# MAJOR ARTICLE

# Detection of *Borrelia burgdorferi* Cell-free DNA in Human Plasma Samples for Improved Diagnosis of Early Lyme Borreliosis

John A. Branda,[1] Jacob E. Lemieux,[2] Lily Blair,[3] Asim A. Ahmed,[3] David K. Hong,[3] Sivan Bercovici,[3] Timothy A. Blauwkamp,[3] Desiree Hollemon,[3] Carine Ho,[3] Klemen Strle,[2] Nitin S. Damle,[4] Timothy J. Lepore,[5] and Nira R. Pollock[6]

[1]Department of Pathology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, USA, [2]Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, USA, [3]Karius Inc, Redwood City, California, USA, [4]South County Internal Medicine Inc, Wakefield, Rhode Island, USA, [5]Nantucket Cottage Hospital, Nantucket, Massachusetts, USA, and [6]Department of Laboratory Medicine, Boston Children's Hospital and Harvard Medical School, Boston, Massachusetts, USA

(See the Editorial Commentary by Pritt on pages e2362–3.)

***Background.*** Laboratory confirmation of early Lyme borreliosis (LB) is challenging. Serology is insensitive during the first days to weeks of infection, and blood polymerase chain reaction (PCR) offers similarly poor performance. Here, we demonstrate that detection of *Borrelia burgdorferi* (*B.b.*) cell-free DNA (cfDNA) in plasma can improve diagnosis of early LB.

***Methods.*** *B.b.* detection in plasma samples using unbiased metagenomic cfDNA sequencing performed by a commercial laboratory (Karius Inc) was compared with serology and blood PCR in 40 patients with physician-diagnosed erythema migrans (EM), 28 of whom were confirmed to have LB by skin biopsy culture (n = 18), seroconversion (n = 2), or both (n = 8). *B.b.* sequence analysis was performed using investigational detection thresholds, different from Karius' clinical test.

***Results.*** *B.b.* cfDNA was detected in 18 of 28 patients (64%) with laboratory-confirmed EM. In comparison, sensitivity of acute-phase serology using modified 2-tiered testing (MTTT) was 50% (*P* = .45); sensitivity of blood PCR was 7% (*P* = .0002). Combining *B.b.* cfDNA detection and MTTT increased diagnostic sensitivity to 86%, significantly higher than either approach alone (*P* ≤ .04). *B.b.* cfDNA sequences matched precisely with strain-specific sequence generated from the same individual's cultured *B.b.* isolate. *B.b.* cfDNA was not observed at any level in plasma from 684 asymptomatic ambulatory individuals. Among 3000 hospitalized patients tested as part of clinical care, *B.b.* cfDNA was detected in only 2 individuals, both of whom had clinical presentations consistent with LB.

***Conclusions.*** This is the first report of *B.b.* cfDNA detection in early LB and a demonstration of potential diagnostic utility. The combination of *B.b.* cfDNA detection and acute-phase MTTT improves clinical sensitivity for diagnosis of early LB.

**Keywords.** Lyme; *Borrelia burgdorferi*; metagenomics; cell-free DNA; next-generation sequencing.

Two-tiered serologic testing remains the most widely used laboratory tool to support the diagnosis of Lyme borreliosis (LB). This approach yields high specificity, and sensitivity is high among patients with advanced LB who have been infected for months to years [1]. Sensitivity is poor in early infection, however, because a detectable antibody response takes time to develop. For example, conventional 2-tiered serologic testing (using an enzyme-linked immunosorbent assay [ELISA] followed by immunoblots) is falsely negative at the time of initial clinical presentation in 70%–80% of patients with localized erythema migrans (EM), a skin rash that is often present during early LB [1]. Serologic testing is also limited by the frequent

persistence of anti–*Borrelia burgdorferi* (*B.b.*) immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies for months to years after infection, despite effective treatment [2].

Potentially, these limitations could be addressed by a test that directly detects the infectious agent [3]. Thus far, this has remained an elusive goal. Although *B.b.* can often be detected using culture or polymerase chain reaction (PCR) of skin biopsies taken from EM lesions [4], this approach is impractical for routine use. Blood PCR has not been very useful; the sensitivity of whole-blood or plasma PCR in patients with EM is usually in the 30%–50% range [5–9], and blood PCR is seldom positive in patients with later manifestations of LB [10]. Similarly, cerebrospinal fluid PCR is poorly sensitive in patients with Lyme neuroborreliosis [11, 12], leaving PCR analysis of joint fluid/synovial tissue from patients with suspected Lyme arthritis as the only clinically useful PCR application.

Here, we report the findings of a study performed in collaboration with colleagues at Karius Inc (Redwood City, California) to analyze plasma samples from patients with physician-diagnosed EM using Karius' quantitative microbial cell-free DNA (cfDNA) detection method. This direct detection method applies unbiased

("shotgun") metagenomic sequencing to human plasma samples for identification and quantitation of microbial sequence reads [13]. Rigorous analytical and clinical validation has established this method's ability to detect cfDNA from a panel of >1000 bacteria, DNA viruses, fungi, and eukaryotic parasites [13]. This is the first report of its performance in the diagnosis of early LB via detection of *B.b.* sensu stricto cfDNA.

