Proceedings of the

Second National Conference on Serologic Diagnosis of Lyme Disease

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SECOND NATIONAL CONFERENCE ON SEROLOGIC DIAGNOSIS OF LYME DISEASE

ASTPHLD Evaluation of Diagnostic Products
Committee--1993-94 and 1994-95

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The Association of State and Territorial Public Health Laboratory Directors (ASTPHLD) represents state and territorial public health laboratory directors throughout the United States. ASTPHLD maintains a Washington D.C. headquarters office and seven Area Resource Offices of the National Laboratory Training Network (NLTN). NLTN is a cooperative training system sponsored in conjunction with the Centers for Disease Control and Prevention (CDC) that forms alliances among federal, state and local health agencies and private sector organizations to develop and deliver localized laboratory training programs based on documented need. ASTPHLD organizes and presents scientific conferences and symposia relevant to testing activities of public health laboratories. The ASTPHLD Mission: "To improve and promote the contributions of public health laboratories in support of the public health objectives of disease prevention and health promotion; and to promote policies, programs, and practices which contribute to improvement in the quality of health and environment laboratory services."

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RECOMMENDATIONS

1. Test Performance and Interpretation

Recommendation 1.1. Two-Test Protocol

All serum specimens submitted for Lyme disease testing should be evaluated in a two-step process, in which the first step is a sensitive serological test, such as an enzyme immunoassay (EIA) or immunofluorescent assay (IFA). All specimens found to be positive or equivocal by a sensitive EIA or IFA should be tested by a standardized Western blot (WB) procedure Specimens found to be negative by a sensitive EIA or IFA need not be tested further.

Recommendation 1.2. WB Controls

Immunoblotting should be performed using a negative control, a weakly reactive positive control, and a high-titered positive control. The weakly reactive positive control should be used to judge whether a sample band has sufficient intensity to be scored. Monoclonal or polyclonal antibodies to antigens of diagnostic importance should be used to calibrate the blots.

Recommendation 1.3. Testing and Stage of Disease

When Western immunoblot is used in the first four weeks after disease onset (early Lyme disease), both IgM and IgG procedures should be performed. Most Lyme disease patients will seroconvert within this four week period. In the event that a patient with suspected early Lyme disease has a negative serology, serologic evidence of infection is best obtained by testing of paired acute- and convalescent-phase samples. In late Lyme disease, the predominant antibody response is usually IgG. It is highly unusual that a patient with active Lyme disease has only an IgM response to *Borrelia burgdorferi* after one month of infection. A positive IgM test result alone is not recommended for use in determining active disease in persons with illness of longer than one month duration, because the likelihood of a false-positive test result is high for these individuals.

Recommendation 1.4. WB Criteria

Use of the criteria of Engstrom *et al.* are recommended for interpretation of IgM immunoblots (Engstrom,S.M., Shoop, E., and Johnson, R.C. [1995]. Immunoblot interpretation criteria for serodiagnosis of early Lyme disease. *J. Clin. Microbiol.*, 33:419-422). An IgM blot is considered positive if two of the following three bands are present: 24 kDa (OspC), 39 kDa (BmpA), and 41 kDa (Fla).

Monoclonal antibodies to these three proteins have been developed and are calibrating immunoblots.[1]

Once antibodies are developed to the 37 kDa antigen, this protein could be use additional band for IgM criteria (≥2 of 4 bands).

Interim use of the criteria of Dressler *et al.* are recommended for interpretation immunoblots (Dressler, F., Whalen, J.A., Reinhart, B.N. and Steere, A.C. [1993]. blotting in the serodiagnosis of Lyme disease *J. Infect. Dis.*, 167:392-400). An IgG considered positive if five of the following ten bands are present: 18, 21 (OspC), 28 (BmpA), 41 (Fla), 45, 58 (not GroEL[2]), 66 and 93 kDa.

Monoclonal antibodies have been developed to the OspC, 39 (BmpA), 41 (Fla), 66, kDa antigens and are suitable for calibrating IgG immunoblots.[1]

The apparent molecular mass of OspC is recorded above as it was denoted in the pu literature. The protein referred to as 24 kDa or 21 kDa is the same, and should be id in immunoblots with an appropriate calibration reagent (see 1.6).

Recommendation 1.5. Reporting of Results

An equivocal or positive EIA or IFA result followed by a negative immunoblot r be reported as negative. An equivocal or positive EIA or IFA result followed by a p immunoblot result should be reported as positive.

An explanation and interpretation of test results should accompany all reports.

Recommendation 1.6. Standardization of WB Nomenclature

The apparent molecular mass of some proteins of *Borrelia burgdorferi* such as Osp vary depending on the *B. burgdorferi* strain and gel electrophoresis system used molecular weights of proteins of diagnostic importance should be identified with mon

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[1]See **ADDENDUM**, Monoclonal antibodies to selected proteins of *Borrelia burgdor* that have been used to calibrate immunoblots.

[2]At the Dearborn conference, this band was referred to as "60 kDa (GroEL)." Since conference, it has been determined that the band of diagnostic significance scored by Dressl *al.* can be distinguished from GroEL, although it is of nearly the same apparent molecular m The band that should be scored is referred to here as "58 kDa" which is consistent with the original nomenclature of Dressler *et al.* and emphasizes that this band is not GroEL.

or polyclonal antibodies (Engstrom et al., 1995). When possible, the molecular weight of the protein should be followed by the descriptive name (e.g. OspC).

Recommendation 1.7. Antibodies to *B. burgdorferi* Antigens

A high priority for industry, possibly through a government contract, is to develop monoclonal or polyclonal antibodies to WB bands of interest. As antibody reagents are developed, they should be made available to researchers and laboratorians through the CDC, NIH, or industry.

There is a priority to resolve the identification of low molecular weight bands with appropriate monoclonal antibodies.

2. Quality Assurance Practices

Recommendation 2.1. *B. burgdorferi* Strain

It is important to use a strain of *B. burgdorferi* (e.g. 2591, low passage 297, or low passage B31) that expresses appropriate amounts of immunoreactive proteins of diagnostic interest. While the selection of a single strain would be desirable, no such strain can be designated at this time. Further evaluations can be carried out by comparisons in proficiency testing programs.

Recommendation 2.2. Test Request Information

In order to assure appropriate test selection and interpretation of test results, complete patient information, including date of onset of disease and date of specimen collection, should be included on the request form.

Recommendation 2.3. Quality Control

Lyme disease testing should be performed only in laboratories that have comprehensive quality assurance programs and trained personnel competent in all aspects of quality control of serologic testing.

Recommendation 2.4. Proficiency Testing

Laboratories performing Lyme disease testing in support of patient diagnosis and treatment should be enrolled and participate satisfactorily in an approved Health Care Financing Administration (HCFA) proficiency testing program.

Serum samples used to evaluate screening tests or Western Blots in prof should cover all stages of Lyme disease, and samples should be representative of population. Each sample should be from a single donor.

Recommendation 2.5. Serum Bank

A repository of serum specimens from patients with well characterized *B. bu* infections (early and late), other spirochetal infections, other infections and infla disorders that have shown cross-reactivity in Lyme disease testing, and normal serum from non-endemic areas should be maintained by the CDC. Industry should resources to develop appropriate serum panels. These panels should be made ava research and development laboratories and to testing laboratories for validation stu

3. New Test Evaluation and Clearance

Recommendation 3.1. New Serologic Methods

Serologic methods based on recombinant antigens or novel technologies may i capabilities to evaluate patients for Lyme Disease. These methods may be devel replace one or both components of the recommended two-test protocol. Before ne can be recommended for diagnostic testing, their specificity, sensitivity, and precision be equal to or better than the performance determined for the recommend procedures.

Recommendation 3.2. Evaluation of New Serologic Methods

All new assays should include, as a step in their evaluation, blind testing ag comprehensive challenge panel as described in Recommendation 5 of Quality Ass Practices.

Recommendation 3.3. Direct Detection Methods

Antigen assays, amplification techniques such as PCR, and other direct detection m must be rigorously evaluated before their potential for diagnostic use can be determine evaluations should be blinded and contain samples from early and late stages of Lyme di Duplicate samples should be included to evaluate precision.

4. Communication of Developments in Lyme Disease Testing

Recommendation 4.1. Conference Proceedings

The proceedings of the Second National Conference on Serologic Diagnosis of Lyme Disease should be made available to all facilities performing Lyme disease testing, to manufacturers of reagents, and to appropriate government agencies.

Recommendation 4.2. Lyme Disease Surveillance Summary

This publication of the Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, CDC, should be widely distributed to serve as a vehicle for communication between industry, governmental agencies, testing laboratories, researchers, and regulators.

ADDENDUM

Monoclonal antibodies to selected proteins of *Borrelia burgdorferi* that have been used calibrate immunoblots

Antibody	Specificity	Isotype	Investigator	Ref No
181.1[1]	93 kDa	IgG1	Benjamin Luft SUNY, Stony Brook, NY	6
8D5[1]	66 kDa	IgG1	Alan Barbour UT Health Sciences Center San Antonio, TX	--
149	GroEL, 62 kDa	IgG1	Benjamin Luft	5
H9724[1]	Fla, 41 kDa	IgG2a	Alan Barbour	1
H1141[1]	BmpA, 39 kDa	IgG2	Thomas Schwan NIH, Rocky Mountain Labs, Hamilton, MT	9
84C	OspB, 34 kDa	IgG2b	Denée Thomas UT Health Sciences Center, San Antonio, TX	
H5332	OspA, 31 kDa	IgG1	Alan Barbour	2
H1C8[2]	OspD, 29 kDa	IgG3	Alan Barbour	8
4B8F4[1]	OspC, 23 kDa	IgG2a	Steven Padula U of Conn Health Center, Farmington, CT	as p 7
CB625	22 kDa	IgG1	Jorge Benach SUNY, Stony Brook, NY	3

[1]These monoclonal antibodies identify antigens of diagnostic importance specified in the recommended criteria fo immunoblot interpretation. The other antibodies have been used as calibration markers, pending development of monoclonals to the antigens recommended for scoring of IgG blots.

[2]Reactive with strain B31, but not with strains 297 and 2591.

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PLENARY PRESENTATIONS

Welcome / Conference Objectives / Review of Progress

Robert Martin, Dr.P.H., President-Elect, ASTPHLD and Laboratory Director, Michigan Department of Health

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Value of Standardization of Serologic Testing in Clinical Diagnosis of *Borrelia burgdorferi* Infections

Article Entitled: Western Blotting in the Serodiagnosis of Lyme Disease[1]

Allen Steere, M.D., Professor of Medicine / Chief of Rheumatology, Tufts, New England Medical Center

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Standardization of Serologic Testing for Epidemiological Purposes

David T. Dennis, M.D., Chief, Bacterial Zoonoses Branch, Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention
(could not attend the conference)

Test Approach and *Borrelia burgorferi* Strain Selection for Standardization of Serodiagnosis of Lyme Disease

Barbara Johnson, Ph.D., Division of Vector-Borne Infectious Diseases, National center for Infectious Diseases, Centers for Disease Control and Prevention

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Recommendations of the Work Group for Standard Criteria for Lyme Serodiagnosis

Arthur Weinstein, M.D., New York Medical College, Division of Rheumatology

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Evaluation and Standardization of New Diagnostic Tests

Raymond J. Dattwyler, M.D., Division of Allergy, Rheumatology and Clinical Immunology, SUNY at Stony Brook
(No paper prepared for the proceedings)

Article Entitled: Immunoblot Interpretation Criteria For The Serodiagnosis of Early Lyme Disease[1]

Russell C. Johnson, Ph.D., Department of Microbiology, Medical School, University of Minnesota

Criteria for FDA Clearance of Diagnostic tests

Roxanne Shively, M.S, M.T. (ASCP), Scientific Reviewer, Office of Device Evaluation, Food and Drug Administration

Importance of Standardization of Laboratory Methods

Kenneth D. McClatchey, M.D, D.D.S., National Committee for Clinical Laboratory Standards
(No paper prepared for the proceedings)

Summary and Future Direction

Duane J. Gubler, Sc.D., Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention

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WORKGROUPS

WORKGROUP A: Standardization and Interpretation

Setting cutoffs for EIA and Western Blot; Are there alternatives to a two-test approach? What should one know about antigens? Use of IgM and IgG blots in diagnosis of early diseases; Band Interpretation.

Moderator: Stanley Inhorn, M.D., Co-Director, Wisconsin State Laboratory of Hygiene

Co-Moderator: Russell C. Johnson, Ph.D., Chair, Department of Microbiology, University of Minnesota, School of Medicine

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WORKGROUP B: Technical Issues in Test Performance

Guidelines for reporting; Proficiency Testing; Education and Training Issues in Lyme Testing; Standardization of Reagents; Personnel Performance; Specimen and Submission Forms; "Home-Brewed" tests versus commercial kits

Moderator: Eric Blank, Dr.P.H., Director, State Public Health Laboratory, Missouri

Co-Moderator: Raymond W. Ryan, Ph.D., Department of Laboratory Medicine, University of Connecticut Health Center

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WORKGROUP C: Certification and New Test Evaluation

What are guidelines for evaluation of new antibody tests? What are guidelines for evaluation of new antigen or DNA direct detection tests? What are criteria for modification of currently approved tests and for a new generation of tests? Criteria for FDA clearance.

Moderator: Ralph J. Timperi, Director, State Laboratory Institute, Massachusetts

Co-Moderator: Alan G. Barbour, M.D., Division of Infectious Diseases, Department of Medicine, University of Texas Health Sciences Center

SECOND NATIONAL CONFERENCE ON SEROLOGIC DIAGNOSIS OF LYME DISEASE

WELCOMING REMARKS

Robert Martin, Dr.P.H.

Welcome to the Second National Conference on Serologic Diagnosis of Lyme Disease. This conference is sponsored by the Association of State and Territorial Public Health Laboratory Directors, the U.S. Centers for Disease Control and Prevention and the Michigan Department of Public Health. Our conference is co-sponsored by the U.S. Food and Drug Administration, the National Institutes of Health, the Council of State and Territorial Epidemiologists and the National Committee for Clinical Laboratory Standards.

The First National Conference on the Serologic Diagnosis of Lyme Disease was held here in 1990. The purpose of that meeting was to bring together research scientists, manufacturers and representatives from state and federal agencies to address problems associated with Lyme disease testing. The format of that meeting was similar to other meeting sponsored by the ASTPHLD. Presentations were made to the entire group to outline the nature of the various issues being dealt with, and then participants were divided into workgroups with the purpose of bringing recommendations on these issues.

At the 1990 meeting, ASTPHLD and CDC reviewed the results of a study examining a variety of commercially available diagnostic test kits. Serum samples used in the evaluation were from clinically diagnosed cases; however, the experience and expertise of the diagnosing physicians varied widely. The results of the examination of test kits revealed fundamental problems with serological testing. Not only was there tremendous variation among tests, but similar variation existed between laboratories, and in some cases, within laboratories using the same test. It was recognized that we had to go back to the beginning and focus on a few major issues.

Recommendations of the first meeting included a recommendation to publish the results of the test kit evaluation, to publish the proceeding of the first meeting, publish a summary in MMWR and to convene a conference with NIH to discuss "state of the art" issues that would help us to move toward better diagnostic tests.

Again, in order to develop better diagnostic tests, we first needed a reference collection of well characterized serum specimens. Two such panels have been established at the CDC in Fort Collins: a large volume panel, available to manufacturers and researchers and a small volume panel of serial specimens.

It was also recognized that in order to have well characterized serum samples, the best specimens would be from those individuals from whom Borrelia had been isolated. At that time, it was believed that the organism was very difficult to cultivate and that a better isolation medium was needed. However, as shown by Berger and Johnson, the problem was not the medium but the specimen. They clarified that the optimal specimen was normal skin at the leading edge of the lesion. The sensitivity of culture of such specimens using BSK medium was approximately 86%.

The third recommendation was to convene a conference on the use of serologic test methods at such a time when new and useful information on the topic was available. We believe that in the past four years a significant amount of new information has been collected to present to this group.

The fourth recommendation addressed the need to convene working groups. A little historical information is useful at this point. In 1982, CDC initiated surveillance for Lyme disease and at that time there were 11 participating states. In 1990, the CSTE/CDC case definition was implemented in 47 states. From 1991 through 1993 Lyme disease became nationally notifiable. In 1992, as a result of this meeting, FDA issued guidelines for manufacturers of Lyme disease assays. These guidelines dealt with issues such as specificity, sensitivity, predictive value and defining the populations in which studies had been done.

Subsequent to the first conference, an Academic Reference Center study was implemented. This study used a panel of well characterized serum samples and as a result of this study a two step process was defined for serological diagnosis; an enzyme immunoassay followed by a Western blot.

In 1993, CSTE and CDC reviewed the case definition, but no changes were made at that time.

In May, 1994, a working group was convened in Fort Collins to address issues pertaining to Western blot. That group consisted of research scientists from CDC, from various medical centers and universities, and ASTPHLD representatives. As a result of that meeting, it was determined that a multicenter study would be done to evaluate IgM criteria for early diagnosis of Lyme disease. During the study, the group recommended following the criteria of Dressler and Steere, requiring a certain number of specific bands in order to be considered a positive test. It was clear from available data that different investigators were identifying the same bands and assigning different molecular weights. In order to clarify this issue, CDC obtained monoclonal antibodies to identify individual protein bands.

That brings us to this conference. As I stated earlier, we feel that a significant amount of additional information is now available to develop new recommendations for serologic diagnosis of Lyme disease.

Workgroup A will be dealing with standardization and interpretation. Among the issues to be discussed in this workgroup are cutoffs for EIA and Western blot, possible alternatives to a two step approach, antigens, IgM and IgG blots in diagnosis of early disease, and band interpretation.

Western Blotting in the Serodiagnosis of Lyme Disease

Frank Dressler,* Jennifer A. Whalen,*
Bruce N. Reinhardt,* and Allen C. Steere

Division of Rheumatology/Immunology, Tufts University Sch Medicine, New England Medical Center, Boston, Massach

There are currently no accepted criteria for positive Western blots in Lyme disease. In a retrospective analysis of 225 case and control subjects, the best discriminatory ability of test criteria was obtained by requiring at least 2 of the 8 most common IgM bands in early disease (18, 21, 28, 37, 41, 45, 58, and 93 kDa) and by requiring at least 5 of the 10 most frequent IgG bands after the first weeks of infection (18, 21, 28, 30, 39, 41, 45, 58, 66, and 93 kDa). When these definitions were tested in a prospective study of all 237 patients seen in a diagnostic Lyme disease clinic during a 1-year period and in 74 patients with erythema migrans or summer flu-like illnesses, the IgM blot in early disease had a sensitivity of 32% and a specificity of 100%; the IgG blot after the first weeks of infection had a sensitivity of 83% and a specificity of 95%. Among patients with indeterminate IgG responses by ELISA, 6 of 9 patients with active Lyme disease had positive blots compared with 2 of 34 patients with other illnesses ($P < .001$). Thus, Western blotting can be used to increase the specificity of serologic testing in Lyme disease.

Lyme disease or Lyme borreliosis is a multisystem infection caused by the tickborne spirochete *Borrelia burgdorferi* [1]. The illness often begins with localized infection of the skin, erythema migrans [2], followed within days to weeks by dissemination of the spirochete to many organs, particularly to other skin sites, the nervous system [3], or joints [4]. Months to years later, usually after periods of latent infection, patients may develop persistent arthritis [4], chronic neurologic involvement [5], or acrodermatitis chronica atrophicans [6].

Because culture or visualization of *B. burgdorferi* from patient specimens has been difficult [7], diagnosis has depended on recognition of a characteristic clinical picture with serologic confirmation. Serologic tests currently available for use in this disorder include ELISA [8–14], indirect immunofluorescence assay (IFA) [8–10], and Western blotting or immunoblotting [15–23]. Antigen preparations for these tests include sonicated spirochetes [8–11, 15–23] or partially purified [12, 13, 15] or recombinant proteins [14]. Serodiagnosis with each of these methods has been com cated by the cross-reactivity of certain spirochetal polyp tides with other antigens [24–26], the delay in the deve ment of the humoral immune response [7, 27, 28], dampening effect of early antibiotic therapy on this respo [11, 29], and the variability of the response in different tients [15, 28]. Furthermore, in a small subset of pati with late Lyme disease who are incompletely treated v antibiotics during the first several weeks of infection, the moral immune response to *B. burgdorferi* may be abor but a cellular immune response to the spirochete may usu be demonstrated in these patients by the T cell prolifera assay [30, 31]. With each of these methods, lack of standa ization has caused significant interlaboratory variation in results [32, 33], which has led to considerable diagno confusion.

The specific immune response in Lyme disease devel gradually over a period of months to years to ≥10 spiroch polypeptides [28]. These antigens include the 31-kDa ou surface protein (Osp) A, the 34-kDa OspB [34, 35], the kDa OspC (Wilske B, Max von Pettenkofer Institut, Muni personal communication), the 41-kDa flagellar prot which is similar to the flagellar antigens of other spiroche [24], and the 58-, 66-, and 74-kDa heat-shock proteins t have homologies with the 60-kDa groEL and the 70-k DnaK heat-shock protein families of *E. coli* [25, 26]. T functions of other prominent antigens, including those at 28, 30, 37, 39, 45, and 93 kDa, are not yet clear.

There are currently no accepted criteria for positive W ern blots in Lyme disease. The purpose of the current stu was to develop such criteria in a retrospective analysis patients with various manifestations of Lyme disease, to termine the sensitivity and specificity of these criteria in p spective studies of patients with early or late manifestatio of the disorder, and to compare the results obtained ELISA and Western blotting.

Received 15 July 1992; revised 15 September 1992.

Presented in part: annual meeting of the American College of Rheumatology, Boston, November 1991 (abstract B94, Arthritis Rheum 1991;34:S113); International Conference on Lyme Borreliosis, Arlington, Virginia, May 1992 (abstract 15).

Informed consent was obtained from patients or their parents, and human experimentation guidelines of the US Department of Health and Human Services were followed.

Financial support: National Institutes of Health (AR-20358, AR-40576); Eshe Fund; Deutsche Forschungsgemeinschaft (1989–1990 research scholarship to F.D.); Becton Dickinson (1991 young investigator award to F.D.).

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Methods

Study patients. For the retrospective study, frozen samples stored at −70°C were selected from our serum bank, in alphabetical order, from the first 25 patients each with erythema migrans, meningitis, arthritis, or encephalopathy or polyneuropathy due to Lyme disease. Patients with erythema migrans were classified as having localized skin infection or disseminated infection according to clinical criteria [36]. The patients with erythema migrans, meningitis, or arthritis had not received prior antibiotic therapy, whereas half of the patients with encephalopathy or polyneuropathy had previously been given antibiotics. For comparison, sera were tested from 25 patients who had participated in an influenza vaccination program (an acute infectious disease antigen), 15 patients with multiple sclerosis and 10 with amyotrophic lateral sclerosis (neurologic diseases), 15 patients with rheumatoid arthritis and 10 with systemic lupus erythematosus (rheumatologic diseases), 25 patients with chronic fatigue syndrome (often misdiagnosed as Lyme disease), and 25 patients with secondary or latent syphilis (another spirochetal infection).

In the prospective study, the sera were tested from all 237 patients evaluated in our weekly diagnostic Lyme disease clinic from July 1990 through June 1991. By clinical criteria, these patients were categorized as having active Lyme arthritis or neuroborreliosis, inactive Lyme disease, or other illnesses. Active Lyme arthritis was defined as brief attacks of oligoarticular arthritis in a few large joints, not caused by other known types of arthritis, in a person from an area endemic for Lyme disease. These patients were required to have objective evidence of joint inflammation at the time of evaluation. Active neuroborreliosis (meningitis, chronic encephalopathy, or polyneuropathy) was diagnosed in patients with meningeal signs, memory impairment, or sensory abnormalities accompanied by a cerebrospinal fluid (CSF) pleocytosis, increased CSF protein, or electromyographic evidence of an axonal polyneuropathy, not caused by other known diseases, in a person from a Lyme disease–endemic area [31]. These patients often had a history of erythema migrans, but this clinical marker was not required for diagnosis. After clinical categorization, all 237 patients were tested for serum antibodies to *B. burgdorferi* by ELISA and Western blotting. To determine the sensitivity and specificity of these tests, results in the 54 patients who met clinical criteria for Lyme disease were compared with those in the 183 patients who did not meet these criteria. If patients met clinical criteria for Lyme disease but were seronegative by ELISA, their cellular immune response to borrelial antigens was determined by the T cell proliferative assay, as previously described [31].

Since patients with early Lyme disease are not commonly seen in this clinic, acute and convalescent sera were tested from all 57 patients with erythema migrans entered into a multicenter antibiotic treatment study in 1989 [36] and from the patients with summer flu-like illnesses evaluated for entry into that same study who were not thought to have Lyme disease. Erythema migrans was defined as an expanding annular skin lesions ≥5 cm in diameter, usually with central clearing and a bright red outer border. Diagnosis was based on observation of this rash by the study physicians.

ELISA methods. The IgG antibody response to *B. burgdorferi* was determined by indirect ELISA, and the specific IgM response was determined by an antibody capture EIA with modifications of previously described methods [8, 11]. For IgG determinations, 96-well microtitration plates (Immulon 1; Dynatech Laboratories, West Chester, PA) were coated with 25 μL of supernatant from sonicated *B. burgdorferi* G39/40 overnight at 4°C. After being washed three times with 0.05% Tween 20 in PBS and again between each step, the plates were blocked with 5% nonfat dried milk in PBS and 0.05% Tween 20 (M-PBS, pH 7.6), incubated with 50 μL of patient sera (1:400 in M-PBS) and with alkaline phosphatase–conjugated goat anti-human IgG (1:1000 in M-PBS; Tago, Burlingame, CA) in each instance for 45 min at 37°C. After a wash with PBS without Tween 20, the substrate, 1 mg/mL *p*-nitrophenyl phosphate (PNPP) with 0.5 mM $ZnCl_2$, was added. For IgM determinations, 96-well plates (Immulon 2; Dynatech) were coated with goat anti-human IgM (1:1000 in 50 m*M* carbonate, pH 9.6; Tago) overnight at 4°C. The plates were blocked with M-PBS followed by the sequential addition (50 μL) and incubation (45 min at 37°C) of the following reagents diluted in M-PBS buffer: patient serum (1:400), normal control serum (1:100), sonicated *B. burgdorferi* antigen (25 μg/mL), rabbit anti–*B. burgdorferi* antibody (1:1000), alkaline phosphatase–conjugated goat anti-rabbit IgG (1:1000; Tago); then the substrate, 1 mg/mL PNPP with 25 μ*M* $ZnCl_2$, was added.

The cutoff optical density readings (405 nm) were 3 SD (IgG) or 5 SD (IgM) above the mean optical density of 8 normal control samples included on the same plate. These samples were representative of 50 previously tested normal control sera. To calculate an antibody titer, we adjusted the value of an unknown sample with a standard curve made from dilutions of the same known positive serum included on the same plate. The patient had had Lyme disease for 3 years at the time the sample was taken; the manifestations of his illness included erythema migrans, meningitis, facial palsy, atrioventricular nodal block, and intermittent attacks of oligoarticular arthritis. For IgG determinations, a titer of ≥1:800 was defined as positive, 1:200–1:400 as indeterminant, and ≤1:100 as negative. For IgM determinations, ≥1:200 was defined as positive, 1:100 as indeterminant, and <1:100 as negative.

