Serodiagnosis of Lyme Disease: Accuracy of a Two-Step Approach Using a Flagella-Based ELISA and Immunoblotting

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The human experimentation guidelines of the US Department of Health and Human Services were followed in the acquisition of serum samples.

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An ELISA containing a purified flagellar antigen from *Borrelia burgdorferi* (FLA-ELISA) was evaluated. The FLA-ELISA, detecting IgM and IgG together, did not have adequate specificity by itself. Good accuracy was obtained, however, when the FLA-ELISA was the first step in a two-step protocol that used immunoblotting as a conditional second test. Samples that scored positive or equivocal by the FLA-ELISA were evaluated with separate IgM and IgG immunoblots. The sensitivity of the two-step process for patients with erythema migrans or with later manifestations of Lyme disease was 64% and 100%, respectively. The specificity for healthy blood donors was 100% and was 90% for the aggregate of all persons with illnesses that may cause serologic cross-reactivity (98% if the samples from relapsing fever patients were excluded). Test precision was 96% overall, 99% for Lyme disease case serum samples, 100% for specimens from blood donors, and 88% for samples from persons with other illnesses.

Lyme disease is caused by *Borrelia burgdorferi*, a spirochete that is transmitted by infected ticks of the *Ixodes ricinus* complex [1]. This disease is found focally in Europe, northern Asia, and North America [2, 3]. More than 71,000 cases were reported in the United States from 1982 to 1994 [4], but the true incidence is probably much higher [5]. Antibiotic therapy is usually curative, particularly early in the illness [6], so it is critical that Lyme disease be correctly diagnosed. Diagnosis of Lyme disease is based fundamentally on the clinical evaluation of patients, where accurate serology can lend support to clinical judgment. In addition, accurate serology is important for determining incidence, prevalence, and geographic spread of the disease and for assessing prevention and control measures.

The 41-kDa flagellin (Fla) antigen of *B. burgdorferi* elicits an early serum immune response [7–9]. Serologic tests based on purified flagella of *B. burgdorferi* (FLA-ELISAs) have been developed by Hansen et al. [10–12] and shown to be both more specific and more sensitive for detecting antibodies to this spirochete in European patients than an ELISA based on a whole cell sonicate (WCS) of *B. burgdorferi* [10–13]. An assay based on an immunodominant, purified protein has the potential to be more readily standardized than one based on a WCS or lysate of the organism. Indeed, many studies have documented serious interlaboratory variability in results when ELISAs based on antigen from whole cells have been used [14–17]. Although flagellin sequences are not invariant, they are fairly well conserved between strains of *B. burgdorferi* [18–25], an important consideration given the diversity of the organisms that cause Lyme disease [26, 27].

The Fla of *B. burgdorferi* has epitopes that are also found in flagellin from numerous other bacteria, particularly at the ends of the molecule [19, 23–25]. Antibodies reactive with *B. burgdorferi* flagellin may, therefore, be nonspecific. In morphologically intact flagella, however, many of the cross-reacting epitopes may not be exposed [10]. Several investigators have mapped the antigenic domains of flagellin to delineate common and specific epitopes [28–30] and have produced truncated recombinant flagellins from the internal portion of the molecule that improve the specificity of serologic tests [31–33] but diminish sensitivity [32]. Internal fragments of Fla have particular promise when combined with other sensitive antigens in recombinant-based assays under development [34].

Since serologic tests based on flagella have been reported to have high accuracy with serum samples from European patients [10–13], we wished to evaluate the performance of a FLA-ELISA with serum from clinically well-characterized patients from the United States and from healthy and diseased controls. We also studied the FLA-ELISA as the first test in a two-step process that used immunoblotting as a conditional second test.