## MATERIALS AND METHODS

### Patients and Control Subjects

Forty-two patients presenting in the summer of 2015 with ≥1 skin lesion meeting the Centers for Disease Control and Prevention (CDC) surveillance case definition of EM [14] were enrolled by investigators on Nantucket Island (T. J. L.) and in Rhode Island (N. S. D.) under a previous research protocol [15]. Among them, 40 patients provided consent for future use of their samples in other research studies and were included in the present study. The parent and current studies were approved by the Partners Healthcare Human Research Committee. At initial presentation, a clinical history was taken and a physical examination was performed. A 2-mm punch biopsy was taken from the leading edge of the skin lesion for *Borrelia* culture [16]. Ethylenediaminetetraacetic acid (EDTA)–whole blood, EDTA-plasma, and serum samples were collected and stored at −80℃. After completion of standard antimicrobial therapy for EM, most enrolled patients returned for a follow-up assessment 3–6 weeks after the initial visit, during which convalescent-phase serum samples were collected.

In addition to patients with physician-diagnosed EM, 3 groups of control subjects were included in the study: (1) T. J. L. enrolled 3 ambulatory subjects on Nantucket during the summer of 2015, who complained of constitutional symptoms but had no skin rash on physical examination. None of these 3 subjects received antimicrobial therapy, and all had improvement of symptoms at their follow-up visit. Baseline and

follow-up blood samples were collected as described above. (2) EDTA-plasma samples were collected by Karius from 684 ambulatory, asymptomatic subjects between 2015 and 2019 as controls for clinical validation of the Karius test. (3) EDTA-plasma samples were submitted to Karius for clinical testing, as ordered by treating physicians, from 3000 hospitalized patients suspected of having acute or chronic infection.

### Laboratory Analysis of Collected Samples
#### *Borrelia* Culture

Cultures were performed as described previously [16]. If spirochetes were detected, *B.b.* PCR targeting the *flaB* gene [17] was performed on pelleted microbes for confirmation. Isolated *B.b.* strains were analyzed by whole-genome sequencing (Supplementary Methods) as a part of an ongoing *B.b.* sequencing effort (J. E. L., K. S., J. A. B., Pardis C. Sabeti, and others) and made available prepublication.

#### *Serologic Testing*

Serum samples were analyzed using the C6 *B.b.* ELISA (Immunetics/Oxford), the ZEUS *B.b.* IgG/IgM ELISA (Zeus Scientific), the ZEUS VlsE1/pepC10 IgG/IgM ELISA (Zeus Scientific), and IgM and IgG ViraStripe ladder immunoblots (Viramed AG) interpreted according to CDC criteria [18]. Conventional and modified 2-tiered testing algorithms [19] were applied to classify each sample as seropositive or seronegative, as described in Tables 1 and 2.

#### *Blood PCR for* B.b. *DNA and Auxiliary Testing for Other Pathogens*

These tests were performed at reference laboratories (Supplementary Methods).

#### *Microbial cfDNA Detection and Analysis*

Frozen aliquots of EDTA-plasma were sent to Karius for processing and analysis. Microbial cfDNA was extracted from

**Table 1.** Clinical Sensitivity of *Borrelia burgdorferi* Cell-free DNA Detection Compared With Standard Diagnostic Methods in Patients With a Clinical Diagnosis of Erythema Migrans

| Patients | | No. (%) Positive | | | | |
|---|---|---|---|---|---|---|
| | *Borrelia burgdorferi* Cell-free DNA | *Borrelia* PCR (Whole Blood) | | Acute-phase Serology | | |
| | | | CTTT[a] | MTTT Algorithm 1[b] | MTTT Algorithm 2[c] | |
| All patients with suspected EM (N = 40) | 19 (48) | 3 (8)[d] | 12 (30) | 18 (45) | 18 (45) | |
| Laboratory confirmed (n = 28) | 18 (64) | 2 (7)[e] | 10 (36) | 14 (50) | 14 (50) | |
| Unconfirmed (n = 12[f]) | 1 (8) | 1 (8)[g] | 2 (17) | 4 (33) | 4 (33) | |

Abbreviations: CTTT, conventional 2-tiered testing; EM, erythema migrans; MTTT, modified 2-tiered testing; PCR, polymerase chain reaction.

[a]Seropositivity by CTTT required a positive or equivocal whole-cell sonicate enzyme-linked immunosorbent assay (ELISA) (Zeus *B. burgdorferi* immunoglobulin G [IgG]/immunoglobulin M [IgM] ELISA) and a positive IgM or IgG immunoblot (ViraStripe, Viramed AG). If only the IgM immunoblot was positive, the individual was considered CTTT seropositive only if symptoms were present for <1 month.

