A First-Tier Rapid Assay for the Serodiagnosis of Borrelia burgdorferi Infection

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Background: The present recommendation for the serologic diagnosis of Lyme disease is a 2-tier process in which a serum sample with a positive or equivocal result by an enzyme-linked immunosorbent assay (ELISA) or immunofluorescent assay is then followed by supplemental testing by Western blot. Our laboratory has developed recombinant chimeric proteins composed of key Borrelia epitopes. These novel antigens are consistent and are easily standardized.

Methods: We adapted these recombinant proteins into a new immunochromatographic format that can be used as a highly sensitive and specific first-tier assay that can be used to replace the ELISA or immunofluorescent assay.

Results: This rapid test was equally sensitive (P > .05) and more specific (P < .05) than a frequently used commercial whole cell ELISA. The overall clinical accuracy achieved on agreement studies among 3 Lyme research laboratories on clinically defined serum panels was shown to be statistically equivalent to the commercial ELISA. The assay can detect anti–Borrelia burgdorferi antibodies in either serum or whole blood.

Conclusion: This sensitive and specific rapid assay, which is suitced for the physician’s office, streamlines the 2-tier system by allowing the physician to determine if a Western blot is necessary at the time of the initial office visit.

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LYME DISEASE is a significant public health concern and is currently the most common vector-borne infectious disease in the United States, where its primary tick vector, Ixodes scapularis, is endemic. Caused by the spirochete Borrelia burgdorferi, this infectious disease may affect multiple organ systems. Except erythema migrans (EM), none of the clinical manifestations of Lyme disease are pathognomonic. In fact, many can be observed in other illnesses. In the absence of observed EM, the diagnosis of Lyme disease is confirmed by the detection of a humoral immune response to B burgdorferi in patients with objective findings suggestive of the disease. Adoption of a conditional 2-step approach to the serodetection of anti–B burgdorferi antibodies has markedly improved the accuracy of laboratory diagnosis. The first step of this 2-step approach is performance of an enzyme-linked immunosorbent assay (ELISA) or immunofluorescent assay; if the results of the assay are positive or equivocal, the second step, performance of a Western blot, follows. Although this 2-step approach improves the accuracy of Lyme disease testing, it has added significantly to the time it takes to finish the laboratory evaluation of patients suspected of having Lyme disease.

During the last few years, our laboratory has developed recombinant chimeric Borrelia proteins (RCBPs) containing key Borrelia epitopes. These novel antigens are consistent, easily standardized, and, by their recombinant nature, easy to manipulate and optimize. In this report, we describe the adaptation of the RCBPs into a new immunochromatographic assay that is rapid, sensitive, specific, simple, and reliable. This rapid test can be used to test for the presence of antibodies to B burgdorferi in serum or whole blood. It can be used in the physician’s office as a first-tier assay, thus streamlining the 2-tier system by allowing the physician to determine if a Western blot is necessary at the time of the office visit.

RESULTS

SENSITIVITY AND SPECIFICITY

To assess the sensitivity of the assay, the panel of 120 serum samples from patients with clinically well-characterized Lyme disease was tested in the recombinant immunochromatographic assay (rapid assay) and a commercial whole cell ELISA and the results are shown in Table 1.
MATERIALS AND METHODS

RCBP-BASED IMMUNOCHROMATOGRAPHIC ASSAY (RAPID ASSAY)

