Predictive value of serology in diagnosing Lyme borreliosis

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Abstract

Aims—To compare the predictive value of immunoblotting and enzyme linked immunosorbent assay (ELISA) in diagnosing Lyme borreliosis.

Methods—An ELISA using a whole cell sonicate of the B31 strain of *Borrelia burgdorferi* was used to screen samples submitted for Lyme borreliosis serology. A total of 1222 serum samples reactive in the ELISA were tested by immunoblotting also using the B31 strain. Patients with other spirochaetal diseases were tested by both methods to assess specificity, while those with erythema migrans were used to evaluate sensitivity. Subjects with different clinical conditions, which may have been associated with Lyme borreliosis, were tested using both techniques.

Results—Only 16.3% of serum samples from patients submitted for Lyme borreliosis serology which were reactive by ELISA were confirmed as positive by immunoblotting. This is unlikely to represent a sensitivity problem as 51% of samples from 53 patients with erythema migrans were detected by immunoblotting compared with only 28% by ELISA. Patients whose samples were negative by ELISA were also negative by immunoblotting. Serum samples from patients with relapsing fever were reactive in both ELISA and by immunoblotting, but for other test groups immunoblotting offered increased specificity.

Conclusions—Not all ELISA results could be confirmed by immunoblotting. Yet immunoblotting was both more sensitive and specific than ELISA techniques. As a result of these observations all ELISA results should be serologically confirmed by immunoblotting. Though immunoblotting is not suited to large scale screening of samples, it can be used satisfactorily in conjunction with ELISA methods to improve the predictive value of serological tests for Lyme borreliosis.

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Serology remains by necessity the preferred method of diagnosing Lyme borreliosis in a laboratory. Enzyme linked immunosorbent assay (ELISA) has been used for serological screening, but this technique is plagued by problems of specificity. Improvements in the

specificity of ELISAs have been explored by using either purified antigen such as the 41 kilodalton flagellin protein¹ or semipurified preparations of *Borrelia burgdorferi*.² Although these second generation ELISAs can reduce the number of serum samples giving false positive results, they do not entirely eliminate the problem.

Serological reactivity can be confirmed by immunoblotting. As the disease progresses, patients produce antibodies against an increasing range of both specific and non-specific borrelial proteins. The interpretation of immunoblot patterns is controversial. Some studies have reported poor specificity of immunoblotting,³ while others maintain that the technique is highly specific.^{4,5} We devised an immunoblotting method to test samples submitted for Lyme borreliosis serology to our reference laboratory and evaluated this with serum samples from patients with Lyme borreliosis, healthy controls, and patients with other disorders.

Methods

Serum samples submitted for Lyme borreliosis serology were initially tested with an ELISA. A total of 1222 samples submitted over four years gave positive values on ELISA and were further tested by immunoblotting. In addition, samples from 53 patients with erythema migrans diagnosed by a dermatologist served as definite clinical cases of Lyme borreliosis. We also had samples from forestry workers (n = 305) from various parts of Britain, who had a high exposure to ticks.

Fifty consecutive samples submitted for serological testing that had negative results on ELISA were tested by immunoblotting to see whether immunoblotting was a worthwhile routine procedure. In addition, 50 samples submitted for routine antenatal serology and 65 samples from healthy subjects that had raised values on ELISA (from an initial group of 1139) served as negative controls.

To compare the specificity of the tests serum samples from subjects with other spirochaetal diseases were studied (louse borne relapsing fever (81 samples from 25 patients); syphilis (20 samples); and samples submitted for Lyme disease serology that were positive for syphilis (25 samples from 24 patients)).

Samples from patients with clinical conditions possibly associated with late borrelial infection and reactive in the ELISA screening test were also tested by immunoblotting.

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These included those from patients with dilated cardiomyopathy or myocarditis (N R B Cary *et al*, fourth international conference on Lyme borreliosis, Stockholm, 1990) (18 positive on ELISA from a study group of 282 patients); scleroderma (24 from a series of 175 patients); and chronic fatigue (five patients from an initial test group of 85).

SEROLOGICAL METHODS

The ELISA used to screen samples and the immunoblotting technique have been described in detail elsewhere.^{6,7} Briefly, wells of an ELISA microtitre plate were coated with sonicated whole cells of *B burgdorferi* (B31 strain) in carbonate buffer. After overnight incubation at 4°C the plates were washed three times and then incubated at 37°C for an hour sequentially with a blocking solution, diluted test serum (1 in 300), and a conjugate of goat antibody to human IgG with horseradish peroxidase; the final incubation was with α -phenylenediamine substrate for half an hour at room temperature before absorbance was read at 492 nm. Absorbance was converted into arbitrary ELISA units, with those above the 95th percentile for healthy control subjects being recorded as weakly positive and those greater than the 98th percentile as positive.

