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Impact of Clinical Variables on *Borrelia burgdorferi*-Specific Antibody Seropositivity in Acute-Phase Sera from Patients in North America with Culture-Confirmed Early Lyme Disease▽

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Erythema migrans, the most common manifestation of Lyme disease, has been associated with highly variable rates of seropositivity for antibodies to *Borrelia burgdorferi*. Differences in the sensitivities of serologic assays for the detection of these antibodies, however, may not be the only or even the primary explanation for this observation. We investigated the impacts of four clinical variables on seropositivity—the duration of erythema migrans, the presence of single versus multiple skin lesions, and the gender and age of the patient. In this analysis, three different serologic tests were performed on acute-phase sera from 175 untreated patients with culture-confirmed erythema migrans: the C6 single-peptide enzyme-linked immunosorbent assay (ELISA), a commercially available ELISA in which a whole-cell sonicate of *B. burgdorferi* was the antigen, and a two-tier procedure. Irrespective of the serologic test performed, the results showed that seropositivity rates increased with the duration of the erythema migrans for patients with single lesions ($P < 0.001$) but not for those with multiple skin lesions. The variability in seropositivity rates was greatest for the two-tier testing strategy, with a >6-fold-higher rate of seropositivity among patients with a single lesion of 22- to 30-day duration than among those whose skin lesion was of 1- to 7-day duration (85.7 versus 14.1%; $P < 0.001$). Rates of seropositivity by each of the testing methods were also significantly higher for patients with multiple skin lesions than for those with single lesions ($P < 0.001$). In contrast, seropositivity rates were not affected by either the gender or the age of the patient. Thus, in patients with erythema migrans, certain clinical variables such as the duration and number of skin lesions had a profound impact on seropositivity rates, irrespective of the serologic assay performed.

Lyme disease is the most common tick-borne infection in North America. Local infection at the site of inoculation with *Borrelia burgdorferi* by a vector tick gives rise to a characteristic skin lesion referred to as erythema migrans. The hematogenous dissemination of *B. burgdorferi* from that site to other areas of the skin may result in additional erythema migrans skin lesions; the spirochete can also spread to extracutaneous sites, such as the joints, nervous system, and heart, and cause a variety of other clinical manifestations (12). Among the several laboratory methods to support a diagnosis of this infection, serologic assays to detect antibodies to *B. burgdorferi* are the most widely used (3). These assays employ a wide variety of antigens, including whole-cell sonicates (WCS) of *B. burgdorferi*, recombinant single proteins, and single-peptide antigens. To improve specificity, in 1995 the Centers for Disease Control and Prevention recommended a two-tier testing strategy in which a determination of seropositivity for *B. burgdorferi* antibodies requires both reactivity in a first-tier assay, such as an enzyme-linked immunosorbent assay (ELISA), and a positive immunoglobulin M (IgM) or IgG immunoblot (6).

Although serologic testing of patients with erythema migrans is not routinely recommended in the management of the care of these patients, many studies described in published reports on the performance of serologic assays used to support a diagnosis of Lyme disease have included serum samples from patients with erythema migrans. There are several reasons for this inclusion. One is that the majority of patients with Lyme disease have this particular type of skin lesion (12). A second is that erythema migrans is the only common manifestation of Lyme disease for which the diagnosis can frequently be confirmed by the microbiologic "gold standard" of recovering *B. burgdorferi* by culture (3). Also, since patients with extracutaneous manifestations of Lyme disease generally test positive in serologic assays, regardless of the methodology, sera from patients with erythema migrans are regarded as being useful to compare the sensitivities of different diagnostic assays (3, 4, 8).

