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Ability of the Borreliacidal Antibody Test To Confirm Lyme Disease in Clinical Practice

Steven M. Callister,[1,2,3]* Dean A. Jobe,[1,3] William A. Agger,[2,3] Ronald F. Schell,[4,5] Todd J. Kowalski,[3] Steven D. Lovrich,[1,3] and Jennifer A. Marks[1,3]

Microbiology Research Laboratory,[1] Section of Infectious Diseases,[2] and Gundersen Lutheran Medical Foundation,[3] Gundersen Lutheran Medical Center, La Crosse, Wisconsin 54601, and Wisconsin State Laboratory of Hygiene[4] and Department of Medical Microbiology and Immunology,[5] University of Wisconsin, Madison, Wisconsin 53706

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Highly specific borreliacidal antibodies are induced by infection with *Borrelia burgdorferi*, and a borreliacidal antibody test (BAT) may be an accurate laboratory procedure for confirming Lyme disease in clinical practice. To investigate this, 34 Lyme disease sera and 34 sera from patients with other illnesses who had presented to a primary-care facility located in an area of borreliosis endemicity were tested by the BAT and Western blotting (WB). The BAT was more sensitive (79% versus 65%; $P = 0.090$), especially in cases in which patients had a single erythema migrans lesion ($P = 0.021$). In addition, the potentially cross-reactive sera were negative by the BAT but WB yielded three (9%) false-positive results. The results from 104 sera from possible Lyme disease patients demonstrated the clinical usefulness of the more sensitive and specific BAT. The BAT was positive for 40 (38%) sera from patients with Lyme disease-related symptoms and appropriate clinical and epidemiological findings. WB confirmed Lyme disease in 30 (75%) of the 40 BAT-positive patients but failed to detect *B. burgdorferi* infection in 10 BAT-positive patients. WB was also positive for 11 BAT-negative sera, but six (55%) patients had case histories which suggested that the results were false positives. Collectively, the results confirm that the BAT is a sensitive and highly specific test and suggest that widespread use would increase the accuracy of serodiagnostic confirmation of Lyme disease.

Lyme disease occurs after the bite of *Borrelia burgdorferi*-infected *Ixodes* spp. ticks. Detecting anti-*B. burgdorferi* antibodies in serum is the most commonly used method for confirming Lyme disease, since detecting the spirochetes by culture or PCR is problematic. The Centers for Disease Control and Prevention (CDC) recommends confirming Lyme disease by a two-tiered system, where serum is screened with an indirect immunofluorescence assay (IFA) or indirect enzyme-linked immunosorbent assay. The serum with equivocal or positive results is then confirmed by immunoglobulin M (IgM) and IgG Western blotting (WB) or IgG WB alone if the illness has been present for less than or more than 1 month, respectively (14).

WB can accurately confirm Lyme disease (21, 34, 35), especially when patients are tested who reside in a focus of endemicity and present with a tick exposure and well-recognized (13) clinical symptoms. However, false-positive WBs can occur (5, 15, 36) and the impact of the nonspecificity is magnified when the patients have less-typical symptoms or little risk of obtaining a tick bite (26, 29, 33, 35). This shortcoming necessitates the development of a more accurate serodiagnostic test.

Infection with *B. burgdorferi* induces the production of borreliacidal antibodies (6, 9–12, 17, 19, 24, 25, 30–32) that activate complement to form a membrane attack complex. The membrane attack complex then kills the spirochetes without the need for phagocytic cells (4, 10, 12, 22–24). Most *B. burgdorferi* proteins expressed on the surface of the spirochetes induce borreliacidal antibodies (4, 10, 11, 16, 18, 19, 24, 27, 28, 32). Unlike other antibodies (e.g., opsonizing), the borreliacidal antibodies are highly specific (9, 11, 12, 19, 31). In earlier studies (9, 11), researchers detected significant amounts (titer, ≥80) of borreliacidal antibodies in only 2 (<0.5%) of 478 potentially cross-reactive sera, many of which yielded positive results when tested by IFA, indirect enzyme-linked immunosorbent assay, or WB.

The borreliacidal antibodies cannot be detected by conventional serodiagnostic tests, since live organisms are necessary for monitoring the ability of the antibodies to kill the spirochetes. A simple, semiautomated borreliacidal antibody test (BAT), in which viable *B. burgdorferi* spirochetes are incubated with serum and complement and then stained with acridine orange, was previously developed (9–11, 30). The acridine orange accumulates in the killed spirochetes, and the fluorescence is evaluated objectively with a flow cytometer. To prevent antimicrobial agents from causing a false-positive result, the serum is treated with nonionic exchange resin prior to testing (20).

