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Original Research

Sanger Sequencing of *Borrelia burgdorferi flaB* Paralogs Detected Spirochetemia at the Early Localized Stage of Lyme Disease

Sin Hang Lee[1,*]

[1]Milford Molecular Diagnostics Laboratory, Milford, CT 06460, USA
*Correspondence: shlee01@snet.net (Sin Hang Lee)
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Abstract

Background: The general assumption that spirochetemia does not occur at the early localized stage of Lyme disease is due to a lack of sensitive and specific methods for molecular diagnosis. **Methods**: During a Lyme disease season in 2023, the platelet-rich plasma specimens of 145 people residing in Lyme disease-endemic areas in the United States were immediately separated from the blood cells following venous blood collection to prevent the spirochetes, if any, from invading the lymphocytes in the test tube. The entire DNA content was extracted from the platelet pellet and used for split sample polymerase chain reaction (PCR) amplification; Sanger sequencing was performed on the nested PCR products to detect the *Borrelia burgdorferi flaB* and *16S rRNA* genes. **Results**: In 98 of the people who were clinically suspected of having early localized Lyme disease irrespective of the presence or absence of a skin lesion, 33 of their blood specimens (33.7%) were positive for *Borrelia burgdorferi* (*B. burgdorferi*), including 17 positive for *flaB* gene only, 15 positive for both the *flaB* and *16S rRNA* genes, and one positive for *16S rRNA* gene only. Eight (17.0%) of the 47 asymptomatic resident controls were positive for *flaB* PCR only. **Conclusions**: The *flaB* gene is a more sensitive chromosomal target than the *16S rRNA* gene for molecular detection of one to three *B. burgdorferi* cells due to spirochetes gaining or retaining *flaB* paralogs at the early localized stage of Lyme disease.

Keywords: Sanger sequencing; spirochetemia; nested PCR; early localized Lyme disease; *flaB* paralogs; SNP in *flaB* gene; single *Borrelia burgdorferi* cell; platelet-rich plasma

1. Introduction

Lyme disease, a systemic bacterial infection caused by *Borrelia burgdorferi* (*B. burgdorferi*), is the most common tickborne disease in the United States. Released 2010–2018 insurance records suggest that each year approximately 476,000 Americans are diagnosed and treated for Lyme disease [1]. But the true number of Lyme disease patients is unknown due to the lack of reliable sensitive and specific routine laboratory diagnostic tools. It is believed that at the early stage of infection this disease can be treated successfully with a proper course of antibiotics, but the spirochete bacteria may disseminate from the site of the tick bite to other regions of the body if not timely and properly treated [2].

It has been assumed that the *B. burgdorferi* spirochetes begin to enter the blood stream at the stage 2 of Lyme disease [3,4] about 3 to 12 weeks after the initial infection [5], and that during the early localized stage of infection, the number of *B. burgdorferi* cells simply increases in the dermal tissue in preparation for dissemination [6]. However, animal experiments showed that blood-borne dissemination of *B. burgdorferi* can be detected at day 2 after intradermal inoculation [7]. Like *Leptospira interrogans*, which enters the blood stream rapidly after invasion into the human body through the skin and mucosa [8], *B. burgdorferi* spirochetemia can occur in early infections based on epidemiological studies [9]. The key to diagnosing Lyme disease spirochetemia at the "early localized" stage of infection is to develop a highly sensitive, specific molecular blood test for the detection of Lyme disease spirochetes in low single-digit numbers.

As previously reported, nested polymerase chain reaction (PCR) amplification of the borrelial *16S rRNA* gene DNA extracted from the spirochetes in the platelet pellet obtained by differential centrifugation followed by Sanger sequencing of the PCR products can diagnose spirochetemias with a bacterial density as low as 25 per mL of whole blood [10]. However, *B. burgdorferi* can actively attach to and invade human lymphocytes during co-incubation of the spirochetes and the blood cells *in vitro* [11]. If there were only a few *B. burgdorferi* spirochetes in the whole blood specimen, delayed exclusion of the blood cells from the platelet-rich plasma may lead to loss of all the spirochetes to the buffy coat by allowing the highly mobile spirochetes to attach to the lymphocytes prior to blood centrifugation.

This research project was designed to prove that immediate separation of the platelet-rich plasma after venous blood collection, using the entire DNA content extracted from the platelet pellet for nested PCR, and targeting the newly found duplicated paralogs of the *flaB* gene for PCR amplification can increase the chances of detecting a single *B. burgdorferi* spirochete in 1 mL of platelet-rich plasma.

While plasmid gene duplication and loss by adaptation to multiple host species are well known phenomena in the life cycle of *B. burgdorferi* [12,13], *flaB* gene duplication in the *B. burgdorferi* chromosome has not been reported.

2. Materials and Methods

2.1 Patients and Specimens

Per agreement, DiaSorin, Inc., Stillwater, MN, USA, enrolled the patients and healthy donor controls and performed the initial specimen preparation for a research project after informed consent was obtained in accordance with applicable regulations in compliance with 21 CFR 812. A total of 98 symptomatic patients clinically suspected of having early localized Lyme disease and 47 asymptomatic residents serving as controls were included in this study. The patients clinically suspected of having early localized Lyme disease were defined as people residing in a known Lyme disease-endemic area in the United States who developed a recent onset of fatigue, skin rash, fever, muscle aches, neck pain, joint pain, or lymphadenopathy during the Lyme disease season in 2023. Their venous blood specimens were drawn for testing when any of these symptoms and signs first appeared. The asymptomatic resident controls, also referred to as healthy donors by DiaSorin, Inc., the specimen supplier, were people residing in the same communities with the symptomatic patients during the Lyme disease season in 2023.

2.2 Platelet-rich Plasma Preparation

Specimen collection was performed under the participating collection site's Institutional Review Board-approved protocols and under informed consent. Preparation of platelet-rich plasma (PRP) followed a standard protocol [14] with slight modification. Specifically, at the participating collection sites, about 3 mL of the patient's venous blood was drawn into a lavender top tube containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. After the whole blood was mixed well for ~5 minutes, the rubber stopper was removed and replaced with a plastic over cap. The whole blood specimen was centrifuged for 15 minutes at 400 ×g within 30 minutes after blood collection; and 1 mL of platelet-rich plasma above the buffy coat was transferred into a labeled plastic vial with screw cap. If the blood collection site did not have centrifugation equipment to produce the platelet-rich plasma, the whole blood specimens were placed in an ice bath immediately and transported to another facility in ice packs for processing on the same day to prevent the spirochetes from invading lymphocytes in the test tube at ambient temperature. All platelet-rich plasma specimens were stored at –20 °C up to 3 months before being sent to Milford Molecular Diagnostics Laboratory (Milford, CT, USA) blind-coded by numbers in dry ice via overnight delivery. Our experience showed that frozen PRP shipped overnight at ambient temperature is suitable for PCR detection of *B. burgdorferi*. EDTA is a known bacteriostatic agent [15] and prevents overgrowth of contaminating bacteria in the PRP.

2.3 Total Platelet Fraction DNA for PCR

This was a modified procedure from a previously published protocol [10]. At Milford Molecular Diagnostics Laboratory, the frozen platelet-rich plasma was defrosted and the entire thawed specimen was centrifuged in a 1.5 mL Eppendorf tube at ~16,000 ×g for 10 min. After the supernatant was discarded, the pellet of platelets was re-suspended in 300 µL of 0.5 M ammonium hydroxide. The mixture was heated at 97 °C for 5 min with closed cap, followed by 10 min with open cap. After the mixture was cooled down to room temperature, 30 µL of 3 M sodium acetate and 700 µL of ice-cold 95% ethanol were added into each tube. The suspension of the crude DNA along with the proteins precipitated was divided into two equal parts in Eppendorf tubes for split sample testing without further purification, one labeled for *B. burgdorferi flaB* gene and the other for borrelial *16S rRNA* gene PCR amplification. All tubes were put in a –20 °C freezer. After 1 hour, the contents of the tubes were centrifuged at ~16,000 ×g for 5 min, and each pellet was washed with 700 µL of ice-cold 70% ethanol.