[b]Seropositivity by MTTT algorithm 1 required a positive or equivocal result in both the Zeus *B. burgdorferi* IgG/IgM ELISA and the Immunetics/Oxford C6 Lyme ELISA.

[c]Seropositivity by MTTT algorithm 2 required a positive or equivocal result in both the Zeus VlsE/pepC10 IgG/IgM ELISA and the Zeus *B. burgdorferi* IgG/IgM ELISA.

[d]One sample was PCR positive; 2 additional samples were PCR equivocal.

[e]One sample was PCR positive; the other sample was PCR equivocal.

[f]Two patients' skin cultures were contaminated with skin flora and were discontinued before the complete incubation period.

[g]This sample was PCR equivocal.

**Table 2. Clinical Sensitivity of *Borrelia burgdorferi* Cell-free DNA Detection Combined with Acute-Phase 2-Tiered Serologic Testing in Patients With a Clinical Diagnosis of Erythema Migrans**

| | No. (%) Positive | | |
| --- | --- | --- | --- |
| Patients | *B.b.* cfDNA or CTTT[a] | *B.b.* cfDNA or MTTT Algorithm 1[b] | *B.b.* cfDNA or MTTT Algorithm 2[c] |
| All patients with suspected EM (N = 40) | 24 (60) | 28 (70) | 28 (70) |
| Laboratory confirmed (n = 28) | 22 (79) | 24 (86) | 24 (86) |
| Unconfirmed (n = 12[d]) | 2 (17) | 4 (33) | 4 (33) |

Abbreviations: *B.b.*, *Borrelia burgdorferi*; cfDNA, cell-free DNA; CTTT, conventional 2-tiered testing; EM, erythema migrans; MTTT, modified 2-tiered testing.

[a]Seropositivity by CTTT required a positive or equivocal whole-cell sonicate enzyme-linked immunosorbent assay (ELISA) (Zeus *B. burgdorferi* immunoglobulin G [IgG]/ immunoglobulin M [IgM] ELISA) and a positive IgM or IgG immunoblot (ViraStripe, Viramed AG). If only the IgM immunoblot was positive, the individual was considered CTTT seropositive only if symptoms were present for <1 month.

[b]Seropositivity by MTTT algorithm 1 required a positive or equivocal result in both the Zeus *B. burgdorferi* IgG/IgM ELISA and the Immunetics/Oxford C6 Lyme ELISA.

[c]Seropositivity by MTTT algorithm 2 required a positive or equivocal result in both the Zeus VlsE/pepC10 IgG/IgM ELISA and the Zeus *B. burgdorferi* IgG/IgM ELISA.

[d]Two skin cultures were contaminated with skin flora and were discontinued before the complete incubation period.

250 µL of EDTA-plasma, next-generation sequencing libraries were prepared, and sequencing was performed on an Illumina NextSeq 500. Sequence reads identified as human were removed, and remaining reads were aligned to a curated pathogen database. Per routine for the Clinical Laboratory Improvement Amendments–certified laboratory diagnostic test, any of >1000 microorganisms in the Karius Test Pathogen List found to have statistically significant levels of cell-free DNA in plasma, relative to real-time negative controls, were reported in molecules of cfDNA/µL (MPM) of plasma as previously described [13]. If the number of cfDNA sequence reads derived from a particular microorganism was too low to reach the predefined statistical threshold, the microorganism was not reported among the test results. However, for all samples included in the present study (both case and control samples), a relaxed statistical threshold was applied for *B.b.*, such that *B.b.* would be reported among the test results if cfDNA derived from this microorganism was observed at any level (investigational use only).

### Statistical Methods

Differences between proportions were considered statistically significant if the 2-tailed *P* value was <.05 as determined using McNemar test.

## RESULTS

### *B.b.* cfDNA Detection in Patients With Physician-diagnosed EM

Among 40 patients in this category, *B.b.* cfDNA was detected in 19 patients (48%; Table 1). Alignment of *B.b.* cfDNA sequence reads to the reference genome (*B.b.* strain B31) resulted in a uniform distribution of alignments spanning the *B.b.* linear chromosome (Figure 1). The number of *B.b.*-specific reads ranged from 1 to 253 per unique patient sample (Supplementary Table 1). When the absolute number of *B.b.* reads per sample was converted to MPM, the range was <1 to 185 MPM, with a median of 2 MPM and an interquartile range of 4.