Immunoblotting methods. SDS-PAGE was done using a miniblot system (Bio-Rad Laboratories, Richmond, CA). Supernatant from sonicated *B. burgdorferi* G39/40 (50 μg for IgG blots, 100 μg for IgM blots) was electrophoresed on a 10% acrylamide gel (10-cm plate; 0.75-mm gel thickness; acrylamide/bis-acrylamide ratio, 30:1) at 20°C and 175 V. Gel proteins were transferred to nitrocellulose paper at 4°C and 100 V for 1 h. The paper was placed in TRIS-buffered saline (TBS: 20 m*M* TRIS, 500 m*M* NaCl, pH 7.6) and 0.1% Tween 20 for 10 min and then cut into 2-mm strips. After being washed three times between each step with 0.1% Tween 20 in TBS, the strips were blocked in 5% nonfat dried milk in TBS and 0.1% Tween 20 (M-TBS) and then incubated with patient sera (1:250 in M-TBS) and with alkaline phosphatase–conjugated goat antibody to human IgM or IgG (1:3000 in M-TBS; Tago), all for 1 h at 20°C. Substrate consisting of 1 mL of 70% *N,N*-dimethylformamide (DMF) with 30 mg of nitroblue tetrazolium chloride and 1 mL of DMF with 15 mg of 5-bromo-4-chloro-3-indolyl phosphate mixed in 100 mL of carbonate buffer (100 m*M* NaHC

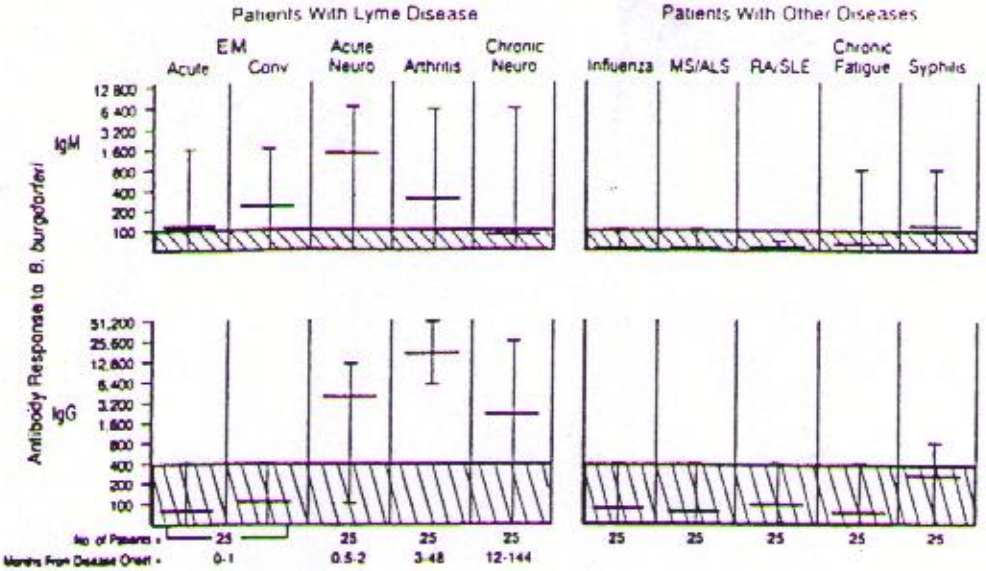

Figure 1. Antibody titers t
burgdorferi by ELISA in pat
with various manifestations
Lyme disease and in control
jects. Horizontal bars = mean;
tical bars = range; hatched are
normal range. Normal range
derived from sera from 50 hea
control subjects. EM, erythema
grans; acute neuro, mening
chronic neuro, encephalopath
polyneuropathy; MS, multiple
rosis; ALS, amyotrophic la
sclerosis; RA, rheumatoid arth
SLE, systemic lupus erythem
sus; conv, convalescent phase.

1 m*M* $MgCl_2$, pH 9.8) was added for 10 min at 20°C. The same positive control sample used in the ELISA and one of the negative control samples from that assay were included with each set of Western blots.

Statistics. The identity of groups was compared in 2 × 2 tables by χ^2 analysis or Fisher's exact test. All *P* values are two-tailed. In the retrospective study, receiver operating characteristic (ROC) curves, which are a plot of sensitivity versus specificity, were constructed for the most common IgM bands in early disease and for the most frequent IgG bands after the first weeks of infection. The ROC area was then determined for each curve. These values described the frequency with which patients with Lyme disease had particular bands that were not found in those who did not have this illness.

Results

Retrospective study. In the retrospective study, the 25 patients with erythema migrans were a mean of 8 days from the onset of symptoms, and 17 (68%) had clinical evidence of disseminated infection. Of the 25 patients, 10 (40%) had positive or indeterminate IgM titers to *B. burgdorferi* (≥1:100) determined by ELISA, and 18 (72%) had such titers during convalescence after antibiotic therapy, 2–4 weeks later (figure 1). The specific IgG responses were minimal at this time. The 25 patients with Lyme meningitis, who were 2 weeks to 2 months from disease onset, had either positive IgM (≥1:200) or IgG responses (≥1:800) to the spirochete. In the 25 patients with Lyme arthritis, who were 3 months to 4 years from disease onset, the IgG titers were markedly elevated in all patients (mean, 1:12,800), and 16 of them had specific IgM responses. Compared with the patients with arthritis, the 25 patients with Lyme encephalopathy or polyneuropathy, who were 1–12 years from disease onset, had a lower mean IgG titer (1:1600); 2 of these patients had ir terminate IgG responses and 2 were seronegative.

Of the 100 control patients who had influenza vacc tions, multiple sclerosis, amyotrophic lateral sclerosis, rh matoid arthritis, systemic lupus erythematosus, or chro fatigue syndrome, 1 had a positive IgM titer (1:800), 5 indeterminate IgM titers (1:100), and 20 had indetermir IgG titers (1:200 or 1:400); the remaining 74 patients w seronegative. Sera from the 25 patients with secondary latent syphilis had a geometric mean titer of 16 in the VD assay, and they had 4+ reactivity by fluorescent treponer antibody absorption test. When these sera were tes against *B. burgdorferi* by ELISA, 13 had low-positive or in terminate IgM titers, and 16 had low-positive or indeter nate IgG titers.

By Western blotting, 11 of the 25 patients with erythe migrans had prominent IgM responses to the 21-kDa prot of the spirochete in acute-phase sera (table 1, figure 2), 15 patients (60%) had this response by convalescence. that time, one-third or more of the patients also had IgM IgG responses to the 18-, 41-, and 58-kDa polypeptic Only 2 of the 8 patients with localized skin infection sho at least 2 IgM bands compared with 13 of the 17 patie with evidence of disseminated infection ($P = .02$). Am the 25 patients with Lyme meningitis, half or more had I or IgG responses to these antigens and to the 28- and 45-k polypeptides. The 25 patients with Lyme arthritis had str IgG responses to ≥10 spirochetal antigens. In addition to responses that were often seen earlier in the illness, mos these patients had reactivity with the 30-, 37-, 39-, 66-, and 93-kDa polypeptides. Furthermore, 11 of them had I and sometimes IgM responses to the 31-kDa OspA and the 34-kDa OspB proteins of the spirochete. In the 25 tients with encephalopathy or polyneuropathy, the IgG

Table 1. Frequency of polypeptide responses in a retrospective analysis of patients with Lyme disease and control subjects.

	IgM band present (%)				IgG band present (%)			
	Erythema migrans (n = 25)							
kDa	Acute	Conv	Meningitis (n = 25)	Control (n = 125)	Meningitis (n = 25)	Arthritis (n = 25)	Late neuro (n = 25)	Controls (n = 125)
18	20	52	8	2	84	100	80	0
21	44	60	68	4	28	48	48	0
28	4	0	52	0	44	88	84	1
30	4	16	8	1	28	100	84	2
31	0	0	12	0	0	44	40	2
34	0	0	12	2	0	60	36	1
37	24	32	28	1	4	44	48	12
39	4	8	0	0	20	92	88	10
41	32	36	52	2	92	96	88	41
45	32	20	28	1	80	84	72	10
58	28	48	36	1	84	100	92	7
66	4	8	24	2	56	92	76	2
74	8	8	8	1	12	68	44	8
93	8	32	20	1	28	100	76	0

NOTE. Conv, convalescent phase; late neuro, encephalopathy and polyneuropathy.

sponses were similar to those in the patients with arthritis, except 4 patients had minimal or absent responses.

Among control subjects, 41% had IgG reactivity with the 41-kDa polypeptide, a response that was particularly prominent in patients with syphilis. Although individual control patients rarely had IgG reactivity with >3 polypeptides, cross-reactivity was found with most of the spirochetal antigens except the 18-, 21-, and 93-kDa polypeptides. IgM reactivity was unusual in control subjects.

To establish criteria for positive Western blots, we constructed ROC curves for the 13 most common IgM bands in early Lyme disease and for the 14 most frequent IgG bands after the first weeks of infection (table 2). For IgM blots, the 8 most common bands gave the greatest ROC area of 0.864; for IgG blots, the 10 most frequent bands gave the greatest ROC area of 0.973 (figure 3). Using these optimal ROC curves, we selected the minimum number of IgM or IgG bands needed to obtain 99% specificity. Thus, in early Lyme disease, at least 2 of the 8 IgM bands at 18, 21, 28, 37, 41, 45, 58, and 93 kDa were required; after the first weeks of infection, at least 5 of the 10 IgG bands at 18, 21, 28, 30, 39, 41, 45, 58, 66, and 93 kDa were needed.

By using the IgM criteria, 40% of the patients with erythema migrans had positive blots in acute-phase sera, and 60% were positive by convalescence (table 3). With the IgG criteria, 64% of the patients with meningitis, 84% with encephalopathy or polyneuropathy, and 100% with arthritis had positive blots. Of the 125 control subjects, 19 (15%) had a single IgM band, and 7 (6%) had 2 or 3 IgM bands. However, only 1 patient with syphilis had 2 bands in the required locations for a positive IgM blot. Eight syphilis patients ≥5 IgG bands, but they were not in the required locat Thus, none of the control subjects had a positive IgG b

Prospective study. In the prospective study, the 57 tients with erythema migrans were seen a mean of 7 after the onset of symptoms, and 41 (72%) had evidenc disseminated disease. Twenty-three patients (40%) had tive or indeterminate IgM titers to *B. burgdorferi* in ac phase sera, determined by ELISA, and 38 (67%) had titers by convalescence, 4 weeks later. In Western blo the acute-phase sera, 22 (39%) had IgM responses to the kDa protein, and 18 (32%) had 2 of the 8 IgM bands requ for a positive blot. By convalescence, 31 (54%) had IgM sponses to the 21-kDa protein, and 25 (44%) had a posi IgM blot. Twenty of the 41 patients with evidence of disse nated infection had positive IgM blots compared with the 16 patients with localized skin infection, but this di ence was not statistically significant. Among the 17 pati with summer flu-like illnesses who were not thought to h Lyme disease, 1 had an indeterminate IgM response ELISA, but none had a positive immunoblot. Thus, in prospective study of early Lyme disease, the sensitivity of IgM Western blot in acute-phase sera was only 32%, but specificity was 100%.

Of the 237 patients seen in our diagnostic Lyme dise clinic from July 1990 through June 1991, 54 met clini criteria for active Lyme arthritis or neuroborreliosis. As termined by ELISA, 39 of the 54 patients had positive I responses to *B. burgdorferi*, 9 had indeterminate IgG sponses, 4 had only positive IgM responses, and 2 were se

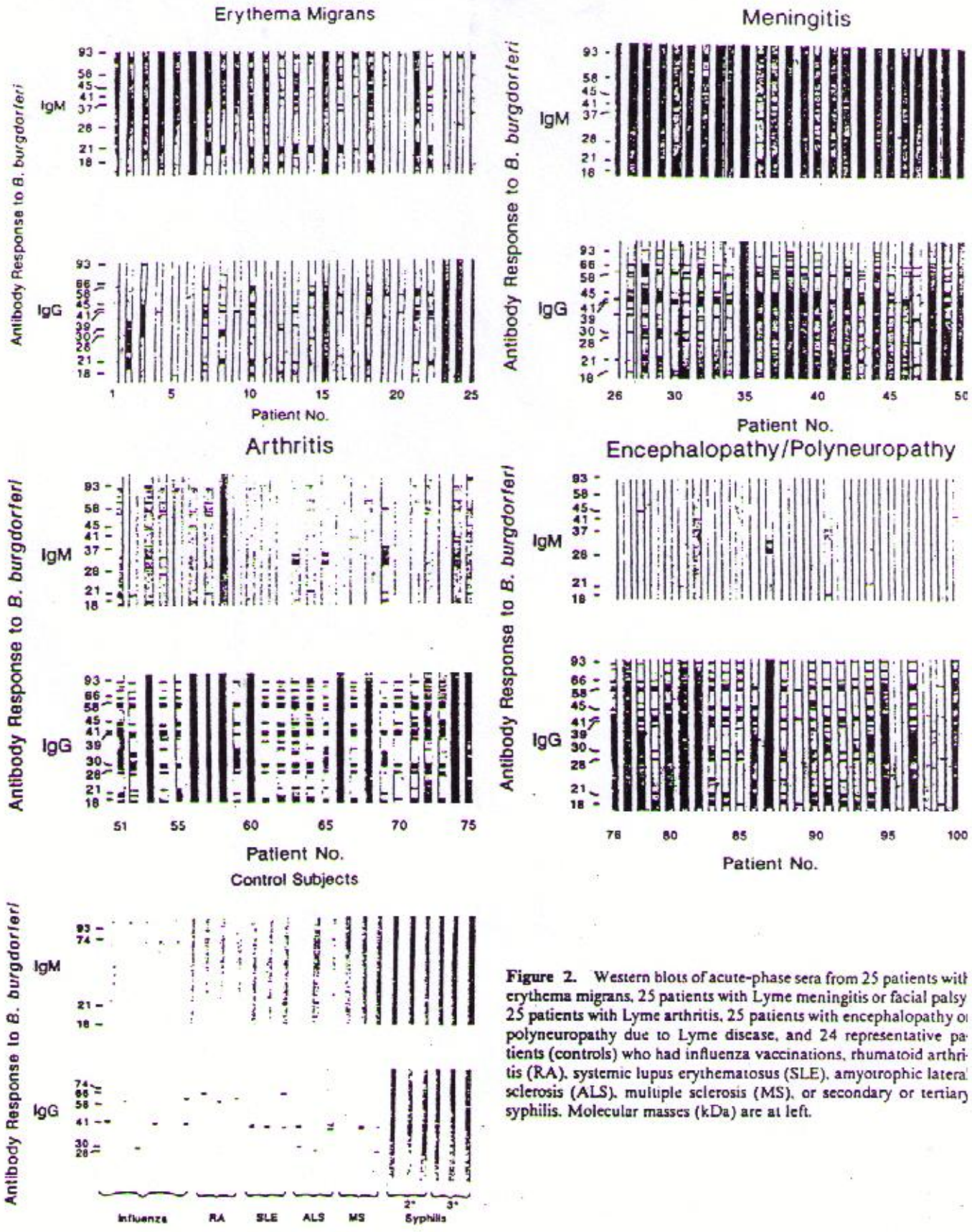

Figure 2. Western blots of acute-phase sera from 25 patients with erythema migrans, 25 patients with Lyme meningitis or facial palsy, 25 patients with Lyme arthritis, 25 patients with encephalopathy or polyneuropathy due to Lyme disease, and 24 representative patients (controls) who had influenza vaccinations, rhumatoid arthritis (RA), systemic lupus erythematosus (SLE), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), or secondary or tertiary syphilis. Molecular masses (kDa) are at left.

Table 2. Receiver operating characteristic (ROC) areas for different numbers of the most common bands.

No. of most common bands	IgM		IgG	
	ROC area	SE	ROC area	SE
2	0.784	0.038	0.921	0.024
4	0.839	0.034	0.964	0.016
6	0.849	0.033	0.968	0.016
8	0.864*	0.031	0.967	0.016
10	0.861	0.032	0.973*	0.013
12	0.859	0.032	0.963	0.015

* Largest ROC areas were obtained by using 8 most common IgM bands in early Lyme disease and 10 most frequent IgG bands after first weeks of infection.

negative (table 4). Of the 48 patients who had positive or indeterminate IgG responses by ELISA, 45 had positive IgG immunoblots: 3 of the 4 patients who had only IgM responses by ELISA had positive IgM blots; the 2 patients who were seronegative by ELISA had negative blots. The 4 patients who had only IgM responses had early neurologic manifestations of Lyme disease, and the 2 patients who were seronegative had had erythema migrans followed by encephalopathy with an elevated CSF protein in 1 and polyradiculopathy demonstrated by electromyography in the other. Both

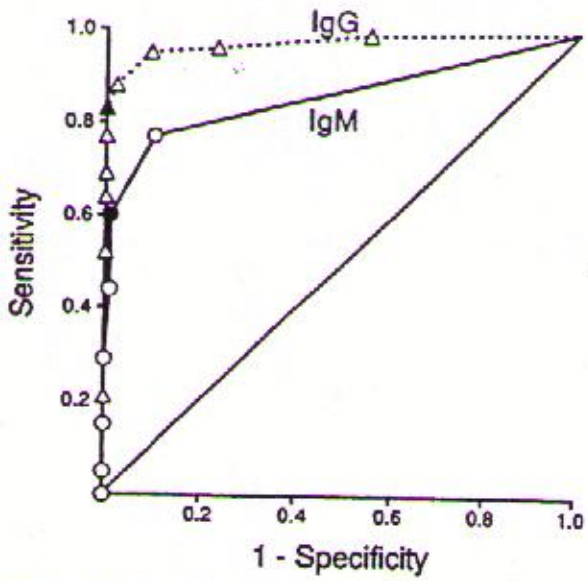

Figure 3. Receiver operating characteristic (ROC) curves for IgM and IgG Western blot criteria that gave largest ROC areas. Circles: sensitivity and 1 − specificity for 8 most common IgM bands in erythema migrans or Lyme meningitis. Triangles: same parameters for 10 most frequent IgG bands in Lyme meningitis, arthritis, or encephalopathy or polyneuropathy. Solid symbols show minimum number of bands needed for 99% specificity: 2 of 8 for IgM and 5 of 10 for IgG.

Table 3. Sensitivity and specificity of Western blotting spective study of patients with various manifestations of ease.

	Positive IgM*		Positiv
	Sensitivity (%)	Specificity (%)	Sensitivity (%)
Erythema migrans			
Acute	40	99	0
Convalescent	60	99	16
Meningitis	80	99	64
Arthritis	16	99	100
Encephalopathy/ polyneuropathy	4	99	84

* 2 of 8 bands at 18, 21, 28, 37, 41, 45, 58, and 93 kDa were r positive IgM blot; 5 of 10 bands at 18, 21, 28, 30, 39, 41, 45, 58, kDa were required for positive IgG blot.

† One patient with syphilis had IgM response to 21-, 41-, a polypeptides.

‡ Of 25 patients with Lyme meningitis, 24 (96%) had positive I blots.

of these patients had a positive T cell proliferative to *B. burgdorferi* antigens (stimulation indices, 35 respectively; normal, <10). Serologic results in th tients with inactive Lyme disease were similar to patients with active Lyme disease, but the percenta tients with indeterminant ELISA results tended to b (table 4).

Table 4. Prospective study of 237 patients seen in a d Lyme disease clinic: number of patients with positive test moral immunity (IgG) to *B. burgdorferi* according to diag

Diagnosis	No. of patients	ELISA	
		Positive	Indeterminate*
Active Lyme disease			
Arthritis	25	22	3
Neuroborreliosis	29	17	6
Total	54	39 (72)	9 (17)
Inactive Lyme disease			
Arthritis	24	18	6
Neuroborreliosis	10	4	5
Erythema migrans	10	5	3
Total	44	27 (61)	14 (32)
Other illnesses			
Fibromyalgia/fatigue	32	0	10
Other rheumatic	62	3	13
Other neurologic	45	2	11
Total	139	5 (4)	34 (24)

NOTE. Data are no. (%). For active Lyme disease vs. other illnes .001 for both tests; for active vs. inactive Lyme disease: *P* not signifi both tests (Fisher's exact test).

* Of 57 patients with indeterminate ELISA IgG titers, 6 of 9 wit Lyme disease and 11 of 14 with inactive infection had positive IgG W blots compared with only 2 of 34 patients with other illnesses ($P <$. both comparisons, Fisher's exact test).

Table 5. Sensitivity and specificity of serologic tests in prospective studies of Lyme disease

	Sensitivity (%)	Specificity (%)
Early Lyme disease*		
IgM ELISA	40	94
IgM Western blot	32	100
Lyme disease after first weeks of infection		
IgG ELISA	89	72
IgG Western blot	83	95

NOTE. Indeterminate ELISA results were considered positive for this analysis.

* Erythema migrans, acute-phase sera.

Among the 139 clinic patients who did not meet clinical criteria for Lyme disease, 5 had positive IgG responses to *B. burgdorferi* by ELISA, and 34 had indeterminate responses. Only 7 of these patients had positive blots; our diagnoses in these patients were gouty arthritis, rheumatoid arthritis, supraspinatus tendinitis, diabetic neuropathy, vascular headache, stroke, or depression. Because these patients came from areas endemic for Lyme disease and had clearly positive blots, we believe that they also had asymptomatic infection with *B. burgdorferi*. The remaining 100 patients were seronegative by all tests. In both case and control subjects, positive and negative serologic results by ELISA and Western blotting were usually concordant. However, among the patients who had indeterminate IgG responses by ELISA, 6 of the 9 with active Lyme disease and 11 of the 14 with inactive infection had positive IgG blots compared with only 2 of the 34 patients who had other illnesses ($P < .001$ for both comparisons).

Sensitivity and specificity of serologic tests. In the prospective studies, the sensitivity of the IgM ELISA in early Lyme disease was 40%, and the specificity was 94%; with Western blotting, the specificity increased to 100% (table 5). After the first weeks of infection, the sensitivity of the IgG ELISA was 89%, and the specificity was 72%; with Western blotting, the specificity increased to 95%.

Discussion

To assess the diagnostic potential of Western blotting in Lyme disease, we first did a retrospective analysis of the humoral immune response in 100 patients with various manifestations of the disorder. Although an initial study emphasized that the first response in patients with erythema migrans was often to the 41-kDa flagellar antigen of the spirochete [29], the most prominent IgM response in the current patients with erythema migrans was to the 21-kDa polypeptide. This protein in our antigen preparation reacts with monoclonal antibody L32 1F8, which is specific for th OspC protein of the spirochete (Wilske B, personal communi cation). This discrepancy between the past and current stud ies is probably due to antigen preparations that may differ i expression of the OspC protein. In a previous study of pa tients followed throughout the course of the illness, the fina point in expansion of the immune response was the develop ment of reactivity with the OspA and OspB proteins of th spirochete months to years after disease onset [37]. In th current study, fewer than half of the patients had these re sponses, even late in the illness, and therefore they were no of much help diagnostically. Cross-reactive IgG responses particularly with the 41-kDa flagellar antigen, were frequen in all control groups. This response may be especially promi nent in patients with syphilis, periodontitis, or acute ulcera tive gingivitis [38]. Reactivity with the 18-, 21-, and 93-kD polypeptides was not found in our control patients, and thes responses may be the most specific for infection with *B. burg dorferi*.

Several previous studies have proposed criteria for a posi tive Western blot in Lyme borreliosis [15–23]. In an initia study of 30 American patients with early Lyme disease, posi tive Western blots were defined by the presence of ≥2 IgM bands alone, ≥4 IgG bands alone, or ≥1 IgM and ≥1 IgG bands [15]. In practice, these definitions, which did not re quire the presence of bands at particular locations, have beer associated with too many false-positive results. As in ou current study, Wilske and colleagues [16, 19] in German reported that common early responses were directed agains the 41-kDa flagellar antigen and the 22-kDa OspC protein o the spirochete; a prominent late response was to a 100-kD polypeptide, which is likely to be the same as the 93-kD protein in our antigen preparation. In a study of Swedisl patients with Lyme meningitis, Karlsson et al. [18] defined positive IgM or IgG immunoblot by the presence of a 41-kD band plus 1 band at 18, 21.5, or 23 kDa. This definition ha a sensitivity of 78% and a specificity of 82%. Zöller et al. [20 in Germany reported that the presence of 4 bands at 21, 30 73, and 93 kDa in late Lyme borreliosis had a specificity o 97% but a sensitivity of only 23%. In a recent report of Ameri can patients, Ma et al. [23] found that those with Lyme dis ease most commonly developed IgG reactivity with the 41 kDa flagellar antigen, but this response was also common ir control subjects. An IgG response to the 39-kDa protein wa second most common and was rare among healthy control but was seen in 11% of patients with syphilis. In our study, a 39-kDa response was found in almost all patients with arthri tis, encephalopathy, or polyneuropathy but only rarely ir patients with earlier manifestations of the illness; it wa found in half of the patients with syphilis.

On the basis of retrospective and prospective analyses, we propose criteria for positive Western blots requiring 2 of the 8 most common IgM bands in early Lyme disease or 5 of the

10 most frequent IgG bands after the first weeks of infection. The IgM criteria should be used with caution after the initial weeks of infection; most patients have an IgG response to the spirochete by that time. We believe that our prospective study provides a rigorous test of sensitivity and specificity because the comparison patients had illnesses that are commonly confused with Lyme disease, and a number of them had previously had positive serologic tests in other laboratories.

The IgG blot in the patients with Lyme disease for at least several weeks was not 100% specific primarily because 4 patients with early neurologic involvement still had only IgM responses to *B. burgdorferi* and 2 patients with late neurologic disease who had been incompletely treated with antibiotics for erythema migrans were seronegative. The IgG blot in the comparison patients was not 100% specific because 7 patients had serologic evidence of exposure to *B. burgdorferi* but clinical pictures of other illnesses. In addition, patients with past Lyme disease usually remain seropositive for years, even after treatment with antibiotics. If patients with inactive Lyme disease or asymptomatic infection have other illnesses, particularly with joint or neurologic symptoms, positive serologic tests for *B. burgdorferi* may cause diagnostic confusion. This may be a greater problem in Europe where the frequency of subclinical infection has been reported to be higher than in the United States [39]. Furthermore, our definitions may not be suitable for European patients because the immune response may be more restricted there [18, 20, 40].

Several technical problems of Western blotting with sonicated *B. burgdorferi* should be stressed. The molecular weight of the same protein may be somewhat different depending on the strain of the spirochete or the conditions of the assay, multiple proteins may comigrate to the same area, the number of bands apparent in the blot is influenced by the concentration of reagents, and the results of Western blotting are observer-dependent. Care must be taken in reading the correct molecular weights of the bands, and faint bands, which we discounted, may pose interpretation difficulties. Video densitometry may help with this problem [20], but it is not suitable for reading miniblots, the method used here, or for reading bands that are close together, a common problem in Lyme disease. Although the use of recombinant borrelial proteins may improve specificity, sensitivity may not be as good.

In this study, the results obtained by ELISA and Western blotting were concordant in patients with clearly positive or negative tests, and Western blotting was of no additional value in these patients. However, among patients who had indeterminate responses by ELISA, Western blots were helpful in identifying false-positive results. We conclude that Western blotting can be used to increase the specificity of serologic testing in Lyme disease.

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STANDARDIZATION OF LYME DISEASE SEROLOGIC TESTING FOR EPIDEMIOLOGIC PURPOSES

David T. Dennis, M.D., M.P.H.

Introduction: The lack of standardized serodiagnostic test methods for Lyme disease has greatly restricted our understanding of the epidemiology of this disease. The following critical epidemiologic functions will be significantly enhanced by the use of standardized serodiagnosis: disease surveillance, outbreak investigations, clinical studies involving case series, clinical trials, decision analyses for diagnosis and management of the disease, and population-based investigations, such as risk factor analyses, intervention studies, and cost-effectiveness analyses.

Surveillance: Disease surveillance is carried out for the purposes of estimating the incidence of disease, identifying the populations at risk, and monitoring trends of occurrence. In the United States, Lyme disease has been a nationally notifiable disease with a uniform case definition for reporting purposes since 1991 (1). Approximately 10,000 cases are reported by states annually to CDC (2). The Lyme disease surveillance case definition requires laboratory "confirmation" of cases when patients present with manifestations of illness other than erythema migrans (EM). The following laboratory criteria are necessary for diagnosis of later-stage illness (3):

- Isolation of *Borrelia burgdorferi* from clinical specimens, or
- Demonstration of diagnostic levels of IgM and IgG antibodies to the spirochete in the serum or CSF, or
- Significant change in IgM or IgG antibody response to *B. burgdorferi* in paired acute-phase and convalescent-phase serum samples.

Although potentially useful in confirming active Lyme disease, neither cultural isolation nor paired serum specimen testing has been much used for validating cases in routine Lyme disease surveillance, since the procedures are not often performed in the general medical setting. Further, test results based on nonstandardized ELISA and IFA testing of single specimens (the usual serodiagnostic approach) have been unreliable (4-6) and have had unknown sensitivity and specificity. Under the circumstances in which these tests are most used, the predictive value of the test results is often too low to be of use in either routine surveillance or the clinical setting. The implementation of standardized test procedures utilizing a two-step approach in which the first test is highly sensitive and the second test is highly specific and confirmatory (7) is expected to provide a level of predictive value high enough that laboratory-based surveillance will become useful and cost-effective. Quality assurance programs that measure the performance of laboratories against these standards will provide state and local disease surveillance programs with information necessary to determine the value, in their respective populations, of using positive test results to trigger case validation investigations.

