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Comparative Evaluation of Three Products for the Detection of *Borrelia burgdorferi* Antibody in Human Serum

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Eighty human serum specimens tested concomitantly by immunoblot and an enzyme-linked immunosorbent assay developed jointly at the University of Connecticut School of Medicine and the Connecticut Agricultural Experiment Station were used to evaluate three commercially available diagnostic products for Lyme borreliosis. The sources of the kits were Hillcrest Biologicals, Cypress, Calif.; Whittaker Bioproducts, Walkersville, Md.; and Cambridge Bioscience, Worcester, Mass. When compared with Western blot analysis, the sensitivities and specificities, respectively, for the diagnostic assays were as follows: Hillcrest Biologicals, 93 and 75%; Whittaker Bioproducts, 73 and 100%; Cambridge Bioscience, 89 and 100%; and University of Connecticut School of Medicine, 96 and 92%.

Since the discovery of *Borrelia burgdorferi* as the causative agent of Lyme disease (2, 12), increasing numbers of human cases have been reported from both Europe and the United States. Because culture and direct visualization of spirochetes are often negative in Lyme disease (1, 11, 12), antibody detection with either immunofluorescence assay or enzyme-linked immunosorbent assay (ELISA) has been the only laboratory method useful for diagnosis (3, 9, 12).

Lyme disease is characterized in its early stages by symptoms of fever, headache, generalized muscle pain, and possibly fatigue and weight loss (13). Approximately 60% of patients will exhibit erythema chronicum migrans, the classic skin lesion which is usually found at the site of the tick bite. Early diagnosis and antibiotic treatment are important for preventing neurologic, cardiac, or joint abnormalities that can occur late in the disease (10). Problems exist in the early diagnosis of Lyme disease, since specific immunoglobulin M (IgM) antibody response to *B. burgdorferi* does not peak until 3 to 6 weeks after onset of the disease and peak IgG antibodies are not observed until after months or years of illness (12).

Presently, there are a limited number of commercially available assays for the diagnosis of Lyme disease. This study determined the sensitivities and specificities of the Hillcrest Biologicals (HB), Cypress, Calif.; Whittaker Bioproducts (WB), Walkersville, Md.; and Cambridge Bioscience (CB), Worcester, Mass., assays compared with Western blot (immunoblot) analysis for the detection of Lyme disease antibody in 80 selected patient serum specimens.

MATERIALS AND METHODS

Patients. Seventy serum specimens from patients clinically suspected of having Lyme disease were tested by Western blot analysis for the presence of either IgM or IgG antibody against *B. burgdorferi*. In addition, the 70 specimens were also tested for rheumatoid factor and nonspecific syphilis antibodies. Both the Venereal Disease Research Laboratory (VDRL) and rheumatoid-factor assays were negative for all 70 specimens. In addition to these 70 specimens, 5 specimens positive for rheumatoid factor and 5 specimens positive by VDRL and the fluorescent treponemal antibody-absorption test (FTA-ABS) were included for analysis. Approximately 90% of the 70 serum specimens were from patients living in an area endemic for Lyme disease. Clinical information was available for 48 of the patients: 10 had erythema chronicum migrans, 1 experienced fever and chills 2 weeks after a tick bite, 3 had neurologic and arthritic complications, 1 had cardiac and arthritic complications, and 33 had complaints of arthritis of unknown origin with no history of a tick bite. The serum specimens were divided into aliquots and frozen at −20°C until tested.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot. Western blot analysis was performed to detect either IgM or IgG antibodies directed against *B. burgdorferi*. *B. burgdorferi* 2591 (supplied by Louis Magnarelli) was grown in modified Kelly medium until a concentration of 10^8 organisms per ml was attained (12). The spirochetes were centrifuged at 10,000 × *g* for 10 min and were washed three times in phosphate-buffered saline (PBS). After the last wash, the spirochetes were resuspended in 10 ml of PBS and sonicated on ice by five, 30-s bursts at 60% intensity (Virsonic cell disrupter; Virtis Co., Gardiner, N.Y.). A 20-μg portion of the spirochetal protein in sample buffer containing 2% sodium dodecyl sulfate and 5% 2-B mercaptoethanol was electrophoresed on a 10% polyacrylamide-sodium dodecyl sulfate gel (30.0:0.08 acrylamide to bis-acrylamide) by the method of Laemmli (7). Prestained molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.; lysozyme, 17,000; soybean trypsin inhibitor, 27,000; carbonic anhydrase, 39,000; ovalbumin, 50,000; bovine serum albumin, 75,000; and phosphorylase *b*, 130,000) and spirochete proteins were boiled for 5 min and electrophoresed in a mini-gel system (Bio-Rad) at 22°C for 1.5 h at a constant current of 30 mA. Proteins were electrophoretically transferred from the gel to nitrocellulose by using a current of 200 mA at 4°C for 1.5 h. Blots were blocked 1 h prior to use by incubation with PBS containing 5% powdered whole milk and then were washed three times with PBS–Tween 20 (0.05%) (Sigma Chemical Co., St. Louis, Mo.) for 10 min at room temperature.

