Comparative Evaluation of Adsorption with E. coli on ELISA Tests for Lyme Borreliosis

PAUL T. FAWCETT, CARLOS D. ROSE, and KATHLEEN M. GIBNEY

ABSTRACT. ***Objective.*** To evaluate prospectively in a clinical setting the use of a soluble fraction of *E. coli* to adsorb nonspecific antibodies which can cause false positive ELISA tests for Lyme borreliosis.
Methods. The patient population tested was obtained from individuals referred to or initially presenting at a pediatric Lyme disease clinic in Wilmington, DE. Patients were followed for a minimum of 6 months subsequent to primary presentation at the clinic.
Results. A total of 209 met criteria for study inclusion, 93 of whom were diagnosed as having Lyme borreliosis and 116 of whom had other diagnoses. Results of ELISA tests were compared with different diagnoses and, when available, ELISA results from commercial laboratories. Findings indicate that some commercial laboratories have excessively high rates of false positive results (>90% of positives were found to be false positives).
Conclusion. Adsorption with *E. coli* antigens effectively removed antibodies causing false positive results including those occurring at commercial laboratories and did not cause any significant reduction in assay sensitivity. (*J Rheumatol 1995;***22:684–8**)

Key Indexing Terms:
LYME BORRELIOSIS ELISA ADSORPTION

It is generally recognized that enzyme linked immunosorbent assays (ELISA) for detecting antibodies to *Borrelia burgdorferi* still yield unacceptably high numbers of false positive results and lack sensitivity for early detection[1,2]. A consequence of this problem is that physicians in our area (endemic for Lyme borreliosis) treat patients for whom ELISA were ordered irrespective of test result. Lack of confidence in serology coupled with potential difficulties in making a diagnosis of Lyme borreliosis on a clinical basis (in absence of erythema migrans) contribute to the reported overdiagnosis of Lyme borreliosis in endemic regions[3,4].

Previous reports indicate that false positive ELISA tests for Lyme borreliosis occur in patients with other bacterial infections, viral infections, and autoimmune diseases[5-7]. Efforts to eliminate these false positives iniclude use of more dilute patient sera, increasing cut off values from 3 standard deviations (SD) above the normal mean to up to 8 SD and addition of adsorbents to remove crossreactive or nonspecific antibodies[8,9]. We have published reports indicating that adsorbent prepared from *E. coli* added to serum diluent effectively reduced false positive test results without adversely affecting assay sensitivity[10,11]. In performing those studies we used sera from a large number of patients having a broad spectrum of disorders that may require serologic tests of Lyme disease in the diagnostic investigation. Our rationale was that use of a large well defined non-Lyme population was necessary to accurately test assay performance in routine clinical use. Our study was performed to prospectively evaluate the performance of *E. coli* adsorption in a clinical setting. All patients for whom results of serology for aid in diagnosing Lyme was available and for whom a clinical diagnosis had been established and 6 months of followup was available were involved in this study. Analysis of data resulting from our study was also reviewed to evaluate our estimates of clinical usefulness determined in prior investigations.

A total of 209 patients seen at our institute's Lyme clinic were enrolled in this study. All patients were tested by ELISA using *E. coli* adsorbent and 45 had ELISA results from tests performed at commercial laboratories available for comparison. Diagnoses for patients in the study included 93 with Lyme borreliosis (30 early localized, 28 early disseminated, 36 chronic) and 116 non-Lyme including diagnosis of chronic fatigue, arthromyalgia, juvenile rheumatoid arthritis and viral infections. Comparison of serologic results obtained at initial presentation to our clinic with final diagnosis after at least 6 months followup indicate that adsorption with *E. coli* maintains adequate assay sensitivity and substantially improves specificity, particularly when compared with results from commercial laboratories.

From the Immunology Research Program, Department of Medical Cell Biology, and the Division of Rheumatology, Alfred I. duPont Institute, Wilmington, DE, and the Department of Pediatrics, Thomas Jefferson Medical College, Philadelphia, PA, USA.

P.T. Fawcett, PhD, Director, Immunology Laboratory, Department of Medical Cell Biology, Alfred I. duPont Institute and Assistant Professor, Pediatarics, Thomas Jefferson University; C. Rose, MD, Division of Rheumatology, Alfred I. duPont Institute and Assistant Professor, Pediatrics, Thomas Jefferson University; K.M. Gibney, BS, Senior Research Assistant, Alfred I. duPont Institute.

