

Immunodiagnostic Tests for Lyme Disease

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Standardized serologic tests for Lyme disease are needed, as isolation or *in situ* demonstration of the spirochete has proved difficult. At the Centers for Disease Control (CDC), an indirect immunofluorescence assay (IFA) was modified from a previously described IFA, and an enzyme-linked immunosorbent assay (ELISA) was developed with soluble spirochetal antigens. Both tests were evaluated with sera from Lyme disease patients, normal controls, and patients with other diseases. They were highly specific for Lyme disease when sera from patients with syphilis were excluded. Sensitivity varied with disease stage: for patients with erythema chronicum migrans alone, the IFA was 53 percent sensitive and the ELISA was 67 percent sensitive. In contrast, all patients with complicated Lyme disease had at least one serum specimen positive in both tests. Twenty-six percent of the sera from 289 patients with suspected Lyme disease that were submitted to CDC in 1983 had IFA titers ≥ 256 and thus were considered positive. Both tests should be useful diagnostic and epidemiologic aids.

Indirect immunofluorescence assays (IFAs) have been used to show that sera from patients with Lyme disease often have antibodies that bind to spirochetes isolated from *Ixodes dammini* ticks [1,2]. Peak IgM titers occur three to six weeks after onset of erythema chronicum migrans (ECM), with a geometric mean titer (GMT) of 178; peak IgG titers with a GMT of 641 occur months later in sera from patients with arthritis [2]. IgG and occasionally IgM titers tend to persist for at least several years in patients with recurrent attacks of arthritis. The potential usefulness of antibody determinations in diagnosing Lyme disease was noted [1,2].

At the Centers for Disease Control (CDC), we standardized an IFA and evaluated an enzyme-linked immunosorbent assay (ELISA) as serodiagnostic aids for Lyme disease [3]. We showed that, with reasonable precautions, either test could be used with a high degree of sensitivity and specificity for diagnosis of complicated Lyme disease.

TEST METHODOLOGY

The IFA used at CDC (Appendix I) differs in several ways from the one described by Steere et al. [2]. First, we used an acetone-fixed, whole-cell antigen prepared from strain B31 (from Barbour) instead of an antigen fixed by freezing. Titers obtained with the acetone-fixed antigen compared favorably with those against the frozen antigen. Second, we made the initial 1:16 serum dilution in 3 percent normal hen's yolk sac suspension to remove nonspecific background fluorescence that can otherwise interfere with determining fluorescence endpoints. Finally, we used a fluorescein isothiocyanate (FITC)-labeled antihuman immunoglobulin (polyvalent)

conjugate instead of anti-IgM and anti-IgG conjugates. To insure adequate test specificity, a titer ≥ 256 was selected as the cutoff level for a positive test result.

The ELISA was developed with a soluble antigen obtained from strain B31 by sonication and an antihuman alkaline phosphatase-labeled Fab, which binds to all immunoglobulin classes (Appendix II). Because optical density (OD) readings can show between-run variation, a high-titered serum was used as an internal positive control in each run. The ratio of the OD of the test serum to the OD of the control serum was determined. A ratio ≥ 0.41 , which corresponded to ≥ 2 standard deviations above the mean OD ratio of 100 normal control sera, was considered a positive test result. Each serum was tested in duplicate at a 1:500 dilution.

EVALUATION OF THE IFA AND ELISA

A total of 138 sera from 45 patients with Lyme disease were generously provided by Allen C. Steere, M.D., and were tested with the IFA and ELISA. Sera from 39 patients were positive in each test, for an overall sensitivity estimate of 87 percent. All 30 patients with cardiac, neuritic, or arthritic complications had positive Lyme serologic results by both tests; in contrast, of 15 patients with ECM alone, only eight (53 percent) and ten (67 percent) had positive test results by IFA and ELISA, respectively (Table 1). Not all sera drawn from each patient with complications showed positive serologic results. Of the 77 sera drawn from the 30 patients with complicated Lyme disease, 73 (95 percent) had IFA titers ≥ 256 , and 75 (97 percent) had ELISA OD ratios ≥ 0.41 .

To determine whether the timing of the serum specimen influenced the low sensitivity of the tests on sera drawn during the ECM stage, we determined the mean day of illness from onset to serum collection for patients with active ECM: 23 days vs. 19 days for positive and negative IFA results, and 21 days vs. 21 days for positive and negative ELISA results. Therefore, the stage of the disease, instead of the timing of the serum specimen, appeared to be the more important determinant of test sensitivity. Similarly, only 27 percent of the patients with ECM alone showed seroconversion by IFA (at least fourfold increase in titer to ≥ 256), but 60 percent of the patients with complicated cases showed seroconversion. Of added interest was the observation that 86 percent of the sera drawn from seven patients while they were in remission from ECM but before complications developed, had positive IFA titers (vs. 56 percent of the ECM patients in whom complications never developed); 100 percent of the sera from the seven patients in remission (vs. 67 percent who had no subsequent disease) had positive ELISA results.

