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# Comparison of Four Immunoserologic Assays for Detection of Antibodies to *Borrelia burgdorferi* in Patients with Culture-Positive Erythema Migrans

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**In view of the significant sequelae associated with Lyme borreliosis, there is a need for timely and accurate diagnosis of erythema migrans (EM). Although *Borrelia burgdorferi* can be cultured from biopsies of EM lesions, immunodiagnostic testing is more widely available. Four immunoserologic methods were studied by using the sera of 51 patients with EM lesions that were culture positive for *B. burgdorferi*. Nineteen patients had single primary lesions, and thirty-two had multiple secondary lesions. At the time of biopsy, 40 patients, 8 with primary lesions and all patients with secondary lesions, were seropositive by an immunoglobulin M (IgM) indirect fluorescent-antibody (IgM IFA) test (Bion Enterprises). Twenty-three patients were seropositive by a whole-cell fluorescence enzyme immunoassay (EIA) (BioWhittaker, Inc.), twenty-two were positive by immunoblotting (ViroStat, Inc.), and one was positive by a P39 recombinant EIA (P39 EIA) (General Biometrics, Inc.). Sera from various patient control groups were tested: rheumatoid arthritis ($n$ = 19), infectious mononucleosis ($n$ = 20), systemic lupus ($n$ = 22), syphilis ($n$ = 13), streptococcal sequelae ($n$ = 20), and healthy subjects ($n$ = 16). None of these sera reacted with the IgM IFA test or P39 EIA. Fifteen reacted with the fluorescence EIA. We conclude that the IgM IFA test is an effective and reliable assay for the diagnosis of EM, particularly for patients with secondary lesions. Immunoblot, fluorescence EIA, and P39 EIA lack the sensitivity to reliably diagnose EM.**

Early diagnosis and treatment of erythema migrans (EM) are necessary to prevent the cardiac, neurologic, and arthritic sequelae of Lyme borreliosis (48, 50, 52). EM is an expanding, erythematous, cutaneous macule that fades centrally over time. It develops around the bite of an *Ixodes* tick infected with *Borrelia burgdorferi* (2, 7, 10, 16, 35, 47). The lesion usually begins within 3 to 5 days following the bite but may be delayed for 4 to 5 weeks. One or more remote secondary cutaneous lesions may develop as a result of spirochetemia. EM is not always a typical, characteristic bull's-eye rash, and many patients do not recall being bitten by a tick (7, 16, 22, 36, 40). Other manifestations, such as fever, lethargy, and arthralgia, are variable (50). Clinical diagnosis may therefore be difficult.

Biopsy of suspected EM lesions with histopathologic and microbiologic studies of tissue has been recommended for diagnosis. Although the findings have been inconsistent, recent culture results in our laboratory (36, 38) and others (8, 9, 46, 54) indicate that culturing can be diagnostically relevant. However, patients may be reluctant to undergo the biopsy procedure or assume the expense. Reliable, immunodiagnostic testing of these patients would be logical and cost-effective.

Immunoserologic testing has been advocated to confirm EM, but most studies have concluded that the immune response is delayed and that currently available methods lack the sensitivity to be of diagnostic value (5, 18, 26, 33). Decreased sensitivity is often method dependent with high cutoff values established to minimize false positives and the lack of appropriate specific antigens in the test system (13, 15, 24, 32, 37). Unavailability of sera from culture-proven cases of EM further limits definition of the diagnostic utility of immunoserologic testing. Most studies have used the sera of patients who were diagnosed by history and clinical findings. Since culture results reveal that not all cutaneous lesions of EM are characteristic and that lesions highly suggestive of EM may have other etiologies (7, 35, 36), there is a need to use well-defined patient sera when determining the sensitivity and specificity of immunoserologic assays.

The purpose of this study was to assess the diagnostic usefulness of four assays for the detection of antibodies to *B. burgdorferi* in patients with culture-positive EM. Commercially available products were evaluated since they are being used by an increasing number of laboratories. They are representative of the methodologies available for the diagnosis of Lyme borreliosis, although the performance characteristics of individual assays and kits using these methods are known to vary (3, 4, 12, 23, 44).

