

Assessment of New Culture Method for Detection of *Borrelia* Species from Serum of Lyme Disease Patients

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A novel method of culturing spirochetes from the serum of U.S. Lyme disease patients was recently reported by Sapi and colleagues to have 94% sensitivity and 100% specificity for *Borrelia* species as assessed by microscopy and DNA sequence analysis of the *pyrG* gene (E. Sapi, N. Pabbati, A. Datar, E. M. Davies, A. Rattelle, and B. A. Kuo, *Int. J. Med. Sci.* 10:362–376, 2013). The majority of the spirochetes described were related by *pyrG* sequences to species of *Borrelia* previously undetected in North American patients without a reported history of travel to Europe or Asia. To better understand these unexpected findings, we determined *pyrG* sequences of the laboratory reference strains used by the investigators for method development and testing of culture medium. Eighty percent (41/51) of the reported patient-derived *pyrG* sequences were identical to one of the laboratory strains, and an additional 12% (6/51) differed by only a single nucleotide across a 603-bp region of the *pyrG* gene. Thus, false positivity due to laboratory contamination of patient samples cannot be ruled out, and further validation of the proposed novel culture method is required.

A new method of culturing Lyme disease bacteria from sera of U.S. patients was recently described by Sapi et al. to have exceptionally high clinical sensitivity (94%, 68/72 cases) and specificity (100%, 0/48 controls) (1). DNA sequencing of a portion of the CTP synthase gene (*pyrG*) was used to determine that the *Borrelia* isolates were closely related to *Borrelia burgdorferi*, *Borrelia garinii*, or *Borrelia afzelii*. These results are at variance with those of numerous previous studies.

Prior to this publication, only *B. burgdorferi* had been reported in U.S. patients without a history of travel to Europe or Asia, where *B. garinii* and *B. afzelii* are endemic (2–4). *B. afzelii* has not been isolated from nature in the Western Hemisphere; *B. garinii* has been detected in this hemisphere only in ticks infesting sea birds on coastal islands (Newfoundland) remote from human habitation (5).

B. burgdorferi has been cultured from the plasma of U.S. erythema migrans patients with close to 50% sensitivity (6–9), whereas culture of *B. burgdorferi* from serum was significantly less successful (17%, $P = 0.005$) (6). In patients with later manifestations of Lyme disease affecting neurologic, cardiac, or musculoskeletal systems, the success of culture from plasma also was below 20%; cultures were obtained only in patients who had later-stage illness for a short time (mean, 14 days; range, 3 to 21 days) (10). The authors of the new culture method did not report the duration or clinical manifestations of Lyme disease in their study population (1), although they stated that all patients were seropositive by standard 2-step testing and had not been treated with antibiotics in the 4 weeks before blood collection.

The Centers for Disease Control and Prevention has received numerous inquiries from national and state health departments and clinicians about the performance of this culture method since Advanced Laboratory Services began offering it in 2012. In view of the novelty and potential clinical significance of the findings, we conducted experiments to better understand the results reported in the study. Using the same primers used by Sapi et al., we sequenced a portion of the *pyrG* gene of each of the laboratory strains used by these investigators for method development and

quality control of culture medium and compared the results with the *pyrG* sequences reported from their patient samples (1).

MATERIALS AND METHODS

Borrelia burgdorferi sensu stricto strain 297 (ATCC 53899), *B. afzelii* strain BO23 (11) (ATCC 51992), and *B. garinii* strain Fuji P1 (11) (ATCC 51991) were purchased from the American Type Culture Collection. Cultures were grown at 34°C in Barbour-Stoenner-Kelly II (BSKII) medium (12) and harvested by centrifugation.

Genomic DNA was extracted from pelleted bacteria using the Promega Wizard genomic DNA purification kit (Promega, Madison, WI) following the manufacturer's protocol for Gram-negative bacteria. *pyrG* was amplified using the *pyrF391/pyrR1190* primer set (13) in a 50- μ l reaction mixture containing Qiagen HotStar *Taq* (2.5 units), HotStar *Taq* buffer (1.5 mM $MgCl_2$, 200 μ M each deoxynucleoside triphosphate [dNTP], and 1.0 μ M each primer), and between 2×10^6 and 2×10^7 genome copies. Template-free PCRs served as negative controls to monitor for DNA/amplicon contamination. The thermocycling conditions were 95°C for 15 min; 5 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s; 35 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 30 s; and 72°C for 5 min. The products were purified using QIAquick PCR purification kits (Qiagen) according to the manufacturer's instructions. Sequencing reactions were run with the primers *pyrF391*, *pyrF448*, *pyrR1154*, and *pyrR1190* (13) and BigDye Terminator ready reaction mix diluted 1:8 following the BigDye Terminator v3.1 cycle sequencing kit reaction mixture and cycle sequencing protocols. Prior to electrophoresis, sequencing reactions were cleaned using the Applied Biosystems BigDye XTerminator purification kit. Electrophoresis and data collection were performed on an ABI 3130xl genetic analyzer. At least 10-fold coverage of the 603-bp sequence analyzed by Sapi et al. was obtained. DNA sequence assembly and alignments were performed using Clustal Omega and DNASTAR LaserGene 9 software.