To estimate test specificity, IFA titers and ELISA OD ratios were determined on sera from 106 patients with similar clinical manifestations, related etiologic agents,

TABLE 1
Reactivity of Sera from Lyme Disease Patients Against Lyme Spirochetal Antigens

Disease Stage	No. of Patients	No. (%) with Positive Test	
		IFA	ELISA
ECM	15	8 (53)	10 (67)
Complications	30	30 (100)	30 (100)

TABLE 2
 Reactivity of Sera from Well Persons or Patients with Other Diseases
 Against Lyme Spirochetal Antigens

Disease Category ^a	No. of Sera	No. with Positive Test	
		IFA	ELISA
Not ill	100	0	4
SLE, RA	32	2	0
RMSF	15	0	0
LD	20	0	0
Lepto	11	0	0
Trep	28	15	9

^a *Abbreviations:* SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; RMSF, Rocky Mountain spotted fever; LD, Legionnaires' disease; Lepto, leptospirosis; Trep, treponemal diseases (syphilis, yaws, and pinta).

or possible cross-reactive antigens (e.g., legionellosis). The only significant cross-reactions were observed with sera from patients with treponemal diseases (Table 2). The IFA was 97 percent specific if these sera were excluded and 84 percent if they were included in the analysis. Similarly, the ELISA was 100 percent specific if the treponemal sera were excluded and 92 percent if they were included. Also shown in Table 2 but not included in the specificity estimates are test results on sera from 100 individuals who were not ill. Further experiments showed that using the Reiter immunosorbent, which is used routinely to lower nonspecific fluorescent treponemal antibody (FTA) titers, also lowered Lyme-specific titers to an unacceptable level. However, syphilis patients could be differentiated from Lyme disease patients with the rapid reagin card test (RPR screening test) or the microhemagglutination (MHA-TP) confirmatory test. Sera from Lyme disease patients gave negative test results in the two tests for syphilis.

EFFECT OF IMMUNOGLOBULIN CLASS, SPIROCHETE STRAIN, AND CUTOFF LEVELS

The major flaw in the serologic tests developed so far for Lyme disease is low test sensitivity for patients with ECM alone. Preliminary experiments designed to improve sensitivity by changing the antigen strain, conjugate, or cutoff level failed. We and others [2] found that spirochetal strains isolated from different sources, including tick and human, gave the same serologic results when they were used as IFA antigens for human serum titrations. Lowering the cutoff level for a positive test result gave minimal improvement in sensitivity, whereas the specificity was lowered by 9 percent for ELISA and 10 percent for IFA. A comparison of IFA titers obtained with class-specific conjugates and the polyvalent conjugate showed that serologic reactivity was primarily due to IgG during complicated Lyme disease, whereas IgM contributed more to the reaction during ECM. This is in agreement with Steere's observations [2]. In our study, titers with the polyvalent conjugate were the same or slightly greater than titers obtained with monovalent conjugates. Therefore, routinely using conjugates specific for IgG or IgM offered no apparent advantage.

FUTURE USE

In 1983, CDC began testing serum specimens submitted through state health laboratories from patients with suspected Lyme disease. By late summer, we had determined IFA titers for 289 patients from 38 states (excluding sera obtained in special surveys). Seventy-four (26 percent) had titers ≥ 256 . As expected, the preponderance of sera were submitted from states known to be endemic for the disease: Massachusetts, New Jersey, Minnesota, and Maryland. Data are being analyzed for the entire 1983 tick season, and the preliminary analyses suggest that Lyme disease may be occurring more widely than has been previously appreciated. All but two (Eastsouth Central and Westsouth Central) of nine U.S. regions submitted at least one positive serum sample. The place of exposure of these patients must still be determined. Because of the difficulty in culturing the spirochete and demonstrating its presence in tissue, serologic testing will be relied on heavily as an aid in diagnosing the disease and also in estimating disease prevalence. Of the two tests developed for this purpose, the ELISA has several advantages over the IFA: greater sensitivity during the ECM stage, less subjective readings, potential for automation, and the possibility of using a purified, specific antigen. Efforts are in progress to further improve the ELISA for routine use.

