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Simple Objective Detection of Human Lyme Disease Infection Using Immuno-PCR and a Single Recombinant Hybrid Antigen

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A serology-based tiered approach has, to date, provided the most effective means of laboratory confirmation of clinically suspected cases of Lyme disease, but it lacks sensitivity in the early stages of disease and is often dependent on subjectively scored immunoblots. We recently demonstrated the use of immuno-PCR (iPCR) for detecting Borrelia burgdorferi antibodies in patient serum samples that were positive for Lyme disease. To better understand the performance of the Lyme disease iPCR assay, the repeatability and variability of the background of the assay across samples from a healthy population (n = 36) were analyzed. Both of these parameters were found to have coefficients of variation of < 3%. Using eight antigen-specific iPCR assays and positive call thresholds established for each assay, iPCR IgM and/or IgG diagnosis from Lyme disease patient serum samples (n = 12) demonstrated a strong correlation with that of 2-tier testing. Furthermore, a simplified iPCR approach using a single hybrid antigen and detecting only IgG antibodies confirmed the 2-tier diagnosis in the Lyme disease patient serum samples (n = 12). Validation of the hybrid antigen IgG iPCR assay using a blinded panel of Lyme disease and non-Lyme disease patient serum samples (n = 92) resulted in a sensitivity of 69% (95% confidence interval [CI], 50% to 84%), compared to that of the 2-tier analysis at 59% (95% CI, 41% to 76%), and a specificity of 98% (95% CI, 91% to 100%) compared to that of the 2-tier analysis at 97% (95% CI, 88% to 100%). A single-tier hybrid antigen iPCR assay has the potential to be an improved method for detecting host-generated antibodies against B. burgdorferi.

Lyme disease is the most commonly reported tick-borne illness in the United States, with approximately 30,000 cases reported to the Centers for Disease Control and Prevention (CDC) each year (1). New preliminary estimates released by the CDC indicate that the number of Americans diagnosed with Lyme disease each year is closer to 300,000, which is roughly 10 times higher than the annual reported number (2). This new estimate supports studies published in the 1990s, which suggested that the number of cases may be between 3- and 12-fold higher than the number of reported cases (3, 4), making Lyme disease a significant health concern in the United States. Accurate diagnosis provides a considerable obstacle for the clinical management of the disease and is necessary in order to differentiate Lyme disease from other diseases with similar clinical presentation. Misdiagnosis is common due to difficulties in detecting Borrelia burgdorferi, the causative agent of Lyme disease (5). Although a wide range of laboratory diagnostic approaches have been explored, the currently accepted method utilizes the detection of serological responses to B. burgdorferi antigens (6).

The currently accepted method for diagnosing Lyme disease in a clinical setting entails a two-tiered approach using a first-tier enzyme-linked immunosorbent assay (ELISA), followed by a second-tier immunoblot assay for both IgM and IgG B. burgdorferi-specific antibodies using whole-cell B. burgdorferi lysates, recombinant antigens, or various combinations, depending on the commercial kit used (7). The ELISA provides an objective and sensitive first-tier screen but lacks the specificity and broad strain applicability (8) required for a standalone test. The second-tier immunoblot provides a higher level of specificity but currently requires somewhat subjective analysis due to its qualitative nature and general lack of automation (9). A tiered approach has to date provided the most effective means of diagnosing Lyme disease in a clinical setting (7).

Other approaches for diagnosing Lyme disease have been developed, including live culture, PCR, and additional molecular-based approaches, with no method surpassing the effectiveness of a serology-based approach. The detection of typical erythema migrans (EM) can be sufficient for a clinical diagnosis of early localized Lyme disease in the absence of laboratory tests (7). However, this manifestation is not present in all patients (7), further highlighting the need for improved methods for early objective diagnosis of Lyme disease. In our previous study, we demonstrated the use of immuno-PCR (iPCR) for detecting host-generated antibodies in a murine model, and we presented preliminary data using serum samples collected from Lyme disease patients and healthy controls (10). Our results indicated that iPCR using B. burgdorferi whole-cell sonicates and a limited number of B. burgdorferi recombinant antigens provided higher sensitivity for detecting B. burgdorferi antibodies in infected mice and an equivalent sensitivity for detecting B. burgdorferi antibodies in Lyme disease patient serum compared to both ELISA and the immunoblot (10).

It is well established that multiple antigens are required for an accurate overall diagnosis of the multiple stages and types of Lyme disease. Therefore, a tiered approach utilizing immunoblotting and ELISA has been the gold standard for the diagnosis of Lyme disease.
Hybrid Antigen iPCR Detection of Lyme Disease