Materials and Methods

**Serum samples.** Lyme disease case serum samples (*n* = 111) were selected for use in this study on the basis of characteristic
clinical manifestations of the patients [2], not on reactivity in any other serologic test. Serum samples were contributed by physicians with extensive experience in the clinical diagnosis of this illness. The clinical manifestations in Lyme disease patients ranged from acute erythema migrans (EM) to late neurologic disease accompanied by arthritis. To increase our confidence that serum samples from persons with clinically diagnosed EM were indeed from Lyme disease cases, we selected samples for which the diagnosis was supported by isolation of *B. burgdorferi* from a skin biopsy specimen when possible (n = 43, 74%). The remaining samples were submitted by physicians who did not culture skin samples (n = 15). Most samples were from persons with localized infection (single EM, n = 50); 4 patients in each group (culture-confirmed and uncultured) had disseminated disease (secondary skin lesions). Samples from EM patients for whom culture results were negative were not included in the panel.

Serum samples from Lyme arthritis patients were from persons with intermittent attacks of asymmetric oligoarticular swelling and pain, affecting primarily the knee. An additional history of EM, meningitis, or facial palsy was documented in 31% (11/36) of arthritis cases, as detailed in table 1. All samples from patients with late neurologic disease, mainly encephalopathy and polyneuropathy, were from persons with a variety of other antecedent manifestations of Lyme disease (itemized in table 1) to support their classification as Lyme disease cases. Although all Lyme disease samples were selected on clinical grounds, in some cases of late disease the patient’s serologic status by another serologic test was known to the submitting physician, so a potential bias toward samples from seropositive persons cannot be excluded. All patients were treated with antibiotics, and most blood samples were collected after therapy was completed.

Pretreatment samples were obtained from 5% of EM patients (n = 3) and 19% of arthritis patients (n = 7). From the 111 case samples, 85 were randomly chosen and repeated in the panel for precision analysis (196 total).

Patients resided in areas in which Lyme disease is endemic: the Northeast (n = 82) and upper Midwest (n = 29) of the United States. Infections were most likely to have been acquired in four states: New York (38%), Wisconsin (23%), Connecticut (12%), and Massachusetts (9%). Case serum was also obtained from patients residing in Rhode Island, Delaware, Maryland, Illinois, and Michigan.

Non-Lyme disease case serum samples (n = 113) for determining test specificity were obtained from unpaid healthy blood donors residing in Cincinnati or Chicago, cities where Lyme disease is not endemic. Samples were randomly chosen and repeated (n = 87) for test precision determinations (total = 200). Potentially cross-reactive samples (n = 111) were obtained from persons with autoimmune disorders, leptospirosis, periodontitis, relapsing fever, syphilis, tularemia, and other illnesses; repeats (n = 85) were randomly selected (total = 196). The specimens designated “other illness” were from persons with Lyme disease–like symptoms who resided in an area where Lyme disease is not endemic (the Rocky Mountain region). This group had the additional selection characteristic of scoring in the positive or equivocal range of an ELISA based on a WCS of *B. burgdorferi* [35].

All test panel serum samples were assigned random numbers and tested blindly (total = 592). For purposes of calculating test sensitivity and specificity, only one result of the duplicated samples was used (the result of the sample with the lower random number of each pair). The results of the sample pairs were used to evaluate test precision.

A second set of serum samples from healthy blood donors (n = 119) was used to establish the positive and negative cutoffs for the FLA-ELISA on each plate. These unpaid donors also were from areas where Lyme disease is not known to occur (Cheyenne, WY, and Cincinnati). The blood bank samples (n = 116) were screened by FLA-ELISA to determine the range of reactivity in this control population. Specimens with optical densities (ODs) ≤1.5 times the interquartile range above the third quartile or ODs ≥1.5 times this range below the first quartile (“outliers”) were eliminated as candidate negative controls. After this exercise, 92% (107/116) of the samples remained in the blood donor control panel. Six serum samples were selected randomly from this panel for use as negative controls on each ELISA plate. The same negative controls were used for all work in this study.

Plate-specific cutoff OD values were necessary to reduce plate-to-plate variability of results. The 6 negative control samples were tested individually, not pooled (mean OD on four plates, 0.120; range, 0.099–0.139). If the 6 negative controls were physically mixed (pooled), the resulting ODs were lower than those obtained with the individual specimens (mean OD on four plates, 0.038; range, 0.022–0.051).

