Use of Western Blot and Enzyme-Linked Immunosorbent Assays to Assist in the Diagnosis of Lyme Disease

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ABSTRACT. Without evidence of erythema chronicum migrans, diagnostic confirmation of Lyme disease may be difficult, particularly if there are conflicting laboratory results. Often, for families and physicians, the clinical dilemma is whether fatigue, arthritis/arthralgias, a positive enzyme-linked immunosorbent assay (ELISA), and tick exposure, but no evidence of erythema chronicum migrans, are sufficient to diagnose and treat Lyme disease. Patients with discordant ELISA and Western blot (WB) assay results for *Borrelia burgdorferi* were studied to determine whether there was sufficient clinical evidence to support a diagnosis of Lyme disease. Of 650 consecutive sera analyzed by ELISA in a laboratory within a 1-year period, 77 were subsequently tested by WB. The clinical data from these patients were then analyzed. The study population was divided into three groups: group 1 (positive ELISA, positive WB), group 2 (positive ELISA, negative WB), and group 3 (negative ELISA, negative WB). Findings included the following: (1) Patients with a strong clinical history of Lyme disease were usually positive by both WB and ELISA (group 1). (2) All patients with erythema chronicum migrans had both positive WB and ELISA tests. (3) Ninety-one percent of group 2 had a rheumatic or inflammatory condition other than Lyme disease. (4) A definite response to antibiotics occurred in 75% of patients wherein both ELISA and WB were positive but in only 11% of cases with a positive ELISA but a negative WB. (5) History of tick exposure and degree of fever were not significantly different among the three serologic groups, and thus they were not diagnostically helpful. It is concluded that (1) positive results from *both* the WB and ELISA assays provide strong diagnostic support for Lyme disease; and (2) a positive ELISA test, particularly if at a low titer and without a positive WB test, is associated with a lack of clinical features of Lyme disease. Thus, Western blotting is helpful in identifying false-positive ELISA results for Lyme disease. *Pediatrics* 1991;88:465–470; *Lyme disease, Western blot, enzyme-linked immunosorbent assay.*

Received for publication May 7, 1990; accepted Sep 28, 1990.
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PEDIATRICS (ISSN 0031 4005).

ABBREVIATIONS. ECM, erythema chronicum migrans; ELISA, enzyme-linked immunosorbent assay; WB, Western blot.

A clinical diagnosis of Lyme disease requires erythema chronicum migrans (ECM), with or without subsequent evidence of rheumatologic, neurologic, or cardiac complications. When a child presents with arthritis, but without evidence of ECM, a diagnosis of Lyme disease may be difficult, particularly if there are conflicting laboratory findings. A clear history of a bite by a deer tick (*Ixodes dammini*) along with direct visualization of the *Borrelia burgdorferi* spirochete in pathologic materials, and/or positive cultures, are both unusual.[1] Further, the rheumatic and neurologic features of stage II and/or stage III Lyme disease will sometimes mimic other musculoskeletal or inflammatory disorders. Therefore, confirmation of Lyme disease often rests on serologic findings. New techniques to detect antibodies directed against *B burgdorferi*, such as the enzyme-linked immunosorbent assay (ELISA), have been developed,[2,3] and improved by preabsorption procedures,[2] but false-positive tests still occur. The Western blot (WB) assay is more sensitive and specific than the ELISA for detecting early Lyme disease (<31 days after ECM),[3] but its

specificity in the patient with late-stage disease or diffuse musculoskeletal complaints is less clear.

We have analyzed the history and physical findings of 58 children and adults with presumptive Lyme disease, whose sera were sent to our laboratory for ELISA and WB testing, to determine whether their clinical features correlated with the serologic findings.

MATERIALS AND METHODS

Between September 1988 and July 1989 we examined more than 650 sera for evidence of antibodies to *B burgdorferi*, using an ELISA[2]; of these, 77 were randomly selected for evaluation by a WB assay using both IgG and IgM monospecific *B burgdorferi* antisera. The clinical observations from these 77 consecutive patients who had WB assays form the basis of this report.