Recently, a panel of sera characterized by culture and WB was blinded and forwarded by the CDC for testing by the BAT. The BAT confirmed infection in the 10 WB-positive Lyme disease patients, but the BAT result was positive earlier in one patient and became negative after two patients were treated. Twenty-one of 25 control sera were also negative by the BAT, but small concentrations (titer, ≤20) of borreliacidal antibodies were detected in 2 normal sera and higher concentrations (titer, ≥80) were detected in sera from two patients with relapsing fever. In addition, the BAT was reproducible, although

* Corresponding author. Mailing address: Microbiology Research Laboratory, Gundersen Lutheran Medical Center, 836 South Ave., La Crosse, Wisconsin 54601. Phone: (608) 782-7300, ext. 2042. Fax: (608) 791-6602. E-mail: Scallist@gundluth.org.

a low level of borreliacidal activity (titer = 10) was detected in two duplicate sera upon retesting. These results, and the findings of studies using well-characterized sera (9–12), provided strong evidence that the BAT is a valuable serodiagnostic test. In this study, we determined the ability of the BAT to confirm Lyme disease in patients who had been evaluated at a primary-care hospital in an area of endemicity in western Wisconsin and compared the results to those obtained using a commercially available WB assay. The results demonstrated that the BAT was more sensitive and specific than WB and suggested that this procedure provided more accurate serodiagnostic confirmation of Lyme disease.

MATERIALS AND METHODS

Serum samples. Gundersen Lutheran Medical Center is located in a Lyme disease focus of hyperendemicity (2) where approximately 45% of nymphal and adult *I. scapularis* ticks are infected with *B. burgdorferi* (7). During 1998, 1,250 Gundersen Lutheran Medical Center patients were screened for Lyme disease with a *B. burgdorferi* IFA and 205 (16%) sera contained antibodies (IgM titer ≥128 and/or IgG titer ≥256). Of the IFA-positive sera, 172 (84%) had sufficient residual volume for additional testing and were blinded in a coded, unlinked fashion for use in this study. Serum from a person not exposed to *B. burgdorferi* was used as a normal control.

Chart reviews. Retrospectively, but blinded to the WB and BAT results, the medical charts of the IFA-positive patients were reviewed and the cases were divided into the following categories based on the chances of a tick exposure and appropriateness of clinical symptoms (13): (i) Lyme disease (tick exposure and erythema migrans (EM) lesion meeting the CDC surveillance criteria), (ii) possible Lyme disease (tick exposure, absence of other illness, and possible Lyme disease symptoms [dependent on laboratory confirmation]), and (iii) other illness (unlikely tick exposure and symptoms unrelated to Lyme disease).

Organisms. *B. burgdorferi* sensu stricto isolates 297 and 50772 were isolated from human spinal fluid and an *Ixodes scapularis* tick, respectively. *B. burgdorferi* 297 expresses OspA and OspB (10), and the 50772 spirochetes lack the *ospAB* operon (3). The spirochetes were serially 10-fold diluted in Barbour-Stoenner-Kelly (BSK) medium capable of supporting growth from a single organism (8). The resultant clonal population of spirochetes was then passaged 10 times in fresh BSK medium at 35°C, dispensed into 200-μl aliquots in 1.5-ml screw-cap tubes (Sarstedt, Newton, N.C.), and stored at −70°C until used.

WB. The IgM or IgG WB was performed using an FDA-approved commercial kit (MRL Diagnostics, Cypress, Calif.). Two experienced laboratory personnel interpreted banding patterns individually. Blots with discrepant results were rescored by a third person, and bands identified by two of the three evaluators were reported. An IgM WB was considered positive if any two of the 23-, 39-, or 41-kDa bands were present within 30 days of the development of symptoms. An IgG WB was considered positive if any five of the 18-, 21-, 28-, 30-, 39-, 41-, 45-, 58-, 66-, or 93-kDa bands were detected (14).