**FLA-ELISA antigen.** Periplasmic flagella were isolated from 2-L cultures of *B. burgdorferi* strain B31 (high-passage) grown in BSKII medium and purified in gradients of CsCl for preliminary experiments, as described by Barbour et al. [36]. Cultures were judged to be free of contaminating microorganisms when no growth was observed after 2 weeks of incubation in four standard bacteriologic media (brain-heart infusion broth and trypticase soy agar, 37°C and ambient temperature; thioglycollate broth, 37°C;
and Sabouraud’s agar, ambient temperature). A larger quantity (20 mg) was prepared subsequently by Kirkegaard & Perry Laboratories (Gaithersburg, MD) by essentially the same procedure, as modified by Hanssen et al. [10]. The yield was 1 mg of protein/L of culture (Bradford method, Bio-Rad protein assay, Richmond, CA). The purity of the flagella was assessed by SDS-PAGE, densitometry, and immunoblotting, using standard methods [37, 38].

The flagellin preparation in immunoblots reacted with anti-Fla monoclonal antibody H9724 [36], with polyclonal hamster anti-Fla antibodies, and with serum from Lyme disease patients. Flagella were dialyzed against TRIS-EDTA buffer, adjusted to a concentration of 1 mg/mL protein, and lyophilized in 0.5-mg portions. Before use in ELISA, macroscopic flagellar aggregates were dispersed in distilled water by mild sonication in an ice bath to facilitate even coating of ELISA plates. Flagellar suspensions were stored at 4°C for up to 2 months.

**FLA-ELISA.** All ELISAs were done by a single investigator (K.R.E.). Polystyrene plates (Immulon 2; Dynatech Laboratories, Chantilly, VA) were coated with flagella in 0.1 M NaCO₃ buffer, pH 9.6, at 4°C overnight. The concentration of the flagella preparation for coating was 125 ng/100 µL/well. The wells were washed five times with TBS-T (0.3 mM TRIS, pH 7.4, 0.14 M NaCl, 2.7 mM KCl, 0.05% vol/vol Tween 20), blocked for 30 min at 37°C with 3% fetal bovine serum in TBS (ELISA block, 300 µL/well), and then washed five times. Test serum samples were diluted 1:500 in ELISA block and added to duplicate wells (100 µL each).

A positive control was added at two duplicated dilutions (1:4000 and 1:8000). Six negative control serum samples were tested at a 1:500 dilution. Duplicate background control wells received ELISA block alone. Plates were incubated at 37°C for 1 h and then washed five times with TBS-T. Bound antibody was detected with goat anti-human IgG plus IgM (heavy and light chain) conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA). The conjugate (diluted 1:10,000 in TBS-T, 100 µL/well) was added and incubated at 37°C for 1.5 h. After the wells were washed five times with TBS-T, substrate was added and incubated at 37°C for 0.5 h (100 µL, 2 mg/mL p-nitrophenyl phosphate [Kirkegaard & Perry] in 0.05 M NaCO₃, pH 9.8, 1 mM MgCl₂). Color development was stopped with 5 N NaOH (100 µL/well).

ODs were read by a microplate reader (EL311; Bio-Tek Instruments, Winooski, VT) at 405 nm (minus the OD at 630 nm). Positive, negative, and equivocal ranges were calculated for each plate. The mean + 1 SD of the 6 negative controls was the negative cutoff; the mean + 3 SD was the positive cutoff. Test serum samples were classified by the mean OD of the duplicates. A sample was retested if duplicates differed by >10% of the mean OD, provided at least one value fell within the equivocal range.

The test serum dilution of 1:500 was chosen after surveying 2-fold serial dilutions (from 1:62.5 to 1:8000) with 4 high-titered Lyme disease case samples and 2 specimens from healthy controls. The 1:500 dilutions gave values in the linear response range of OD with dilution and good positive/negative ratios (data not shown). Variation in the ODs of the 6 plate negative controls used to calculate cutoffs was assessed for 27 plates, assayed on separate days; mean ODs ranged from 0.144 to 0.258.