MATERIALS AND METHODS

Healthy human sera. The current study was approved by the University of Central Florida’s institutional review board (UCF IRB) (FWA0000351 and IRB00001138). All procedures and investigators involved in the sample collection process were approved by the UCF IRB with Collaborative Institutional Training Initiative (CITI) training. All donors provided written consent to participate in the study. Sample collection was undertaken at the University of Central Florida campus. UCF is a diverse community of nearly 60,000 students and approximately 8,000 faculty and staff members of various ages and ethnic and racial backgrounds. Individuals were included in the study if they had not been previously diagnosed with and/or treated for Lyme disease, received a Lyme disease vaccination, or lived within the past 10 years in a state with a high incidence of Lyme disease (Connecticut, Delaware, Maine, Maryland, Massachusetts, Minnesota, New Hampshire, New Jersey, New York, Pennsylvania, Vermont, Virginia, and Wisconsin). Approximately 10 ml of blood was sampled, according to the IRB-approved protocol, from 36 individuals into serum separator tubes, inverted five times to mix the clot activator with the blood, and allowed to clot for 10 min. The serum was further clarified by centrifugation at 9,100 g for 30 min. Serum fractions were collected by taking at the University of Central Florida campus. UCF is a diverse community of nearly 60,000 students and approximately 8,000 faculty and staff members of various ages and ethnic and racial backgrounds. Individuals were included in the study if they had not been previously diagnosed with and/or treated for Lyme disease, received a Lyme disease vaccination, or lived within the past 10 years in a state with a high incidence of Lyme disease (Connecticut, Delaware, Maine, Maryland, Massachusetts, Minnesota, New Hampshire, New Jersey, New York, Pennsylvania, Vermont, Virginia, and Wisconsin). Approximately 10 ml of blood was sampled, according to the IRB-approved protocol, from 36 individuals into serum separator tubes, inverted five times to mix the clot activator with the blood, and allowed to clot for ≥30 min. Serum fractions were collected by centrifugation at 1,200 × g for 10 min. The serum was further clarified by centrifugation at 9,100 × g for 5 min to remove any insoluble material and stored at 4°C for short-term or −80°C for long-term storage.

Lyme disease human serum panel. The CDC research panel I consisted of patient serum samples collected from 32 individuals, including patients with stage 1, 2, or 3 Lyme disease (n = 12), look-alike diseases, including fibromyalgia, rheumatoid arthritis, multiple sclerosis, mononucleosis, syphilis, and severe periodontitis (n = 12), as well as healthy individuals from areas of endemicity (n = 4) and nonendemicity (n = 4) for Lyme disease. All Lyme disease patients were diagnosed by a physician, stage 1 and 2 patients were confirmed by culture and/or PCR detection of B. burgdorferi, and stage 3 patients were positive by two-tiered testing. The CDC-recommended two-tiered testing algorithm (6) was performed using FDA-cleared assays for Lyme disease and consisted of a first-tier whole-cell sonicate enzyme immunoassay (VIDAS Lyme IgM and IgG polyvalent assay; bioMérieux, Inc., Durham, NC), followed by second-tier IgM and IgG immunoblots (IB) (MarDx Diagnostics, Inc., Carlsbad, CA), and/or western blotting (WB) (Euroimmun, Inc., Lübeck, Germany). The blinded CDC research panel II consisted of serum samples collected from 92 individuals, including patients with stage 1, 2, or 3 Lyme disease (n = 32), look-alike diseases, including fibromyalgia, rheumatoid arthritis, multiple sclerosis, mononucleosis, syphilis, and severe periodontitis (n = 36), as well as healthy individuals from areas of endemicity (n = 12) and nonendemicity (n = 12) for Lyme disease. The laboratory support of Lyme disease diagnosis was the same as for CDC research panel I. Prior to analysis, all serum samples were clarified by centrifugation at 9,100 × g for 5 min to remove any insoluble material and put in the short-term storage at 4°C.

Cloning and expression of recombinant antigens lacking GST fusion tags. Recombinant glutathione S-transferase (rGST)-BmpA and rGST-OspC were constructed as previously described (10). In-frame glutathione S-transferase (GST) fusion proteins for BBK19, OspA, DbpA, RevA, Crasp-2, and BBK50 were generated by PCR amplification of the corresponding coding regions, without the signal sequences from B. burgdorferi genomic DNA, using primer pairs 1147 and 1148 (BBK19), 1151 and 1152 (OspA), 1145 and 1146 (DbpA), 1143 and 1144 (RevA), 1149 and 1150 (Crasp-2), or 1043 and 1044 (BBK50) engineered with BamHI and SalI or XhoI restriction sites (Table 1) and Phusion polymerase. The PCR products were purified (Qiagen, Valencia, CA), digested with the appropriate restriction enzymes (New England Biolabs, Ipswich, MA), and cloned into BamHI- and SalI- or

### TABLE 1 iPCR DNA oligonucleotide sequences used in this study

| Oligo no. | Oligo ID | Sequence (5’ to 3’) |
|-----------|----------|---------------------|
| T1 | Template 1 (IgG coupled) | Biotin-agcctgacaagaagacaagccagaactgcctcgacagtgtgctgcactgcctacacaagactctagctacaggtcc |
| T1F | Template 1 forward | agcctgacaagacagagaac |
| T1R | Template 1 reverse | ggcaccacagaggtactgtgg |
| T1P | Template 1 probe | FAM-agcctgacaagacagagaac |
| T2 | Template 2 (IgM coupled) | Biotin-agcctgacaagacagagaac |
| T2F | Template 2 forward | agcctgacaagacagagaac |
| T2R | Template 2 reverse | ggcaccacagaggtactgtgg |
| T2P | Template 2 probe | FAM-agcctgacaagacagagaac |
| 1147 | BBK19 F | CCGGATCCtttatactagagtccatatcttgcaattt |
| 1148 | BBK19 R | CCGGATCCtttatactagagtccatatcttgcaattt |
| 1151 | OspA F | CCGGATCCcaagaaatgtactagagtccatatcttgcaattt |
| 1152 | OspA R | CCGGATCCcaagaaatgtactagagtccatatcttgcaattt |
| 1145 | DbpA F | CCGGATCCggcctagagaaagagaaaag |
| 1146 | DbpA R | CCGGATCCagcctagagaaagagaaaag |
| 1143 | RevA F | CCGGATCCcaagaaatgtactagagtccatatcttgcaattt |
| 1144 | RevA R | CCGGATCCcaagaaatgtactagagtccatatcttgcaattt |
| 1149 | Crasp-2 F | CCGGATCCagcctagagaaagagaaaag |
| 1150 | Crasp-2 R | CCGGATCCagcctagagaaagagaaaag |
| 1043 | BBK50 F | CCGCCTCGAGctataataaagtttgcttaatagctttataag |
| 1044 | BBK50 R | CCGCCTCGAGctataataaagtttgcttaatagctttataag |
| 1084 | DbpA_PEPC10 F | CCGCCTCGAGctataataaagtttgcttaatagctttataag |
| 1085 | C6_PEPC10 F | CCGCCTCGAGctataataaagtttgcttaatagctttataag |