Detection and removal of antimicrobial agents. There were no indications in the clinical records that patients had received antimicrobial agents prior to collection of the sera. However, the serum samples were screened for antimicrobial agents, which were removed as described previously (20) to ensure the accuracy of the BAT results. Briefly, wells (5-mm diameter) were cut into Mueller-Hinton agar plates containing 10^6 *Bacillus subtilis* spores per ml with the nondispensing end of a sterile 2-ml serological pipette. A 50-μl amount of each serum was loaded into individual wells, and the plates were incubated for 4 to 6 h at 37°C. When a zone of inhibition was detected, a 500-μl amount of the serum, diluted fivefold in phosphate-buffered saline (0.01 mol/liter, pH 7.2), was combined with 1 gram of Amberlite XAD-16 nonionic polymeric beads (Sigma Chemical Co., St. Louis, Mo.) and incubated at room temperature for 20 min with occasional shaking. The serum was then retested to confirm removal of the antimicrobial agent. Antimicrobial agents were present in 12 (7%) of the 172 sera, and anti-50772 or -297 borreliacidal antibodies were detected in 5 of the 12 treated sera after the antimicrobial agents had been removed.

Detection of borreliacidal antibodies. The BAT was performed as previously described (9, 11). Briefly, a frozen 200-μl aliquot of *B. burgdorferi* 50772 or 297 was thawed, inoculated into 6 ml of fresh BSK medium, and incubated for 72 h at 35°C. After incubation, the concentration of spirochetes was determined using a Petroff-Hausser counting chamber and adjusted to 5×10^5 organisms per milliliter by the addition of fresh BSK. Serum samples were also diluted 1:40 in fresh BSK and sterilized by passage through a 0.2-μm microcentrifuge filter (Corning Costar, Cambridge, Mass.). A 200-μl amount of the diluted serum was transferred to a 1.5-ml screw-cap microcentrifuge tube (Sarstedt) and heat inactivated at 56°C for 10 min. The heat-inactivated serum was then serially diluted (1:40 to 1:5,120) with fresh BSK, and a 100-μl amount was combined with a 100-μl aliquot of the *B. burgdorferi* 50772 or *B. burgdorferi* 297 suspension (5×10^4 organisms) and 15 μl of guinea pig serum (≥200 50%-hemolytic complement units per ml; Sigma). The suspensions were mixed gently and incubated for 16 to 24 h at 35°C.

Following incubation, 100 μl was removed from each assay and mixed with 400 μl of phosphate-buffered saline containing 1 μg of acridine orange/ml. Dead, blebbed borrelia were then detected with a FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, Calif.). Spirochetes were isolated by gating (CELLQuest software; Becton-Dickinson) and analyzed for 1 to 2 min with the flow rate set at low (12 μl/min). Borreliacidal antibodies were detected by monitoring the increased side scatter and fluorescence intensity that occurs when the acridine orange intercalates into the blebbed, nonviable spirochetes. Samples that yielded a ≥13% increase in fluorescence intensity compared to a normal serum control were considered positive (9). In addition, positive assays were examined by dark-field microscopy to confirm the presence of characteristic blebbed, nonmotile spirochetes (10).

Statistical analysis. A binomial comparison was used to determine the significance of differences in sensitivity.

RESULTS

Sensitivity and specificity of BAT and WB. Thirty-four sera were from patients with early Lyme disease (EM lesions), and an additional 34 sera were from patients with no evidence of tick exposure and with symptoms unrelated to Lyme disease. The BAT was significantly more sensitive ($P = 0.090$) than WB for detecting early Lyme disease. Twenty-seven (79%) and 22 (65%) of the 34 Lyme disease sera were positive by the BAT or WB, respectively (Table 1). The BAT-positive Lyme disease sera all contained high levels of borreliacidal anti-50772 antibodies (average titer, 4,886). One (3%) of these sera also contained borreliacidal anti-297 antibodies (titer, 160). The BAT was positive in one additional serum when the patients had multiple EM lesions ($n = 12$), but the increased sensitivity was most significant when the patients had a single EM lesion ($n = 22$). The BAT was positive in 16 (73%) sera from these patients, while only 12 (55%) were positive by WB ($P = 0.021$).

Borreliacidal antibodies were not detected in the sera from the 34 patients with little risk of tick exposure and symptoms unrelated to Lyme disease. In contrast, WB confirmed Lyme disease in 3 (9%) of these patients. One patient had cold symptoms, joint stiffness, and shortness of breath, and two patients had single episodes of joint swelling. One was a 73-year-old patient with a swollen knee and no recollection of tick exposure. The other was a 55-year-old patient with a diffuse erythematous rash, papules over the entire body, and swollen hands, feet, arms, and legs.