## MATERIALS AND METHODS

**Study population.** Sera from 51 patients with culture-proven EM were included in the study. Nineteen patients presented with single primary lesions, and thirty-two presented with multiple secondary lesions. The sera were obtained on the same day lesions were biopsied for culture. The skin biopsy procedure and culture methods have been described previously (36, 38).

**Control population.** Sera from various patient control groups were used to determine specificity. They were residents of Wisconsin with diagnoses of rheumatoid arthritis ($n$ = 19), syphilis ($n$ = 13), systemic lupus ($n$ = 22), infectious mononucleosis ($n$ = 20), and group A streptococcal infection ($n$ = 20). Sera from 16 subjects without evidence of any disease were

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also tested. These subjects resided in central Wisconsin, were actively involved in outdoor activities conducive to tick exposure, but gave no pertinent history suggestive of Lyme borreliosis.

**IgM IFA test.** Commercially prepared substrate slides with strain B31 (Bion Enterprises, Chicago, Ill.) and fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin M (IgM) (Kallestad Diagnostics, Chaska, Minn.) diluted 1:100 were used in the IgM indirect fluorescent-antibody (IFA) test. Serum dilutions of 1:32 and 1:64 were tested. Reactivity observed as heterogeneously stained borrelias with an intensity of 3+ to 4+ at a 1:64 dilution of patient serum was interpreted as positive.

**IgG-IgM fluorescence EIA.** The IgG-IgM fluorescence enzyme immunoassay (EIA) was supplied in kit form (3M Diagnostics, Santa Clara, Calif.; currently BioWhittaker, Inc., Walkersville, Md.) and used according to the manufacturer's instructions. Test values are calculated on the basis of the reactivities of reference sera supplied with the kit. Results of <10% of the reference sera were interpreted as negative for antibodies to *B. burgdorferi*, results of 10 to 15% were interpreted as equivocal, and results of >45% were interpreted as positive.

**P39 EIA.** The P39 recombinant EIA (P39 EIA) was supplied in kit form (General Biometrics, Inc., San Diego, Calif.) and used according to the manufacturer's instructions. The sera were absorbed with a blocking solution containing *Escherichia coli* proteins (19, 20). The conjugate was goat anti-human IgG, IgM, and IgA. Results of <0.12 (normalized absorbance) were interpreted as negative for antibodies to the 39-kDa antigen of *B. burgdorferi*, results of 0.12 to 0.21 were interpreted as equivocal, and results of >0.21 were interpreted as positive.

**Western blot (immunoblot) assay.** The Western blot assay components were supplied in kit form (ViroStat, Inc., Portland, Maine) and used according to the manufacturer's instructions. The conjugate was goat anti-human IgG (heavy and light chains). Immunoblots were considered positive when two or more bands corresponding to species-specific antigens (22-, 31-, 34-, 39-, or 83-kDa proteins) were present and were considered equivocal or negative if one or none of these bands were present, respectively. Reactivities to other antigens usually observed in immunoblotting, i.e., 41- and 60-kDa proteins, were considered nonspecific.

## RESULTS

Selected patient and culture information is presented in Table 1. Approximately one-third of the patients had a tick bite that occurred from several days to 4 weeks prior to seeking medical care. Given this limited information, it was difficult to relate the time of tick bite and the occurrence of EM with the detectable immune responses. One patient with a primary lesion and one with multiple secondary lesions had received antimicrobial therapy prior to being biopsied.

Table 2 summarizes the immunoserologic test results. The IgM IFA test was the most sensitive assay, being positive for all 32 patients with secondary EM (sensitivity, 100%) and for 8 of the 19 patients with primary EM (sensitivity, 42%). The sensitivity of the P39 EIA was 2%. The sensitivities of the fluorescence EIA (primary, 26%; secondary, 56%) and immunoblot (primary, 10%; secondary, 63%) were comparable, and both assays gave a number of equivocal reactivities, particularly for patients with secondary EM. The IgM IFA test and P39 EIA were highly specific (100%) in that none of the control sera were reactive. The fluorescence EIA was less specific (86%), with positive reactivities observed with several sera from patients with infectious mononucleosis, rheumatoid arthritis, systemic lupus, and syphilis.