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DNA was amplified and sequenced from *B. burgdorferi* strain 297 two times independently to verify the microheterogeneity observed at position 546 of the 603 bp analyzed.

Nucleotide sequence accession numbers. The sequences described here were submitted to GenBank and assigned accession numbers [KF170280](#) (strain BO23), [KF170281](#) (strain 297), and [KF170282](#) (strain Fuji P1).

RESULTS

Sapi et al. characterized *Borrelia* cultured from serum samples by sequencing a 603-bp portion of the *pyrG* housekeeping gene for 51 of 68 culture-positive patients (1). This region of *pyrG* concatenated with portions of 7 other housekeeping genes has been used previously in multilocus sequence typing to study the population structure of *Borrelia* species in Europe and North America (13). Sapi et al. compared the *pyrG* gene sequences of *Borrelia* cultured from Lyme disease patients with *pyrG* of *B. burgdorferi* strain B31 as a reference. Because polymorphism in most patient samples was observed (up to 51 nucleotide differences from strain B31 out of 603 nucleotides analyzed), the cultured strains were judged to indicate active infection of patients.

We examined the 51 sequences reported from patient samples and compared them with sequences of the 4 laboratory strains used by this group (*B. burgdorferi* strains B31 and 297, *B. garinii* strain Fuji P1, and *B. afzelii* strain BO23). Of these 4 strains, the *pyrG* gene sequence had been reported only for strain B31 (GenBank [E000783.1:588066.0.589667](#)) (14). Accordingly, we determined the relevant portion of the *pyrG* DNA sequences of the other laboratory strains and deposited them in GenBank ([KF170280](#) [strain BO23], [KF170281](#) [strain 297], and [KF170282](#) [strain Fuji P1]).

The 603-bp region of *pyrG* of *B. burgdorferi sensu stricto* strain 297 is identical to the homologous region of strain B31 with the exception of a single polymorphism at nucleotide position 546. Approximately half of the amplicons contained a T at this position, and the remainder contained a C; C is also present at position 546 in strain B31.

A comparison of the DNA sequences associated with Lyme disease patients to those of the laboratory strains used during method development and quality control is shown in [Table 1](#). The majority of the patient-related sequences described by Sapi et al. (53%, 27/51) indicate infection by *B. garinii*, a species of *Borrelia* not found in the United States. When the nucleotide sequences were compared, 20/27 clones were identical to each other and to the Japanese *B. garinii* Fuji P1 strain used by these authors. Of the other *B. garinii* sequences (7/27), five had single nucleotide differences from the laboratory strain, one had two nucleotide differences from the laboratory strain, and one had three nucleotide differences from the laboratory strain used by the authors during method development.

The second most common group of patient sequences (41%, 21/51) was related to *B. burgdorferi*. In 20 of these patients, the nucleotide sequences match the laboratory strain B31 exactly over the 603-bp region studied. One patient-associated sample contained a single nucleotide difference from the *B. burgdorferi* reference strain.

Sequences of two clones from patient samples (4%, 2/51) were most closely related to *Borrelia afzelii*, a second Eurasian genospecies of *Borrelia* that is not found in the United States. We determined that the *pyrG* sequence of the German strain BO23 is iden-

tical to one patient-associated clone and differs by two nucleotides from a second patient sequence.

Only one patient sequence (JX87376.1) was highly divergent. It aligned most closely with *B. burgdorferi* and showed 21 nucleotide differences from strain B31, 29 differences from *B. garinii* Fuji P1, and 31 differences from *B. afzelii* BO23. This sequence might indicate patient infection by a distinctive strain of *Borrelia*; however, the sequence is consistent with sample contamination by two laboratory strains. Of the 21 nucleotide differences between JX87376.1 and strain B31, 20 agreed with the *B. afzelii* strain BO23 sequence at the relevant positions. Trace files of the primary sequence data should reveal two nucleotide peaks at the divergent positions if mixed amplicons were sequenced. If evidence of two templates is lacking in the trace files, a more robust genetic analysis, such as sequence typing using multiple polymorphic genes, can clarify the phylogeny and clinical significance of this sample.