2.4 Primary PCR Amplification

All Eppendorf tubes containing the 70% ethanol-washed pellets were put in an 85 °C heating block for 2 min with opened cap to evaporate the residual ethanol. Into each tube, 25 µL of complete "*flaB* primary PCR mixture" or 25 µL of complete "borrelial *16S rRNA* gene primary PCR mixture" was added. The tubes were heated with closed caps at 85 °C for another 3 min, then vortexed to dissolve the nucleic acids into the PCR mixture.

After centrifugation at ~16,000 ×g for 5 min, the entire liquid portion in the centrifuge tube was transferred to a 0.2 mL PCR tube. The thermocycling steps for PCR amplification were programmed to 30 cycles at 85 °C for 30 s, 50 °C for 30 s, and 65 °C for 1 min after an initial heating for 10 min at 85 °C, with a final extension at 65 °C for 10 min.

The 25 µL of complete "*flaB* primary PCR mixture" contained 20 µL of ready-to-use LoTemp® PCR mix (HiFi DNA Tech, LLC, Trumbull, CT, USA), 3 µL of molecular grade water, 1 µL of 10 µmolar *flaB* outer forward (FOF) primer (5′-GCATCACTTTCAGGGTCTCA-3′), and 1 µL of 10 µmolar *flaB* outer reverse (FOR) primer (5′-TGGGGAACTTGATTAGCCTG-3′) to amplify a 503-bp segment of the *B. burgdorferi flaB* gene [16].

The 25 µL of complete "borrelial *16S rRNA* gene primary PCR mixture" contained 20 µL of ready-to-use LoTemp® PCR mix, 3 µL of molecular grade water, 1 µL of 10 µmolar M1 forward primer (5′-ACGATGCACACTTGGTGTTAA-3′), and 1 µL of 10 µmolar M2 reverse primer (5′-

TCCGACTTATCACCGGCAGTC-3′) to amplify a 357/358-bp segment of the borrelial *16S rRNA* gene [10]. DNA extracts from the cultured cells of *Borrelia burgdorferi* (ATCC 53210) and of *Borrelia coriaceae* (ATCC 43381) were used as the positive control for *flaB* amplification and as the positive control for borrelial *16S rRNA* gene amplification, respectfully.

Borrelia coriaceae is rarely found in human specimens and its *16S rRNA* gene has the same M1 and M2 primer sites as those of the pathogenic *Borrelia* species. Using *B. coriaceae* instead of *B. burgdorferi* as the positive control helps monitor bench work contamination. Contamination by *B. coriaceae 16S rRNA* gene DNA will be recognized easily at the stage of Sanger sequencing, thus avoiding false-positive results due to control contamination. However, the *flaB* gene sequences of various *Borrelia* species are heterogeneous. In the United States, it is necessary to use *B. burgdorferi* sensu stricto *flaB* DNA as the positive control for *flaB* PCR amplification.

2.5 Nested PCR Amplification

Transferring of post-PCR materials from one test tube to another was accomplished by using a micro-glass rod in a 32″ PCR workstation (AirClean Systems, Raleigh, NC, USA) to avoid aerosols, which may occur in routine micropipetting. In order to minimize the potential for contamination, DNA extractions, PCR setup, and agarose gel electrophoresis were performed in three separate rooms.

A trace (about 0.2 µL) of each of the primary PCR products was transferred by a calibrated micro-glass rod to another 25 µL complete PCR mixture containing 20 µL of ready-to-use LoTemp® PCR mix, 3 µL of molecular grade water, 1 µL of 10 µmolar forward primer and 1 µL of 10 µmolar reverse primer for nested PCR or same-nested PCR amplification with identical thermocycling steps as for the primary PCR.

The primers for the *flaB* nested PCR amplification were the *flaB* inner forward (FIF) primer (5′-CTTTAAGAGTTCATGTTGGAG-3′) and the *flaB* inner reverse (FIR) primer (5′-TCATTGCCATTGCAGATTGT-3′) to amplify a 447-bp segment of the *B. burgdorferi flaB* gene [16].

The primers used for the *16S rRNA* gene nested PCR amplification were the same M1 and M2 primers used in the borrelial *16S rRNA* gene primary PCR mixture [10].

All nested PCR products, including those of the positive controls and negative controls, were tested by agarose gel electrophoresis. The nested PCR products showing a DNA band at agarose gel electrophoresis without further purification were subjected to bidirectional Sanger sequencing to verify the authenticity of the PCR-amplified products.

Based on a study reported by other investigators, the limit of detection of *16S rRNA* gene in human blood samples by nested PCR assays was approximately 2 (≥1.93) template copies per reaction [17].

Using one identical pair of primers for nested and primary amplification of borrelial *16S rRNA* genes takes advantage of a unique 7-base discriminatory sequence immediately downstream of the M1 PCR primer between the *Borrelia burgdorferi* sensu lato complex and the relapsing fever borrelia group. The M1/M2 PCR primer pair can amplify all known pathogenic *Borrelia 16S rRNA* genes, including those of *B. burgdorferi* and *B. miyamotoi*. With these 2 PCR primers, all *B. burgdorferi* sensu lato complex members will generate a 357-bp amplicon while all members of the relapsing fever borrelia group will generate a 358-bp amplicon [18]. Further increasing the size of the amplicon will reduce detection sensitivity. Moving the primers inward for nested PCR will lose the discriminatory power of Sanger sequencing.

2.6 Automated Sanger Sequencing

A trace (about 0.2 µL) of the nested PCR product showing a band at agarose gel electrophoresis was transferred by a calibrated micro-glass rod from the nested PCR tube into a Sanger reaction tube containing 1 µL of 10 µmolar sequencing primer, 1 µL of the BigDye® Terminator (v 1.1/Sequencing Standard Kit, Applied Biosystems, Foster City, CA, USA), 3.5 µL 5× buffer (Applied Biosystems, Foster City, CA, USA), and 14.5 µL molecular grade water in a total volume of 20 µL for 20 enzymatic primer extension/termination reaction cycles according to the protocol supplied by the manufacturer (Applied Biosystems, Foster City, CA, USA). After a dye-terminator cleanup, the reaction mixture was loaded in an automated ABI SeqStudio for sequencing. Sequence alignments were performed against the standard sequences stored in the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) by Basic Local Alignment Search Tool (BLAST) on-line analysis.

2.7 Methodology Validation

To verify that Milford Molecular Diagnostics Laboratory is capable of using the described methods to detect borrelial spirochetes causing clinical Lyme disease in platelet-rich plasma specimens before launching this research project, DiaSorin, Inc. sent 20 blind-coded platelet-rich plasma specimens, some of which were spiked with cultured cells of *Borrelia burgdorferi*, *Borrelia miyamotoi*, a mixture of the two, and none of the two, to Milford Molecular Diagnostics Laboratory on June 29, 2023. Milford Molecular Diagnostics Laboratory was required to use the methods described above to identify all the blind-coded specimens correctly without cross contamination. However, the purpose of this work was to detect 1–3 *Borrelia burgdorferi* cells in 1 mL of patient platelet rich plasma at the early localized stage of infection. It is technically difficult to spike a sample with 1–3 spirochetes from a pure

culture. As a result, these 20 blind-coded specimens did not mimic real clinical conditions.

