JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1984, p. 181–184
0095-1137/84/080181-04$02.00/0

Vol. 20, No. 2

# Comparison of an Indirect Fluorescent-Antibody Test with an Enzyme-Linked Immunosorbent Assay for Serological Studies of Lyme Disease

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Received 24 February 1984/Accepted 26 April 1984

**An enzyme-linked immunosorbent assay was compared with an indirect fluorescent antibody test for its ability to detect antibodies to the Lyme disease spirochete in sera of naturally infected humans, dogs, and white-footed mice and experimentally infected Swiss mice. Ninety-five percent of the total 123 sera analyzed reacted similarly in both tests. For 36 human sera, the correlation coefficient ($r = 0.47$) for logarithmic transformations of indirect fluorescent antibody and enzyme-linked immunosorbent assay titers was significant at $P < 0.01$. Within each mammalian species, mean titers for indirect fluorescent antibody and enzyme-linked immunosorbent assay antibodies were within three-fold. Comparisons of different naturally infected mammals revealed relatively higher average titration endpoints in both tests for patients with Lyme disease. Human sera also had the widest range of titers. Both methods proved satisfactory for serological confirmation of prior spirochetal infections.**

Lyme disease, a newly described illness in humans, is caused by spirochetes transmitted by the ticks *Ixodes dammini* and *Ixodes pacificus* in the United States (4, 7, 14). This malady typically begins with an expanding skin lesion, erythema chronicum migrans (ECM), at the site of a tick bite and may be followed weeks or months later by arthritic, cardiac, or neurological disorders (13, 14). A related disease occurs in Europe, where the suspected vector is *Ixodes ricinus* (8). To date, the Lyme disease spirochete has been isolated not only from ticks (1, 2, 7, 8, 14) and humans (4, 14) but from a number of hosts, including white-tailed deer (*Odocoileus virginianus*) (6), a meadow vole (*Microtus pennsylvanicus*) (6), a raccoon (*Procyon lotor*) (2), and white-footed mice (*Peromyscus leucopus*) (2, 6).

The indirect fluorescent antibody (IFA) test has been successfully employed to detect antibodies to this organism in sera from patients clinically diagnosed as having Lyme disease (2, 7, 14) and from wild and domestic mammals living in Lyme disease foci (11, 12). The enzyme-linked immunosorbent assay (ELISA) has a number of advantages for serodiagnosis, including sensitivity and ease of automation (5, 19). We report here the development of an ELISA to detect antibodies to this spirochete and compare this test with the IFA for its use in serological studies of Lyme disease.

## MATERIALS AND METHODS

**Sera.** Acute and convalescent-phase serum samples from persons exhibiting clinical signs and symptoms typical of Lyme disease were provided by the Virology Laboratories of the Connecticut Department of Health. Detailed information on ECM, an important clinical marker for Lyme disease, and on other characteristic manifestations was obtained from the attending physicians. Of the 36 serum samples in our positive group, 27 were drawn either within 5 to 21 days after the onset of ECM ($n = 16$) or after the onset of joint pains or arthritis ($n = 11$). The remaining nine sera were obtained 1 to 9 months after the onset of ECM. These serum samples were initially screened by IFA and found to be positive for antibodies to the Lyme disease spirochete. Our group of negative control sera included 12 samples from healthy children ≤15 years old living in an area of western Connecticut where no cases of Lyme disease have been reported and 14 serum samples from adults (living at scattered sites in the state) who had no history of ECM or arthritis. An additional 14 samples from people living in Lyme disease foci and having rashes or arthritis but who were shown to be negative for antibodies by IFA were tested by ELISA. There was no history of syphilis in either the control or positive test groups.

Serum samples from naturally infected dogs and white-footed mice were obtained from southcentral and southeastern Connecticut, where *I. dammini* and cases of Lyme disease in humans are prevalent. Specimens were also collected from western Connecticut, where this tick and the disease are rare. Details on sampling methods have been reported (11).

Reference antisera to spirochetes used as antigens in IFA and ELISA tests were prepared in Swiss mice. Samples consisted of pooled sera of challenged and uninoculated mice (2).