Overall, 80% (41/51) of the patient-associated gene sequences are identical to those of the laboratory controls, and the majority of the remaining patient sequences (6/10) differ from one of the laboratory strains by only a single nucleotide.

DISCUSSION

The methods used by Sapi et al. to assess test specificity were inadequate to rule out false positivity due to contamination by cultures of laboratory strains or PCR amplicons. Patient cultures were subjected to nested PCR, a contamination-prone method that is unnecessary when bacteria are numerous enough to be seen by microscopy. Samples from healthy blood donors were judged to be negative only by dark-field microscopy and antibody staining. PCR testing of these control samples was not performed. Furthermore, these authors reported that all 68 cultures from Lyme disease patients were stained by a monoclonal antibody that bound to *B. burgdorferi* strains B31 and 297 and that this antibody did not bind *B. garinii* Fuji P1 or *B. afzelii* BO23. However, 56% (29/51) of the patient-associated cultures group phylogenetically with *B. garinii* or *B. afzelii* and should not have been stained by a monoclonal antibody reportedly specific for *B. burgdorferi*. Thus, microscopy results contradict the sequence-derived phylogenetic determinations.

All patient-associated sequences were compared with *B. burgdorferi* strain B31 to assert that numerous polymorphic sites existed in *pyrG* sequences (Table 3 of reference 1). Since polymorphism is a measure of within-species variation, this analysis implies that the authors believe that the patients became infected with various strains of the species *B. burgdorferi*, not with other species of Lyme disease agents. The lack of reported history of foreign travel for the patients and the absence of a protocol to exclude foreign travelers from eligible enrollees reinforce this interpretation. Nevertheless, the majority of patient-associated sequences (29/51) are grouped with Eurasian *Borrelia* species in Fig. 9 of reference 1. Furthermore, these patients were reported to be seropositive by standard 2-tiered serologic tests used in the United States. Conventional U.S. 2-tiered serology is significantly less sensitive in diagnosing Lyme disease acquired in Europe than tests specifically designed for use in Europe (15), where *B. garinii* and *B. afzelii* are the predominant etiologic agents. Selection of the majority of patients from a larger cohort of foreign travelers would have been required to obtain 29 seropositive patients infected with Eurasian *Borrelia*, but selection was not described.

The few nucleotide differences observed between patient sam-

TABLE 1 Comparison of patient-associated *pyrG* gene sequences with those of laboratory strains of *B. burgdorferi sensu lato*

