Borrelia-ELISA dokumentsamling v/ Alex H. 2015.04.05

Borrelia-ELISA 100%, realitet eller myte ?

Er en ELISA Borrelia-test altid 100% efter 8 ugers infektion – det er spørgsmålet…

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Serodiagnosis of Erythema Migrans and Acrodermatitis Chronica Atrophicans by the *Borrelia burgdorferi* Flagellum Enzyme-Linked Immunosorbent Assay

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The diagnostic performance of an enzyme-linked immunosorbent assay (ELISA) using purified *Borrelia burgdorferi* flagella as test antigen was compared with that of a *B. burgdorferi* sonic extract ELISA. We tested sera from 200 healthy controls, 107 patients with erythema migrans (EM), 50 patients with acrodermatitis chronica atrophicans (ACA), and 98 patients with various dermatological disorders without clinical evidence of active Lyme borreliosis. The flagellum ELISA was significantly more sensitive than the sonic extract ELISA. With sera from patients with EM, the diagnostic sensitivity for immunoglobulin G (IgG) antibody detection increased from 11.2 to 35.5% ($P < 0.001$) and for IgM antibody detection it increased from 16.6 to 44.8% ($P < 0.001$). In the flagellum ELISA, the number of positive tests increased significantly ($P < 0.005$) when the duration of EM exceeded 1 month, but still only about 50% of patients with longstanding (1 to 12 months) untreated EM were IgG seropositive. Concomitant general symptoms did not affect the antibody level, whereas patients with multiple erythema were more frequently seropositive. All sera from patients with EM which were positive in the sonic extract ELISA were also positive in the flagellum ELISA. Not only did the overall number of positive tests increase, but the flagellum ELISA yielded a significantly better quantitative discrimination between seropositive patients and controls ($P < 0.002$). IgG antibodies to the *B. burgdorferi* flagellum were found in all sera from patients with ACA, indicating persistence of an antiflagellum immune response in late stages of Lyme borreliosis. IgM reactivity in sera from patients with ACA was shown to be unspecific and the result of IgM rheumatoid factor. A rheumatoid factor was detected in sera from 32% of patients with ACA, compared with 7.5% of patients with EM. The improved diagnostic performance, the ease of standardization of the flagellum antigen, and the lack of strain variation make the *B. burgdorferi* flagellum a needed reference antigen for growing routine serology in Lyme borreliosis.

Serological tests for antibodies to *Borrelia burgdorferi* have been available since 1982 (9). The presently used tests, indirect immunofluorescence assay and enzyme-linked immunosorbent assay (ELISA), use whole cells or whole-cell sonic extracts as antigen. These tests are of limited diagnostic value in early disease, since they yield low diagnostic sensitivities. Only 10 to 40% of patients with erythema migrans (EM) are reported to be seropositive (1, 3, 5, 10, 20–22, 26). A low antigen load, a late and slow humoral immune response, and the quality of the test antigen used may be responsible. *B. burgdorferi* shares immunogenic antigens with many other bacteria, not only spirochetes (7, 14). The inclusion of such cross-reactive antigens in the test antigen may be responsible for the low diagnostic specificity of the presently used tests. A single, immunodominant, and more specific *Borrelia* antigen should be used for a serodiagnostic assay. Several Western (immuno-) blotting (WB) studies have shown that the human immune response in Lyme borreliosis early and constantly recognizes the 41-kilodalton band corresponding to the flagellum (7, 11–13, 17, 25). In a recent study, we showed that the use of purified *B. burgdorferi* flagellum as ELISA antigen significantly improved immunoglobulin G (IgG) and IgM serodiagnosis, especially in early cases of lymphocytic meningoradiculitis (Bannwarth's syndrome) (15).

The aim of the present study was to investigate the diagnostic efficiency of the *B. burgdorferi* flagellum ELISA in patients with EM, the primary stage of Lyme borreliosis, and in patients with the late dermatological manifestation of *B. burgdorferi* infection, acrodermatitis chronica atrophicans (ACA), which may start 0.5 to 8 years after EM (2).

MATERIALS AND METHODS

Patients. A total of 107 patients presenting with a typical, clinically uncomplicated EM entered the study. There were 26 males and 81 females. They were aged 6 to 83 years (median age, 54 years). Ninety-one patients had a solitary EM; sixteen experienced multiple erythema. Thirty-eight patients reported accompanying constitutional symptoms (low-grade fever, headache, musculoskeletal pain), and sixty-nine had only the skin lesion. The time from onset of the EM until blood sampling took place ranged from 2 days to 12 months, with a median duration of 3.5 weeks.

Another 50 patients with ACA, including 16 males and 34 females, were investigated. They were aged 28 to 89 years (median age, 61 years). The duration of the ACA in these patients ranged from 0.5 to 20 years (median duration, 2.5 years).

The diagnosis of EM and ACA was based on clinical evidence and in every case was made by one of us (E.Å.). The clinical diagnosis of ACA was confirmed by histopathology (2). All the patients were from the Stockholm area and were seen between 1984 and 1987. All serological measurements were done on pretreatment sample 1.

Controls. Sera from 200 healthy Danish controls were used for determination of the 95% specific cutoff level in both

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According to text
107 Total, all with EM
69 EM only, *107-69 = 38 left for other symptoms*
38 EM and Low-grade fever, headache, musculoskeletal pain *(the 38 with other symptoms)*
50 EM and ACA *(where do they fit? if ME only, 107-69 = 38 left for other symptoms)*

More likely the case ?
107 Total, all with ME *(38+50=88 likely with borreliosis)*
19 EM only
38 EM and Low-grade fever, headache, musculoskeletal pain
50 EM and ACA

tests. The Swedish control group consisted of 98 patients with various dermatological disorders without clinical evidence of an active *B. burgdorferi* infection. This was to assure the applicability of the cutoff level defined by the Danish controls to the Swedish patient population. Sera from patients and controls were stored at −20°C until use.

***B. burgdorferi* test antigens.** A Swedish *B. burgdorferi* strain, ACA-1, isolated from the skin of a patient with ACA (4) was used for all antigen preparations. Spirochetes were grown for 5 days in BSK II medium (6) at 32°C to a cell density of 10^8/ml. The sonic extract antigen for ELISA was prepared as described previously (15). The spirochetes were washed three times in phosphate-buffered saline (PBS; pH 7.4) and sonicated on ice by seven 15-s blasts with an MSE 150 W ultrasonic disintegrator (Manor Royal, Crawley, England). The sonic extract was centrifuged (10,000 × *g*, 30 min), and the supernatant primarily containing the soluble antigens was used for ELISA. This sonic extract contained flagellar components according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and WB examination.

Isolation of *B. burgdorferi* flagellum. The purification procedure for the *B. burgdorferi* flagellum was recently reported in detail (15). Briefly, the spirochetes were subjected to a mild-ionic detergent for removal of the outer envelope. The detergent-insoluble material containing the protoplasmic cylinders with the periplasmatic flagella attached was sheared in a blender to achieve a mechanical detachment of the flagella from the cell bodies. The sheared material was then subjected to several differential centrifugations. The final and most important step in the purification was a banding on a CsCl density gradient. Visible bands were isolated separately and examined by WB to assess the yield and purity of the 41-kilodalton flagellum antigen. Flagella containing bands were pooled and dialyzed against PBS. The *B. burgdorferi* flagellum antigen prepared in this way proved to be highly pure by electron microscopy, WB, and crossed immunoelectrophoresis, as shown previously (15).

ELISA procedure. The *B. burgdorferi* sonic extract ELISA and the flagellum ELISA were performed identically except for the antigen. Flat-bottomed polystyrene microwell plates (Immunoplates, code 2-69620; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μl of antigen diluted in PBS. The optimal coating concentration was defined as the antigen dilution resulting in the highest ratio of the optical densities (ODs) between positive and negative control sera. Unspecific protein binding was blocked with 1% (wt/vol) bovine serum albumin in PBS. The wells were washed, and 100 μl of serum diluted 1:200 in PBS with 0.5% (wt/vol) bovine serum albumin and 0.05% (vol/vol) Tween 20 was added to the wells and incubated for 2 h at 20°C. After washing, 100 μl of peroxidase conjugate was added, either rabbit anti-human IgG or anti-human IgM (codes P-214 and P-215; Dakopats, Copenhagen, Denmark) diluted 1:10,000 and 1:1,000, respectively, in PBS with 0.05% (vol/vol) Tween 20. After incubation for 2 h at 20°C, the plates were washed, and 200 μl of the substrate *o*-phenylenediamine (0.41 mg/ml; Sigma Chemical Co., St. Louis, Mo.) in citrate buffer (pH 5) with 0.04% (vol/vol) H_2O_2 was added to each well. After 15 min under protection from light, the enzymatic reaction was stopped by the addition of 50 μl of 3 M H_2SO_4. The OD at 492 nm was read by a colorimeter (Immuno Reader NJ 2000; Nippon, InterMed, Tokyo, Japan). All washings were done three times with 0.56% (wt/vol) NaCl containing 0.05% (vol/vol) Tween 20. Positive and negative control sera were included on every plate. Samples were tested in duplicate, and the mean value was calculated. If the two values differed more than 10% from the mean, the sample was retested. To eliminate plate-to-plate and day-to-day variations, samples of three serum pools with known low, medium, and high titers were included on every plate for construction of a standard curve. The OD value of every sample was adjusted to this standard curve.

Measurement and absorption of IgM RF. All samples from patients with EM and ACA were investigated for IgM rheumatoid factor (RF). An ELISA with human IgG was used, and the results were evaluated according to the 95% specific upper limit of the assay, 8 IU/ml (16). To eliminate unspecific IgM reactivity resulting from IgM RF, all IgM-reactive samples from patients with ACA were subjected to IgM RF absorption with a commercially available kit (RF Absorbans; Behringwerke, Marburg, Federal Republic of Germany).

Statistical analysis. The diagnostic sensitivities of the sonic extract and flagellum ELISAs were compared by using McNemar's test, assuming a binominal distribution of paired data. Nonpaired data were compared by using the chi-square test. Furthermore, the precise quantitative discrimination of the two assays between controls and seropositive patients was estimated. For each individual sample that was positive in both ELISAs, the distance from the achieved OD value to the cutoff level was calculated for both assays. These differences were compared by using Wilcoxon's rank sum test for paired data.

RESULTS

Measurement of IgG antibodies to the flagellum of *B. burgdorferi* in the sera of 200 healthy controls demonstrated a significant increase in specificity compared with IgG measurement with a sonic extract ELISA (Fig. 1). Using a 95% specific upper limit in both tests, the diagnostic cutoff level could be lowered from 0.400 to 0.160 OD value in the flagellum assay. For IgM antibodies in healthy controls, there was no significant increase in specificity, since the 95% specific cutoff levels were 0.260 and 0.230 OD values, respectively (Fig. 2).

The investigation of the Swedish control group consisting of patients with various dermatological disorders assured that the diagnostic 95% specific cutoff level in both assays based on the Danish control population was also representative and suitable when Swedish patients were studied (Fig. 1 and 2).

When IgG and IgM antibodies in sera from 107 EM patients were measured, the flagellum ELISA showed overall increases in diagnostic sensitivity from 11.2 to 35.5% ($P < 0.001$) for IgG and from 16.6 to 44.8% ($P < 0.001$) for IgM detection compared with the sonic extract ELISA (Table 1). A separate evaluation of the 70 patients with an EM duration of less than 1 month and the 37 patients with an EM duration of more than 1 month revealed similar significant increases in diagnostic sensitivity of the IgG and IgM flagellum ELISA ($P < 0.001$) at both times. Not only did the overall number of positive results increase significantly, but so did the quantitative discrimination between controls and patients. This was the result of a generally higher signal obtained in the flagellum ELISA in addition to the lower cutoff level (Fig. 3a and b).

All sera that were reactive in the sonic extract ELISA were also reactive in the flagellum ELISA (Fig. 3). The sera of 69 patients (64.5%) were either IgG or IgM reactive in the flagellum ELISA, compared with the sera of 28 patients (26.2%) in the sonic extract ELISA. If the assay sensitivity

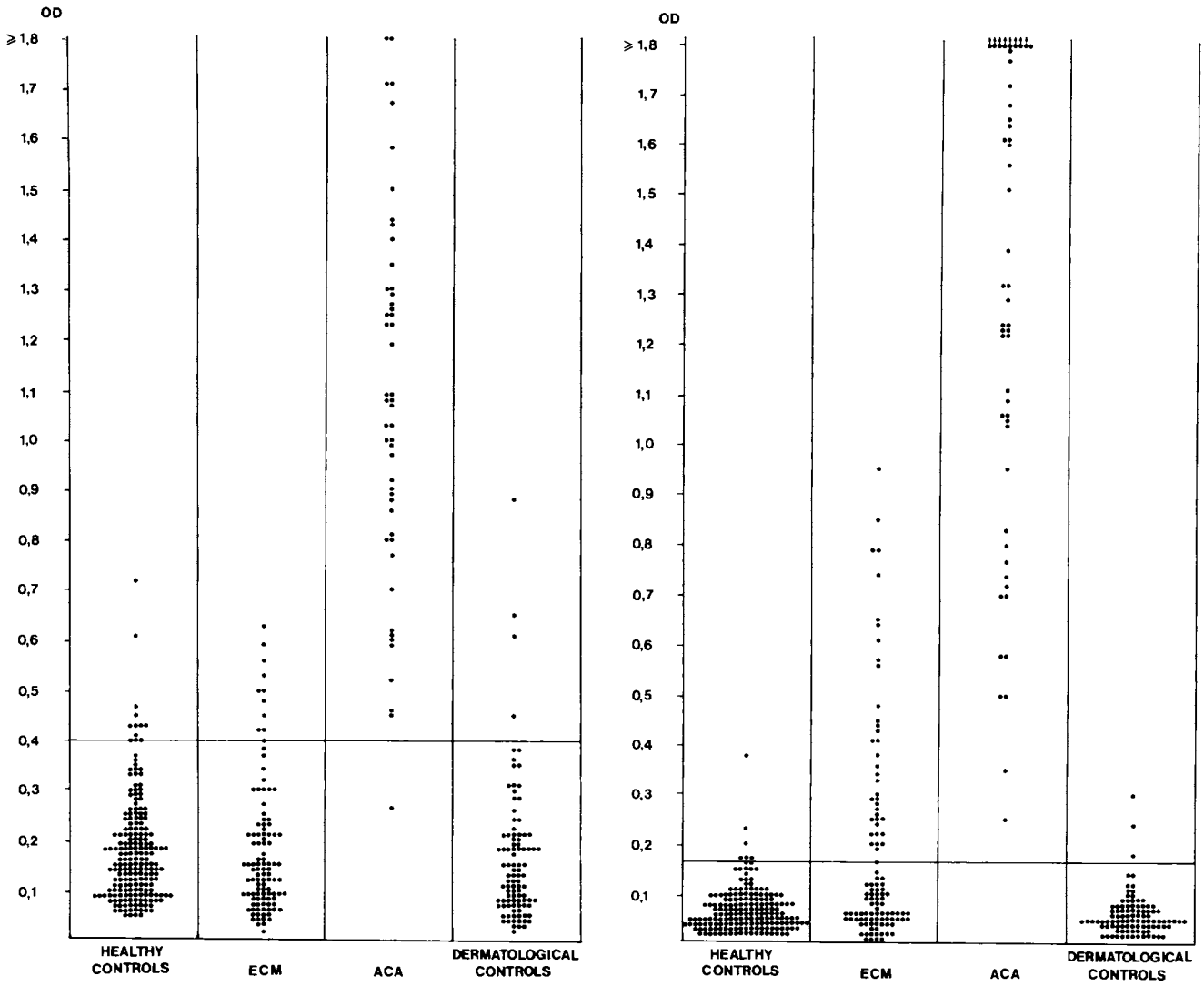

FIG. 1. IgG levels measured by *B. burgdorferi* sonic extract ELISA (left) and *B. burgdorferi* flagellum ELISA (right) in sera of 107 patients with EM (ECM) and 50 patients with ACA. Serving as controls were 200 healthy individuals and 98 patients with various dermatological disorders. The horizontal lines mark the diagnostic 95% specific cutoff levels.

is expressed as a combined evaluation of IgG and IgM data, e.g., either IgG or IgM reactivity, it must be remembered that the diagnostic specificity will decrease to about 90%, since 10% of all controls were either IgG or IgM positive. The sera of 15 patients (14.0%) were both IgG and IgM reactive in the flagellum ELISA, compared with the sera of only 2 patients (1.9%) in the sonic extract ELISA. None of the 298 control individuals was IgG and IgM seroreactive.