In comparison, a commercial pan-*Borrelia* whole-blood PCR assay (Supplementary Methods) was positive or equivocal in 3 of 40 samples from the same cohort (8%; *P* = .0004; Table 1). When the pan-*Borrelia* PCR-positive (n = 1) or PCR-equivocal (n = 2) DNA extracts were further analyzed using *B.b.* species–specific and *Borrelia miyamotoi*–specific PCR assays, none tested positive. Serologic testing for LB using the conventional 2-tiered testing (CTTT) algorithm (a whole-cell sonicate ELISA followed by immunoblots) was positive in 12 of 40 acute-phase serum samples from the same patients (30%; *P* = .15 for the comparison with *B.b.* cfDNA detection). Using
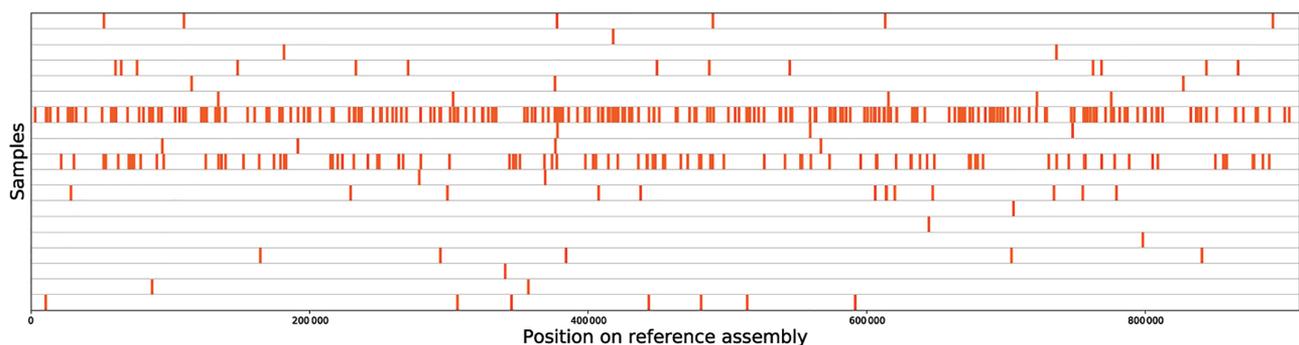


**Figure 1.** Alignment of cell-free DNA reads to *Borrelia burgdorferi* (*B.b.*) reference genome. Sequence reads from microbial cell-free DNA aligning to assembly accession GCF_000008685.2 (*B.b.* strain B31) were plotted along the genome, with each row representing a unique patient sample (n = 19). Alignments were uniform across the genome and were of high percentage identity to the reference. None of the reads that aligned to *B.b.* aligned to any assembly outside of the *Borrelia* genus.

a modified serologic testing algorithm involving a whole-cell sonicate (WCS) ELISA followed by a C6 ELISA (MTTT 1), without the use of immunoblots, 18 of 40 patients were seropositive in the acute phase of illness (45%; *P* = 1.0 when compared with *B.b.* cfDNA detection). A second MTTT algorithm, using a VlsE/pepC10 ELISA followed by a WCS ELISA (MTTT 2), also demonstrated seroreactivity in 18 of 40 patients (45%) using acute-phase serum samples (Table 1).

### *B.b.* cfDNA Detection in Patients With Laboratory-confirmed EM

Among 40 patients with physician-diagnosed EM, the diagnosis was confirmed in 28 (70%) either by recovering *B.b.* in skin biopsy culture (n = 18), by demonstrating seroconversion between acute- and convalescent-phase serum samples (n = 2), or both (n = 8). *B.b.* cfDNA was detected in 18 of 28 such patients (64%; Table 1). In comparison, using a commercial pan-*Borrelia* whole-blood PCR assay, 2 of 28 samples were positive or equivocal (7%; *P* = .0002). Whereas both samples were negative when further analyzed using a *B.b.*-specific PCR assay, both were positive for *B.b.* cfDNA. Acute-phase serologic testing using the CTTT algorithm was positive in 10 of 28 patients (36%; *P* = .08 for the comparison with *B.b.* cfDNA detection). Using either MTTT algorithm 1 or 2, acute-phase serology was positive in 14 of 28 patients (50%; *P* = .45 compared with *B.b.* cfDNA detection; Table 1).

We aligned *B.b.* cfDNA sequence reads from individual patients against whole-genome sequence reads generated from the same individual's cultured *B.b.* isolate, in cases where cfDNA sequencing yielded sufficient *B.b.*-specific reads for such an analysis. In multiple individuals, *B.b.* cfDNA sequences from plasma precisely matched strain-specific genomic sequence of the *B.b.* isolate cultured from that individual's skin biopsy (Figure 2), meaning the paired reads shared single-nucleotide polymorphisms (SNPs) not present in other strains, or lacked SNPs found in other strains.