Antigen Immobilization

The RCBPs used in this assay were cloned, expressed, and purified as described previously. Briefly, RCBPs containing epitopes from OspA, OspB, OspC, flagellin, and p93 were generated. Portions of DNA of these sequences were cloned in tandem in an expression vector that could produce recombinant fusion proteins. Recombinant proteins OspB-OspC-Fla (B-Fla, 64 kd), OspA-p93 (A-93, 97 kd), and 2 OspC (22 kd) proteins from different genospecies (Borrelia afzelii and Borrelia garinii) were used. A protein cocktail composed of a mixture of the RCBPs was made at a concentration of 2.3 mg/mL, which included 10µM B-Fla, 12µM A-93, and 9.5µM each OspC (plus 0.1mM diethylenetriol, 1.0mM phenylmethylsulfonyl fluoride (PMSF), and 0.02% sodium azide in 20mM phosphate-buffered saline, pH 7.2). The protein concentration was checked by the Bradford method (Bio-Rad Laboratories, Hercules, Calif) and by direct visualization on a Coomassie-stained sodium dodecyl sulfate-polyacrylamide electrophoresis gel. Then, 100 µg of protein was loaded onto the test zone of a nitrocellulose membrane (Chembio Diagnostics, Medford, NY) on a thin-band layer (Camag Linomat IV; Camag, Muttenz, Switzerland). Staphylococcus aureus protein A antigen (2.0 mg/mL in 50mM Tris-hydrochloride, pH 8.0, and 150mM sodium chloride) was loaded a third of an inch (~1 cm) away from the B burgdorferi test antigen on the control stripe of the membrane. Antibody binding protein conjugated to colloidal gold, which interacts with immunoglobulins, notably IgG, in a nonantibody-type pseudo immune reaction, was applied onto the sample zone of the membrane. The assay is polyclonal. The membrane strip was air dried and was packed into a plastic device containing 3 windows labeled sequentially: sample, test, and control (Chembio Diagnostics). The test device was stored at room temperature in a dry area until used (up to 1 year).

Procedure

The standard procedure for the rapid assay was as follows: 5 µL of serum or 10 µL of whole blood was added to the sample window, and 250 µL of diluent substrate (Chembio Diagnostics) was added to the sample. The diluent substrate dissolves on the sample pad and, in the presence of the serum immunoglobulins (IgG and IgM), forms a color-labeled complex with the antibody binding protein that, by capillary, flows upward across the membrane and binds to the control stripe. If B burgdorferi antibodies are present in the sample serum, they attach to the color-labeled antibody binding protein, which in turn will bind to the RCBP antigen immobilized on the membrane in the test window, producing a pink-purple band. In the absence of antibodies to B burgdorferi, this color-labeled complex will bind only to the control stripe, demonstrating that the reagents are functioning properly. A test result is positive when 2 bands (test and control) develop and negative when only 1 hand (control) develops (Chembio Diagnostics) (Figure). When a weak band develops in the test window, this is also considered a positive result.

COMMERCIAL ELISA AND WESTERN BLOT

ELISA kits using whole B burgdorferi as the antigen source were obtained from Wampole Laboratories, division of Carter-Wallace, Inc (Palatine, Ill). The test is polyclonal, measuring both IgM and IgG, and was performed according to the manufacturer’s instructions. Values greater than 3 SDs over the mean are considered positive. Values between 2 and 3 SDs are considered equivocal. The B burgdorferi Western blot assays were purchased from MarDx Diagnostics, Inc (Carlsbad, Calif) and were performed and read according to the manufacturer’s recommendations.

STUDIES

The primary objective of these studies was to determine the efficacy of the rapid assay in detecting antibodies to B burgdorferi. The primary outcome measure was the ability of the rapid assay to identify individuals with well-characterized Lyme disease. Standard whole B burgdorferi ELISAs and Western blots were used as comparison.

Serologic Studies

Serum Banks. The Lyme Disease Center at the State University of New York (SUNY) at Stony Brook has access to a large bank of sera that were obtained from patients with well-characterized clinical disease. Many of these patients participated in large multicenter clinical trials conducted by this center. A total of 406 individual samples were used in these studies. Of these, 188 were clinically classified as Lyme disease (120 samples characterized clinically and in the laboratory, 42 samples from the Centers for Disease Control and Prevention [CDC], and 26 samples of whole blood

WHOLE BLOOD TESTING

Twenty-one of the 26 patients with EM presenting to the SUNY at Stony Brook Lyme Disease Center had B burgdorferi isolated from culture of their skin biopsy specimens. The initial capillary (fingerstick) blood from these 21 patients with culture-confirmed early (<1-month onset) Lyme disease tested positive in 13 samples (62%) in the recombinant rapid assay, while the serum samples obtained from these patients tested positive for 10 samples and equivocal for 1 (52%) by whole cell ELISA. All the blood samples from the 5 patients with EM in which
from patients with EM presenting to the SUNY at Stony Brook Lyme Center); 118 were classified as potentially cross-reactive and 100 were classified as asymptomatic healthy controls from an endemic area.