Antigens for the ELISA and WB assays were prepared from *B burgdorferi* strain B 31 (American Type Culture Collection). The spirochetes were grown at 34°C in Barbour Stoenner Kelly (BSK) medium.[4] Briefly, sera were diluted 1:80 with either a 10% suspension of heat-killed *Escherichia coli* in phosphate-buffered saline, or with phosphate-buffered saline alone. Following incubation and washing, diluted sera were added to microtiter wells containing a 10 000 × g supernatant fraction of sonicated *B burgdorferi*. After another incubation and washing, peroxidase conjugated anti-human IgG antiserum (Cappel) was added to each well for a 30-minute incubation. Wells were then washed, substrate 2,2′-azinobis(3-ethylbenzothiazoline) sulfonic acid added, and the optical density was determined using a Titertek Miniskan at 405 nm. An optical density of 0.2, which we have previously determined to be equivalent to a titer of ≥1:80, was considered positive.[2]

IgG and IgM antibodies to *B burgdorferi* antigens were measured, by WB analysis, in the same serum samples, as follows: spirochetal antigens were prepared by the method of Laemmli and Fayre[5] and electrophoretically separated on a single-trough (22 × 10 cm), thin-layer, horizontal 10% polyacrylamide gel. Separated antigens were transferred from the polyacrylamide gel electrophoresis gel to nitrocellulose strips using a Nova blot transfer system (LKB Laboratories). The nitrocellulose strips were then blocked with 0.5% bovine serum albumin, dried, and stored desiccated until needed. Patient sera were diluted 1:100 with blotting buffer (phosphate-buffered saline with 0.5% bovine serum albumin) and incubated with the nitrocellulose strips at room temperature for 1 hour. After washing, a 1:1000 dilution of biotinylated goat anti-human IgG or IgM (Kirkergaard and Perry Laboratories Inc) was added, and incubated for 1 hour. The strips were then washed and a 1:1000 dilution of peroxidase conjugated streptavidine (Kirkergaard and Perry Laboratories Inc) was added for a 1-hour incubation. Substrate (4-chloro-1-naphthol) was then added to visualize antibody binding, and reactivity was determined by comparison with controls. The presence of four or more reactive bands was considered a positive test. Examples of positive and negative blots are displayed in Fig 1.

Chart Analysis

Clinical data from patient records were obtained by a rheumatologist who was "blind" to the serologic results. Eleven medical records (15%) were not available, and 8 records (9.5%) contained insufficient information. The remaining 58 patients (75%) are the subject of the present study. There were 28 children from tertiary and primary care facilities and 30 adults from a primary care clinic. Table 1 describes the clinical review criteria and disease definitions used.

To correlate serologic and clinical observations, the study population was divided into three groups,

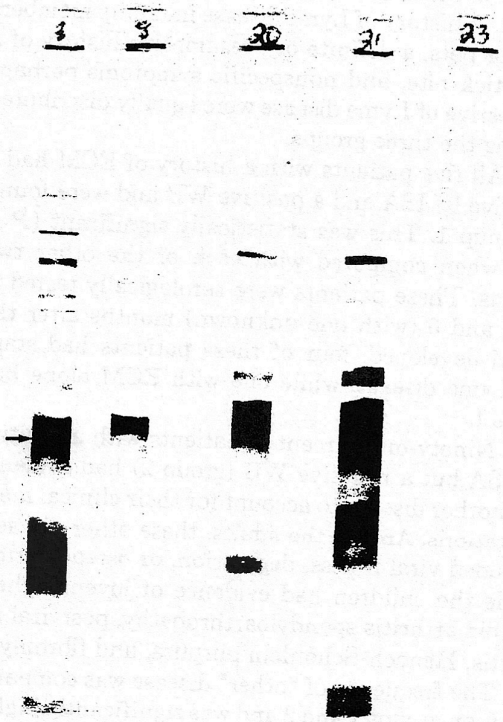

Fig 1. Western blot strips showing different degrees of reactivity. The arrow shows the 41-kd band. The last strip on the far right is the negative control.

according to serologic results: group 1, positive ELISA/positive WB; group 2, positive ELISA/negative WB; group 3, negative ELISA/negative WB.