**Notes:**

- **ID:** identification.
- **Upper case letters indicate nontemplate sequence used for the addition of terminal restriction sites, epitope tags, or synthetic assembly.**
- **FAM:** 6-carboxyfluorescein; **BHQ1:** black hole quencher 1.
Xhol-digested pGEX-6P-1 (GE Healthcare, Piscataway, NJ) to generate translational fusions with GST at the N terminus. Subsequent clones were selected and the sequence confirmed by sequence analysis. pGEX-6P-1 plasmids carrying the bmpA, ospC, bkb19, ospA, dbpA, revA, crasp-2, and bkb50 genes were transformed into Escherichia coli strain BL21 (Novagen, Billerica, MA). Protein expression was induced by the growth of BL21 cells containing the expression construct for each B. burgdorferi antigen in 50 to 100 ml of MagicMedia E. coli expression medium, according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA) for 24 h at 37°C with aeration. Recombinant protein purification was performed according to the procedures outlined in the bulk GST purification module (GE Healthcare). The purified proteins were dialyzed in Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl [pH 7.5]) overnight at 4°C using D-Tube dialyzers (EMD Millipore Chemicals, Philadelphia, PA) and two buffer exchanges to remove excess glutathione. The dialyzed proteins were subjected to protease cleavage of the GST tag overnight at 4°C, according to procedures outlined in the PreScission protease kit (GE Healthcare). Cleaved proteins were purified from GST and excess protease using two rounds of bulk GST purification (GE Healthcare) and collection of the eluent. Purified proteins lacking a GST tag were concentrated using Amicon Ultra-2 centrifugal filter devices (EMD Millipore Chemicals) to a volume of approximately 80 μl and stored at 4°C. The total protein content was quantified by absorbance spectrophotometry at a wavelength of 280 nm. Recombinant protein purity and seroreactivity were determined by Coomassie gel staining and immunoblot using infected mouse serum. Briefly, 100 ng of each recombinant protein was separated by 12.5% polyacrylamide gel electrophoresis. For Coomassie staining, the gels were incubated in Imperial protein stain (Thermo Scientific, Rockford, IL) for 1 h and destained in deionized water for 1 h prior to imaging. For immunoblot analysis, the proteins were transferred to a nitrocellulose membrane, and the membrane was blocked in 5% skim milk and incubated for 1 h with mouse serum samples collected 3 weeks postinoculation with wild-type B. burgdorferi, as previously described (10), diluted 1:200 in Tris-buffered saline–0.05% Tween (TBST) (pH 7.6), washed twice with TBST, incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG/IgM (Chemicon International, Billerica, MA) for 1 h, washed twice with TBST, and the signal was detected using the SuperSignal West Pico chemiluminescent substrate kit (Thermo Scientific).

Cloning and expression of the recombinant DOC antigen. An in-frame glutathione S-transferase (GST) fusion protein for the DOC hybrid protein was generated using two distinct PCR amplification steps. First, the corresponding coding regions for DbpA and the C6 peptide of VlsE (11) were amplified separately from B. burgdorferi strain B31 genomic DNA, and the PEPC10 sequence (12) was added to each amplicon using the primer pairs 1145 and 1023. The final constructs were sequenced and described above for the other B. burgdorferi antigens.

iPCR reagents, assay, and signal amplification. iPCR reagents were prepared and the assays conducted as previously described (10), with minor modifications. Briefly, iPCR assays were assembled in a two-sided (sandwich) manner, as detailed in Fig. 1A, with the capability to simultaneously capture and report both IgM and IgG host-generated antibodies (Fig. 1B). Recombinant antigens lacking fusion tags were used to coat magnetic beads for host antibody capture using 10 to 20 μg of antigen per mg of beads. The beads were resuspended in 500 μl TBST for secondary antibody incubation. Signal amplification by real-time quantitative PCR was accomplished as previously described (10), and the quantification cycle (Cq) for each reaction was determined using a manual baseline determination (cycle 10 to 20) and a manual threshold setting of 1.0. The PCR plate set-ups for all experiments included, in duplicate, a PCR-negative template control consisting of water and an iPCR bead processing negative control that contained the TBST stock used for processing to determine the sample-to-sample contamination. Additionally, each PCR run included calibrator plasmids carrying the cloned template for the IgM or IgG reporter oligonucleotides that were used to account for run-to-run variation in the threshold calculation between the PCR plates. Briefly, the baseline was manually adjusted such that the Cq values for the calibrator plasmids were set at a constant value for each plate to account for minor variability in the threshold setting.