3. Results

3.1 Total DNA Nested PCR Detection of Spirochetemia in Early Localized Lyme Disease

3.1.1 Detection of B. Burgdorferi Cells in Low Single-digit Numbers by Chromosomal DNA Nested PCR

The overall results of split sample testing on the venous blood specimens of 98 patients clinically suspected of having early localized Lyme disease showed that 65 specimens were negative for both *flaB* gene and *16S rRNA* gene PCR amplifications and that 33 specimens (33.7%) were PCR-positive, including 17 specimens positive for the *flaB* gene only, and 15 specimens positive for both the *flaB* gene and *16S rRNA* gene. One specimen was positive for the *16S rRNA* gene only, indicating that this sample contained only two copies of *B. burgdorferi* chromosome and both copies were aliquoted for *16S rRNA* gene PCR amplification by chance during sample splitting. Split sample testing on the venous blood specimens of 47 asymptomatic resident controls generated 8 positives (17.0%), all positive for *flaB* PCR only.

3.1.2 Decoupling of flaB Gene From 16S rRNA Gene in Split Sample PCR Testing

When tested in groups of 10, many specimens in each group only showed a *flaB* gene PCR product without a concomitant *16S rRNA* gene amplification. The results of 20 selected consecutive nested PCRs from the symptomatic patients and 20 selected consecutive nested PCRs from the asymptomatic residents, respectively, are presented in Fig. 1. This decoupling of two chromosomal gene amplifications in split sample PCR testing suggested that there were more copies of the *flaB* gene than the *16S rRNA* gene in one chromosome and this gene copy number difference became apparent only when split sample nested PCR testing of a specimen containing spirochetes in low single-digit numbers was carried out.

3.2 Sanger Sequencing Detection of flaB Mutations and Paralogs

All nested PCR products showing a band at agarose gel electrophoresis were verified by Sanger sequencing without purification. A typical specimen positive for *B. burgdorferi* (case No. M23-167) containing a single-nucleotide silent mutation in the *flaB* gene (Figs. 2,3) and

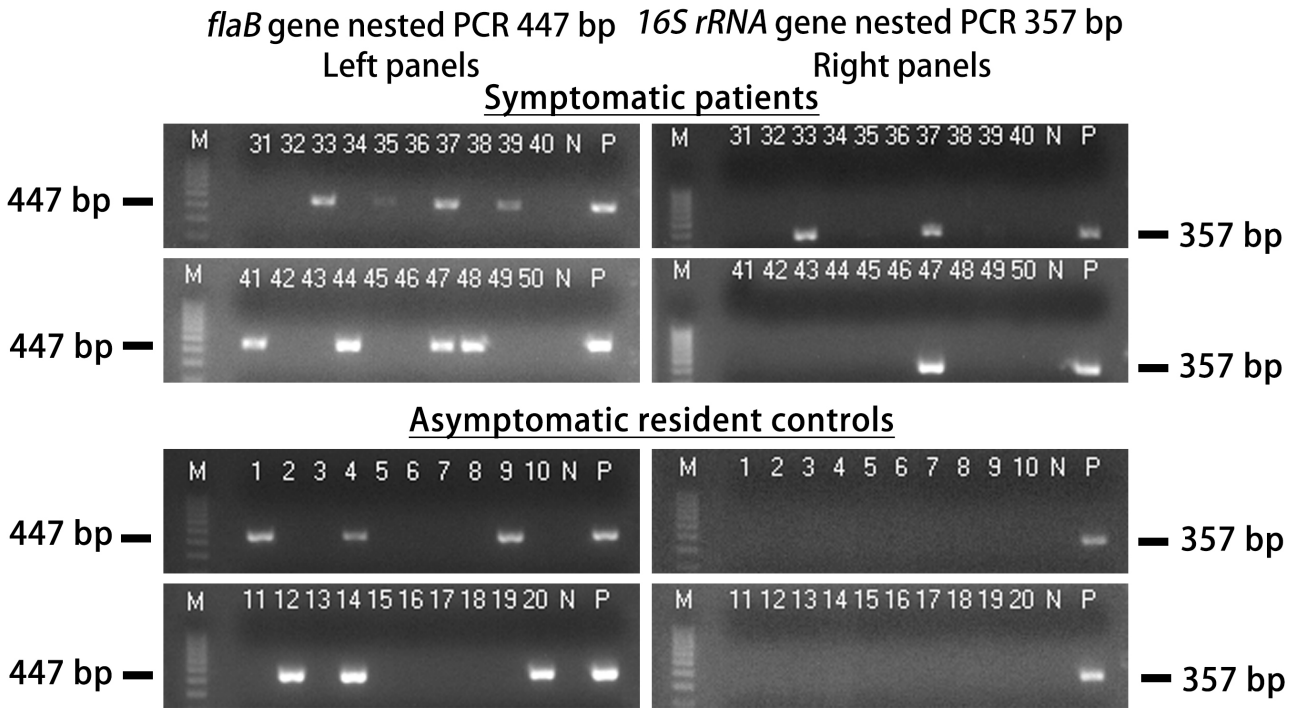

Fig. 1. Decoupling of *flaB* gene from *16S rRNA* gene in split sample polymerase chain reaction (PCR). Images of agarose gel electrophoresis of nested PCR products showing split sample testing results on 20 blood specimens taken from 98 symptomatic patients suspected of having early localized Lyme disease and 20 blood specimens taken from 47 asymptomatic residents living in the same communities during a Lyme disease season. The left panels show a 447-bp *flaB* nested PCR product band in 13 specimens. But only 3 of the 13 *flaB*-positive samples were associated with a concomitant 357-bp *16S rRNA* gene same-nested PCR product band in the right panels, and all 3 were in the group of symptomatic patients. Lane numbers = specimen No. N = negative water control. P = DNA extract from cultured *Borrelia burgdorferi* (*B. burgdorferi*) cells (ATCC 53210) in the left panels, and DNA extract from cultured *B. coriaceae* cells (ATCC 43381) in the right panels.

Fig. 2. Single-nucleotide silent mutation in the *flaB* gene forward sequence. Image of a *flaB* gene forward sequencing electropherogram, using the *flaB* inner forward (FIF) nested PCR primer as the sequencing primer, shows an A-to-G (indicated by an arrow) single-nucleotide silent mutation in the 447-bp *flaB* gene PCR amplicon. In this FlaB protein, the 306th amino acid alanine was encoded by codon GCG instead of GCA as annotated in the GenBank database for all wild-type *B. burgdorferi* isolates.

a positive *B. burgdorferi 16S rRNA* gene (**Supplementary Fig. 1**) was chosen for illustration.

Nucleotide substitutions in *flaB* were found in 5 of the 32 (15%) *flaB*-positive specimens derived from symptomatic patients, and in 1 of 8 (12%) *flaB*-positive specimens derived from asymptomatic residents.

All the single nucleotide polymorphisms (SNPs) and nucleotide substitutions were verified by bidirectional Sanger sequencing in Figs. 2,3,4,5,6 and **Supplementary Fig. 2**.

Of the 41 positive specimens, 6 (one in Fig. 2, four in Fig. 4 and one in Fig. 5) showed 8 single- nucleotide mutations or substitutions (summarized in Fig. 7) all of which have not been annotated in the GenBank sequence database in the 447-base segment of the *flaB* DNA selected for PCR amplification and sequencing. Six of these 8 single-nucleotide mutations occurred as SNPs with two overlapping peaks due to the presence of duplicated *flaB* paralogs pointed by arrows (four in Fig. 4A,F,H, and two in Fig. 5), and two as single-nucleotide substitutions that resulted in one silent mutation (Fig. 2) and one missense mutation (Fig. 4D). Two of the SNPs (one in Fig. 4H and one in Fig. 5) also resulted in missense mutations.