**Spirochete cultures.** Antigens were prepared from spirochetes originally isolated from *I. dammini* and from the blood of a white-footed mouse (2). All spirochetes were maintained in fortified Kelly medium (10, 18). Antigens tested in the present study included strains from *I. dammini* (no. 2356, no. 2898) collected in East Haddam, Conn., and Prudence Island, R.I., respectively, and an isolate from a white-footed mouse (no. 2591) caught in East Haddam. All of these isolates were previously shown to be serologically indistinct from the prototype Lyme disease spirochete (1, 2).

**Immunofluorescence.** Procedures for IFA tests with fluorescein isothiocyanate-labeled goat anti-human total immunoglobulins and specific immunoglobulin M (IgM) with spi-

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TABLE 1. Comparison of IFA and ELISA results for mammalian sera with immunoglobulin or IgG conjugates

| Test group | Conjugate | Total no. of serum samples assayed | No. | | % Agreement | + IFA/− ELISA[b] | + ELISA/ − IFA[c] |
|---|---|---|---|---|---|---|---|
| | | | Positive[a] in both tests | Negative in both tests | | | |
| Humans | Immunoglobulin | 76 | 34 | 40 | 97 | 2 | 0 |
| Swiss mice | IgG | 9 | 5 | 4 | 100 | 0 | 0 |
| White-footed mice | IgG | 18 | 7 | 9 | 89 | 2 | 0 |
| Dogs | Immunoglobulin | 20 | 5 | 13 | 90 | 1 | 1 |

[a] IFA positive was ≥1:64 and ELISA positive was ≥1:160 for humans, Swiss mice, and dogs. White-footed mouse positive values were ≥1:32 (IFA) and ≥1:80 (ELISA).
[b] Positive by IFA/negative by ELISA.
[c] Positive by ELISA/negative by IFA.

rochetes have been described (2, 11). Conjugated rabbit antihuman IgG (Accurate Chemical and Scientific Corp., Westbury, N.Y.) was included in the present study for comparison. Working antigens were prepared by centrifuging 8 ml of 2-week-old cultures at 35,000 × *g* for 45 min and resuspending the pellet with equal parts (1:1) of 5% yolk sac diluted in phosphate-buffered saline (PBS) containing 0.01% sodium azide. Extensive tests of control sera in our laboratory led us to adopt titers ≥1:64 as positive for human, Swiss mouse, and dog sera and reactions ≥1:32 as significant for white-footed mice.

**ELISA.** Test procedures were essentially those of Voller et al. (19). Two-week-old cultures, each containing 8 ml of media with actively growing spirochetes, were centrifuged at 35,000 × *g* for 45 min. Pellets were washed twice in 8 ml of PBS (pH 7.2) before being resuspended in 3 ml of PBS containing 0.01% aqueous thimerosal, 100 U of potassium penicillin G, and 100 μg of streptomycin to inactivate the spirochete. Preparations were stored at 4°C and remained stable as ELISA antigens for at least 3 months. Duplicate culture tubes of uninfected medium were processed similarly and used as negative (control) antigens. Protein assays (Bio-Rad Laboratories, Richmond, Calif.) were conducted to estimate antigen concentrations, and different lots of antigens were equated for protein content before coating on microtiter plates. Optimum concentrations for all reagents were determined in a series of preliminary studies by checkerboard titrations.

Positive and negative (control) antigens were added in alternate rows (50 μl per well) to 96-well, flat-bottomed, polystyrene plates (Dynatech Laboratories, Alexandria, Va). After incubation for 18 to 20 h at 37°C (at which time the wells were dry), 200 μl of 0.5% donor horse serum in PBS was added to each well to block binding sites not covered with antigen. Plates were incubated for 1 h at 37°C and washed three times with PBS–0.05% Tween 20.