Rapid Assay Sensitivity. All serum samples used in this analysis were obtained from patients with clinically and laboratory well-characterized Lyme disease (n=120). The criteria for positive serum samples included (1) early localized infections typified by the presence of well-defined EM in patients from an endemic area (n=30); (2) early disseminated infections typified by EM and 1 or more of the following (n=60): additional EM lesions, atrioventricular block, neurologic abnormalities (eg, seventh nerve palsy), or meningitis; and (3) late Lyme disease (occurring more than 3 months after onset) with neurologic or arthritic manifestations and a positive serology test result for \textit{B burgdorferi} as defined by CDC criteria (n=30). For patients with EM, culture isolation of \textit{B burgdorferi} from the skin lesion or photographic documentation was required. A blinded (coded), clinically characterized, and stratified Lyme disease sera panel (n=42) was obtained from the CDC as a means to convey further information on the performance of the rapid assay.

Rapid Assay Cross-reactivity (Specificity). Serum samples from patients with diseases known to produce false-positive results were tested (n=118). The panel included the following: 20 samples from patients with various stages of syphilis; 20 samples from patients with connective tissue disorders (rheumatoid arthritis or systemic lupus erythematosus); 13 samples from patients with other infectious diseases associated with false-positive test results (eg, Epstein-Barr virus, cytomegalovirus); 20 samples from patients hospitalized with acute or chronic disorders (infectious diseases, inflammatory conditions, neurologic disorders); 10 samples from patients with parvovirus infection with secondary rheumatologic manifestations; and 10 samples from patients with other tickborne infectious diseases (human granulocytic ehrlichiosis); 25 samples from patients with \textit{Helicobacter pylori} were tested only on the rapid assay. In addition, as controls, serum samples from 100 asymptomatic healthy people, without Lyme disease or objective evidence of other diseases known to cause false-positive ELISA results, were tested.

Testing of Whole Blood

Twenty-six patients presenting to the SUNY at Stony Brook Lyme Center with EM were prospectively studied. At the time of their initial presentation, punch biopsy specimens (2 mm) were obtained from the leading edge of the erythema and cultured in BSK-H (BSK II with high sodium bicarbonate) media (Sigma-Aldrich, St Louis, Mo). Blood was collected from a fingerstick and tested by the rapid assay, and each subject donated a sample of venous blood. The correspondent serum was tested by ELISA. Seven healthy controls were tested using both capillary (fingerstick) blood and venous blood by the rapid assay to assess for reproducibility.

Comparative Studies

Clinical Accuracy of the Rapid Test in Lyme Disease Reference Laboratories. Serum samples were tested at 3 Lyme disease reference laboratories (sites A, B, and C), and the agreement among the results obtained in each site was evaluated. The same 120 samples from patients with Lyme disease used in the serologic sensitivity studies and 100 samples from asymptomatic healthy donors used in the specificity studies were used. Rapid assay results obtained in site A were compared with the clinical diagnosis and ELISA results obtained at site A; rapid assay results obtained in sites B and C were then compared with the rapid assay results obtained in site A. The agreement, or frequency of concordant positive or negative results in the rapid assay, was determined among the sites. The rapid assays were done in duplicate.

Clinical Accuracy of the Rapid Test in Physician Office Locations. Three physician office locations (POLs) tested 25 clinically defined serum samples with the rapid test. The serum samples used in this study were selected from the blinded panels used before in the sensitivity and specificity studies and sent to the POL sites. At each of the sites, the tests were done by untrained office personnel with a minimum of a seventh-grade reading level following the written instructions only. The differences among the sites were statistically accessed by the Fisher exact test and the t test (1-tailed).