Statistical Analysis

Pairs of counts for each clinical variable were compared, between groups, using the χ^2 test. A P value of $\leq .05$ was considered significant. ELISA values between groups were tested for significance using the Mann-Whitney nonparametric U test.

RESULTS

Among the 58 patients, there were 28 children (age range: 6 months–16 years) and 30 adults (age range: 17–74 years); there were 33 males and 25 females. Forty percent of the children and 10% of the adults were treated by rheumatologists, 30% of the children and 50% of the adults were followed up by infectious disease specialists, and the remainder were cared for by adult or pediatric general practitioners.

Group 1 (n = 16) consisted of 10 children and 6 adults, group 2 (n = 23) contained 11 children and 12 adults, and group 3 (n = 19) had 7 children and 12 adults. Their clinical features are presented in Table 2. Findings are briefly summarized below:

1. A history of Lyme disease in family members and/or pets, a definite or presumptive history of a deer tick bite, and nonspecific symptoms perhaps suggestive of Lyme disease were equally distributed among the three groups.

2. All five patients with a history of ECM had a positive ELISA and a positive WB and were found in group 1. This was statistically significant ($P \leq .05$) when compared with each of the other two groups. These patients were serologically tested 1, 4, 6, and 6 (with one unknown) months after the ECM developed; four of these patients had stage III Lyme disease, while one with ECM alone had stage I.

3. Ninety-one percent of patients with a positive ELISA but a negative WB (group 2) had evidence of another disease to account for their clinical manifestations. Among the adults, these other diseases included viral illness, depression, or osteoarthritis, while the children had evidence of juvenile rheumatoid arthritis spondyloarthropathy, postviral arthritis, Henoch-Schönlein purpura, and fibromyalgia. The frequency of "other" disease was compared between groups 1 and 2 and was significantly higher for group 2 (positive ELISA/negative WB) ($P < .01$). The two group 1 patients with "other" diseases were a child receiving anticonvulsants in whom antinuclear antibodies and knee effusions developed and a child who presented with antinuclear antibody-positive, polyarticular arthritis.

4. A history and clinical findings compatible with Lyme disease were found in 100% of group 1 patients (positive ELISA/positive WB). This was statistically significant at the $P \leq .01$ level. The clustering of patients with arthritis in group 1 was considered a probable consequence of referral bias.

TABLE 1. Clinical Review Criteria and Disease Definitions

A. Patient exposure to Lyme disease (LD): clear-cut episode of bite by a deer tick or history of LD in pets or family members.
B. Nonspecific symptoms of LD: illness of ≥2 wk duration and having 4 or more of headache/stiff neck, arthralgias, fever, fatigue, myalgias, and/or conjunctivitis.
C. Erythema chronicum migrans (ECM): convincing history, or observed by physician (spreading red rash, central clearing, mostly symptomless).
D. Arthritis, possibly due to LD: definite monoarticular or pauciarticular arthritis; no other defined cause.
E. Clinically likely LD: sequence of signs and symptoms compatible with LD (could include ECM and/or arthritis), based on physician chart review, blinded to serology test results.
F. Disease exclusions:
   1. Other rheumatic disorders, which fulfill standard diagnostic criteria and alternatively explain symptoms.
   2. Other inflammatory, nonrheumatic, noninfectious diseases, diagnosed at any time, which account for present signs/symptoms.
   3. Viral illness: short-duration disease which did not create diagnostic dilemma for the primary physician, who ordered Lyme tests because of patient request or for other unspecified reasons.
G. Antibiotic treatment response:
   1. A clearly recorded physician statement of response vs no response, at ≥8 wk, was evidence of "information available."
   2. A positive and significant treatment response was defined as ≥8 wk without symptoms, following an appropriate course of antibiotics.