When the 107 patients with EM were divided into four groups according to the duration of the erythema, the frequency of seropositive samples increased after a disease duration of more than 1 month (Table 1). This increase was statistically significant only in the IgG flagellum ELISA ($P < 0.005$).

There were 16 patients with multiple erythema. When the serological findings for these patients were compared with those for patients with a solitary EM, an increased IgM reactivity was shown (68.8 versus 39.5%) ($P < 0.05$) (Table 2). Three patients with multiple erythema had a disease duration of more than 2 months; they were all IgG seroreactive. There was no correlation of the specific antibody level with the occurrence of general symptoms concomitant to the EM (Table 2).

Fifty serum specimens from patients with ACA were tested. For IgG antibodies, the diagnostic sensitivities of the sonic extract and flagellum ELISA were identical, 98 and 100%, respectively. As in EM, the individual OD signal obtained was significantly higher in the IgG flagellum ELISA (Fig. 3c). The number of IgM-seroreactive patients with ACA was reduced from 22 to 12% by the flagellum ELISA.

To rule out the possible role of an IgM RF as the cause of unspecific IgM reactivity, all sera from patients with EM and ACA were investigated for IgM RF. A total of 16 (32%) of 50 patients with ACA and 8 (7.5%) of 107 patients with EM had IgM RFs in the ranges of 10 to 150 and 10 to 35 IU/ml, respectively. Nine of eleven patients with ACA whose sera were IgM reactive in the sonic extract ELISA had detectable IgM RF, including all sera with OD values of >0.3. The sera of all six patients with ACA that were IgM reactive in the flagellum ELISA had an IgM RF. Absorption of the RF abolished IgM reactivity in all IgM-reactive samples from patients with ACA in both assays. Since only 3 of 48

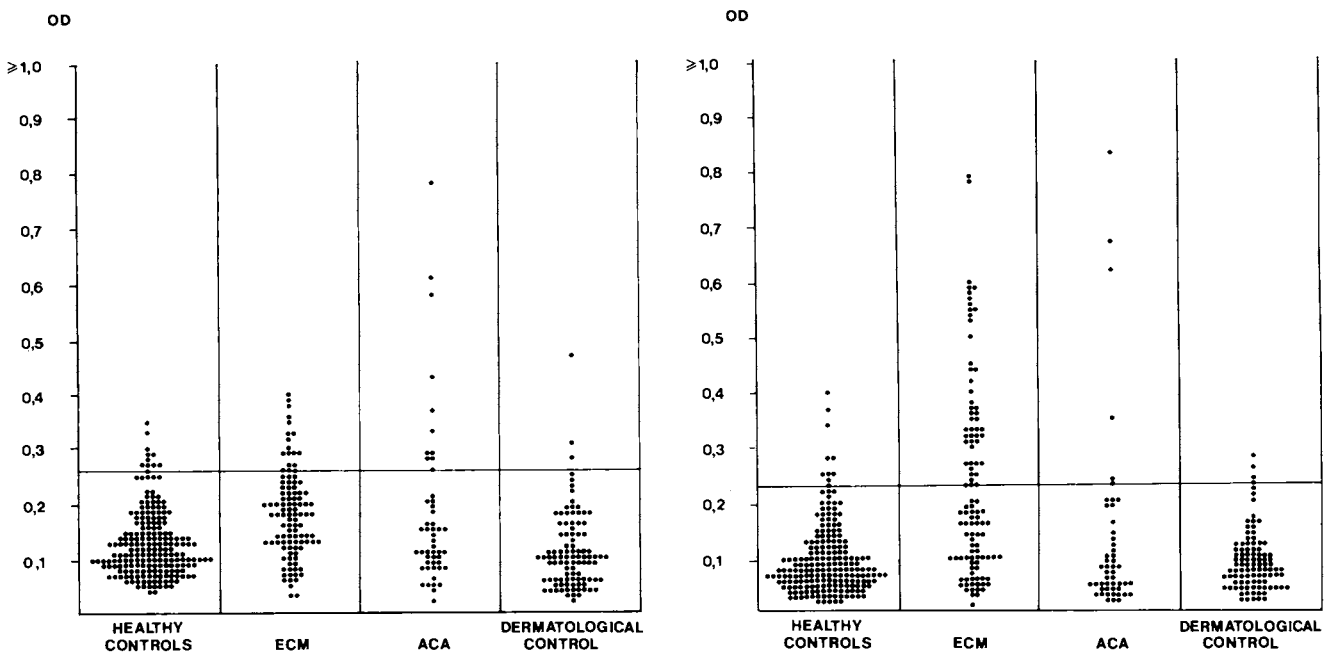

FIG. 2. IgM levels measured by *B. burgdorferi* sonic extract ELISA (left) and *B. burgdorferi* flagellum ELISA (right) in sera of 107 patients with EM (ECM) and 50 patients with ACA. Serving as controls were 200 healthy individuals and 98 patients with various dermatological disorders. The horizontal lines mark the diagnostic 95% specific cutoff levels.

IgM-reactive serum specimens from patients with EM showed an IgM RF, no absorption of the RF was done with these specimens.

DISCUSSION

In a previous study, the *B. burgdorferi* flagellum ELISA was shown to be clearly superior to a sonic extract ELISA in testing serum and cerebrospinal fluid samples from patients with lymphocytic meningoradiculitis (15). A comparable increase in the diagnostic performance of the flagellum ELISA was found in the present investigation of sera from 107 patients with EM, the early and first-stage manifestation of Lyme borreliosis.

A main advantage of the purified antigen was a greatly increased specificity of the IgG ELISA, since the OD values in the control groups were significantly lower. When the diagnostic cutoff level was adjusted to be 95% specific, the gained specificity was converted into a marked increase in diagnostic sensitivity. The diagnostic specificity of the IgM flagellum ELISA was almost unaltered, whereas the diagnostic sensitivity increased significantly.

TABLE 1. Diagnostic sensitivity of *B. burgdorferi* sonic extract and flagellum ELISAs

Wk after onset of EM (no. of patients)	No. (%) of positive samples			
	Sonic extract ELISA		Flagellum ELISA	
	IgG	IgM	IgG	IgM
≤1 (22)	2 (9.1)	2 (9.1)	6 (27.2)	7 (31.8)
2–4 (48)	3 (6.3)	11 (22.9)	12 (25.0)	22 (45.8)
5–12 (32)	6 (18.8)	5 (15.6)	17 (53.1)	17 (53.1)
≥12 (5)	1 (20.0)		3 (60.0)	2 (40.0)
Total (107)	12 (11.2)	18 (16.6)	38 (35.5)	48 (44.8)

The improved diagnostic performance of the flagellum ELISA compared with the sonic extract ELISA is most likely the result of the early and strong antibody response to the 41-kilodalton antigen recognized in several WB studies (7, 11–13, 17, 25) and the elimination of irrelevant cross-reacting antigens which are contained in the whole-cell sonic extract. *B. burgdorferi* shares antigenic epitopes with other spirochetes (7, 15, 18) and (and this may be of more practical importance to serology) also with many remotely related and common bacteria, including the normal human flora. Such an antigen is the immunogenic 60-kilodalton protein of *B. burgdorferi* which recently was shown to be the widely cross-reacting common antigen (14). This protein is soluble and is present in every sonic extract of *B. burgdorferi*.

A recent ELISA study (13) evaluated a sonic extract antigen versus a flagellum-enriched but not purified flagellum antigen preparation for ELISA. Possibly because of many residual antigens in the antigen preparation, no significant increase in the number of seropositive patients was found. In agreement with two previous studies (11, 15), Grodzicki and Steere (13) noticed a clearly improved discrimination between controls and patients, since positive sera generally gave considerably higher OD signals when either the flagellum-enriched or purified flagellum antigen was used. This observation is of great importance, especially in growing routine serology, since borderline values in sonic extract ELISAs are frequent and difficult or impossible to interpret.

In patients with a disseminated infection, as in lymphocytic meningoradiculitis, the magnitude of the antibody response to *B. burgdorferi* is correlated with the duration of the disease (12, 15, 21, 23). IgM titers show a maximum at 5 to 7 weeks after onset, whereas IgG titers increase gradually and are positive in almost every untreated case after 2 months (15). In EM, only the use of the flagellum ELISA showed a significant increase ($P < 0.005$) in the number of IgG-positive tests when the duration of an untreated EM

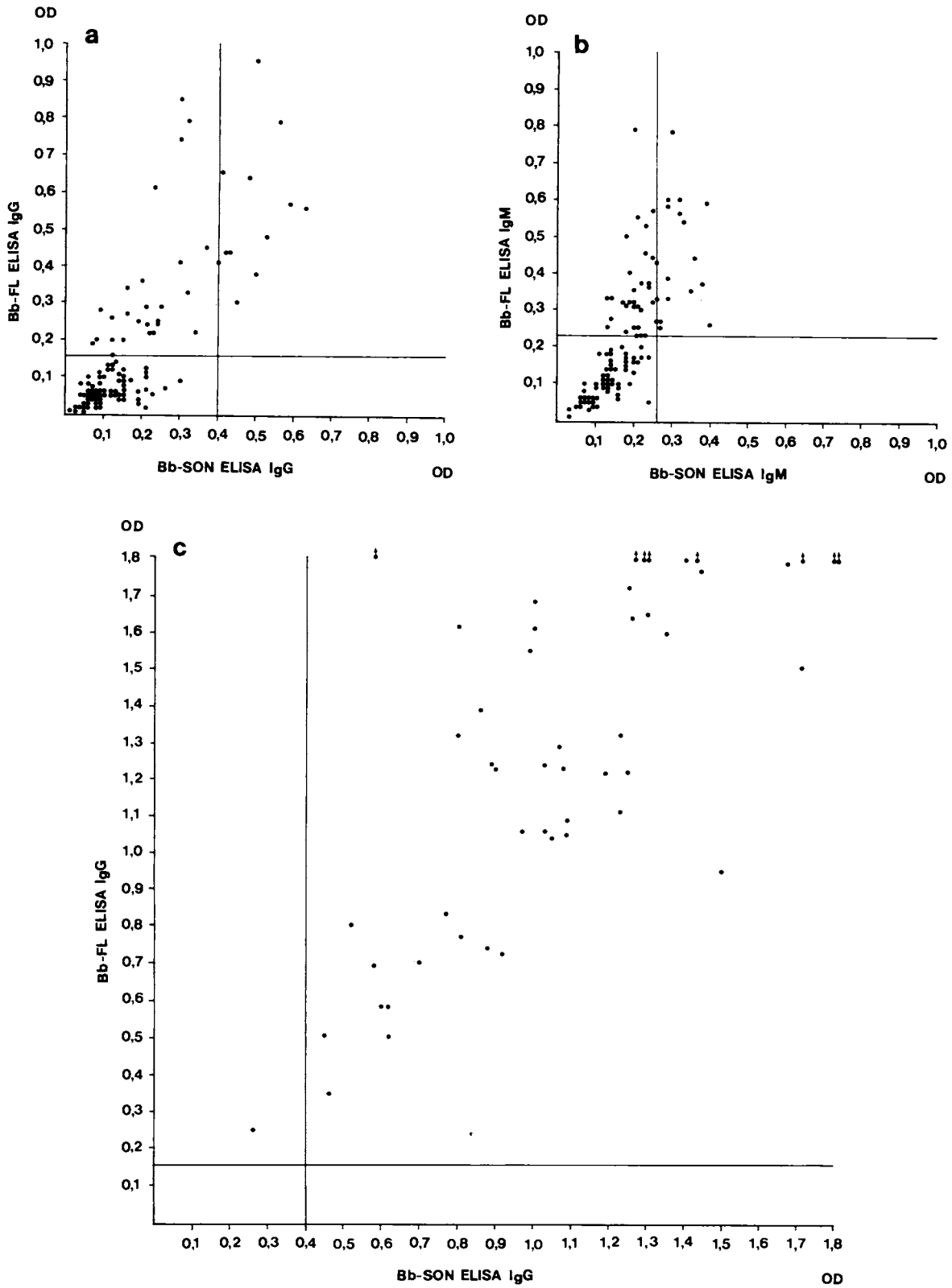

FIG. 3. Correlation of IgG (a) and IgM (b) measurement in sera of 107 patients with EM and of IgG measurement in sera of 50 patients with ACA (c) by the *B. burgdorferi* sonic extract and flagellum ELISAs. The vertical lines represent the cutoff levels in the sonic extract ELISA, and the horizontal lines mark the cutoff levels in the flagellum ELISA. The flagellum ELISA improved the quantitative discrimination between controls and seropositive samples significantly, as estimated by comparing the distances of the achieved OD values from the cutoff level in each test using Wilcoxon's rank sum test for paired data: panel a, $P = 0.002$; panel b, $P = 0.001$; panel c, $P = 0.001$.