Confirmation of *B. burgdorferi* infection in possible Lyme disease patients by BAT and/or WB. The BAT and WB confirmed *B. burgdorferi* infection in 40 (38%) and 41 (39%) of the 104 sera from the patients with possible Lyme disease. These sera were from patients with well-accepted but less-specific Lyme disease symptoms that required confirmation by serodiagnostic testing for an accurate diagnosis (13). Thirty-four (85%) of the BAT-positive sera contained high levels of borreliacidal anti-*B. burgdorferi* 50772 antibodies (average titer, 2,059), and three also contained borreliacidal anti-297 antibodies (average titer, 1,333) (Table 2). The remaining 6 BAT-positive sera contained only high concentrations of borreli-

TABLE 1. Sensitivity and specificity of the WB and BAT using sera from patients with early Lyme disease or other illnesses

Serum	WB	BAT		
	No. positive (%)	No. anti-50772 positive (%)	No. anti-297 positive (%)	Total (%)
Lyme disease				
Single EM (n = 22)	12 (55)	16 (73)	1 (5)	16 (73)[a]
Multiple EM (n = 12)	10 (83)	11 (92)	0	11 (92)
Total (n = 34)	22 (65)	27 (79)	0	27 (79)[b]
Other illness				
Non-Lyme symptom(s) (n = 24)	1 (4)	0	0	0
Non-Lyme arthritis (n = 10)	2 (20)	0	0	0
Total (n = 34)	3 (9)	0	0	0

[a] Binomial comparison; P = 0.021.
[b] Binomial comparison; P = 0.090.

acidal anti-297 antibodies (average titer, 1,584). Thirty (75%) of the BAT-positive sera were also positive by WB, including the BAT-positive sera from patients with recurrent arthritis or facial palsy. However, there were 21 (20%) discrepant results in the sera from patients with less-characteristic symptoms including atypical lesions, arthralgia, and myalgia. Eleven (11%) sera were WB positive and BAT negative, and 10 (10%) sera were BAT positive and WB negative.

Clinical validity of test results. Because of the divergent confirmatory BAT and WB results, the medical histories of the possible Lyme disease patients with positive BAT or WB results were reexamined. No clinical or epidemiological findings inconsistent with a diagnosis of Lyme disease were detected in the case histories of the patients with positive BAT results. The 31 patients with anti-*B. burgdorferi* 50772 borreliacidal antibodies had likely tick exposures and recent onsets of skin lesions, headache, fever, stiff neck, arthralgia, myalgia, joint swelling, or extremity paresthesias. The three sera that contained borreliacidal anti-50772 and anti-297 antibodies were from a patient with multiple skin lesions and a several-month history of arthralgia and myalgia and from two patients with recurrent arthritis. The six sera that contained only borreliacidal anti-297 antibodies were from two patients with recurrent arthritis, three patients with several-month histories of arthralgia and myalgia, and one patient with headache and paresthesia of the face, hands, and feet. In contrast, the sera of 6 (55%) of the 11 patients with WB-positive and BAT-negative results had laboratory or clinical results that could cast doubt on a diagnosis of Lyme disease. Three patients with arthralgia and myalgia were positive by the IgG WB, despite having had symptoms for less than 1 week. The skin lesion of another patient appeared after a documented spider bite, and the lesion of an additional patient was more consistent with cellulitis. The sixth patient had fever, headache, fatigue, and arthralgia but was also infected with cytomegalovirus.

DISCUSSION

Researchers (9–12, 17, 19, 24, 25, 30, 31) have shown that patients infected with *B. burgdorferi* produce high concentrations of highly specific borreliacidal antibodies. Researchers (9, 11, 30) have also demonstrated that borreliacidal antibodies can be accurately detected in well-characterized culture- or case-defined Lyme disease sera by using a flow-cytometric BAT. In this study, we determined how the BAT would perform in a primary-care practice located in a region where Lyme disease is common (2, 7).