Serosurveys: Serologic tests with defined, high levels of sensitivity and specifity can be important tools in understanding the distribution of disease in communities by providing estimates of point and period prevalence and the incidence of infection. Estimates of community seroprevalence are important, along with clinical presentation and history of exposure, in determining the pretest probability of disease in patients being diagnostically evaluated for suspected Lyme disease. When serodiagnosis is used as a screening tool, the pretest probability of the disease is equal to the prevalence of the disease in the population; the predictive value of test results, therefore, depends on a knowledge of the underlying seroprevalence (8).

CDC has used the combination of a sensitive, standardized enzyme-linked immunosorbent assay (ELISA), followed by standardized Western immunoblotting to conduct seroepidemiologic studies of defined populations in several Lyme disease-endemic communities in the northeastern region of the United States and in Sonoma County, California. These populations represent varying levels of endemicity. In a collaborative study with the Maine Medical Center, Portland, the seropositvity of specimens from 272 mostly elderly and retired persons living in a low enzootic area of coastal Maine was compared with the seropositivity of specimens from 100 controls living in a nonenzootic area of northern Maine. Seropositivity rates were 2% or less in both populations when serum samples yielding ELISA results in the equivocal range (between 1 and 3 standard deviations greater than the mean optical density of the normal non-disease controls) were then tested by a confirmatory Western blot procedure. When serum samples that were ELISA positive were tested by Western immunoblotting, the seropositivity of the exposed group remained at 2%, while that of the nonexposed group fell to 0% positivity. This indicates a very high specificity of serodiagnosis in this seroepidemiologic study (9). In contrast, serologic studies of nearly 800 persons living in two highly endemic neighborhoods in Westchester County, New York, demonstrated a baseline seroprevalence of 10.7% and a seroincidence over a single transmission season of about 4% (10). A serosurvey in a residential semirural community in Sonoma County, California, identified only 3/219 (1.4%) of persons with diagnostic Lyme disease seroreactivity, although seropositivity to other tick-borne pathogens, *Ehrlichia spp.* and *Babesia spp.* was 4.7% and 18%, respectively (11). In each of the serosurveys, the seropositivity rates are what would be expected based on clinical and ecologic findings. Experience with the two-test approach in the above serosurveys identified the need to confirm both seropositive and equivocal results obtained on the first-test with Western immunoblotting to avoid false positivity. As expected, the proportion of false positive results was greatest in those populations with the lowest expected prevalence, e.g., in Maine, 0/4 ELISA positive serum specimens from the control area were confirmed as positive by Western immunoblotting; among the serum samples from Sonoma County residents, 7/9 (77.8%) ELISA positive specimens were false positive, and only 1/39 (2.6%) ELISA equivocal specimens was positive as determined by Western immunoblot. In the serosurvey of residents of a highly endemic community in Westchester County, New York described above, 56/72 (77.8%) ELISA positives were false positive, and only 21/164 (11.4%) ELISA equivocals were positive as determined by Western immunoblot testing (10).

Epidemiologic Studies: The standardized two-test serodiagnostic approach has also been used by CDC in support of analytic epidemiologic studies. The two-test approach was used to test

serum specimens collected in 1993 from incident case-patients and community controls in Hunterdon County, New Jersey, a known Lyme disease-endemic area (12). Both ELISA equivocal and ELISA positive serum specimens were tested by Western blot. Seropositivity was found in 9/37 (24.3%) convalescent-phase serum samples from case-patients treated several months previously for erythema migrans, and in 2/44 (4.5%) non-case controls. Using the Western blot as a confirmatory test, 20/29 (69%) ELISA positives among cases were false positives, and only 2/11 (15%) ELISA equivocals were Western blot positive. In contrast, a study in 1994 of persons with suspected Lyme disease and their neighborhood or family controls in two presumably nonendemic counties in West Virginia revealed no seropositivity among 30 case-patients and 30 controls tested. These findings suggest that it may now be appropriate in epidemiologic studies to use serologic test results to identify case-patients and controls.

Clinical Studies: Clinical diagnosis of Lyme disease is often made difficult by the nonspecificity of many of the signs and symptoms of this disease, by a lack of a solid history of exposure to tick vectors of this disease, and a poor understanding of the prevalence of the disease in the population represented by the patient. The rational approach to diagnosis makes use of the likelihood ratio, a measure of the accuracy of the test procedure determined by comparing the likelihood of a obtaining a certain test result when testing a group of true positives to the likelihood of obtaining the same test result when testing a group of true negatives. The post test probability (the predictive value) is determined by applying the pretest probability of the disease tested for and the likelihood ratio for the test employed (13). The more sensitive the test, the better the negative predictive value of the test; the more specific the test, the better its positive predictive value. Likelihood ratios indicate by how much a given diagnostic test result will raise or lower the pretest probability of the target disease. A likelihood ratio of 1 means that the post test probability is the same as the pretest probability. The greater the likelihood ratio the greater the probability that the target disease is present. Likelihood ratios describe the performance of a diagnostic test, summarize the same information as sensitivity and specificity, and are used to estimate the probability of disease after a positive or negative test result (14, 15).

Cost-Effectiveness Determinations: Determinations of test-treatment thresholds also depend on an understanding of standardized test performance. Decision analyses carried out to assess the relative cost-effectiveness of various management options are based on models that incorporate the prevalence of disease and the performance characteristics (sensitivity and specificity) of the tests used in diagnostic support, along with such factors as adherence to therapy, complications of treatment, effectiveness of therapy, the probability of complications of the disease itself, and, of course, the direct and indirect costs (16). The use of standardized tests with known sensitivity and specificity allows the development of test-treatment thresholds to guide clinical management decisions.

Intervention Studies: There are two basic intervention approaches in Lyme disease: curative, i.e., antimicrobial treatment to eliminate infection and reduce or eliminate morbidity, and preventive. There are several primary prevention modalities, including avoidance of tick-infested areas, and the use of repellents, protective clothing, and environmental interventions to reduce exposure

to infective tick bites, early detection and removal of attached ticks, as well as the use of prophylactic antimicrobial treatment and vaccination to prevent infection. Early detection and treatment of disease prevents secondary morbid sequelae. Standardized serodiagnostic tests will be useful to identify case-patients who have been infected with *B. burgdorferi* and to determine eligibility for enrollment in antibiotic treatment trials. These tests will also be needed to identify persons who have had a prior exposure, as well as those who develop infection with *B. burgdorferi* following intervention, in assessing the effectiveness of prophylactic antibiotics and vaccines in preventing infection and disease. Ultimately, seroepidemiologic evaluations of communities will be important to define populations at high risk in order to best target intervention efforts and to evaluate the cost-effectiveness of these interventions.

Conclusions: The use of reliable, accurate serodiagnostic tests, monitored by programs of quality assurance, will significantly increase our ability to conduct surveillance activities and epidemiologic studies, improve decision-making for patient diagnosis and clinical management, and accelerate the development and evaluation of Lyme disease control and prevention strategies, including the use of vaccines, personal protective measures, and environmental management activities.

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TEST APPROACH AND *BORRELIA BURGORFERI* STRAIN SELECTION FOR STANDARDIZATION OF SERODIAGNOSIS OF LYME DISEASE

Barbara J.B. Johnson, Ph.D.

In May 1994, a Work Group on Standardization of Serodiagnosis of Lyme Disease* was convened at the Centers for Disease Control and Prevention (CDC), Division of Vector-Borne Infectious Diseases. This work group recommended adoption of a two-step approach to Lyme disease serologic testing: use of a sensitive, relatively inexpensive first test, such as an enzyme immunoassay, followed by immunoblotting of specimens scored positive or equivocal in the first test. The value of a two-step approach was supported by a comparison of testing methodologies in five academic reference centers (ARC) and CDC. An abstract of the ARC/CDC study by Craven et al. is presented below (1).

The work group also identified the use and interpretation of immunoglobulin M (IgM) immunoblots as a high priority for further study. Accordingly, a multi-centered evaluation of IgM test performance and interpretation was designed and performed. The results of this investigation are reported elsewhere in these proceedings (2). Since the impact of *Borrelia burgdorferi* strain variability on diagnostic test sensitivity, particularly on IgM detection in early disease, had not yet been adequately assessed, CDC investigators agreed to compare the performance of antigens from eight strains chosen to represent the major Lyme disease-endemic areas of the United States and the strains most commonly used for serodiagnosis in this country.

Experimental Design, ARC/CDC Study (1)

Participating laboratories. Five academic laboratories (located at the Tufts/New England Medical Center, Boston, MA; Marshfield Clinic, Marshfield, WI; University of Connecticut Health Center, Farmington, CT; State University of New York at Stony Brook, Stony Brook, NY; and the Robert Wood Johnson Medical School, New Brunswick, NJ) and the diagnostic laboratory of the Division of Vector-Borne Infectious Diseases, within the National Center for Infectious Diseases, CDC tested the serum panel.

Serum samples. Each laboratory tested 600 blind-coded serum samples. The samples were from patients with clinically well defined Lyme disease (n=200), healthy blood donors from areas in which Lyme disease is not endemic (n=200), and persons from such areas who had reactive antibodies in a *B. burgdorferi* whole-cell sonicate enzyme-linked immunosorbent assay (ELISA) (n=200). Each group of 200 samples consisted of 113 individual specimens and 87 random duplicates for precision analyses.

Test methods. Each investigator used the testing method(s) currently employed in his or her laboratory. All participants used whole-cell sonicate ELISAs. Three ARC investigators used both ELISAs and immunoblotting. CDC also used a flagellar antigen ELISA.

Results

The most accurate results were obtained in the three laboratories that used an ELISA supplemented by immunoblotting. The results in these laboratories were similar: sensitivities for the aggregate of all Lyme case specimens, 73%-79%; specificities, 98%-99.5%; and precisions, 82%-99%. Test performance in the three laboratories that used ELISAs alone ranged from fair to poor.

Conclusions

A two-step approach to serologic testing that uses ELISA supplemented by immunoblotting was more accurate than ELISAs alone. This approach is suitable for the first efforts to standardize Lyme disease serologic testing. A panel of well-defined, blind-coded serum specimens large enough to afford good statistical power is a critical tool in developing and standardizing serologic tests.

Experimental Design, Antigen Comparison Study (3)

Immunoblot antigens. Antigens were prepared from four strains of *B. burgdorferi* commonly used for Lyme disease serology: 2591, G39/40, 297, and a low passage of strain B31 (4-7). Additional strains, all isolated from humans and representing the major Lyme disease-endemic areas, were NY 92-0094, NY HB-19, WI MC-23, and CA 92-0953. Immunoblot strips of each strain were commercially prepared (MarDx Diagnostics, Inc., Carlsbad, CA) under uniform conditions.

Serum samples. The serum panel consisted of 212 specimens, randomized and blind coded. Paired serum samples were obtained from 70 patients with erythema migrans (EM) residing in endemic areas of the northeastern or upper midwestern United States. An acute-phase sample was obtained on the day the patient was first seen at the physician's office; a convalescent-phase specimen was obtained 2-4 weeks after the first sample. All patients were treated with antibiotics at the first visit. Skin lesions were cultured and confirmed to harbor *B. burgdorferi* in 50 of the 70 patients with EM; skin was not cultured from the remaining 20 patients, who were judged to have early Lyme disease by the characteristic appearance of their EM. Thirty-six samples were from healthy blood donors from nonendemic areas (OH and WY). An additional 36 samples were duplicates to assess the precision of the assays, 12 each from acute-phase patients, convalescent-phase patients, and healthy blood donors.

Calibration of immunoblots. A panel of monoclonal antibodies recognizing 10 antigens of *B. burgdorferi*, a strongly reactive positive control, and a weakly reactive positive control were used to standardize blot development and interpretation.

Data analysis. The frequency of recognition of all proteins was calculated. Antigens were ranked in order of diagnostic utility by their ability to distinguish Lyme disease cases from

controls (chi-square) for each strain. Test interpretation criteria were derived that produced the best test performance.

Results

Three of the strains in common diagnostic use--2591, and low passages of 297 and B31--performed equally well. The criteria for blot interpretation proposed by Engstrom *et al.* (8) (two or more of the following three bands must be present for a blot to be judged positive: OspC, P39, and Fla) resulted in reasonable test accuracy. The sensitivity was 43%-44% for acute specimens and 71%-72% at convalescence; specificity was 98%-100%. If reactivity with P37 was added to the scoring criteria (two or more bands of OspC, P37, P39, and Fla present), the sensitivity increased slightly to 43%-48% for acute specimens and 71%-74% at convalescence. Specificity was unchanged.

One of the strains currently in common use for serodiagnosis of Lyme disease (G39/40) did not efficiently express the P39 antigen. This strain did not perform as well as those listed above when the criteria of Engstrom *et al.* were applied. The IgM blot scoring system of Dressler *et al.* (5) (two or more bands present from a set of eight: 18, 21, 28, 37, 41, 45, 58, and 93 kDa) was more suitable for this strain (sensitivity 44%, acute; 77%, convalescent.) The specificity achieved with this strain was 94%.

Use of antigen from any of the other four strains examined did not result in more accurate test performance.

Conclusions

The work group judged strain 2591 and low passages of strains 297 and B31 to be equally suitable for use as antigen in immunoblots. The group wished to standardize the outcome, that is, test performance, rather than methodology *per se*. The criteria of Engstrom *et al.* are both simple to apply and standardizable; monoclonal antibodies are available to all three antigens of diagnostic significance. Use of these criteria for blot interpretation is recommended. When an antibody marker for P37 becomes available, this protein may be included as an antigen to be scored.

Other *B. burgdorferi* strains also may be suitable for diagnostic purposes. Investigators should first demonstrate that the strain that they have chosen expresses appropriate amounts of the immunoreactive proteins of diagnostic interest.

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RECOMMENDATIONS OF THE WORK GROUP FOR STANDARD CRITERIA FOR LYME DISEASE SERODIAGNOSIS

Arthur Weinstein, M.D. and Barbara J.B. Johnson, Ph.D.

Introduction

The CDC/ASTPHLD Work Group on the standardization of serodiagnostic testing for Lyme disease sought to develop criteria for a positive immunoblot in both early and late disease. This was of high priority since immunoblotting was to be the confirmatory test in a two-step approach to diagnosis.

Prior studies had suggested that immunoblotting could provide sufficient sensitivity and specificity to be used in Lyme disease diagnosis (1,2). Dressler et al. conducted a retrospective analysis of 225 Lyme disease case and control subjects from the United States and proposed criteria for positive immunoblots incorporating both the number and location of bands (3). However, since monoclonal antibodies were not readily available at that time, it was not possible to identify proteins of diagnostic importance whose locations in gels depend on both experimental conditions and *Borrelia burgdorferi* strain choice. For example, Padula et al. reported that the OspC antigen is of diagnostic importance, especially in early Lyme disease, and this protein may be poorly expressed in a number of North American *B. burgdorferi* strains (4). Despite these limitations, the proposed criteria for a positive immunoblot in late Lyme disease-- at least 5 of 10 specified IgG bands --seemed to stand up reasonably well in other laboratories (5). Therefore, the Work Group proposed that the criteria of Dressler et al. be used to interpret IgG immunoblots in the interim, pending further studies.

The Work Group focused on the standardization of the IgM immunoblot for early Lyme disease. Preliminary data, presented by Russell Johnson, suggested that relatively simple criteria could be used for IgM immunoblot interpretation (6). Accordingly the Work Group conducted two studies:

1. A multi-centered, blind-coded study of the IgM response in early disease, comparing the performance of the criteria of Dressler et al. and Engstrom et al. for immunoblot interpretation (3,6).

2. A comparison of the expression of proteins of diagnostic importance by *B. burgdorferi* strains commonly used for Lyme disease serology.

Multi-centered Study of IgM Response in Early Lyme Disease

Design

Participating Laboratories. Five academic laboratories tested the serum panel. These laboratories are located at the University of Minnesota, Minneapolis, MN; Tufts/New England Medical Center, Boston, MA; University of Connecticut Health Center, Farmington, CT; New York Medical College, Valhalla, NY; and State University of New York at Stony Brook, Stony Brook, NY.

Serum Samples. Each laboratory tested 212 blind-coded serum samples. Acute and convalescent samples (140) were obtained from 70 patients with erythema migrans, 50 of whom were confirmed to have *B. burgdorferi* infection by culture of skin biopsy specimens. The remaining 72 specimens were duplicates of the above and controls. Further characteristics of this serum panel are described elsewhere in these Proceedings (7).

Test Methods. Based upon the results of an Academic Reference Center/ CDC study, each laboratory used a two-test approach for analysis of serum samples (7). The first test was to be a sensitive "in house" IgM ELISA. Samples that were judged positive or equivocal by ELISA were to be tested by IgM immunoblot. Each laboratory employed the methods and strain of *B. burgdorferi* routinely used by that laboratory for both tests. All protein bands scored positive on the immunoblots were reported to the CDC.

Data Analysis. The frequency of recognition of all proteins was calculated. Antigens were ranked in order of diagnostic utility by their ability to distinguish Lyme disease cases from controls (chi square) for each laboratory. The performance criteria for blot interpretation of Dressler et al. and Engstrom et al. were compared. The performance of many other criteria were also examined, the two best of which are reported below.

Results

The ELISA and immunoblot results of three of the five laboratories were used in the data analysis. The results of one laboratory were not used because a single recombinant antigen was used in the ELISA; the results of a second laboratory were not used because specialized equipment (an image intensity analyzer) was employed in the test. A different strain of *B. burgdorferi* was used by each of the three laboratories (297, 2591, and G39/40).

IgM ELISA RESULTS ALONE
Number Positive, Number Positive + Equivocal
(Percent Positive, Percent Positive + Equivocal)

Lab	Strain	Acute n=82*	Convalescent n=82*	Healthy Controls n=48*
1	297	33, 40 (40, 49)	67, 73 (82, 89)	0, 4 (0, 8)
2	2591	46, 46 (56, 56)	71, 71 (87, 87)	3, 3 (6, 6)
3	G39/40	32, 54 (39, 66)	72, 76 (88, 93)	0, 3 (0, 6)

* Includes 12 duplicate specimens in each group

Sensitivity (mean % positive, mean % [positive + equivocal]) acute: 45%, 57%; convalescent: 86%, 90%.

Specificity (1 - mean % positive, 1- mean [% positive + equivocal] controls): 98%, 93%

IgM IMMUNOBLOT RESULTS
(ELISA POSITIVE AND EQUIVOCAL SPECIMENS)

Criteria: ≥2 of (OspC, 41, 39 kDa) bands present
(Engstrom et al.)

Number Positive (Percent Positive)

Lab	Strain	Acute n=82	Convalescent n=82	Healthy Controls n=48
1	297	32 (39)	64 (78)	0 (0)
2	2591	43 (52)	70 (85)	2 (4)
3	G39/40	26 (32)	45 (56)	1 (2)

Sensitivity (mean % positive): acute, 41%; convalescent, 73%

Specificity (1 - mean % controls): 98%

Criteria: ≥2 of (18, OspC, 28, 37, 41, 45, 58, 93 kDa) bands present (Dressler et al.)

Number Positive (Percent Positive)

Lab	Strain	Acute n=82	Convalescent n=82	Healthy Controls n=48
1	297	35 (43)	66 (81)	0 (0)
2	2591	42 (51)	68 (83)	2 (4)
3	G39/40	30 (37)	55 (67)	1 (2)

Sensitivity (mean % positive): acute, 44%; convalescent, 77%
Specificity (1 - mean % positive controls): 98%

Criteria: ≥2 of (OspC, 41, 37 kDa) bands present

Number Positive (Percent Positive)

Lab	Strain	Acute n=82	Convalescent n=82	Healthy Controls n=48
1	297	35 (43)	65 (79)	0 (0)
2	2591	42 (51)	66 (81)	2 (4)
3	G39/40	28 (34)	50 (61)	1 (2)

Sensitivity (mean % positive): acute, 43%; convalescent, 74%
Specificity (1 - mean % controls): 98%

Criteria: ≥2 of (OspC, 41, 39, 37 kDa) bands present

Number Positive (Percent Positive)

Lab	Strain	Acute n=82	Convalescent n=82	Healthy Controls n=48
1	297	35 (43)	66 (81)	0 (0)
2	2591	43 (52)	70 (85)	2 (4)
3	G39/40	28 (34)	50 (61)	1 (2)

Sensitivity (mean % positive): acute, 43%; convalescent, 76%
Specificity (1 - mean % controls): 98%

Precision analysis. There was a very high concordance rate on immunoblotting for the 12 pairs of

duplicate specimens in each group. For this analysis, the criteria for a positive immunoblot employed were those of Engstrom et al (2 of Osp C, 39, 41 kDa bands). One laboratory had 100% concordance on all duplicate specimens. On the acute specimens, one laboratory had one discordant result (97% concordance). On the convalescent specimens, one laboratory had 2 discordant results (94% concordance). On the control specimens, one laboratory had one discordant result (97% concordance). The overall concordance rate for the testing of all the duplicate specimens in all the laboratories was 96%.

Discussion

The purpose of this study was to determine the performance of various criteria for a positive IgM immunoblot in early Lyme disease for those specimens which previously had tested positive or equivocal by IgM ELISA. The desire was to combine good performance with simplicity. In order to permit uniform application of diagnostic criteria between laboratories, it was also deemed desirable to adopt standards which employed proteins for which monoclonal antibodies existed.

The results of this study confirm that, in general, antibody testing is an imperfect method to diagnose Lyme disease in its earliest stages. Specimens from patients with acute Lyme disease demonstrated less than 50% seropositivity no matter which criteria were employed. This low frequency, also observed by others, reflects the absence of detectable antibodies in the first days of infection using methods that afford acceptable specificity (8,9). In some cases, it may represent abrogation of the antibody response by appropriate and successful antibiotic therapy since only approximately 75% of the convalescent specimens were positive by immunoblotting.

The sensitivities and specificity obtained in this study were very similar to that obtained by Johnson et al. in comparing the same three strains using the criteria of Engstrom et al. (7). This suggests that the true sensitivity and specificity are very close to what we have observed (sensitivity acute 41-44%, convalescent 73-77%; specificity 98%) and that the differences among the laboratories to some degree reflect differences in the strains employed.

In general, there was good concordance of results among the 3 laboratories and high precision (96% overall). The laboratory utilizing the G39/40 strain performed less well, especially with the criteria of Engstrom et al. This is a reflection of the inefficient expression of the P39 antigen in that strain, as demonstrated elsewhere in these Proceedings (7). The more complicated criteria of Dressler et al., developed for use with strain G39/40, performed better than those of Engstrom et al. when this strain was used as antigen. If the presence of ≥ 2 bands of (Ospc, 41, 37 Rda) was used to judge an IgM blot positive, test performance was essentially equivalent to that using the criteria of Engstrom et al. (6). There was a slight improvement in sensitivity using the criteria of ≥ 2 of 4 bands (OspC, 41, 39, 37 kDa), but this improvement was not statistically significant (chi-square).. A disadvantage of the criteria employing P37 is that there is no monoclonal antibody yet available for this antigen.

Regardless of the criteria utilized, the specificity of the two-step approach to IgM testing in the multi-centered study was very high (98%). However, this specificity was calculated using serum

samples from only normal controls (healthy blood donors). With normal and disease controls, th specificities of the IgM criteria of Dressler et al. were 100% (n=125) and 99% (n=100), respectively, for the two-step procedure (3). Engstrom et al. also evaluated their IgM immunoblot criteria with serum samples from both healthy blood donors (n=75) and disease controls (n=84). The specificity for healthy donors was 100% and for disease controls was 95% (80/84) for the two-step approach (6). False-positivity occurred with serum from patients with infectious mononucleosis (2/12) and tick-borne relapsing fever (1/2), specimen types not represented in the study of Dressler et al., as well as with rheumatoid arthritis (1/16). Additional data on the specificity of the two-step approach, using a sensitive ELISA based on a flagellar preparation is included in the addendum (CDC, unpublished). In this study, the specificity of the IgM tests using the criteria of Engstrom et al. was 100% for healthy blood donors (200/200) and 93% for disease controls (183/196). If samples from patients with tick-borne relapsing fever were excluded from the calculation, the two-test approach was 98% specific. The specificity of IgM immunoblots for disease controls would have been only 89% if an ELISA had not been performed as a first step (data not shown). Similarly, the specificity of IgM testing of healthy blood donors by Engstrom et al. would have been reduced from 100% to 92% (59/65) if the immunoblot results of all samples were included in the calculations, not just those that were positive or borderline by ELISA. It is important to screen out weakly reactive samples by ELISA, especially for IgM, when blots are scored visually.

The multi-centered study was not intended to compare ELISA testing to immunoblotting. However, the results show a higher frequency of true positive ELISA tests, especially in the convalescent specimens. The laboratories that participated in this study have extensive experience using ELISA testing for Lyme disease, which may account for the high sensitivity and specificity of the ELISA in this study. Specificity was lower (93%) if both positive and equivocal ELISA results were used and would likely be even lower if control serum from patients with infectious and autoimmune diseases had been included (10). The use of ELISA testing alone has been previously noted to lack specificity and to exhibit significant interlaboratory variability (11). Furthermore, as reported in these Proceedings, the ARC/CDC study showed that the laboratories which employed a two-test approach, using ELISA and immunoblotting, achieved a higher accuracy than those using ELISA alone (7).

Conclusion

A two-step approach utilizing a sensitive ELISA test and a confirmatory immunoblot increases the accuracy of Lyme disease serodiagnosis. The simple criteria for a positive IgM immunoblot of Engstrom et al., which use three antigens for which monoclonal antibodies are available, performs as well as the more complicated criteria of Dressler et al. Therefore, it is recommended that the criteria for a positive IgM immunoblot for early Lyme disease be the presence of ≥ 2 of 3 bands (OspC, 41, 39 kDa). IgG immunoblotting can be used in the diagnosis of both early and late Lyme disease. Interim use of the criteria of Dressler et al. to interpret IgG immunoblots is recommended.

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ADDENDUM

(Unpublished data, CDC)

Specificity of the two-test approach: immunoblotting of samples positive or equivocal by FLA-ELISA.[1]
[IgM immunoblot interpretation criteria of Engstrom *et al.* and IgG criteria of Dressler *et al.*]

Serum Source	Specificity, % (reactive samples/total[2])		Comments
Autoimmune Disorders [3]			
SLE	100	(0/41)	
RA	97	(1/35)	1 IgM+, 0 IgG+
SS	100	(0/10)	
FS	100	(0/2)	
Leptospirosis	94	(2/32)	1 IgM+, 1 IgG+
Periodontitis	100	(0/9)	
Relapsing Fever [4]	52	(11/23)	10 IgM+, 4 IgG+, 3 Both+
Syphilis	94	(1/17)	1 IgM+, 0 IgG+
Tularemia	100	(0/10)	
Other [5]	100	(0/17)	
Total, All Diseases	92	(15/196)	13 IgM+, 5 IgG+, 3 Both+
Total, All Diseases Except Relapsing Fever	98	(4/173)	3 IgM+, 1 IgG+
Healthy Blood Donors	100	(0/200)	

1 ELISA antigen was a flagellar preparation from *B. burgdorferi* strain B31.

2 Sample size: 111 individual disease controls and 85 repeated specimens (n=196); 113 individual healthy blood donors and 87 repeated specimens (n=200) for precision analysis.

3 SLE = systemic lupus erythematosus
RA = rheumatoid arthritis
SS = Sjögren's syndrome
FS = Felty's syndrome

4 Tick-borne relapsing fever

5 Includes various rheumatologic and dermatologic conditions. Patients were from CO, MT, and WY, areas where Lyme disease is not known to be endemic.