The amino acid codons in the 447-base *flaB* gene segment affected by the single-nucleotide mutations described above and their relationship to the outer and inner PCR primers were summarized in Fig. 7.

3.3 Sequence of the 503-base Segment of the flaB Gene in the Cultured B. burgdorferi Cells

In bacterial genetics, the DNA sequence of a gene in *Borrelia burgdorferi* strain B31 is considered the "wild type". Any base changes in a gene sequence from the wild type are mutations. Since the *flaB* gene in *B. burgdorferi* strain 31 cultured cells (ATCC 53210) is well characterized, the sequence of the amplicon generated by the PCR primers listed in Fig. 7 was used as the wild type comparator sequence for investigation of the *flaB* gene complex found in

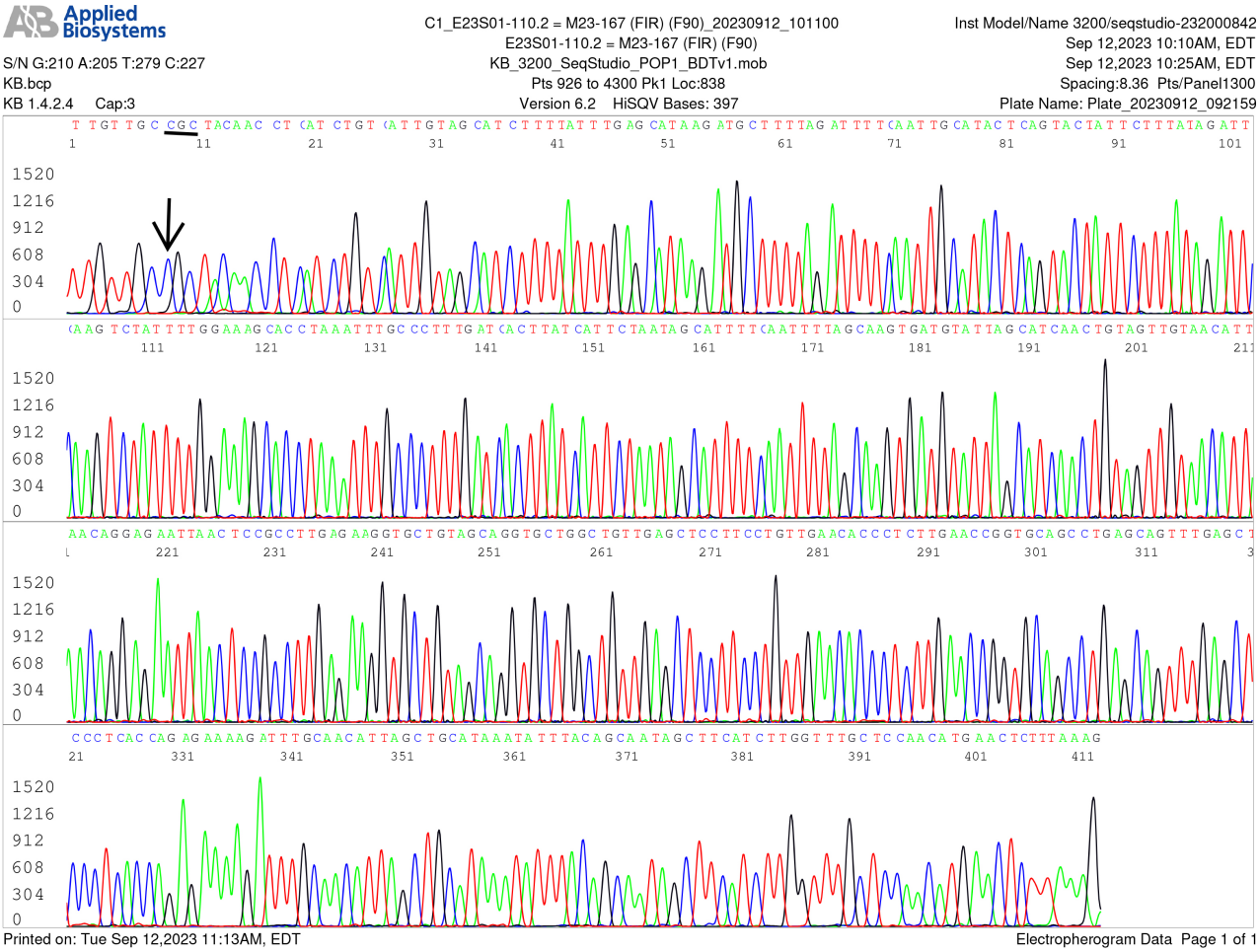

Fig. 3. Single-nucleotide silent mutation in the *flaB* gene reverse sequence. Image of a *flaB* gene reverse sequencing, using the *flaB* inner reverse (FIR) nested PCR primer as the sequencing primer, of the same nested PCR product used to generate the electropherogram shown in Fig. 2, confirming the A-to-G mutation in the *flaB* gene (the reverse complementary base C is indicated by an arrow).

the spirochetes in the positive samples. The bidirectional sequencing electropherograms of the 503-base wild type *B. burgdorferi flaB* gene sequence defined by both outer PCR primers are presented in **Supplementary Figs. 3**,**4** as reference. Concatenation of the two bidirectional sequences shown in **Supplementary Figs. 3**,**4** generated one contiguous 503-base DNA sequence identical to that illustrated in Fig. 7 (range 147673 to 148175, Sequence ID: CP019767).

To confirm that decoupling of *flaB* gene from *16S rRNA* gene does not occur in cultured *B. burgdorferi* PCRs, two-fold serial dilutions of a DNA extract from cultured *B. burgdorferi* cells (ATCC 53210) were made and used as the templates for *flaB* gene and *16S rRNA* gene nested PCRs in parallel. Since both the *flaB* gene and *16S rRNA* gene nested PCRs invariably ended at the same endpoint dilution (**Supplementary Fig. 5**), the data indicated that the chromosome of cultured *B. burgdorferi* indeed contains a single *16S rRNA* gene and a single *flaB* gene as reported in literature.

4. Discussion

4.1 Timely Isolation of Platelet-rich Plasma is Crucial in Sanger Sequencing-based Diagnosis of Lyme Spirochetemia

The results of this study confirm that if the platelet-rich plasma of the patients is separated immediately from the red cells and the white cells after venous blood collection before the spirochetes can attach to the lymphocytes in the test tube [11] and the entire DNA content extracted from the platelet pellet obtained by differential centrifugation is used to initiate a primary PCR, Sanger sequencing of the nested PCR products can accurately diagnose *B. burgdorferi* spirochetemia at the early localized stage of Lyme disease when the density of spirochetes is in the low single-digit numbers (1–3) per mL of platelet-rich plasma. A highly sensitive reliable nucleic acid-based blood test is also needed for identification of the post-treatment Lyme disease syndrome patients whose infected tissues continue releasing a small number of spirochetes into the circulating blood [10]. In contrast to metagenomic (next-generation) sequencing detection of *B. burgdorferi*