### Combination of *B.b.* cfDNA Detection With 2-Tiered Serologic Testing

Among 40 patients with physician-diagnosed EM, 24 (60%) were positive for *B.b.* cfDNA detection and/or were seropositive in the acute phase of illness using the CTTT algorithm (Table 2). The combined sensitivity of 60% was significantly higher compared with acute-phase CTTT alone (30%; *P* = .002) and approached significance when compared with *B.b.* cfDNA detection alone (48%; *P* = .07). When acute-phase serologic testing using either MTTT algorithm 1 or 2 was combined with *B.b.* cfDNA detection, 28 patients (70%) were positive. The combined sensitivity of 70% was significantly higher than either MTTT algorithm 1 or 2 alone (45% using either algorithm; *P* = .004) or *B.b.* cfDNA detection alone (48%; *P* = .008).
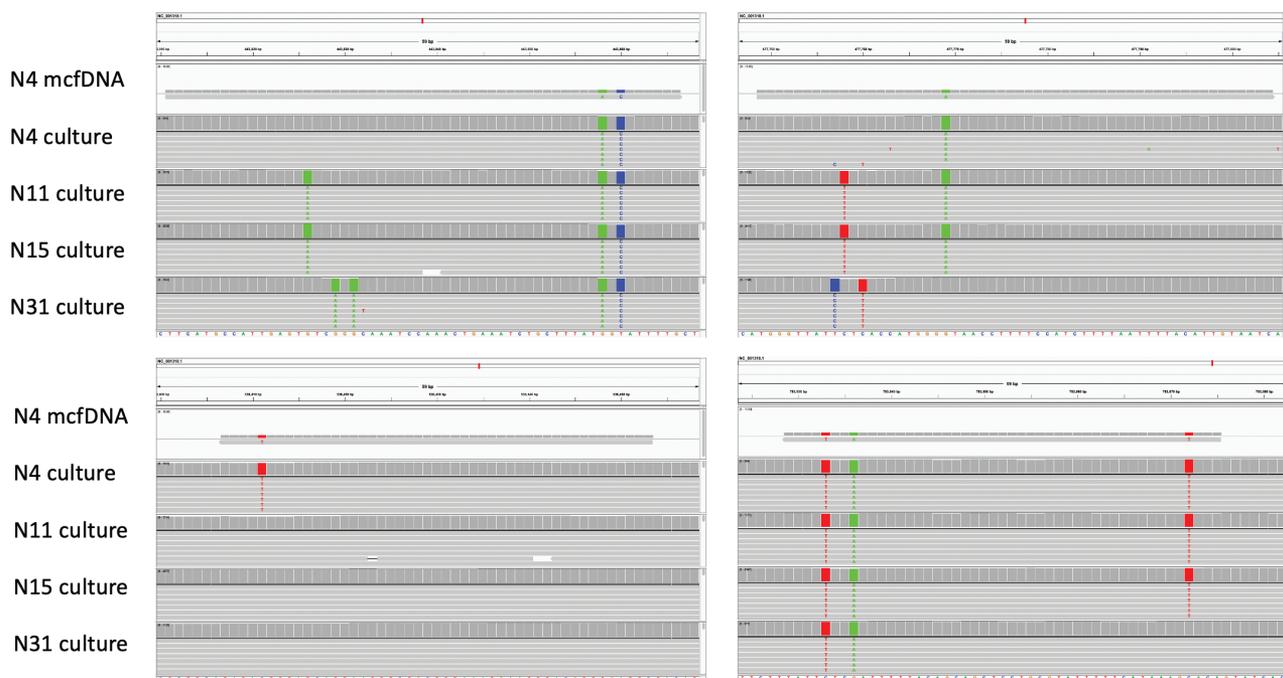


**Figure 2.** Alignment of *Borrelia burgdorferi* (*B.b.*) cell-free DNA (cfDNA) reads to strain-specific genome sequence from matched culture isolates. The *B.b.* cfDNA sequence reads match with reads from the source patient's infecting *B.b.* strain and differ from reads obtained from other study patients' cultured strains. Four informative *B.b.* cfDNA reads from patient N4's plasma sample, along with sequence reads from representative *B.b* strains isolated from skin biopsy specimens, are shown aligned to the B31 reference genome. Abbreviation: mcfDNA, microbial cell-free DNA.

Among patients with laboratory-confirmed EM, 22 of 28 (79%) were positive using *B.b.* cfDNA detection and/or were seropositive in the acute phase of illness using CTTT (Table 2). This test combination was significantly more sensitive compared with acute-phase CTTT alone (36% sensitivity; $P = .002$), but not compared with *B.b.* cfDNA detection alone (64%; $P = .13$). When combining *B.b.* cfDNA detection with acute-phase MTTT, 24 of 28 (86%) of patients were positive by 1 or both diagnostic approaches, exceeding the sensitivity of acute-phase MTTT alone (50% using either algorithm 1 or 2; $P = .004$) or *B.b.* cfDNA detection alone (64%; $P = .04$).

### *B.b.* cfDNA Detection in Control Subjects

In a retrospective review of 3000 hospitalized patients who recently underwent microbial cfDNA testing at Karius for clinical care, *B.b.* cfDNA was detected in 2 individuals, with concentrations of 9 and 195 MPM. Both patients had clinical features consistent with acute early LB (A. A. A., personal communication). Also, in a cohort of 684 asymptomatic ambulatory control subjects, sequence reads aligning with *B.b.* reference genomes were not detected at any concentration. We also analyzed 3 control samples collected from ill ambulatory subjects without rash on Nantucket (see Methods); none of the 3 seroconverted and all 3 were negative for *B.b.* cfDNA.