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Immunoblot Interpretation Criteria for Serodiagnosis of Early Lyme Disease

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We monitored the antibody responses of 55 treated patients with early Lyme disease and physician-documented erythema migrans. Six sequential serum samples were obtained from patients before, during, and until one year after antibiotic therapy and analyzed by in-house enzyme-linked immunosorbent (ELISA) and immunoblot assays. An immunoblot procedure utilizing a gradient gel and an image analysis system was developed. A relational database management system was used to analyze the results and provide criteria for early disease immunoblot interpretation. Recommended criteria for the immunoglobulin M (IgM) immunoblot are the recognition of two of three proteins (24, 39, and 41 kDa). The recommended criteria for a positive IgG immunoblot are the recognition of two of five proteins (20, 24 [>19 intensity units], 35, 39, and 88 kDa). Alternatively, if band intensity cannot be measured, the 22-kDa protein can be substituted for the 24-kDa protein with only a small decrease in sensitivity. Monoclonal antibodies were used to identify all these proteins except the 35-kDa protein. With the proposed immunoblot interpretations, the sequential serum samples were examined. At visit 1, the day of diagnosis and initiation of treatment, 54.5% of the serum samples were either IgM or IgG positive. The peak antibody response, with 80% of the serum samples positive, occurred at visit 2, 8 to 12 days into treatment. The sensitivities of the IgM and IgG immunoblot for detecting patients that were seropositive into the study period were 58.5 and 54.6%, respectively, at visit 1 and 100% at visit 2. Twenty percent of the patients remained seronegative throughout the study. The specificities of the IgM and IgG immunoblots were 92 to 94% and 93 to 96%, respectively. The IgM immunoblot and ELISA were similar in sensitivities, whereas the IgG immunoblot had greater sensitivity than the IgG ELISA ($P = 0.006$).

Lyme disease, a multisystem disorder caused by infection with the spirochete *Borrelia burgdorferi*, is the most common vector-borne disease in the United States today. The diagnosis of early Lyme disease is usually based on the presence of an expanding erythematous lesion, erythema migrans (EM). However, this clinical marker may be absent in approximately 20 to 40% of patients. Although the diagnosis is primarily based on clinical findings, it may be assisted by the results of serological tests. The enzyme-linked immunosorbent assay (ELISA) has been widely used for detecting antibodies to *B. burgdorferi*. These assays are not standardized, resulting in tests with various levels of sensitivity and specificity. Some of these tests may result in false-positive reactions, especially when sera are from persons with other illnesses such as syphilis, sarcoidosis (18), or viral illnesses (21, 26).

The Western immunoblot has also been used by investigators to study the antibody response to infection with *B. burgdorferi*, with variable results. This test has been reported to be more sensitive than ELISA for immunoglobulin M (IgM) detection (11, 14, 22) and can identify false ELISA reactions (11, 27). Karlsson et al. (15) reported that the immunoblot was more sensitive but not more specific than whole-cell ELISA in diagnosing early Lyme disease in Swedish patients with neurological involvement. In contrast, Dressler et al. (6) reported that the immunoblot can be used to increase the specificity of current serological testing for Lyme disease and have proposed interpretation criteria. However, their proposed IgM interpretation criteria resulted in a low sensitivity (32%) in early disease. Aguero-Rosenfeld et al. (1) used a commercial Ig immunoblot to test patients with EM and reported that the immunoblot was more sensitive than the ELISA.

Differences in the interpretation criteria and antigen source for the immunoblot have led to confusion about the usefulness of this test for the diagnosis of Lyme disease. The difficulty of immunoblot interpretation is compounded by the problems of identification of protein bands and of defining when to consider a weak band present or absent. Some researchers discount all weak bands but fail to define intensity quantitatively (6). Densitometric studies (36) have attempted to define the significance of strong versus weak bands, yet no standard method for counting or discounting a band has been proposed.

Standardization of the methodology and interpretation of immunoblots is necessary for the effective use of this assay in the serodiagnosis of Lyme disease. We describe and compare immunoblot and ELISA results of sequential serum samples from 55 patients with early Lyme disease who presented with EM, utilizing an image analysis system and a database management system. Immunoblot results were examined statistically, and various interpretation criteria were evaluated for their sensitivity and specificity for detecting antibodies in early Lyme disease. Criteria for the interpretation of the IgM and IgG immunoblot for the serodiagnosis of early Lyme disease are proposed.

MATERIALS AND METHODS

Bacterial culture and antigen preparation. *B. burgdorferi* sensu stricto 297, a human spinal fluid isolate from Connecticut (32), was cultured at 30°C in Barbour-Stoenner-Kelly medium (2) with minor modifications (3). Low-passage 297 (fewer than 10 passages in vitro) was used for the immunoblot, and high-passage

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TABLE 1. ELISA serology results for sera from persons with early Lyme disease, healthy blood donors, and persons with illnesses other than Lyme disease

Sample group	Visit	n	% Positive (no. of persons positive) by:			
			IgM	IgG	IgG or IgM	IgG and IgM
Lyme disease	1	55	34.5 (19)	23.6 (13)	45.5 (25)	12.7 (7)
	2	55	63.6 (35)	41.8 (23)	76.4 (42)	29.1 (16)
	3	54	61.1 (33)	40.7 (22)	72.2 (39)	31.5 (17)
	4	54	48.1 (26)	31.5 (17)	59.3 (32)	20.4 (11)
	5	53	47.1 (24)	17.6 (9)	50.9 (27)	9.4 (5)
	6	52	12.2 (6)	8.2 (4)	19.2 (10)	1.9 (1)
Healthy donors		75	0 (0)	1.3 (1)	1.3 (1)	0.0 (0)
Other illnesses		84	12.7 (10)[a]	20.2 (17)	29.1 (23)[a]	5.1 (4)[a]

[a] n = 79 for this group; ELISA IgM was not tested for 5 of the 84 serum samples from persons with other illnesses.

297 (more than 50 passages in vitro) was used for the ELISA. Early-log-phase bacteria (5 to 7 days) were harvested by centrifugation at 4°C. The pellet was washed three times with ice-cold phosphate buffered saline (PBS; pH 7.2). The final suspension in 0.063 M Tris was mixed well, and aliquots (50 μl) were stored at −70°C. The protein content (micrograms per microliter) was determined with a detergent-compatible protein assay (Bio-Rad, Hercules, Calif.) according to manufacturer's instructions.

Serum samples. Six sequential serum samples per patient (55 sets, a total of 318 serum samples) were available from an early Lyme disease treatment study (23). Patients enrolled in the study had physician-documented EM and received antibiotic treatment for 20 days (500 mg of cefuroxime twice a day or 100 mg of doxycycline three times a day). Serum samples were collected at visit 1 (V1, the day treatment was initiated), V2 (day 8 to day 12 into treatment), V3 (day 1 to day 5 posttreatment [PT]), V4 (day 30 PT), V5 (day 90 PT), and V6 (1 year PT). All except five patients responded satisfactorily to antibiotic treatment as assessed at V4. Serum samples from healthy donors (normals) were collected by the Red Cross from an area of nonendemicity for Lyme disease (n = 75). Potentially cross-reactive samples (n = 84) were collected from patients with rheumatoid arthritis (n = 16), systemic lupus erythematosus (n = 5), multiple sclerosis (n = 10), syphilis (n = 29), relapsing fever (n = 2), infectious mononucleosis (n = 12), leptospirosis (n = 6), and group A streptococcal sequelae (n = 4).

MAbs. Monoclonal antibodies (MAbs) were used to identify proteins of *B. burgdorferi* 297. H9724 (flagellin), H5332 (OspA), H5TS (OspB), and P39 (39 kDa) were kindly provided by T. Schwan, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Mont.; CB312 (DnaK), CB625 (22 kDa), and CB49 (19 kDa) were provided by J. Benach, New York Department of Health, Stony Brook; P20a (22 kDa) and O62a (62 kDa) were provided by T. Masuzawa, University of Shizuoka, Shizuoka, Japan; D4 (82 to 93 kDa) was provided by D. Volkman, State University of New York, Stony Brook; L22 1F8 (OspC) was provided by B. Wilske, Pettenkofer-Institut, University of Munich, Munich, Germany; 86 DN-1 (25 kDa) was provided by P. Duffey, Department of Health Services, Berkeley, Calif.; 184.1 (OspA, 22 kDa) was provided by B. Luft, State University of New York, Stony Brook; and 240.7 (7.5 kDa) was provided by G. Habicht, State University of New York, Stony Brook.

ELISA. ELISA was performed as described previously (10) on Lyme disease samples (55 on V1, 55 on V2, 54 on V3, 54 on V4, 53 on V5, and 52 on V6), healthy blood donors, and persons with other illnesses. Briefly, a whole cell sonicate filtrate of *B. burgdorferi* 297 was the antigen source. The 96-well microtiter plates were coated with 0.5 μg of protein per well of the sonicate filtrate (0.22 μm) and dried overnight uncovered at 37°C. The plates were blocked with 1% horse serum, washed four times with 0.05% Tween 20–PBS, incubated with a 1:200 dilution of patient serum for 1 h, and washed five times with 0.05% Tween 20–PBS. After this, the wells were incubated with horseradish peroxidase-conjugated anti-human IgM or IgG, washed five times, and incubated with peroxidase substrate, and the optical density was determined at 405 nm. An optical density of 3 standard deviations or greater above the mean optical density of 200 serum specimens from Red Cross healthy blood donors was considered positive. Between two and three standard deviations above the same mean was considered borderline. Less than two standard deviations above the mean was considered negative.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described previously (13). Briefly, proteins were separated with a linear gradient polyacrylamide gel (7.5 to 15%) (12) with a thickness of 0.75 mm. The gels were poured in a multiple gel caster (Hoefer Scientific Instruments, San Francisco, Calif.) and stored at 4°C for up to 1 month. On the day of electrophoresis, a 3.75% stacking gel was poured with a comb with a 12.2 cm trough and one 1.5 cm well. A cell suspension of low-passage *B. burgdorferi* 297 was thawed, diluted appropriately in sample buffer consisting of 0.063 M Tris, 2% SDS, 15 mM dithiothreitol, 25% sucrose, and 0.002% bromophenol blue, and boiled for 2 min. Three hundred micrograms of protein was loaded onto the gel, and 2 μl of low-molecular-weight standards (Pharmacia LKB) was loaded into the single lane. Electrophoresis by the discontinuous buffer system of Laemmli (30) was carried out at a 35-mA constant current per gel at room temperature for approximately 1.5 h until the dye front reached the bottom of the gel. Following electrophoresis, proteins were transferred to Immobilon P (Millipore Corp., Bedford, Mass.) at 1 A for 30 min by the method of Towbin et al. (33). The standard lane and the sides of the trough were cut off the membrane, washed, and stained with India ink. The center of the membrane containing the antigen section was dried on filter paper and stored in a dry dark place for up to 4 weeks at room temperature.

Western immunoblot. Human serum samples, including those from Lyme disease patients (55 on V1, 55 on V2, 54 on V3, 54 on V4, 51 on V5, 49 on V6), from healthy blood donors, and from persons with other illnesses, were examined by immunoblot for reactivity to proteins of *B. burgdorferi* 297. Two serum samples from V5 and three samples from V6 tested by ELISA were unavailable for immunoblot testing. Prior to immunoblot, the antigen section was marked at the top and bottom, with the India ink-stained sections as guides, for later trimming. A 10.6-cm height, which contained proteins ranging from less than 10 kDa to greater than 150 kDa, was kept. The antigen section was rewet with 0.5% Tween 20–PBS for 25 min. The blot was then blocked for 1 h with 0.5% instant nonfat dry milk in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl [pH 7.5]) and washed for 45 min with 0.1% Tween 20–TBS. After being blocked, the blot was trimmed at the markings on the top and bottom. The remaining section was cut into 3- to 4-mm strips (approximately 10 to 11 μg of protein per strip), which were numbered and incubated for 1 h with human serum samples at a dilution of 1:400 (5 μl of serum plus 2 ml of 0.5% instant nonfat dry milk in TBS) in trays (Accutran disposable incubation trays; Schleicher and Schuell, Keene, N.H.). The strips were then washed twice for 5 min each with 0.1% Tween 20–TBS, after which they were incubated with 2 ml of alkaline phosphatase-conjugated goat anti-human IgG (1:6,000) or IgM (1:5,000) (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) in 0.5% instant nonfat dry milk in TBS for 1 h. The strips were washed twice for 5 min with 0.1% Tween 20–TBS, twice for 5 min with TBS, and twice for 5 min with barbital buffer (150 mM sodium barbital [pH 9.6]). Color was developed for 20 min at 22 to 23°C with a developing solution containing 66 μg of nitroblue tetrazolium per ml, 150 μg of 5-bromo-4-chloro-3-indolyl-phosphate per ml, and 813 μg of $MgCl_2 \cdot 6H_2O$ per ml in barbital buffer. Control of immunoblot intensity development time was kept consistent by the use of moderate IgM- and IgG-positive human control sera with band reactivities of various intensities. Strongly positive control sera were unsatisfactory for this purpose because color development occurred too quickly and led to difficulty in assessing color development with sera containing lower levels of antibodies. To facilitate easier and more accurate band identification, control strips were placed at the first, middle, and last strips of the immunoblot.

Immunoblot analysis. A video optical scanning system consisting of a video camera (500 lines resolution; Panasonic WVBL600), videodigitizing circuit, microcomputer (MacIntosh IIci), Image public domain software (20, 29), and National Center for Supercomputing Applications (NCSA) GelReader software were utilized. NCSA GelReader was used to obtain the molecular weight and intensity of each protein on each immunoblot strip. The intensity was set on a gray scale of 1 to 256 units. The peak of each band was selected for the intensity reading. Three positive IgG control strips on each blot were used to identify nine different proteins of *B. burgdorferi* (88, 72, 58, 41, 39, 34, 31, 24, and 20 kDa), which were used as size markers. These nine proteins had been previously identified with MAbs. The lowest intensity reading (background) level on each blot was set as 1 intensity unit, and all other readings were adjusted accordingly. Band and intensity information and ELISA serology results for all patient samples were stored in an Ingres relational database management system. This database management system was used because its querying capabilities allow immunoblot band patterns common to a set of patients to be extracted from the database and summarized.

Statistical analysis. To determine which proteins were significant markers of early Lyme disease, the frequencies of reactivity for each protein with Lyme disease sera or normal sera were compared in 2-by-2 contingency tables by χ analysis.

RESULTS

We examined sequential serum samples from 55 patients with early Lyme disease and physician-documented EM by ELISA and immunoblot. This patient set was selected from a larger group of 123 patients from a 1-year clinical antimicrobial trial (23). The sample set was not randomly chosen; patients with serum samples available from each of the six visit points (V1 to V6) were more likely to be included in the study

TABLE 2. ELISA and immunoblot results for persons with illnesses other than Lyme disease

Illness	n	No. positive by[a]			
		IgM ELISA	IgG ELISA	IgM immunoblot[b]	IgG immunoblot[c]
Syphilis	20	3[d]	15	0	0
Rheumatoid arthritis	16	1 (25)	0	1 (25)	1 (25)
Infectious mononucleosis	12	4 (23, 24, 65, 67)	0	2 (24, 67)	0
Multiple sclerosis	10	0	0	0	2 (80, 102)
Leptospirosis	6	1 (62)	0	0	1 (61)
Systemic lupus erythematosus	5	0	0	1 (36)	1 (35)
Group A strep sequelae	4	0	0	0	0
Relapsing fever	2	1 (68)	2 (68, 69)	1 (68)	1 (69)

[a] Numbers in parentheses are individual patient identification numbers.
[b] According to the positive IgM immunoblot interpretation criterion two of three proteins (41, 39, and 24 kDa).
[c] According to the positive IgG immunoblot interpretation criterion two of five proteins (88, 39, 35, 24 [>19 intensity units], and 20 kDa).
[d] All three syphilis patients testing ELISA IgM positive were also IgG ELISA positive.

ELISA. Prior to treatment (day treatment was initiated at V1), 25 of the 55 patients (45.5%) had a positive IgM or IgG antibody response by ELISA (Table 1). Twelve (21.8%) of the patients had a positive response only for IgM, and six (10.9%) patients had a positive response only for IgG. Seven patients (12.7%) were positive by both IgM and IgG ELISA. Seven patients (12.7%) had either a borderline IgM or a borderline IgG response, and 23 (41.8%) had a negative response at V1). After 8 to 12 days of treatment (V2), 32.7% (18 of 55) of patients seroconverted to either a positive IgM or a positive IgG ELISA result. The percent of patients with a positive IgM or IgG ELISA response increased from 45.5% (25 of 55) at V1 to 76.4% (42 of 55) at V2, and this was the maximum number of patients with a positive IgM or IgG ELISA response during the 1-year study (Table 1). Also, between V1 and V2 the percent of patients with both a positive IgM response and a positive IgG response increased from 12.7% (7 of 55) to 29.1% (16 of 55). After V2, the percent of patients with a positive IgM or IgG response declined with each subsequent visit, reaching a low of 19.2% (10 of 52) at V6, 1 year PT. At 1 year PT, 12.2% (6 of 52) of the patients were IgM positive and 8.2% (4 of 52) were IgG positive. Twenty percent (11 of 55) of the patients did not respond with either a positive IgM or a positive IgG ELISA at any of the six visits.

Only one of 75 healthy blood donors had a positive IgG ELISA result, and none had a positive IgM ELISA result (Table 1). However, persons with illnesses other than Lyme disease were more likely than healthy blood donors to have positive ELISA results. Of persons with other illnesses, 12.7% (10 of 79) had positive ELISA IgM results, including three with syphilis, one with rheumatoid arthritis, four with infectious mononucleosis, one with leptospirosis, and one with relapsing fever (Table 2). Twenty percent (17 of 84) of persons with other illnesses had a positive IgG ELISA. Fifteen of these patients had syphilis, and two patients had relapsing fever.

Immunoblot. We next investigated the use of the immunoblot in combination with the image analysis system to monitor the antibody response in early Lyme disease. MAbs were used to identify the location of various proteins of *B. burgdorferi* 297 separated by a 7.5 to 15% linear gradient polyacrylamide gel (Table 3). With this gradient gel, the 39-kDa protein was clearly resolved from the 41-kDa flagellin protein (Fig. 1). The following proteins were identified with MAbs and used as reference markers for the immunoblot: 88, 72, 58, 41, 39, 34, 31, 24, and 20 kDa. By the image analysis system and with moderately positive IgG control serum, the mean intensity reading for each of the nine reference proteins on each immunoblot was calculated. The 88-kDa protein had the lowest average intensity reading, 15 U, and the 39-kDa protein had the highest average intensity reading, 92 U. A standard deviation was calculated for the intensity readings of each reference protein and used to assess the reproducibility of this assay. The assay was very reproducible, with only 1 of the 43 immunoblots having two reference proteins with an intensity reading greater than 3 standard deviations from their mean intensity readings. Of the 43 immunoblots, 6 had one of the nine reference proteins outside of 3 standard deviations.

The number of *B. burgdorferi* 297 proteins reactive with sera of early Lyme disease patients was determined for each of the six visits (Table 4). Although the standard deviation is large, the pattern observed was an increase in the number of protein bands reactive with IgM and IgG from V1 (5.8 and 6.7, respectively) to V2 (9.3 and 8.7, respectively) followed by a gradual decrease in the number of reactive protein bands to a low of 4.3 on IgM and 6.7 on IgG at V6. Lower numbers of reactive protein bands were present in immunoblots of healthy blood donors and patients with other illnesses (Table 4). The numbers of IgM- and IgG-reactive bands were 3.0 and 4.1 for healthy blood donors and 2.1 and 3.7 for patients with other illnesses, respectively.

The frequencies at which the various proteins of *B. burgdorferi* 297 were reactive with the sera of ELISA-positive early Lyme disease patients were compared with those of normal

TABLE 3. Protein location as determined by MAb reactivity

MAb	MAb specificity[a]	Size of our corresponding blot protein (kDa)
D4	82–93	88
CB312	DNaK	72
O62a	62	58
H9724	41	41
P39	39	39
H5TS	OspB	34
H5332	OspA	31
86 DN-1	25	NR[b]
L22 1F8	OspC	24
184.1	OspA, 22	31
CB625	22	22
P20a	22	20
CB49	19	20
240.7	7.5	20, 10

[a] Proteins are identified by size (in kilodaltons) or by name.
[b] NR, nonreactive.

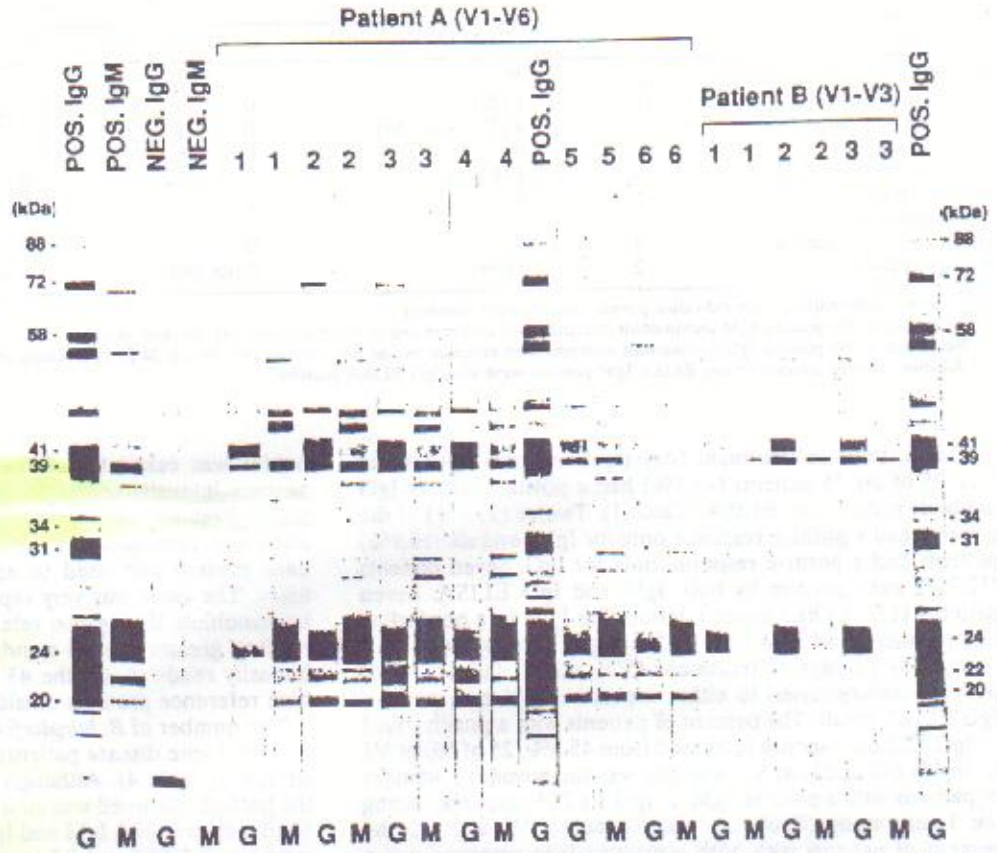

FIG. 1. A representative immunoblot of serum samples from two patients with early Lyme disease. All six visits are shown for patient A, and V1 to V3 are sh for patient B. The corresponding ELISA results for patient A are V1, IgM positive and IgG negative; V2, IgM positive and IgG positive; V3, IgM positive and positive; V4, IgM positive and IgG negative; V5, IgM positive and IgG negative; and V6, IgM positive and IgG negative. For patient B, the corresponding ELISA re are V1, IgM negative and IgG negative; V2, IgM positive and IgG negative; and V3, IgM positive and IgG negative. Markers are identified in kilodaltons on the and right sides. On the bottom of the figure, G indicates IgG and M indicates IgM. On the top of the figure, numbers are patient visit numbers (V1 to V6). Co sera are labeled as positive IgM, positive IgG, negative IgM, and negative IgG.

blood donors by χ^2 analysis. Arbitrarily, proteins reactive with samples from fewer than 35% of ELISA-positive early Lyme disease patients were not studied further. The frequencies of recognition of *B. burgdorferi* 297 proteins by IgM and IgG of ELISA-positive early Lyme disease patients (V1 and V2) and healthy blood donors are shown in Tables 5 and 6. Proteins of significance ($P < 0.01$) for the IgM immunoblot include the following, in decreasing order of significance: 39, 41, 23, 44, 88, 56, 24, 37, 20, 46, and 58 kDa (Table 5). The 39-kDa protein, in addition to being the most significant protein, was also the protein most frequently reactive on IgM immunoblot for patients with early Lyme disease. This protein was recognized by 84% of samples from these patients at V1 and by 94% at V2. In contrast, only 6.7% of the healthy blood donors' samples were reactive with the 39-kDa protein.

Eight proteins were found to be significant for the IgG immunoblot in early Lyme disease. In decreasing order of significance, they are 39, 20, 35, 22, 56, 72, 23, and 88 kDa. Again, the 39-kDa protein was the most significant, and early Lyme disease patients' sera reacted with this protein with a high frequency.

Some proteins were recognized by healthy blood donors frequently but at a very low intensity (weak band), while showing a more intense reaction with sera of early Lyme disease patients. We examined the possibility that the statistical significance of these proteins could be increased by establishing an intensity reading cutoff that would discount reactivities at background levels (Table 7). Utilizing an intensity read cutoff of greater than 19 U proved useful for three prot (46, 41, and 24 kDa) on IgG immunoblot. OspC (24 kDa) frequently recognized at a low intensity by sera of m healthy blood donors (49%, 37 of 75) but only by 5.3% (75) with an intensity reading of greater than 19 U. Sim results were seen for healthy blood donors with respec

TABLE 4. Average numbers of bands seen on immunoblot

Group	Visit	No. of bands of indicated (mean ± SD)	
		IgM	Ig
Lyme disease	1	5.8 ± 6.3	6.7 ±
	2	9.3 ± 7.1	8.7 ±
	3	8.1 ± 6.2	8.6 ±
	4	6.5 ± 5.4	8.2 ±
	5	5.3 ± 4.8	7.4 ±
	6	4.3 ± 3.7	6.7 ±
Healthy donors		3.0 ± 2.6	
Other illnesses		2.1 ± 3.0	3.7 ±

TABLE 5. Frequency of recognition of proteins of *B. burgdorferi* 297 by IgM of ELISA-positive early Lyme disease sera and healthy donor sera

Protein[a]	Frequency (% reactive) of recognition by IgM of:			*P* value[b]
	Early Lyme disease sera[c]		Sera of healthy human donors (n = 75)	
	V1 (n = 19)	V2 (n = 35)		
88*[d]	42	49	2.7	6.4×10^{-7}
58*	48	54	17	0.0057
56*	32	35	1.3	7.3×10^{-6}
46*	74	83	37	0.0044
44*	42	37	1.3	6.9×10^{-8}
41*	68	77	9.3	1.9×10^{-8}
39*	84	94	6.7	4.2×10^{-13}
37*	53	80	19	0.0024
35	47	63	24	0.0443
24*	84	91	44	0.0017
23*	37	40	0.0	4.7×10^{-8}
22	42	57	29	0.2861
20*	68	77	31	0.0025

[a] Identified by size in kilodaltons.
[b] Sera from healthy human donors were compared with early Lyme disease sera from V1.
[c] Data shown are for proteins having frequencies (percent reactive) of at least 35% at V1 or V2 and for sera positive by IgM ELISA.
[d] *, statistically significant for early Lyme disease ($P < 0.01$).

flagellin (41 kDa) and the 46-kDa protein, for which *P* values were decreased to less than 0.01 with an intensity reading cutoff of greater than 19 U. Intensity reading cutoffs were not useful for any proteins on IgM immunoblot.

Using proteins that were statistically significant on the basis of comparison with those of healthy blood donors for IgM and IgG immunoblot, we examined the numbers of these proteins

TABLE 6. Frequency of recognition of proteins of *B. burgdorferi* 297 by IgG of ELISA-positive early Lyme disease sera and healthy donor sera

Protein[a]	Frequency (% reactive) of recognition by IgG of:			*P* value[b]
	Early Lyme disease sera[c]		Sera of healthy human donors (n = 75)	
	V1 (n = 13)	V2 (n = 23)		
88*[d]	23	35	5.3	1.6×10^{-4} (V2)
72*	31	44	6.7	0.0081
58	54	70	45	0.5700
56*	39	44	5.3	2.7×10^{-6}
46	100	70	71	0.0241
41	85	91	49	0.0183
39*	85	91	4.0	2.2×10^{-13}
35*	46	57	9.3	5.5×10^{-4}
24	77	83	49	0.0656
23*	31	35	0.0	8.8×10^{-7}
22*	46	52	9.3	5.5×10^{-6}
20*	69	83	13	1.9×10^{-10}

[a] Identified by size in kilodaltons.
[b] Sera from healthy human donors were compared with early Lyme disease sera from V1, unless specified otherwise.
[c] Data shown are for proteins having frequencies (percent reactive) of at least 35% at V1 or V2 and for sera positive by IgG ELISA.
[d] *, statistically significant for early Lyme disease ($P < 0.01$).