**STATISTICAL ANALYSIS**

Whenever appropriate, the McNemar exact test for correlated proportions was used to evaluate the statistical significance of the data obtained. The variance in the agreement among the results obtained at the different clinical laboratory sites where the rapid assay was tested was calculated by the normal theory method for obtaining 95% confidence intervals (CIs). The Fisher exact test and t test were used to evaluate the variance in the agreement among the results obtained at the different POLs.
atic healthy donors) were tested in 3 different Lyme disease reference laboratories (sites A, B, and C) (Table 4). The rapid assay results obtained in site A were compared with the clinical diagnosis and with the ELISA results obtained at the same site; rapid assay results obtained in sites B and C were then compared with the rapid assay results obtained in site A, including 95% CIs. The agreement, or frequency of concordant positive or negative results in the rapid assay, was determined among the sites. The overall sensitivity between ELISA (site A) and clinically positive results was 70.8% (85/120) (95% CI, 62.5%-79.1%); the overall sensitivity between rapid assay results obtained at site A and clinically positive results was 72.5% (87/120) (95% CI, 64.3%-80.7%). The overall agreement between rapid assay results from sites B and A was 83.9% (73/87) (95% CI, 76.0%-91.8%); the

Table 1. Comparison of Recombinant Rapid Assay and a Commercial Whole Cell Borrelia (WCB) Enzyme-Linked Immunosorbent Assay (ELISA) for Sensitivity in Detecting Antibodies to Borrelia burgdorferi Infection

<table>
<thead>
<tr>
<th>Group*</th>
<th>WCB ELISA Sensitivity, % (No.)</th>
<th>Rapid Assay Sensitivity, % (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early Lyme disease (n = 30)</td>
<td>50.0 (15/30)</td>
<td>53.6 (16/30)</td>
</tr>
<tr>
<td>Early disseminated infection (n = 80)</td>
<td>66.7 (40/60)</td>
<td>68.3 (41/60)</td>
</tr>
<tr>
<td>Late Lyme disease (n = 30)</td>
<td>100 (30/30)</td>
<td>100 (30/30)</td>
</tr>
<tr>
<td>Overall positive†</td>
<td>70.8 (85/120)</td>
<td>72.5 (87/120)</td>
</tr>
</tbody>
</table>

*Early Lyme disease is defined as disease for 0 to 1 month; early disseminated infection, disease for 0 to 3 months; and late Lyme disease, disease for more than 3 months.
†P>.05 (McNemar exact test).

Table 2. Comparison of Recombinant Rapid Assay and Centers for Disease Control and Prevention (CDC) Enzyme-Linked Immunosorbent Assay (ELISA) and Western Blot for Sensitivity in Detecting Antibodies to Borrelia burgdorferi Infection Stratified by Time After Onset

<table>
<thead>
<tr>
<th>Time After Onset</th>
<th>No. of Samples</th>
<th>CDC ELISA Sensitivity, % (No.)</th>
<th>CDC Western Blot Sensitivity, % (No.)</th>
<th>Rapid Assay Sensitivity, % (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1 mo</td>
<td>5</td>
<td>60 (3/5)</td>
<td>60 (3/5)</td>
<td>80 (4/5)</td>
</tr>
<tr>
<td>1-2 mo</td>
<td>10</td>
<td>90 (9/10)</td>
<td>90 (9/10)</td>
<td>100 (10/10)</td>
</tr>
<tr>
<td>3-12 mo</td>
<td>19</td>
<td>84 (16/19)</td>
<td>68 (13/19)</td>
<td>95 (16/19)</td>
</tr>
<tr>
<td>&lt;1 y</td>
<td>6</td>
<td>100 (6/6)</td>
<td>100 (6/6)</td>
<td>100 (6/6)</td>
</tr>
</tbody>
</table>