Test sera were diluted in twofold steps starting at 1:80 in a dilution buffer of PBS–0.05% Tween 20 containing 5.0% horse serum and 50 μg of dextran sulfate per ml (analytical grade; ICN Pharmaceuticals, Cleveland, Ohio). Sera were added in 60-μl volumes to each well, and after 1 h of incubation at 37°C, the plates were washed four times with PBS–0.05% Tween 20. Subsequently, in all tests except those for the white-footed mice, 60 μl of horseradish peroxidase-conjugated anti-species antisera was added to each well. We used goat anti-human immunoglobulin (Tago, Inc., Burlingame, Calif.) diluted to 1:1,000 in dilution buffer, goat anti-dog immunoglobulin (Cappel Laboratories, West Chester, Pa.) at 1:800, or goat anti-mouse IgG (Tago) at 1:800 for ELISA studies of different species. When testing sera from white-footed mice, unconjugated rabbit anti-*P. leucopus* immunoglobulins, prepared at the Centers for Disease Control (9), were diluted to 1:200 and added in 60-μl volumes to each well. This necessitated an additional step of adding 60 μl of horseradish peroxidase-conjugated goat anti-rabbit IgG (Tago) at 1:800 to each well. In all cases, the incubation period for each step was 1 h at 37°C, followed by four washes with PBS–0.05% Tween 20.

Sixty microliters of commercially prepared 2,2′-azino-di-(3-ethyl-benzthiazoline sulfonate) substrate (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) were delivered to each well. Plates were then incubated for 30 min before determining absorbance values.

Absorbance values (optical densities) of all preparations were recorded at 414 nm by using a microplate spectrophotometer (Multiskan; Flow Laboratories, Rockville, Md.). For each serum dilution, a net absorbance value (representing the difference in optical densities between positive and negative antigens) was calculated. Each plate contained a positve serum dilution and a series of known negative control serum dilutions. The average net absorbance values for the known negative serum dilutions were analyzed statistically to determine significant titers for positive reactions. A serum dilution was considered positive if it yielded a net absorbance greater than the total derived by adding three standard deviations to the mean ($[SD \times 3] + \bar{x}$) of the absorbance for the group of negative serum dilutions. After preliminary tests of nonspecific background reactivity, we

TABLE 2. Mean and range differences in reciprocal antibody titers in IFA and ELISA tests

| Test group | IFA antibody titers | | | ELISA antibody titers | | |
|---|---|---|---|---|---|---|
| | *n*[a] | Mean | Range | *n* | Mean | Range |
| Humans | 36 | 1,246 | 64–8,192 | 34 | 3,238 | 160–10,240 |
| Swiss mice | 5 | 10,650 | 4,096–16,384 | 5 | 6,656 | 2,560–10,240 |
| White-footed mice | 9 | 92 | 32–256 | 7 | 171 | 80–320 |
| Dogs | 5 | 666 | 256–2,048 | 5 | 1,792 | 640–2,560 |

[a] *n*, Number of positive serum samples tested.

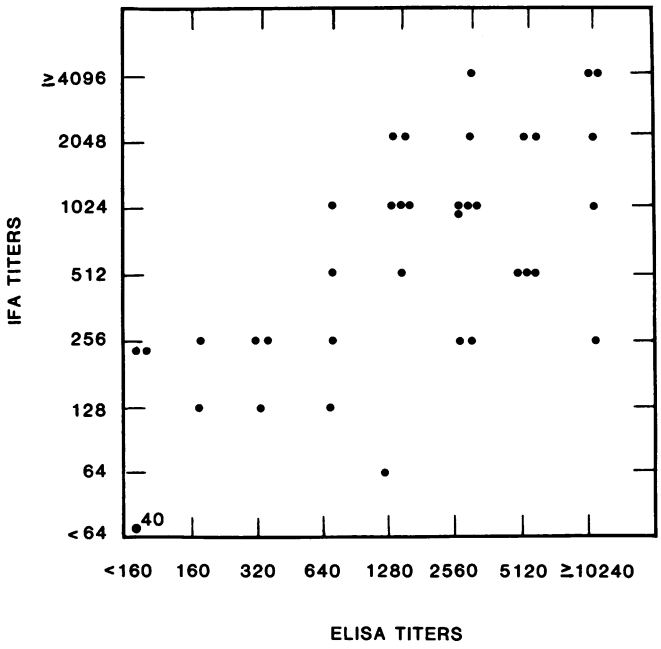


FIG. 1. Relationship between IFA and ELISA antibody (immunoglobulin) titers for human sera.

considered ≥1:160 as positive for human, Swiss mouse, and dog sera, whereas titers ≥1:80 were significant for *P. leucopus* samples.