The reported rates of seropositivity among North American patients with erythema migrans in published studies of the performance of individual whole-cell ELISAs have varied from 33 to 86% (3). Although it is tempting to ascribe these differences to the sensitivity of the particular laboratory assay performed, limited evidence suggests that a number of other factors need to be considered before such a conclusion is justified (1, 2, 4, 10). These factors include whether the sera tested were obtained before antibiotic treatment, during the acute phase of the infection, or were acquired posttreatment, during the con-

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TABLE 1. Comparison of the rates of seropositivity in relationship to the duration of erythema migrans

Erythema migrans presentation	Serologic test	No. (%) of samples positive among samples collected during postonset days[d]:				*P* value[a]	*P* value for trend test[b]
		1 to 7	8 to 14	15 to 21	22 to 30		
Single lesion	C6 ELISA	29 (45.3)	20 (66.7)	16 (76.2)	7 (100.0)	0.003	<0.001
	WCS ELISA[c]	34 (53.1)	23 (76.7)	19 (90.5)	7 (100.0)	0.001	<0.001
	Two-tier serology	9 (14.1)	10 (33.3)	7 (33.3)	6 (85.7)	<0.001	<0.001
Multiple lesions	C6 ELISA	22 (84.6)	14 (87.5)	8 (100.0)	3 (100.0)	0.81	0.26
	WCS ELISA[c]	23 (88.5)	16 (100.0)	8 (100.0)	3 (100.0)	0.50	0.20
	Two-tier serology	17 (65.4)	10 (62.5)	6 (75.0)	1 (33.3)	0.72	0.67

[a] Calculated by Fisher's exact test for comparison across the four time points.
[b] Calculated by exact logistic regression for the comparison of proportions of positive samples across the four time points.
[c] The classification WCS ELISA positive includes both positive and indeterminate results. No C6 ELISA results were indeterminate.
[d] For patients with single lesions, the total numbers of samples were as follows: from days 1 to 7, 64; from days 8 to 14, 30; from days 15 to 21, 21; and from days 22 to 30, 7. For patients with multiple lesions, the total numbers of samples were as follows: from days 1 to 7, 26; from days 8 to 14, 16; from days 15 to 21, 8; and from days 22 to 30, 3.

valescent phase (1, 2, 4, 9, 11); how long the erythema migrans lesions were present when the acute-phase serum samples were obtained (1, 2, 4); and whether the patients had single or multiple erythema migrans skin lesions (1, 10). From many published reports, it is impossible to determine the degree of homogeneity of the serum samples studied with regard to these variables or infer the significance of these factors for the results, since the investigators do not provide sufficient data to characterize the samples in this manner.

We examined the results of a recent serologic evaluation of acute-phase sera from 175 untreated patients with culture-confirmed erythema migrans to test the hypothesis that seropositivity is directly related to certain clinical variables, regardless of the laboratory assay method employed.

MATERIALS AND METHODS

Serologic assays. The C6 Lyme ELISA kit, a test kit approved by the Food and Drug Administration (FDA), was used according to the instructions of the manufacturer (Immunetics, Inc., Boston, MA). In place of the original cutoff formula based on a Lyme disease-positive serum sample, a simplified cutoff formula based on a negative control and validated by the manufacturer in a separate study was employed. The C6 peptide used as the antigen in the kit is derived from the *B. burgdorferi* B31 strain sequence, which differs from the originally described *B. garinii* IP90 sequence by four amino acids. The kit is formatted as an indirect ELISA in which both IgG and IgM antibodies to the C6 peptide are detected by an enzyme conjugate. Two-tier serology was performed using an IgG/IgM ELISA kit from Wampole Laboratories (now Inverness Professional Diagnostics, Cranbury, NJ), followed by Lyme IgG and IgM immunoblot kits from MarDx/Trinity Biotech (Carlsbad, CA). The ELISA and immunoblot kits for two-tier testing were also FDA approved for in vitro diagnostic use, and testing was performed according to the manufacturers' directions. Both the ELISA and immunoblot kits used a WCS preparation of *B. burgdorferi* B31 as the source of antigen. A determination of positivity by two-tier testing required a positive or indeterminate ELISA result and a positive IgM or IgG immunoblot, interpreted according to recommended criteria (6).