TABLE 2. Diagnostic sensitivity of *B. burgdorferi* flagellum ELISA for 107 patients with EM

Positive reaction	% of patients				
	Multiple erythema (n = 16)	Solitary erythema (n = 91)	Concomitant general symptoms (n = 38)	No concomitant general symptoms (n = 69)	Avg (n = 107)
IgM	68.8	39.5	44.7	43.5	44.8
IgG	37.5	36.3	39.5	34.8	35.5

exceeded 1 month. Still, only slightly more than 50% of the patients with longstanding EM (1 to 12 months) were IgG seropositive (Table 1). The reason for this discrepancy is unknown. It may be because uncomplicated EM is an infection restricted to the skin, whereas a disseminated infection leads to a more predictable and stronger immune response. This explanation is supported by the observation of higher antibody levels in patients with multiple skin lesions, which are most likely the result of hematogenous spread. The restriction of a *B. burgdorferi* infection to a solitary skin lesion may be controlled by host factors and properties of the spirochete that determine its pathogenicity. The spontaneous course of the infection, as well as the production of antibodies, seems to be a question not only of disease duration. Therefore, the diagnostic sensitivity of serological tests must be defined for cases of EM and separately for cases of systemic *Borrelia* infection and not for cases of unspecified Lyme borreliosis.

Many serological studies using indirect immunofluorescence assays or sonic extract ELISAs have been reported since 1982. The diagnostic sensitivities achieved vary considerably. The results are often not comparable for several reasons: (i) unequal selection or lack of classification of patients according to the type of manifestation and disease duration; (ii) combined evaluation of IgM and IgG results, e.g., determining the number of patients who are either IgM or IgG seropositive without noting that this procedure reduces the diagnostic specificity significantly; expression of the diagnostic sensitivity of a test as the rate of either IgG or IgM reactivity demands at least a 97.5% specific cutoff level in both the IgG and IgM assays; (iii) inclusion of several samples from one patient or consideration of only the highest titer measured in a patient and not the first, diagnostic, pretreatment sample; (iv) different criteria for size and composition of the control group; and (v) very different cutoff levels. In a diagnostic or screening test, the cutoff level should be at least 95% specific based on a large and not serologically preselected control group.

There were no differences regarding the IgM or IgG antibody levels in 200 Danish and 98 Swedish controls representing two independent populations from distant regions. Such differences could have been the result of regional differences in tick or *B. burgdorferi* exposure of the populations or differences in the regional prevalences of certain strains. Previous indirect immunofluorescence assay studies that tested sera with American and European *B. burgdorferi* strains did not show any strain-dependent differences (1, 3, 19, 26). The use of *B. burgdorferi* flagellum as test antigen will further diminish the possible influence of strain variation on serological results, since it is a genuswide antigen (8).

IgG antibodies to the *B. burgdorferi* flagellum were found in all sera from patients with ACA. This proves the persistence of an antibody response to the flagellum even into the late stages of the disease.

IgM RF may cause false-positive IgM reactivity in an indirect ELISA (24). Therefore, samples from patients with EM and ACA were investigated for an IgM RF. Of the samples from patients with ACA, 32% had a detectable IgM RF, including 9 of 11 serum samples that were reactive in the sonic extract ELISA for IgM and 6 of 6 samples that were reactive in the flagellum ELISA for IgM. In accordance with a previous study (26), pretreatment of the sera with IgM RF Absorbans abolished the IgM reactivity in all IgM-reactive samples from patients with ACA. False IgM reactivity caused by IgM RF in EM is probably rare, since only 3 of 48 IgM-reactive serum samples had a demonstrable RF. IgM RF is frequently found in various acute and chronic infectious and immunological disorders. For routine serology, we therefore recommend that the use of the indirect IgM ELISA be restricted to probable early cases of Lyme disease and, in particular, to the investigation of cerebrospinal fluid (15).

The *B. burgdorferi* flagellin (the reduced protein subunit of the flagellum antigen) has genus-specific epitopes (8) but also some more conserved epitopes cross-reacting with other spirochetal flagellins, especially from treponemas. Therefore, the main limitation of the diagnostic specificity of the *B. burgdorferi* flagellum as a test antigen is a partial cross-reactivity with the *Treponema pallidum* flagellum (15). The new test does not permit serological discrimination between patients with Lyme borreliosis and syphilis, although serological cross-reactivity was reduced compared with that of the sonic extract ELISA (15). The magnitude of this problem in routine serology is limited. During one year, 1987, when a sonic extract ELISA was still being run, the Copenhagen Borrelia Laboratory received 5,264 samples. Only four were false-positive because of syphilitic infections. Furthermore, patients with Lyme borreliosis and syphilis are easily differentiated clinically and serologically by the nontreponemal syphilis serological tests (19).

On the basis of the present study of sera of patients with EM and ACA and a previous investigation of sera and cerebrospinal fluid of patients with lymphocytic meningoradiculitis (15), we conclude that the *B. burgdorferi* flagellum ELISA is for the time being the most sensitive and specific quantitative serological test. WB does not seem to be a practicable method for routine serology. Neither a single *B. burgdorferi*-specific band nor a combination of such bands with a high diagnostic sensitivity that would allow the discrimination of specific and unspecific antibody reactivities has been identified (13, 17). The flagellum ELISA is of particular value when the specific antibody level is low, as in many cases of EM and in the early secondary stage of Lyme borreliosis (15). A low but specific antibody response is often missed by a sonic extract ELISA because it is hidden by the necessarily high cutoff level. The antigenic composition of a whole-cell sonic extract may vary considerably, depending on the strain and the preparation technique. The *B. burgdorferi* flagellum is easy to standardize as a test antigen. These properties make the *B. burgdorferi* flagellum a suitable and needed reference antigen for routine serodiagnosis in Lyme borreliosis.

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SERONEGATIVE LYME DISEASE

Dissociation of Specific T- and B-Lymphocyte Responses to *Borrelia burgdorferi*

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Abstract The diagnosis of Lyme disease often depends on the measurement of serum antibodies to *Borrelia burgdorferi*, the spirochete that causes this disorder. Although prompt treatment with antibiotics may abrogate the antibody response to the infection, symptoms persist in some patients.

We studied 17 patients who had presented with acute Lyme disease and received prompt treatment with oral antibiotics, but in whom chronic Lyme disease subsequently developed. Although these patients had clinically active disease, none had diagnostic levels of antibodies to *B. burgdorferi* on either a standard enzyme-linked immunosorbent assay or immunofluorescence assay. On Western blot analysis, the level of immunoglobulin reactivity against *B. burgdorferi* in serum from these patients was no greater than that in serum from normal controls.

The patients had a vigorous T-cell proliferative response to whole *B. burgdorferi*, with a mean (±SEM) stimulation index of 17.8±3.3, similar to that (15.8±3.2) in 18 patients with chronic Lyme disease who had detectable antibodies. The T-cell response of both groups was greater than that of a control group of healthy subjects (3.1±0.5; $P<0.001$).

We conclude that the presence of chronic Lyme disease cannot be excluded by the absence of antibodies against *B. burgdorferi* and that a specific T-cell blastogenic response to *B. burgdorferi* is evidence of infection in seronegative patients with clinical indications of chronic Lyme disease. (N Engl J Med 1988; 319:1441-6.)

THE best clinical marker of acute Lyme disease is a characteristic skin lesion, erythema chronicum migrans (ECM).[1-4] In many patients, initial infection progresses to chronic Lyme disease, a spectrum of clinical signs and symptoms characterized by persistent musculoskeletal, cardiac, and neurologic involvement.[4-10] Since *Borrelia burgdorferi* was discovered to be the etiologic agent of this infectious disease, the demonstration of specific antibodies to this spirochete has been considered to be the best indicator of exposure to the organism and has become a prerequisite for the diagnosis of chronic Lyme disease.[11-15] A vigorous humoral response against *B. burgdorferi* develops during the course of natural infection. In chronic Lyme disease, *B. burgdorferi* persists, inducing an ongoing inflammatory response.[16,17] The absence of a continuing humoral response in patients treated with antibiotics during the ECM phase of Lyme disease has been believed to indicate that the organism had been effectively eradicated.[18-20] However, many patients given oral antibiotics at the ECM stage have reported persistence of symptoms, including severe chronic fatigue, numbness of the extremities, memory loss, and joint pain.[18-21] In the absence of elevated levels of antibodies to *B. burgdorferi*, these symptoms have been attributed to a post–Lyme disease syndrome rather than considered as evidence of persistent infection and failure of the initial antibiotic regimen to treat the disease effectively.

In persons exposed to *B. burgdorferi*, an early, vigorous, and sustained specific T-lymphocyte response develops that often precedes a measurable antibody response.[22,23] In this study we used borrelia-specific T-cell immune responses to document exposure to *B. burgdorferi* in patients with symptoms of chronic Lyme disease who lacked diagnostic levels of specific serum antibodies to the organism. It is noteworthy that all these patients had received oral antibiotics early in the course of infection. Thus, these findings exemplify an infectious disease in which removal of most of the microbial antigens at an early point in its course appears to abrogate the development of sustained humoral immunity despite evidence of persistent infection.

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METHODS

Subjects

Patients were classified as either seropositive or seronegative on the basis of their serum antibody reactivity to *B. burgdorferi* in a standardized enzyme-linked immunosorbent assay (ELISA). Serum samples were considered positive if the concentrations of antibodies to *B. burgdorferi*, expressed as optical-density values, were more than 3 SD above the mean values for a panel of samples obtained from a group of healthy adults with no history of *B. burgdorferi* infection. Samples with optical-density values less than 3 SD above the mean were considered negative.

The present study was based on findings in 17 patients with signs, symptoms, and a history compatible with the diagnosis of chronic Lyme disease who had specific optical-density values in the negative range (<3 SD above the mean). All were from Suffolk County, New York, an area in which Lyme disease is highly endemic. Eighteen other patients with comparable signs, symptoms, and histories of chronic Lyme disease who had diagnostic levels of antibodies to *B. burgdorferi* on ELISA served as positive controls, and 17 healthy adults with no history of Lyme disease or any other rheumatic or immune disorders served as negative controls. The clinical manifestations of both the seronegative and seropositive patients are shown in Table 1. Of the 17 seronegative patients, 15 had well-documented histories of ECM, the best clinical marker of *B. burgdorferi* infection. In each of these patients an influenza-like illness had accompanied the ECM. The other two patients had a similar history of an influenza-like illness that occurred soon after a tick bite. All 17 patients had been treated with oral antibiotics for at least 10 days at the time of their initial illness. Twelve had received tetracycline (1 to 2 g per day), four penicillin (1 to 2 g per day), and one erythromycin (1 g per day).

At the time of their initial presentation at the Lyme Disease Clinic at our institution, all 17 seronegative patients had systemic

symptoms, including fatigue, arthralgias, headaches, and cognitive dysfunction. Fever was not noted. The incidence of arthritis or a history of arthritis in this group (12 of 17 patients) was similar to that observed in the seropositive group (15 of 18 patients). In both the seronegative and the seropositive groups the arthritis was characterized by pain occurring when the affected joint was moved, periarticular soft-tissue swelling, synovial thickening, or small effusions. In all cases it was mild, oligoarticular, and nonerosive and its course contained remissions and relapses. The arthritis generally involved large joints, especially the knee. Nine of the seronegative patients had peripheral neuropathy. Four had entrapment neuropathies, documented by nerve-conduction studies, and seven had measurable abnormalities of peripheral nerves other than entrapment neuropathy, also documented by nerve-conduction studies. The neurophysiologic abnormalities of the peripheral nerves were indicative of an axonal neuropathic process. Demyelinating neuropathy was not observed. None of the patients had a history of recurrent infections. At the time of entrance into the study none were taking glucocorticosteroids or other immunosuppressive agents.

After the 17 seronegative patients received treatment with parenteral antibiotics (either penicillin [24 million units per day in divided doses for 10 days] or ceftriaxone [2 to 4 g per day for 14 days]), all had marked improvement. Objective evidence of active disease, including arthritis, resolved in all patients over a three-month period, with the exception of peripheral neuropathy, which resolved more slowly. None of the patients had a recurrent episode of arthritis or a symptomatic relapse during an eight-month follow-up period.

ELISA for Antibodies to *B. burgdorferi*

The ELISA was performed as previously described,[19] with minor modifications. In brief, 96-well microtiter plates (Flow Laboratories, McLean, Va.) were coated with a sonicate of *B. burgdorferi* (5 μg per milliliter) for 18 hours in 0.05 M sodium carbonate (pH 9.6) and then washed three times with phosphate-buffered saline (pH 7.2) containing 0.05 percent Tween 20 (PBS/Tween). Excess binding sites on the wells were blocked by postcoating the wells with 250 μl of phosphate-buffered saline containing 1 percent bovine serum albumin. The plates were washed as described above. Serum from all three study groups was diluted to 1:500 in PBS/Tween. Aliquots of 0.2 ml were added to duplicate wells and incubated for one hour at 37°C. The plates were washed, and alkaline phosphatase–conjugated goat antihuman immunoglobulin specific for both light-chain and heavy-chain determinants was added to detect bound immunoglobulin of all isotypes. After incubation for one hour at 37°C, the wells were again washed three times in PBS/Tween. *p*-Nitrophenyl phosphate (Sigma, St. Louis) was added at a concentration of 0.66 mg per milliliter in 0.15 M sodium bicarbonate containing 1 mM magnesium chloride, to each well. Plates were incubated two hours at room temperature, and the reaction was stopped with 30 μl of 3 *N* sodium hydroxide. The optical density of each well at 405 nm was measured on an ELISA reader (Bio-Tek, Winooski, Vt.).

Immunofluorescence Assay

Indirect immunofluorescence assays for the detection of both IgM and IgG antibodies to *B. burgdorferi* were performed according to standard techniques. In brief, slides coated with *B. burgdorferi* (Diagnostic Technology, Hauppauge, N.Y.) were incubated with serum at serial dilutions beginning at 1:64. After 60 minutes at room temperature, the slides were washed three times in phosphate-buffered saline and then incubated for 30 minutes with fluorescein-labeled antihuman immunoglobulin, washed, and examined on a fluorescence microscope (Nikon-Photomat, Garden City, N.Y.).