Address reprint requests to Dr. P.T. Fawcett, Alfred I. duPont Institute, Research Department, P.O. Box 269, Wilmington, DE 19899.

Submitted July 14, 1944 revision accepted September 27, 1994.

MATERIALS AND METHODS

Patient selection. Patient data used for this study were obtained from patients seen at our pediatric Lyme clinic during 1990, 91 and 92. Inclusion in the study required that the following criteria were met (1) Patient was seen and evaluated by either a rheumatologist or infectious disease specialist at our clinic. (2) Medical histories or records were reviewed by a rheumatologist with expertise in the diagnosis of Lyme borreliosis. (3) A patient diagnosis remained unchanged for at least 6 months following sample test date. (4) A serum specimen was tested by ELISA, using *E. coli* antigens in serum diluent, for antibodies to *B. burgdorferi*. A total of 209 patients met the criteria for inclusion in this study. Diagnosis for 93 patients was Lyme borreliosis (30 early localized, 28 early disseminated and 35 chronic). Patients diagnosed with Lyme borreliosis met Centers for Disease Control criteria for case definition and reporting[12]. The other 116 patients had other diagnoses which are given in the results.

Adsorbent preparation. Adsorbent was prepared from *E. coli* (ATCC strain 25.922). The bacteria were grown to late log phase at 37°C in nutrient broth (Difco, Detroit, MI) and harvested by centrifugation in a refrigerated centrifuge at 10,000 g for 15 min. Pelleted bacteria were then washed 5 times by centrifugation in phosphate buffered saline (PBS). Resuspended *E. coli* were then disrupted by sonication on ice with 6 ten second blasts at a setting of 3 using a cell disrupter (Heat Systems-Ultrasonics, Formingdale, NY). Sonicated bacteria were then centrifuged at 10,000 for 20 min, the supernatant harvested, centrifuged again for 20 min and saved. Protein concentration of the supernatant was determined using the BioRad microassay (a modified Bradford procedure) and adjusted to 0.7 mg/ml of *E. coli* protein in PBS containing 0.5% bovine serum albumin (BSA). Frozen stock was stable for 6 months at −70°C.

Borrelia antigen preparation. Borrelia burgdorferi (ATCC strain B31) were grown to late log phase at 35°C in Barbour Stoenner Kelly medium. Spirochetes were harvested by centrifugation at 10,000 g for 30 min at 4°C, washed 3 times in PBS (pH 7.4). Pelleted spirochetes were resuspended in cold PBS and sonicated on ice by six 10 second blasts of a cell disrupter at a setting of 6. The sonicate was then centrifuged at 10,000 g for 30 min at 4°C, the supernatant collected, protein content determined using a BioRad protein assay kit and protein content adjusted to 400 μg/μl in PBS. The soluble *Borrelia* antigen stock was then diluted to 5 μg/μl in PBS and 100 μl added to microtiter wells (Immulon-1, Dynatech, Alexandria, VA) and incubated at 37°C for 2 h. Wells were then washed 3 times with PBS containing 0.05% Tween 20 and 0.5% BSA, fixed with 95% methanol for 10 min, washed 3 times as above and blocked by incubation with 300 μl of 1% BSA at 37°C for 1 h.

ELISA assay. Patient sera were diluted 1:80 with PBS containing 0.7 mg/ml *E. coli* protein and 0.5% BSA. A 100 μl aliquot of diluted sera was then added to appropriate microtiter wells and incubated for 1 h at 37°C. Wells were then washed 3 times with PBS containing 0.5% BSA and 100 μl of 1:1000 dilution of peroxidase conjugated goat antihuman IgG (Cappel, West Chester, PA) was added to each well for a 30 min incubation at 37°C. Wells were then washed as above and 100 μl of ABTS substrate was added for a 10 min incubation at room temperature. The reaction was stopped by addition of 50 μl of 250 mM oxalic acid and the optical density (OD) of each well at 405 nm was determined using a Titerteck Multiscan (Flow Laboratories, Helsinki, Finland). Results were converted to titer units for reporting purposes from a standard curve prepared from sera of known reactivity.