Using sera from patients with early Lyme disease characterized by a classic EM lesion (13) or from patients with clinical signs and symptoms that were not Lyme disease related, we confirmed that the BAT was sensitive (79%) and highly specific (100%). The high sensitivity and specificity were almost identical to those of previous findings (9–11, 30). In addition, antimicrobial agents could be easily removed prior to testing with little or no apparent effect on the borreliacidal antibodies. The collective results from previous studies, the CDC evaluation,

TABLE 2. Positivity and intertest agreement of the WB and BAT using sera from patients with possible Lyme disease

Primary symptom	WB	BAT			No. of discrepant results (%)
	No. positive (%)	No. anti-50772 positive (%)	No. anti-297 positive (%)	Total (%)	
Recurrent arthritis (n = 10)	8 (80)	6 (60)	3 (30)	8 (80)	0
Facial palsy (n = 6)	3 (50)	3 (50)	0	3 (50)	0
Skin lesion (n = 39)	17 (44)	15 (38)	1 (3)	16 (42)	9 (23)[b]
Other[a] (n = 49)	13 (27)	10 (20)	5 (10)	13 (27)	12 (24)[c]
Total (n = 104)	41 (39)	34 (33)	9 (9)	40 (38)	21 (20)

[a] Primarily arthralgia, myalgia, or fatigue.
[b] Five WB positive and BAT negative and four WB negative and BAT positive.
[c] Six WB positive and BAT negative and six WB negative and BAT positive.

and this study confirm that the BAT can be used to accurately detect infection with *B. burgdorferi* in Lyme disease patients who are evaluated at a primary-care facility.

The results also showed that the BAT was more sensitive and specific than the widely used WB. The potential benefit of the increased accuracy was suggested by the results obtained using the sera from the possible Lyme disease patients. The 40 BAT-positive sera contained high concentrations of borreliacidal antibodies, which eliminated the possibility that the results were due to cross-reactive antibodies. Additionally, these patients had tick exposures, well-accepted symptoms (13), and no additional findings sufficient to refute a diagnosis of Lyme disease. Thirty (75%) of the 40 BAT-positive sera were positive by the WB, which strengthened further the argument that these BAT-positive patients had Lyme disease. However, 21 (20%) of the WB results were questionable. The decreased sensitivity may have been responsible for the negative WB results for 10 (25%) of the BAT-positive sera. A likely explanation is that these Lyme disease patients had developed an antibody response that was insufficiently diverse to yield reactivities against the multiple proteins necessary for confirmation by WB. In addition, assays of several sera that tested positive by WB and negative by BAT appeared to have yielded false positives. Some sera did not contain borreliacidal antibodies, despite WB-detectable reactivities against multiple species-specific *B. burgdorferi* proteins known to induce borreliacidal antibodies (4, 10, 11, 16, 18, 19, 24, 27, 28, 32). As further support, several patients had inappropriate laboratory results (e.g., IgG antibodies) or clinical or epidemiological findings that suggested an illness other than Lyme disease. Unfortunately, it was impossible to determine with certainty whether the BAT-positive or WB-positive patients were Lyme disease patients, since the *B. burgdorferi* infections were not confirmed directly by PCR or culture. However, the high concentrations of borreliacidal antibodies and the absence of contrary clinical findings in the BAT-positive patients provided strong evidence that the BAT could be used alone or in conjunction with WB to increase the accuracy of serodiagnostic confirmation of Lyme disease.

Researchers previously showed that borreliacidal anti-*B. burgdorferi* 297 antibodies are common in Lyme arthritis patients (9–11) but are rarely detected during an early infection (1, 9). In contrast, borreliacidal anti-50772 antibodies, which are specific for proteins other than OspA and OspB, were detected during all stages of Lyme disease (9, 30). The results in this study confirmed these findings. Sixty-one (91%) sera contained borreliacidal anti-50772 antibodies, but 6 (9%) sera from patients with long-standing clinical complaints had only borreliacidal anti-297 antibodies. Thus, *B. burgdorferi* 50772 could be used to confirm most cases of Lyme disease, but the sensitivity of the BAT was maximized by also using *B. burgdorferi* 297.

The technical complexity of the BAT has hindered widespread use, but the increased sensitivity and specificity should be of sufficient value to warrant availability. The test requires a flow cytometer, live and specific isolates of *B. burgdorferi*, and removal of antimicrobial agents from sera, but laboratories with experience in flow cytometry could easily perform these tasks. The time required to prepare live organisms, remove antimicrobial agents from sera, set up the test, and evaluate the flow cytometric results is comparable to the time necessary to perform a WB.

In summary, additional prospective studies remain necessary for completely defining the full utility of the BAT. However, our results demonstrate that the BAT is a sensitive and highly specific confirmatory test for Lyme disease that can be used in clinical practice.

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