Electrophoresis and Immunoblotting

Qualitative antibody analyses were performed as previously described,[24] with slight modifications. In brief, *B. burgdorferi* organisms of the B31 strain were grown in BSK II media[25] and washed three times in phosphate-buffered saline. Spirochetes were resuspended in 2 ml of phosphate-buffered saline containing 10 mM ethylenediaminetetraacetic acid and 1 nM *p*-methylsulfonylfluoride and sonicated in an Ultra tip Labsonic System (Lab-Line Instruments, Melrose Park, Ill.) for five minutes. The protein concentration was measured according to the Bradford procedure.[26] The mixture was concentrated to 2.5 percent in sodium dodecyl sulfate and 2.5 percent in 2-mercaptoethanol, heated at 100°C for three minutes, and adjusted to a concentration of 0.4 to 0.6 mg of protein per milliliter. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed with a Mighty Small II apparatus (Hoeffer, San Francisco) at 60 V for two hours. Blots were left overnight at 4°C in TRIS buffer (50 mM TRIS–150 mM sodium chloride) containing 1 percent bovine serum albumin. They were then incubated with serum diluted with TRIS buffer for two hours at 37°C. Serum was assayed at dilutions of both 1:200 and 1:100. The blots were washed for 10 minutes with TRIS buffer containing 0.5 percent Tween-20 and then for 10 minutes in TRIS buffer alone. Bound immunoglobulin was detected with goat antihuman IgG, IgM, or IgA conjugated to alkaline phosphatase (Sigma). After a two-hour incubation at 37°C with a 1:5000 dilution of the conjugates, the blots were again washed in a two-step procedure and then incubated with the BICIP-Substrate System (5-bromo-4-chloro-3-indole phosphate–nitroblue tetrazolium; Kirkegaard and Perry Laboratories, Gaithersburg, Md.) for 20 minutes. The reaction was stopped by rinsing the blots in distilled water.

Lymphocyte-Proliferation Assays

The lymphoproliferative response of peripheral-blood mononuclear cells to whole *B. burgdorferi* organisms was assessed during the initial evaluation for chronic Lyme disease. Proliferation assays were performed as previously described.[22] Mononuclear cells were isolated by Ficoll–Hypaque (Pharmacia, Piscataway, N.J.) density-gradient centrifugation and washed three times. They were adjusted to a final concentration of 1×10^6 cells per milliliter in RPMI-1640 supplemented with 10 percent human AB serum, 100 U of penicillin, and 100 μg of streptomycin per milliliter, and 2 mM L-glutamine. A total of 1×10^5 cells per well were pipetted into a 96-well microtiter plate (Costar, Cambridge, Mass.) and stimulated (in triplicate) with whole *B. burgdorferi* at a concentration of 1×10^6 organisms per well. Control wells received medium only. Cell cultures were incubated in a humidified atmosphere of 5 percent carbon dioxide at 37°C. [^{3}H]Thymidine (specific activity, 6.7 Ci per millimole [New England Nuclear, Boston]) was added to the wells (1 μCi per well) 18 hours before harvesting at five days. Cells were collected with a cell harvester (Skatron, Sterling, Va.) onto glass-fiber filters, and the radioactivity associated with the cells was counted in a liquid scintillation system (LS7500, Beckman Instruments, Fullerton, Calif.). Counts were expressed as disintegrations per minute (dpm). The triplicate values were averaged, and a stimulation index (SI) was calculated for each patient, according to the formula, SI = dpm (stimulated)/dpm (unstimulated).

Statistical Analysis

The Student one-tailed t-test was used to compare the T-cell proliferative responses in the three groups tested.

Results

At presentation, 12 of the 17 seronegative patients had evidence of active arthritis on physical examination, and 9 had a peripheral neuropathy that was confirmed by nerve-conduction studies. All 17 reported that they had had severe chronic fatigue and recurrent headaches, 16 that they had had arthralgias, and 15 that they had had difficulty with short-term memory and cognition; all had objective evidence of active disease (Table 1). Eleven of the patients were evaluated with an extensive battery of neuropsychological tests,[27] including the California Verbal Learning Test (a test of memory) and the booklet version of the Category Test[28]; all 11 had evidence of intellectual

Table 1. Characteristics of Patients Seropositive and Seronegative for Chronic Lyme Disease.

	Seropositive Patients (N = 18)	Seronegative Patients (N = 17)
Mean age (yr)	36.6 (16–72)	38.0 (10–65)
Sex (M/F)	9/9	11/6
History of ECM	14	15
Initial influenza-like illness	17	17
Initial treatment		
Tetracycline	1	12
Penicillin	1	4
Cephalexin	1	—
Erythromycin	—	1
Duration of illness (mo)*	8.4±2.5 (1–24)	17.6±5.4 (8–72)
Clinical manifestations		
Arthritis	15	12†
Peripheral neuropathy	7	9
Symptoms‡	18	17

*Period (mean ±SD) from initial illness to diagnosis of chronic Lyme disease.

†Three other patients had a history of arthritis.

‡Includes headaches, fatigue, arthralgia, and cognitive dysfunction.

impairment. The results of serologic tests for antinuclear antibodies and rheumatoid factor, VDRL testing, and C1q binding assay, and serum protein electrophoresis patterns were all negative or normal.

Table 2 compares the results on ELISA in the seronegative and seropositive patients with Lyme disease. None of the 17 seronegative patients had levels of antibodies to *B. burgdorferi* that were more than 3 SD above the mean level in the negative controls. Immunofluorescence assays using whole *B. burgdorferi* organisms were also negative at serum dilutions above 1:64 (minimum positive titers in our laboratory, ≥1:256). Western blot analyses of solubilized whole *B. burgdorferi* antigens were performed to confirm the absence of specific anti-borrelia antibodies. Figure 1A shows IgG immunoblots for six seropositive patients. Their serum samples were assayed at a 1:200 dilution; all samples showed reactivity against distinct borrelia antigens. The samples differed in the number of antigens detected. The longer the Lyme disease was untreated in these seropositive patients, the greater the number of antigens against which their serum samples reacted (lanes d through f). Figure 1B shows representative IgG blots for five of the seronegative patients. Lane a represents serum from a seropositive patient that was run as a positive control. Lanes b through f represent samples from seronegative patients that were run at twice the concentration of the samples from the seropositive patients in order to detect low levels of borrelia-reactive antibodies. The most consistent indicator reactivity observed in the samples from the seronegative patients was a faint band corresponding to the 41-kd borrelia flagella antigen. Similar reactivity was observed in blots for 10 other seronegative patients that were run in separate assays (data not shown). This pattern of reactivity against spirochetal antigens, especially the 41-kd protein, was also observed in samples from the majority of controls (Fig. 1C). When the blots for the seronegative patients were stained for IgM or IgA antibodies, little specific reactivity was observed. A faint IgM band of reactivity against the protein associated with the 41-kd flagella was detectable in 2 of the 14 patients tested. Faint bands of IgA reactivity were also detected against the 41-kd and 66-kd antigens in all of five seronegative patients tested. This pattern of low-level reactivity of IgA and IgM antibodies was also observed in the normal controls and was readily distinguishable from the pattern observed in the seropositive patients, both in terms of the number of antigens recognized and the intensity of the bands. Thus, immunoblot analysis was consistent with the results obtained with the ELISA and the immunofluorescence assay, and demonstrated a profoundly blunted response to *B. burgdorferi* in the patients with clinically active disease.

T-cell immune responses specific for *B. burgdorferi* were assessed according to a standard proliferative assay using whole *B. burgdorferi* as the stimulatory antigen. These results are shown in Figure 2. Of note is the finding that the 17 seronegative patients with clinical Lyme disease had proliferative responses that were virtually identical to those of the seropositive patients. The stimulation index in the seronegative patients ranged from 2.7 to 58.0, with a mean (±SEM) of 17.8±3.3. The average number of counts was 19,455 dpm. The group of 18 seropositive patients had similar blastogenic responses to *B. burgdorferi*, with a mean stimulation index of 15.8±3.2. In contrast, the 17 healthy controls had stimulation indexes that ranged from 0.5 to 7.8, with a mean of 3.1±0.5, and an average count of 2932 dpm. Statistical analysis did not demonstrate any significant difference between the seronegative and the seropositive patient groups. However, the proliferative responses in each patient group were significantly different from those in the normal control group ($P<0.001$).

Discussion

Infection with *B. burgdorferi*, the etiologic agent of Lyme disease, is associated with the development of a vigorous T-cell response to this organism.[22,23] In this study we have shown that a specific T-cell response can occur independently of a diagnostic humoral response to *B. burgdorferi*. Although our 17 chronically ill patients had clinical histories, signs, and symptoms

Table 2. Reactivity for *B. burgdorferi* Antibodies on ELISA.*

	Normal Controls (N = 10)	Seronegative Patients (N = 17)	Seropositive Patients (N = 18)
Mean serum antibody concentration	0.44	0.55	3.81
Standard deviation	0.19	0.24	0.62

*Values are expressed as normalized values for optical density (donor value in subject ÷ [mean value among normal controls +3 SD]).

compatible with the diagnosis of active Lyme disease, their levels of antibodies to *B. burgdorferi* as measured by ELISA or by immunofluorescence assay were indistinguishable from those of normal controls. This lack of reactivity was confirmed by Western blot analysis. Serum from these 17 patients primarily exhibited only weak reactivity to the 41-kd flagella antigen, an antigen sharing epitopes with many other spirochetes, including both other borrelia species[20] and treponema species. In contrast, 14 of the 17 patients had a marked T-cell response to this organism. Three patients had low T-cell responses (stimulation indexes of 2.7, 4.1, and 4.9) that overlapped with the range of the normal controls. Each of these three patients, however, had a history of ECM that had been observed by a physician, early antibiotic therapy, a clinical course compatible with Lyme disease, and improvement after administration of parenteral antimicrobial agents known to be effective against *B. burgdorferi*. A similar range of T-cell reactivity was also observed in the seropositive patients. Once established, this T-cell reactivity in both seronegative and seropositive individuals was reproducible and persistent. None of the 17 patients had a history suggestive of a primary immune defect, although the possibility of a specific abnormality in the ability to respond to discrete spirochetal antigens or an immunoglobulin-subclass deficiency was not ruled out.

The disorder in these seronegative patients reflected a dissociation between T-cell and B-cell immune responses, in which the cell-mediated arm of the immune response was intact yet the humoral portion of the response to *B. burgdorferi* appeared to be blunted. This diminished antibody response is in contrast to the T-cell anergy commonly observed in several chronic infections (e.g., infection with *Mycobacterium leprae* or *M. marinum*, filariasis, and some chronic fungal infections[29-33]). It has previously been found that some patients who received antibiotics for ECM had low or undetectable levels of anti-borrelia antibodies.[15,20] This condition may be analogous to syphilis, in which early antimicrobial therapy can abort the development of a measurable humoral response. Since help from T cells is usually required to produce a vigorous antibody response to protein antigens and to augment the response to most carbohydrate antigens,[34] the development of a measurable T-cell response probably occurs before an antibody response becomes fully manifest. In previous studies, we showed that patients with ECM can mount specific T-cell responses to *B. burgdorferi* before antibodies to this organism become detectable by routine ELISA.[22,23]

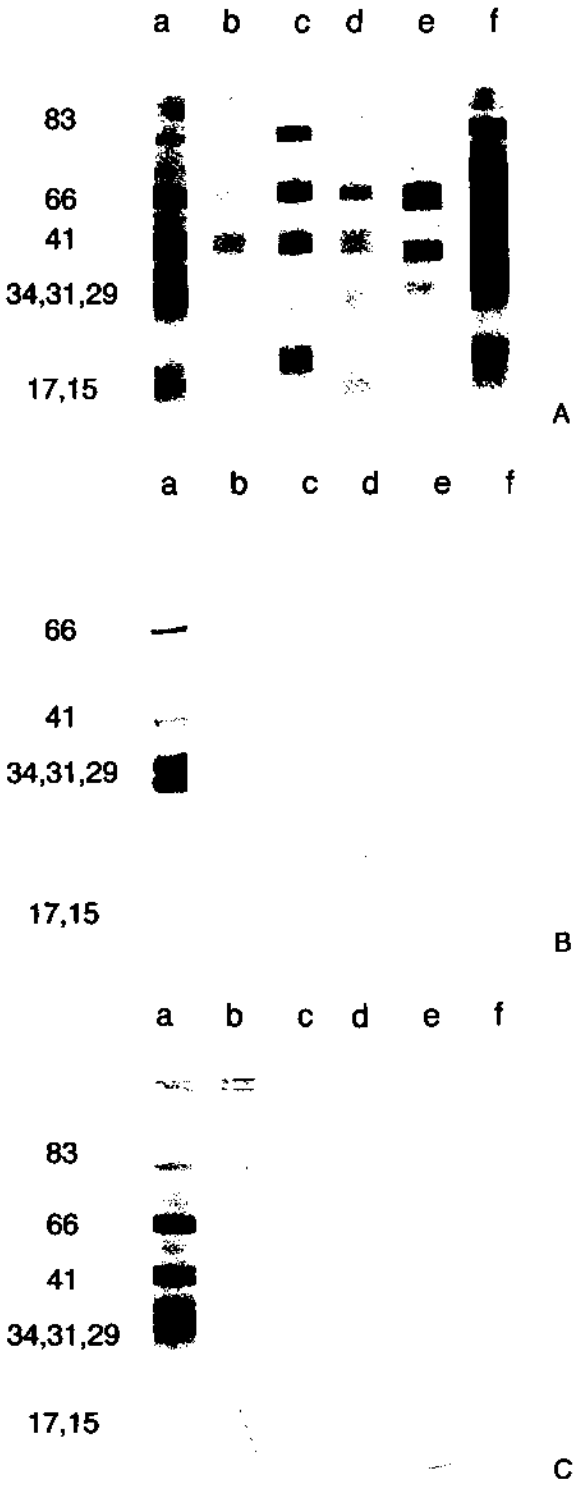
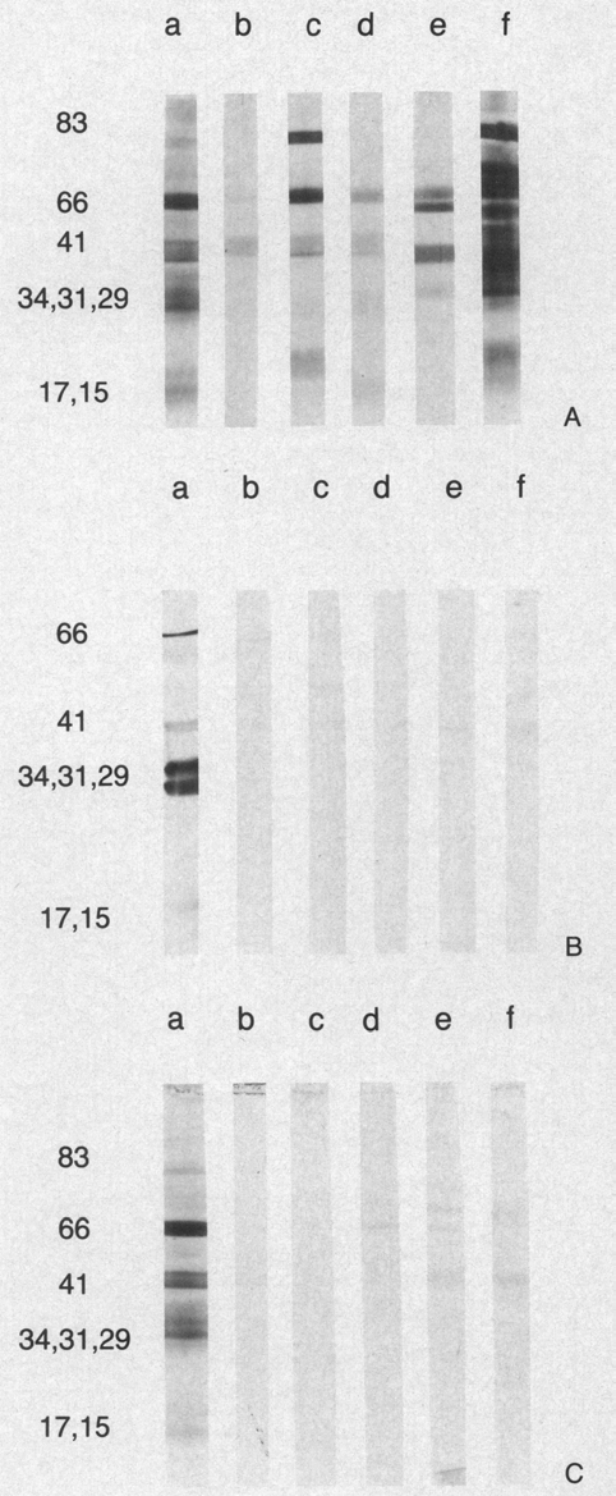


Figure 1. Immunoblots of IgG Antibodies to *B. burgdorferi* Antigens.