TABLE 2. Summary of Clinical Findings, by Serologic Group

Variable	Group 1 (n = 16)	Group 2 (n = 23)	Group 3 (n = 19)
Possible Lyme exposure	3	4	8
Nonspecific symptoms	8	6	4
Erythema chronicum migrans	5*	0	0
Arthritis	11*	6	5
Other disease	2†	21	13
Clinically likely Lyme disease	16†	5	3

* $P < .05$ when compared with group 2 and group 3.
† $P < .01$ when compared with group 2 and group 3.

5. Sufficient data from charts were available to accurately assess antibiotic treatment responses in 50% and 45% of patients in groups 1 and 2, respectively. All group 1 and group 2 patients received antibiotics. Group 3 patients were not treated with antibiotics because they were believed not to have Lyme disease. Seventy-five percent of the patients in group 1 but only 11% of the patients in group 2 appeared to benefit from treatment. Although the number of patients with sufficient data to assess treatment efficacy was relatively small, the poor treatment responses in group 2 suggest that these patients may have had a disorder other than Lyme disease.

6. Mean ELISA test values (expressed in optical density units) were 1.019 (±0.131 SEM) in group 1 patients (ELISA positive/WB positive) and 0.509 (±0.031 SEM) among group 2 patients (ELISA positive/WB negative). This was a statistically significant intergroup difference ($P \leq .01$) (see Table 3 and Fig 2).

DISCUSSION

Without evidence of ECM, clinical confirmation of Lyme disease may be difficult because of the low specificity of common complaints such as fever, headaches, neurologic "abnormalities," and arthralgias/arthritis. In one large report of the clinical manifestations of Lyme disease seen in Connecticut, 83% of a combined group of children and adults had evidence of ECM[6]; however, 79% of the patients in this study who had arthritis did not have antecedent ECM. In a related study from Connecticut, of 1149 serologically confirmed Lyme disease patients 20 years of age or younger, only 10% of those with arthritis also had evidence of ECM.[7] Similarly, in a prospective evaluation of normal Swiss adults, 21 of 964 were found to have high titer anti-IgG anti-borrelia antibodies, but only 11% of these had prior evidence of ECM.[8] Only 31% of our patients with both a positive ELISA and a positive WB, and none in the other two serologic groups, presented with ECM.

TABLE 3. Enzyme-Linked Immunosorbent Assay Titers (Mean Optical Density) by Serologic Group

Group	n	Optical Density*		Low Value	High Value
		Mean	SE		
1	16	1.019	0.131	0.210	1.980
2†	23	0.509	0.031	0.221	0.810
3	19	0.111	0.013	0.000	0.193

* Optical density values ≥0.2 are considered positive.
† Mean of optical density values from group 2 was significantly lower ($P \leq .01$) than the mean of values from group 1.

In one recent report, among 25 children with definite Lyme arthritis as the study entry criterion, ECM was noted in only 44% of cases, and a history of tick bite was found in only 49%.[9] In a similar investigation of 43 children with proven Lyme arthritis, only 20 (47%) had evidence of ECM, while 19 (44%) recalled being bitten by a tick.[10] In another early investigation of 23 children with Lyme disease, the frequency of ECM was 74%, but only 22% had arthritis.[11] In our study, among 11 patients with acute or subacute onset arthritis and a positive WB, only 4 had ECM. Overall, fewer than half of adults or children with Lyme disease who first present with arthritis will have prior historical or clinical evidence of ECM; this emphasizes the utility of serologic testing for Lyme disease in patients with acute or subacute onset arthritis, but without ECM.

In the present investigation, performed in an endemic region of the country, we found no differences in the frequency of reported tick bites among the three different serologic groups. In two other studies of children with definite Lyme arthritis, recall of a tick bite was noted in 44%[10] and 48%[9] of cases.