### Non-*B.b.* Microbial cfDNA Detection in Cases and Controls

Among 40 patients with physician-diagnosed EM, cfDNA from microorganisms other than *B.b.* was detected in the plasma of 12 (30%). In most cases, the reads aligned with genomic DNA from commensal flora of the human skin, gut, or oropharynx (Supplementary Table 1), although some likely pathogens were also detected (eg, *Helicobacter pylori*). In 2 patients with laboratory-confirmed EM, cfDNA derived from *Rickettsia felis*, a flea-transmitted pathogen, was detected at concentrations of 405 and 55 MPM. To investigate this finding, PCR assays capable of detecting *R. felis* were performed at CDC using genomic DNA extracts prepared from whole-blood samples of both patients. The PCR results were negative, however, and serologic tests for *R. felis* infection were not available to adjudicate the discrepancy.

Among 3 control samples collected from ill, ambulatory subjects without rash on Nantucket in whom LB was not suspected clinically, 1 produced sequence reads from *Francisella tularensis* (52 MPM). The sample was collected from a 62-year-old woman presenting in July 2015 with fever/chills, headache, malaise, fatigue, myalgias, and arthralgias. When the cfDNA result was obtained, we performed *F. tularensis* serology. Results were negative (titer <1:128) using serum collected at the time of initial presentation, but strongly positive (titer = 1:2048) using a sample collected 32 days later, confirming the diagnosis of acute tularemia.

## DISCUSSION

This study demonstrates that *B.b.* is detectable using unbiased metagenomic sequencing of cfDNA in acute-phase plasma samples from patients with EM, the most common manifestation of early LB. The cfDNA sequence reads were uniformly distributed across the *B.b.* reference genome, indicating that the findings are not artifactual. Moreover, *B.b.* cfDNA sequence reads precisely matched the strain-specific genome sequence generated from the *B.b.* isolate cultured from the same individual, proving that the *B.b.* cfDNA sequence detected in plasma was derived from infecting *B.b.* spirochetes and confirming the validity of this diagnostic approach.

Detection rates of *B.b.* cfDNA were higher in the subcategory of patients with laboratory-confirmed EM, compared with those in whom physician-diagnosed EM could not be confirmed by skin biopsy culture or seroconversion. Speculatively, this may be due to differences in *B.b.* organism burden between the 2 groups, or may reflect incorrect clinical diagnosis in some of the unconfirmed cases. At the time of clinical presentation, EM lesions often do not have central clearing or a classical "bullseye" appearance [20, 21] and other entities can look similar.

*B.b.* cfDNA detection also appears to complement acute-phase serologic testing as a diagnostic tool in early LB. The combination of modified 2-tiered serologic testing and *B.b.* cfDNA detection produced significantly higher sensitivity compared with either method alone—reaching 86% in patients with laboratory-confirmed EM. Potentially, this phenomenon could be explained by variability in duration of infection prior to testing; it is often the case that infectious agents are detected directly in blood at a higher rate prior to development of a humoral immune response. There was no significant difference in the mean duration of EM prior to presentation between the groups of patients with discordant results (positive *B.b.* cfDNA detection but negative MTTT, and vice versa; data not shown), although this measurement does not capture the incubation period and relies on patient self-reporting.

Detection of *B.b.* cfDNA was significantly more sensitive compared with blood PCR performed at a commercial reference laboratory, both in the cohort with physician-diagnosed EM and in the subset with laboratory-confirmed EM. The relatively low yield of blood PCR in early- or late-stage LB is well described, whereas our findings suggest that cfDNA metagenomic sequencing is a more promising direct pathogen detection method. Among patients with laboratory-confirmed EM, *B.b.* cfDNA detection was 64% sensitive, compared with 7% using blood PCR. Notably, this was achieved without prior capture/enrichment of *B.b.* DNA targets from the plasma samples, and without optimization of the Karius microbial cfDNA sequencing method to increase detection of *B.b.* DNA. Adding these steps is expected to improve sensitivity further [3], and future studies will address this.

It is unlikely that the difference in sensitivity between *B.b.* cfDNA detection and blood PCR is explained by differences in the volume of primary sample analyzed. For *B.b.* cfDNA detection, each DNA extraction was performed on 250 μL of EDTA-plasma, whereas for PCR the starting sample volume was 500 μL of EDTA-whole blood, which should translate to at least 250 μL of plasma on average. (As an extracellular bacterium, *B.b.* is present in the plasma fraction of blood.) More likely, the results are attributable to another methodological difference: *B.b.* PCR methods rely on detection of specific, intact DNA targets that are present in limited quantity within each spirochete (typically 1 copy per organism), whereas cfDNA detection is unbiased and can reveal an organism's presence if informative DNA sequence is produced from anywhere in the genome, even if the DNA is fragmented.

