gyrB*) (D) simultaneously detected a single genotype of *Borrelia burgdorferi* on day 0. (C and E) Detection of two genotypes of *B. burgdorferi* for these targets on day 21. Paired peaks correspond to the forward and reverse strands of the PCR amplicons, which separate under the conditions of electrospray ionization.

In addition to PCR/ESI-MS analysis, two-tier antibody testing for *B. burgdorferi* infection was performed by Quest Diagnostics (Madison, NJ) from serum collected at days 0 and 21, and results were interpreted according to CDC recommendations (12) (Table 2). For all test specimens, the second-tier IgM/IgG Western blots were performed regardless of the enzyme-linked immunosorbent assay (ELISA) result. Patient B tested positive at day 0, whereas patients A and C seroconverted; however, the unusual pattern of reactivity shown by patient C suggested prior exposure to *B. burgdorferi*. Serological tests for patient D were negative at both day 0 and day 21 (Table 2).

Unexpectedly, both patients who tested positive by PCR/ESI-MS were coinfected with more than one *B. burgdorferi* genotype. However, we have seen coinfecting

Patient	Day	Major Genotype	Minor Genotype	Level	BCT3511 (*gyrB*)	BCT3517 (*Flagellin*)	BCT3518 (*ospC*)	BCT3519 (*hbb*)	BCT3520 (*hbb*)	BCT6092 (*rplB*)	BCT6095 (*rpoC*)	BCT6101 (*leuS*)
A	0	25	ND	15	A36 G30 C14 T36	A38 G28 C24 T31	A37 G23 C14 T36	A41 G31 C19 T44	A58 G22 C15 T46	A23 G25 C21 T23	A44 G35 C12 T42	A40 G28 C24 T40
	1	ND[1]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	8	6	25	1	A37 G29 C14 T36	A38 G28 C24 T31	A38 G22 C14 T36	A41 G31 C19 T44 A41 G30 C20 T44	ND	A23 G25 C21 T23	ND	A40 G28 C24 T40 A41 G27 C24 T40
	21	6	25	5	A36 G30 C14 T36 A37 G29 C14 T36	A38 G28 C24 T31	A38 G22 C14 T36	A41 G30 C20 T44	A58 G22 C15 T46	A23 G25 C21 T23	ND	A40 G28 C24 T40 A41 G27 C24 T40
B	0	7	77	60	A37 G29 C14 T36 A36 G30 C14 T36	A38 G28 C24 T31	A38 G22 C14 T36 A39 G21 C14 T36	A41 G30 C20 T44 A42 G30 C19 T44	A58 G23 C15 T45	A23 G25 C21 T23	ND	A41 G27 C24 T40 A40 G28 C24 T40
	1	77	7	2	A37 G29 C14 T36	A38 G28 C24 T31	A38 G22 C14 T36	A41 G30 C20 T44	A58 G23 C15 T45	A23 G25 C21 T23	ND	A40 G28 C24 T40 A41 G27 C24 T40
	2	77	7	2	A37 G29 C14 T36	A38 G28 C24 T31	A38 G22 C14 T36	A42 G30 C19 T44 A41 G30 C20 T44	ND	A23 G25 C21 T23	ND	A41 G27 C24 T40
	4	77	7	1	A37 G29 C14 T36	A38 G28 C24 T31	A38 G22 C14 T36	A42 G30 C19 T44 A41 G30 C20 T44	ND	A23 G25 C21 T23	ND	ND
	8	77	ND	2	A37 G29 C14 T36	A38 G28 C24 T31	A38 G22 C14 T36	A41 G30 C20 T44	ND	A23 G25 C21 T23	ND	ND
	21	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

FIG 2 *Borrelia burgdorferi* detections and genotypes with PCR amplicon base-count signatures from serial whole-blood collections. Base counts with the largest amplitude are shown on the bottom for cells with multiple base counts. Each base-count signature per column is differentiated by color. ND, no detection; level, relative intensity based on calibrant copies per reaction.

