A Limitation of 2-Stage Serological Testing for Lyme Disease: Enzyme Immunoassay and Immunoblot Assay Are Not Independent Tests

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To improve the accuracy of testing for antibody to *Borrelia burgdorferi*, 2-stage conditional testing has been recommended, in which sera that yield positive or equivocal results in a first-stage test (e.g., an ELISA) are then tested by immunoblot assay. The increased specificity anticipated with sequential testing, however, depends on immunoblot assays and ELISAs being independent tests. To examine whether they are independent, control serum samples were tested with 2 different commercially available IgM ELISAs and with an IgM immunoblot assay kit. The frequency of false-positive IgM immunoblot assays was significantly higher with ELISA-reactive than with ELISA-negative serum samples ($P \leq .001$). In addition, there was a highly significant direct correlation between the number of reactive bands on IgM blotting and the rate of false-positive results by IgM ELISA ($P < .0001$). These observations demonstrate that IgM ELISAs and IgM immunoblot assays for antibodies to *B. burgdorferi* are not independent tests. Therefore, when used in sequential testing for Lyme disease, the immunoblot assay should be considered a test that supplements rather than confirms an ELISA.

Serological tests for detection of antibodies to *Borrelia burgdorferi* (usually to whole-cell sonicates of *B. burgdorferi*), include ELISAs or immunofluorescence assays. Of the 2 test methods, the ELISA is more widely used because it is easier to perform. The reported specificity of ELISA for detection of antibodies to *B. burgdorferi* is 72%–94% [1]. Alternatively, antibodies to *B. burgdorferi* may be detected by a third method, immunoblot assay. Immunoblot assay appears to be at least as sensitive and specific as ELISA but is more difficult to perform and is more expensive, and its interpretation is subjective [1–3].

In 1995, to improve the accuracy of serological testing for Lyme disease, the Centers for Disease Control and Prevention (CDC) and the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD) recommended 2-test (sequential) antibody testing, in which separate IgM and IgG immunoblot assays are done for patients with a positive or equivocal ELISA (or immunofluorescence assay) [4]. To support the diagnosis of Lyme disease in persons ill for ≤1 month, either the IgM immunoblot assay or the IgG immunoblot assay may be positive, whereas for patients with illness of >1 month's duration, the IgG immunoblot assay specifically must be positive.

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Although limited data are available on the specificity of the immunoblot assay in control populations without Lyme disease, no information is available on its specificity among patients whose ELISA results are false-positive. Since immunoblot assay is not recommended for ELISA-negative samples, the specificity of this test on ELISA-positive or -equivocal samples is an important concern. A key question is whether immunoblot assay and ELISA are independent tests. If the tests are independent, the frequency of false-positive immunoblot assays on sera from patients without Lyme disease would be the same for ELISA-positive samples, compared with ELISA-negative samples, and conversely. This study addresses the question of whether IgM ELISA testing and IgM immunoblot assays for serological diagnosis of Lyme disease are independent tests.

Subjects and Methods

Serum samples from subjects without Lyme disease. Serum samples were collected from healthy subjects who lived in areas of the United States where Lyme disease is endemic and who had volunteered for a Lyme disease vaccine trial. Serum samples were obtained before receipt of the experimental vaccine, from 4 February to 27 April 1994, a period during which early Lyme disease does not occur. In addition, none of these subjects had clinical evidence of Lyme disease in the 2-month period preceding the time of serum collection. Serum samples were frozen at −70°C until they were tested (see below). For calculations of specificity, reactive serological tests in this group were regarded as false positives [3].

ELISA. All sera were tested by 2 kits: kit A was the Lyme IgM ELISA Test System (Zeus Scientific, Raritan, NJ), and kit B was the Lyme Disease EIA IgM Test System (MarDx Diagnostics,

Table 1. Specificity of IgM immunoblot assay of sera collected during the winter and early spring months from volunteers without clinical evidence of Lyme disease, in areas of the United States where the disease is endemic.

Serum group, assay result	Specificity of IgM immunoblot assay, %
Consecutive specimens (n = 270)	97
False-positive, kit A (n = 9)[a]	66.7
False-positive, kit B (n = 49)[a]	87.8
Consecutive plus 60 selected specimens[b]	
False-positive, kit A (n = 23)[a]	47.8
False-positive, kit B (n = 84)[a]	73.8

NOTE. Kit A, Lyme IgM ELISA Test System (Zeus Scientific, Raritan, NJ); kit B, Lyme Disease EIA IgM Test System (MarDx Diagnostics, Carlsbad, CA).

[a] Positive or equivocal results.