**Immunoblots.** Serum samples from all patients were tested blindly using separate IgM and IgG commercial immunoblots (MarDx Diagnostics, Carlsbad, CA), according to the manufacturer’s instructions, by a single investigator (B.-L.C.). Serum was blotted at a dilution of 1:100. Immunoblot strips contained antigen from a low passage of *B. burgdorferi* strain B31. The blind-coded immunoblots were scored visually by one reader (B.J.B.J.) and interpreted by the criteria of Engstrom et al. [39] (IgM) and Dressler et al. [40] (IgG), as recommended by the Second National Conference on the Serologic Diagnosis of Lyme Disease [41]. IgM immunoblots were considered positive if two of the following three bands were present: 24 kDa (OspC; denoted 23 kDa on MarDx strips), 39 kDa (BmpA), and 41 kDa ( Fla) [39]. IgG blots were considered positive if 5 of the following 10 bands were present: 18, 21, 39 (OspC; denoted 23 kDa on MarDx strips), 28, 30, 39 (BmpA), 41 (Fla), 45, 58, 66, and 93 kDa [40].

The positive control in each set of immunoblots was calibrated with monoclonal antibodies (MAbs). Antibodies recognizing the diagnostically significant proteins in IgM blots were 4B8F4 (anti-OspC), prepared as per Padula et al. [42]; H1141 (anti-BmpA) [43]; and H9724 (anti-Fla) [36]. MAbs were available to 5 of the 10 diagnostically important proteins scored in IgG blots. In addition to the above three antibodies, MAbs 181.1 (anti−93 kDa) [44] and 8D5 (anti−66 kDa) (provided by A. Barbour, University of Texas Health Science Center, San Antonio) were used. Lacking MAbs to the other 5 proteins scored in IgG blots, we used the following additional calibration markers: MAb 149 (anti-GroEL, 62 kDa on MarDx strips) [45, 84C (anti-OspB) [46], H5332 (anti-OspA) [47], H1C8 (anti-OspD, 29 kDa) [48], and CB625 (anti−22 kDa) [49]. To have greater confidence that we were scoring the 10 IgG bands intended by Dressler et al. [40], we obtained the positive control serum used by those investigators (provided by A. Steere, Tufts, New England Medical Center, Boston). The decision whether a band was of sufficient intensity to be scored was subjective, based on the judgment and experience of the blot reader. A weak positive control for setting intensity cutoffs was not provided with the commercial blot strips that we used.

**Results**

**Purity of ELISA antigen.** Isolated flagella were denatured and electrophoresed, as shown in figure 1. The major component in this preparation was flagellin, which has an apparent molecular mass of 41 kDa. Although faint minor bands were visible in the Coomassie-stained gel, a densitometric scan (fig-
Table 2. Performance of FLA-ELISA alone for serodiagnosis of Lyme disease.

<table>
<thead>
<tr>
<th>Serum source</th>
<th>FLA-ELISA result, no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Lyme disease cases</td>
<td>75 (68)</td>
</tr>
<tr>
<td>Healthy blood donors</td>
<td>5 (4)</td>
</tr>
<tr>
<td>Patients with other illnesses*</td>
<td>20 (18)</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
</tr>
</tbody>
</table>

NOTE: IgM and IgG antibodies were assayed together.
* See table 4 for types of illness.