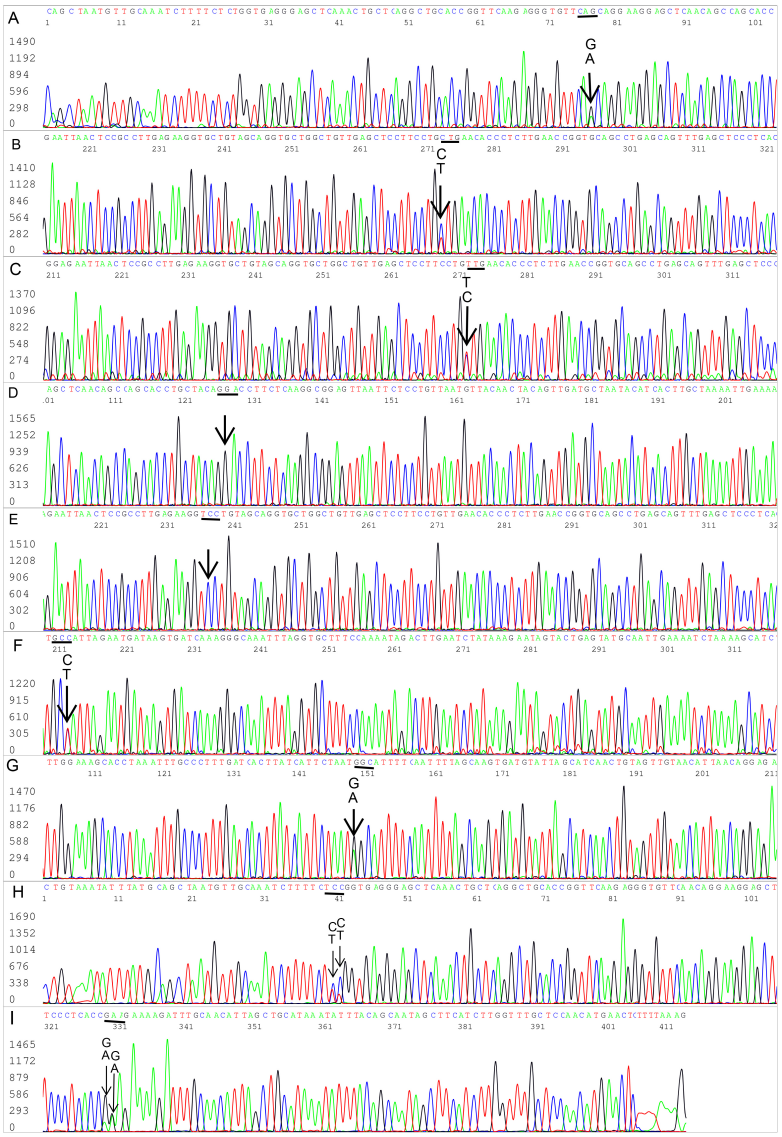

Fig. 4. Four cases of single nucleotide polymorphisms (SNPs) or nucleotide substitutions verified with bidirectional sequencing. (A–I) Bidirectional Sanger sequences excised from 9 electropherograms show 5 *B. burgdorferi flaB* gene single-nucleotide mutations (all indicated by arrows) in 4 patient blood specimens. (A) There were two copies of *flaB* gene with a silent mutation. In one copy, the codon for the 216th amino acid glutamine was CAA as annotated in the GenBank sequence database. In the other copy, the 216th amino acid glutamine codon was CAG (not annotated in the GenBank database). (B,C) Reverse complement sequencing of the same nested PCR product used for (A), confirming the 216th amino acid glutamine was encoded by 2 codons CAG (B) and CAA (C), called by the computer base-caller as CTG and TTG in reverse complement when the sequencing was repeated. (D) There was a C-to-G single-nucleotide missense mutation. The 228th amino acid alanine encoded by codon GCA has been converted to glycine encoded by codon GGA (not annotated in the GenBank database). (E) Reverse complement sequencing of the same nested PCR product used for (D), confirming the 228th amino acid codon was GGA (TCC in reverse complement). (F) There were two copies of *flaB* gene with a silent nutation. In one copy, the codon for the 208th amino acid alanine was GCT. In the other copy, the codon was GCC (not annotated in the GenBank database). (G) Reverse complement sequencing of the same nested PCR product used for (F), confirming 2 copies of *flaB* in one of which the 208th amino acid alanine was encoded by GCT while the other by GCC. (H) There were two C/T SNPs in the underlined codon TCT, encoding the 199th amino acid serine. When the third nucleotide of the codon changed from T to C, the result was a silent mutation. When the second nucleotide changed from C to T, it caused a missense mutation, turning the amino acid serine into phenylalanine encoded by codon TTT or TTC (a phenylalanine in this amino acid position has not been annotated in the GenBank database). (I) Reverse complement sequencing of the same nested PCR product used for (H), confirming the two C/T SNPs in the codon encoding the 199th amino acid. The existence of two SNPs in one codon verified by Sanger sequencing raised the possibility of more than one duplicated *flaB* paralog in a chromosome.