Serum was tested at a 1:100 dilution in PBS containing

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0.05% Tween 20 and 5% powdered whole milk by overnight incubation with the nitrocellulose strips at 4°C with gentle agitation. After three washings of 10 min each in PBS–0.05% Tween 20 (Sigma), the nitrocellulose strips were incubated for 1 h at 22°C with a 1:500 dilution of peroxidase conjugated anti-human goat antibody specific for either IgM or IgG (Sigma). The strips were then washed once in PBS–0.05% Tween 20 and three times in PBS for 10 min in each wash. Strips were then incubated with diaminobenzidine (DAB; Sigma) at 5 mg/10 ml in PBS for 1 h at 22°C to visualize the bands. Bands used for diagnosis included the 17,000-molecular-weight protein (17K) and 25K, 31K, 34K, 41K, 55K, 58K, and 66K proteins of *B. burgdorferi*. Patient serum was considered positive if it reacted with two or more bands in the IgM assay, four or more bands in the IgG assay, or at least one band in both the IgM and IgG assay (5).

ELISA. The HB and CB diagnostic kits were based on ELISA methods. Both systems used antigen-coated microdilution plates and were essentially identical in procedure. Assays were performed according to the directions of the manufacturer. Briefly, 100 μl of a 1:100 dilution of the patients' serum was added to previously coated wells, and they were incubated for 1 h at 22°C. The plates were washed five times in wash buffer. A total of 100 μl of peroxidase conjugated anti-human goat antibody specific for both IgG and IgM was added to each well, and the plates were incubated for 30 min at 22°C. After incubation, the plates were washed five times in wash buffer. Substrate was added, and the plates were incubated for 10 min at 22°C. Stop solution (1 N H_2SO_4, CB; 1 N HCl, HB) was added, and the optical density was read at 492 nm for the HB kit and 450 nm for the CB kit. The HB kit supplied a high- and low-titered positive control and a negative control. Interpretation with the HB kit was based on the optical density ratio of the patient specimen to the low-titered positive control. A Lyme index ratio of greater than or equal to 1.0 was considered positive. The CB kit supplied only a positive and negative control. Serum was considered positive if it had an optical density reading of 0.20 or greater. (R.D.F., R.W.R., and R.C.T. participated in the development of the CB assay for Lyme disease.)

UC ELISA. The University of Connecticut School of Medicine (UC) ELISA was similar to that published by Magnarelli et al. (8). A 50-μl portion of a suspension containing 2×10^6 organisms per ml in PBS was added to alternate wells of a round-bottom microdilution plate (Nunc-ImmunoPlate; Marsh Biomedical Products, Rochester, N.Y.). A 50-μl portion of PBS was added to the remaining wells as a nonspecific control. Plates with antigen were then incubated overnight at 37°C and stored at −20°C until used. The antibody assay was performed as follows. The plates were blocked for 1 h at 37°C with 200 μl of PBS containing 0.05% horse serum and 0.01% dextran sulfate (Sigma). The plates were washed five times with PBS–0.05% Tween 20. Patient sera were serially diluted twofold and tested at dilutions of 1:160 through 1:5,120. Positive and negative control sera were included in each assay. The positive control was diluted in a manner similar to that described for the patient sera, and the negative control was tested at 1:160. A 60-μl portion of each serum dilution was added both to an antigen well and to a nonspecific well. After serum addition, plates were incubated for 1 h at 37°C and then washed five times in PBS–0.05% Tween 20. A 60-μl portion of a 1:500 dilution of peroxidase conjugated antihuman goat antisera specific for either IgM or IgG was then added, and they were incubated for 1 h at 37°C. The plates were washed five times in PBS–0.05% Tween 20. A 60-μl portion of substrate chromagen (equal volumes of 2,2′ anzino-di-[3-ethylbenzthiazolinesulfonate] and hydrogen peroxide; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) was added to each well. The plates were checked spectrophotometrically at 414 nm until the optical density reading of the 1:160 dilution of the positive control minus the nonspecific background was equal to 1.0 for IgG or 0.5 for IgM, and they were then read immediately. A serum dilution was considered positive if the net absorbance (antigen minus nonspecific well) was 3 standard deviations or more above the mean absorbance of the negative control wells.