RESULTS

A total of 209 patients seen at our pediatric Lyme clinic were included in this study. Lyme borreliosis was diagnosed in 93 of the patients and 116 had Lyme borreliosis ruled out as a diagnosis. All sera were tested for IgG antibodies to *B. burgdorferi* by ELISA using *E. coli* antigens in the serum diluent. Results shown in Figure 1 indicate that ELISA using

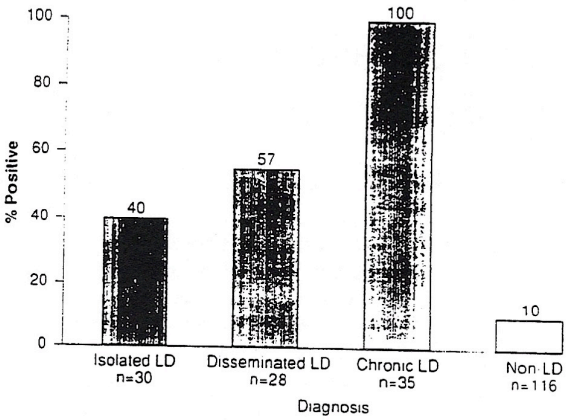

Fig. 1. Comparison of adsorbed ELISA test results with diagnostic category for Lyme and non-Lyme patients.

adsorption performs reasonably well in detecting *B. burgdorferi* specific antibody in sera from patients with early localized [primarily presenting with erythema migrans (EM) at the time of sample acquisition] and patients with early disseminated Lyme borreliosis (primarily mild neurologic, febrile, or mild muscular/articular involvement) with 40 and 57% of patients testing positive. All of the chronic Lyme patients (primarily Lyme arthritis) tested positive using the *E. coli* antigen adsorption ELISA. Ten percent of the non-Lyme patients (12 of 116) produced sufficient reactivity in the adsorbed ELISA to test positive. Titers for this group ranged from 1:80 to 1:320 (1 patient). All of the patients tested by ELISA were also tested by Western blot for both IgG and IgM antibodies to *B. burgdorferi*. Interpretive criteria for the Western blot required detection of 4 bands including one at the 41 kDa position and one band in the 60 kDa range as described[13,14]. No non-Lyme patients tested positive by Western blot; however, about 50% had at least one band on blots, the majority occurring at the 41 and 60 kDa locations. Preliminary work (unpublished) indicates the adsorption with *E. coli* does not prevent binding to these bands in non-Lyme sera.

A subset of our patient population had been tested by referring physicians for antibodies to *B. burgdorferi* at commercial laboratories. Data from the commercial laboratories were available for 12 of the Lyme borreliosis patients and for 45 of the non-Lyme patients. Figure 2 shows a comparison of the commercial laboratory results with results achieved using *E. coli* adsorbed ELISA. Results achieved for patients with Lyme borreliosis were identical for both assays. Results for the non-Lyme patients were dramatically different, however, with 3 of the 45 non-Lyme patients testing positive by our *E. coli* adsorbed ELISA and 42 of the 45 non-Lyme patients testing positive at commercial laboratories. The median time elapsed between acquisition of samples for reference laboratories and our assays was less than 2 weeks. These results suggest that over 90% of false positive test

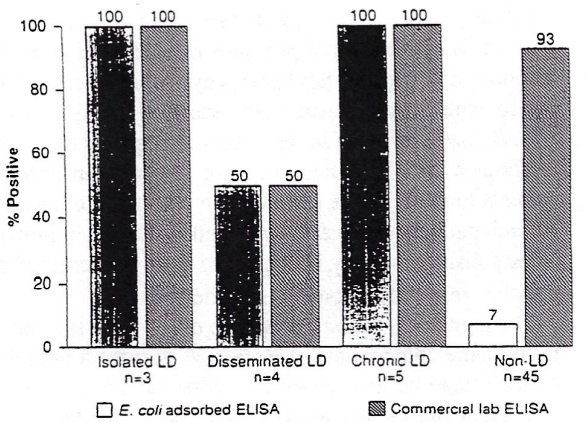

Fig. 2. Data presented depicts results of a comparison between commercial laboratories and *E. coli* adsorbed ELISA for Lyme and non-Lyme populations tested. The 2 patients classified as disseminated Lyme disease testing positive by our assay were the same 2 who tested positive at commercial laboratories.

results for antibodies to *B. burgdorferi* are removed by addition of *E. coli* antigens to serum diluent.