Immunoblotting also used the B31 strain of *B burgdorferi*, which was separated by discontinuous sodium dodecyl sulphate with polyacrylamide gel electrophoresis with 12.5% gels and transferred to nitrocellulose. Sheets of nitrocellulose were blocked overnight with dried milk powder and then washed and cut into strips. These strips were reacted with 1 in 200 dilutions of the patient's serum overnight and then washed, and incubated with goat antibody to human IgG conjugated with alkaline phosphatase. Bands were detected using p-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt substrate.

Interpretation of IgG banding patterns was based on comparing the immunoblot profiles of healthy subjects with the profiles of patients with clinical Lyme borreliosis. This led to the development of a subjective system. Bands of 93, 31 (OspA), 30 (OspA leading front), and 20 kilodaltons were found in patients with Lyme borreliosis and considered to be specific; bands of 82, 58–56 doublet, 52, 50, 48, 39, and 34 (OspB) were often seen but were occasionally found in samples from patients with other disorders and thus only considered to be semispecific; and, finally, bands including 76, 66, 62, 60, 41, and 15 kilodaltons did not correlate with disease. Typically healthy subjects had bands at 41 and 60 kilodaltons. This method was later adapted with numerical values assigned to reflect the relative importance of immunoblot bands.⁷

Additional strains of Lyme borreliosis spirochaetes were tested by immunoblotting using samples from patients who had acquired their illness in Europe (6), the United States (9), or the United Kingdom (11). These sam-

ples were tested in parallel with the B31 strain of *B burgdorferi*, together with the Swedish ACA isolate (ACA1), American human blood isolate (HB19), American CSF strain (297), and United Kingdom tick isolate (UK).

Results

Immunoblotting of 50 antenatal serum samples from London failed to produce any positive results. Typically, these samples only reacted to a band of 76 kilodaltons (nine), the 60 kilodaltons common antigen (21), 41 kilodalton flagellum protein of *B burgdorferi* (36). Similarly, samples from 50 consecutive patients submitted for Lyme borreliosis serology that had given negative results on ELISA were all negative by immunoblotting. Patients with established Lyme borreliosis usually showed reactivity to a much wider range of proteins.

When the results of ELISA and immunoblotting were compared in the samples submitted for Lyme borreliosis serology that had given positive results on ELISA only 199 of 1222 (16.3%) samples were positive by immunoblotting (table 1). When weakly reactive serum samples were removed the proportion of ELISA results positive by immunoblotting rose only to 24.4%.

Geographical differences were seen in the United Kingdom. The proportion of samples from forestry workers in Scotland that could be confirmed by immunoblotting was smaller than the proportion from similar groups in England (table 2).

No difference in immunoblot interpretation as positive, equivocal, or negative was observed in samples from patients giving travel histories in America and Europe, or those who had not left Britain, when tested using either the American B31 reference strain, 297, or HB19, the Swedish skin isolate ACA1, or a British tick isolate of *B burgdorferi*, although minor differences in banding profiles were seen—for example, in possession of a 39 kilodalton band in the first of the British patients illustrated in the figure.

Table 1 Results on immunoblotting in 1222 samples positive by ELISA. Values are numbers (percentages) of samples

ELISA result	Immunoblotting result	
	Negative	Positive
Weakly positive (n = 608)	559 (91.9)	49 (8.1)
Strongly positive (n = 614)	464 (75.6)	150 (24.4)
Total (n = 1222)	1023 (83.7)	199 (16.3)

Table 2 Positive results on ELISA and immunoblotting in British forestry workers

Group	No of workers	No (%) on ELISA	No (%) on immunoblotting
New Forest rangers	42	12 (28.6)	5 (11.9)
Thetford Forest	78	16 (20.5)	11 (14.1)
Scottish foresters	142	23 (16.2)	2 (1.4)
Scottish deer workers	43	7 (16.3)	2 (4.6)
Total	305	58 (19)	20 (6.6)

When individual serological tests were evaluated with known cases of erythema migrans authenticated by a dermatologist, serology at its best could only detect half of the cases (table 3). Lowering the diagnostic cut off points of the ELISA would not resolve this

problem as no significant difference could be detected when absorbance values from 51 patients with erythema migrans were compared with those from 120 healthy blood donors (Mann-Whitney U test, $p = 0.498$, data not shown).