TABLE 1. Selected patient information and culture results of patients with EM lesions that were culture positive for *B. burgdorferi*

| Characteristic | No. of patients |
|---|---|
| Age | |
| 2 to 19 yr | 9 |
| 20 to 39 yr | 15 |
| 40 to 59 yr | 13 |
| 60 to 76 yr | 14 |
| Gender | |
| Male | 32 |
| Female | 19 |
| EM | |
| Primary | 19 |
| Secondary | 32 |
| Culture positivity | |
| ≤3 days | 22 |
| 4 to 6 days | 23 |
| 7 to 10 days | 2 |
| 11 to 16 days | 4 |

By Western immunoblot, 2 of the 19 patients with primary EM had antibody responses to the 41-kDa protein as well as to multiple *B. burgdorferi*-specific antigens, i.e., 22-, 31-, 34-, and 39-kDa proteins. Three additional patients demonstrated equivocal reactivities, with responses to the nonspecific 41-kDa protein and only one specific protein, i.e., the 31- or 39-kDa protein. Sera from 10 patients reacted only with the 41-kDa protein, and no reactivities were observed with sera from 4 patients. Twenty of the thirty-two patients with multiple secondary lesions demonstrated antibodies to the 41-kDa protein and at least two species-specific antigens. Although the patterns observed among these patients were variable, immune responses to the 22-kDa antigen, the 39-kDa antigen, the 31-kDa antigen, and the 34-kDa antigen were observed in 16, 12, 14, and 8 patients, respectively. Variable responses were also seen with sera from the five patients with equivocal immunoblots. Of the seven immunoblot-negative patients, six demonstrated reactivity only to the 41-kDa protein and one demonstrated reactivity to the 41- and 60-kDa proteins.

## DISCUSSION

In this study, we evaluated four commercially available immunoserologic assays for the detection of antibodies to *B. burgdorferi* in patients with early Lyme borreliosis. Whereas previous studies used sera from clinically diagnosed patients, we used sera from patients with EM lesions that were culture positive for *B. burgdorferi*. Since there are cutaneous lesions that can mimic EM, using sera from culture-proven cases provided us with a means to more definitively assess the diagnostic value of these assays.

The IgM IFA assay was the most sensitive of the assays evaluated, and no cross-reactivities were observed with the sera of the control population. Sera from the 11 patients with primary EM that were seronegative did not demonstrate reactivity, even at the lower 1:32 dilution. When these 11 sera were tested by immunoblot, two sera gave no reactivity, seven reacted with the 41-kDa antigen, one reacted with the 31- and 41-kDa antigens, and one reacted with the 39- and 41-kDa antigens.

IFA methodology was used initially to establish the etiologic relationship of *B. burgdorferi* with Lyme arthritis (11, 51) and subsequently as a diagnostic aid (25, 34, 42). Within several

TABLE 2. Reactivities of patient and control sera in selected immunoserologic assays

| Patient group | No. of patients | No. with positive reactivity by[a]: | | | |
|---|---|---|---|---|---|
| | | IgM IFA | P39 EIA | Fluorescence EIA | Western blot[b] |
| Culture positive | | | | | |
| Primary EM | 19 | 8 | 1 | 5 | 2 (3) |
| Secondary EM | 32 | 32 | 0 (1) | 18 (9) | 20 (5) |
| Control | | | | | |
| Infectious mononucleosis | 20 | 0 | 0 | 4 | |
| Rheumatoid arthritis | 19 | 0 | 0 | 3 | |
| Systemic lupus | 22 | 0 | 0 | 1 | |
| Syphilis | 13 | 0 | 0 | 7 | |
| Streptococcal sequelae | 20 | 0 | 0 | 0 | |
| Healthy subjects | 16 | 0 | 0 | 0 | |

[a] Numbers in parentheses are the numbers of equivocal reactivities.
[b] None of the controls were tested by Western blot.

years, a number of Food and Drug Administration-approved IFA kits were marketed and many laboratories began using these reagents for diagnostic testing. It soon became apparent that the test reagents varied from one manufacturer to another, the technical expertise necessary to perform the assays was lacking, and reagents of different lot numbers from the same manufacturer gave variable performance characteristics. One major observation that we made when evaluating kits from several suppliers was the variability in the morphologic and antigenic properties of the borrelias used as a substrate. These technical problems became magnified when indiscriminate testing of patients became widespread. Although these limitations are well-known and have recently been reiterated through proficiency testing programs (3, 4), these test kits remain available.