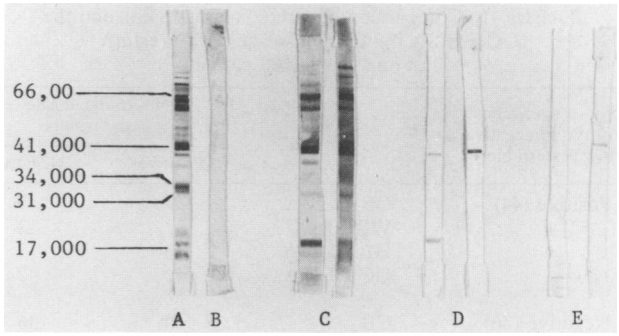

FIG. 1. Western blot analysis. (A) Positive IgG control with molecular weights of some characteristic *B. burgdorferi* antigens used in the criteria for determining seropositivity or seronegativity. (B) Negative control. (C) Patient 1, Strongly positive serum (IgM on left and IgG on right). (D) Patient 2, Weakly positive serum (IgM on left and IgG on right). (E) Patient 3, FTA-ABS-positive serum negative for Lyme disease antibodies (IgM on left and IgG on right).

FIAX-WB. FIAX is a solid-phase fluorescence immunoassay in which the sampler has two sides of nitrocellulose, one coated with purified *B. burgdorferi* antigen and the other coated with culture medium to measure specific and nonspecific binding of antibody. The assay was performed according to the directions of the manufacturer. The sampler was incubated with a 1:100 dilution of the patient's serum, washed, and then exposed to fluorescein isothiocyanate-labeled goat antibody to human IgG and IgM. After the sampler was washed, the intensity was measured on both the specific and nonspecific sides by using a FIAX fluorometer 100 and the results were analyzed by a FIAX 850 computer (Hewlett-Packard Co., Palo Alto, Calif.). The difference in fluorescence units (ΔFSU) between specific and nonspecific sampler sides was calculated. Duplicate samples of negative and high-positive control serum were tested to generate a two-point calibration curve which related ΔFSU to titer. Duplicate samples of a low-positive serum were tested to determine that their measured ΔFSU fell within acceptable specified limits when using the calibration curve. Each patient specimen was run twice, once in each of two separate assays. The ΔFSU of each patient specimen was translated into a FIAX titer and was interpreted as negative if the titer was below 50, as positive if the titer was above 75, and as equivocal if the titer was between 50 and 75.

Statistical tests. Sensitivity and specificity were determined by the method of Galen and Gambino (4).

RESULTS

Western blot analysis (Fig. 1) was used as the reference method to which other assays were compared. Of the 80 specimens tested, 44 were positive and 36 were negative. Of

TABLE 1. Comparison of three diagnostic kits and the UC ELISA for Lyme disease with Western blot analysis for 80 patients

No. of specimens positive or negative by Western blot	Diagnostic assay used	No. of diagnostic assay results	
		Positive	Negative
Positive (44)	CB ELISA	39	5
	WB FIAX	32	12
	HB ELISA	41	3
	UC ELISA	42	2
Negative (36)	CB	0	36
	WB	0	36
	HB	9[a]	27
	UC	3[b]	33

[a] Includes three VDRL- and FTA-ABS-positive and two rheumatoid factor-positive serum specimens.
[b] Includes two VDRL- and FTA-ABS-positive and one rheumatoid factor-positive serum specimens.

the 44 Western blot-positive specimens, 42 were positive in the UC ELISA; 9 were positive only for IGM; 16 were positive only with low IgG titers of 320 to 640; 10 were positive only with high IgG titers of 1,280 or greater; and 7 were positive for both IgG and IgM. The two Western blot-reactive specimens found negative by the UC ELISA method appeared to be predominantly IgM, since they reacted with more than four bands in the Western blot IgM assay and fewer than two bands in the Western blot IgG assay.