## RESULTS

**Comparison of IFA and ELISA.** Reactions of positive and negative serum samples were similar in both IFA and ELISA tests for all mammalian species (Table 1). Ninety-five percent (117 and 123) of the serum samples were either positive or negative by both procedures. Five of the six samples having inconsistent results were positive by IFA and negative by ELISA. IFA titration endpoints for these serum samples (i.e., IFA positive and ELISA negative) ranged from 1:64 to 1:256; at least one serum sample from each group of animal sera had a titer of 1:256. Only one serum sample, from a dog, was positive by ELISA (1:640) and negative by IFA. For each species, the mean values for IFA and ELISA antibody titers were within threefold (Table 2). The human sera had the widest range of titers in both tests.

Sera of humans and Swiss mice were analyzed to determine test-to-test variation and to assess reproducibility. Of the 31 human serum samples retested by IFA, differences in titers were less than or equal to twofold for 26 samples and less than or equal to fourfold for 30 samples. An eightfold drop in titer was recorded for one specimen. Despite changes in endpoints, all serum samples remained positive in duplicate tests. Similar IFA tests of Swiss mouse antisera also revealed a two- to fourfold variation in reactions (titers were 1:4,096 to 1:16,384). ELISA antibody titers could be reproduced within fourfold for a group of six serum samples, four from humans and two from Swiss mice.

The relationship between IFA and ELISA titration endpoints for 76 serum samples from humans is shown in Fig. 1. For the 36 IFA-positive serum samples, the correlation coefficient ($r$ = 0.47) of logarithmic transformations of ELISA and IFA values was statistically significant at $P < 0.01$.

**Temporal development of antibodies.** In a preliminary study of 25 human serum samples, we used IFA to detect the presence of total immunoglobulin, IgM, and IgG antibodies and found that IgM appeared within 3 weeks after onset of ECM (Table 3). Only 4 of 16 samples drawn 1 to 3 weeks after ECM had detectable amounts of IgG antibodies. Of the 25 patients with ECM, 16 also developed arthralgias ≤10 weeks after the appearance of these skin lesions. Although in each case the precise time of tick bites was unknown, the utilization of polyvalent conjugates, which react with both IgM and IgG, in IFA and ELISA revealed antibodies in patients seen by physicians during early and late periods of illness.

Serum samples from an additional 11 patients were obtained during the same time intervals after attacks of arthritis (no records of ECM). Serological tests revealed IgM antibodies in seven samples (1:128 to 1:512) and IgG antibodies (1:128 to 1:16,384) in all samples.

## DISCUSSION

The results show that both IFA and ELISA methods were satisfactory for serological confirmation of Lyme disease in humans, for the assessment of immune status in naturally infected dogs and white-footed mice, and for antibody detection in Swiss mice inoculated with spirochetes. With few exceptions, both methods compared favorably in the separation of positive and negative sera. The disparity in the results for six samples and differences in titration endpoints could be due to variability associated with methods of antigen preparation, quality of serum samples and reagents, and the subjective grading of immunofluorescence tests.

Previous studies of spirochetes isolated from *I. dammini* and mammals, including the original Shelter Island, N.Y., strain, showed that all antigens reacted similarly in IFA tests (2, 14). Using murine monoclonal antibodies, Barbour et al. (3) reported that spirochetes isolated from humans, ixodid ticks in North America and Europe, white-footed mice, and a raccoon share a common but specific surface antigen, designated as the H5332 epitope. Thus, similarities in immunological and morphological properties (1–4, 7, 14) suggest that spirochetes recovered thus far from ticks and mammals belong to the same (currently unnamed) species. The taxonomic status of these organisms is under review.