Table 3. Comparison of the Rapid Assay and a Commercial Whole Cell Borrelia (WCB) Enzyme-Linked Immunosorbent Assay (ELISA) for Specificity of Potentially Cross-reactive Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Rapid Assay Specificity, % (No.)</th>
<th>WCB ELISA Specificity, % (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syphilis</td>
<td>100 (0/20)</td>
<td>65 (7/20)</td>
</tr>
<tr>
<td>Autoimmune</td>
<td>100 (0/20)</td>
<td>100 (0/20)</td>
</tr>
<tr>
<td>Epstein-Barr virus and cytomegalovirus</td>
<td>100 (0/13)</td>
<td>100 (0/13)</td>
</tr>
<tr>
<td>Parvovirus</td>
<td>100 (0/10)</td>
<td>100 (0/10)</td>
</tr>
<tr>
<td>Neurologic disease</td>
<td>95 (1/20)</td>
<td>95 (1/20)</td>
</tr>
<tr>
<td>Human granulocytic ehrlichiosis</td>
<td>90 (1/10)</td>
<td>80 (2/10)</td>
</tr>
<tr>
<td>Paired overall negative†</td>
<td>97.8 (91/93)</td>
<td>89.2 (83/93)</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>97 (3/100)</td>
<td>95 (5/100)</td>
</tr>
</tbody>
</table>

*Only neurologic disease and human granulocytic ehrlichiosis samples tested positive on both the rapid assay and the Western blot.
†Statistically significant difference between rapid assay and whole cell ELISA, P<.001 (McNemar exact test).

Table 4. Agreement of Rapid Assay Results Obtained at 3 Different Sites With Enzyme-Linked Immunosorbent Assay (ELISA) Results and Clinical Diagnosis

<table>
<thead>
<tr>
<th>Clinical Diagnosis*</th>
<th>ELISA (Site A) vs Clinical</th>
<th>Rapid Site A vs Clinical</th>
<th>Rapid Site B vs Rapid Site A</th>
<th>Rapid Site C vs Rapid Site A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early Lyme disease (n = 30)</td>
<td>50 (31.7-68.3)</td>
<td>53 (35.1-71.6)</td>
<td>62.5 (38.3-86.7)</td>
<td>68.8 (45.6-91.9)</td>
</tr>
<tr>
<td>Early disseminated infection (n = 80)</td>
<td>66.5 (51.4-83.9)</td>
<td>69 (51.4-85.3)</td>
<td>83 (66.7-97.2)</td>
<td>85.2 (69.9-98.9)</td>
</tr>
<tr>
<td>Late Lyme disease (n = 30)</td>
<td>100 (90.1-100)†</td>
<td>100 (90.1-100)†</td>
<td>96.7 (90.1-100)</td>
<td>86.7 (74.3-99.1)</td>
</tr>
<tr>
<td>Healthy controls (n = 100)</td>
<td>95 (88.9-100)</td>
<td>97 (92.2-100)</td>
<td>97 (92.2-100)</td>
<td>97 (92.2-100)</td>
</tr>
</tbody>
</table>

*Early Lyme disease is defined as disease for 0 to 1 month; early dissemination infection, disease for 0 to 3 months; and late Lyme disease, disease for more than 3 months.
†One false discrepant result was assumed in the 95% confidence interval calculation.
agreement between rapid assay results from sites C and A was 82.8% (72/87) (95% CI, 74.7%-90.9%). For the healthy donors, the overall specificity between ELISA and clinically negative results was 95% (95/100) (95% CI, 88.9%-100%); the specificity between rapid results obtained in site A and clinically negative results was 97% (97/100) (95% CI, 92.2%-100%). The overall agreement between rapid results in sites B and A and sites C and A was 97% (97/100) (95% CI, 92.2%-100%). Sensitivity for positive samples and specificity for negative samples were shown to be statistically equivalent to the commercial ELISA.