Table 3. Sensitivity of FLA-ELISA alone for serodiagnosis of Lyme disease when specificity was 96%.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Sensitivity (%)</th>
<th>No. positive/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythema migrans, all</td>
<td>47</td>
<td>27/58</td>
</tr>
<tr>
<td>Culture positive</td>
<td>44</td>
<td>19/43</td>
</tr>
<tr>
<td>Culture not done</td>
<td>53</td>
<td>8/15</td>
</tr>
<tr>
<td>Early neurologic (meningitis/facial palsy)</td>
<td>100</td>
<td>3/3</td>
</tr>
<tr>
<td>Lyme arthritis</td>
<td>89</td>
<td>32/36</td>
</tr>
<tr>
<td>Late neurologic (encephalopathy/polyneuropathy)</td>
<td>93</td>
<td>13/14</td>
</tr>
<tr>
<td>All specimens</td>
<td>68</td>
<td>75/111</td>
</tr>
</tbody>
</table>

NOTE: Specificity was determined with serum samples from healthy blood donors residing in areas in which Lyme disease is not endemic. Equivocal FLA-ELISA results were considered negative for purposes of table, resulting in specificity of 96% (table 2).

Performance of the FLA-ELISA combined with immunoblotting. The FLA-ELISA was examined to see if it could be a sensitive first test in a two-step protocol. Immunoblotting results were used as the second test for specimens that scored as positive or equivocal by ELISA, to determine whether greater test accuracy could be achieved. A positive test was defined as one with a positive or equivocal FLA-ELISA and a positive immunoblot, either IgM or IgG.

IgM and IgG blots (total = 1184) were done and interpreted. We used the calibrating monoclonal antibodies shown in figure 2, as well as the positive control of Dressler et al. [40] (data not shown), to identify the IgM and IgG bands of diagnostic importance. The anti-22 kDa monoclonal antibody developed by Coleman and Benach [49] recognized a protein that migrated only slightly slower in this gel system than the 18 kDa diagnostic antigen (figure 2).

Both sensitivity and specificity were improved when a two-step protocol was adopted. The increase in sensitivity for all cases from 68% (75/111, table 3) to 81% (90/111, table 1) was statistically significant ($\chi^2 = 5.31, P = .02)$. Specificity for serum from healthy donors (table 4) increased from 96% to 100% (Fisher’s exact test, two-tailed, $P = .06$).

The sensitivity of the two-test approach is summarized by stage of Lyme disease in table 1. The overall sensitivity for persons with EM was 64% (37/58). If only those samples that were equivocal by FLA-ELISA were immunoblotted (i.e., ELISA-positive samples were considered positive), the sensitivity would have been 69% (40/58). This small increase in sensitivity is not statistically significant.

A significant difference in seropositivity ($\chi^2 = 4.58, P = .03)$ was found between 2 subgroups of EM patients, those in whom *B. burgdorferi* infection had been confirmed by culture of skin biopsy samples (56% positive) and those from whom biopsies were not obtained (87% positive) (table 1). This difference in serologic reactivity was related to the duration of EM.
The culture-positive group had EM of shorter duration before the initiation of antibiotic therapy (mean = 7 days) than the group that was not cultured (mean = 14 days). Of the patients from whom *B. burgdorferi* was cultured from a skin biopsy specimen, 79% (34/43) had EM of ≤7 days' duration at the time of biopsy and therapy, whereas in the uncultured group this proportion was 53% (8/15). The group with the shorter duration of EM also had a lower incidence of disseminated infection as manifested by secondary EM (9%, 4/43, vs. 27%, 4/15, for the uncultured group).

The sensitivity of the combined protocol was 100% (53/53) for early neurologic and late Lyme disease. The clinical manifestations of this group of patients included meningitis, facial palsy, Lyme arthritis, or encephalopathy/polynuropathy (table 1). All final results were the same whether both those FLA-ELISA positive or equivocal were blotted or only those with equivocal FLA-ELISA results.

The specificity (table 4) was highest when both those FLA-ELISA positive or equivocal were blotted (100%, 0/113 positive). If FLA-ELISA-positive samples were considered positive and not blotted, the specificity dropped to 96% (5/113 positive) for healthy blood donors. Similarly, blotting of FLA-ELISA-positive samples from persons with autoimmune disorders and leptospirosis improved specificity slightly. The greatest value of blotting FLA-ELISA-positive specimens was observed with syphilis patients. The specificity of the two-test protocol improved dramatically from 9% to 91% (1/11 positive) when this was done. The overall specificity of the two-test approach was 90% (11/111 positive) for serum from persons with the spectrum of illnesses shown in table 4. When samples from patients with tickborne relapsing fever caused by other *Borrelia* species were excluded from the test panel, the specificity was 98% (2/97 positive). False-positive results were twice as likely to be due to IgM than IgG immunoblots (data not shown).