Sera from patients with Lyme disease. A convenience sample of serum specimens that had been stored at −80°C for 3 to 16 years was tested by both the C6 and two-tier methods as part of a performance comparison. The sera were originally obtained between June 1991 and August 2004 from patients with erythema migrans whose clinical diagnosis was confirmed by the growth of *B. burgdorferi* upon the culture of a skin biopsy or blood sample by previously reported methods (14). Sera were obtained prior to antibiotic treatment. The patients who provided the sera were subjects in approved research studies at New York Medical College, Valhalla, NY, who were evaluated at the Lyme disease practice of the Westchester Medical Center. Information on demographic and clinical variables was obtained from study records. The length of time that the erythema migrans lesion was present was determined based on what the patient reported to the study investigators, whereas the number of erythema migrans skin lesions was determined based on the physical examination performed by the study investigators.

Statistical methods. Differences in the proportions of positive samples were assessed using Fisher's exact test. A test for a trend in the proportions of positive samples relative to the durations of skin lesions was conducted using exact logistic regression with conditional scores tested for *P* value determination. Analyses were performed with software version 10.0 from Stata (College Station, TX). A *P* value of <0.05 was considered to be significant.

RESULTS

The seropositivity of serum samples from 175 untreated patients with culture-confirmed erythema migrans of ≤30 days' duration was evaluated. Ninety-six of the patients were male, and 79 were female; the patients' mean age ± standard deviation was 47.5 ± 13.5 years (median, 47.1 years), with a range of 16.1 to 76.2 years. The sera were tested by three different methods: the C6 ELISA, in which a single peptide was the antigen; a commercially available ELISA in which a WCS of *B. burgdorferi* was the antigen (the WCS ELISA); and a two-tier testing strategy in which sera found to be reactive by the WCS ELISA were then retested by using separate IgM and IgG immunoblots. The impacts of the following four clinical variables on seropositivity rates were evaluated: the duration of the erythema migrans skin lesion, the presence of single versus multiple erythema migrans skin lesions, and the age and gender of the patient.

Seropositivity rates in patients with single lesions increased consistently and substantively with the increasing duration of the skin lesions, irrespective of the testing method. The rates determined for these patients were 45.3 to 100% by the C6 ELISA ($P < 0.001$), 53.1 to 100% by the WCS ELISA ($P < 0.001$), and 14.1 to 85.7% ($P < 0.001$) by two-tier testing (Table 1). The same relationship, however, was not found for patients with multiple erythema migrans skin lesions (Table 1).

Rates of seropositivity by each of the testing methods also varied significantly between patients with single erythema migrans skin lesions and those with multiple lesions (Table 2) ($P < 0.001$). In patients with multiple lesions compared to those with single erythema migrans skin lesions, the rate of C6 ELISA seropositivity increased to 88.7% from 59.0% ($P < 0.001$), the rate of WCS ELISA seropositivity increased to

TABLE 2. Comparison of rates of seropositivity in relationship to single versus multiple erythema skin lesions

Serologic test	% Positive results among samples from patients with:		*P* value[a]
	Single erythema migrans (n = 122)	Multiple erythema migrans (n = 53)	
C6 ELISA	59.0	88.7	<0.001
WCS ELISA[b]	68.0	94.3	<0.001
Two-tier serology	26.2	64.2	<0.001

[a] Calculated by Fisher's exact test.
[b] The classification WCS ELISA positive includes both positive and indeterminate results.

TABLE 4. Comparison of rates of seropositivity in relationship to genders of patients

Serologic test	% Positive results among samples from:		*P* value[a]
	Males (n = 96)	Females (n = 79)	
C6 ELISA	68.8	67.1	0.87
WCS ELISA[b]	76.0	75.9	1.0
Two-tier serology	33.3	43.0	0.21

[a] Calculated by Fisher's exact test.
[b] The classification WCS ELISA positive includes both positive and indeterminate results.

94.3% from 68.0% ($P < 0.001$), and the rate of seropositivity by two-tier testing increased to 64.2% from 26.2% ($P < 0.001$). Seropositivity was not affected by either the ages or the genders of the patients (Tables 3 and 4).