Positive threshold value and data analysis. The results of the Lyme disease iPCR assay were reported as ΔCq values. The ΔCq value was calculated as the difference between the antigen-/isotype-specific background threshold Cq value and the Cq value of the sample. The antigen-/isotype-specific background threshold Cq values were calculated as the mean Cq value of each antigen-isotype combination for a group of 16 healthy individuals minus a specific multiple of the standard deviation (SD) of the mean. The antigen-specific multiplier was set at a minimal value (1.9 to 6.6 for IgM and 3.1 to 5 for IgG), such that the samples from all individuals without Lyme disease in CDC research panel I resulted in a Lyme disease iPCR ΔCq value of <0. Using these antigen-/isotype-specific thresholds, any sample that resulted in a Lyme disease iPCR ΔCq of ≥0 was called iPCR positive. The coefficient of variation (CV) was calculated as the ratio of the SD to the mean. Assay sensitivity and specificity and the associated 95% confidence intervals were calculated using GraphPad Prism 5.0 for Windows (GraphPad Software).

RESULTS

Lyme disease iPCR demonstrates strong within-assay precision and reproducible background across a sample population of healthy individuals. We previously demonstrated proof-of-principle for iPCR detection of human host-generated B. burgdorferi antibodies using VlsE C6 peptide-coated magnetic beads and a panel of serum samples (n = 36) from Lyme disease-positive and
Lyme disease-negative patients and healthy controls (10). This feasibility study was accomplished using a small number of samples from healthy controls (n = 5) to determine test efficiency and background threshold levels. In an effort to establish a better understanding of the performance of the Lyme disease iPCR assay, including the repeatability and the variability of the background of the assay across a healthy population, the number of replicates and overall sample size of healthy individuals were expanded. Prospective blood samples were collected from consenting individuals without a history of Lyme disease under the approval of the University of Central Florida’s institutional review board. To assess assay repeatability, a serum sample from a single healthy individual was tested 18 times using the same reagent preparation lots, including DbpA antigen-coated beads and oligonucleotide-labeled secondary antibodies. The DbpA protein was selected as a representative in vivo-expressed B. burgdorferi antigen. The results of this analysis demonstrated low within-assay variability for both the IgM- and IgG-specific detection reagents, as indicated by standard deviation values for each data set of 0.39 and 0.73, respectively, and coefficient of variation values for each data set of 1.34% and 2.30%, respectively (Fig. 2).

To determine the variability in the background of the Lyme disease iPCR assay across a healthy human population, the serum samples from 36 healthy individuals were tested in duplicate using magnetic beads coated with the DbpA antigen and the oligonucleotide-labeled secondary antibodies. The DbpA protein was selected as a representative in vivo-expressed B. burgdorferi antigen. The results of this analysis demonstrated low within-assay variability for both the IgM- and IgG-specific detection reagents, as indicated by standard deviation values for each data set of 0.39 and 0.73, respectively, and coefficient of variation values for each data set of 1.34% and 2.30%, respectively (Fig. 2).

FIG 3 Lyme disease immuno-PCR demonstrates reproducible background across a healthy human population for both IgM and IgG isotypes using the DbpA antigen. Serum samples from 36 healthy individuals were assayed in duplicate by multiplex iPCR using both IgM (A) and IgG (B) secondary antibodies and recombinant DbpA antigen-coupled magnetic beads. Each dot represents a single replicate per individual, with the horizontal lines representing the mean value for duplicate serum samples from each individual. The y axis represents the quantification cycle (Cq) determined by real-time quantitative PCR. (C) The mean, standard deviation (SD), range, and coefficient of variation (CV) (calculated as the ratio of the SD to the mean) is listed for each isotype.

Mean and standard deviation background values across a population of healthy individuals are unique for each Lyme disease iPCR assay antigen-isotype combination. The analysis of the Lyme disease iPCR assay repeatability and population variability using DbpA-coupled magnetic beads demonstrated that the mean background value for the detection of IgM versus IgG antibodies differed by as much as ~2.5 Cq values (Fig. 2 and 3). Based on this observation, we predicted that depending on the different antigen used, each Lyme disease iPCR assay would result in a distinct mean background Cq value. If true, this finding would
impact the determination of the background threshold setting for the assay, making it necessary to assign a distinct background threshold for each antigen-isotype combination. To test this hypothesis, we compiled a list from the literature of B. burgdorferi proteins that are known or hypothesized to be seroreactive in humans (13–27). From this list, a subset of 8 B. burgdorferi antigens was selected for further analysis in our assay due to their ability to be produced in large quantities as recombinant in-frame N-terminal glutathione S-transferase (GST) fusion proteins in E. coli. To eliminate any possibility of antibody cross-reactivity to the GST tag, this sequence was proteolytically removed. The purity and antigenicity of each recombinant antigen were demonstrated by SDS-PAGE, followed by Coomassie brilliant blue staining and immunoblot analysis using pooled sera collected from B. burgdorferi-infected mice (see Fig. S1 in the supplemental material).

Each antigen was coupled to magnetic beads and examined by Lyme disease iPCR for both IgM and IgG background reactivities across 16 serum samples collected from healthy individuals. As predicted, all antigen-isotype combinations demonstrated unique background values that ranged from a mean $C_q$ of 26.09 to 32.46 for IgM and 25.30 to 36.62 for IgG and a standard deviation of 0.40 to 1.53 for IgM and 0.37 to 1.47 for IgG (Fig. 4).