Patient no.	Patient-associated <i>pyrG</i> sequence	Laboratory strain to which sequence was mapped	Fractional identity	% identity
1	JX867375.1	<i>B. garinii</i> Fuji P1	603/603	100.00
2	JX867401.1	<i>B. garinii</i> Fuji P1	603/603	100.00
3	JX867402.1	<i>B. garinii</i> Fuji P1	603/603	100.00
4	JX867403.1	<i>B. garinii</i> Fuji P1	603/603	100.00
5	JX867404.1	<i>B. garinii</i> Fuji P1	603/603	100.00
6	JX867406.1	<i>B. garinii</i> Fuji P1	603/603	100.00
7	JX867407.1	<i>B. garinii</i> Fuji P1	603/603	100.00
8	JX867408.1	<i>B. garinii</i> Fuji P1	603/603	100.00
9	JX867409.1	<i>B. garinii</i> Fuji P1	603/603	100.00
10	JX867410.1	<i>B. garinii</i> Fuji P1	603/603	100.00
11	JX867412.1	<i>B. garinii</i> Fuji P1	603/603	100.00
12	JX867413.1	<i>B. garinii</i> Fuji P1	603/603	100.00
13	JX867414.1	<i>B. garinii</i> Fuji P1	603/603	100.00
14	JX867415.1	<i>B. garinii</i> Fuji P1	603/603	100.00
15	JX867416.1	<i>B. garinii</i> Fuji P1	603/603	100.00
16	JX867418.1	<i>B. garinii</i> Fuji P1	603/603	100.00
17	JX867420.1	<i>B. garinii</i> Fuji P1	603/603	100.00
18	JX867421.1	<i>B. garinii</i> Fuji P1	603/603	100.00
19	JX867422.1	<i>B. garinii</i> Fuji P1	603/603	100.00
20	JX867423.1	<i>B. garinii</i> Fuji P1	603/603	100.00
21	JX867378.1	<i>B. garinii</i> Fuji P1	602/603	99.80
22	JX867379.1	<i>B. garinii</i> Fuji P1	602/603	99.80
23	JX867380.1	<i>B. garinii</i> Fuji P1	602/603	99.80
24	JX867411.1	<i>B. garinii</i> Fuji P1	602/603	99.80
25	JX867417.1	<i>B. garinii</i> Fuji P1	602/603	99.80
26	JX867394.1	<i>B. garinii</i> Fuji P1	601/603	99.70
27	JX867393.1	<i>B. garinii</i> Fuji P1	600/603	99.50
28	JX867374.1	<i>B. burgdorferi</i> B31	603/603	100.00
29	JX867377.1	<i>B. burgdorferi</i> B31	603/603	100.00
30	JX867381.1	<i>B. burgdorferi</i> B31	603/603	100.00
31	JX867382.1	<i>B. burgdorferi</i> B31	603/603	100.00
32	JX867383.1	<i>B. burgdorferi</i> B31	603/603	100.00
33	JX867384.1	<i>B. burgdorferi</i> B31	603/603	100.00
34	JX867385.1	<i>B. burgdorferi</i> B31	603/603	100.00
35	JX867386.1	<i>B. burgdorferi</i> B31	603/603	100.00
36	JX867387.1	<i>B. burgdorferi</i> B31	603/603	100.00
37	JX867388.1	<i>B. burgdorferi</i> B31	603/603	100.00
38	JX867389.1	<i>B. burgdorferi</i> B31	603/603	100.00
39	JX867390.1	<i>B. burgdorferi</i> B31	603/603	100.00
40	JX867391.1	<i>B. burgdorferi</i> B31	603/603	100.00
41	JX867392.1	<i>B. burgdorferi</i> B31	603/603	100.00
42	JX867395.1	<i>B. burgdorferi</i> B31	603/603	100.00
43	JX867396.1	<i>B. burgdorferi</i> B31	603/603	100.00
44	JX867397.1	<i>B. burgdorferi</i> B31	603/603	100.00
45	JX867399.1	<i>B. burgdorferi</i> B31	603/603	100.00
46	JX867400.1	<i>B. burgdorferi</i> B31	603/603	100.00
47	JX867405.1	<i>B. burgdorferi</i> B31	603/603	100.00
48	JX867419.1	<i>B. burgdorferi</i> B31	602/603	99.80
49	JX867376.1	<i>B. burgdorferi</i> B31	582/603	96.50
50	JX867398.1	<i>B. afzelii</i> BO23	603/603	100.00
51	JX867424.1	<i>B. afzelii</i> BO23	601/603	99.70

ples and laboratory strains are of unknown diagnostic significance. Some microheterogeneity in gene sequence is expected since the ATCC-supplied *Borrelia* species stocks of strains 297, Fuji P1, and BO23 were not reported to have been cloned at the cellular level. In fact, we detected one polymorphism in the *pyrG* gene of the seed stock of strain 297 after a single passage in our laboratory. Additional microheterogeneity could have been selected for and enriched by passage of strains in culture, since the

authors reported maintaining *Borrelia* cultures for as long as 8 months (1). Finally, since Sapi et al. did not report the number of times each patient-associated DNA strand was sequenced, sequencing errors cannot be excluded. Errors are more likely if sequencing coverage was low.

To establish that the culture and sequence results differ from those for laboratory strains, evaluation of all patient samples using multiple highly polymorphic gene targets or equally powerful an-

alytic methods is essential. To demonstrate that a new culture method is superior to standard culture in BSKII medium, side-by-side comparison of the two methods is required. Without a full description of the patient population, including Lyme disease stage and treatment and patient travel history, comparisons with standard culture methods cannot be made. Cultures should be archived for independent study, especially when they are thought to be *genospecies* isolated for the first time in North America.

Taken together, our data and those of Sapi et al. indicate that laboratory contamination was the probable source of the borrelial DNA found in the patient samples. The vast majority of patient *pyrG* sequences (41/51) are indistinguishable from the laboratory strains used by the investigators. The clinical relevance of the other *pyrG* sequences (10/51) is unclear; these findings also may be consistent with laboratory contamination. Before use in clinical medicine, novel findings must be independently verified. Independent verification is particularly critical when claims are at odds with a large body of other scientific work and when they may trigger unnecessary antibiotic treatment of patients. We caution clinicians and patients to wait for independent verification by scientifically sound methods before using this culture service for diagnostic purposes.

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The findings and conclusions are those of the authors and do not necessarily represent the official position of the agency.

The authors report no potential conflicts of interest.

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