Reactivity of British patients (A), European patients (B), and American patients (C) with Lyme borreliosis strains isolated from America, Europe, and Britain. Lane 1 = tick strain B31; lane 2 = Swedish ACA1; lane 3 = human blood isolate HB19; lane 4 = CSF isolate 297; and lane 5 = UK tick strain.

Syphilitic samples, with the exception of one sample, gave negative immunoblot patterns. The remaining sample gave an equivocal result with bands at 76, 60, 41, 39, 30, and 22 kilodaltons. This sample was submitted for Lyme borreliosis serology without clinical details. This patient may have had two spirochaetal diseases.

Serum from patients with relapsing fever showed the greatest degree of cross reactivity by immunoblotting. Magnarelli *et al* noted a high degree of reactivity for patients with relapsing fever in indirect immunofluorescence, ELISA, and microscopic agglutination tests, but they did use immunoblotting.⁸ Of the 81 samples tested by immunoblotting, 45 (55.6%) would have been reported as positive, while in the ELISA 49 samples (60.5%) were reactive. Eight patients showing immunoblot seroconversion from negative to positive generally did so between the third and fifth day after treatment, possibly as a result of liberated antigenic components of killed borreliae.

Of the 18 samples from patients with heart disease that had given positive values on ELISA, only one gave a positive immunoblot profile. The 24 patients with scleroderma and positive results on ELISA, likewise, only produced two positive immunoblots. None of the

samples from five patients with chronic fatigue that had been positive by ELISA could be confirmed by immunoblotting.

Discussion

A major problem encountered with serological tests for Lyme borreliosis is the lack of specificity. Patients with a variety of other spirochaetal diseases and unrelated clinical conditions have been reported to give false positive serological results. Samples may cross react by binding to non-specific antigens of *B burgdorferi* such as the 60 kilodalton common antigen or heat shock related proteins.^{9 10} Immunoblotting has been regarded as a "gold standard" method for laboratory confirmation of Lyme borreliosis.⁴ As the disease progresses antibodies are produced against a range of borrelial proteins, both specific and non-specific. The interpretation of immunoblot patterns is controversial, with many different schemes available. Some studies have reported poor specificity of immunoblotting,³ while others maintain that the technique is highly specific.⁴ These discrepancies largely result from differing methods used for interpretation. The principal methods used for the interpretation of immunoblots are those based on band counting, which includes both specific and non-specific proteins,⁴ or those which require possession of selected bands such as a single band of 18, 21.5, or 23 kilodaltons in conjunction with the 41 kilodalton flagellum.¹¹ Some studies have proposed that quantification of bands may be valuable in interpreting immunoblot bands.⁵ This type of interpretation was not evaluated in this study,

Table 3 Comparison of positive results in serological tests in 53 patients with erythema migrans

Serological test	No (%) of patients seropositive
ELISA	15 (28)
Immunoblotting	27 (51)

in part owing to the variability sometimes observed in band intensity, despite using the same batch of antigen, the same amount of protein loaded on to the gel, and standardised electrophoretic and blotting conditions. Other potential problems which may arise using immunoblotting semiquantitatively are standardisation of the antigen preparation. Differences have been reported in both the quantities of outer surface proteins expressed by cells and whether or not the protein is expressed at all. Some of these differences may result from in vitro selective pressure, or modulation or loss of antigens by *B burgdorferi*.¹² Additionally, borrelial strains may be heterogeneous in their antigenic composition, and comprise several different species,¹³ which may result in greater band intensity when a patient is tested with a homologous strain to that with which they have been infected.¹¹ We prefer to use a weighted numerical system for interpretation, with greater value being attached to bands considered to be specific and other bands considered to be less important being awarded lesser values. With numerical weighting features of both the band counting and possession of specific bands can be combined to form a simplified method for interpreting immunoblots.⁷

The results from this study showed that only 16.3% of samples that were positive on ELISA could be confirmed as genuinely positive by immunoblotting. Therefore, what do the remaining samples with positive results on ELISA and negative results on immunoblotting represent? These may illustrate a specificity problem of the ELISA, with patients producing antibody against non-specific borrelial proteins. As the ELISA utilises 95th and 98th percentiles of healthy individuals for its test cut off values, it follows that 2% of well ambulatory subjects will be strongly seropositive and 3% weakly seropositive. When ELISA was used to test 1139 healthy individuals from various geographical locations in the United Kingdom, 65 (5.7%) gave positive ELISA values. None of these individuals could be confirmed as having Lyme borreliosis by immunoblotting.