Data presented in Table 1 shows results for both *E. coli* adsorbed and commercial ELISA performed on non-Lyme patients. The most common diagnoses for non-Lyme patients in this study were chronic fatigue and arthromyalgia. Of the 31 patients with chronic fatigue tested by our adsorbed ELISA only 1 was considered positive in comparison to 14 out of 15 chronic fatigue sera testing positive by commercial laboratories. Two of 15 patients with arthromyalgia (13%) tested positive using our ELISA versus 6 of 6 by commercial laboratories. Viral infection (Epstein-Barr virus, Parvovirus B19 and Varicella) caused the greatest percentage of false positives (29%) for *E. coli* adsorbed ELISA and also caused false positives for commercial laboratories (6 of 6 for 100%). The overall false positive rate for ELISA using *E. coli* adsorption was 10% while that for commercial laboratories was 93%. The false positive rate for *E. coli* adsorbed ELISA on samples from patients tested by reference laboratories was 7%. Of the 42 patients who tested positive at commercial laboratories only 3 tested positive when *E. coli* antigens were added to serum diluent demonstrating that over 90% false positive may be eliminated by using this adsorption procedure. Results obtained in this study are similar to those reported in a previous retrospective study which included more patients with non-Lyme autoimmune and inflammatory conditions[11,14].

DISCUSSION

Reliability, reproducibility, and diagnostic significance of results from assays detecting antibodies to *B. burgdorferi* remain subjects of contention[1,5]. This despite availability of FDA approved assays and commercial laboratories to perform such tests.

Our data demonstrate an effective and relatively simple technique that could be adapted to most enzyme assay tests and that improves specificity without sacrificing sensitivity. Perhaps more important results from this study concur with our earlier work[11]. This demonstrates that our validation study design, in which we included extensive testing of disease controls, was effective at predicting assay performance in routine clinical use. Results from commercial laboratories suggest that certain factors included in our validation study are not, though should be, included as part of such studies for serologic test performance. Those factors are (1) use of appropriate non-Lyme control populations for assay validation studies, (2) use of appropriate and rigorously defined Lyme disease patient sera for positive controls in validation studies, and (3) realistic expectations of performance capabilities for antibody detection assays.

Reports on evaluation/validations of commercial and in-

Table 1. *Comparison of adsorbed and commercial ELISA results with diagnoses of non-Lyme patients*

		Number Tested		Number Positive		
Diagnosis	n	Ads	Comm	Ads	Comm	Both
Chronic fatigue	31	31	15	1	14	0
Arthromyalgia	15	15	6	2	6	0
Fever	13	13	5	1	3	0
Tick	7	7	1	0	1	0
Viral infection	14	14	6	4	6	1
Rash	5	5	1	0	1	0
Bell's palsy	5	5	1	0	1	0
Arthritis	3	3	0	1	0	0
Headache	4	4	1	1	1	0
No symptoms	6	6	2	0	2	0
Other	13	13	7	2	7	2
Total	116	116	45	12*	42**	3

* The 12 samples that tested positive using the adsorbed ELISA did not include any of the 3 that tested negative at commercial laboratories. No non-Lyme patient tested was positive for either IgG or IgM antibodies measured by Western blotting.

** The majority of specimens testing positive at commercial laboratories were in the "low" positive range.

house developed ELISA tests for detection of antibodies to *B. burgdorferi* usually indicate that distinguishing negative from borderline and positive reactions is based on determining 3 or more standard deviations of reactivity above the mean for normal sera. Many reports also indicate that several or more autoimmune or syphilitic sera were tested to assess cross reactivity. Use of sera from healthy normals to establish cutoff values is a valid and necessary step in the development of an assay; however, determination of assay efficacy for routine clinical use should involve more extensive testing of a disease control population in addition to testing sera from patients with diseases known to cause false positive test results in many antibody detection based assays. Control patient populations can be best determined by noting what symptoms are likely to lead to suspicion of Lyme disease and hence testing for antibodies to *B. burgdorferi* or by testing patients presenting for evaluation at a Lyme disease clinic.