[b] An additional 60 sera were known to have ≥2 bands on IgM immunoblots.

Carlsbad, CA). Assays were performed according to the manufacturers' instructions.

Immunoblot assay. The IgM immunoblot assay (MarDx Diagnostics) was used to test all serum specimens according to the manufacturer's instructions. In accordance with the criteria proposed by the CDC/ASTPHLD, the immunoblot assay was considered positive if 2 of the following 3 bands were present: 24 kDa (OspC), 39 kDa, and 41 kDa [4]. For the purpose of this analysis, the only bands considered reactive were those with intensity equal to or greater than that of the 41-kDa band of the weakly positive control provided by the test manufacturer.

Calculation of positive predictive value. The positive predictive value for sequential testing with immunoblot assay was calculated by determining the proportion of positive tests that were true positives (the number of true positives/[number of true positives + the number of false positives]) for populations with a varied prevalence of early Lyme disease. The predictive value of positive results was determined both by assuming the independence of tests, as well as by utilizing the observed specificity. The sensitivity of all tests was assumed to be 50%.

Statistics. Categorical variables were compared by use of the Fisher's exact test (2-tailed) and, when indicated, the χ^2 test for trend (2-tailed). CIs were calculated with the assumption of a Gaussian distribution.

Results

We determined the apecificity of the IgM ELISA kits and of the IgM immunoblot assay kit for 270 consecutive serum specimens collected during the winter and early spring months from healthy volunteers living in areas where Lyme disease is endemic. Nine of these sera (3.3%) tested either positive (n = 6) or equivocal (n = 3) with IgM ELISA kit A, yielding a specificity value of 96.7% (95% CI, 94.6%–98.8%). Forty-nine (18.1%) tested either positive (n = 24) or equivocal (n = 25) by IgM ELISA test kit B, yielding a specificity of 81.9% (95% CI, 77.3%–86.5%). Eight (2.9%) tested positive by IgM immunoblot assay on the basis of CDC/ASTPHLD criteria, yielding a specificity of 97% (95% CI, 95%–99%). The specificity of ELISA kit B was significantly less than that of either kit A or the immunoblot assay ($P < .0001$).

Three (33.3%) of the 9 serum samples that yielded false-positive or equivocal results with kit A were positive by immunoblot assay; similarly, of the 49 sera yielding false-positive or equivocal results with kit B, 6 (12.2%) were also positive by immunoblot assay. The frequency of false-positive immunoblot assays was significantly higher among ELISA-reactive than ELISA-negative serum samples with both kit A ($P = .001$) and kit B ($P < .001$). Conversely, of the 8 sera that were false-positive by the immunoblot assays, 3 (37.5%) were reactive with kit A ($P = .001$, compared with negative immunoblot assays) and 6 (75%) were reactive with kit B ($P < .001$, compared with negative immunoblot assays). Collectively, these results suggest that the 2 methods of serological testing are not independent.

To examine this issue with larger numbers of reactive sera, an additional 60 winter-collected sera were selected for study on the basis of the knowledge that these sera had at least 2 bands on the IgM immunoblot assay; 27 (45%) fulfilled CDC/ASTPHLD criteria for positivity. These sera were then tested by both IgM ELISA kits. The results of testing for this group were combined with the results for the 270 consecutively tested specimens (table 1). Twenty-three of the 330 samples were positive or equivocal with kit A, and 84 were positive or equivocal with kit B. The specificity of the immunoblot assay for kit-A–reactive sera was just 48% (1 − [12/23]; 95% CI, 27.4%–68.2%), whereas for kit-B–reactive sera it was 74% (1 − [22/84]; 95% CI, 64.4%–83.2%; $P < .03$). The specificity of the immunoblot assay was significantly lower for both kit-A–reactive and kit-B–reactive sera than for sera that were nonreactive with these ELISAs ($P < .0001$).

The dependence of ELISA and immunoblot assay test results was also evident from the highly significant direct correlation between the number of bands on IgM immunoblotting and the rate of false-positive reactivity with kits A and B (table 2; $P < .0001$).

Discussion

This study demonstrates that IgM ELISA and IgM immunoblot assay for antibodies to *B. burgdorferi* are not indepen-

Table 2. Correlation between the number of bands on IgM immunoblot and the reactivity of IgM ELISA kits A and B on sera of persons without Lyme disease.