As expected, we found nonspecific signs and symptoms to be equally distributed among our three study groups, and thus of little help in confirming a diagnosis of Lyme disease. Although fever and/or a preceding febrile illness were found in 37% of a group of children with Lyme disease described by Culp and coworkers,[10] these are also commonly observed in viral, postviral, and other reactive arthropathies. Other clinical features that develop either prior to, or coexistent with, an episode of Lyme-related arthritis are usually nonspecific (eg, fever, headache, conjunctivitis) or uncommon (Bell's palsy, aseptic meningitis).[7,9,10]

The arthritis associated with Lyme disease is usually transient, monoarticular or oligoarticular, and most often affects large joints such as the knees. If untreated, Lyme arthritis may be recurrent, and rarely it can become erosive.[12,13] However, in the absence of ECM the arthritis associated with Lyme disease is often nonspecific, and it is not easily differentiated from juvenile or adult rheumatoid arthritis, spondyloarthritis, postinfectious reactive arthritis, or other rheumatic disorders.[14] Further, routine synovial fluid analysis was not found to be helpful for diagnosis of Lyme disease in 4 children studied by Eichenfield et al,[9] and the histopathologic findings are similar in adult rheumatoid and Lyme synovitis.[15] Given this lack of specificity of presenting symptoms, it could be expected that significant numbers of patients might be misclassified as having Lyme disease.

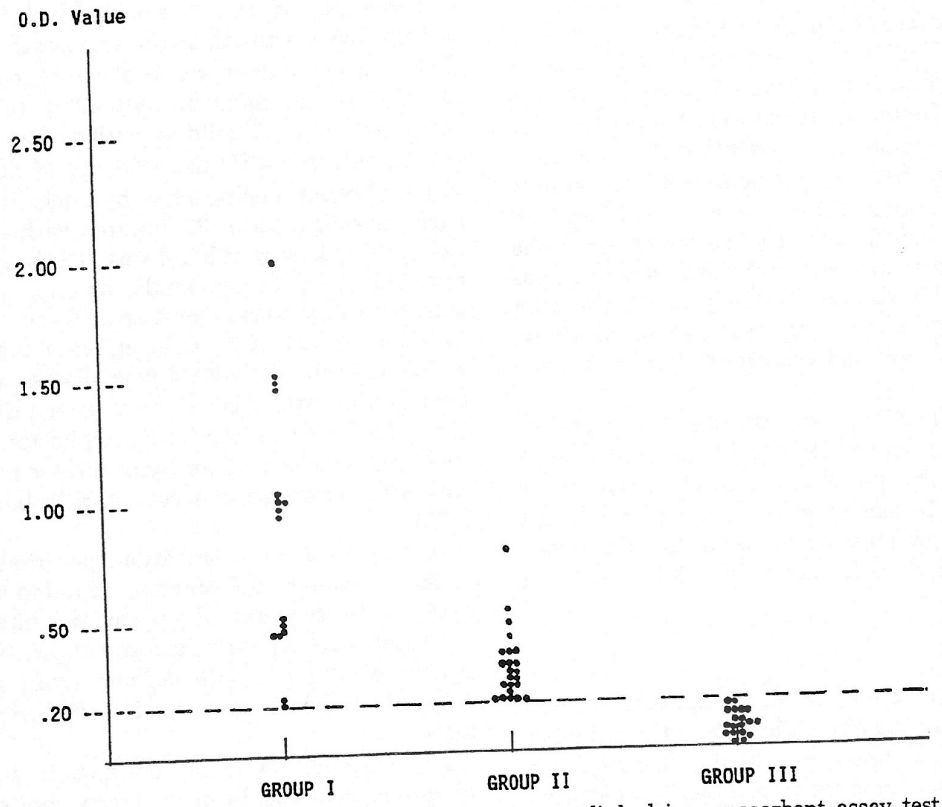

Fig 2. Optical density (OD) values for the enzyme-linked immunosorbent assay test displayed by patient groups. The dotted line represents a cutoff at 0.20 (equivalent to titer of 1:80). Each point represents one patient's OD value.