Selection of patient sera for antibody positive controls should require that patients not only meet accepted critera for a diagnosis of Lyme borreliosis based on clinical findings but that the patients have been followed for a sufficient time to assess outcome following treatment. Ideally one should try to obtain positive control sera from culture confirmed cases. However, such cases are relatively rare. Well documented (by experienced physicians) erythema migrans, classical disease symptoms, and chronology representative of disease stage and confirmation from 1 or more already evaluated assays should suffice for documentation of positive control sera.

The 3rd factor to consider is performance expectation of serologic assays. Detection of IgG antibodies and in some circumstances IgM antibodies to *B. burgdorferi* in sera from patients with chronic Lyme arthritis presents little or no problem. However, in cases with vague symptoms and no history of EM or when samples are obtained soon after infection (before or concurrent with EM), detection of IgG antibodies is unlikely and even IgM antibody detection should be interpreted with caution. The time course for generating a humoral immune response for both IgG and IgM antibodies has been fairly well established as has the persistence of IgG antibodies and usually rapid decrease in IgM antibodies. Results in some reports and case reports of patients from whom College of American Pathologists proficiency survey samples were prepared appear to deviate from what is considered the norm for humoral responses[15]. While it may be that *B. burgdorferi* possesses unique immunopotentiating properties that contribute to generation of a vigorous immune response, positive antibody tests for IgG occurring sooner than expected after infection, or in the case of IgM presenting at significant levels for up to a year following infection and successful treatment should be viewed with skepticism[16-18]. We feel this to be a particularly significant problem as false positives are common (in this study almost all of the positive referrals were false positive) for Lyme borreliosis. In addition, the presence of an EM rash or a tick bite does not preclude false positivity. Indeed there is no reason to expect patients with very recent infection (< 1 week) with *B. burgdorferi* to be less likely to possess crossreactive antibodies and yield false positive results than non-Lyme patients for whom false positives are relatively common. Use of such patients with very early well documented infections to establish sensitivity limits could lead to increased false positive results for non-Lyme patients.

The high background prevalence of IgM and IgG antibodies to some components of *B. burgdorferi* most notably the 41 kDa flagellar antigen in non-Lyme patients seems likely to result from natural antibodies as suggested by Cook, *et al*[19,20]. If this is indeed the case it would help to explain why IgM responses tend to persist for prolonged periods of time in patients with Lyme disease and remain detectable in non-Lyme patients. In the absence of classical symptoms, IgG response or favorable response to treatment, distinguishing between IgM *B. burgdorferi* specific and natural antibodies, known to be broadly reactive, is difficult[21].

When first investigating the use of *E. coli* to adsorb cross reactive antibodies we used a well defined panel of 20 positive reactive control sera as well as a selection of sera from patients with autoimmune diseases. We next tested a large patient population ($n = 200$) of control sera obtained from patients with well defined diagnoses (other than Lyme) for whom extensive (min 6 months) followup was available. This 2nd phase of testing was used as our validation study. Our report presents results obtained for use of *E. coli* adsorption on patients presenting or seen at our pediatric Lyme clinic over a 2-year period. Our findings with regard to sensitivity and specificity are very similar to that obtained by our validation study that was designed taking into account the factors discussed in the preceding paragraphs. There is no doubt that the results obtained from commercial laboratories which we presented in comparison with our own were achieved using assays that had passed a validation study. We acknowledge that the extremely high false positive rate (93%) results in part from sample bias as patients with a positive serology were more likely to be referred to our clinic than were negative. However, the fact that over 90% of the false positives were correctly identified by our assay demonstrate that commercial assays were not properly validated.