As performed in our laboratory, the IFA methodology remains an effective and reliable diagnostic test. The substrate prepared by Bion Enterprises is antigenically and morphologically consistent from lot to lot. The reason for the increased sensitivity of the IgM IFA test compared with those of the other assays is unknown. However, the expression of unique epitopes on intact borrelias used as a substrate in the IFA test may be responsible for recognizing acute-stage antibodies to the 41-kDa antigen. These particular epitopes may not be present in the whole-cell sonicates or extracts used in EIA and immunoblot methodologies.

An important technical advantage of immunofluorescence is that the experienced microscopist is able to visualize the reactivity and in turn make interpretive decisions. Immunofluorescence microscopy becomes less subjective with experience. The observation of heterogeneously stained borrelias in the IgM IFA assay is highly characteristic in early cases of Lyme borreliosis. Laboratories need to become familiar with this pattern and intensity of staining and to recognize nonspecific fluorescence that may be misinterpreted as positive reactivity.

The 39-kDa antigen of *B. burgdorferi* has been cloned in *E. coli* and shown to be species specific and highly conserved among North American and European isolates of *B. burgdorferi* (45, 49). These findings indicated that this recombinant protein (P39) might be a useful antigen in immunoserologic assays. Although several studies have determined that an immune response to the 39-kDa antigen is uniformly observed in patients with later-stage Lyme borreliosis, very few patients with clinically diagnosed EM have been found to have antibodies to this antigen (21, 29, 44).

Using a commercially available EIA for the quantitative detection of antibodies to P39, we confirmed these observations in that only one of our patient serum samples was positive and only one was equivocal. The lack of sensitivity of the P39 EIA could not be totally attributed to the absence of an immune response to the 39-kDa antigen. Sera from 15 patients that were negative by the P39 EIA were found to contain antibodies to the 39-kDa antigen by immunoblot. Two of these patients had primary EM, and thirteen had multiple secondary lesions. Since immunoblot reactivity was discernible as a distinct band, it may be necessary to establish a lower cutoff value for the P39 EIA to increase sensitivity. It is also possible that different epitopes of this antigen are being recognized in these assays, thus explaining the variable reactivities.

The fluorescence EIA utilizes a whole-cell sonicate of *B. burgdorferi* as an antigenic extract. As noted in Table 2, sera from 5 of the 19 patients with primary EM and from 18 of the 32 patients with secondary lesions were positive by this assay. An additional nine patients gave equivocal results. The decreased sensitivity of this and other EIAs is in part attributable to the cutoff values that have been established to decrease false positivity. The 41-kDa flagellar antigen is an immunodominant protein, and antibodies to this antigen are a hallmark of the primary immune response. Consequently, manufacturers of diagnostic assays have emphasized the importance of using this antigen in their assays for increasing sensitivity. This antigen is not specific for *B. burgdorferi*, and antibodies directed to this antigen can be found in 40 to 60% of sera from healthy individuals (1, 17, 18, 25, 31, 39). Specificity has, in many instances, been sacrificed for sensitivity, contributing to overdiagnosis and misdiagnosis.

Western immunoblotting has been used for diagnostic purposes and to characterize the immune responses in patients with Lyme borreliosis (1, 14, 17, 54, 55). Although not currently approved by the Food and Drug Administration, commercially prepared materials and reagents are available; most laboratories prepare their own test components. There are currently no accepted criteria for the preparation of reagents used in these test systems or for interpretation of immunoblot test results. Immunoblotting has the advantage of providing more specific and detailed information about immunoreactivities in that it is possible to discern which antigens are reacting with a given patient's serum. Although analyses have identified at least 30 major antigens of *B. burgdorferi*, approximately 18 are detectable when sera from patients with progressive Lyme borreliosis are used. On the basis of current information, the 31- and 34-kDa outer surface proteins, OspA and OspB,

respectively, the 39-kDa polypeptide, the 83-kDa extracellular protein, the 94-kDa protoplasmic protein, and the 22-kDa OspC protoplasmic protein appear to be specific to *B. burgdorferi*. The 41-kDa flagellar protein, the 60-kDa common antigen, and a number of 58- to 74-kDa heat shock proteins are not considered to be species specific.