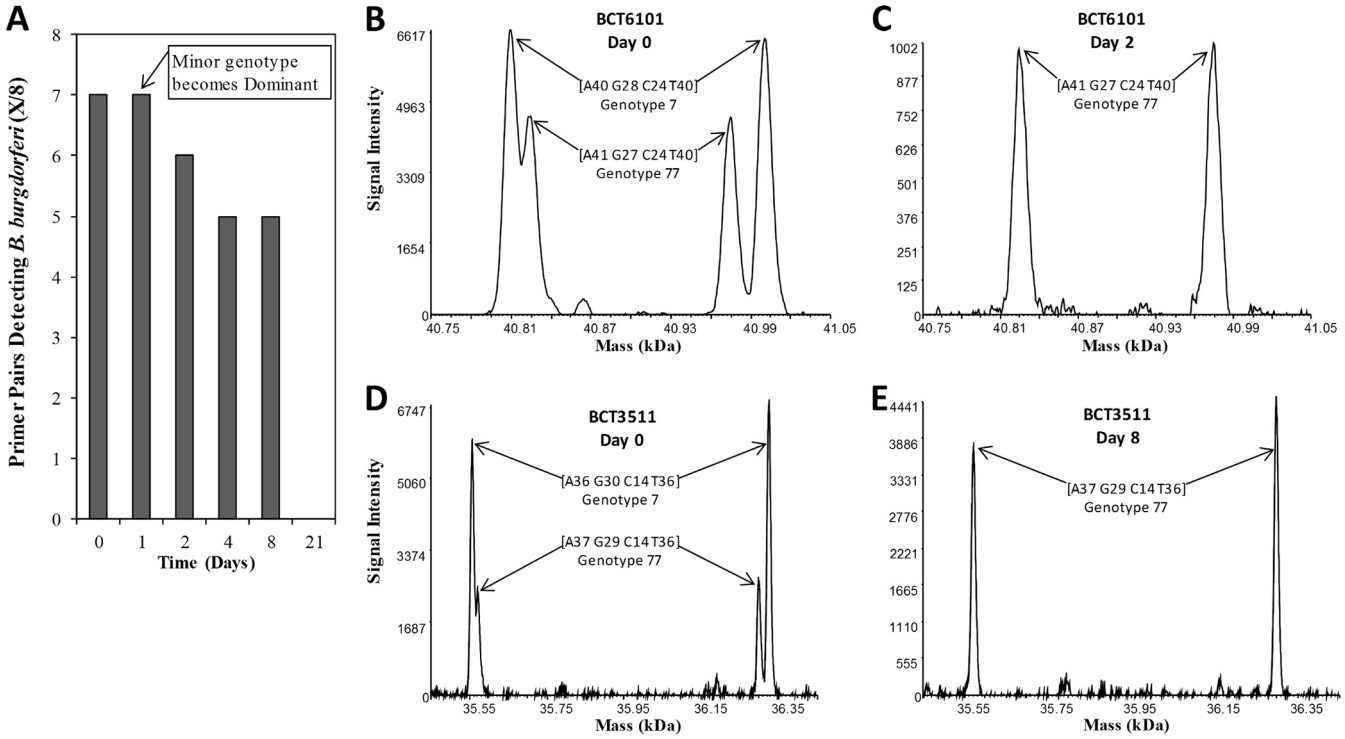

FIG 3 *Borrelia burgdorferi* primer pair detections and deconvolved spectral data of PCR amplicons derived from patient B. PCR/ESI-MS was performed on serial whole blood from a patient with clinically diagnosed early Lyme disease with EM. Specific primer pairs detecting *B. burgdorferi* isolates are plotted over time (A), displayed as cumulative detections from the four 5-ml aliquots of 20 ml blood. Primer pairs BCT6101 (*leuS*) (B) and BCT3511 (*gyrB*) (D) simultaneously detected two genotypes of *Borrelia burgdorferi* on day 0. (C and E) The last time points, days 2 and 8, where one genotype of *B. burgdorferi* was detected for these targets. Paired peaks correspond to the forward and reverse strands of the PCR amplicons, which separate under the conditions of electrospray ionization.