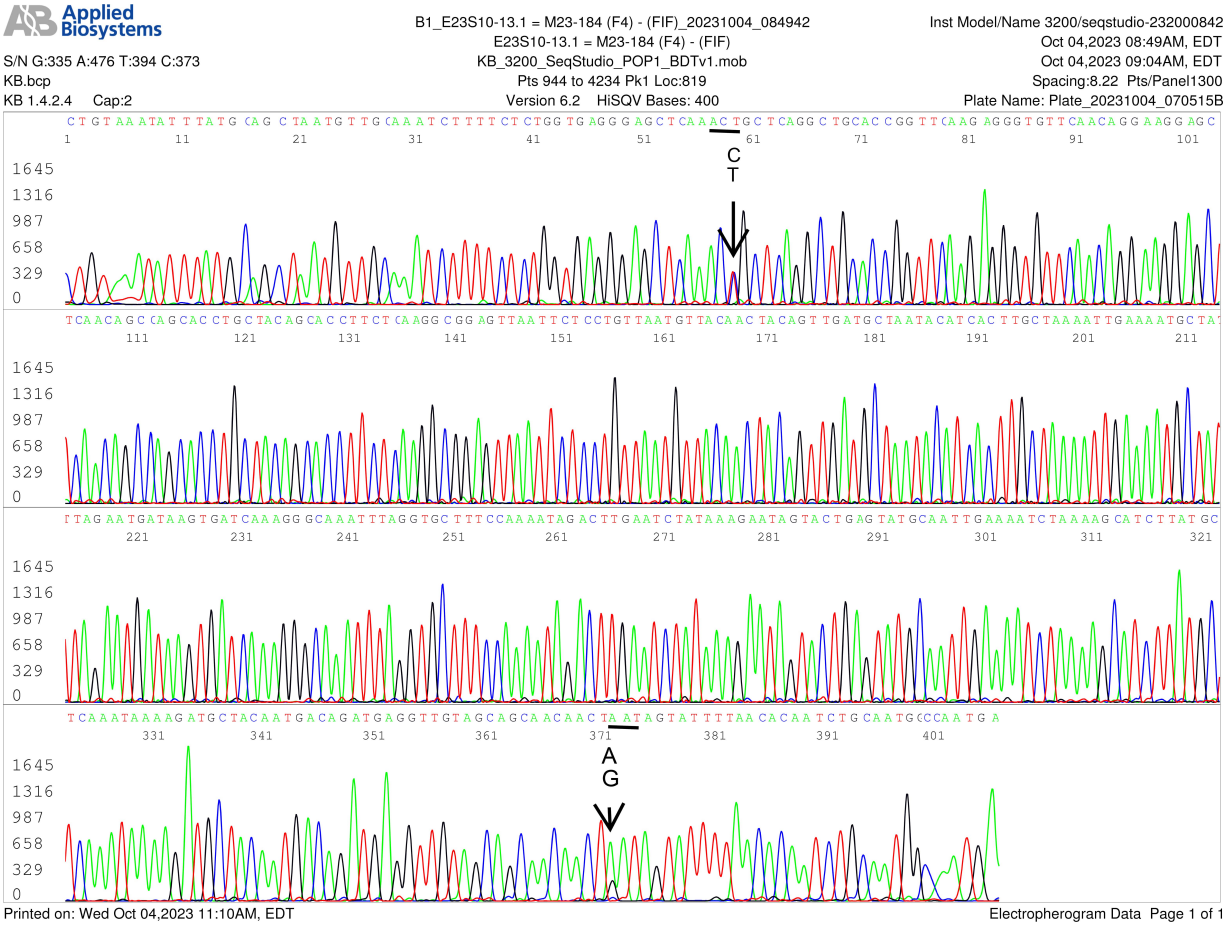

Fig. 5. SNPs in *flaB* gene of *B. burgdorferi* in blood of an asymptomatic resident-forward sequencing. This electropherogram shows a forward sequencing of the *flaB* nested PCR product illustrated in Fig. 1, Left Panel, Lane 4 of an asymptomatic resident control. In this specimen, there were more than one paralogous *flaB* gene in the *B. burgdorferi* chromosome. The first T/C SNP in this electropherogram (pointed by an arrow) indicates that there were at least two copies of *flaB* gene in one of which the 205th amino acid threonine was encoded by codon ACT, and in the other the 205th amino acid threonine was encoded by codon ACC, a silent mutation, which has not been annotated in the GenBank sequence database. In addition, there was an A/G SNP at position 371 (also pointed by an arrow), indicating that there was another duplicate paralog in which the 310th amino acid asparagine encoded by codon AAT was changed to aspartate encoded by codon GAT, a missense mutation. The co-existence of AAT and GAT coding the 310th amino acid was confirmed by a repeated forward sequencing electropherogram (Fig. 6) and a reverve complement sequencing electropherogram presented in **Supplementary Fig. 2**. A strain of *Borrelia burgdorferi* containing a flagellin B protein with aspartate in its 310th amino acid position has not been annotated in the GenBank database.

cell-free DNAs [19] that can be continuously detected even after the *B. burgdorferi* cells have been eliminated by antibiotics [20,21], Sanger sequencing of a segment of the chromosomal DNA of the *B. burgdorferi* cells spun down in the pellet of the platelet fraction can diagnose active infections in patients with persistent Lyme disease. Dead bacteria in the circulating blood are quickly removed and digested by the reticuloendothelial system (RES), particularly in the liver and the spleen [22]. In practice, next-generation sequencing is unable to detect *B. burgdorferi* cell-free DNA in the plasma of patients with active *erythema migrans* rashes and is considered unlikely to be helpful in diagnosing early localized Lyme disease [23].

4.2 FlaB is a More Sensitive Chromosomal Target Than 16S rRNA Gene for Detection of Spirochetemia in Early Localized Lyme Disease

The finding of 33 spirochetemic cases (33.7%) among 98 symptomatic patients clinically suspected of having early localized Lyme disease was a surprise because it is generally assumed that spirochetemia only begins at the "early disseminated stage" [3,4]. Given the fact that spirochetemia can occur at the early localized stage of Lyme disease even without an *erythema migrans*, a highly sensitive and specific diagnostic laboratory test for detecting very low-density *B. burgdorferi* cells in the blood specimen is needed to diagnose Lyme disease patients for early, proper treatment to prevent deep tissue damage.

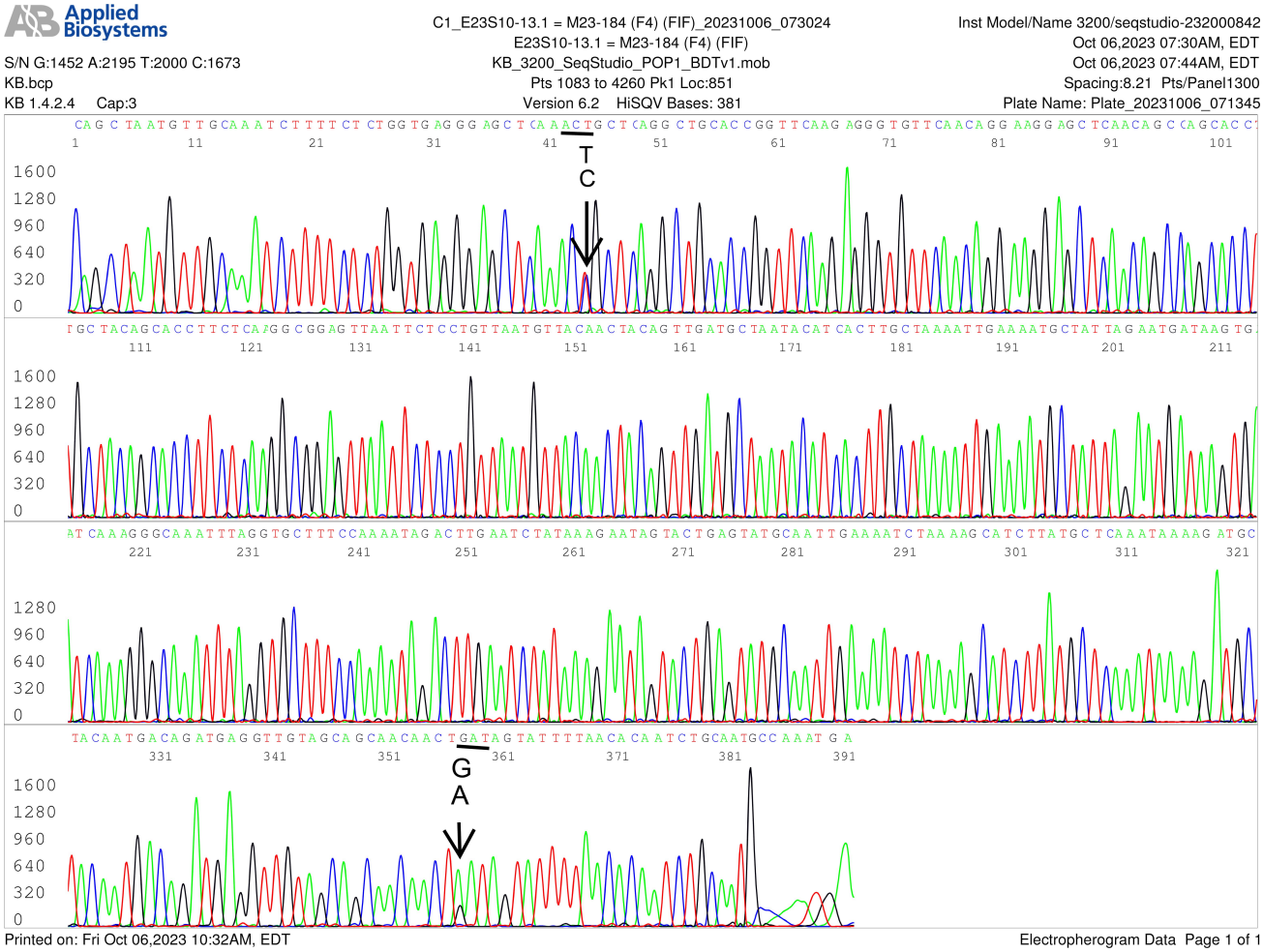

Fig. 6. SNPs in *flaB* gene of *B. burgdorferi* in blood of an asymptomatic resident-repeated sequencing. Electropherogram of re-sequencing of the nested PCR product used to generate the sequence shown in Fig. 5, confirming an A/G SNP in position 358, pointed by a GA arrow. The computer base-caller called the codon as GAT in this repeated sequencing instead of AAT. The computer base-caller failed to consistently determine whether "A" or "G" was the dominant base in this position even though the "G" peak appears lower than the "A" peak in the A/G SNP in the electropherograms. Sanger sequencing is designed for qualitative analysis, peak heights in Sanger sequencing analysis are not necessarily quantitative and may not indicate the amount of DNA.