**Multiplex iPCR detection of IgM and/or IgG host response antibodies against B. burgdorferi using a panel of antigens has the potential for improved sensitivity compared to 2-tier testing.** Most existing protocols for Lyme disease diagnostics require the use of multiple antigens to diagnose the disease. In an effort to further explore the application of iPCR as a Lyme disease diagnostic, we sought to develop a similar methodology that utilizes a combination of results for different antigens to facilitate diagnosis. The panel of eight B. burgdorferi antigens was tested against the CDC research panel I collection of sera using multiplex iPCR for the simultaneous detection of IgM and IgG host-generated antibodies. The same human serum panel was previously tested according to CDC guidelines by a commercial enzyme-linked immunosorbent assay (ELISA), followed by IgM and IgG immunoblot (IB), and classified for 2-tier testing status (see Table S1 in the supplemental material). Samples were considered positive by iPCR if they resulted in a $\Delta C_q$ value that was $\geq 0$ for IgM or IgG for one or more of the eight antigens tested. The $\Delta C_q$ value was calculated as the difference between the antigen-/isotype-specific background threshold $C_q$ value and the $C_q$ value of the sample. The antigen-/isotype-specific background threshold $C_q$ values were calculated as the mean $C_q$ value of each antigen-isotype combination for a group of 16 healthy individuals minus a specific multiple of the standard deviation (SD) of the mean (Fig. 4). Each antigen-specific multiplier was set at a minimum value (1.3 to 6.6 for IgM and 2.8 to 5 for IgG; see Table S2 in the supplemental material), such that the samples from all individuals without Lyme disease in CDC research panel I resulted in a Lyme disease iPCR $\Delta C_q$ value of $<0$. Using these criteria, iPCR testing provided similar results to those of 2-tier testing for the Lyme disease patient samples, with one exception (see Table S1 in the supplemental material). A single early Lyme disease patient sample that was deemed negative by 2-tier testing was positive by iPCR (see Table S1, sample A4). It should also be noted that no single antigen provided iPCR-positive results across all Lyme disease patient samples, which comprised different stages and clinical presentations of disease.
of a single hybrid antigen for iPCR detection of host-generated antibodies against B. burgdorferi infection, we synthetically constructed a novel hybrid antigen composed of full-length DbpA, the PEPC10 peptide (OspC) (12), and the C6 peptide (VlsE) (11), referred to as the DOC antigen (Fig. 5A). Similar to the previous eight recombinant antigens, we determined the protein purity and seroreactivity toward B. burgdorferi-infected mouse sera of the hybrid protein (see Fig. S1 in the supplemental material). The range of the background reactivity of the DOC antigen in the iPCR assay was determined using the serum from a group of 16 healthy individuals (Fig. 5B). The results of the between-sample variability analysis demonstrated a standard deviation across the population of 0.57 for the background detection of IgM antibodies and 0.51 for the background detection of IgG antibodies; the coefficients of variation were 2.31% and 1.94%, respectively. Using iPCR, we then tested the hybrid antigen in duplicate against the CDC research panel I for IgM and IgG reactivity, utilizing the results to establish the positive call threshold as described above. The DOC antigen IgG results confirmed all 2-tier-positive results (Fig. 6A). Interestingly, the Lyme disease iPCR assay using the DOC antigen tested negative for the detection of host-generated IgM antibodies for all human samples analyzed (Fig. 6B).

Although early, specific diagnosis is the primary goal for any Lyme disease diagnostic, determining the stage of disease progression would provide additional information to aid in the treatment of the disease. It is logical to assume that the amount of host-generated B. burgdorferi antibody will increase with further disease progression. Due to the quantifiable nature of iPCR test-
ing, we hypothesized that the amount of anti-DOC host-generated IgG antibody correlates with disease stage. The mean ± SD iPCR value was −1.61 ± 0.36 for stage 1 acute early Lyme disease patients, 0.67 ± 0.38 for stage 1 convalescent early Lyme disease patients, and 2.39 ± 0.64 for stage 2/stage 3 Lyme disease patients, for a total of n = 4 samples per group. These data may suggest a correlation of increasing antibody capture with disease progression; however, further evaluation with an increased number of clinically defined samples is required to support this finding. It should also be noted that the number of EM rashes documented clinically defined samples is required to support this finding.

**DISCUSSION**

There is an urgent need to develop new tools for improved diagnosis of Lyme disease. This study describes an objective Lyme disease diagnostic method using iPCR detection of host IgG antibody binding to a single recombinant hybrid antigen.

Repeatability is a key parameter of any newly developed diagnostic test that provides confidence the test will identify individuals as disease positive or negative in a reproducible manner across the inherent variability of a human population. iPCR has been shown to be a reproducible approach for detecting other targets (28, 29), although this method generates a background signal in the absence of the analyte being detected (30). The background signal has been attributed to nonspecific binding of the oligonucleotide-labeled secondary antibody, similar to the results observed for other immunodiagnostics (31). Although a number of approaches have been proposed to minimize the level of background amplification (32–34), no approach to date has proven successful at completely eliminating the background signal. For diagnosing Lyme disease, we propose that the iPCR background signal provides an intrinsic advantage due to the fact that a positive result is a relative measure above the established background threshold, thereby limiting the potential contribution of contamination, whereas a positive result for standard PCR is an absolute measure that can be highly sensitive to low-level laboratory contamination (7). The baseline level of amplification using iPCR for a negative sample far surpasses any low-level laboratory contamination that commonly results in false-positive detection for PCR-based clinical diagnostic tests. As a result, the level of PCR contamination required to produce a false positive above background for iPCR is orders of magnitude above that for standard PCR. In addition, critical to the success of this approach is a constant background that remains consistent between sample replicates and is standardized across a healthy human population.