In summary, we report on an adsorption procedure which utilizes soluble antigens prepared from *E. coli* that remove antibodies that can crossreact with *B. burgdorferi*. The manufacture and use of this adsorbent is relatively simple and adds no additional incubation or timed steps to our ELISA for *B. burgdorferi* specific antibodies. Comparison of our results with those obtained at commercial laboratories on the same patients clearly demonstrates the value of incorporating this adsorption step. Finally, we suggest that proper validation studies that include appropriately selected

sera should be required prior to instituting tests for antibodies to *B. burgdorferi.*

REFERENCES

1. Bakken LL, Case KL, Callister SM, Bourdeau NJ, Schell RF: Performance of 45 laboratories participating in a proficiency testing program for lyme disease serology. *JAMA 1992;*268:891–5.
2. Corpuz M, Hilton E, Lardis P, Singer C, Zolan J: Problems in the use of serologic tests for the diagnosis of Lyme disease. *Arch Intern Med 1991;*151:1837–40.
3. Steere AC, Taylor E, McHugh GL, Logigian EL: The overdiagnosis of Lyme disease. *JAMA 1993;*269:1812–6.
4. Ostrov BE, Athreya BH: Lyme disease: Difficulties in diagnosis and management. *Pediatr Clin North Am 1991;*38:535–53.
5. Golightly MG: Laboratory considerations in the diagnosis and management of Lyme borreliosis. *Am J Clin Pathol 1993;*99:168–74.
6. Rahn DW, Malawista SE: Lyme disease: Recommendations for diagnosis and treatment. *Ann Intern Med 1991;*114:472–81.
7. Raoult D, Hechemy KE, Baranton G: Cross-reaction with *Borrelia burgdorferi* antigen of sera from patients with human immunodeficiency virus infection, syphilis, and leptospirosis. *J Clin Microbiol 1989;*27:2152–5.
8. Magnarelli LA, Miller JN, Anderson JF, Riviere GR: Cross-reactivity of nonspecific treponemal antibody in serologic tests for Lyme disease. *J Clin Microbiol 1990;*28:1276–9.
9. Magnarelli LA, Anderson JF: Adsorption and biotin-streptavidin amplification in serologic tests for diagnosis of Lyme borreliosis. *J Clin Microbiol 1991;*29:1761–4.
10. Fawcett PT, O'Brien AE, Doughty RA: An adsorption procedure to increase the specificity of enzyme-linked immunosorbent assays for Lyme disease without decreasing sensitivity. *Arthritis Rheum 1989;*32:1041–4.
11. Fawcett PT, Gibney KM, Rose CD, Klein JD, Doughty RA: Adsorption with a soluble *E. coli* antigen fraction improves the specificity of ELISA tests for Lyme disease. *J Rheumatol 1991;*18:705–8.
12. Centers for Disease Control: Case definitions for public health surveillance. *MMWR 1990;*39:19–21.
13. Rose CD, Fawcett PT, Singsen BH, Dubbs SB, Doughty RA: Use of Western blot and enzyme-linked immunosorbent assays in the diagnosis of Lyme disease. *Pediatrics 1991;*88:465–70.
14. Fawcett PT, Gibney KM, Rose CD, Dubbs SB, Doughty RA: Frequency and specificity of antibodies that crossreact with *Borrelia burgdorferi* antigens. *J Rheumatol 1992;*19:582–7.
15. College of American Pathologists: WSLH/CAP Lyme disease survey. *CAP Surveys 1993;*LY-A:1–12.
16. Feder HM, Gerber MA, Luger SW, Ryan RW: Persistence of serum antibodies to *Borrelia burgdorferi* in patients treated for Lyme disease. *Clin Infect Dis 1992;*15:788–93.
17. Dorward DW, Huguenel ED, Davis G, Garon CF: Interactions between extracellular *Borrelia burgdorferi* proteins and non-*borrelia* directed-immunoglobulin M antibodies. *Infect Immun 1992;*60:838–44.
18. Luft BJ, Dunn JJ, Dattwyler RJ, Gorgone G, Gorevic PD, Schubach WH: Cross-reactive antigenic domains of the flagellin protein of *Borrelia burgdorferi. Res Microbiol 1993;*144:251–7.
19. Cooke WD, Bartenhagen NH: Seroreactivity to *Borrelia burgdorferi* antigens in the absence of Lyme disease. *J Rheumatol 1994;*21:126–31.
20. Cooke WD, Orr AS, Wiseman BL, Rouse SB, Murray WC, Ranck SG: Human cord blood contains an IgM antibody to the 41 KD Flagellar antigen of *Borrelia burgdorferi. Scand J Immunol 1993;*38:407–9.
21. Gergely J: Multifunctional IgG and IgG-binding receptors. *Res Monogr Immunol 1988;*10:84–7.