When subjected to a two-test protocol that included immunoblotting, all samples from the Rocky Mountain region of the United States, denoted as “other,” were negative (table 4), a result compatible with what is currently known of the epidemiology of Lyme disease.

**Precision of the two-test protocol.** Precision was calculated by comparing the results of the duplicated specimens in each serum category. Because all samples had been assigned random numbers and were tested blindly, duplicates were not tested next to each other. In some cases, immunoblots were developed months apart. The overall precision of the two-test approach for the entire serum panel was 96% (246/257 pairs). For the Lyme disease case group, identical final results were obtained for 84/85 serum pairs (99% precision). For samples from healthy blood donors, precision was 100% (87/87). Discordant results were obtained most frequently with samples from persons with illnesses other than Lyme disease. The overall precision for this group was 88% (75/85). Of the 10 discordant results with samples from persons with other illnesses, 6 occurred with serum pairs from tickborne relapsing fever patients.

Discordant test results (11 pairs) were analyzed to determine the likely source of error in each case. Discrepancies had diverse causes. About half (6/11) of the variable results were due to differences in sensitivity of the immunoblots, apparently caused by lot-to-lot differences in the strips themselves; three tickborne relapsing fever samples did not produce an OspC band when strips from one gel were used but clearly did react with OspC on other strips. Three discrepancies were due to lack of reproducibility in interpretation by the reader of the blots. Very faint bands were not scored as reactive. The demarcation between a band to be scored and one to be ignored was based on the judgment and experience of the reader. Two additional errors were attributed to the laboratorian. As with specificity problems, lack of precision occurred twice as often with IgM as IgG blots.

**Discussion**

Flagella of *B. burgdorferi* have been demonstrated to be a useful antigen for serodiagnosis of Lyme disease, affording improvements in both specificity and sensitivity over tests based on sonicated whole cells [10–13, 50]. We confirm the value of this test antigen with serum samples from US patients. The flagellar antigen, however, is not completely *B. burgdorferi*-specific. When separate indirect FLA-ELISAs for IgM and IgG were developed, using healthy control subjects from Denmark or Sweden, cutoffs giving 95% specificity were established [10, 11] (a μ-capture ELISA was subsequently developed and set for 98% specificity [12]).

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**Table 4. Specificity of two-test approach for serodiagnosis of Lyme disease: FLA-ELISA plus conditional immunoblotting.**

<table>
<thead>
<tr>
<th>Serum source</th>
<th>Only ELISA-equivocal samples blotted</th>
<th>ELISA-positive and -equivocal samples blotted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoimmune disorders, all</td>
<td>94 (3/47)</td>
<td>100 (0/47)</td>
</tr>
<tr>
<td>SLE</td>
<td>95 (1/22)</td>
<td>100 (0/22)</td>
</tr>
<tr>
<td>RA</td>
<td>95 (1/19)</td>
<td>100 (0/19)</td>
</tr>
<tr>
<td>SS</td>
<td>100 (0/5)</td>
<td>100 (0/5)</td>
</tr>
<tr>
<td>FS</td>
<td>0 (1/1)</td>
<td>100 (0/1)</td>
</tr>
<tr>
<td>Leptospirosis</td>
<td>89 (2/19)</td>
<td>95 (1/19)</td>
</tr>
<tr>
<td>Periodontitis</td>
<td>100 (0/5)</td>
<td>100 (0/5)</td>
</tr>
<tr>
<td>Relapsing fever</td>
<td>36 (9/14)</td>
<td>36 (9/14)</td>
</tr>
<tr>
<td>Syphilis</td>
<td>9 (10/11)</td>
<td>91 (1/11)</td>
</tr>
<tr>
<td>Tularaemia</td>
<td>100 (0/6)</td>
<td>100 (0/6)</td>
</tr>
<tr>
<td>Other*</td>
<td>100 (0/9)</td>
<td>100 (0/9)</td>
</tr>
<tr>
<td>Total, all diseases</td>
<td>78 (24/111)</td>
<td>90 (11/111)</td>
</tr>
<tr>
<td>Total, all diseases except relapsing fever</td>
<td>85 (15/97)</td>
<td>98 (2/97)</td>
</tr>
<tr>
<td>Healthy blood donors</td>
<td>96 (5/113)</td>
<td>100 (0/113)</td>
</tr>
</tbody>
</table>