Another unexpected finding was that 25 of the 41 split samples tested positive for *flaB* gene without a concomitant *16S rRNA* gene PCR amplification. Since the chromosome of the cultured "wild type" *B. burgdorferi* is known to contain a single copy of *flaB* gene [24] and a single copy of *16S rRNA* gene [25], a 1:1 gene ratio, which is also supported by simultaneously testing these two genes on double serial dilutions of the DNA extract from cultured *B. burgdorferi* cells (**Supplementary Fig. 5**), the *B. burgdorferi* spirochetes invading the blood stream of the patients at the early localized stage of Lyme disease must have more than one copy of *flaB* gene in the chromosome through gene duplication to account for the split sample discordance between these two gene PCR test results (Fig. 1). Other investigators also reported that when the synovial fluid DNA of Lyme disease patients was tested for both the *16S rRNA* gene and flagellin gene, the PCR targeting the *16S rRNA* gene yielded amplification in only 8 cases while the PCR targeting the flagellin gene yielded amplification in 9 cases, a phenomenon referred to as "target imbalance". Such target imbalance could not be demonstrated when serial dilutions of *B. burgdorferi* DNA extracted from cultured organisms were tested [26], as observed in the current study.

4.3 The Presence of Paralogs Makes flaB a More Sensitive Target for Molecular Detection of Spirochetemia in Early Localized Lyme Disease

The findings of 6 SNPs in the form of 2 overlapping nucleotide peaks in 4 of the 40 specimens positive for *flaB* gene PCR (Fig. 4A,F,H, and Fig. 5) provided evidence for gene duplication in the chromosomal *flaB* gene of the *B. burgdorferi* cells invading the blood stream of the patients during the early localized stage of Lyme disease. Since duplicate genes initially have identical sequences but diverge

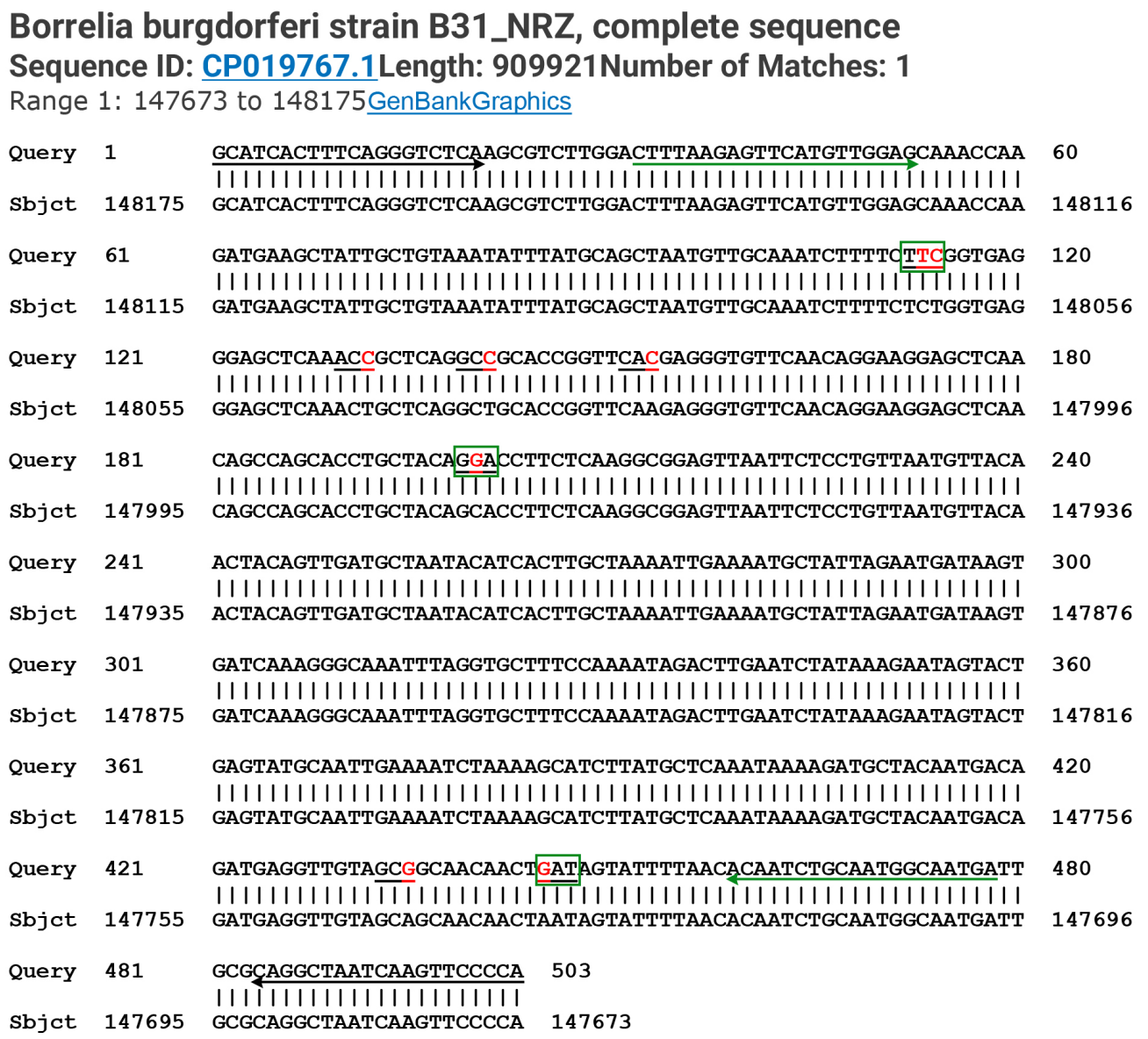

Fig. 7. Summary of the amino acid codons affected by the SNPs verified by Sanger sequencing. A modified Basic Local Alignment Search Tool (BLAST) report from the GenBank including a 503-base Query sequence defined by the *flaB* outer forward (FOF) PCR primer (marked by a left-to-right black arrow) and the *flaB* outer reverse (FOR) PCR primer (marked by a right-to-left black arrow) showing the positions of the 8 newly discovered single-nucleotide mutations or SNPs (8 red letters in the 7 underlined codons) in the 447-base segment of the *B. burgdorferi flaB* gene defined by the *flaB* inner forward (FIF) nested PCR primer (marked by a left-to-right green arrow) and the *flaB* inner reverse (FIR) nested PCR primer (marked by a right-to-left green arrow). A single-nucleotide change in the 3 codons framed by green rectangles resulted in missense mutations. The single-nucleotide changes in the 4 unframed codons are silent mutations. The first green-framed codon encoding the 199th amino acid of the FlaB protein had two SNPs one of which also resulted in a silent mutation (see Fig. 4H).

in coding and regulatory regions during subsequent evolution [27], the spirochetes in the other 36 *flaB*-positive specimens without a SNP in their *flaB* gene sequence probably also had more than one identical *flaB* gene copy in the form of paralogs in their chromosome at the early stage of duplication. When duplicated paralogs with an identical sequence are amplified by a pair of PCR primers, only one PCR amplicon will be generated for Sanger sequencing.