No. of bands on IgM immunoblot	No. of serum samples	No. (%) reactive[a]	
		Kit A	Kit B
0	216	0 (0)	21 (9.7)
1	44	5 (11.4)	21 (47.8)
2	43	8 (18.6)	23 (53.5)
≥3	27	9 (33.3)	19 (70.4)

NOTE. Kit A, Lyme IgM ELISA Test System (Zeus Scientific, Raritan, NJ); kit B, Lyme Disease EIA IgM Test System (MarDx Diagnostics, Carlsbad, CA).

[a] Positive or equivocal results. For all values, $P < .0001$ (χ^2 test for trend, 2-tailed).

dent tests. Reactivity of 1 test method is associated with reactivity of the second test method (tables 1 and 2). Since these immunoassays use similar antigens, this finding should not be unexpected. Therefore, when the 2 assays are used in sequential testing for Lyme disease, the immunoblot assay should not be considered a confirmatory test for ELISA but, instead, a supplementary test, in the same way that the immunoblot assays for antibodies to hepatitis C and HIV are regarded as supplementary tests to the ELISA for diagnosis of infections with these viruses [5–7].

Although not an independent test, the IgM immunoblot assay to detect antibodies to *B. burgdorferi* is also not a completely dependent test. If it were, all control sera reactive by an IgM ELISA would be positive by immunoblot assay. As shown in this study, the degree of independence of these test methods is a function of the specific test kits used (table 1). The overall specificity of sequential testing for Lyme disease with IgM ELISA kit A, followed by the IgM immunoblot assay used in this study, can be calculated to be 98.3% (vs. 96.7% for kit A alone, or 99.9% if the immunoblot assay were an independent test), and the specificity with kit B, 95.3% (vs. 81.9% for kit B alone, or 99.5% if the immunoblot assay were an independent test).

Although the reduction in specificity due to the lack of test independence is relatively small (1.6%–4.2%), differences of this magnitude will have a substantial impact on the positive predictive value of sequential serological testing when the pretest probability of Lyme disease is low (table 3) [3]. The calculated specificity values for sequential testing should be interpreted cautiously, however, since the number of false-positive samples in our study was relatively small, and selection bias may have been introduced by including the results of testing 60 serum samples already known to be reactive by immunoblot assay.

The explanation(s) for the IgM serological reactivity in our group of healthy volunteers are not known. Because the volunteers were from an area where Lyme disease is endemic, it is possible that some had had previously treated or spontaneously resolved *B. burgdorferi* infection. IgM bands may be detectable for >1 year after apparently successful treatment of early Lyme disease [8, 9]. However, of the 35 volunteers whose IgM immunoblot assays were positive, only 3 (9%) also had a positive IgG immunoblot assay, on the basis of CDC/ASTPHLD criteria (data not shown) [4]. Furthermore, since the presence of IgM antibodies to *B. burgdorferi* is of diagnostic significance only for patients with early disease within 1 month of onset of symptoms [4], IgM seropositivity in our healthy volunteers during the winter months would be a false-positive result, even if the reactivity were attributable to a prior *B. burgdorferi* infection. Current guidelines for *B. burgdorferi* antibody testing apply to the use of serology only for diagnostic purposes and not for assessing past infection or immune status.

Testing for IgG antibody to *B. burgdorferi* does not have a 1-month time limit for diagnostic utility. Consequently, this study did not attempt to determine the relationship between the results of ELISA and immunoblot assay for detection of IgG antibody. However, analogous to IgM testing, similar or identical antigens of *B. burgdorferi* are used in these IgG antibody assays. This suggests that ELISA and immunoblot assay for IgG antibodies to *B. burgdorferi* are also unlikely to be independent tests.

Table 3. Positive predictive value of sequential testing in early Lyme disease, with IgM ELISA followed by IgM immunoblot assay (IB).

Test: probability before testing, %	Probability after testing, %[a]	Probability if tests were independent, %[a]
Test kit A plus IB		
1	19	72
4.2	50	92
10	72	97
20	85	98
Test kit B plus IB		
1	10	32
8.1	50	80
10	56	84
20	74	92

NOTE. Kit A, Lyme IgM ELISA Test System (Zeus Scientific, Raritan, NJ); kit B, Lyme Disease EIA IgM Test System (MarDx Diagnostics, Carlsbad, CA).
[a] Assumes a sensitivity of 50% for each IgM ELISA and for the immunoblot assay (see Subjects and Methods section).

In conclusion, in areas where Lyme disease is endemic, IgM ELISA and IgM immunoblot assay are not independent tests for detection of antibodies to *B. burgdorferi*. This information should be considered when clinicians estimate the probability of Lyme disease after testing. The recommendation by the American College of Physicians to limit serological testing to patients with at least a 20% pretest probability of Lyme disease is supported by the results of this study [10].

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