*NOTE.* Data are specificity, % (no. positive/total). SLE = systemic lupus erythematosus; RA = rheumatoid arthritis; SS = Sjögren’s syndrome; FS = Felty’s syndrome.

*Includes various rheumatologic and dermatologic conditions. Patients were from Colorado, Montana, or Wyoming, areas where Lyme disease is not known to be endemic.
The volume of Lyme disease testing in the United States is much greater than in Scandinavia. Although precise figures are not available, market analysts estimated that ~2.8 million tests would be done in 1995 [51]. Patients often are tested when the prior probability of them having Lyme disease is low. Treatment of unsubstantiated Lyme disease with intravenous ceftriaxone has resulted in serious complications [52] and expense. In this environment, a testing approach with very high specificity is essential.

The FLA-ELISA as done here (IgM and IgG tested together) was not suitable as a “stand-alone” test. The purified and standardizable flagellar antigen was valuable, however, for a sensitive and quantitative first test of a two-step protocol. Higher specificity was achieved by immunoblotting of samples that had at least a minimal level of reactivity (ELISA-positive and -equivocal samples). A two-step approach permits use of a simple and relatively low-cost ELISA for initial evaluation of serum, followed by blotting of a fraction of samples submitted.

Although IgM and IgG immunoblots were done on all samples in this study design, in practice there is no need to perform IgM blots in late Lyme disease. All samples from patients with late Lyme disease had positive IgM immunoblots and, as noted earlier, IgM blots had a higher rate of false-positivity. IgM blots are of significant value, however, in early Lyme disease. One-third of the seropositive EM cases (11/37) would have been missed if only IgG blots had been done.

The sensitivity overall for patients with EM was 64%. Serologic reactivity in these patients was directly related to the duration of EM, as has been previously observed [11, 53]. The sensitivity was 100% for all other manifestations of Lyme disease examined. The test specificity for serum samples from healthy blood donors was 100% and 98% for serum from persons with a variety of illnesses, excluding tickborne relapsing fever. The specificity determined for the latter group is, of course, dependent on the constitution of the serum panel. Immunoblotting of ELISA-positive samples from syphilis patients in particular resulted in a marked gain in specificity (from 9% to 91%). The two-test protocol did not accurately distinguish Lyme disease from tickborne relapsing fever. However, this limitation is not a serious practical problem in the United States, since tickborne relapsing fever generally does not occur in the areas of the United States in which Lyme disease is endemic and it can be readily distinguished clinically [3]. The cost-benefits of immunoblotting ELISA-positive samples as well as ELISA-equivocal ones have not been determined, although gains in specificity suggest benefits of this higher level of testing in seroepidemiologic studies and in testing populations for whom autoimmune disorders or syphilis would be included in the differential diagnosis.

Although reproducibility is an essential feature of any serodiagnostic test, precision analysis has not been reported routinely in the Lyme disease literature. The precision of this two-step protocol was 96% for all samples evaluated. Precision was excellent for Lyme disease cases (99%) and healthy blood donors (100%) and good for persons with diseases that could potentially induce cross-reactive antibodies (88%).

Over the course of this study, two different commercial lots of flagella were used. One preparation yielded higher average ODs in ELISA than the other. A study comparing the two lots with 184 samples had 97% concordance of results (data not shown). The flagellar antigen preparation can be standardized and cost ~$0.08 (US) per well coated (125 ng/well, $500/mg) at the time it was produced.