TABLE 7. Use of intensity reading cutoffs to increase the statistical significance of some proteins of *B. burgdorferi* 297 for Lyme disease on IgG immunoblot

Protein[a]	% of samples of indicated type found reactive at indicated intensity reading cutoff				*P* value[b]
	Early Lyme disease sera (V1)[c]		Sera of healthy human donors		
	All levels	>19 U	All levels	>19 U	
46	100	77	71	11	4.6×10^{-8}
41	85	62	49	19	9.8×10^{-4}
24	77	46	49	5.3	1.9×10^{-5}

[a] Identified by size in kilodaltons.
[b] The *P* value was calculated by comparison of early Lyme disease and healthy serum reactivities, with intensity reading cutoffs set at >19 U.
[c] IgG ELISA positive: n = 13.

with which samples from Lyme disease patients reacted regardless of the pattern of reactivity (Table 8). We found interpretation criteria of this type to be useful when the early Lyme disease group was compared with healthy blood donors (data not shown) but not useful in comparisons with persons with illnesses other than Lyme disease. For example, at V1, 63.2% of ELISA IgM-positive early Lyme disease serum samples reacted with any 5 of the 11 significant proteins for IgM immunoblot, but only 30.9% of all early Lyme disease patients reacted with any 5 significant proteins. This criterion was not sensitive, although it was specific for early Lyme disease as only 6.0% of persons with other illnesses reacted. Similar results were found for IgG immunoblot with any 4 of 11 significant proteins, such that 69.2% of ELISA IgG-positive early Lyme disease serum samples and 36.4% of all early Lyme disease serum samples at V1 reacted and only 8.3% of samples from persons with other illnesses reacted.

Since the above criteria could provide specificity but lacked a high sensitivity, we examined the reactivity of specific combinations of significant bands of *B. burgdorferi* to establish the most sensitive and specific interpretation criteria for serodiagnosis of early Lyme disease (Table 9). The results of this investigation showed that for the IgM response, the most sensitive and specific criterion was recognition of two of three bands (41, 39, and 24 kDa). When this criterion was used, 43.6% (24 of 55) of early Lyme disease patients were positive at V1 (the day treatment was initiated) and 74.5% (41 of 55) were positive at V2 (8 to 12 days of treatment). In fact, eight patients having negative IgM ELISA results at V1 were positive by IgM immunoblot according to these criteria. Only one patient converted on IgM immunoblot from negative to positive after V2, compared with 17 patients converting between V1 and V2. This IgM interpretation pattern was specific (92 to 94%) for early Lyme disease, since only 8% (6 of 75) of healthy blood donors and 6.0% (5 of 84) of persons with illnesses other than Lyme disease were positive. Persons with other illnesses recognizing two of three (41, 39, and 24 kDa) proteins on IgM immunoblot included two with infectious mononucleosis and positive ELISA IgM serology, one with rheumatoid arthritis and positive ELISA IgM serology, one with relapsing fever and positive ELISA IgM and IgG serology, and one with systemic lupus erythematosus and negative ELISA serology (Table 2). Although not statistically significant, the IgM immunoblot interpretation criteria appeared to be more sensitive in detecting antibodies in early Lyme disease than IgM ELISA since at V1 43.6% (24 of 55) of patients were positive by IgM immunoblot versus 34.5% (19 of 55) positive by ELISA IgM. This same

blood donors with positive IgG immunoblots results was observed with these two interpretations.

DISCUSSION

Increased use of the immunoblot has resulted from some of the uncertainties associated with the results obtained with the variety of ELISA and indirect immunofluorescent assays presently available. The immunoblot has the potential of being more sensitive and specific than the ELISA and indirect immunofluorescence assay and has been used to confirm results obtained with these assays. However, the lack of standardization of antigen preparations, techniques, and interpretation has limited its usefulness. In an attempt to improve the sensitivity and specificity of the immunoblot, we used a linear gradient gel for better resolution of proteins and an image analysis system and database management system to develop interpretation criteria. The availability of six sequentially collected serum specimens before, during, and after antibiotic treatment from 55 patients with early Lyme disease (physician-documented EM) provided the opportunity to monitor the antibody profiles of these patients in detail.

We first determined which proteins of *B. burgdorferi* 297 that reacted with the antibodies of patients with early Lyme disease were significant. Next, we examined several interpretation criteria for the immunoblot, utilizing our relational database management system. We found that the number of significant proteins a person's serum reacted with was not generally useful since the number of reactive bands required for good specificity resulted in a low level of sensitivity. The immunoblot interpretation recommended by Dressler et al. (6) and used by Aguero-Rosenfeld et al. (1) advanced the following criteria for positive immunoblots: for the IgM immunoblot, at least 2 of 8 common bands in early disease (18, 21, 28, 37, 41, 45, 58, and 93 kDa) and for the IgG immunoblot, at least 5 of 10 bands (18, 21, 28, 30, 39, 41, 45, 58, 66, and 93 kDa) after the first weeks of infection. It was not possible for us to accurately match a number of our protein bands with those described by Dressler et al. (6). For example, we found that our 88- and 24-kDa proteins correspond to their 93- and 21-kDa proteins. However, we were uncertain as to which of our proteins correspond to their 18-, 30-, and 45-kDa proteins. Accordingly, we were not able to satisfactorily apply their interpretation criteria to our immunoblots. This difficulty in matching protein bands is probably due to the use of different strains of *B. burgdorferi* as the antigen source and the use of different acrylamide gel concentrations for protein separation. It also emphasizes the importance of using MAbs for identification of protein bands.

Our study of the use of the immunoblot for the serodiagnosis of early Lyme disease demonstrated that relatively simple criteria can be used for the interpretation of IgM and IgG immunoblots. Only two of three proteins (24 [OspC], 39, and 41 kDa) need be recognized for a positive IgM immunoblot. Recognition of just two of five proteins (20, 24 [>19 intensity units], 35, 39, and 88 kDa) will satisfy the requirements for a positive IgG immunoblot. If a laboratory is not equipped to measure intensity, then the 22-kDa protein can be substituted for the 24-kDa protein. This substitution only slightly decreases the sensitivity of the IgG immunoblot. In addition to the requirement under the proposed interpretation for the identification of only 6 or 7 proteins, MAbs are available for all the proteins except the 35 kDa. As discussed previously, the use of MAbs is of critical importance for the standardization of the immunoblot assay. On the basis of the analysis of a limited number of serum specimens, we found that the criterion we developed for the positive IgG immunoblot in early Lyme disease could also be applied to late Lyme disease. Although antibodies to the 31-kDa (OspA) and 34-kDa (OspB) proteins occur relatively infrequently, these proteins can be included among the significant proteins reactive in late disease because of their high levels of specificity, especially when they occur in tandem. In addition, MAbs for identification of these proteins are available.

The flagellin protein (41 kDa) has been shown to be one of the first proteins that antibodies are directed against after infection with *B. burgdorferi* (4, 5, 11, 17, 22, 36). We found the flagellin protein to be significant for the IgM immunoblot. It was not significant for the IgG immunoblot unless an intensity reading cutoff was used. This was due to the presence of low levels of IgG directed against the flagellin in sera from healthy blood donors and persons with illnesses other than Lyme disease. However, the inclusion of the 41-kDa protein with an intensity cutoff in the criterion for a positive IgG immunoblot did not improve the specificity or sensitivity of the proposed interpretation. Similar results were observed with the 46-kDa protein, which required an intensity level cutoff in order to be significant for the IgG immunoblot.

Another dominant antibody response in early Lyme disease is directed against OspC. The European investigators Wilske et al. (35) and Fuchs et al. (8) were the first to describe and characterize this immunodominant antigen, originally referred to as pC. North American investigators have also described an early and dominant antibody response to this protein in patients with Lyme disease (1, 6, 9, 24). However, the expression of this protein is variable, and it may be poorly expressed in strains that have been passaged many times in vitro (19, 25, 28, 34). We have used low-passage strain 297 for our immunoblot studies, because OspC is the major protein expressed whereas our high-passage 297 expresses this protein at a low level. Using this low passage strain, we observed a strong, specific, and frequent IgM response to the 24-kDa protein (OspC). The IgG response to OspC was also strong and frequent in patients with early Lyme disease, but a response also occurred at a low intensity in healthy blood donors and persons with other illnesses. In order to include OspC as a significant reactive protein for the IgG response, it was necessary to use an intensity reading cutoff of greater than 19 U.

Simpson et al. (31) reported that the 39-kDa protein is specific for *B. burgdorferi* and is strongly serologically reactive. Ma et al. (17) found the 39-kDa protein to be the most significant marker for Lyme borreliosis, with approximately 50% of serum samples from patients with early disease reacting with this protein. Aguero-Rosenfeld et al. (1) reported that antibodies against the 39-kDa protein were observed in 35% of IgM and 26% of IgG immunoblots during the acute phase of early disease. In contrast, Dressler et al. (6) did not observe antibodies reactive with the 39-kDa protein in patients with early Lyme disease. We found the 39-kDa protein to be the most common specific marker for early Lyme disease on both IgM and IgG immunoblots. These variable results could be attributed to the lack of resolution of the 41- and 39-kDa proteins in gel concentrations of less than 12.5%. Variations in the expression of this protein in the strains used may also be important.

We found that the immunoblot was both very sensitive and specific for detecting antibodies in patients with early Lyme disease. Results from our proposed criteria for immunoblot interpretation were compared with results from our in-house ELISA. We found the immunoblot to be more specific than the ELISA. In our control group of 84 serum specimens from patients with illnesses that might resemble Lyme disease, the specificities of the IgM ELISA and the IgG ELISA were 87

TABLE 8. Numbers of significant *B. burgdorferi* proteins recognized on immunoblot by sera of persons with early Lyme disease or other illnesses

No. of significant proteins recognized[a]	Frequency (% reactive) of recognition by Ig of indicated type of serum samples from donors with disease of indicated type					
	IgM			IgG		
	Early Lyme disease (V1; $n = 55$)	ELISA IgM positive early Lyme disease (V1; $n = 19$)	Other illnesses ($n = 84$)	Early Lyme disease (V1; $n = 55$)	ELISA IgG positive early Lyme disease (V1; $n = 13$)	Other illnesses ($n = 84$)
2	72.7	94.7	21.4	61.8	84.6	50.0
3	50.9	84.2	13.1	45.5	84.6	22.6
4	38.2	80.0	11.9	36.4	69.2	8.3
5	30.9	63.2	6.0	25.5	53.8	4.8
6	23.6	63.2	3.6	18.2	53.8	2.4
7	21.8	63.2	2.4	16.4	46.2	1.2
8	14.5	42.1	1.2	12.7	38.5	0.0

[a] Significant IgM proteins include 88, 58, 56, 46, 44, 41, 39, 37, 24, 23, and 20 kDa; significant IgG proteins include 88, 72, 56, 46 (>19 U), 41 (>19 U), 39, 35, 24 (>19 U), 23, 22, and 20 kDa.

trend was seen at V2, when 74.5% (41 of 55) of early Lyme disease patients were IgM immunoblot positive versus 63.6% (35 of 55) positive by ELISA IgM.

The most sensitive and specific interpretation criterion for IgG immunoblot in early Lyme disease was found to be the recognition of two of the following five proteins: 88, 39, 35, 24 (>19 intensity units), and 20 kDa. According to this criterion, 43.6% (24 of 55) of Lyme disease patients were positive by immunoblot at V1 versus 23.6% (13 of 55) positive by ELISA IgG ($P = 0.026$). At V2, 80.0% (44 of 55) of early Lyme disease patients were IgG immunoblot positive compared with 41.8% (23 of 55) positive by IgG ELISA ($P < 0.001$). Few healthy blood donors or persons with other illnesses tested IgG immunoblot positive with this IgG interpretation criterion (4.0 and 7.1%, respectively). Persons with other illnesses testing IgG immunoblot positive included two with multiple sclerosis and negative ELISA serology, one with systemic lupus erythematosus and negative ELISA serology, one with leptospirosis and negative ELISA serology, one with relapsing fever and positive ELISA IgG serology, and one with rheumatoid arthritis and positive IgM ELISA serology (Table 2). Overall, one patient with rheumatoid arthritis had both IgM and IgG immunoblot and ELISA tests positive.

Since the IgG immunoblot criterion requires an image analysis system to analyze the intensity of the 24-kDa protein, it may not be useful to laboratories without this ability. We found that recognition of two of the five proteins 88, 39, 35, 22, and 20 kDa was only slightly less sensitive than the previously mentioned IgG immunoblot interpretation criterion. This pattern replaces recognition of the 24-kDa protein at an intensity greater than 19 U with recognition of the 22-kDa protein at any intensity. According to this IgG immunoblot interpretation, 40% (22 of 55) and 76.4% (42 of 55) of V1 and V2 serum samples were positive, respectively. This compares favorably with the previous pattern incorporating intensity measurements, according to which 43.6% (24 of 55) and 80% (44 of 55) of serum samples at V1 and V2 were positive. According to this modified IgG immunoblot interpretation, one additional patient with other illnesses became positive, resulting in an 8.3% (7 of 84) reactivity. No difference in the numbers of healthy

TABLE 9. Use of patterns for interpretation of IgM and IgG immunoblots

Group	Visit	ELISA result[a]	% Immunoblot positive (no. positive/no. tested)		
			IgM[b]	IgG[c]	IgM or IgG[b,c]
Early Lyme disease	1	Pos., bord., or neg.	43.6 (24/55)	43.6 (24/55)	54.5 (30/55)
	1	Pos.	84.2 (16/19)	76.9 (10/13)	NA[d]
	1	Bord.	0.0 (0/1)	66.7 (2/3)	NA
	1	Neg.	22.9 (8/35)	30.8 (12/39)	NA
	2	Pos., bord., or neg.	74.5 (41/55)	80.0 (44/55)	80.0 (44/55)
	2	Pos.	97.1 (34/35)	91.3 (21/23)	NA
	2	Bord.	66.7 (4/6)	90.0 (9/10)	NA
	2	Neg.	21.4 (3/14)	63.6 (14/22)	NA
	3	Pos., bord., or neg.	72.2 (39/54)	79.6 (43/54)	80.0 (43/54)
	4	Pos., bord., or neg.	59.3 (32/54)	77.8 (42/54)	80.0 (43/54)
	5	Pos., bord., or neg.	41.2 (21/51)	68.6 (35/51)	72.5 (37/51)
	6	Pos., bord., or neg.	26.5 (13/49)	49.0 (24/49)	59.2 (29/49)
	1–2	Pos., bord., or neg.	74.5 (41/55)	80.0 (44/55)	NA
	1–4	Pos., bord., or neg.	76.4 (42/55)	81.8 (45/55)	NA
Healthy donors		Pos., bord., or neg.	8.0 (6/75)	4.0 (3/75)	12.0 (9/75)
Other illnesses		Pos., bord., or neg.	6.0 (5/84)	7.1 (6/84)	11.9 (10/84)

[a] Refers to corresponding ELISA IgM or IgG results of samples; pos., positive; bord., borderline; neg., negative.
[b] Positive interpretation criterion: two of three proteins (41, 39, and 24 kDa).
[c] Positive interpretation criterion: two of five proteins (88, 39, 35, 24 [>19 intensity units], and 20 kDa).
[d] NA, not analyzed.

and 80%, respectively, whereas the specificities of the IgM and IgG immunoblots were 94 and 93%, respectively ($P = 0.0806$). The IgM ELISA and immunoblot were similar in sensitivity for detecting antibodies in early Lyme disease. Although the IgM immunoblot was slightly more sensitive than the IgM ELISA, the difference was not statistically significant. There was a significant difference between the sensitivities of the IgG ELISA and immunoblot. At V1 and V2 (acute- and convalescent-phase specimens), 34.5 and 63.6% of the patients were ELISA IgG positive whereas 43.6 and 80.0% were positive by IgG immunoblot. The finding that the IgG immunoblot was already positive for 80.0% of the patients after only 8 to 12 days of treatment was unexpected. Generally, the IgG response at this early stage of the disease was thought to be of little diagnostic value. For example, Dressler et al. (6) reported that their 25 patients with EM had only a minimal specific Ig response 2 to 4 weeks after antibiotic therapy and did not analyze these serum samples with the IgG immunoblot. Our results suggest that the IgG response can be of considerable value in the serodiagnosis of early Lyme disease. In addition, some patients who are reinfected may have an IgG response only in early disease. It is possible that the greater sensitivity of the immunoblot versus the ELISA may be due in part to the use of high-passage *B. burgdorferi* 297 in the ELISA and low-passage 297 in the immunoblot.

The highest level of positive serology was seen at V2 (day 8 to day 12, during a 20-day antibiotic treatment regimen), when 76.4% (42 of 55) of patients were positive by ELISA IgG or IgM and 80.0% (44 of 55) were positive by IgG or IgM immunoblot. Since 93% of the seroconversions occurred by V2, it may be of value for physicians to test a patient approximately 2 weeks into treatment, if serology is negative at the patient's first consultation. This may minimize problems later if treatment is unsuccessful or symptoms reoccur by providing serologic confirmation of the earlier diagnosis of Lyme disease.

The length of time antibodies persist will depend on the serological assay used. The ELISA measures antibody concentration, and 1 year after treatment 17.1% (6 of 35) of the IgM ELISA-positive patients and 17.4% (4 of 23) of the IgG ELISA-positive patients remained positive. The immunoblot as routinely used measures only the presence of antibodies, and accordingly more patients remained antibody positive with this assay. One year after therapy, 31.7% (13 of 41) of the IgM immunoblot- and 54.6% (24 of 44) of the IgG immunoblot-positive patients remained positive. Five patients in this study group did not have a satisfactory response to antibiotic therapy, as assessed at V4. Neither the ELISA nor the immunoblot was helpful in identifying these patients. Feder et al. (7) examined the persistence of antibodies in patients with a variety of clinical manifestations (EM, arthritis, and neuropathy) who received appropriate antibiotic therapy. They reported that bands on the IgG immunoblot could be detected as long as 3 years after therapy and that the ELISA and immunoblot were not helpful for identifying patients with persistent or recurrent symptoms.

It has been suggested that early antibiotic therapy may abort the antibody response in patients treated soon after infection (30). Aguero-Rosenfeld et al. (1) reported that 13% (8 of 59) of treated early-disease patients did not seroconvert and two of these patients had culture-positive EM lesions. We found that 20% (11 of 55) of our patients with early Lyme disease did not develop either a positive IgM or a positive IgG immunoblot during the duration of this study. It is possible that the clinical diagnosis of EM for some of these patients was incorrect since culture of the skin was not conducted.

On the basis of our study of 55 clinically and serologically well-defined patients with early Lyme disease, several recommendations for standardization and use of the immunoblot for the serodiagnosis of Lyme disease can be made. (i) MAbs should be available and used for identification of proteins that are of diagnostic importance, since they may vary in molecular weight in different isolates of *B. burgdorferi*. The use of MAbs will allow the comparison of the results of immunoblots from different laboratories. (ii) Since there is variation in the expression of some proteins by *B. burgdorferi* strains, only those that adequately express the proteins of diagnostic importance should be used. (iii) The concentration of acrylamide gel used must be sufficient to allow resolution of proteins of similar molecular masses, such as the 39- and 41-kDa proteins. These two proteins are not well separated on 10% acrylamide gels. (iv) Both IgM and IgG assays should be conducted on sera from patients with early Lyme disease. Most patients with early disease have a good IgG response, and some reinfected patients may have only an IgG response. (v) Patients seronegative at the time treatment is initiated should be retested 2 weeks later if serological confirmation of the clinical diagnosis is desirable.

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Plenary Presentations and Workgroups

CRITERIA FOR FDA CLEARANCE OF LABORATORY TEST KITS

Roxanne Shively, M.S., M.T., SC (ASCP)

The Federal Food Drug and Cosmetic Act (FD&CA), by amendments passed in 1976, provides for regulation of in vitro diagnostic laboratory test kits. Additionally, the safe Medical Devices Act of 1990 enacted new regulations that apply to in vitro diagnostic laboratory test kits. The regulations specify three regulatory classes for medical devices according to the level of controls deemed necessary to assure device safety and effectiveness: Class I devices require general controls and are legally marketed by Section 510 (k) notification; Class II devices require special controls and premarket approval (PMA). A PMA is designed to establish that all aspects of a device are reasonably safe and effective for its intended use. A 510 (k) establishes substantial equivalence to a pre-amendments device and also (following enactment of SMDA) may require clinical data to provide a reasonable assurance of safety and effectiveness. Legally, all devices are considered Class III until determined substantially equivalent, at which time they are classified.

Commercial test kits for detecting antibodies to *B. burgdorferi* are cleared for legal marketing by 510 (k) notification. They are classified under treponemal test reagents (21 CFR Section 866.3830) as Class II devices. A 510(k) is required when a device is introduced into the market for the first time, a new device is being introduced that may already be marketed by another manufacturer, a device currently distributed is to be significantly changed or modified, or there is a change of intended use. Substantial equivalence is having the same intended use and the same technological characteristics as a predicate; or having different technological characteristics but performance data demonstrates safety and effectiveness equivalent to a predicate, and that there are no new issues of safety and efficacy.

The FDA review of test kits is not a certification nor does it include laboratory testing by the FDA. The review is a scientific assessment of the clinical utility, relevant medical literature, supporting performance data, and labeling information. Recommendations from NCCLS and other professional groups are often embraced. For example, the proposed guidelines Specifications for Immunological Testing for Infectious Diseases (NCCLS I/LA18-P) has been an invaluable resource for FDA in reviewing all serological assay devices.

IFA type serological tests for antibodies to *B. burgdorferi* have been cleared for marketing since 1987, EIA formats since 1988. Today legally marketed commercial kits are available from 14 different manufacturers in 35 different formats (IFA and EIA for IgG, IgM, and total antibody). At this time, no Western blot methods have been cleared for marketing. Other than indirect serological assays, no direct detection tests using antigen detection, capture, or nucleic acid hybridization are cleared by the FDA for marketing. Serological assays are not considered diagnostic as a stand-alone test; they are only useful for testing patients with suggestive signs and symptoms. As with most serological assays, disadvantages include insensitivity in early disease stages and poor reproducibility between methods, inter-, and intra- laboratory.

Since 1991, FDA review of serological assays is based on an internal guidance document that is available to the public: "Review Criteria for Assessment of Serological in vitro Diagnostic Devices for Detection of Serum Antibodies to *B. burgdorferi*." The NCCLS document previously mentioned is also used. The FDA internal guidance document outlines the types of information (descriptive and data) that should be included in a submission of a 510 (k) notification; it also includes recommendations for the labeling (package insert). The guidance document is available from the Division of Small Manufacturers Assistance (telephone: 800-638-2041) by requesting the name of the document (dated 9/12/91) and coded GDL-0133.

SUMMARY AND FUTURE DIRECTION OF SEROLOGIC DIAGNOSIS OF LYME DISEASE

Duane J. Gubler, Sc.D.

Approximately 5 years ago, the Centers for Disease Control and Prevention (CDC) embarked on a program to better define Lyme disease and its public health importance in the United States. We outlined several major objectives: 1) to better define the epidemiology and ecology of the disease, 2) to implement standardized national surveillance, 3) to implement improved, standardized diagnostic testing for *Borrelia burgdorferi*, and 4) to develop more effective prevention and control strategies for Lyme disease. Clearly, a great deal of research was needed before we could achieve any of these objectives, especially standardized diagnostic testing. With the help and partnership of the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD), the Council of State and Territorial Epidemiologists (CSTE), and health professionals all over the country, significant progress has been made.

An evaluation of diagnostic test kits sold in this country in 1989-90 culminated in the First National Conference on Lyme Disease Testing in November 1990, where I gave a talk by the same title as I have today.[1] At that time, I was particularly discouraged because an evaluation of available Lyme disease serologic tests had confirmed what many of you already knew, that the tests were inaccurate and that concordance in test results between laboratories was poor.[2] I concluded at that time that there were fundamental problems with the serologic tests used for Lyme disease and that we must go back to basic bacteriology and attempt to confirm *Borrelia burgdorferi* infection in patients by culturing the spirochete. Only then could we be sure that the serum specimens used to develop serologic tests came from persons actually infected with *B. burgdorferi*.

At that meeting in 1990, I committed CDC to obtaining a reference collection of serum specimens from patients whose infections were bacteriologically confirmed and clinically well characterized, and to using that reference collection to develop new and more accurate diagnostic tests.[1] As you have heard over the past 2 days, tremendous progress has been made, primarily because of the partnerships and collaborations between academic research centers, commercial manufacturers, ASTPHLD, and CDC. The unselfish commitment and dedication of these groups and many others not mentioned have resulted in significant progress and an approach for serologic diagnosis of Lyme disease that, while still not perfect, has come a long way in terms of accuracy.

As you have heard, a two-test approach to serologic diagnosis of Lyme disease, using a sensitive ELISA as the front-line test and the Western Immunoblot as a confirmatory test on all serum specimens that show ELISA positive or equivocal results, provides relatively high sensitivity (dependent on stage of disease) and specificity >98%.[3] Of significant importance is that these two tests can be fully standardized so that serologic results will be interpreted the same way regardless of where in the country the person is infected. As has been discussed at this meeting, geographic variation has been documented among strains of *B. burgdorferi*, and the full

implications of this observation on serologic test performance and interpretation need further study.[3] While the two-test approach that we propose is not the gold standard, it does provide a reliable and reasonably accurate laboratory diagnosis that can be used anywhere in the country. In the meantime, as you have also heard, there are many exciting developments on the horizon. I think we can expect in the not too distant future, the development of new tests that use a cocktail of recombinant antigens or chimeric antigens that will improve the sensitivity and specificity of serologic tests for *B. burgdorferi*.

As with all infectious diseases, it is difficult to achieve an unequivocal serologic diagnosis of *B. burgdorferi* infection in the acute stage of disease. Culture of the spirochete or direct detection methods must be used for this, and as we all know, these methods are not always practical. As a result, physicians must continue to rely on their clinical judgement in deciding whether to treat a patient for Lyme disease. However, sensitive and specific laboratory diagnostic tests are critical to help physicians better define the clinical spectrum of *B. burgdorferi* infection and to help the epidemiologists gain a better understanding of the epidemiology and risk factors associated with transmission.

So where do we go from here? The multicenter studies, using large panels of coded serum specimens collected from persons with clinically and bacteriologically well-characterized illness, have given us the confidence to come to you this week and propose that we move forward with standardized serologic testing on a national level. We recognize that the approach is still not the best we can achieve, but it is good enough to move ahead. There may be better tests that we do not know about, and we have heard at this conference in the past 2 days about many tests that have potential. We encourage continued development of these tests, but it should be pointed out that they must be properly evaluated and they must be standardizable if they are to be used for serologic diagnosis of Lyme disease.

There are two basic approaches to achieving national standardized serologic testing. The first, and one that has been followed by default, has been to wait until the perfect test has been developed before implementing the program. However, we feel this approach is not in the interest of public health. A second approach is to move ahead and implement national standardized testing with the two-test approach proposed at this conference. The rationale for this is that implementation of standardized testing will take some time. If we move now to put the process in place, as new and better tests are developed and evaluated, they can be integrated into the system without delay. In the meantime, we will all be able to interpret results and understand each other when we talk about serologically positive Lyme disease, regardless of where we are in the country.

We must remember that even sensitive and specific tests are only as good as the persons and the laboratory conducting the test. Some questionable findings have been published in recent years, and we as a group must be vigilant to police ourselves and critically evaluate our work. It helps no one to have inaccurate and uncontrolled laboratory tests that mislead the physician in prescribing treatment. I implore you all to insist on quality control and to help us bring Lyme

disease laboratory diagnostic testing up to the level that we demand of diagnostic testing for other infectious diseases.

It has been agreed at this conference that a rigorous national proficiency testing program must be implemented and that the emphasis of this program should be on outcome. Many of you have suggested that CDC be responsible for implementing this program. Unfortunately, CDC does not have the resources to implement such a program alone, but will work with ASTPHLD to make sure that a quality proficiency testing program is developed. Given proper support from the Lyme disease community, CDC can play a leadership role in coordination and supervision of this program.

In conclusion, I want to thank all of you for your attendance and participation in this important Second National Conference on Lyme Disease Serologic Testing. I think we can return home from this conference feeling much better than we did 4 years ago. Many, but not all, of the fundamental problems mentioned earlier have been solved.

However, we have much work to do. I urge everyone who has an interest in this disease to participate in this exercise of national standardization of Lyme disease serologic testing. Working alone, ASTPHLD and CDC will fail. Working in partnership with the entire Lyme disease community as a team and with the guidance of scientists conducting research on diagnostic test development, however, I feel will ensure success.