TABLE 3. IFA and ELISA antibody titers in relation to onset of ECM[a]

| Time of sera collection (weeks after onset of ECM) | Total no. of serum samples tested | No. positive (reciprocal titer ranges) by: | | | |
|---|---|---|---|---|---|
| | | IFA | | | ELISA (immunoglobulin) |
| | | IgM | IgG | Immunoglobulin | |
| 1 to 3 | 16 | 15 (128–2,048) | 4 (512–1,024) | 16 (64–2,048) | 16 (160–10,240) |
| 4 to 6 | 3 | 0 | 3 (512–2,048) | 3 (128) | 3 (320–2,560) |
| 7 to 9 | 1 | 0 | 1 (4,096) | 1 (256) | 0 |
| ≥10 | 5 | 0 | 3 (512–1,024) | 5 (128–4,096) | 4 (640–10,240) |

[a] Four patients with ECM received penicillin or tetracycline 2 to 7 days before serum collections. All had IFA titers of 1:256 to 1:512.

A wide range of antibody titers was recorded for human sera in both tests. Steere et al. (14) analyzed paired serum samples from persons with Lyme disease and likewise reported various titers by IFA. We attribute this to differences in immune response, dissimilarities in times when blood samples were obtained, effects of antibiotics, or all three. Therapy with penicillin or tetracycline early in the illness not only shortens the duration of ECM and reduces the chances of subsequent arthritis but may also abort or curtail the antibody response (14, 17). Only four of the patients in the current study received antibiotics before serum samples were obtained, and we were unable to determine if antibody development was affected. Nonetheless, with adequate clinical data, low-titered immunoglobulin, IgM, or IgG reactions may still provide important evidence of prior spirochetal infections.

Detectable amounts of IgM and in some cases IgG antibodies were present in sera obtained within 3 weeks after onset of ECM. A previous report (14) indicates that titers of IgG antibodies rise later than IgM titers. The presence of IgG immunoglobulins shortly after ECM might be due to an early production of these antibodies.

Information on ECM, a unique clinical marker for Lyme disease (13, 15, 16), is of paramount importance for the diagnosis of human cases. Serological confirmation can be made by either IFA or ELISA, but the specificities of these tests are unknown. For example, Steere et al. (14) detected elevated titers of IgM antibodies in sera of 3 of 20 persons with infectious mononucleosis. In particular, studies are needed to determine cross-reactivity among the Lyme disease spirochete and *Borrelia*, *Treponema*, and *Leptospira* antigens.

Our investigation of patients who did not have or report skin lesions but who sought medical attention for arthritic disorders revealed IgM or IgG antibodies, or both, to *I. dammini* spirochetes. Even without records of ECM, serum samples from persons who live in endemic areas and show arthritic or neurological sequelae consistent with Lyme disease should be serologically evaluated.

With comparable sensitivities, IFA and ELISA using polyvalent conjugates or class-specific reagents to detect IgM and IgG antibodies are both suitable for serological diagnoses of Lyme disease. Spectrophotometric determinations of absorbance values and statistical analyses of ELISA yielded quantitative results, an improvement over the subjective grading of fluorescence. With automation, ELISA procedures should enable investigators to screen many more samples in surveillance programs.

## ACKNOWLEDGMENTS

We thank Deborah Shea, Carol Lemmon, Rose Ann LaBarre, James Brassard, and Deborah Winograd for their invaluable technical assistance. Mary Ann Markowski of the Virology Laboratories, Connecticut Department of Health, provided the human sera and Leo L. Lieberman, Waterford, Conn., and George D. Whitney, Orange, Conn., obtained the dog sera. We are especially grateful to the many physicians who provided clinical information and James G. Olson of the Yale Arbovirus Research Unit for performing the correlation analyses.

This work also benefited from Department of Defense contracts NOOO14-78-C-0104 and DAMD-17-81-C-1156 and from Public Health Service Grant NIH-AI-10984 from the National Institutes of Health awarded to the Yale Arbovirus Research Unit, Yale School of Medicine.

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