genotypes in ticks (7, 13, 14). While unlikely, it cannot be ruled out that a second infection occurred during these two patients' antibiotic treatments, because the study was carried out in an area where Lyme disease is endemic during tick-transmission season. Interestingly, the present work suggests that the ratios of coinfecting *B. burgdorferi* genotypes can change over time during antibiotic treatment. To date, few studies have focused on the ratio of genotypes in coinfections. Recently, Rynkiewicz et al. (15) showed that infection of mice with two strains of *B. burgdorferi* isolates resulted in similar fitness in single infections of each strain and asymmetric competition in coinfections. Moreover, two studies showed an apparent random founder effect, where some *B. burgdorferi* isolates dominated over others in murine models of *B. burgdorferi* infection (16, 17). As an alternative to the founder effect hypothesis, the host immune

TABLE 2 *B. burgdorferi* two-tier serology results from patients with clinically diagnosed early Lyme disease with EM

Patient	Day	2-Tier result[a]	ELISA	IgM bands	IgG bands
A	0[b]	Neg	0.94	0/3	1/10
	21	Pos	≥5.00	2/3	3/10
B	0	Pos	≥5.00	3/3	6/10
	21	Pos	≥5.00	3/3	6/10
C	0	Neg	≤0.90	0/3	5/10
	21	Pos	1.18	0/3	5/10
D	0	Neg	≤0.90	0/3	0/10
	21	Neg	1.22	0/3	1/10

[a]Neg, negative; Pos, positive. Results reported according to CDC recommendations (12).
[b]Day 0, initial doctor's visit.

system or differential antibiotic susceptibility might have played a role in the observed genotypic shift (18).

Our study does have limitations, most obviously that we relied on a small convenience sample from four participants. However, our PCR/ESI-MS method used for detection of *B. burgdorferi* isolates has been utilized to great extent (11, 19–23), including genotyping *B. burgdorferi* and detecting other vector-borne pathogens from ticks and clinical specimens (7, 8, 13, 14, 24–26). An additional limitation is that we did not follow participants beyond the 21-day study period to systematically capture longer-term clinical or microbiological outcomes. This would have been of particular interest for patient A, who had detectible infection at the day 21 visit. Although not followed beyond the 21-day study period, none of the participants self-reported significant lingering subjective symptoms at the final study visit, nor did any of the participants present to us for further clinical evaluation.

Direct diagnostic tests have the advantage of being able to measure response to treatment by demonstrating clearance of the pathogen. Nucleic acids are an excellent analyte for direct diagnostic tests and are the basis of all molecular diagnostics because DNA/RNA is quickly cleared from the human body. A study measuring fetal DNA in the bloodstream of mothers carrying male fetuses before and after giving birth has shown that the mean half-life of circulating male fetal DNA in the bloodstream is 16.3 min (27). Moreover, a study where heat-killed *B. burgdorferi* isolates were injected under the skin of mice found that *B. burgdorferi* became virtually undetectable after 8 h (28). However, only culture and not the presence of *B. burgdorferi* nucleic acids can confirm the presence of viable organism (29). Other studies have shown that *B. burgdorferi* isolates are detectable by PCR in synovial fluid or synovial membranes after antibiotic treatment (30, 31), and one study found PCR positivity in plasma months after treatment (32). These findings and ours may suggest that the bacteria reside in parts of the body that are not readily cleared, and bacterial remnants may continue to leak into the circulatory system after antibiotic treatment (33).

The present work demonstrates the utility of a direct molecular test that can both detect and genotype *B. burgdorferi* isolates from serially collected specimens. Currently, there is no FDA-approved direct diagnostic test for Lyme disease, due to challenging low levels of *B. burgdorferi* isolates in clinical specimens (34). We previously demonstrated direct molecular detection of *B. burgdorferi* isolates in 1.25 ml of whole blood collected from patients with early Lyme disease by PCR/ESI-MS with a sensitivity of 62% (13/21) (8). To further increase sensitivity, we recently increased the blood volume to 20 ml, similar to the typical volume of blood used for bacterial culture (20 to 30 ml) (35). The continued development of a direct molecular test that can both detect and genotype *B. burgdorferi* isolates is paramount, not only to promptly diagnose early Lyme disease in patients, but to provide a tool for testing new antibiotics and to further our understanding of infection by *B. burgdorferi* genotype(s) and their impact on the human immune system and illness.

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