B. burgdorferi proteins were transferred to nitrocellulose and probed with diluted serum (either 1:200 for ELISA-positive samples or 1:100 for negative samples). Bound IgG was detected by goat antihuman gamma-chain antibody conjugated to alkaline phosphatase. Blots were developed with the BICIP-substrate system.

Panel A shows blots for six patients with chronic Lyme disease who were seropositive on ELISA. Panel B shows representative blots for five of the seronegative patients (lanes b through f), and Panel C immunoblots of serum from five of the normal controls (lanes b through f). Lane a in both Panel B and Panel C represents serum from a seropositive patient, shown for comparison. Molecular-weight markers are shown to the left.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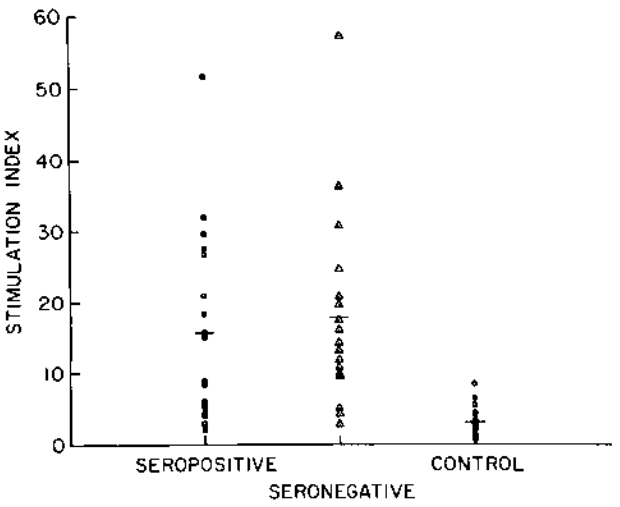

Figure 2. Lymphoproliferative Responses to *B. burgdorferi*. Isolated peripheral-blood mononuclear cells were stimulated with whole *B. burgdorferi* organisms as outlined in the Methods. The cells were cultured in flat-bottomed microtiter wells, at a concentration of 10^5 cells per well, pulsed with 1 μCi of [^{3}H]thymidine for 18 hours, and harvested on day 5. Values for the stimulation index are shown on the abscissa. The groups studied were patients with positive values on ELISA for *B. burgdorferi* (n = 18), patients without serologic evidence of Lyme disease (n = 17), and normal controls (n = 17). Bars represent the arithmetic mean response in each group.

The precise mechanism of the observed humoral anergy has not yet been delineated. However, each of the 17 seronegative patients was given antibiotics early in the course of infection. Although the standard antibiotic therapy for Lyme disease appears to eradicate the organism in most instances,[18] we have found that in some patients the response to treatment is incomplete and late sequelae develop.[21] In the seronegative patients whom we describe, it may well be that antibiotic therapy resulted in the elimination of most spirochetes at a critical early stage of the immune response, before a sustained B-cell response had developed. A precedent for the abrogation of a mature antibody response by early treatment exists not only in *Treponema pallidum* infection[35] but also in Rh incompatibility, in which the infusion of antibody to the Rh antigen clears the antigen and selectively inhibits the maternal antibody response.[36] Current concepts of immune responsiveness hold that T cells initially recognize foreign antigen presented by cells expressing Class II major histocompatibility antigens, such as macrophages and dendritic cells.[37] After recognition, antigen-specific T cells proliferate and mature to become effector cells. These effector T cells can then interact with other cells in the immune system that express the same HLA-D–region gene products. In antigen-specific systems, an additional requirement for cell–cell interactions is the presence of antigen to provide a close physical linkage between the T effector cell and the other cells. Antigen-specific B cells, having both dense Ia and specific antigen receptors on their surface, are exquisitely suited to present antigen to T cells.[38] In the absence of antigen, cognate recognition is not established and T cells and B cells do not interact effectively. Thus, according to this model of immune interaction, after an initial proliferative response by B cells that is independent of the response by T cells, the continued presence of the antigen is required for the development of a mature humoral response.

There is an apparent inconsistency in this model of seronegative Lyme disease and the generally held concept of the pathogenesis of chronic Lyme disease. If the cognate recognition of B cells by T cells fails to occur because the spirochetes have been removed, how can continued disease activity be explained? Although all the patients described were given antibiotics that could effectively eradicate the bulk of *B. burgdorferi* organisms from most sites of infection, the central nervous system and perhaps other privileged sites may not have received adequate treatment. Currently recommended regimens of oral tetracycline and penicillin fail to produce drug concentrations in the central nervous system that are high enough to reach the mean inhibitory concentrations for the majority of strains of *B. burgdorferi*.[21,39,40] Thus, spirochetes reaching this immunologically privileged site may remain viable despite standard therapy. A similar phenomenon has been observed in patients in whom therapy believed to be curative for *T. pallidum* infection was administered yet in whom active neurosyphilis later developed.[41-43] Consistent with chronic central nervous system infection is our recent observation that some patients with chronic Lyme disease have appreciable abnormalities of the white matter on magnetic resonance imaging.[27] In addition, three separate groups of investigators have reported local production of anti-borrelia antibody in the central nervous system in patients with neurologic symptoms of Lyme disease in the absence of diagnostic levels of serum antibodies.[44-46]

In summary, the diagnosis of chronic *B. burgdorferi* infection should be considered in any patient with a history of ECM and signs and symptoms compatible with chronic Lyme disease. Serologic assays remain the best tests for screening for exposure to *B. burgdorferi*. Although the true incidence of seronegative disease is unknown, it is not common. Seronegative patients represent less than 5 percent of the more than 200 patients with Lyme disease who are referred to our group each year. Assessment of T-cell blastogenesis should be limited to symptomatic patients who have been exposed to ticks, who have objective evidence of active disease, who have received antibiotics early in the course of their infection, and who have nondiagnostic levels of antibody systemically and locally. Although chronic fatigue is a common finding in chronic Lyme disease, we do not believe that chronic fatigue as an isolated symptom should be considered to be indicative of *B. burgdorferi* infection. It is important to diagnose chronic

Lyme disease with its accompanying neurologic and rheumatologic sequelae correctly, since this systemic spirochetosis can be effectively treated with appropriate intravenous antibiotics.[41,47-49] However, it is also important to remember that immunologic tests, whether they measure antibodies or cellular immune responses, are indicators of exposure and do not by themselves prove that the patient has an active infectious process.

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Antibodies against Whole Sonicated *Borrelia burgdorferi* Spirochetes, 41-Kilodalton Flagellin, and P39 Protein in Patients with PCR- or Culture-Proven Late Lyme Borreliosis

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The sensitivities and specificities of three enzyme-linked immunosorbent assays (ELISAs) for *Borrelia burgdorferi* antibodies were compared for 41 patients presenting with symptoms compatible with late Lyme borreliosis (LB) and 37 healthy controls. All subjects were living in southwestern Finland, where LB is endemic. Only patients with culture- or PCR-proven disease were enrolled in the study. The antigens of the ELISAs consisted of sonicated spirochetes, 41-kDa flagellin, and recombinant P39 protein of *B. burgdorferi*. Fifteen patients had strongly or moderately positive results in the serological assay(s), 19 patients had only weakly positive or borderline antibody levels, and the remaining 7 patients were seronegative by ELISA. The sensitivities of the ELISAs were 78.0% with sonicate antigen, 41.5% with 41-kDa flagellin, and 14.6% with P39 protein. The specificities of the tests were 89.2, 86.5, and 94.6%, respectively. The sonicate antigen ELISA seems to be an effective screening method. These results show that antibodies to *B. burgdorferi* may be present in low levels or even absent in patients with culture- or PCR-proven late LB. Therefore, in addition to serological testing, the use of PCR and cultivation is recommended in the diagnosis of LB.

Borrelia burgdorferi is difficult to isolate from body fluids, because the numbers of spirochetes are low and bactericidal factors may suppress their growth (1, 20, 34). PCR is extremely sensitive, detecting even dead spirochetes (25). However, suitable body fluids or tissue biopsy specimens are not always available for PCR or culture. Therefore, serological tests remain important screening methods in the diagnosis of Lyme borreliosis (LB) (5, 17).

B. burgdorferi is an antigenically complex microorganism with epitopes common to other microbes (18) and host tissue components (31). On the other hand, antibody responses to *B. burgdorferi* can be weak, delayed, or even absent during different stages of the disease (3, 4, 7, 20, 34, 38). The 41-kDa flagellin has shown promise in the diagnosis of early and also late stages of LB (9, 11–13, 34). Furthermore, recent reports have suggested that antibodies to the 39-kDa antigen of *B. burgdorferi* may be important markers for LB (8, 32, 33).

In a recent study, Mitchell et al. compared results of immunoserologic assays for patients with culture-positive erythema migrans (EM) and found that an immunoglobulin M (IgM) indirect fluorescent-antibody assay detected antibodies to *B. burgdorferi* in 78% of cases, while other assays tested were substantially less sensitive (21). The diagnostic sensitivity of serological tests is considered to be better in later stages of LB than in early disease. However, several reports about seronegative patients with definite late LB have been published (6, 7, 10, 16, 24, 26, 29). In the present study, we concentrated on testing the sensitivities of three enzyme-linked immunosorbent assays (ELISAs) in late LB. Only PCR- or culture-positive patients from a larger group of Lyme disease patients were included. This enabled us to measure antibody levels in patients who have live spirochetes or borrelia DNA in their bodies and to avoid patients with post-Lyme syndrome and patients with old serological scars months or years after infection.

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MATERIALS AND METHODS

Patients. The study included 41 patients with late LB from southwestern Finland and 37 healthy controls from the same region. Table 1 shows the demographic data for the patients and controls. All subjects were living in an area where LB is endemic. Thirty-four of the patients were seen by one of the authors (J. Oksi) and seven were seen by other clinicians at Turku University Hospital in 1991 to 1992. The diagnosis of LB was based on clinical symptoms and positive culture and/or PCR. Past histories and clinical manifestations (Table 1) indicated that 26.8% of the patients recalled a tick bite(s), and 31.7% had a history of EM. Two patients had developed EM around the area of a fly bite. All patients had been suffering from LB symptoms for more than 3 months, 78.0% had had LB symptoms for more than 6 months, 53.7% had had LB symptoms for more than 1 year, and many patients had had symptoms for several years. The clinical criteria for LB in our patients follow the case definition for Lyme disease developed by the Centers for Disease Control and Prevention (2, 27). Table 1 shows the manifestations and their frequencies. Detailed case reports for two of these patients have been previously published (23, 35).

All patients were positive by culture and/or PCR (12 positive by culture, 39 positive by PCR, and 10 positive by both tests). The numbers of specimens positive by culture and/or PCR were as follows: peripheral blood, 14; serum, 15; cerebrospinal fluid, 11; aspirates, 5 (including 4 synovial fluid samples and 1 bone marrow aspirate); and biopsy material, 6. For six patients, PCR or culture was positive for two or more body fluid or biopsy specimens. Plasma or serum samples were positive by culture or PCR for 28 patients (68.3%).

The control material consisted of 37 serum specimens. Of these, 30 were obtained from healthy blood donors and 7 were obtained from patients with suspected allergy but with negative radio-allergo-sorbent tests.

ELISAs. IgM and IgG antibodies against whole-cell sonicated *B. burgdorferi* were measured by an in-house ELISA (SA-ELISA) (36). *B. burgdorferi* B31 (ATCC 35210; high passage) was grown in BSK-II medium, harvested by centrifugation (10,000 × *g* for 30 min), washed with 5 mM $MgCl_2$ in phosphate-buffered saline (PBS), and sonicated in an ice bath four times for 30 s at 30 W (Sonifier cell disrupter model B15; Branson Sonic Co. Danbury, Conn.). The protein concentration of the sonicate was adjusted to 20 μg/ml in 0.05 M PBS for attachment to microwells. All steps of the SA-ELISA, including coating of the

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TABLE 1. Demographic data and clinical symptoms of patients with culture- or PCR-proven late LB and control subjects

Characteristic	Value for group	
	Patients (n = 41)	Controls (n = 37)
Men/women	19/22	12/25
Mean age, yr (range)	37.6 (4–76)	32.2 (1–59)
Tick bite (%)	11 (26.8)	
History of EM (%)	13 (31.7)	
Tick bite and/or EM (%)	17 (41.5)	
Other manifestations (%)		
Skin, other than EM[a]	10 (24.3)	
Musculoskeletal[b]	31 (75.6)	
Neurological[c]	24 (58.5)	
Cardiac[d]	6 (14.6)	
Ocular[e]	5 (12.2)	
Recurrent fever episodes	19 (46.3)	
Hepatitis	2 (4.9)	
Other symptoms or findings[f]	5 (12.2)	

[a] Numbers of patients (n) with the indicated symptoms: panniculitis, 3; unspecific dermatitis, 1; vasculitis, 2; secondary EM, 2; and chronic urticaria, 2.

[b] n for the following symptoms: arthritis, 17; arthralgia, 9; myositis, 4; myalgia, 4; multiple-site osteomyelitis, 1; tendinitis, 3; and fibromyalgia, 1.