DISCUSSION

This study demonstrates that seropositivity rates determined with acute-phase sera from untreated patients with erythema migrans are profoundly affected by two particular clinical features of the patients: the presence of single versus multiple skin lesions and, for patients with a single skin lesion, the duration of the lesion. Wide disparities in the seropositivity rates determined using three different serologic testing methods were consistently observed. Variability in seropositivity rates based on the durations of single skin lesions was greatest for the two-tier testing method, with a >6-fold-higher rate of seropositivity among patients whose lesion was of 22- to 30-day duration than among those whose skin lesion was of 1- to 7-day duration. In contrast, neither the ages nor the genders of the patients significantly affected seropositivity rates. Although higher rates of seropositivity among older individuals have been observed in seroprevalence surveys of certain groups of healthy individuals (7), there was no a priori reason to believe that the ages of our patients or their genders would have influenced the humoral immune response to active infection with *B. burgdorferi*.

The observations made in the present study confirm earlier observations made by us and by others with smaller numbers of serum samples (1, 2, 4, 10). The duration of infection is a well-recognized variable that affects the humoral response to infectious agents (5). The explanation for why patients with multiple erythema migrans lesions are more likely to be seropositive than those with localized infection may be the greater contact of the pathogen with the immune system and the engagement of a larger number and variety of antigen-presenting cells in patients with multiple lesions, larger antigen loads in these patients, differences in the virulence of the infecting strains of *B. burgdorferi*, or a combination of factors (5).

The findings in this study emphasize the importance of meticulously characterizing the serum samples that are used in studies on the performance of serologic assays for the detection of antibodies to *B. burgdorferi*, at least with regard to samples from patients with erythema migrans. It is also clear that the relative sensitivities of different serologic assays for patients with erythema migrans cannot be determined based on independent studies of each specific test. Furthermore, even head-to-head comparisons may be skewed by the selection of serum samples. For example, it may not be appreciated that a particular assay is actually more sensitive than another for patients with erythema migrans of short duration if the majority of serum samples studied were obtained from patients with relatively long illnesses. This issue is well-illustrated by the finding that the rates of seroreactivity based on either the C6 or the WCS ELISA results were much more similar to the results of two-tier testing for patients with a single erythema migrans lesion of 22- to 30-day duration than for those with a single lesion of 1- to 7-day duration (Table 1).

In our experience, however, untreated patients with erythema migrans of such a long duration are uncommon. Thus, the longer duration of infection is probably much more likely to confound seropositivity rates when convalescent-phase rather than acute-phase serum samples are tested, since the humoral response to *B. burgdorferi* typically expands for a period of up to several weeks after antibiotic therapy is initiated (1, 2). Furthermore, a variety of other variables not considered here, such as genetic differences in the strains of *B. burgdorferi* causing infection (13); the severity, number, or nature of systemic symptoms (1, 4); and presumably, factors related to the patient's immunologic response, are also likely to be pertinent to the development of seropositivity. Thus, the

TABLE 3. Comparison of rates of seropositivity in relationship to ages of patients

Serologic test	% Positive results among samples from patients of:		*P* value[a]	% Positive results among samples from patients of:		*P* value[a]
	≤50 yr (n = 102)	>50 yr (n = 73)		≤40 yr (n = 53)	>60 yr (n = 34)	
C6 ELISA	65.7	71.2	0.51	64.2	67.6	0.82
WCS ELISA[b]	74.5	78.1	0.72	71.7	79.4	0.46
Two-tier serology	37.3	38.4	1.0	41.5	41.2	1.0

[a] Calculated by Fisher's exact test.
[b] The classification WCS ELISA positive includes both positive and indeterminate results.

most meaningful evaluations of the sensitivities of serologic assays for patients with erythema migrans need to include an appropriate number of serum specimens that are representative of the variety of patients with single or multiple erythema migrans skin lesions seen in clinical practice. Sets of acute-phase sera from patients with a single erythema migrans skin lesion should include specimens obtained from patients who had various durations of illness at the time of presentation.

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