The direct visualization of *B burgdorferi* in tissue specimens, and/or isolation in cultures, is extremely unusual.[15–19] Thus, laboratory confirmation of diagnosis is presently limited to serologic tests. Although the sensitivity of immunofluorescent and ELISA assays for patients with early Lyme disease (mostly manifest as ECM) can be as low as 14% to 30%,[6] sensitivity can be as high as 91% in a combined population of patients with stage II and stage III Lyme disease.[20] One study compared the immunofluorescent assay, ELISA, and T*reponema phagedenis* preabsorbed ELISA assays, using sera from confirmed Lyme disease and from other autoimmune and infectious diseases, and showed the preabsorbed test to be more sensitive (86%) and more specific (94%) than the other two assays when testing sera from non-Lyme infectious disorders such as Epstein-Barr virus, syphilis, etc.[21] Both forms of the ELISA were better than the immunofluorescent assay when sera from autoimmune disorders were tested. Conversely, Craft et al[20] were able to block cross-reactivity to the Lyme spirochete by preabsorption of test sera with *Borrelia hermsii* but not with *Treponema reiter*. We have been able to show that preabsorption with *E coli*, of sera from patients with Lyme disease, prior to performing ELISA testing leads to increased specificity and higher sensitivity when compared with the ELISA performed on intact sera.[2] In the present study, all ELISAs were done on preabsorbed sera.

The definition of a positive test for Lyme disease has not been fully established for either the ELISA[22] or the WB assay. Complete agreement on how many, or which specific, band(s) should be required to determine positivity in the WB assay may not be possible as long as individual laboratories use different methods of antigen preparation and separately determine assay sensitivity and specificity.[23–25] Presently, we consider the presence of four reactive bands to be a positive result, while one to three reactive bands is considered a "gray" zone, indicative of possible disease.

In our series, the patients with the most convincing signs and symptoms suggestive of Lyme disease were limited to group 1, who exhibited both positive ELISA and positive WB tests. It is also notable that a high percentage of group 2 patients (positive ELISA, negative WB) had some better alternative explanation for their symptoms. The association of a positive WB, presence of the pathognomonic

ECM, and perhaps a better response to antibiotic therapy, suggests that a positive WB assay provides strong evidence for a diagnosis of Lyme disease. This correlation may also be strengthened by our observation that antibody levels detected by ELISA, expressed as optical density, were significantly higher in patients who were WB positive than in those who were WB negative. This suggests that low titer ELISA values should be further evaluated with a WB prior to offering either a diagnosis or treatment to a patient with suspected Lyme disease.

The underlying mechanism(s) for discordant assay results in some patients is not clear. Our ELISA and WB both use the same antigen source (sonicated *B burgdorferi*). It is conceivable, however, that some antinucleic acid or antiphospholipid antibodies reacting with components of the *Borrelia* could cause discordant results.[26] In a related area, we have found that patients who are antinuclear antibody positive are more likely to demonstrate a false-positive ELISA; this association between antinuclear antibodies and ELISA has been previously noted.[2] Further work is needed to clarify these issues.

In summary, we have demonstrated that the occurrence of Lyme disease in the absence of a positive WB test is very unlikely. A positive ELISA test, particularly at a low titer and without a positive WB, is not associated with a clinical diagnosis of Lyme disease. Finally, the combination of a positive ELISA and a positive WB is strongly associated with the presence of clinical Lyme disease.

ACKNOWLEDGMENTS

This study was supported, in part, by a grant from the Delaware Chapter of the Arthritis Foundation.

We gratefully acknowledge Dr George R. Hilty III for allowing us to include his patients; Kathy Gibney, RT, for performing the ELISA tests; Gail McIlvain-Simpson, MSN, RN, for coordination of patient care; and Eleanor Wyatt for secretarial assistance.