I would like to finish with a quote from Lewis Carroll's Alice in Wonderland, which says, "If you don't know where you are going, any road will take you there." This quote typifies research on serologic diagnosis of Lyme disease in this country, with everyone going in different directions to presumably the same destination. However, I believe we have now identified a single road that we can all follow. I would urge all of you to join us on that road to standardization of serologic testing for Lyme disease.

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Abstracts

(These abstracts did not go through a peer review process)

WORKGROUP A: Standardization and Interpretation

Paul T. Fawcett, Ph.D., Alfred I. DuPont Institute, Wilmington, Delaware
Cross Reactivity and Antigen (Band) Identification by Western Blotting

Arthur Markovits, MSPH, MarDx Diagnostics, Carlsbad, California
Standardization of Lyme Western Blot Interpretation

Dr. Edward Guy, Public Health Laboratory, Swansea, United kingdom
Standardization of the Laboratory Diagnosis in Lyme Borreliosis - Suggestions from Europe

Frank Dressler, M.D., Kinderklinik NHH, Hannover, Germany
Standardization of the Laboratory Diagnosis in Lyme Borreliosis - Suggestions from Europe, part 2.

Maria E. Aguero-Rosenfeld, M.D., et. al., New York Medical College, Westchester County Medical Center, Valhallia, New York
Seroconversion in Treated Patients with Culture Positive Erythema Migrans.

R.H. Seder, M.D., M.P.H., Vice President, Imugen, Norwood, Mass.
Accuracy of Diagnosis and Staging of Well Characterized Lyme Disease patients (CDC) by an Antibody Capture / Western Blot Panel. Compared with IgM and IgG ELISA, and IgM and IgG Western Blotting and a Flagellin-Enriched ELISA.

Kay Case, Immunology Laboratory, Lutheran Hospital, LaCrosse, WI
Use of a Monoclonal Standard Curve and Uniform Band Designations

Sunil K. Sood, M.D., Zemel, L.S., M.D. and Ilowite, N.T., M.D., Pediatric Lyme Disease Center, Division of Infectious Diseases and Division of Rheumatology, Schneider Children's Hospital, Long Island Jewish Medical Center, the Long Island Campus for Albert Einstein College of Medicine, New Hyde Park, NY; and University of Connecticut School of Medicine and Division of Rheumatology, Newington Childrens Hospital, Newington, CT.
Interpretation of Lyme Borreliosis Immunoblot in Pediatrics

Workgroup A: Moderator: Stanley Inhorn, M.D., Co-Director WI State Laboratory of Hygiene

Cross Reactivity and Antigen (Band) Identification by Western Blotting

Submitted by: Paul T. Fawcett, Ph.D., Head of Immunology Laboratory at the Alfred I. DuPont Institute, 1600 Rockland Road, P.O. Box 269, Wilmington, DE 19899

This presentation will focus on the types of cross reactive binding detected on Western blots of *B. burgdorferi* when sera from normal and disease control patients are tested. Banding patterns from patients with well documented Lyme disease will also be presented to demonstrate potential problems associated with identification of antibodies to specific antigens by KD.

Data to be presented were selected from a Lyme disease clinic population and control patient population over a 4 year period. Normal controls were obtained from autodonors for elective orthopedic surgery and healthy blood bank donors. Disease control sera was obtained from patients with a variety of confirmed autoimmune and infectious diseases for whom Lyme borreliosis was ruled out. Sera from Lyme borreliosis patients was obtained from cases which met CDC criteria.

Results demostrate which blotted antigens of *B. burgdorferi* are most likely to bind cross reactive antibodies (primarily 41 and 60 range KDs) and which Ags of *B. burgdorferi* appear to have high diagnostic specificity as well as their frequency of detection.

Results to be presented on banding patterns demostrate the intra and inter lot variability in antigen location on Western blot as well as providing an indication of patient to patient variability in Ag recognition.

The objective of this presentation is to demonstrate some of the technical problems which should be recognized and taken into account when attemping to standized and establish interpretive criteria for Western blots of *B. burgdorferi*.

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The Second National Conference on Serologic Diagnosis of Lyme Disease
Dearborn, Michigan

October 28, 1994

A Technical Report
Lyme Western Blotting of the CDC ARC Panel

A clinical study consisting of 500 clinically defined sera from four stages of Lyme Borreliosis, normal specimens from non-endemic geographic locations, and sera from patients found positive by tests other than Lyme.

Introduction

In this blind study from the CDC, Division of Vector-Borne Infectious Diseases, Fort Collins, CO., 500 clinically defined specimens (CDC ARC Panel) were tested by MarDx Lyme IgM and IgG Western Blot. Banding results and interpretations were processed, entered into a spreadsheet and sent to the statistician, Mr. Ray Bailey of the CDC, for decoding.

The retrospective study, contained 200 clinically defined Lyme specimens, sera from 200 healthy blood donors residing in non-endemic areas, and 100 potential cross-reactive serum specimens from patients with:

1. Systemic lupus (SLE)
2. Rheumatoid arthritis
3. Felty's syndrome
4. Leptospirosis
5. Syphilis
6. Periodontal disease
7. Tularemia
8. Equivocal EIA results from patients which did not meet the CDC surveillance case definition
9. Tick-borne Relapsing Fever were analyzed using the MarDx Lyme IgM and IgG Western Blot Test system.

It was the goal of this study to evaluate the MarDx Western Blot test systems and interpretation criteria using the CDC ARC Panel. Sensitivity and specificity of the various case and control groups were determined.

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MarDx IgG Positive Criteria

IgG Blot Positive / Any 5 of 12 bands:

18, 23,28,30,31,34***,39,41,45,58,66,or 93kDa
*** Bolded molecular weights are new bands added to Dressler-Steere criteria

MarDx IgM Positive Criteria

IgM Blot Positive /

23 plus one of the following:
18,31,34,37,39,41,93kDa

-or-

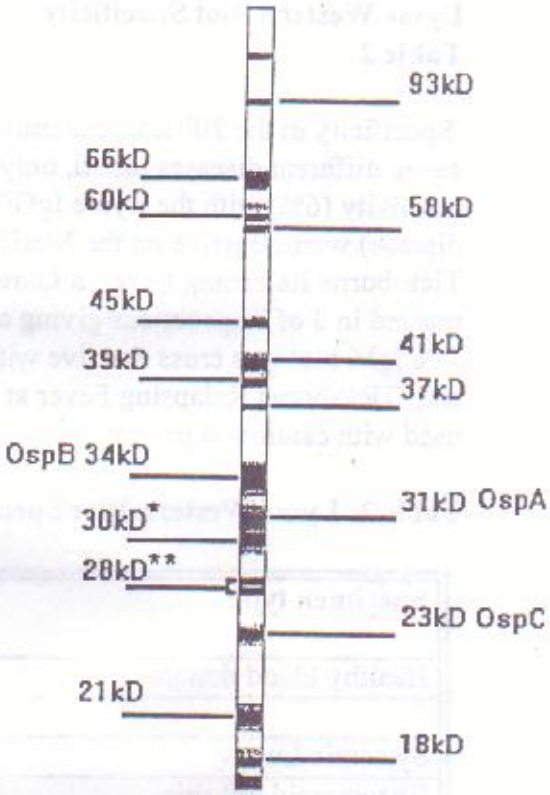
37 plus one of the following:
18,23,31,34,39,41,93kDa

NEGATIVE

Any pattern that does not meet the positive criteria.

[1]The 93kD band and the 83kD referred to in the literature is the same band and will be referred to as the **93kD** band.

[2]The Osp C band referred to in the literature as 25kDa, 23kDa, or 21kDa is the same band and will be referred to as the **23kDa Osp C** band.



* The 30kDa band may be diffuse or have multiple thin bands.
** The 28kDa (s) band can be seen with either one of the two bands or both bands.

Conclusions

Results of this study provide evidence for the intended use of separate MarDx Lyme IgM and IgG Western Blot Test Systems. When properly used with staged sera from suspected *B. burgdorferi* infected individuals, sensitivity and specificity of the test can be 95% and 100% respectively. These tests must not be used on first stage EM patients (4 weeks after onset) or patients known to have had Tick-borne Relapsing Fever. Periodontal disease is not seen to be cross-reactive with this test and therefore does not present a cause for false positive results. Specificity of this test is greater than 94% with all diseases tested, SLE, RA, Felty's syndrome, Leptospirosis, Syphilis, Periodontal disease, and Tularemia.

MarDx would like to thank Dr. Barbara Johnson, Mr. Ray Bailey, Dr. May Chu, and Dr. Martin Schriefer for the opportunity to participate in the CDC ARC panel study for Lyme Western Blot.

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Lyme Western Blot Specificity
Table 2

Specificity in the 200 non-endemic normal specimens was 97% for IgG and 87% for IgM. Of the seven different diseases tested, only Syphilis serum specimens were found to have any cross-reactivity (6%) with the Lyme IgG Western Blot. All other six diseases (including periodontal disease) were negative on the MarDx Lyme IgG Western Blot Test, 100% specificity.
Tick-borne Relapsing Fever, a known serologic cross-reactive organism with *B. burgdorferi*, cross reacted in 3 of 7 specimens giving a false positive rate of 42%.
The IgM blot was cross reactive with Systemic Lupus (27%), Leptospirosis (9%), Syphilis (6%), and Tick-borne Relapsing Fever at (72%). The MarDx Lyme Western Blot Test Systems must be used with caution in patients known to have had Tick-borne Relapsing Fever.

Table 2: Lyme Western Blot Specificity

Specimen type	Number Tested	Specificity IgM		Specificity IgG	
Healthy blood donors	200	(26/200)	87%	(6/200)	97%
Systemic Lupus	11	(3/11)	73%	(0/11)	100%
Rheumatoid arthritis	7	(0/7)	100%	(0/7)	100%
Felty's syndrome	2	(0/2)	100%	(0/2)	100%
Leptospirosis	22	(2/22)	91%	(0/22)	100%
Syphilis	17	(1/17)	94%	(1/17)	94%
Periodontal disease	9	(0/9)	100%	(0/9)	100%
Tularemia	8	(0/8)	100%	(0/8)	100%
Tick-borne Relapsing Fever	7	(5/7)	28%	(3/7)	58%
"Equivocal" EIA specimens	17	(2/17)	88%	(0/17)	100%

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Sensitivity: Table 1

Erythema migrans

0 to 4 weeks after onset

During the erythema migrans phase (clinically classified) the IgM blot had a sensitivity of 73% and the IgG sensitivity was 50%. During the erythema migrans phase (culture positive) the IgM blot had a sensitivity of 70% and the IgG sensitivity was 43%.

Meningitis

8 weeks after onset

Although both the IgM and IgG blots were efficacious in the detection of early stage neurologic manifestations, all seven 100% of the patients were positive by IgM blotting. The IgG sensitivity for this phase with a limited population sample of seven was 87%.

Arthritic Borreliosis

month to years after onset

From this stage forward the IgG blot had a high sensitivity while the IgM sensitivity predictably decreased. The IgM sensitivity for this phase was 48% while the IgG sensitivity was 97%.

Chronic neuro-borreliosis

years after onset

As during the arthritic stage of this disease, the IgG blot had maximum sensitivity at 100% while the IgM sensitivity for this phase was 69%.

Table 1: Lyme Western Blot Sensitivity

Specimen type	Number Tested	Sensitivity IgM		Sensitivity IgG	
EM Clinically Classified	26	(19/26)	73%	(13/26)	50%
EM Culture Positive	70	(49/70)	70%	(30/70)	43%
Meningitis Stage	7	(7/7)	100%	(6/7)	87%
Arthritic Stage	62	(30/62)	48%	(60/62)	97%
Neuro-borreliosis	25	(17/25)	69%	(25/25)	100%

The IgG blot was most sensitive during the arthritic and neuro-borreliosis stages of the disease (97% and 100% sensitivity respectively). The IgM blot was most sensitive during the meningitis stage of the disease (100%)

STANDARDISATION OF THE LABORATORY DIAGNOSIS IN LYME BORRELIOSIS-SUGGESTIONS FROM EUROPE

Edward Guy, Public Health Laboratory, Swansea, UK, and Frank Dressler, Kinderklinik MHH, Hannover, Germany, for the European Union-Funded Research Network "Risk Assessment of Lyme Disease'

A European Union-funded research network (EU-network) for 'Risk Assessment of Lyme Disease' was set up to promote the coordinated study of ecological, epidemiological and clinical aspects of Lyme borreliosis across Europe. One of the research groups within this network deals with the laboratory diagnosis in specimens from humans and animals.

The clinical presentation of Lyme borreliosis shows certain differences in Europe and in North America. For example, borrelial lymphcytoma and acrodermatitis chronica atrophicans occur much more frequently in Europe. The greater variation of *Borrelia burgdorferi* strains in Europe, including the isolation of three of the four known different species from patients, may be responsible for some or all of these differences. Within Eurpoe there are significant differences in the vector ticks (mainly *Ixodes ricinus* and *ixodes persulcatus)*, the rate of tick infection by the spirchete and the frequencies and clinical presentations of Lyme borriliosis.

Any attempts at standardisation of laboratory diagnostic criteria for Lyme borreliosis in Europe have to take such aspects of variation into account. In an initial study an interlaboratory comparison was undertaken in 5 laboratories in Austria, France, Great Britian, Italy, and The Netherlands. Sera from 100 patients (with erythema migrans or with clinically diagnosed late Lyme borreliosis, and healthy and disease controls including patients with syphilis were investigated in a blinded fashion. Each laboratory used its own serological test, usually an elisa, with or without a confirmatory second test, usually Western blot.

Preliminary analysis of the data indicates a significant discrepancy between the sensitivity and specificity of some of the tests employed. The laboratories with the highest sensitivities did not achieve the highest specificities, and visa versa. The highest specificities (up to 100%) were achieved by laboratories employing Western blots.

Considering the large number of laboratories using a wide range of commercial or 'in-house' diagnostic tests and the apparent confusion among many clinicians caring for patients with positive Lyme serologies, we have concluded that a larger European study is indicated. One important question that must be addressed is whether serodiagnostic methods can be improved by incorporation of antigen from more than one species of *B. burgdorferi*. The strategy for such a study will be the subject of a special session of the EU-network in Vienna on October 16th. The results of this meeting, including our proposed plan, will be presented in Dearborn for discussion.

STANDARDISATION OF THE LABORATORY DIAGNOSIS IN LYME BORRELIOSIS-SUGGESTIONS FROM EUROPE (PART 2)

Frank Dressler, MD, kinderklinik MHH; 30623 Hannover; Germany; for the European Union-Funded Research Network "Risk Assessment of Lyme Disease'

A European Union-funded research network for 'Risk Assessment of Lyme Disease' was set up to promote the coordination study of ecological, epidemiological and clinical aspects of Lyme borreliosis across Europe (chairman: Dr. J. Gray, Dublin, Ireland). One of the research groups within this network deals with the laboratory diagnosis in specimens from humans and animals (chairman: Dr. E. Guy, Swansea, UK).

The clinical presentation of Lyme borreliosis shows certain differences in Europe and in North America. For example, borrelial lmphocytoma and acrodermatitis chronica atrophicans occur more frequently in Europe. The greater variation of *Borrelia burgdorferi* strains in Europe, including the isolation of three of the four known differences in the vector ticks(mainly *Ixodes ricinus* and *Ixodes persulcatus*), the rate of tick infection by the spirochete and the frequencies and clinical presentations of Lyme borreliosis.

Attempts at standardisation of laboratory diagnostic criteria for Lyme borreliosis in Europe have to take these aspects of variation into account. At a meeting of the collaborators of the Concerted Action in Vienna on October 16 and 17 (organized by Dr. G. Stanek, Vienna, Austria) the following steps were discussed: First, there is a need for more specific case definitions based on clinical criteria and well established laboratory tests, such as culture, histology, and serological methods. New criteria for erythema migrans, acrodermatitis chronica atrophicans, neuroborreliosis, and Lyme arthritis were discussed and will be published after futher review. Second, there is a need to establish a bank of specimens from European patients with well -established Lyme borreliosis. This bank may be developed under the guidance of the WHO in Geneva. Third, there was general agreement that attempts at standardisation of laboratory tests should start with serological methods.

A questionnaire will be sent to a large number of European laboratories, in order to identify which methods are currently used, how the cut-offs are determined and the results are interpreted, what antigens are used.

Next, we discussed three kinds of studies needed in the next years: First, single laboratory studies using sera from different parts of Europe could establish Western blot criteria suitable for European patients, test the value of antigens from each of the three strains of *B. burgdorferi* in serodiagnosis in Europe, or the value of recombinant antigen preparations in sera from our patients. Second, a blinded study involving the laboratories of the Concerted Action collaborators as well as a number of other leading European laboratories could investigate, whether the two-test approach advocated by the CDC for use in North America is suitable in Europe or whether a one-test method can yield similar results. Finally, a quality assurance program with a small panel of high volume sera should be developed to be used by any European laboratory willing to participate in such an effort.

The members of the Concerted Action welcome the participation of all colleagues interested in the standardisation of Lyme testing in Europe.

SEROCONVERSION IN TREATED PATIENTS WITH CULTURE POSITIVE ERYTHEMA MIGRANS.

Aguero-Rosenfeld, M.E., J. Nowakowski, S. Bittker, D. Cooper, R. Nadelman, and G.P. Wormser.
Departments of Pathology and Medicine, New York Medical College, Westchester County Medical Center, Valhalla, NY

The development of IgM and IgG antibodies to *B. burgdorferi* in patients with erythema migrans (EM) correlates directly with the duration of disease prior to antibiotic treatment. We present our experience with 46 patients with culture positive EM attending the Lyme Disease Diagnostic Center at Westchester County medical Center, Valhalla, NY, during the 1991 and 1992 seasons. These patients were periodically evaluated after their initial visit and sera collected at each visit. Sera were tested by commercial IgM and IgG immunoblots (MarDx diagnostics) and polyvalent ELISA (Whittaker Bioproducts). Blot results were interpreted according to the criteria of Dressler et al.

At baseline 38% of patients with EM< 7days duration (n=29) had a positive IgM immunoblot (IB) and 10% a positive ELISA; patients with EM of 7-14 ds duration (n-11) were positive in 73% and 55% by IgM IB and ELISA respectively and all 6 patients with EM>14 ds duration (range 16-23 ds) had a positive ELISA and IgM IB (5 patients also had a positive IgG IB). As we previously reported, the IgM bands most frequently seen at baseline were 23-kDa (ospC) (63%) and 41-kDa (54%).

Seroconversion Sera collected 8-14 days after initial visit showed seroconversion by IgM IB and ELISA in 83% of patients initially testing negative. An additional 3% of negative patients seroconverted at day 20 or day 30 post initial visit. Greater intensity and number of bands were also observed in IgM IB of sera collected at day 8 - 14 post baseline. Most frequently observed bands in IgM IB blots in these sera were: 23 and 41 kDa (86%), 37-kda (68%), 39-kDa (54%) and 60-kDa (43%). Although IgG bands appeared and/or increased in number and intesity in majority of blots at some point during follow-up, only 20% of patients with EM <7ds and 36% of patients with EM-7 14 ds duration could be considered positive by Dressler's criteria.

In conclusion: IgM IB appears to be more sensitive thatn polyvalent ELISA in patients with EM of <7ds duration, IgM reactivity to the 23-kda is the single most frequent band in blots at baseline;We concur with the CDC recommendation of 2 of 3 bands in IgM IB for positivity, but being then; 23, 41 and 37-kDa; and finally we recommend that serological follow-up for seroconversion in patients receiving antimicrobials should be done 8-14 days after the initial visit rather than after 1 month when antibody reactivity may already be decreasing.

From the Dearborn booklet re IMUGEN

ACCURACY of DIAGNOSIS and STAGING of WELL-CHARACTERIZED LYME DISEASE PATIENTS (CDC) by an ANTIBODY CAPTURE/WESTERN BLOT PANEL, COMPARED with IgM and IgG ELISA, and IGM and IgG WESTERN BLOTTING and a FLAGELLIN-ENRICHED ELISA. RH SEDER and VP BERARDI, IMUGEN, NORWOOD, MA 02062

Blinded testing of a CDC-assembled and documented serum set from well characterized patients was performed by IMUGEN using its panel of antibody capture assays and Western blotting, by the laboratory of a community hospital using commercial kit IgM and IgG ELISA assays, and by laboratories of the CDC using IgM and IgG Western blots and a flagellin-enriched ELISA. Striking performance differences occurred on the sera from 22 patients with early Lyme disease, all but one of them culture positive for *B. burgdorferi*, as shown:

		POSITIVE RESULTS ONLY				POSITIVE/BORDERLINE			
Laboratory	Test Method	No.	%	Chi^2	p-value	No.	%	Chi^2	p-value
IMUGEN	M-Capture	20	91						
HOSPITAL	M-ELISA	7	32	16.2	<.0001	8	36	14.1	<.0001
	G-ELISA	12	55	7.8	<.01	14	64	4.6	<.05
	M or G-ELISA	12	55	7.8	<.01	14	64	4.6	<.05
CDC	M-Blot	13	59	5.9	<.01				
	G-Blot	3	14	26.5	<.0001				
	M or G-Blot	14	64	4.6	<.05				
	Flagellin ELISA	2	9	29.4	<.0001	7	32	16.2	<.0001
	ELISA/Blot POS[1]	2	9	29.4	<.0001	6	27	18.4	<.0001
	Any test(s) POS	14	64	4.6	<.05	15	68	3.5	<.05

1. blot performed only to "confirm" a positive ELISA

Ablility of the IMUGEN algorithm to identify a patient's stage of Lyme disease was also demonstrated on the 36 positive patients:

CLINICAL STAGE BY CDC DATA	I	II	III
n	24	3	9
No. CORRECTLY STAGED by IMUGEN	21	3	7
% STAGE CORRECTLY IDENTIFIED	88	100	78

The clinical and public health implications of these findings will be discussed.

Accuracy of Dresser method = 14%

Western Blot Standardization -Kay L. Case, Immunology Laboratory, Lutheran Hospital, La Crosse, WI.

Current Center for Disease Control recommendations advocate the use of the Western blot as the confirmatory test for Lyme disease. However, results from the Lyme Disease Proficiency Testing Survey provided by the Wisconsin State Laboratory of Hygiene and the College of American Pathologists highlight the need for standardization of Western blotting procedures before guidelines for interpretation are recommended. Significant procedural differences (eg. gel concentrations, isolates) currently exist among testing laboratories. These and other factors have contributed to cause wide variations in molecular weight standard curves. In addition, monoclonal antibodies for detecting positions of several significant *B. burgdorferi* proteins are not widely available. Consequently, detection of significant band reactions identified in the scientific literature is often difficult. The CDC has proposed standardizing the molecular weight designations of significant proteins by averaging the values reported in the literature. This non-standardized approach will likely generate additional confusion.

The designation of significant Western blot antigen-antibody reactions is also puzzling. For example, the CDC interpretive guidelines proposes inclusion of the highly nonspecific 41kD band while omitting consideration of the more specific 31kD (OspA) band. Several investigators have recently confirmed that patients produce antibody to OspA during both early and late Lyme disease. In a recent in-house study using 65 case-defined sera from patients with all stages of Lyme disease and 200 control sera, antibodies against OspA were detected in 14(22%) and 9(14%) of Lyme sera using IgM or IgG Western blotting, respectively. Results from this study also demonstrated a highly significant decrease in sensitivity when the proposed CDC criteria were applied for interpretation. Using an in-house interpretation, the sensitivity for IgM and IgG Western blotting was 86% and the specificity was 91%. The specificity using the proposed CDC criteria was 100%. However, the sensitivity was only 34% for IgM-specific tests and 22% for IgG-specific tests.

In conclusion, these results strongly suggest that establishing guidelines for interpretation may be inappropriate until standardized procedures such as calculating standard curves based on monoclonal antibodies are established. In addition, the sensitivity of the recommended Western blot criteria highlights the need to carefully consider the consequence of promoting Western blotting as the confirmatory methods in Lyme disease serology.

From Dearborn Conference summary

50 kd is specific in children

See second page

INTERPRETATION OF LYME BORRELIOSIS IMMUNOBLOT IN PEDIATRICS

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Pediatric Lyme Disease Center, Division of Infectious Diseases and Division of Rheumatology, Schneider Children's Hospital, Long Island Jewish Medical Center, the Long Island Campus for Albert Einstein College of Medicine, New Hyde Park, NY; and University of Connecticut School of Medicine and Division of Rheumatology, Newington Children's Hospital, Newington, CT.

The Pediatric Lyme Disease Center at the Schneider Children's Hospital of Long Island Jewish Medical Center receives referrals from a wide endemic area, including children with non-specific, often long standing symptoms, with positive results on a EIA or IFA for Lyme disease. We have used the immunobot as an aid in the diagnosis of *B. burgdorferi* infection in these children. However, we have lacked uniformly accepted criteria for a positive immunoblot.

Children differ from adults with regard to their antigenic exposure experience and in their level of maturation of the immune system, therefore criteria derived in adults may not necessarily be extrapolated to children. Children experience fewer periodontal infections and syphilis is rare, and both are conditions in which cross reactive antibodies to the 41 kD *B. burgdorferi* flagellum polypeptide occur frequently. Also, children are less likely to have experienced past *B. burgdorferi* infection, which could result in persistent antibodies. Conversely, they experience multiple viral infections, e.g. infection with the Epstein Barr virus which is known to stimulate a non-specific polyclonal antibody response. We sought to determine band patterns in control sera, this being the background against which a positive immunoblot is interpreted. We hypothesized that bands would be present infrequently in control pediatric sera, chiefly due to the less frequent occurance of other spirochetal infections and because children are less likely to have experienced *B. burgdorferi* infection in the past.

Another common pediatric problem is that of episodic oligoarthritis. Using earlier proposed criteria,[1] children with a clinical diagnosis of JRA and positive EIA for *B. burgdorfei* antibodies were found to be immunoblot negative.[2] Thus we showed that the immunoblot is potentially a useful assay to exclude Lyme disease.

Our experience with immunoblots performed on sera from control children, children with JRA and children with Lyme arthritis[3] is summarized herein. A comparison of the band patterns seen in these three groups enabled us to derive criteria for positive immunoblot for pediatric Lyme arthritis.

METHODS

Sera

162 sera submitted for other (non-Lyme) tests from children aged 2 mo to 18 yr in an endemic area, sera from 99 children with immunoblot. 6.2% of controls, 10% of JRA specimens, and all Lyme arthritis specimens (by definition) were positive by EIA.

Immunoblot Conditions

Sonicated *B. burgdorferi* B31 sodium dodecyl sulfate (SDS)-glycerol-mercaptoethanol

Larry Zemel UCONN Health Ctr.

COC Dearborn Conference Booklet

buffer was applied at a protein concentration of 15 μg/lane on an 11% SDS-PAGE gel (Mini-protean); 1:100 dilutions of sera; IgG detection with goat anti-human alkaline phosphatase conjugate.

RESULTS (Figures 1-3)

87% of normal control sera revealed no bands on immunoblot, 10% had 1 band, 2% had 2 bands. One serum specimen each had 3 (18/21, 41, 55 kD) and 4 (31, 34, 55, 60 kD) bands. Bands most frequently present were 41 kD and 54kD. 82% of JRA sera revealed no bands on immunoblot, 13% had 1 bands, 8% had 2 bands. One had 3 bands(18/21, 41, 55 kD). Bands most frequently seen were 41 kD and 66 kD. Thus all non-Lyme sera had $\leq$ 4 bands, and the most frequent bands were those representing antibodies to the 41 kD polypeptide and to higher molecular weight polypeptides.

All Lyme arthritis sera revealed $\geq$5 bands (mean 8.4, range 5-13 bands). Bands of molecular weight 25-OspC, 28, 39, 47, 50, 93 kD were seen in patients and not in controls. In no specimen was an IgM band present where there was no correlation between duration of arthritis or activity of arthritis at presentation and number of bands present.

DISCUSSION

We hypothesized that the interpretation of immunoblot in pediatrics may be different because of the different antigentic experience and relative immaturity of the immune response of children. We found a relative paucity of bands in pediatric control and JRA sera, despite a substantial number of sera that were positive by EIA. This is probably due to a lower prevalence of cross-reactive antibodies. Additionally, past *B. burgdorferi* infection is less likely because of their younger age, therefore specific bands are less likely to be encountered. The lower "noise" facilitates the interpretation of an immunoblot in children. As a consequence, pediatric criteria may not need to be as stringent as those derived in adult sera, as the presence of multiple bands is more likely to represent true *B. burgdorferi* infection. In clinical practice in an endemic area, the knowledge that non-specific bands are infrequent, and, when present, few, is useful when interpreting an immunoblot in children with non-specific symptoms and positive EIA.