In an effort to determine the consistency of the background amplification for the technique, we tested the serum from a single healthy individual over 18 replicates using iPCR and found the standard deviations of the mean C_q values to be 0.39 and 0.73 for IgM and IgG, respectively, with corresponding coefficients of variation of 1.34% and 2.30%, respectively. The accepted value for PCR sampling error is ~1 C_q (35), and the coefficient of variation for an ELISA-based test is considered good at <15% (36). These data indicate that our iPCR protocol can provide highly consistent and repeatable results across multiple replicates of a single sample. We proceeded to test serum samples collected from 36 healthy individuals in duplicate for IgM and IgG reactivity using the same antigen to determine the variability of the background across a healthy population. As expected, compared to the within-sample repeatability analysis, we observed a slightly higher standard deviation of the mean C_q values of 0.79 and 0.84 for IgM and IgG, respectively, as well as slightly increased corresponding coefficients of variation of 2.66% and 2.63%, respectively. These data demonstrate that the assay maintains strong repeatability even when compounded with normal human population serum variability. Taken together, these results indicate that the background variability for iPCR detection of host-generated antibodies within and across a healthy human population is within acceptable levels for the technique.

Previous studies using recombinant antigens have indicated that no single antigen tested to date has the capability to diagnose Lyme disease across its multiple stages and disease manifestations (7). A panel of eight antigens was generated for use in the iPCR assay. These proteins were selected based on previous studies that identified B. burgdorferi immunoreactive antigens (13–27). We provided the necessary parameters, including the mean background C_q value and the standard deviation of that mean for determining an
| Sample group          | Sample ID | DOC IgG | Interpretation for 2-Tier ELISA | Tier 1 bands detected for: |
|-----------------------|-----------|---------|---------------------------------|----------------------------|
| **Lyme disease stage 2** |           |         |                                 |                            |
| Early Lyme-EM<sup>d</sup> |           |         |                                 |                            |
| **B1**                | 2.24      | Pos     | Pos                             | 41, 39, 23, 58, 41, 39, 23, 18 |
| **B2**                | 2.00      | Pos     | Pos                             | 23, 66, 45, 41, 39, 23, 18  |
| **B3**                | 2.07      | Pos     | Pos                             | 41, 39, 23, 41, 23           |
| **B4**                | 2.05      | Pos     | Pos                             | 41, 58, 45, 41, 39, 23, 18   |
| **B5**                | 1.59      | Pos     | Pos                             | 41, 23, 41, 23               |
| **B6**                | 1.45      | Pos     | Pos                             | 41, 39, 23, 66, 45, 41, 39, 23, 18 |
| **B7**                | 1.08      | Pos     | Pos                             | 41, 39, 23, 41, 23           |
| **B8**                | 0.80      | Pos     | Pos                             | 41, 23, 41                  |
| **B9**                | 0.52      | Pos     | Neg                             | 23, 66, 41, 23               |
| **B10**               | 0.08      | Pos     | Neg                             | Equ                        |
| **B11**               | (0.08)    | Neg     | Neg                             | Pos                        |
| **B12**               | (0.27)    | Neg     | Neg                             | 66                         |
| **B13**               | (0.58)    | Neg     | Neg                             | Pos                        |
| **B14**               | (0.91)    | Neg     | Pos                             | 23, 41, 23                 |
| **B15**               | (1.00)    | Neg     | Neg                             | 67                         |
| **B16**               | (1.01)    | Neg     | Nq                              | 39, 23, 23                 |
| **B17**               | (1.22)    | Neg     | Neg                             | 23                         |
| **B18**               | (1.48)    | Neg     | Nq                              | 23                         |
| **B19**               | (1.50)    | Neg     | Neg                             | 23                         |
| **B20**               | 1.14      | Pos     | Neg                             | 41, 41, 23, 18             |
| **Lyme disease stage 2** |           |         |                                 |                            |
| Neuroborreliosis       |           |         |                                 |                            |
| **B21**               | 2.64      | Pos     | Pos                             | 41, 23, 45, 41, 23          |
| **B22**               | 2.01      | Pos     | Pos                             | 41, 39, 23, 41, 39, 23      |
| **B23**               | 0.00      | Pos     | Pos                             | 41, 39, 23, 41, 23          |
| **B24**               | (0.26)    | Neg     | Neg                             | 41, 23, 41                 |
| **Lyme carditis**      |           |         |                                 |                            |
| **B25**               | 2.83      | Pos     | Pos                             | 41, 39, 23, 66, 45, 41, 23, 18 |
| **B26**               | 1.37      | Pos     | Pos                             | 41, 39, 23, 66, 45, 41, 23, 18 |
| **Lyme disease stage 3** |           |         |                                 |                            |
| Lyme arthritis         |           |         |                                 |                            |
| **B27**               | 3.44      | Pos     | Pos                             | 23, 93, 66, 58, 45, 41, 39, 30, 28, 23, 18 |
| **B28**               | 2.96      | Pos     | Pos                             | 41, 93, 66, 58, 45, 41, 39, 30, 28, 23, 18 |
| **B29**               | 2.67      | Pos     | Pos                             | 41, 23, 93, 66, 58, 45, 41, 39, 30, 28, 23, 18 |
| **B30**               | 2.62      | Pos     | Pos                             | 23, 66, 58, 45, 41, 39, 28, 23, 18 |
| **B31**               | 2.09      | Pos     | Pos                             | 23, 58, 41, 39, 23, 18      |
| **B32**               | 1.84      | Pos     | Pos                             | 93, 66, 58, 41, 39, 30, 23, 18 |
| **Non-Lyme**          |           |         |                                 |                            |
| Fibromyalgia           |           |         |                                 |                            |
| **B33**               | (0.28)    | Neg     | Neg                             | 23                         |
| **B34**               | (0.81)    | Neg     | Neg                             | 39, 58, 41                 |
| **B35**               | (1.70)    | Neg     | Neg                             | 41                         |
| **B36**               | (1.89)    | Neg     | Neg                             | 41                         |
| **B37**               | (1.93)    | Neg     | Neg                             | 41                         |
| **B38**               | (2.30)    | Neg     | Neg                             | 41                         |
| **Rheumatoid arthritis** |         |         |                                 |                            |
| **B39**               | (0.90)    | Neg     | Neg                             | 41                         |
| **B40**               | (1.17)    | Neg     | Neg                             | 41                         |
| **B41**               | (1.56)    | Neg     | Nq                              | 41, 23                     |
| **B42**               | (1.73)    | Neg     | Pos                             | 41, 23                     |
| **B43**               | (1.77)    | Neg     | Neg                             |                            |
| **B44**               | (2.05)    | Neg     | Neg                             |                            |
| **Multiple sclerosis** |           |         |                                 |                            |
| **B45**               | (0.55)    | Neg     | Neg                             | 39, 23, 41                 |
| **B46**               | (0.78)    | Neg     | Pos                             | 41, 23                     |
| **B47**               | (1.09)    | Neg     | Neg                             |                            |
| **B48**               | (1.11)    | Neg     | Neg                             | 39                         |
| **B49**               | (1.75)    | Neg     | Neg                             |                            |
| **B50**               | (2.05)    | Neg     | Neg                             | 66                         |