**Clinical Accuracy of the Rapid Test in POLs**

Three POLs tested 25 previously clinically defined and blinded serum samples with the rapid test. Of 16 positive samples, POL site 1 detected 14 as positive, POL site 2 detected 16 as positive, and POL site 3 detected 13 as positive. Of 9 negative samples, POL site 1 detected 9 as negative. POL site 2 detected 8 as negative (this site was missing 1 sample), and POL site 3 detected 9 as negative. The agreement was 92% (23/25) for POL site 1, 100% (24/24) for POL site 2, and 88% (22/25) for POL site 3. The differences in the agreement between the 2 most sparse sites, site 2 vs site 3, were not significant by both the Fisher exact (P=.12) and the t tests (P=.15).

This new recombinant rapid assay for the detection of antibody to key *B. burgdorferi* antigens offers several advantages over the other first-tier assays. The most obvious advantage is the fact that this assay yields results in 20 minutes or less. However, there are other important advantages.

Because virtually all the current first-tier anti-*B. burgdorferi* assays, immunofluorescent assays or ELISAs, use cultured *B. burgdorferi* as their antigen source, a high degree of variability and a large number of proteins that contain cross-reactive epitopes are included in these assays. The spirochete is known to undergo adaptation changes in culture, introducing variability in the proteins expressed. Furthermore, since the whole cell tests used as first-tier assays are not standardized, there are significant differences in how they are performed and reported from different laboratories. These problems, particularly the low specificity of whole cell assays in addition to the poor results in terms of the accuracy, precision, and concordance among the various test kits on the market, led the CDC to issue the recommendation that all positive or equivocal whole cell assay results should be followed by an immunoblot assay.

Because the recombinant proteins were designed to limit cross-reactive epitopes, the specificity of the assay is higher. Furthermore, by their very nature, the use of single or combined recombinant proteins markedly improves the uniformity of the test, which can be translated to assay standardization. In addition, an issue that is not addressed in assays using cultured *B. burgdorferi* is the serotypic variability of *B. burgdorferi* in North America. To deal with this serotypic variability of OspC and improve the sensitivity of the assay, proteins containing epitopes from different OspC serotypes were included in our recombinant assay.

Although the 2-tier recommendation markedly improved the accuracy of serologic testing for anti-*B. burgdorferi* antibodies, it made the process more cumbersome, expensive, and time-consuming. It frequently takes more than a week before the results of the first test are available and a second blood sample is sent to the laboratory. This rapid test can yield results in less than half an hour.

The adaptation of these recombinant antigens to create a rapid immunochromatographic assay has produced a test that has significantly superior specificity compared with the most commonly used commercial ELISA. The sensitivity of the rapid assay remained equivalent to the commercial ELISA tested. The ability of the rapid assay to identify individuals with clinically defined Lyme disease was further supported by the accuracy of the test in detecting antibodies to *B. burgdorferi* in the comparative studies done at 3 different Lyme disease reference laboratories and at 3 POLs. In these studies, the differences observed among the detection of positive results among the sites can be due to the variability in the way different laboratories interpret weakly positive results. However, in the first study, the agreement between the serologic tests and the clinical diagnosis indicates that the clinical accuracy of the rapid assay was statistically equivalent to the commercial ELISA. In the second study, the rapid test was performed at 3 different POLs by untrained users and gave the expected results. The observed differences were minimal and not statistically significant, which indicates that this test is suited for use at the physician’s office.

Both capillary and venous blood testing indicated that the rapid test is reproducible and further suggested that the test is appropriate for use at the physician’s office. This new standardized immunochromatographic assay is an excellent alternative to the existing first-tier tests to detect the presence of *B. burgdorferi* antibodies. The use of this rapid assay in the physician’s office streamlines the 2-tier system by allowing the physician to determine if a Western blot is indicated at the time of the initial office visit. This approach will decrease the turnaround time for final results, which could potentially increase the acceptance and use of the 2-tiered system that has been shown to improve the accuracy of serologic testing and most importantly improve patient care.

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The recombinant proteins (RCBPs) and technology described in this article are subjects of patent application 08/235,836.

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