Our criteria for a positive immunoblot based on the number and molecular weights of bands seen in our laboratory are the presence of 5 or more bands, of which at least one is an apparently specific band (25-OspC, 28, 39, 47, 50 or 93 kD). This "pool" of apparently the 39kD band was present in commonly in adults with syphilis[4], but was not seen in our controls, thus constituting a specific band in pediatrics.

Whereas the presence of 5 or more bands in children may be sufficient to confirm Lyme arthritis, we recommend that the presence of at least one specific band be required in addition, to account for the theoretical possibility of encountering 5 or more bands non-specifically e.g. in EBV infection or in JRA. We conclude that non-specific symptoms, as well as JRA, in children with positive EIA, can be distinguished from Lyme arthritis by immunoblot. An additional conclusion from these data is that there appears to be no utility in performing separate Igm and IgG blots for Lyme arthritis.

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4. Dressler F, Whalen JA, Reinhardt BN, Steere AC: Western blotting in the serodiagnosis of Lyme disease. *J Infect Dis* 1993; 167:392-400.

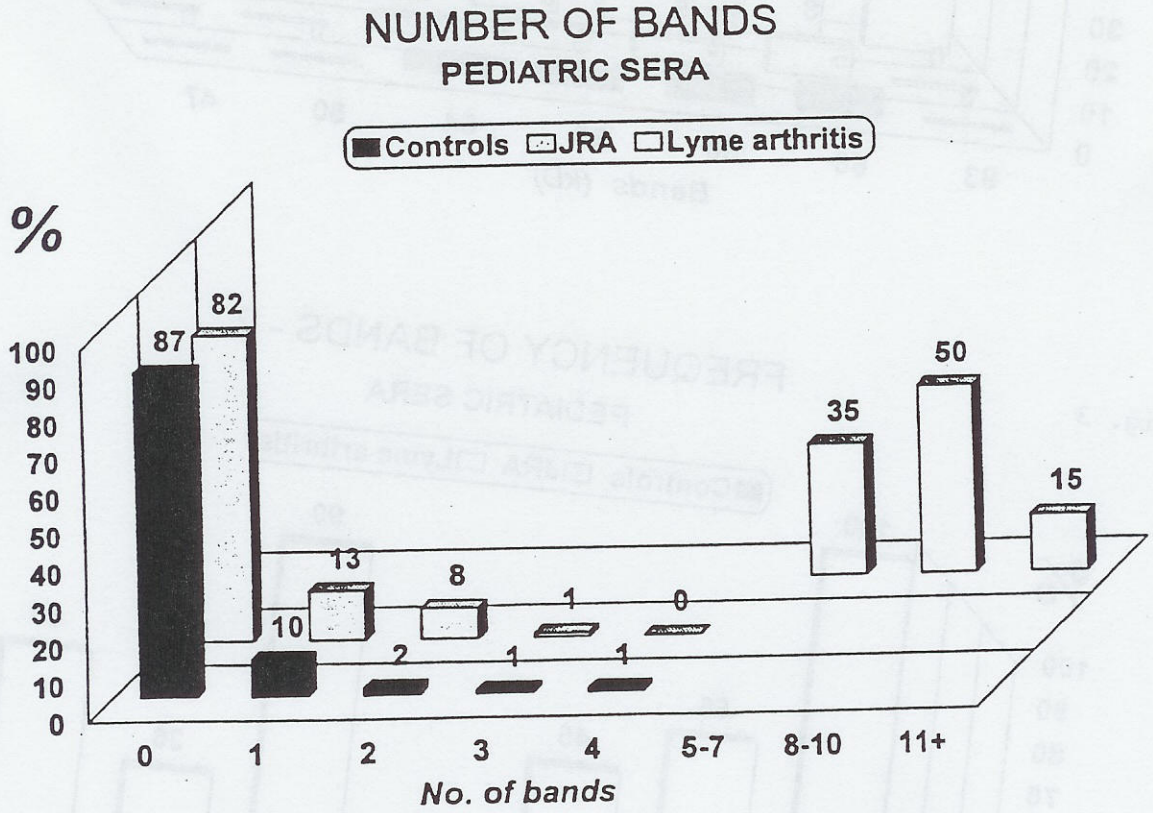


Fig. 1

Fig. 2

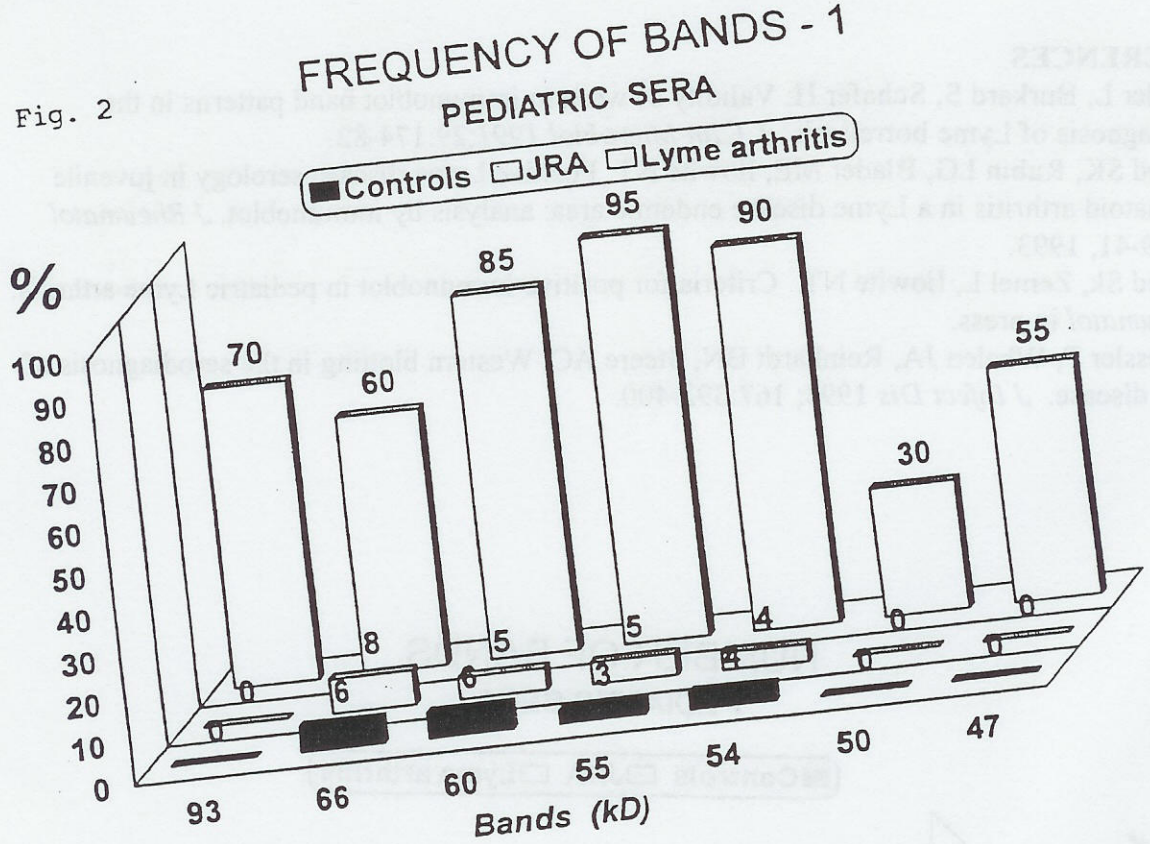
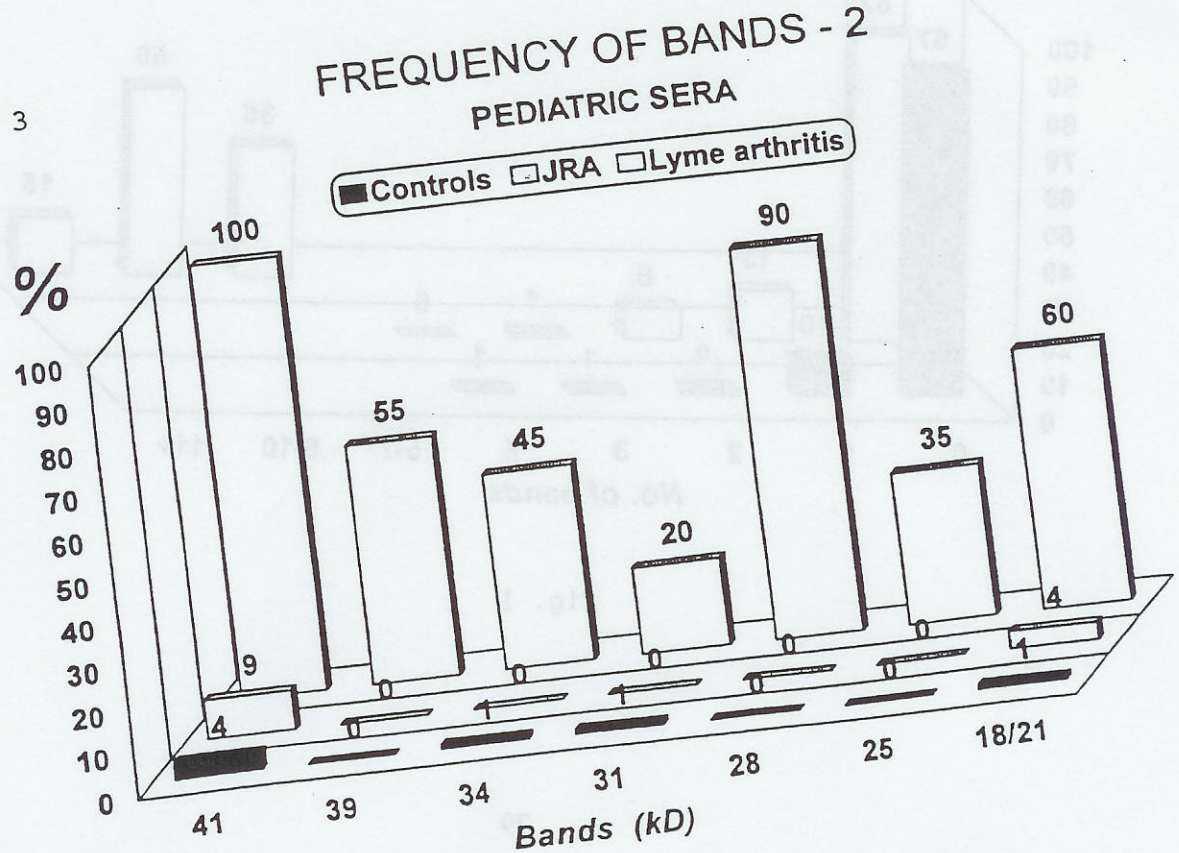

FREQUENCY OF BANDS - 1
PEDIATRIC SERA
Controls JRA Lyme arthritis
%
100
90
80
70
60
50
40
30
20
10
0
70
60
85
95
90
30
55
0
8
5
5
4
0
0
6
6
3
4
0
0
93
66
60
55
54
50
47
Bands (kD)

Fig. 3


FREQUENCY OF BANDS - 2
PEDIATRIC SERA
Controls JRA Lyme arthritis
%
100
90
80
70
60
50
40
30
20
10
0
100
55
45
20
90
35
60
9
0
0
0
0
0
4
4
0
1
1
1
0
1
41
39
34
31
28
25
18/21
Bands (kD)

WORKGROUP B and C:
Technical Issues and Test Performance:
Certification and New Test Evaluation

Dr. Janet N. Robertson, UK Lyme Disease Reference Unit, Southhampton.
Nine Years of Serologic Testing at the Southampton Public Health Laboratory

Ann M. Johnston, Ph.D., Roche Biomedical Laboratories, Raritan, New Jersey
Summary of Lyme Testing Study of 1992.

Karim E. Hechemy, Rory A. Duncan, Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY.
Reporting of Digitized Immunoblot Scan Test Results for Lyme Disease to Clinicians

E.K. Hofmeister, J.E. Childs, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland, and division of Viral and Rickettsial Diseases, CDC, Atlanta, Georgia.
Sensitive and Specific Detection of Antibodies to *Borrelia burgdorferi* by Imunoblotting in Naturally Infected *Peromyscus leucopus* Captured at a Lyme Disease Enzootic Site in Maryland.

Ching Y. Lo, Robert H. Notenboo, Laboratory Services Branch, Ontario Ministry of Health, Toronto, Canada
Lyme Borreliosis in Ontario.

Nick S. Harris, Ph.D., Igenex, Inc. Reference Laboratory, Palo Alto, California
A Brief view of Antibody Diversity in Western Blots of Lyme Patients and an Overview of the Data of an Antigen-Capture Test for *Borrelia burgdorferi.*

Lori Bakken, M.S., Wisconsin State Laboratory of Hygiene
Experience with Proficiency Testing in the United States in 1993. The CAP WSLH PT Program

Dr. Janet Robertson (UK Lyme disease reference Unit, Southampton)

The Southampton Public Health Laboratory (PHL), located in one of the key UK endemic areas for Lyme disease, has approximately 9 year's experience of providing both screening and reference serological testing for the UK. The laboratory also organized the recent EU research network study on ser-standardization among five 'regional' and national European Lyme disease reference laboratories.

The widely reported problems of low sensitivity of serological tests, particularly in early Lyme disease, and cross-reactivity among the normal population is apparent in the UK also. The strategies employed by Southampton PHL in attempting to overcome the limitations of the available serological methods, and to optimize the diagnostic value to clinicians of test results, will be discussed.

The currently favored protocol for serological testing consists of either a commercial enriched antigen ELISA or an 'in-house' ELISA screen with confirmatory testing of all positive results by immunoblot. In addition, immunoblot is also performed on all samples where symptoms are consistent, with early Lyme disease. Current criteria for positivity by immunoblot will be present together with a summary of the relative merits of both methods, based on the experience of Southampton PHL.

Summary of Lyme Testing Study of 1992
Roche Biomedical Laboratories
Immunology Department

Our laboratory produces a large volume of Lyme Disease tests each year using enzyme immunoassays and Western blot kits provided by Cambridge Biotech. In the absence of any definitive criteria for positivity we use a modification of those recommended by the vendor and we have added an equivocal category. The criteria used are relatively non-stringent.

We have a large data bank of lyme test results but do not have access to patient histories. In an effort to correlate positive Western blot results with a final diagnosis of Lyme Disease we sent questionnaires to referring physicians during 1992 requesting information about patient symptoms, test results, diagnosis, and treatment. This information was voluntary and kept confidential once received in our laboratory.

We received eighty-one responses to the questionnaire, in many instances we had total antibody EIA and/or the IgM specific EIA results, some of which were from tests performed at RBl and others from external laboratories. Twenty four of the patients were reported to have shown erythema migrans(EM). The most commonly reported symptoms were flu-like illnesses, and joint pain usually of the knee.

The number of Western blot bands in putative positive patients varied but in general exceeded the number required by our criteria. A few patients showed the minimal number of bands for positivity (two for IgM and three for IgG) but most showed multiple bands. Twenty eight percent were positive for every possible IgG band. Others were positive by IgM Western blot and equivocal by the IgG blot suggested of early disease.

Although these data are just a "snapshot" of our population we are hopeful that they may aid in the development of consensus criteria for Lyme Western blot interpretation. Presentation and discussion of these data at the ASTPHLD sponsored meeting of Serological Diagnosis of Lyme Disease may contribute towards this end.

Submitted by Anne M. Johnston, Ph.D.

Reporting of Digitized Immunoblot Scan Test Results for Lyme Disease to Clinicians

Karim E. Hechemy and Rory A. Duncan

Wadsworth Center for Laboratories and Research

New York State Department of Health

Albany, N.Y., 12201-0509

We are reporting the immunoblot assay results for antibodies to *Borrelia burgdorferi* to clinicians along with the screening test results. The immunoblot is scanned and the picture digitized and reproduced on the test results report. The actual reporting of the immunoblot profile instead of just the number of bands and/or identity of the bands, allows the clinician to better evaluate the profile of the antibody response to the various antigens of *B. burgdorferi*. The following is the test algorithm used in our laboratory: The IBA is used to confirm reactive or equivocal test results obtained with the standard screening serodiagnostic assay(s). The bands that are lighted with IBA are rated 1+ to 4+. Only those bands that are rated ≥2+ are scored. The serum's IBA test results are interpreted according to the "Report of the CDC/ASTPHLD Working Group on Standardization of Imunoblotting for Serodiagnosis of Lyme Disease". Serum specimens are first tested with IBA using the polyvalent probe. If the serum scores R (≥5 bands) or NR, no further tests are performed. Sera that score EQ in the IBA with the polyvalent probe are tested in the IBA with both IgG and IgM probes.

Sensitive and specific detection of antibodies to *Borrelia burgdorferi* by immunoblotting in naturally infected *Peromyscus leucopus* captured at a Lyme disease enzoatic site in Maryland.

E. K. Hofmeister and J.E. Childs, Johns Hopkins University School of Hygiene and Public Health Baltimore, MD, and Division of Viral and Rickettsial Disease, Centers for Disease Control and Prevention, Atlanta, GA.

In a prospective study on the maintenance of infection with *B. burgdorferi* in wild-caught *P. leucopus*, in which infection status was primarily determined through culture and PCR of ear biopsy samples, immunoblotting was utilized to determine the infection status of mice which were captured, but not biopsied. The immunoblot method was validated by testing captured and sacrificed wild-caught mice of which 34 were determined to be culture-positive for the organism in ≥ one organ or ear biopsy sample and 22 were culture-negative, and by testing 10 colony reared *P. leucopus* which were negative for the organism by testing ear and/or organ tissues by PCR. *B. burgdorferi* strain 910255, isolated from one of the sacrificed mice, was utilized as the antigen, after first determining the identity of the spirochete by comparison with strain B31 by SDS-PAGE, monoclonal antibody reactivity, and PCR. Whole cell lysates were electrophoresed by proteins with molecular weights of approximately 16, 17.9 and 21.5 kDa (the latter specifically identified as OspC by reaction with a monoclonal antibody) were selected from the major protein bands recognized by mouse serum for determination of infection status in mice of unknown infection status. The 16 kDa band was detected by 27 of 34 (79%) of the infected and 1 of 22 (5%) uninfected wild-caught mice and in none of the uninfected wild-caught mice, and 1 of 10 (10%) of the colony reared mice. OspC was detected by 25 of 34 (74%) of the infected mice and by none of the uninfected wild-caught mice or the colony reared mice. Reactivity with two out of three of these proteins was considered positive for infection with *B. burgdorferi* in wild-caught mice resulting in an overall sensitivity of between 74-79% and a specificity of > 99%. No other major protein bands were as clearly diagnostic as the bands selected due to co-migration of non-specific proteins. The use of a 10-20% gradient (10x14 cm) gel enabled the clear visualization of low molecular weight bands which resulted in a sensitive and highly specific immunoblot detection of antibodies to *B. burgdorferi* in wild-caught mice. We believe this approach may be successfully applied in detection of antibodies to *B. burgdorferi* in other species as well.

LYME BORRELIOSIS IN ONTARIO: Ching Y. Lo and Robert H. Notenboom, Serology Department, Laboratory Services branch, Ontario Ministry of Health, Toronto, Ontario, Canada

Objective: To estimate the number of cases of Lyme borreliosis diagnosed in the Province of Ontario in the year 1992.

Methods: Reported cases and test results in 1992 were reviewed. Sera and Cerebral Spinal Fluids were screened for antibodies to *Borrelia burgdorferi* by ELISA(Lymestat, Whittaker) and confirmed by Western Blot (Accublot-G and-M, Whittaker). ELISA was repeated on any positive or borderline specimen.

Results: In 1992, 6057 clinical specimens (including follow-ups) were tested by ELISA among which 58 (0.96%) were ELISA positive, 10(0.17%) were borderline, and 90 (1.49%) were irreproducible. Western blots were positive in 38 of the 58 (66%) ELISA positive specimens. This represent 36 patients confirmed by Western Blots.

In 1992, 22 new cases of Lyme borreliosis were studied, all were sero-positive by ELISA and by Western Blots. Nineteen of them were contracted in the USA, one in Long Point Ontario. The exposure risk was unknown in the remaining 2 cases. After exposure in endemic areas, antibodies were detectable as early as 1 week (1 case) of half a month (8 cases).

Summary: Indigenous Lyme disease is rare in Ontario. The National Wildlife Area and Provincial Park at Long Point remain the only endemic area identified up to the Spring of 1993.

Overview of an Antigen-Capture Test for *B. burgdorferi*

Nick S. Harris, Ph.D., ABMLI, IgeneX, Inc. Reference Laboratory, Palo Alto, CA.

The Lyme Urine Antigen Test (LUAT) offered by IgeneX, Inc. is a second generation antigen capture test which was developed by 3M Diagnostic Systems (3MDS) of Santa Clara, CA. 3MDS was a wholly owned subsidiary of 3M Corporation, St.Paul, MN. The first generation antigen test was developed by 3M Corporation, St. Paul, Mn. It is important to make the distinction between both tests, because while both tests are covered under the same patent, they are considerably different. The first test used a single monoclonal antibody to 31KD to capture antigen on a paper membrane in an assay somewhat analogous to a dot blot. Because of the design of the first generation assay, the results were influenced by the protein concentration of the urine. This assay was available from an East Coast laboratory for a short period of time (1989-1990), before it was taken off the market.

The new second generation assay, the LUAT, is a "capture" and competitive inhibition assay, which uses a specially absorbed polyclonal antibody. The Electron Microscope capture assay, developed at the NIH-Rocky Mountain National Laboratory, also uses a polyclonal antibody. In order to have specificity for those antigens most unique in infections with *B. burgdorferi*, the antibody was absorbed with non-specific bacteria obtained from urine of Lyme-negative individuals. Western Blot analysis of this absorbed antibody indicate reactivity only against the 31KD , 34KD, 39KD and 93 KD antigens of *B.burgdorferi*. In the assay (Figure 1), antigen in urine competes with antigen bound on the solid phase. Captured antigen in the urine thus blocks the bindings of the antibody to the solid phase and gives a lower signal in a fluorescent Elisa assay.

The initial studies of the LUAT were designed to examine negative control groups, as well as patients suspected of Lyme disease. The following data was obtained from more than 1000 patients and controls.

The initial part of the studies with the LUAT focussed on Lyme negative controls (Table 1) as well as, blocking and interference studies. Two hundred eight (208) non-Lyme healthy controls from California were studied by LUAT. That study appeared to have a 3% false positive rate. Those seven controls, who tested positive, were lost to additional clinical follow-up.

Because of the high percentage of Lyme patients with arthritic symptoms a new control study was recently initiated. Those results are also presented in Table 1. In this new control study, the urine from 150 patients with arthritis and arthralgials were studied from the presence of Lyme antigens. Patients were excluded from this study if they had Lyme disease, syphilis, SLE or scleroderma. Only one arthritic control exhibited a positive antigen value. Upon further study this individual has a urinary tract infection, which is a listed contraindication to LUAT testing.

Numerous blocking, interference and recovery studies were performed and demonstrated that blood, serum, and urinary protein levels neither caused false positive nor negative values nor effected the quantitative recovery of antigen spiked into urine.

Serum and urine was obtained from four hundred and twenty five (425) Lyme disease patients located in endemic areas of New Jersey, New York, Connecticut and Massachusetts. This group was further refined to reflect only those patients meeting the CDC surveillance criteria, at the time. The remaining group of 251 patients, Table 2, had a physician diagnosed erythema migrans (EM) rash and at least three of the critical signs and symptoms of Lyme disease. It was observed that 30% of this group had a positive LUAT. However, only 8% of the group had a concurrent positive serology. This observation, troubling at the time, became the focus of another clinical study.

The new study attempted to observe and evaluate the temporal relationship of the antigenuria seen in patients with Lyme Disease and the serological response in the patients. The study followed a group of patients for a two month period after the appearance of an EM diagnosed in a physicians office. Since all patients presented with an EM, all patients were put on oral antibiotics at the initial visit. Urine was obtained at this first visit and every other day for two months. A LUAT (Lyme Urine Antigen Test) was performed on these urines. Serum was also obtained at this first visit and weekly thereafter, for two months. An ELISA IgG/IgM Lyme serological assay was performed on the serum. During the first visit, and weekly thereafter, the patient's various symptoms were recorded and scored by a physician. A symptom algorithm was then developed to quantitate the symptoms and severity. Figure 2 is an example of the data sheet by which symptoms were analyzed.

Of the ten patients in the study, one patient did not make an antibody response of excrete Lyme antigen. Three patients (examples, Figures 3 and 4) had an antibody response, by no significant antigen was detected in the urine during the two month period. The antibody response, scaled on the right side of the graph, was judged positive at greater than 8.5%. The antigen response, scaled on the left side of the graph, was considered positive at greater or equal to 46 ng/ml.

Six patients excreted Lyme antigen, but made no significant antibody as seen in the examples from Figures 5, 6 and 7. It was noticed that antigenuria was not present every day and in some patients it would last for a few weeks, then disappear only to reappear again. It was also observed in this preliminary study that the average symptom score for the patients with antigenuria was 3.5 times higher than the patients with an antibody response.

The preliminary data suggests a potential dichotomy of response to *B. burgdorferi*. During the limited time of this study, some patients made the typical antibody response to infection. Another group of patients, had antigenuria and developed no detectable serological response. It was observed that the patients with early antigenuria and no antibody, had more clinical symtoms and appeared "sicker" than the patients that made an antibody to *B. burgdorderi*.

While these single-blinded studies are interesting and suggest that *B. burgdorferi* antigen may be another laboratory marker for Lyme disease, more patients need to be studied. It is important to try and understand the physiology which leads to the conditions for antigenuria. It is also important to investigate if other antigens, such as osp-C, are also detectable and whether they are present in the same pattern as the osp-A, osp-B, 39Kd and 93Kd moieties.