[c] n for the following symptoms: meningitis, 2; myelitis, 1; radiculitis, 1; paresthesia, 3; encephalitis, 2; dizziness, 7; memory impairment or encephalopathy, 7; epilepsy, 1; multiple cerebral infarcts, 1; transient hemiparesis, 1; hemisyndrome, 1; ataxia, 2; cephalalgia, 6; involvement of III, VI, or VII cranial nerve, 6, including 1 case of bilateral facial palsy; dementia, 2; and brain abscess, 1.

[d] n for the following symptoms: transient third-degree atrioventricular block, 1; myocarditis, 2; pancarditis, 1; endocarditis, 1; and cardiomyopathy, 1.

[e] n for the following symptoms: iritis, 3; chorioretinitis, 1; (kerato)conjunctivitis, 2; and chronic photophobia, 1.

[f] n for the following symptoms: lymphadenopathy, 1; and prolonged fatigue, 4.

solid phase with antigen, were carried out automatically by an Auto-EIA II instrument (Labsystems, Helsinki, Finland). Serum samples were tested at a dilution of 1:100. Alkaline phosphatase-conjugated swine anti-human IgG or IgM antibodies (Orion Diagnostica, Espoo, Finland) and *p*-nitrophenylphosphate substrate were used for detection of bound antibodies. A standard curve was drawn by using a strongly positive patient serum specimen as a standard. The results were expressed as relative ELISA units. Seropositivity was determined by comparing antibody results for test serum samples with those for 110 healthy controls. The cutoff values were the mean + 2 standard deviations (SD) of the controls for weakly positive results, the mean + 4 SD for positive results, and the mean + 6 SD for strongly positive results.

Commercial kits were used for measurement of antibodies against 41-kDa flagellin of *B. burgdorferi* (FL-ELISA) (Lyme borreliosis ELISA kit, second generation; DAKO A/S, Glostrup, Denmark) (11) and against recombinant P39 protein (P39-ELISA) (ImmunoWeLL Recombinant P39 [Lyme] test; General Biometrics, Inc., San Diego, Calif.) (33). FL-ELISA measures both IgM and IgG antibodies, whereas P39-ELISA does not differentiate between immunoglobulin isotypes. Interpretation of the results obtained by the kits was done as instructed by the manufacturers. Borderline or positive results with FL-ELISA were recorded as weakly positive, strongly positive ones were considered positive, and very strongly positive ones were classified as strongly positive. Results with P39-ELISA were classified as negative, weakly positive, or positive.

Cultivation. The specimens (e.g., skin biopsy specimens, cerebrospinal fluids, or blood or serum samples) were inoculated into tubes containing BSK-II medium and incubated at 30°C. The tubes were examined macroscopically twice a week and passaged once a week for at least 2 months. Dark-field microscopy was carried out if the color of the culture medium indicated growth. The final identification of cultured spirochetes was based on PCR.

Extraction of DNA for PCR. One milliliter of sample (plasma, serum, cerebrospinal fluid, or synovial fluid) was centrifuged (Eppendorf Microfuge, 13,000 rpm, 10 min), 800 μl of supernatant was removed, and the remaining 200 μl was mixed with 300 μl of sodium dodecyl sulfate (SDS) solution (0.1 M NaOH, 2 M NaCl, and 0.5% SDS). After incubation at 95°C for 15 min, 200 μl of 0.1 M Tris-HCl (pH 8) was added. After SDS treatments, DNA was extracted with phenol-chloroform, precipitated with ethanol, and finally dissolved in water.

PCR. A 5-μl volume of extracted DNA was added to the reaction tube. Our target sequence for the PCR was the *fla* gene. The PCR was run in two steps, first with external primers prB31/41-4 and prB31/41-5 (37), resulting in a 730-bp PCR product, and then with nested primers WK1 and WK2 (14), resulting in a 290-bp fragment. Each PCR run included a positive control containing DNA extracted from reference strain B31 of *B. burgdorferi* (ATCC 35210). Furthermore, every fifth tube of each run was used as a negative control subjected to all sample treatment procedures. The PCR products were detected by gel electrophoresis on a 1.5% agarose gel with ethidium bromide staining.

RESULTS

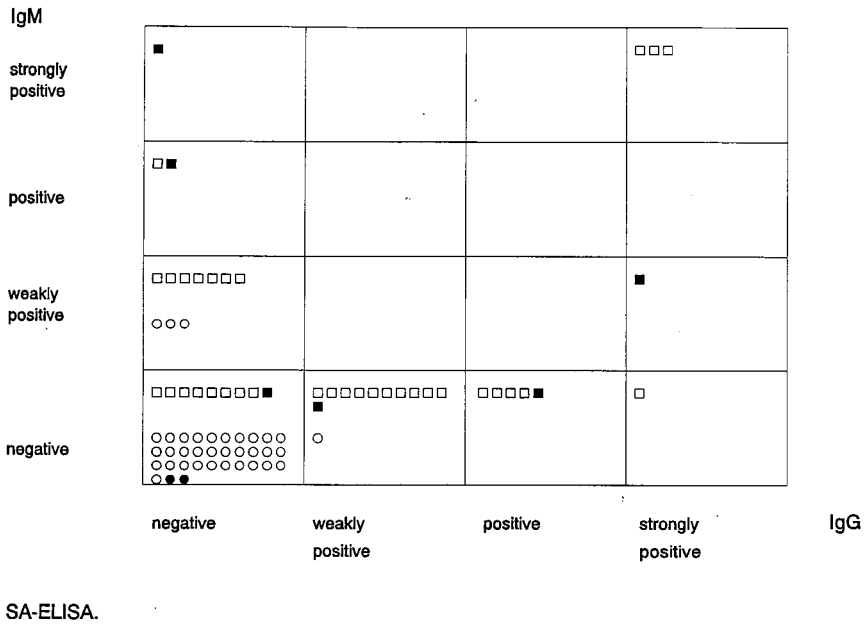
In determining the sensitivities and specificities of the three ELISAs, three different levels of positivity (weakly positive, positive, and strongly positive) were considered positive results. Table 2 shows ELISA results with three different antigens for culture-positive patients, only-PCR-positive patients, and control subjects. For all 41 patients presenting with symptoms compatible with late LB, the sensitivity of SA-ELISA was 78.0%, that of FL-ELISA was 41.5%, and that of P39-ELISA was 14.6% (Table 2). Both FL-ELISA and P39-ELISA detected only one positive specimen which had gone undetected by the other two tests. In the analysis of 37 healthy controls, the test specificities were 89.2% for SA-ELISA, 86.5% for FL-ELISA, and 94.6% for P39-ELISA (Table 2). The respective positive predictive values were 88.9, 77.3, and 75.0%. The respective negative predictive values were 78.6, 57.1, and 50.0%.

The sensitivities achieved with different combinations of the three tests were as follows: SA-ELISA plus FL-ELISA, 80.5%; SA-ELISA plus P39-ELISA, 80.5%; FL-ELISA plus P39-ELISA, 51.2%; and SA-ELISA plus FL-ELISA plus P39-ELISA, 82.9%. Thus, for 34 of the 41 patients, diagnosis could be confirmed by serological tests.

Although all patients suffered from late LB, 10 (24.4%) and 8 (19.5%) of the patients had only IgM antibodies as measured by SA-ELISA and FL-ELISA, respectively. The corresponding figures for IgG antibodies were 17 (41.5%) and 4 (9.8%). Both IgM and IgG antibodies were detected in 5 patients (12.2%) by both SA-ELISA and FL-ELISA. The levels of antibodies against *B. burgdorferi* in patients and controls are shown in Fig. 1.

TABLE 2. Results of SA-ELISA, FL-ELISA, and P39-ELISA for patients with culture- or PCR-proven late LB

Group	No. of results					
	SA-ELISA (IgM, IgG, or both)		FL-ELISA (IgM, IgG, or both)		P39-ELISA	
	Positive	Negative	Positive	Negative	Positive	Negative
Patients						
Culture positive (n = 12)	11	1	6	6	5	7
Only PCR positive (n = 29)	21	8	11	18	1	28
Total	32	9	17	24	6	35
Controls (n = 37)	4	33	5	32	2	35



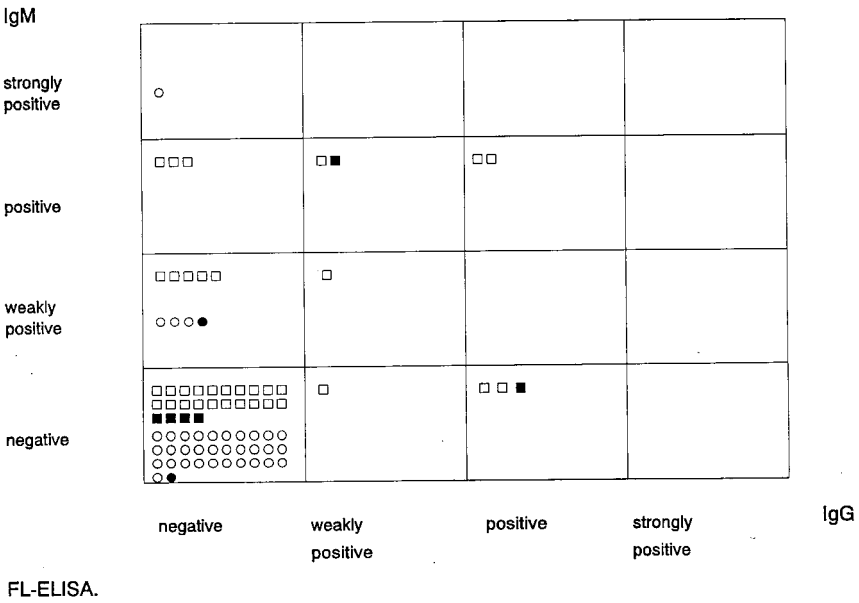


FIG. 1. Levels of IgM and IgG antibodies in patients with PCR- or culture-proven late LB (squares) and control subjects (circles) as measured by SA-ELISA and FL-ELISA. Closed squares and closed circles represent sera which had borderline or positive results by P39-ELISA.

DISCUSSION

This study shows that patients with late LB who have live spirochetes or borrelial DNA in their body fluids may have low or negative levels of borrelial antibodies in their sera. This emphasizes that an efficient diagnosis of LB has to be based on culture, PCR, and serology, because even the combined sensitivity of the three ELISA modifications tested was only 83%. If serological means alone are used, a considerable proportion of LB patients may not be diagnosed and treated. It might be possible that LB patients with weak or no humoral immune responses against the spirochete develop even more serious disease than the patients with strong antibody responses (15). Our results also show that plasma and serum samples are suitable specimen types for the detection of circulating spirochetes or their structures also in late disease.

In this study, multiple organs were frequently involved. Recurrent fever episodes were seen in nearly half of the patients, neurological symptoms were seen in more than half of the patients, and musculoskeletal manifestations were seen in three-fourths of the patients. Moreover, most of these manifestations were long-lived. In spite of this, several patients were

seronegative and most seropositive patients had only weakly positive antibody levels.

Several antigenic components of *B. burgdorferi* have been tested in an attempt to improve the diagnostic efficacy of serological tests. We tested one purified borrelia antigen, 41-kDa flagellin, and one recombinant borrelia antigen, P39 protein, in serological diagnosis of late LB. These antigen types have recently shown promise in the diagnosis of early and late stages of LB (8, 9, 11, 12, 22, 32–34). Compared with the results of these earlier studies, our results are disappointing. In our study, SA-ELISA was far more sensitive than FL-ELISA. The sensitivity of P39-ELISA was 14.6%, which is substantially lower than those in one earlier study with this commercial kit giving sensitivities of 8% for early and 39% for late disease (28). However, in the culture-positive subgroup of our patients, 5 of 12 (41.7%) had borderline or positive results as determined by P39-ELISA. Our results with P39-ELISA were not due to any technical errors, because the positive controls of the kits repeatedly gave absorbance values within the limits indicated by the manufacturer. Furthermore, the specificity advantage obtained by the components was limited.

One reason for the disappointingly low sensitivity obtained by the two separate component antigen tests may be the difference in expression of antigens by various strains and, possibly even more importantly, the differences in the host ability to develop an immune response to a given antigen (i.e., the immunogenicity of the antigen). It is also evident that tests relying upon single antigenic components are far more sensitive to the differences in host immune responses to those antigens than tests using crude antigen extracts. Crude extracts always contain such a broad spectrum of antigens that differences in host immune reactions to some antigens do not affect test sensitivity. In fact, this hypothesis is supported by the study of Magnarelli et al. in which roughly similar antibody titers were obtained by a whole-cell ELISA using different *B. burgdorferi* strains (19). However, the present study design may give a too pessimistic estimation of the sensitivity of the serological techniques tested, because we excluded patients with diagnoses based only on serological laboratory evidence.

Low or even undetectable antibody levels in late LB may be caused by formation of circulating immune complexes (29). Immune complexes are formed especially in the presence of excess antigen. Furthermore, circulating immune complexes may be a sign of active disease. Schutzer and coworkers demonstrated complexed antibody against *B. burgdorferi* in almost all of their patients with active symptoms of LB and its absence in a group of recovering patients (30). We did not assess circulating immune complexes or antibodies after dissociation of immune complexes. However, 68% of our patients had borrelia DNA or cultivatable spirochetes in the serum or plasma. This shows that borreliae or their structures are frequently present in the circulation of patients with late LB, permitting complex formation. It is possible that for patients without borrelia DNA in their circulation, the sensitivity of serology may be greater than observed in our study.

Elevated IgM antibody levels without a concomitant rise in IgG levels are generally considered a sign of a primary immune response. Our results provide further support for earlier studies showing that antibody responses against *B. burgdorferi* can be restricted to IgM even in late LB (23, 35). The persistence of the IgM response can be explained either by a disability to switch antibody production from IgM to IgG or by a continuous appearance of new antigenic epitopes on the spirochetes during the infection (4). The isolated occurrence of IgM without the presence of IgG was also detected by FL-ELISA. This indicates that host factors are more important than microbial factors for IgM persistence, because there are no data showing antigenic variation in flagellin, whereas the variation of the whole antigenic mosaic of *B. burgdorferi* can be abundant.

We conclude that antibodies to *B. burgdorferi* often are present in only low levels or are even absent in culture- or PCR-positive patients who have been suffering for years from symptoms compatible with LB. Therefore, in addition to serological testing, the use of PCR and cultivation in the diagnosis of LB is recommended. Furthermore, the use of the two kits using component antigens tested in this study does not solve the problems of serological diagnosis of LB, at least in northern Europe. SA-ELISA seems to be an effective method for screening purposes, especially in late disease.