APPENDIX I

Indirect immunofluorescence assay (IFA) for Lyme disease [3]

1. Grow spirochetes in suitable medium, such as BSK [4] for five to seven days.
2. Harvest cells by centrifugation at 21,000 g, 15°C, for 30 minutes.
3. Wash cells three times in 0.01 M phosphate-buffered saline, pH 7.2 (PBS).
4. Dilute cells in PBS to yield approximately 100 organisms per 40 × microscopic field.
5. Apply antigen suspension to glass slides and allow to air-dry. It is convenient to use acetone-resistant multiwell fluorescent antibody slides. Place 20 μ l of antigen in each well, and remove excess liquid with a Pasteur pipette. After the slide has dried, fix in acetone for 10 minutes and allow to air-dry.
6. For each serum to be tested, prepare twofold dilutions in PBS from an initial 1:16 dilution in 3 percent normal hen's yolk sac suspension (NYS). The NYS decreases nonspecific fluorescence that interferes with reading endpoints.
7. Place a drop (20 μ l) of each serum dilution $\geq 1:64$ on an antigen-coated well. Incubate in a moist chamber for 30 minutes at 37°C.
8. Rinse slides, then soak them in PBS for 10 minutes. Blot dry.
9. Place a drop (20 μ l) of FITC-labeled antihuman immunoglobulin (Ig) (polyvalent conjugate) on each well. Incubate, rinse, and dry as above. Alternatively, use Ig class-specific conjugates, but only if class-specificity has been evaluated.
10. Cover slides with carbonate-buffered mounting fluid, pH 9, and coverslips.
11. Read fluorescence intensity on a fluorescence microscope equipped for fluorescein. At CDC, the titer is expressed as the reciprocal of the highest dilution of serum giving 1+ (barely visible) blue-green fluorescence of at least 50 percent of the spirochetes per microscopic field. We use a Leitz Dialux 20 fluorescence microscope equipped with an HBO-100 mercury in-

cident light source, I cube filter system, 40 × dry objective, and 6.3 × binoculars (final magnification, 315 ×).¹

12. A titer ≥ 256 is considered a positive test result.

APPENDIX II

Enzyme-linked immunosorbent assay (ELISA) for Lyme disease [3]

1. Grow spirochetes in suitable medium, such as BSK [4], for five to seven days. For the ELISA, approximately 350 ml of broth medium is required.
2. Harvest cells by centrifugation at 21,000 g, 15°C, for 30 minutes.
3. Wash cells three times in 0.01 M phosphate-buffered saline, pH 7.2 (PBS).
4. Resuspend washed cells in 15 ml of PBS and mix with an equal volume of 2M NaCl in PBS.
5. Sonicate the suspension in an ice bath for 10 minutes at 60 percent of maximum setting on a Biosonik IV Sonifier (or comparable settings on different models).¹
6. Centrifuge sonicate at 21,000 g, 4°C, for 30 minutes.
7. Wash sediment once with 5 ml of 1 M NaCl. Pool both supernatant fluids and dialyze against several changes of distilled water and then against PBS.
8. Determine protein concentration of antigen, and adjust its concentration to 5 μg of protein per ml of 0.1 M carbonate buffer, pH 9.6.
9. Place 25 μl of antigen in each well of a U-bottom microtiter plate (Dynatech, Cambridge, MA).¹ Incubate overnight at 4°C.
10. Wash plate three times with 0.9 percent NaCl containing 0.05 percent Tween 20 and 0.02 percent NaN_3 .
11. To each well, add 25 μl of a 1:500 dilution of test serum in PBS containing Tween 20 and 0.02 percent NaN_3 (PBS-TA). Incubate at 37°C for one hour.
12. Wash plate three times as above.
13. To each well, add 25 μl of antihuman immunoglobulin alkaline phosphatase conjugate, diluted in PBS-TA. Incubate at 37°C for three hours and wash as before.
14. To each well, add 25 μl of p-nitrophenyl phosphate substrate (2 mg/ml in 0.05 M carbonate buffer, pH 9.8, containing 0.001 M MgCl_2). Incubate at 37°C for one hour.
15. To stop the reaction, add 25 μl of 5 N NaOH to each well. Read OD at 405 nm.
16. At CDC, the ratio of the OD of the test serum to the OD of a high-titered serum from a patient with Lyme disease is determined (OD ratio). An OD ratio ≥ 0.41 (≥ 2 SDs above the mean OD ratio of 100 normal control sera) is considered a positive test result. Results are expressed as the mean of duplicate tests.

¹Use of trade name is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

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