TABLE 1

CONTROL POPULATIONS

Apparent False Positive Rate
Lyme Antigen Test

Normal Controls	
Endemic (n=139) and Non Endemic (n=69)	
1992	7/208=3%
Arthritic and Arthralgic Controls (n=150)	
1994	1/150=<1%

NORMAL RANGE

Mean +3SD = 46ng/ml

TABLE 2

Lyme Patients with a Physician Diagnosed EM.
n=251

History of Tick Bite	133/251	53%
>3 other symptoms	204/251	81%
History of arthritis	177/251	71%
Positive concurrent serology	19/251	8%
Positive Lyme Urine Antigen Test (LUAT)	75/251	30%
Antibiotic treatment	159/251	63%

Lyme Urine Antigen Test (LUAT)

CAPTURE STAGE

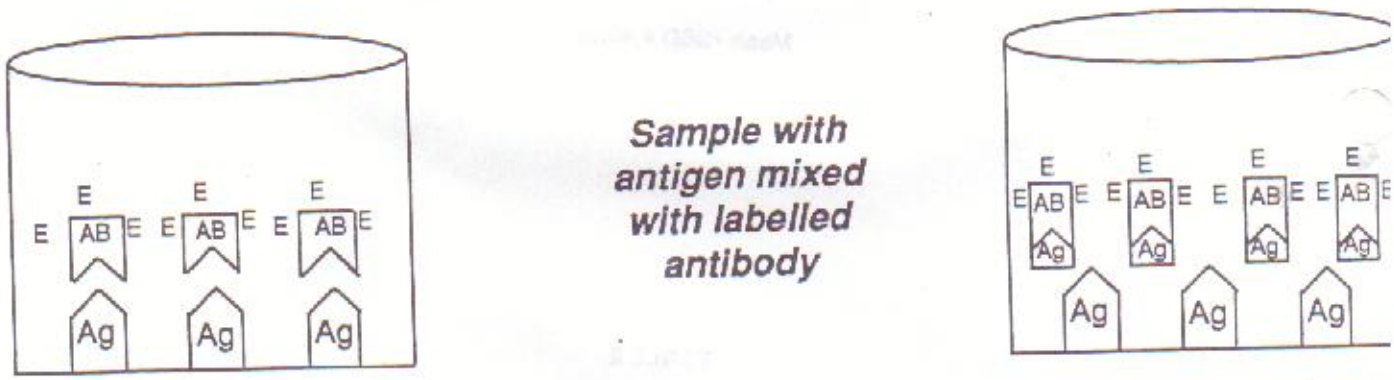


Plate washed

COMPETITIVE INHIBITION

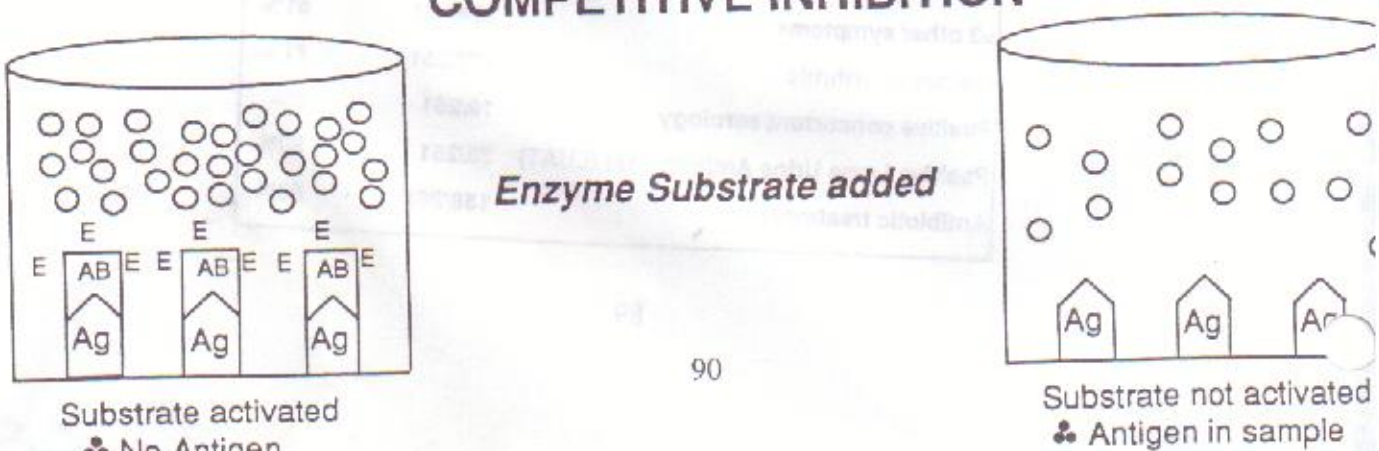


FIGURE 1

U.S. Patent Number 4,888,276

HISTORY AND SYMPTOM FORM

EM rash was observed on: ___/___/___

Date of tick bite: ___/___/___

Where was patient when tick bite occured? City _____ State ____

Does anyone in the patient's family have Lyme Borreliossis? No____ Yes ____

1. Brother 2. Father 3. Son 4. Husband 5. Sister 6. Mother 7. Daughter 8. Wife

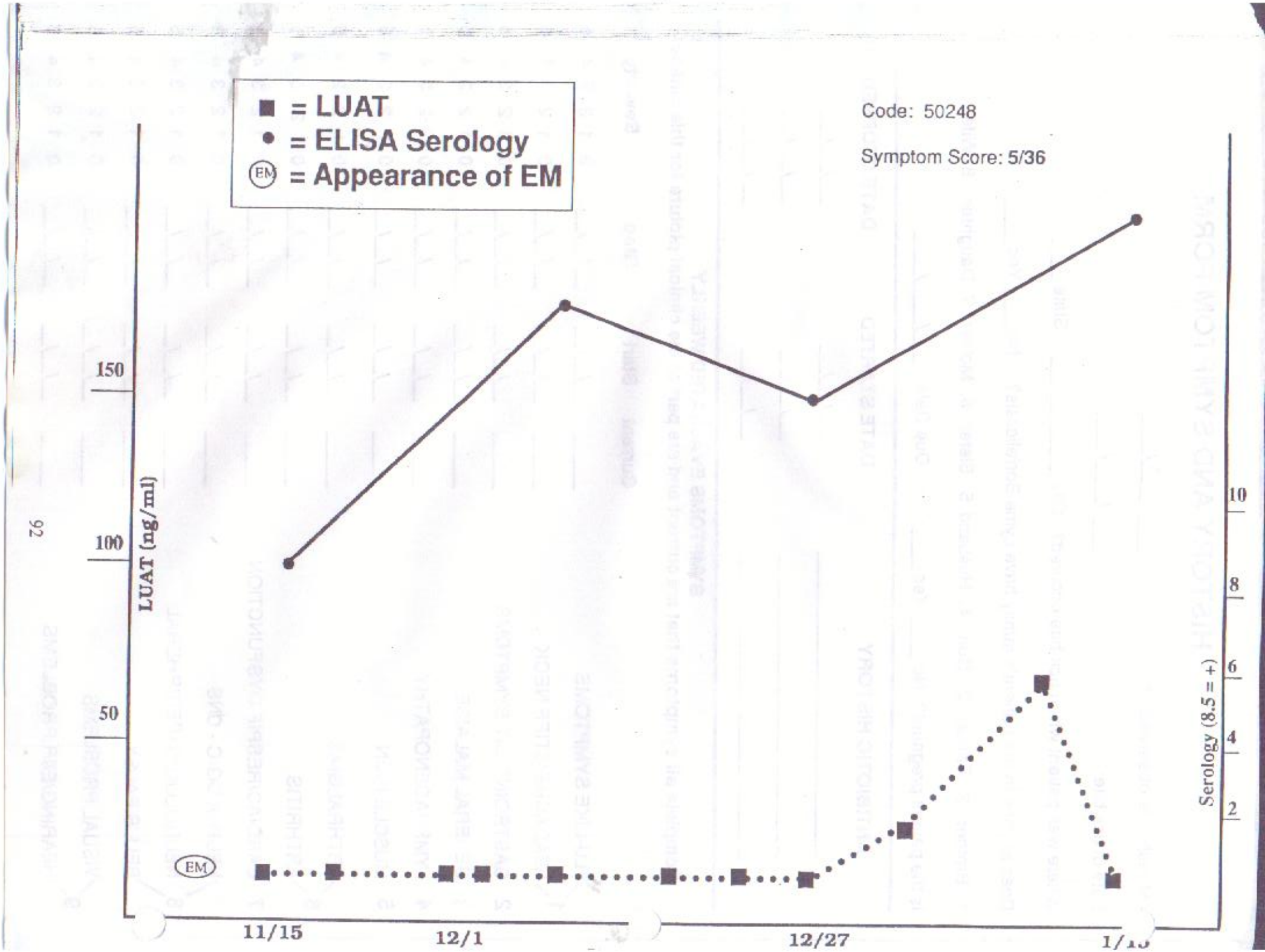
Is the patient pregnant? No ____ Yes ____ Due Date ___/___/___

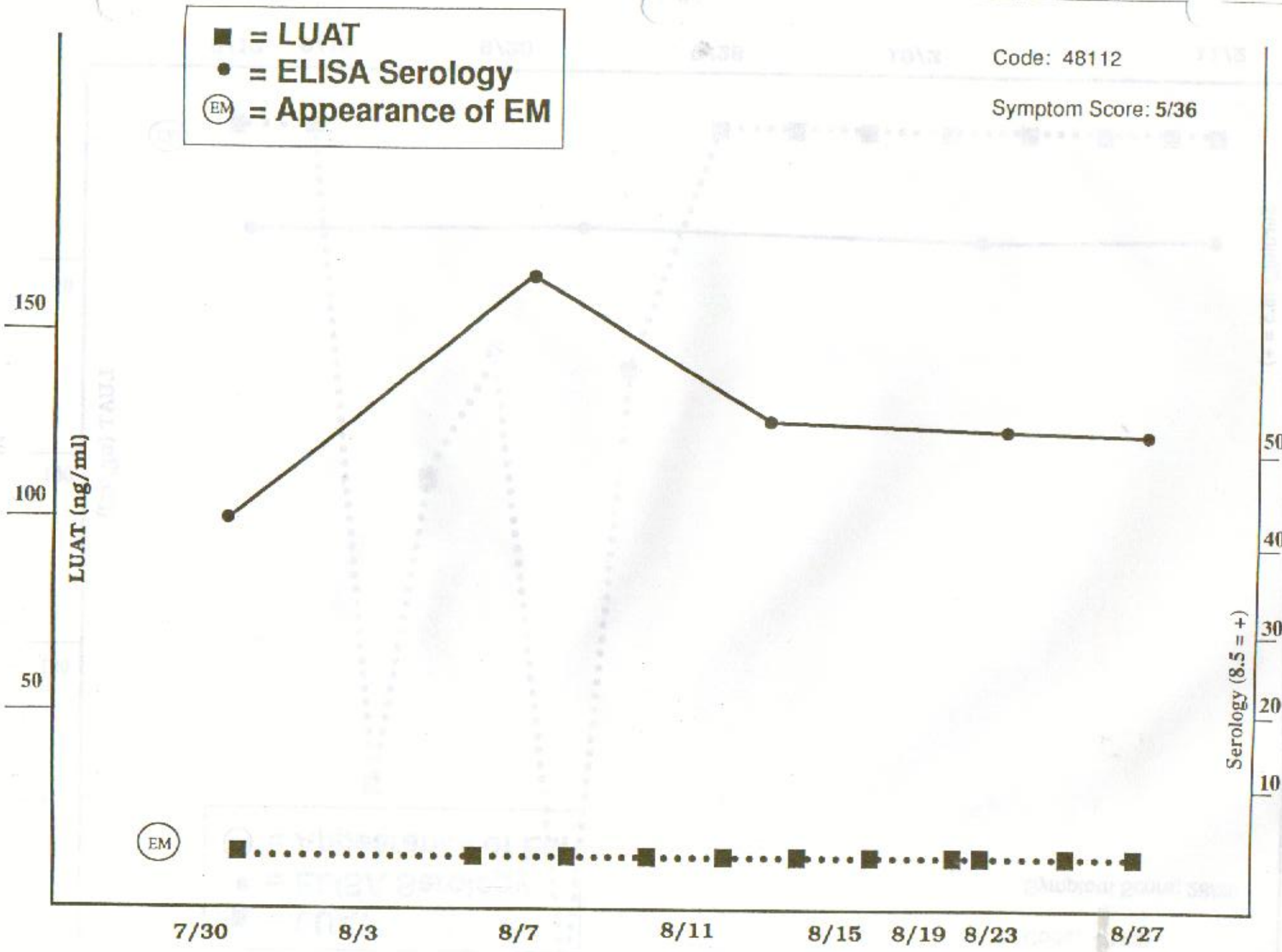
ANTIBIOTIC HISTORY	DATE STARTED	DATE STOPPED
_____	__/__/__	__/__/__
_____	__/__/__	__/__/__
_____	__/__/__	__/__/__

SYMPTOMS EVALUATED WEEKLY

Complete all symptoms that are current and are part of the clinical picture for this illness

		Current	Start	Stop	Severity
1	FLU-LIKE SYMPTOMS	____	__/__/__	__/__/__	0 1 2 3 4 5
	HEADACHE/STIFF NECK	____	__/__/__	__/__/__	0 1 2 3 4 5
2	GASTROINTEST SYMPTOMS	____	__/__/__	__/__/__	0 1 2 3 4 5
3	GENERAL MALAISE	____	__/__/__	__/__/__	0 1 2 3 4 5
4	LYMPHADENOPATHY	____	__/__/__	__/__/__	0 1 2 3 4 5
5	MUSCLE PAIN	____	__/__/__	__/__/__	0 1 2 3 4 5
6	ARTHRALGIAS	____	__/__/__	__/__/__	0 1 2 3 4 5
	ARTHRITIS	____	__/__/__	__/__/__	0 1 2 3 4 5
7	CARDIAC/RESPIF DYSFUNCTION	____	__/__/__	__/__/__	0 1 2 3 4 5
8	NEUROLOGIC - CNS	____	__/__/__	__/__/__	0 1 2 3 4 5
	NEUROLOGIC -PERIPHERAL	____	__/__/__	__/__/__	0 1 2 3 4 5
	BELL'S PALSY	____	__/__/__	__/__/__	0 1 2 3 4 5
9	VISUAL PROBLEMS	____	__/__/__	__/__/__	0 1 2 3 4 5
	HEARING/EAR PROBLEMS	____	__/__/__	__/__/__	0 1 2 3 4 5


■ = LUAT
• = ELISA Serology
EM = Appearance of EM
Code: 50248
Symptom Score: 5/36
LUAT (ng/ml)
150
100
50
Serology (8.5 = +)
10
8
6
4
2
EM
11/15
12/1
12/27
1/15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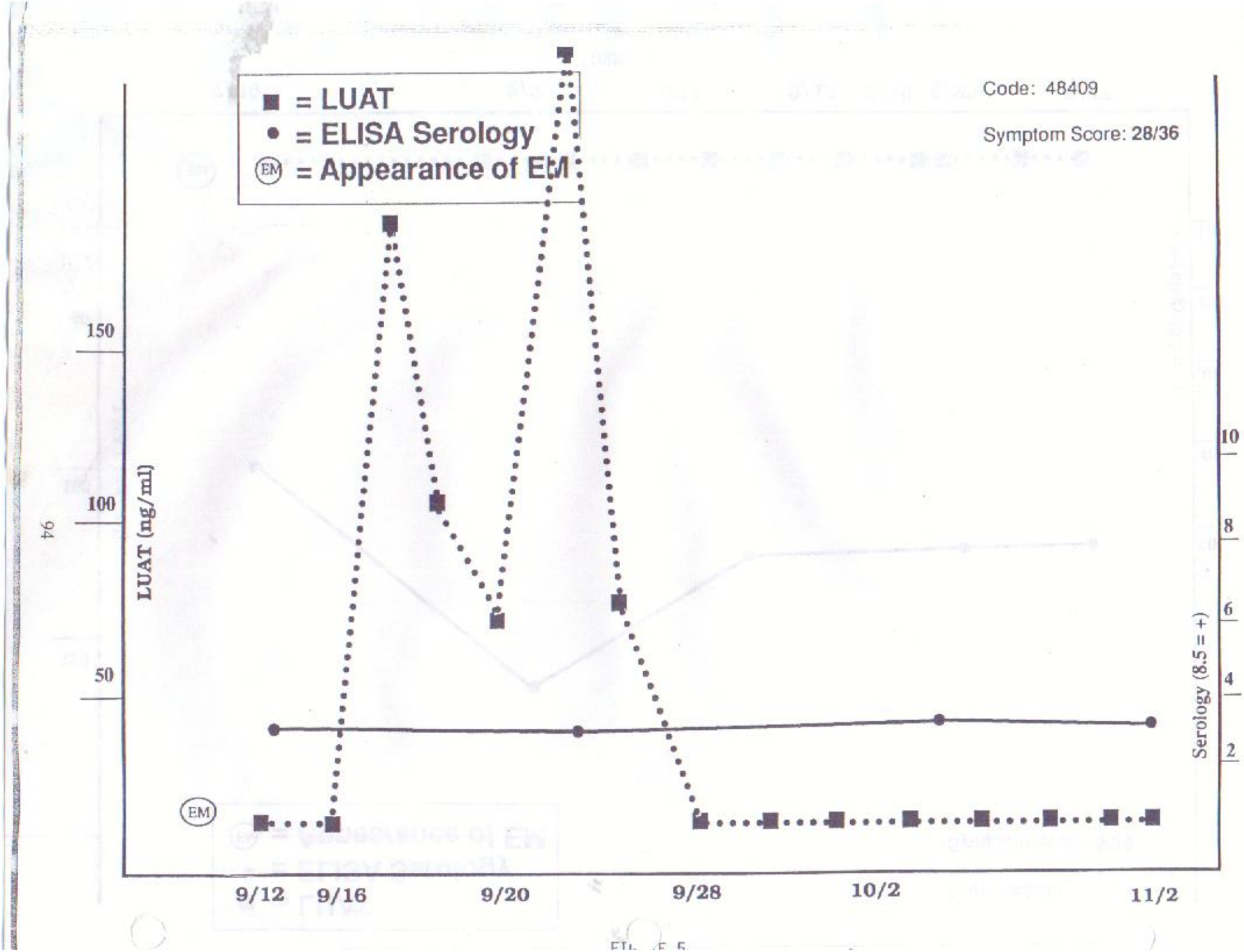
● = ELISA Serology
EM = Appearance of EM
Code: 48112
Symptom Score: 5/36
LUAT (ng/ml)
150
100
50
Serology (8.5 = +)
50
40
30
20
10
EM
7/30
8/3
8/7
8/11
8/15
8/19
8/23
8/27

FIGURE 4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EM = Appearance of EM
Code: 48409
Symptom Score: 28/36
LUAT (ng/ml)
150
100
50
EM
9/12
9/16
9/20
9/28
10/2
11/2
Serology (8.5 = +)
10
8
6
4
2

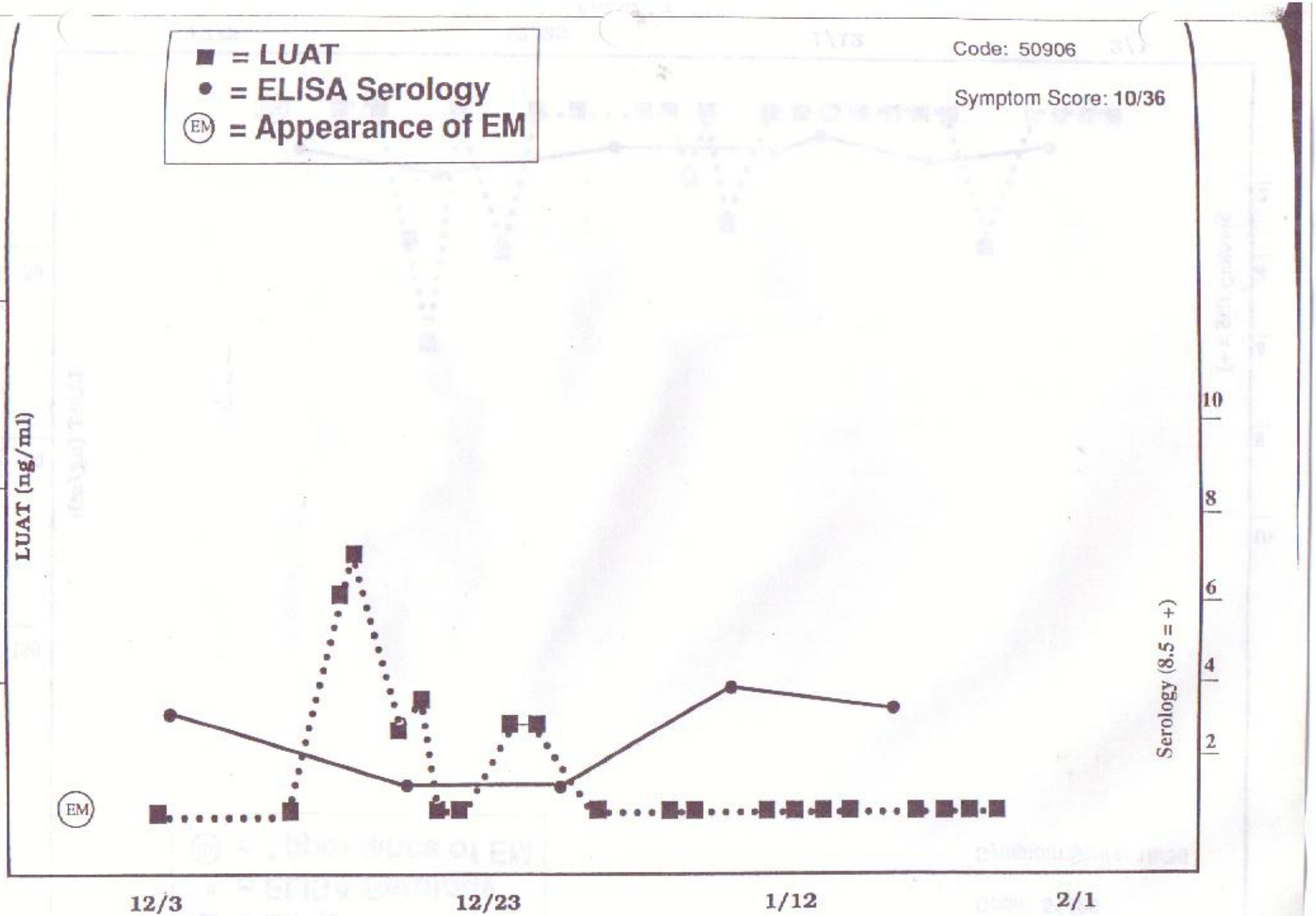
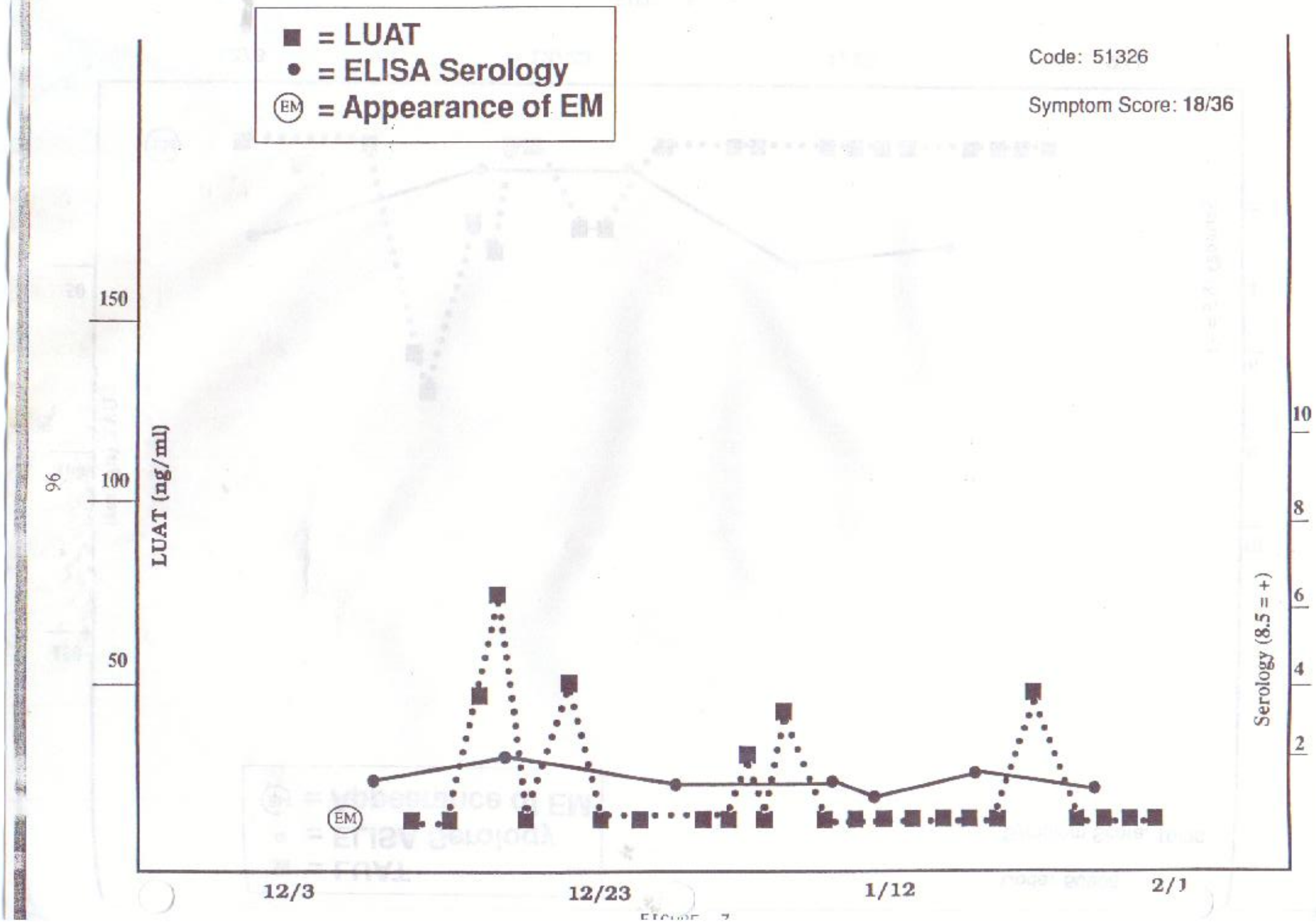

■ = LUAT
• = ELISA Serology
EM = Appearance of EM
Code: 50906
Symptom Score: 10/36
LUAT (ng/ml)
150
100
50
Serology (8.5 = +)
10
8
6
4
2
EM
12/3
12/23
1/12
2/1

FIGURE 6


■ = LUAT
● = ELISA Serology
EM = Appearance of EM
Code: 51326
Symptom Score: 18/36
LUAT (ng/ml)
150
100
50
96
Serology (8.5 = +)
10
8
6
4
2
EM
12/3
12/23
1/12
2/1

A Report on the Experience of the WSLH/CAP Proficiency Testing Program for Lyme Disease

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EXTENDED ABSTRACT

The Wisconsin State Laboratory of Hygiene and the College of American Pathologists provide the Lyme Disease Proficiency Testing Survey to approximately 600 laboratories nationwide. In 1993, fifteen serum samples were sent to participants including seven from patients with Lyme disease (CDC case definition), six from healthy individuals, one from a reactive pool for syphilis and one containing rheumatoid factor. The duration between onset of disease and collection of serum samples ranged from one to six months. Approximately fifty of the participants performed Western immunoblots. Twenty-five laboratories utilized commercial assays while the remainder employed a variety of procedures with different preparations of antigens.

The frequency of bands identified in case-defined and normal serum samples was compared for IgM- and for IgG-specific Western immunoblots. The 41 kDa protein was reported with a frequency of 85% and 95% for IgM and IgG tests with case-defined serum samples. In normal serum samples, the 41 kDa protein was reported with a frequency of 70% for IgM-specific tests and 82% for IgG-specific tests. In case sera, IgM bands for the 25-, 31-, 34- and 39- kDa proteins were reported with a frequency of 23%, 41%, 28%, and 48%, respectively. The 18-, 21-, 22-, 75-, 83- and 93 kDa bands were reported with a frequency less than or equal to ten percent in case-defined and normal serum samples with IgM tests. The 60- and 66 kDa IgM bands were reported with a higher frequency in normal serum samples (18% and 34%, respectively) than case-defined serum samples (16% and 25%, respectively). IgG bands to the 21-, 25-, 31-, 34-, 39-, 60-, 66- and 75 kDa proteins were reported with frequencies between 20% and 48%. These bands were also identified with frequencies less than or equal to twenty percent in normal sera.

Subsequently, the case defined Lyme disease serum samples were separated by
l definition into early (≤2 months) or late disease. In early disease, the 41 kDa
n band was reported by >95% of the laboratories using IgG or IgM conjugates. Bands
: IgM-specific tests were reported more frequently (20-45%) than IgG bands (<10%)
: 31-, 39- and 83 kDa proteins. The 18-, 21-, 22-, 25-, 34-, and 60 kDa proteins were
ed with a frequency less than or equal to 20% by participants using a IgG or IgM
;ate.

In late disease, the band for the 41 kDa protein was reported with a frequency of 95%
G and 70% for IgM. A greater number of bands were reported more frequently in late
: than those reported in early disease. The 25-, 31-, and 34- kDa bands were reported
early equal frequencies for IgG and IgM-specific tests. IgG bands to the 18 and 21
roteins were reported by 30% and 38% of the participants, respectively. IgM bands to
ne proteins were reported with a frequency of less than ten percent. Bands reported
: 75- and 83 kDa proteins were predominately IgG (30% and 22%, respectively). The
)- and 66 kDa bands were observed at a frequency of 55%, 38% and 45% for IgG and
15% and 15% for IgM, respectively.

Based on the participants' interpretation of their immunoblots, the specificity for IgG
M assays was 85%. IgM tests had a sensitivity of 81%, while IgG tests had a
vity of 69%. When the proposed CDC criteria were applied for interpretation of the
the specificity of Western immunoblot was 99%. The sensitivity of the immunoblot
this interpretive criteria was 32% for IgM-specific tests and 15% for IgG-specific tests.

In conclusion, the sensitivity of the Western immunoblot to identify *B. burgdorferi*
is is limited. For example, less than 50% of the participants identified *B. burgdorferi*

specific proteins (39-, 34-, 31- and 22 kDa) in case-defined Lyme serum samples. These results strongly suggest that standardization of Western blotting procedures must be established before guidelines for interpretation are recommended. Since few laboratories have experience with performing Western immunoblot, the impact of using this test must be carefully considered before it is promoted as the confirmatory method in Lyme serodiagnosis.

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