Alternatively, patients with positive results on ELISA but negative results on immunoblotting may not have had the disease sufficiently long to produce a positive pattern on immunoblotting. Not all patients may be able to produce an immune response to diagnostic bands and early antibiotic treatment may prevent a positive immunoblot pattern developing. In addition, the diversity of borrelial strains causing Lyme borreliosis may result in false negative immunoblots if a different strain or species is used as antigen.

The ELISA used in this study gave comparable findings with other ELISAs for Lyme borreliosis that are available commercially (data not shown).¹⁴ Second generation ELISAs still showed cross reactivity with patients with other spirochaetal diseases and produced positive findings which could not be confirmed by immunoblotting.

Considerable diversity exists between

strains of *B burgdorferi*, especially among European isolates, resulting in the recent proposition of new genomic species.¹³ This may account for differences in seropositivity reported, depending on which strain of borrelia was used as antigen, and highlights the problem of which isolate should be used for diagnostic purposes. Possibly a panel of different isolates should be used for preparing antigens for serodiagnosis?

Use of strains other than the American B31 reference strain did not significantly alter the interpretation of immunoblot results in this study (figure). The strains tested might all belong to the same borrelial species.¹³ The UK isolate used differed from the B31 strain in its failure to react with the "species specific" monoclonal antibody H5332, but patients who acquired Lyme borreliosis in America, the United Kingdom, or Europe gave similar immunoblot results regardless of the strain used as antigen (figure). Although the UK and B31 strains differed in reactivity to monoclonal antibody H5332 (S J Cutler *et al*, fourth European congress of clinical microbiology, Nice, 1989), both strains still possessed OspA with which patients' antibodies could react. Different immunoblot reactivity has been reported by Wilske *et al* using a different strain as antigen,¹⁵ but whether this would have caused results to be erroneously recorded as positive or negative was not disclosed.

Immunoblotting in this study was more sensitive than ELISA, detecting 50.9% of patients with erythema migrans compared with the 28.3% found by ELISA. Specificity was also superior using immunoblotting; none of the 65 samples that were positive on ELISA from healthy subjects gave a positive result on immunoblotting and only one of the 45 samples that were positive on ELISA from patients with syphilis gave a positive result on immunoblotting. The 81 samples from patients with relapsing fever were highly cross reactive in both ELISA and immunoblot analysis, with 49 and 45 respectively giving falsely positive results.

Patients with cardiac disease, scleroderma, or chronic fatigue, which may have been associated with chronic borrelial infection and which had given positive ELISA results, had essentially negative results, suggesting that *Borrelia* sp does not cause these conditions.

Problems common to all serological tests are that the IgG response to *B burgdorferi* rises slowly, often not achieving significant concentrations until the sixth week after infection. Titres of IgG can remain raised years after clinical remission or after asymptomatic exposure,¹⁶ which complicates diagnosis in patients with unrelated clinical presentation. Additionally, some patients with clinical Lyme borreliosis fail to mount a clinically significant antibody response. This may result from prompt treatment which has been reported to abrogate the antibody response.¹⁷ Alternatively, antibody production may be restricted to the site of active disease—for example, in neuroborreliosis the cerebrospinal

fluid may give positive results while serum gives negative results.¹⁸ Antibody may remain sequestered in immune complexes and therefore not detectable by commonly used assay methods. Cases have been reported with no significant B cell response, although specific T cell proliferative responses were detected.¹⁹ The humoral response, unlike the cellular immune response, may be selectively suppressed during early borreliosis.²⁰

Immunoblotting was more sensitive than ELISA in detecting disease in patients with early Lyme borreliosis as well as more specific: the only cross reaction was with samples from subjects with relapsing fever. This technique, however, is not readily automated and is too expensive to use as a screening method for serodiagnosis of Lyme borreliosis. Alternatively, use of ELISA alone would result in a large number of false positive results. Although some improvement is offered with second generation ELISAs, specificity is still a problem. ELISA is best suited to screen for samples giving negative results, while immunoblotting should be reserved for confirming serological reactivity and for cases with a high index of clinical suspicion.

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