4.4 Re-amplification of the Primary PCR Products is Needed to Generate an Amplicon for Sanger Sequencing-based Diagnosis of Spirochetemia

Using PCR amplification of chromosomal genes for laboratory diagnosis of *B. burgdorferi* spirochetemia at the early localized stage of Lyme disease has been hampered by its low sensitivity [28]. When agarose gel electrophoresis is used as the tool for detection of PCR products, ap-

proximately 10^4 copies of target DNA are required as the template to generate a visible PCR product band after 25–30 cycles of PCR amplification [29] because in testing for a pathogen's genomic DNA in human specimens PCR amplification never reaches its theoretical 100% efficiency due to the presence of PCR inhibitors among many other interfering factors [30,31]. In the current nested PCR study, the primary PCR mixture contained all the PCR inhibitors, especially the mitochondrial DNA, derived from the platelets isolated from about 2 mL of whole blood. The primary PCR needed at least two copies of target DNA [17] to initiate an exponential amplification to deliver the required copy number of target DNA in about 0.2 µL for the nested PCR to generate a visible product band at agarose gel electrophoresis after 30 cycles of nested PCR amplification.

Using the same pair of primers for nested PCR, referred to as same-nested PCR in this report, is generally not advisable because one pair of PCR primers can generate many unwanted PCR products after 40 cycles of DNA amplification, including exponential amplification of sequences with "*Taq* errors". However, when a high-fidelity DNA polymerase is chosen for the same-nested PCR, a segment of borrelial *16S rRNA* gene and a segment of *flaB* gene of *Borrelia burgdorferi* can be amplified after 60 cycles of PCR for Sanger sequencing without ambiguous base-calling sequences as reported previously [10,18] and illustrated in **Supplementary Figs. 3**,**4**, respectively. Comparing the results of the conventional nested PCR amplification of the *flaB* gene and those of the same-nested PCR amplification of the *16S rRNA* gene of cultured *Borrelia burgdorferi* cells, there is no observed difference in sensitivity between the two nested PCRs (**Supplementary Fig. 5**).

4.5 Split Sample PCR for Detection of Spirochetemia May Lose Sensitivity

When each of the two split sample primary PCR mixtures contained only one copy of *B. burgdorferi* chromosome, the split sample nested PCR testing would generate a negative *16S rRNA* gene amplification and a positive *flaB* gene amplification because each chromosome contained only one copy of *16S rRNA* gene and at least two copies of *flaB* gene, one in the form of paralog. It required at least 3 copies of chromosome, one copy aliquoted to the *flaB* gene amplification and two copies aliquoted to the *16S rRNA* gene amplification, to generate a positive *flaB* gene PCR and a positive *16S rRNA* gene PCR. When there was only one copy of chromosome in the entire platelet-rich plasma specimen, the specimen would test positive for *B. burgdorferi* if this single copy of chromosome was aliquoted for the *flaB* primary PCR during sample splitting. However, if this single copy of chromosome was aliquoted for the *16S rRNA* gene primary PCR by chance during sample splitting, the same specimen would generate a false-negative result.

If the entire platelet pellet DNA extract containing a single copy of *B. burgdorferi* chromosome were used to initiate a primary *flaB* PCR without sample splitting, the false-negative result would be avoided. However, the *flaB* gene PCR primers are designed specifically for *B. burgdorferi* assays, and will fail to amplify the *flaB* genes of the relapsing fever *Borrelia* species, including the *flaB* gene of *Borrelia miyamotoi*. Since the *16S rRNA* gene PCR primers used in this study are designed to amplify a highly conserved segment of the *16S rRNA* gene of all known pathogenic *Borrelia* species [18], a split sample testing to include the *16S rRNA* gene PCR can detect spirochetemia caused by a relapsing fever *Borrelia* species, a *B. burgdorferi* sensu lato species, or a mixture of the two [10,32]. The trade-off for ensuring detection of a single *B. burgdorferi* cell by allocating the entire DNA extract to a *flaB* gene PCR without a concomitant *16S rRNA* gene PCR is to risk missing spirochetemic cases caused by a tick-borne relapsing fever *Borrelia* species.

The presence of SNPs in a sequencing electropherogram may indicate mixed infections with two species of *Borrelia* as reported previously [10] rather than duplicate paralogs. However, in the current study SNPs were present in 3 specimens that were positive for *flaB* gene PCR and negative for *16S rRNA* gene PCR in split sample testing. One of these 3 cases is shown in Fig. 1 lanes 4, Figs. 5,6 and **Supplementary Fig. 2**. Since the *16S rRNA* nested PCR was negative, the number of chromosome copies aliquoted to each split sample PCR in these 3 cases must be less than 2, which practically rules out mixed *Borrelia* infections being the cause for the detected SNPs.

4.6 Sanger Sequencing Confirms Asymptomatic Early Lyme Disease Patients May Have Spirochetemia

The finding of one to two (1–2) *B. burgdorferi* cells, detected by *flaB* sequencing only, in 1 mL of platelet-rich plasma among 17.0% of asymptomatic people residing in Lyme disease-endemic areas in the United States is not a surprise since spirochetemia among asymptomatic patients in early Lyme disease has been reported to be 22.9% by blood cultures [33]. Blood culture seems to be more effective for the diagnosis of spirochetemia in asymptomatic Lyme disease patients than the *flaB* gene nested PCR amplification method (22.9% versus 17.0%). However, this difference might be due to different criteria being used for the selection of asymptomatic patients. Since *B. burgdorferi* does not have lipopolysaccharides, *B. burgdorferi* spirochetemia, unlike other Gram-negative bacteremias, may generate very mild nonspecific symptoms that can be ignored by some patients. To what extent the human innate immunity can clear the *B. burgdorferi* cells from early Lyme disease patients' blood needs further study. The biological significance of the duplicate *flaB* gene paralogs is totally unknown. The *B. burgdorferi* spirochetes may gain these *flaB* gene paralogs at certain stage of their life cycle for a specific function and lose the paralogs when the spirochetes are cultured in artificial media. Since almost all DNA se-

quencing works on *B. burgdorferi* were carried out on pure cultures, no *flaB* gene paralog sequences have been published.

5. Conclusions

This study shows that *B. burgdorferi* cells causing spirochetemia at the early localized stage of Lyme disease have more than one copy of *flaB* gene due to the presence of *flaB* paralogs. Targeting a 447-base segment of the *flaB* gene for nested PCR amplification followed by Sanger sequencing of the PCR product for verification can detect a single *B. burgdorferi* cell spun down in the platelet pellet derived from about 2 mL of whole blood with ~100% specificity. Since the highly mobile *B. burgdorferi* spirochetes can invade co-incubated lymphocytes in the test tube at ambient temperature, the spirochetes along with the platelets must be separated from the whole blood by differential centrifugation as soon as possible after blood collection to prevent losing the limited number of spirochetes to the buffy coat.

Availability of Data and Materials

Data is available upon written request sent to the author.

Author Contributions

SHL developed the methodology, supervised the testing, analyzed the PCR and Sanger sequencing results, wrote the initial manuscript and revised the manuscript after peer reviews. The author has participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

DiaSorin, Inc., Stillwater, MN, USA enrolled the patients and healthy donor controls and performed the initial specimen preparation for a research project after informed consent was obtained in accordance with applicable regulations in compliance with 21 CFR 812. Written document is available upon request. The study was carried out in accordance with the guidelines of the Declaration of Helsinki.

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Conflict of Interest

The author declares no conflict of interest despite the author's affiliation is a commercial entity performing tests for fees. The author had full access to all of the data in this study and takes complete responsibility for the integrity of the data and the accuracy of the data analysis.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBS31280.

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