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Clinical
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Case Report

Seronegative Lyme Arthritis caused by *Borrelia garinii*

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Abstract: A case of a female patient suffering from Lyme arthritis (LA) without elevated antibody levels to *Borrelia burgdorferi* sensu lato is reported. Seronegative Lyme arthritis was diagnosed based on the classic clinical manifestations and DNA-detected *Borrelia garinii* in blood and synovial fluid of the patient, after all other possible causes of the disease had been ruled out. The disease was resistant to the first treatment with antibacterial agents. Six months after the therapy, arthritis still persisted and DNA of *Borrelia garinii* was repeatedly detected in the synovial fluid and the tissue of the patient. At the same time, antigens or parts of spirochaetes were detected by electron microscopy in the synovial fluid, the tissue and the blood of the patient. The patient was then repeatedly treated by antibiotics and synovectomy has been performed.

Keywords: *Borrelia garinii*; Lyme arthritis; Therapeutic failure

Introduction

Lyme borreliosis (LB) is a multisystemic disease caused by a Gram-negative spirochaete, *Borrelia burgdorferi* sensu lato (*B. burgdorferi*). The infection is usually transmitted through an infected tick bite [1]. The first common sign of the disease, so-called erythema migrans, is localised on the skin. After several days or even weeks, dissemination of the infection to various organs is observed. Several months or years later, the organs in which the infection had been previously localised, show a chronic involvement. The nervous system and joints are affected most often. Rarely, the involvement of other organs has also been reported [2].

The development of species-specific DNA amplification methods has made it possible to differentiate eight genotypes of *B. burgdorferi* sensu lato. It has been suggested so far that LB can be caused by *B. burgdorferi* sensu stricto, *Borrelia afzelii*, *Borrelia garinii* and *Borrelia valaisiana*. In other species, no association, with LB has been proven [3–5]. It is not clear whether individual species have a special affinity for different organs [6,7]. It is commonly stated that *Borrelia afzelii* is responsible for cutaneous manifestations, *Borrelia garinii* is often associated with neurological affections, and *B. burgdorferi* sensu stricto affects joints [8]. The results of the majority of European works studying the representation of individual species in LB patients with joint involvement suggest that *B. burgdorferi* sensu stricto prevails in these patients [9–11].

In the initial phase LA is associated with migrating arthralgia and/or arthritis. In the course of the disease, an intermittent monoarthritis or oligoarthritis may develop and progress into a chronic arthritis. The classic clinical picture of LA is represented by monoarthritis of the knee, which occurs in more than 85% of patients. The diagnosis of LA is made based on classic-clinical manifestations, endemic area exposure and on laboratory findings of elevated anti-*Borrelia* IgG antibodies in the patient's serum [12]. The prognosis of patients with LA is usually good, although in 10%–20% arthritis is resistant to treatment with antibiotics [13–15].

We present a case of a female patient diagnosed with a seronegative LA. The diagnosis was based on a positive epidemiological history, typical clinical manifestations and repeated evidence of *Borrelia garinii* DNA in the blood and the synovial fluid of the patient. The first treatment with antibiotics proved unsuccessful. The

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failure of the treatment could be established with regard to the clinical state of the patient, as well as the presence of *Borrelia garinii* in the synovial fluid and synovial tissue of the patient 6 months after therapy. At the same time spirochaetes were found in the patient's synovial fluid, synovial tissue and blood.

Case Report

A 27-year-old agricultural engineer presented with recurrent attacks of oligoarthritis limited in time, with a dominant affection of the knee joints, and was admitted to the Institute of Rheumatology, Prague, to establish the diagnosis.

Her past history indicated that she worked in the country. Contact with ticks cannot be ruled out, as the patient had been repeatedly exposed but was not aware either of a tick bite or of erythema migrans. She reported that she repeatedly suffered from gynecological and urological infections which were treated with antimicrobial agents. No medical documentation from this period is available and information about causative agents from that time is also missing. No other serious conditions were reported.

The present disease began in 1992 and presented with painful swellings of the right wrist and both knee joints. The patient was treated with amoxycillin and non-steroidal anti-inflammatory drugs on a short-term basis and her condition gradually improved. In 1993, arthritis of the knee joints recurred and lasted 2 months. Since then, until the year 2000, bilateral gonitis recurred several times; in between the attacks the patients had no complaints. The patient had been treated by her rheumatologist and orthopaedist with non-steroidal anti-inflammatory drugs, and repeatedly with intra-articular corticosteroids. Sulfasalazine was also administered for 9 months. However, the condition did not react to the treatment. The medical documentation from that period is not available and we presume that the therapy with sulfasalazine was introduced as a therapeutic trial which was ineffective.

In July 2000 the patient was admitted to our clinic. At that time she was experiencing a disturbing pressure in her knee joints, without any other complaints. physical examination showed an effusion in the knee joints, as well as mild warmth and painful sensations on movement to border positions. Both popliteal areas showed a swelling indicative of Baker's cysts. Other findings were normal. Laboratory data showed normal values of acute-phase reactants, and the blood picture and clinical chemistry were normal. Serum examination detecting antibody response to arthritogenic agents, anti *Yersinia* anti-*Salmonella* and anti-*Chlamydia trachomatis* antibodies was negative and no *Chlamydia trachomatis* on other pathogens were found in urine or cervical smears. Immunological tests were normal, apart from one finding of a slightly increased titre of antinuclear antibodies, which was not confirmed when repeated. HLA-B27 antigen was not detected. HLA-DR4 and HLA-DR2, which are assumed to be associated with resistance to treatment in LA, were not detected [13]. The patient has HLA-DR 01 and HLA-DR 08 alleles.

ELISA did not show an elevation in serum levels of antibodies to *B. burgdorferi*. Immunoblot analysis only confirmed a borderline reactivity against *B. garinii* (Western blot IgM and IgG anti-*B. garinii*, Biowestern dg.and Immunoblot *B. garinii* IgG, Euroimmun, Ltd) in the IgG class (P83 $\pm$, P60+, P56+, Osp C $\pm$, P14 $\pm$).

Cytology of the synovial fluid revealed signs of mild inflammation (leukocytes 1.3 $\times$ 10^9/l, erythrocytes 0.1 $\times$ 10^9/l, leukocyte count showed 2% neutrophil segments, 60% lymphocytes and 38% monocytes). Cultivation of the synovial fluid for the detection of a specific and non-specific infection proved negative. The examination of anti-*Borrelia* antibodies using an immunoblot analysis (Biowestern) was negative in the right knee joint and borderline IgG reactivity was detected in the left knee joint (83 $\pm$, Osp A $\pm$). With regard to the clinical manifestations indicative of LA, we proposed a polymerase chain reaction (PCR) examination of the DNA of *B. burgdorferi* sensu lato with species-specific primers to amplify the Osp As of strains [16,17] in the patient's synovial fluid and blood. This examination proved positive both in the synovial fluid and the blood, and indicated the presence of DNA of *Borrelia garinii* Osp A-type 5. PCR fragments were purified and directly sequenced by the dideoxychain termination procedure using CEQ 2000 Dye terminator Cycle (Hulínská, unpublished).

X-rays of the knees, hands, feet and sacroiliac joints were normal. Ultrasound examination of the knee joints showed hypertrophy of synovial tissue, synovial fluid excess and bilateral Baker's cyst. The findings were more pronounced in the left knee. Findings from MRI examination of the knee joints correlated with the ultrasound examination. Electrocardiography and echocardiography findings were normal.

With regard to the above-mentioned findings, the diagnosis of LA was made and intravenous ceftriaxon administered for 14 days in a daily dose of 2 g. After the treatment, a gradual improvement of the condition in the right knee joint was observed, but the left-sided gonitis persisted.

In January 2001 the patient was again hospitalised. Arthritis of the left knee persisted and the condition was confirmed by ultrasound examination. *Borrelia garinii* Osp A-type 5 was again detected in the synovial fluid.

Also, in the blood and synovial fluid antigens and altered parts of spirochaetes were detected using monoclonal antibody in immunosorbent method (ISEM) and negative staining on electron microscopy (Figs 1 and 2). Treatment with intravenous ceftriaxon was initiated, followed by oral administration of doxycyclin in standard dosage. Synovectomy was indicated for the left knee joint. Histology of the synovial tissue showed a non-specific synovitis. In the synovial tissue sections, spirochaetes as partly helical or convoluted forms were found, using electron microscopy (Figs 3 and 4). PCR examination detected DNA of *Borrelia garinii* in the

Fig. 1. Immunocytochemistry of human blood sample with monoclonal antibody anti-Osp A *Borrelia garinii* – anti-mouse, labelled with gold (Janssen GoAM/IgGAu). Expression of antigens on surface of alterated spirochaetes. Particle size 10 nm. (Negative staining with 1% phosphotungstic acid, magnification 82 000 × .)

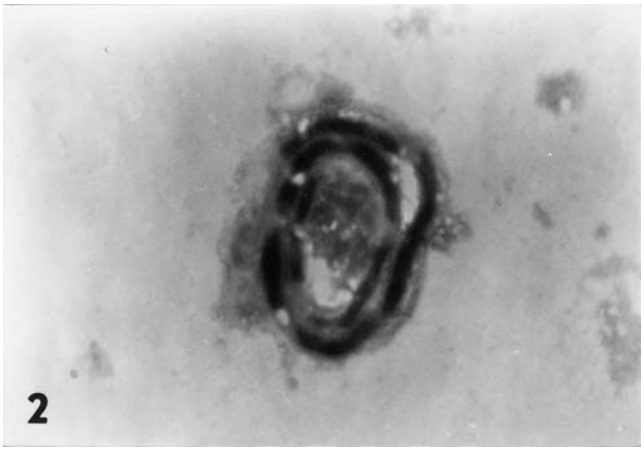

Fig. 2. Altered spirochaete coiled inside granular material, which could be debris of a human cell or rest of cyst in synovial fluid sample. (1% phosphotungstic acid, magnification 25 200 × .)

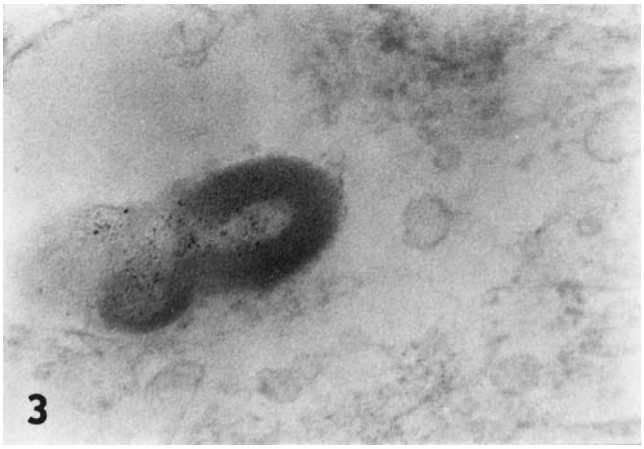

Fig. 3. Electron microscopy of synovial tissue samples stained with uranyl acetate and lead citrate (UaLc). Tissues were fixed in 6% glutaraldehyde; after fixation the tissues were treated with 1% OsO4. Cross-section of convoluted spirochaete (Magnification 58 000 × .)

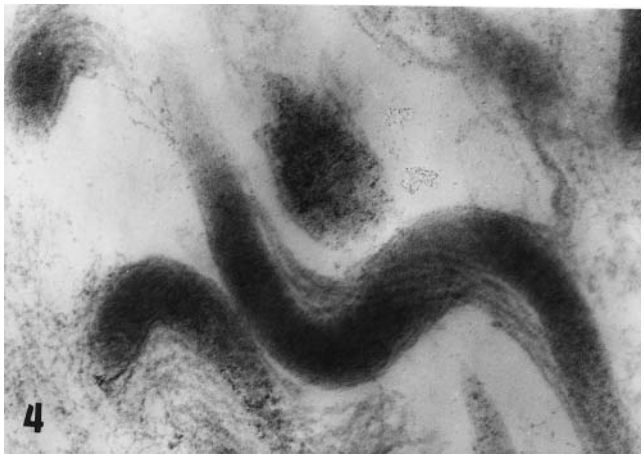

Fig. 4. Synovial tissue. Tangential section of apical site of synovial membrane shows central and apical parts of spirochaetes. Alteration of synovial cell and partial lysis of reticular fibrils but no borrelial cells are seen. (UaLc, magnification 72 000 × .)

synovial tissue with different set of primers instead of the target Osp A gene (Hulínská, unpublished).

Six months after the second treatment, the patient's condition showed no signs of clinical or laboratory recurrence of the disease.

Discussion

We present the case of a female patient with LA that showed certain interesting features. The first surprising finding was a long-term absence of antibody reactivity against *B. burgdorferi* sensu lato antigens in the patient's serum and synovial fluid that for a long time made it difficult to diagnose the condition. It is anticipated that antibody response to the *Borrelia* antigens plays an important role in the pathogenesis of LA [12]. However, a number of studies suggest that in patients insufficiently treated with antibacterial agents a seronegative LA develops. This therapy may suppress the immune response of the affected body, and the infection itself may persist [18]. This may explain the seronegativity of our patient, who had been repeatedly treated with antibiotics for short periods of time owing to the urogenital infections. Because of the patient's exposure and the clinical finding of arthritis of the knee joints with effusions causing popliteal cysts, the diagnosis of LA could not have been omitted in differentials, leading to the use of a direct method capable of detecing the *Borrelia* infection. The results of the examination were positive in both the synovial fluid and the blood of the patient.

It is also interesting to note that arthritis in this patient has been associated with *Borrelia garinii*, as there has been only rare evidence of this causality in connection with arthritis in Europe [7,16].

The first treatment with antibiotics proved unsuccessful. Insufficient response to the therapy may have a

number of reasons, one of which may be a repeated intra-articular application of corticosteroids into the knees in the course of the treatment. Another possibility may be the choice of antibiotics. The treatment with ceftriaxon, which possesses mostly extracellular activity, may have led to a temporary eradication of the infection while intracellular infection persisted. Experimental studies suggest that *B. burgdorferi* sensu lato may penetrate into cells, including synovial cells. This mechanism protects *Borrelia* against the effect of prevalently extracellular antibiotics [19,20]. Also, a number of other factors may be responsible for the failure of the antibiotic treatment. Experimental studies in vitro document the potential of *B. burgdorferi* sensu lato to transform itself into a metabolically inactive cystic form under unfavourable conditions. Antibiotic therapy can represent such an unfavorable condition, and it is anticipated that under the influence of antibiotics *B. burgdorferi* sensu lato is able to transform into a cystic forms. However, active bacterial metabolism is a prerequisite for an effective antimicrobial treatment. The above-mentioned mechanism is probably used by some spirochaetes to avoid the effect of antibiotics. These metabolically inactive forms are capable of transforming into a metabolically active form under certain favourable conditions, and this may be responsible for treatment-resistant conditions in some patients with LB [21]. Animal studies also document the possibility of survival of *Borrelia* infection after antimicrobial treatment. In experimentally infected animals the antibiotic therapy suppresses clinical manifestations; nevertheless, the DNA of *B. burgdorferi* sensu lato can be isolated from the tissues of some animals several months after treatment [19,22,23]. Also, in human there is evidence that in a number of patients with chronic arthritis the DNA of *B. burgdorferi* sensu lato can be detected in joints despite repeated antibiotic therapy [24,16]. However, this does not necessarily mean that *Borrelia* is still alive. The vitality of the strain can only be detected by cultivation, but this method has an extremely low sensitivity. It is also very difficult to detect an intra-articular infection caused by *B. burgdorferi* by means of electron microscopy [25,26,16]. There exist only few reports documenting success in detecting the presence of spirochaetes in the synovial fluid of a patient using electron microscopy [26,27].