(Continued on following page)
individual call threshold for each antigen-isotype combination. The call thresholds were established as the mean background Cq value minus a multiple of the standard deviation. The multiplier of standard deviation was unique for each antigen-isotype combination and established based on the minimum multiplier that resulted in no false-positive calls for the CDC research panel I, which served as the training set for optimizing our assay. The \( C_q \) was calculated as the established threshold call \( C_q \) minus the \( C_q \) value of the sample. A sample with a \( \Delta C_q \) value of \( \geq 0 \) was deemed positive by iPCR. Using the panel of eight antigens, this approach duplicated 2-tier testing results with a single early Lyme patient sample (culture positive) testing positive by iPCR that was negative by 2-tier testing. Samples from individuals in the later stages of disease (neurologic and arthritis) tended to test positive for multiple antigens.

In addition to detecting the presence of host antibodies as laboratory support of an exposure to \( B. burgdorferi \), it would be de-

### TABLE 2 (Continued)

| Sample group | Sample ID | DOC IgG | Interpretation for: | Tier 2 bands detected for: |
|--------------|-----------|---------|---------------------|---------------------------|
|              |           |         | iPCR 2-Tier Tier 1 ELISA IgM IgG |
| Mononucleosis| B51       | (0.09)  | Neg Neg Neg          | 39                         |
|              | B52       | (0.28)  | Neg Neg Pos          | 41, 39                     |
|              | B53       | (0.58)  | Neg Neg Pos          |                            |
|              | B54       | (0.77)  | Neg Neg Equ          | 41                         |
|              | B55       | (0.78)  | Neg Neg Neg          |                            |
|              | B56       | (1.25)  | Neg Neg Neg          |                            |
| Syphilis     | B57       | (0.56)  | Neg Neg Pos          |                            |
|              | B58       | (0.75)  | Neg Neg Pos          | 41                         |
|              | B59       | (0.96)  | Neg Neg Pos          | 41                         |
|              | B60       | (1.01)  | Neg Pos Pos          | 39, 23                     |
|              | B61       | (1.38)  | Neg Neg Pos          | 41                         |
|              | B62       | (1.47)  | Neg Neg Neg          |                            |
| Severe periodontitis | B63 | (0.22) | Neg Neg Neg          |                            |
|              | B64       | (0.29)  | Neg Neg Neg          |                            |
|              | B65       | (0.56)  | Neg Neg Neg          |                            |
|              | B66       | (0.90)  | Neg Neg Neg          | 45, 41                     |
|              | B67       | (1.03)  | Neg Neg Neg          | 66                         |
|              | B68       | (3.04)  | Neg Neg Neg          |                            |
| Healthy controls | B69 | 0.23    | Pos Neg Neg          | 23                         |
| From areas of endemicity | B70 | (0.04) | Neg Neg Pos          | 41, 66                     |
|              | B71       | (0.53)  | Neg Neg Pos          | 41, 23                     |
|              | B72       | (0.87)  | Neg Neg Neg          | 23                         |
|              | B73       | (0.87)  | Neg Neg Equ          | 23                         |
|              | B74       | (1.11)  | Neg Neg Neg          | 45, 41                     |
|              | B75       | (1.16)  | Neg Neg Neg          |                            |
|              | B76       | (1.37)  | Neg Neg Neg          |                            |
|              | B77       | (1.42)  | Neg Neg Neg          |                            |
|              | B78       | (1.49)  | Neg Neg Neg          | 66, 41                     |
|              | B79       | (1.95)  | Neg Neg Neg          | 23                         |
|              | B80       | (2.47)  | Neg Neg Pos          | 23, 58, 41, 39, 18         |
| From areas of nonendemicity | B81 | (0.53) | Neg Neg Neg          | 41                         |
|              | B82       | (0.60)  | Neg Neg Neg          | 41, 23                     |
|              | B83       | (0.78)  | Neg Neg Equ          |                            |
|              | B84       | (0.80)  | Neg Neg Pos          |                            |
|              | B85       | (0.86)  | Neg Neg Neg          |                            |
|              | B86       | (0.90)  | Neg Neg Neg          | 58, 45                     |
|              | B87       | (1.09)  | Neg Neg Neg          | 66, 58, 45, 41             |
|              | B88       | (1.15)  | Neg Neg Neg          | 41                         |
|              | B89       | (1.17)  | Neg Neg Neg          | 41                         |
|              | B90       | (1.77)  | Neg Neg Neg          | 23                         |
|              | B91       | (2.06)  | Neg Neg Neg          | 23                         |
|              | B92       | (2.09)  | Neg Neg Neg          |                            |