Table 1 presents the results of the three commercial products and the UC ELISA to Western blot analysis for the 70 serum specimens of patients clinically suspected of having Lyme disease, the 5 serum specimens positive for rheumatoid factor, and the 5 VDRL- and FTA-ABS-positive serum specimens. By using Western blot analysis as the reference method to compare all 80 serum specimens, the sensitivities for the CB, WB, HB, and UC assays were 89, 73, 93, and 96%, respectively, and their specificities were 100, 100, 75, and 92%, respectively.

DISCUSSION

Although *B. burgdorferi* has been identified and cultured from several sites, the isolation of the spirochetes from patients is difficult (1, 9, 13) and not yet practical for routine diagnosis. The clinical diagnosis should be confirmed by determining the antibody response to the spirochete. However, physicians depend heavily on clinical symptoms for diagnosis of early (stage 1) Lyme disease, due to the low sensitivities of most assays routinely used. Reports of low sensitivity (20 to 60%) when tested 2 to 3 weeks after exhibiting erythema chronicum migrans (6), as well as problems with specificity (3, 5, 8), demonstrate the need for a simple, reliable diagnostic test for Lyme disease.

Western blot analysis was chosen as the reference method because of its increased sensitivity and specificity as compared with both whole-cell- and periplasmic-flagellum-enriched ELISA for the detection of antibodies to *B. burgdorferi* (5). In a retrospective study, Grodzicki and Steere (5) demonstrated that immunoblot was more sensitive than ELISA (53 versus 30%) for detection of early Lyme disease. Immunoblot was also more specific than ELISA (4 of 64 false-positive results by immunoblot compared with 11 of 64 false-positive tests with ELISA [5]). In a limited study done at the University of Connecticut Health Center, an increase in sensitivity (91 versus 73%) of immunoblot as compared with ELISA was observed on 11 clinically known positive specimens.

Three commercially available test products and the UC ELISA were compared with Western blot analysis to determine their sensitivity and specificity in diagnosing Lyme disease. Serum specimens were obtained from patients with clinical symptoms of Lyme disease, as well as from five rheumatoid factor-positive and five VDRL- and FTA-ABS-positive patients. Sensitivities of the three diagnostic products and the UC ELISA ranged from 73 to 96%, as compared with Western blot. Two patients, one who had erythema chronicum migrans and another who presented with fever and chills 2 weeks after a tick bite, tested positive by Western blot but negative by the UC ELISA. In both patients, the IgM Western blot was positive but the IgG Western blot was negative.

A total of 12 serum specimens tested falsely negative in the three commercial products (5 by CB, 12 by WB, and 3 by HB). Of the 12 total specimens, 9 were shown to be predominantly IgM positive when compared with the UC ELISA and Western blot. Details of clinical symptoms were available for seven of these serum specimens, and they supported the diagnosis of Lyme disease. Only Western blot and the UC ELISA test individually for either IgG or IgM, while the three commercial products determine total antibody. To increase the sensitivity of these commercial kits, it may be necessary to modify the assay to better detect IgM.

Specificity ranged from 75 to 100% for the three diagnostic products and the UC ELISA when compared with immunoblot. Two diagnostic products, the CB and WB, were 100% specific on the limited number of negative specimens tested. The UC ELISA incorrectly identified three false positives (two VDRL- and FTA-ABS-positive specimens and one rheumatoid factor-positive specimen). The HB ELISA incorrectly identified nine false positives (three VDRL and FTA-ABS positives and two rheumatoid factor positives; the remaining four were considered false positives on the basis of a negative Western blot, since detailed clinical history was unavailable). Western blot analysis of the five VDRL- and FTA-ABS-positive and five rheumatoid factor-positive specimens proved to be negative by the criteria of Grodzicki and Steere (5); however, nonspecific binding of antibody to the 25K, 41K, and 66K proteins was observed not only for these specimens but also in 39% of the other specimens which were considered negative by the criteria of Grodzicki and Steere (5). The false reactivity of the HB kit may be a problem in the interpretation of results as specified by the product insert. Specificity could be increased from 75 to 83% without a loss in sensitivity if the Lyme index considered positive was increased from 1.0 to 1.25.

In summary, the CB, HB, and UC ELISAs are comparable in their sensitivities when the Western blot is used as the reference assay, while the WB FIAX is the least sensitive. However, when specificity is considered, both CB and WB assays are highly specific, while HB is the least specific of the commercially available kits when compared with Western blot.

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