With the immunoblot assay, antibodies to the 41-kDa antigen were the most frequently observed reactive antibodies in our patients. All patients with secondary lesions ($n = 32$) had antibodies to this antigen, as did 15 of the 19 patients with primary EM. Sera from 16 patients, 10 with primary lesions and 6 with secondary lesions, demonstrated reactivity only to the 41-kDa antigen. Considering the nonspecificity of these antibodies, reactivity to this antigen alone could not be considered diagnostic of current infection. This is substantiated by our observation that 96 of 174 (55%) consecutive serum samples submitted to our clinical laboratory for immunoblot testing demonstrated antibodies only to the 41-kDa antigen (unpublished observations). Sera from 30 patients, 5 with primary lesions and 25 with secondary lesions, demonstrated reactivity to the 41-kDa antigen as well as to at least one species-specific antigen. Eight of these patients demonstrated reactivity to only one of the species-specific antigens, which we considered to be equivocal. Twenty-two of the thirty positive and equivocal patient sera reacted with the 22- and/or 39-kDa antigens, indicating that there is an early immune response to these antigens and that antigenic preparations used in other methods should contain these specific antigens.

Many studies using the immunoblot assay have examined sera with alkaline phosphatase-conjugated goat antibody to human IgM or IgG (1, 6, 17, 27, 28, 30, 31, 41, 43, 54, 55). When we examined a number of our patient sera with conjugated anti-IgM, fewer reactivities were observed than with an anti-IgG serum, decreasing the sensitivity of this particular assay. Therefore, we chose to evaluate the assay by using the anti-IgG serum.

Since our results indicated that the IgM IFA test was the most sensitive of the four methods, we used this assay to test convalescent-phase sera from eight patients who were IgM IFA negative at the time of biopsy. Four patients remained seronegative while four seroconverted. However, three of the four patients remaining seronegative demonstrated typical IgM reactivity at a 1:32 dilution. The patient who remained IgM IFA negative presented with a single primary lesion that was not typical of EM and had received 5 days of antimicrobial therapy. Sera taken at the time of biopsy gave significantly elevated fluorescent EIA and P39 EIA values, and strong reactivities to the 39- and 41-kDa antigens were observed by immunoblot. On questioning, the patient could not provide a prior history suggestive of Lyme borreliosis.

During our study, 12 additional patients who presented with clinical findings suggestive of early Lyme borreliosis and lesions suspected of being EM were biopsied. The cultures of seven were contaminated within several days, and five were negative following a 3-month incubation. Eight of the twelve patients were seropositive by the IgM IFA test at the time of biopsy. The four seronegative patients presented with single primary lesions. This information indicates that culture is not always reliable for providing an etiologic diagnosis and that immunoserologic testing is called for. We found that primary EM is a diagnostic challenge when patients lack other manifestations or symptoms and have nondiagnostic lesions (36). Contrary to our expectations, primary EM is usually a circular or oval area of solid erythema that may resemble a hypersensitivity reaction or bacterial cellulitis. Lesions may be annular with a bull's-eye, especially when lesions are large, but they may also be vesicular with lymphangetic streaks. On the basis of these experiences, both culture and IgM IFA testing can often aid in a definitive diagnosis of localized Lyme borreliosis with only primary EM.

The cultivation of *B. burgdorferi* from skin lesions of EM allowed us to assess the value of immunoserologic assays for diagnosing early Lyme borreliosis. The IgM IFA assay, as performed in our laboratory, is sensitive and specific. Although patients with single, primary lesions may be seronegative by this test, preliminary observations indicate that seroconversion can usually be detected within 1 to 2 weeks. For diagnosis of EM, the fluorescence EIA, P39 EIA, and Western immunoblot lack the sensitivity to be used exclusively. The specificity of EIA using whole-cell preparations, comparable to that used in the fluorescence EIA, is problematic and contributes to false positivity.

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