REFERENCES

1. Steere AC. Lyme disease. *N Engl J Med.* 1989;321:586–596
2. 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;32:1041–1044
3. Grodzicki RL, Steere AC. Comparison of immunoblotting and indirect enzyme-linked immunosorbent assay using different antigen preparations for diagnosis of early Lyme disease. *J Infect Dis.* 1988;157:790–797
4. Barbour AG, Burgdorfer W, Hayes SF, et al. Isolation of a cultivatable spirochete from *Ixodes ricinus* ticks of Switzerland. *Curr Top Microbiol Immunol.* 1983;8:123–126
5. Laemmli UK, Fayre M. Maturation of the head of bacteriophage T4. *J Mol Biol.* 1973;80:575–599
6. Centers for Disease Control. Lyme disease—Connecticut: MMWR 1988. *JAMA.* 1988;259:1147–1148
7. Petersen LR, Sweeney AH, Checko PJ, et al. Epidemiological and clinical features of 1149 persons with Lyme disease identified by laboratory-based surveillance in Connecticut. *Yale J Biol Med.* 1989;62:253–262
8. Fahrer H, VD Linden S, Sauvain M, Gerber N. "Lyme borreliosis": an epidemiologic study in a Swiss risk population. *Arthritis Rheum.* 1987;30:S50
9. Eichenfield AH, Goldsmith DP, Benach JL, et al. Childhood Lyme arthritis: experience in an endemic area. *J Pediatr.* 1986;109:753–758
10. Culp RW, Eichenfield AH, Davidson RS, Drummond DS, Christofersen MR, Goldsmith DP. Lyme arthritis in children: an orthopedic prospective. *J Bone Joint Surg Am.* 1987;69A:96–99
11. Cristofaro RL, Appel MH, Gelb RI, et al. Musculoskeletal manifestations of Lyme disease in children. *J Pediatr Orthop.* 1987;7:527–530
12. Steere AC, Malawista SE, Snydman DR, et al. Lyme arthritis. *Arthritis Rheum.* 1977;20:7–17
13. Steere AC, Schoen RT, Taylor E. The clinical evolution of Lyme arthritis. *Ann Intern Med.* 1987;107:725–731
14. Dlesk A, Bjarnason DF, Mitchell P, McCarty P. Lyme disease presenting as seronegative rheumatoid arthritis. *Ann NY Acad Sci.* 1989;539:454–455
15. Steere AC, Duray PH, Butcher EC. Spirochetal antigens and lymphoid cell surface markers in Lyme synovitis. *Arthritis Rheum.* 1988;31:487–495
16. Snydman DR, Scheinkein DP, Berardi VP, Lastavica CC, Pariser KM. *Borrelia burgdorferi* in joint fluid in chronic Lyme arthritis. *Ann Intern Med.* 1986;104:798–800
17. Atlas E, Novak SN, Duray PH, Steere AC. Lyme myositis: muscle invasion by *Borrelia burgdorferi*. *Ann Intern Med.* 1988;109:245–246
18. Benach JL, Bosler EM, Hanrahan JP, et al. Spirochetes isolated from the blood of two patients with Lyme disease. *N Engl J Med.* 1983;308:740–742
19. Valesova M, Trnavsky K, Hulinska D, et al. Detection of *Borrelia* in the synovial tissue from a patient with Lyme borreliosis by electron microscopy. *J Rheumatol.* 1989;16:1502–1505
20. Craft JE, Grodzicki RL, Steere AC. Antibody response in Lyme disease: evaluation of diagnostic tests. *J Infect Dis.* 1984;149:789–795
21. Mertz LE, Wobig GH, Duffy J, Katzmann JA. A comparison of test procedures for the detection of antibody to *Borrelia burgdorferi*. *Ann NY Acad Sci.* 1988;539:474–475
22. Schwartz BS, Goldstein MD, Ribeiro JMC, et al. Antibody testing in Lyme disease: a comparison of results in four laboratories. *JAMA.* 1989;262:3431–3434
23. Karlsson M, Mollegard I, Stiernstedt G, Wretlind B. Comparison of Western blot and enzyme-linked immunosorbent assays for diagnosis of Lyme borreliosis. *Eur J Clin Microbiol Infect Dis.* 1989;8:871–877
24. Guy EC, Martyn CN, Bateman DE, et al. Lyme disease: prevalence and clinical importance of *Borrelia burgdorferi* specific IgG in forestry workers. *Lancet.* 1989;1:484–486
25. Fister RD, Weymouth LA, McLaughlin JC, et al. Comparative evaluation of three products for the detection of *Borrelia burgdorferi* antibody in human serum. *J Clin Microbiol.* 1989;27:2834–2837
26. Mackworth-Young CG, Harris EN, Steere AC, et al. Anticardiolipin antibodies in Lyme disease. *Arthritis Rheum.* 1988;31:1052–1056