\( ^a \) ID, identification.

\( ^b \) Values shown represent a \( \Delta C_q \) in reference to the antigen/isotype background threshold \( C_q \) value determined using an antigen-specific multiplier of the standard deviation (SD) above the mean value for a set of healthy individuals for each antigen/isotype combination, as described in Materials and Methods. The values in parentheses represent negative iPCR \( \Delta C_q \) values.

\( ^c \) Two-tier results were established by standard ELISA and IgG/IgM immunoblot (IB) protocols. Pos, positive; Neg, negative; Equ, equivocal.

\( ^d \) EM, erythema migrans.

\( ^e \) Bold type indicates positive assay results/interpretations.
with all 2-tier positives identified as positive by iPCR. The quantification of the ΔCq for Lyme disease patients showed a trend of increasing average values from early Lyme acute (−1.61) to convalescent early Lyme (0.67) to late-stage Lyme (2.39), suggesting a correlation in the amount of detectable *B. burgdorferi* antibody with disease stage. Interestingly, DOC iPCR IgM was negative for all samples tested, including Lyme disease patient samples. The full-length DbpA antigen alone resulted in a low-positive IgM iPCR value (0.69) for only a single Lyme disease patient sample. iPCR testing using the full-length OspC antigen resulted in a number of IgM iPCR-positive samples, suggesting that the antibodies detected in these samples may have resulted from OspC epitopes other than the PEPC10 sequence. It is also possible that in the context of the DOC hybrid antigen, the PEPC10 sequence lacks the conformational epitope(s) required for IgM recognition. It is well documented that the VlsE antigen primarily generates IgG rather than IgM antibodies early in infection (41). Therefore, it may not be surprising that the DOC antigen detects IgG antibodies only. These results indicate that testing only the IgG fraction using the DOC hybrid antigen was necessary to achieve a level of sensitivity equivalent to that of 2-tier testing, which required IgM for positive detection in some samples. Given the small sample size, these findings do not rule out the possibility that IgM antibodies might be detected with the DOC iPCR assay in some Lyme disease patient serum samples. Moreover, the additional optimization of the hybrid antigen to include the specific detection of IgM antibodies may contribute to further improved sensitivity for detecting disease in patients with early Lyme disease. Nonetheless, IgM detection has been problematic and controversial due to its contribution to false-positive results and the requirement that IgM testing be used only within the first 4 weeks of infection (7), suggesting that an assay that does not use IgM may represent an improvement over the current methods of testing for Lyme disease. In addition, our data suggest that there exists the potential to determine the stage of disease based on the ΔCq value of the DOC iPCR assay, which represents another possible improvement over current Lyme disease diagnostics.

iPCR testing of the anti-*B. burgdorferi* IgG antibody fraction using the DOC hybrid antigen was successful at duplicating the 2-tier testing results for a small panel of samples. We then proceeded to test a larger blinded panel of 92 samples composed of serum samples from Lyme patients (early, early disseminated with cardiac or neurological involvement, and Lyme arthritis), those with look-alike diseases (fibromyalgia, mononucleosis, multiple sclerosis, rheumatoid arthritis, severe periodontitis, and syphilis), and healthy (from areas of endemicity and nonendemicity) individuals (CDC research panel II). iPCR demonstrated 69% sensitivity and 98% specificity compared to 59% and 97%, respectively, for 2-tier testing. A single neurologic Lyme patient tested negative by both iPCR and 2-tier testing. This result is most likely due to the fact that the serum sample was taken 7 days post-erythema migrans (EM), which was likely too early in the infection process to produce an adequate immune response.

Currently, the DOC hybrid antigen is composed of *B. burgdorferi* B31 sequences. Amino acid sequences can vary between strains and species of Lyme disease borreliae by as much as 24% for VlsE C6 (11), 10% for OspC PEPC10C (12), and 44% for DbpA (42). This may be limiting if an individual is infected with other strains or species. It is likely that the incorporation of additional protein/peptide sequences from other species, such as *Borrelia afzelii* or...
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