Standardization of immunoblotting remains a practical problem. Significant advances have been made both in defining a positive blot [39–41] and in calibrating blots with monoclonal antibodies [42–49]. Nevertheless, calibration antibodies are not readily available to all of the proteins of diagnostic interest. Quality assurance practices, such as enrollment in a proficiency testing program, will be important tools for raising the general standard of immunoblotting.

Immunoblotting has been reported to be more sensitive than a commercial polyvalent ELISA in persons with EM of short duration (<1 week), although this increased sensitivity was not statistically significant [53]. Since reactivity with OspC and P37 is prominent in this period, it is possible that the FLA-ELISA may fail to detect antibodies in persons in the earliest stage of Lyme disease. To evaluate this possibility, we calculated the sensitivity using only the results of immunoblotting for all patients with EM. Immunoblotting alone detected 72% of EM patients (42/58, data not shown), whereas 64% (37/58, table 1) were detected by the two-step protocol. This difference (5/58) may represent cases missed by using flagellar antigen alone, although this difference was not statistically significant (χ² = 0.99, P = .32). A similar result was found when only patients who had EM for ≤7 days at the time of diagnosis and treatment were included in the calculations. Immunoblotting alone detected 79% (27/34) of these patients, whereas the two-step procedure identified 71% (24/34) (χ² = 0.71, P = .40). Serology remains an imperfect means of detecting Lyme disease in its earliest stage.

A consequence of foregoing the FLA-ELISA as a first step was a decrease in specificity for both healthy blood donors (from 100% to 98.5%) and persons with other diseases (from 92% to 88%). Although neither of these decreases in specificity was statistically significant, performing a quantitative test first (ELISA) to eliminate samples with very low levels of reactivity may reduce the number of blots that are difficult to interpret owing to the presence of only faint bands. False-positive results were about twice as common with IgM immunoblots as IgG blots. Similarly, the specificity of IgM testing of healthy blood donors by Engstrom et al. [39] would have been reduced from 100% to 92% (69/75) if the immunoblot results of all samples were used, not just those for samples that were positive or borderline by ELISA (Fisher’s exact test, two-tailed, P = .03).

The performance characteristics of the FLA-ELISA combined with immunoblotting compared favorably with results obtained in two academic reference centers using ELISAs based on disrupted whole cells of B. burgdorferi and immunoblotting [39, 40]. Although a detailed comparison of our results with those of the academic laboratories is not possible
without testing identical serum panels and analyzing all results by a two-step protocol, overall sensitivity and specificity were roughly comparable. Engstrom et al. [39] detected 72% of persons with physician-diagnosed EM by whole cell sonicate ELISA when serum was collected 1–5 days after treatment. This ELISA also scored 29% of persons with other illnesses as positive. When immunoblot analysis was done, higher specificity was obtained (92%–94% for IgM blots and 93%–96% for IgG blots) and sensitivity for early disease after treatment was ~80%. Dressler et al. [40] detected 100% of arthritis and 84% of encephalopathy/polyneuropathy patients in a retrospective study, using IgG immunoblot criteria that were 100% specific. When prospectively applied, these IgG criteria detected 83% of Lyme disease patients after the first weeks of infection with 95% specificity.

Initial steps toward national standardization of testing were outlined at the Second National Conference on the Serologic Diagnosis of Lyme Disease [41]. This conference adopted the interim recommendation that a two-test protocol for Lyme disease testing be followed. The first test should be a sensitive EIA or immunofluorescence assay and the second should be immunoblotting. New technologies may improve laboratory tests for Lyme disease and replace one or both components of the recommended two-test protocol. Before new tests can be recommended for general adoption, however, their accuracy and precision should be demonstrated to equal or exceed the performance of the recommended two-test procedures. Blind testing of comprehensive serum panels will be necessary to evaluate new serologic methods. The two-test approach described herein, as well as new tests under development, should be validated in a series of patients with suspected Lyme disease seen in clinical practice.

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References