Besides *B.b.*, 2 other vector-borne pathogens were detected by metagenomic sequencing in our study. *Francisella tularensis* cfDNA reads were detected in plasma from 1 ill ambulatory control subject, and this finding's significance was confirmed by demonstration of seroconversion using an *F. tularensis* microagglutination assay. *Rickettsia felis* cfDNA reads were detected in 2 patients with laboratory-confirmed EM, but subsequent efforts to confirm *R. felis* infection using whole-blood PCR assays were unsuccessful. Unfortunately, a validated serologic assay for *R. felis* is not available to assess for seroconversion in these patients, and it is possible that blood PCR assays for *R. felis* are simply less sensitive compared with microbial cfDNA detection (as with *Borrelia* blood PCR). Overall, the clinical significance of this finding remains uncertain, although it warrants future investigation. While human infection with *R. felis* is most common in sub-Saharan Africa, cases have been documented in Europe and the United States [22]. Our findings raise questions about the potential for unappreciated exposure to this emerging pathogen in temperate climates, and the significance of its detection in patients with concurrent infections.

A potential limitation of our study is the use of relaxed criteria for reporting *B.b.* among microorganisms detected using cfDNA metagenomic sequencing. To control for environmental contamination, the routine Karius test (offered for clinical diagnostic purposes) contrasts the quantity of sequence reads observed for each microbe against the quantity observed in a set of negative control samples; microorganisms are only reported if a statistically significant difference between the 2 quantities is observed. While this approach is critical for achieving high analytical specificity across a broad spectrum of microbes, not all microbes pose the same level of risk. For *B.b.*, we hypothesized that the usual statistical threshold was not needed, as *B.b.* is not a known environmental contaminant, nor does it exhibit high homology with other free-living contaminants. As such, incidental *B.b.* cfDNA detection in human plasma is assumed to be highly unlikely. Thus, in a modification of the clinical Karius

test method made for investigational use only, we considered *B.b.*-specific reads to be reportable in any quantity for cases and control subjects, and dispensed with the statistical threshold (only for *B.b.*). This resulted in higher sensitivity for *B.b.* detection than would have been achieved with the clinical version of the test. The validity of this approach is supported by our retrospective review of results from 684 asymptomatic control subjects and 3000 hospitalized patients, which demonstrated that *B.b.* cfDNA reads were not detected in any quantity except in 2 hospitalized individuals, both of whom had a clinical syndrome compatible with early LB.

Our study is also limited by its focus on patients with EM, without inclusion of patients with other LB manifestations (or other vector-borne infections). Although blood PCR techniques have proven insensitive for the diagnosis of acute neuroborreliosis or Lyme arthritis, we have demonstrated that *B.b.* cfDNA detection has higher sensitivity than blood PCR in EM, and its potential use in the diagnosis of noncutaneous LB should be investigated. Indeed, previous studies have shown that microbial cfDNA sequencing is capable of detecting pathogen DNA in plasma from patients with deep-seated infection at distant sites [23–26]. Also, our detection of *F. tularensis* in a symptomatic control subject suggests that microbial cfDNA detection for diagnosis of tick-borne illnesses more broadly may have utility. Future studies will address whether this single platform could be applied as a robust syndromic test for direct detection of a wide array of pathogens in patients with suspected tick-borne illness.

In summary, unbiased metagenomic sequencing applied to acute-phase plasma samples can detect *B.b.* cfDNA in patients with EM. This approach was significantly more sensitive compared with whole-blood PCR, both in the full cohort of patients with physician-diagnosed EM and in the subset of patients with laboratory-confirmed EM. When *B.b.* cfDNA detection was combined with acute-phase serologic testing using MTTT algorithms, 86% of patients with laboratory-confirmed EM were positive by 1 or both diagnostic methods, demonstrating that prompt, timely, and accurate test results can potentially be obtained in early LB by pairing a direct detection method with an indirect detection method at the time of initial clinical presentation. Technical modifications to the *B.b.* cfDNA detection assay will be pursued in an attempt to further improve sensitivity.

## Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

## Notes

## References

1. Steere AC, McHugh G, Damle N, Sikand VK. Prospective study of serologic tests for Lyme disease. Clin Infect Dis **2008**; 47:188–95.
2. Feder HM Jr, Gerber MA, Luger SW, Ryan RW. Persistence of serum antibodies to *Borrelia burgdorferi* in patients treated for Lyme disease. Clin Infect Dis **1992**; 15:788–93.
3. Schutzer SE, Body BA, Boyle J, et al. Direct diagnostic tests for Lyme disease. Clin Infect Dis **2019**; 68:1052–7.
4. Nowakowski J, Schwartz I, Liveris D, et al; Lyme Disease Study Group. Laboratory diagnostic techniques for patients with early Lyme disease associated with erythema migrans: a comparison of different techniques. Clin Infect Dis **2001**; 33:2023–7.
5. Liveris D, Schwartz I, McKenna D, et al. Comparison of five diagnostic modalities for direct detection of *Borrelia burgdorferi* in patients with early Lyme disease. Diagn Microbiol Infect Dis **2012**; 73:243–5.
6. Liveris D, Schwartz I, McKenna D, et al. Quantitation of cell-associated borrelial DNA in the blood of Lyme disease patients with erythema migrans. Eur J Clin Microbiol Infect Dis **2012**; 31:791–5.
7. Coulter P, Lema C, Flayhart D, et al. Two-year evaluation of *Borrelia burgdorferi* culture and supplemental tests for definitive diagnosis of Lyme disease. J Clin Microbiol **2005**; 43:5080–4.
8. Jones KL, Glickstein LJ, Damle N, Sikand VK, McHugh G, Steere AC. *Borrelia burgdorferi* genetic markers and disseminated disease in patients with early Lyme disease. J Clin Microbiol **2006**; 44:4407–13.
9. Snyder JL, Giese H, Bandoski-Gralinski C, et al. T2 magnetic resonance assay-based direct detection of three Lyme disease-related *Borrelia* species in whole-blood samples. J Clin Microbiol **2017**; 55:2453–61.
10. Pícha D, Moravcová L, Zdárský E, Maresová V, Hulínský V. PCR in Lyme neuroborreliosis: a prospective study. Acta Neurol Scand **2005**; 112:287–92.
11. Dumler JS. Molecular diagnosis of Lyme disease: review and meta-analysis. Mol Diagn **2001**; 6:1–11.
12. Avery RA, Frank G, Eppes SC. Diagnostic utility of *Borrelia burgdorferi* cerebrospinal fluid polymerase chain reaction in children with Lyme meningitis. Pediatr Infect Dis J **2005**; 24:705–8.
13. Blauwkamp TA, Thair S, Rosen MJ, et al. Analytical and clinical validation of a microbial cell-free DNA sequencing test for infectious disease. Nat Microbiol **2019**; 4:663–74.
14. Council of State and Territorial Epidemiologists. Revised national surveillance case definition for Lyme disease. **2007**. Available at: https://cdn.ymaws.com/www.cste.org/resource/resmgr/PS/07-ID-11.pdf. Accessed 24 February 2020.
15. Branda JA, Strle K, Nigrovic LE, et al. Evaluation of modified 2-tiered serodiagnostic testing algorithms for early Lyme disease. Clin Infect Dis **2017**; 64:1074–80.
16. Vaz A, Glickstein L, Field JA, et al. Cellular and humoral immune responses to *Borrelia burgdorferi* antigens in patients with culture-positive early Lyme disease. Infect Immun **2001**; 69:7437–44.
17. Liveris D, Schwartz I, Bittker S, et al. Improving the yield of blood cultures from patients with early Lyme disease. J Clin Microbiol **2011**; 49:2166–8.
18. Centers for Disease Control and Prevention. Recommendations for test performance and interpretation from the Second National Conference on Serologic Diagnosis of Lyme Disease. MMWR Morb Mortal Wkly Rep **1995**; 44: 590–1.
19. Mead P, Petersen J, Hinckley A. Updated CDC recommendation for serologic diagnosis of Lyme disease. MMWR Morb Mortal Wkly Rep **2019**; 68:703.
20. Smith RP, Schoen RT, Rahn DW, et al. Clinical characteristics and treatment outcome of early Lyme disease in patients with microbiologically confirmed erythema migrans. Ann Intern Med **2002**; 136:421–8.
21. Wormser GP, Masters E, Nowakowski J, et al. Prospective clinical evaluation of patients from Missouri and New York with erythema migrans-like skin lesions. Clin Infect Dis **2005**; 41:958–65.
22. Angelakis E, Mediannikov O, Parola P, Raoult D. *Rickettsia felis*: the complex journey of an emergent human pathogen. Trends Parasitol **2016**; 32:554–64.
23. Farnaes L, Wilke J, Ryan Loker K, et al. Community-acquired pneumonia in children: cell-free plasma sequencing for diagnosis and management. Diagn Microbiol Infect Dis **2019**; 94:188–91.
24. Hong DK, Blauwkamp TA, Kertesz M, Bercovici S, Truong C, Banaei N. Liquid biopsy for infectious diseases: sequencing of cell-free plasma to detect pathogen DNA in patients with invasive fungal disease. Diagn Microbiol Infect Dis **2018**; 92:210–3.
25. Vudatha V, Ranson M, Blair L, Ahmed AA. Rapid detection of bacille Calmette-Guérin-associated mycotic aortic aneurysm using novel cell-free DNA assay. J Vasc Surg Cases Innov Tech **2019**; 5:143–8.
26. Rossoff J, Chaudhury S, Soneji M, et al. Noninvasive diagnosis of infection using plasma next-generation sequencing: a single-center experience. Open Forum Infect Dis **2019**; 6:ofz327.