The presented case documents the possible persistence of *B. burgdorferi* sensu lato (*Borrelia garinii* in this case) in joints after ceftriaxon treatment.

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hemoglobin levels, in our view, is the clinical significance of any toxicity. The abnormalities were reversible. In the patients for whom 4-week values were available, median creatinine and hemoglobin values were the same for patients in both trial arms. Of the 5 patients in the high-dose arm who discontinued study drugs early, 4 were alive and well and receiving antiretroviral therapy at 6 months, and 1 died of culture-proven tuberculosis.

For settings in developed countries, the current Infectious Diseases Society of America guidelines [3] for treatment of HIV-associated cryptococcal meningitis suggest a dose range for AmB of 0.7–1 mg/kg per day combined with flucytosine. The updated guidelines will retain this dose range (J. R. Perfect, personal communication). Our study [2] provides the first comparative data for making a choice of dose within this range. At the higher dose, clinicians will know that they can achieve more-rapid clearance of infection. In addition, complementary data on the toxicity of AmB at 1 mg/kg per day for a larger number of patients will be available from a trial in Vietnam in which all patients received the higher dose [4].

In the many settings in which flucytosine is not yet generally available—and resource limitations may make a full 2 weeks of induction treatment difficult—toxicity issues may be reduced, and the importance of more-rapid initial clearance, in the absence of flucytosine, is increased. In such settings, our study [2] and an earlier study [5] provide evidence to support the use of the 1 mg/kg dose of AmB. Thus, South African guidelines advocate AmB at 1 mg/kg per day for 7–14 days [6].

Routine, frequent monitoring and saline fluid loading during administration of AmB, at any dose, are essential. In Kampala, Uganda, with use of AmB at 0.7 mg/kg per day in 2 observational study cohorts in 2001 and 2006, 2-week mortality was reduced from 42% in 2001 to 20% in 2006, a reduction that may have been associated, at least in part, with more-frequent monitoring (3 times weekly vs. once weekly) and routine fluid loading in the later cohort [7]. Indeed, in settings in which frequent routine monitoring and transfusion, if occasionally needed, are not available, it is possible that an optimized oral treatment regimen could give results comparable to the results of treatment with AmB. In response to specific questions, none of our patients were excluded on the basis of previous adverse reactions to AmB, saline preloading was given for all doses, and AmB infusions were over 4 h.

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Serologic Tests for Lyme Disease: More Smoke and Mirrors

To the Editor—The article by Steere et al. describing serologic testing for Lyme disease contains the following conclusion: "the sensitivity of 2-tier testing in patients with later manifestations of Lyme disease was 100%, and the specificity was 99%" [1, p. 192]. This conclusion is both disingenuous and misleading.

Steere et al. [1] classified 44 patients as having disseminated (stage 2) or persistent (stage 3) infection due to *Borrelia burgdorferi*, the spirochetal agent of Lyme disease. The mandatory inclusion criteria for these categories were neurologic, cardiac, or joint involvement and a serologic result positive for *B. burgdorferi* by ELISA and Western blot [2]. Thus, by definition, all patients with disseminated or persistent Lyme disease were required to have a positive serologic test result. It is disingenuous to define a condition by a positive test result and then state that the test has 100% sensitivity. The true sensitivity of the 2-tier test system has been estimated to be 44%–56% when standard commercial Lyme testing was evaluated in clinical practice [3–5]. In fact, on the basis of a recent molecular diagnostic study, the sensitivity of this testing approach may be as low as 7.5% [6]. Thus, the sensitivity data presented by Steere et al. [1] is not realistic.

In the study by Steere et al. [1], 14 pa-

tients were classified as having "post–Lyme disease symptoms," with persistent symptomatic manifestations after receiving "recommended antibiotic therapy" for Lyme disease. Among these patients, 36% had serologic evidence of persistent infection due to *B. burgdorferi,* as defined by the Centers for Disease Control and Prevention criteria of positive results of ELISA and IgG Western blot [2]. Recent studies have revealed that "post–Lyme disease symptoms" may represent failure of short-course antibiotic therapy and persistent infection due to the Lyme spirochete, and this chronic illness may respond to a longer duration of antibiotic treatment [7–11]. Thus, the test results in patients with "post–Lyme disease symptoms" may reflect the true sensitivity of 2-tier testing for persistent Lyme disease, and the 36% sensitivity reported by Steere et al. [1] is consistent with the poor results of previous studies [3–5]. The VlsE C6 peptide ELISA was not significantly better, with a test sensitivity of only 43% for patients with persistent Lyme disease symptoms.

In summary, the sensitivity data presented by Steere et al. [1] reflect both circular reasoning in the context of disseminated infection and poor results in the context of persistent Lyme disease. Better tests are needed for diagnosis of this elusive tick-borne illness.

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Reply to Stricker and Johnson

To the Editor—Stricker and Johnson [1] maintain that the frequency of seropositivity among patients with disseminated or persistent Lyme disease is lower than the frequency reported in our prospective study of serologic testing for this infection. As stated in our article [2], it is problematic to determine the frequency of seroreactivity among patients with neurologic, cardiac, or joint manifestations of Lyme disease, because serologic confirmation is a part of the case definition [3]. However, it has not been possible to confirm *Borrelia burgdorferi* infection by other methods, such as culture or PCR, in all patients with the aforementioned manifestations of Lyme disease. Nevertheless, *B. burgdorferi* DNA can be detected by PCR of joint fluid specimens obtained before antibiotic therapy for the majority of patients with Lyme arthritis [4, 5], and it has been detected by culture or PCR in some patients with neuroborreliosis [6, 7]. In our experience, all such patients have had samples that were seropositive for *B. burgdorferi.* Moreover, in animal models of Lyme disease, spirochetes have been seen in and cultured from CNS, heart, or joint lesion specimens, and animals with spirochetes were seropositive for *B. burgdorferi* [8, 9]. Therefore, on the basis of current knowledge, all patients with objective neurologic, cardiac, or joint abnormalities of Lyme disease have serologic responses to *B. burgdorferi.*

Serologic testing for Lyme disease is insensitive during the first several weeks of infection in patients with the initial skin lesion erythema migrans, but the frequency of seropositivity is low during this period only [10, 11]. In our study, 29% of patients with erythema migrans had acute-phase samples with positive IgM or IgG antibody responses to *B. burgdorferi,* and 64% had convalescent-phase samples with positive responses 3–4 weeks later [2]. After that time, the sensitivity of 2-tier testing (ELISA and Western blot) for patients with disseminated or persistent Lyme disease was 100%, and the specificity was 99% [2]. Others have reported similar results [11], and similar results were found with a newer serologic test, the VlsE C6 peptide ELISA [2, 11, 12].

In our study, 36 (47%) of the 76 patients with erythema migrans had blood samples obtained during the acute phase of the illness that were positive for *B. burgdorferi* by PCR [2]. Other researchers have found similar results of blood cultures for patients with erythema migrans [13]. After this early period, results of culture and PCR testing of blood samples for *B. burgdorferi* DNA are almost always negative.

Finally, 10 (71%, not 36%) of 14 patients with post–Lyme disease symptoms

Listeriosis

Clinical description

Infection caused by *Listeria monocytogenes*, which may produce any of several clinical syndromes, including stillbirths, listeriosis of the newborn, meningitis, bacteremia, or localized infections

Laboratory criteria for diagnosis

- Isolation of *L. monocytogenes* from a normally sterile site

Case classification

Confirmed: a clinically compatible case that is laboratory confirmed

Lyme Disease

Clinical description

A systemic, tick-borne disease with protean manifestations, including dermatologic, rheumatologic, neurologic, and cardiac abnormalities. The best clinical marker for the disease is the initial skin lesion, erythema migrans, that occurs among 60%-80% of patients.

Clinical case definition

- Erythema migrans, or
- At least one late manifestation, as defined below, and laboratory confirmation of infection

Laboratory criteria for diagnosis

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

Unlikely

Case classification

Confirmed: a case that meets one of the clinical case definitions above

Comment

This surveillance case definition was developed for national reporting of Lyme disease; it is NOT appropriate for clinical diagnosis.

Definition of terms used in the clinical description and case definition:

2 Wald DS, Bestwick JP, Wald NJ. Child-parent screening for familial hypercholesterolaemia: screening strategy based on a meta-analysis. *BMJ* 2007;335:599. (22 September.)
3 Is cascade testing a sensible method of population screening? *J Med Screen* 2004;11:57-8.
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5 Hopcroft KA. Child-parent screening may have adverse psychological effects. *BMJ* 2007:335:683. (6 October.)

LYME WARS

Let's tackle the testing

The two tier testing system endorsed by the Centers for Disease Control and Prevention (CDC) has a high specificity (99%) and yields few false positives. But the tests have a uniformly miserable sensitivity (56%)—they miss 88 of every 200 patients with Lyme disease (table). By comparison, AIDS tests have a sensitivity of 99.5%—they miss only one of every 200 AIDS cases. In simple terms, the chance of a patient with Lyme disease being diagnosed using the commercial tests approved by the Food and Drug Administration and sanctioned by the CDC is about getting heads or tails when tossing a coin, and the poor test performance assures that many patients with Lyme disease will go undiagnosed.

Sensitivity and specificity of commercial two tier testing for Lyme disease

Study	Sensitivity	Specificity
Schmitz et al. *Eur J Clin Microbiol Infect Dis* 1993;12:419-24	66%	100%
Engstrom et al. *J Clin Microbiol* 1995;33:419-27	55%	96%
Ledue et al. *J Clin Microbiol* 1996;34:2343-50	50%	100%
Trevejo et al. *J Infect Dis* 1999;179:931-8	29%	100%
Nowakowski et al. *Clin Infect Dis* 2001;33:2023-7	66%	99%
Bacon et al. *J Infect Dis* 2003;187:1187-99	68%	99%
Mean of all studies	56%	99%

Until we scrap the worthless commercial tests for Lyme disease and find a better way to make the diagnosis of this protean illness, the "Lyme wars" will continue unabated.[1]

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Competing interests: RBS serves on the advisory panel for QMedRx.

1 Tonks A. Lyme wars. *BMJ* 2007;335:910-2. (3 November.)

RESUSCITATION DECISIONS

Yes, but who is in charge?

I definitely support nurses' involvement in resuscitation decisions.[1] I have worked on teams where nursing staff were always involved before a "not for resuscitation" decision was made, and if they did not agree the patient would remain "for resuscitation." I have also worked for consultants who will not make patients not for resuscitation.

What will happen if the consultant responsible for a patient wishes them to remain for resuscitation but a senior nurse feels that they should not be for resuscitation? Other doctors may agree with the nurse's decision but in the end I would want clarification as to who has the final say.

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Competing interests: None declared.

1 White C. Cardiopulmonary resuscitation decisions should be extended to nurses. *BMJ* 2007;335:901. (3 November.)

RESISTANCE TO HIV DRUGS

Detainees are affected

The problem of discontinuous antiretroviral therapy in the prison healthcare system leading to increased viral resistance applies also to detainees held by the immigration authorities. Many such detainees are held for a long time after the initial decision to deport, pending the resolution of legal challenges.[1] Detainees are often moved between detention centres and immigration removal centres as their cases are considered, and their antiretroviral drugs often do not follow them. It may be weeks before the local genitourinary clinic is made aware that a transfer has taken place, which causes a large gap in treatment.

Consequently, when these patients are eventually deported, they have developed a resistance pattern that requires second or third line antiretrovirals, which may not be available in the countries to which they are deported, even if those countries have some provision of antiretrovirals. It is a serious failure of the healthcare systems provided by the Border and Immigration Agency that the treatment of HIV positive detainees is undermined.

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Competing interests: None declared.

1 Laurent C. Prisoners are developing resistance to HIV drugs because their care is fractured. *BMJ* 2007;335:583. (22 September.)

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ALCOHOL CONFUSION

What is a unit?

A recent *BMJ* editorial[1] discussed the World Cancer Research Fund (WCRF) report on cancer[2] and commented on the report's recommendation that men should drink no more than two units of alcohol a day and women no more than one unit a day. These recommendations are much lower than current government advice in Britain.[2] This highlights a widespread confusion regarding units of alcohol and "standard" drinks—WCRF "drinks" contain 10-15 g of ethanol and British units contain 8 g.

Although a unit is often taken as one drink (half a pint of beer or one glass of wine), this is not the case. One pint of beer (4.2%) contains 2.4 units and a 175 ml glass of wine (12%) contains 2.1 units. The Department of Health leaflet, *How Much is Too Much?*, promises information on the number of units in alcoholic drinks. It advises using smaller glasses, stating that a 125 ml glass of wine contains one unit.[3] This would be true if the alcohol content was 8%, but at a more typical 12% it contains 1.5 units.

Furthermore, the standard drink varies across the world. The WCRF report is an international publication, which may explain the wide range of ethanol contents per drink (10-15 g) in the recommendation.

In 1991, Miller et al issued "a plea for consistency" regarding alcohol content.[4] Given the ambiguity present in the WCRF report, and the confusion evident even in a *BMJ* editorial, it is surely time to heed that plea.

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Competing interests: None declared.

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4 Miller WR, Heather N, Hall W. Calculating standard drink units: international comparisons. *Br J Addiction* 1991;86:43-7.

The Western Immunoblot for Lyme Disease: Determination of Sensitivity, Specificity, and Interpretive Criteria with Use of Commercially Available Performance Panels

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Recent recommendations for the serological diagnosis of Lyme disease include statements on quality assurance and the use of performance panels to assess laboratory competency. We used two performance panels—one from the Centers for Disease Control and Prevention (CDC) and one from Boston Biomedica Inc. (West Bridgewater, MA)—to evaluate the sensitivity and specificity of four western blot kits. We used the same panels to compare the interpretive criteria for western blots as proposed by participants in the Centers for Disease Control and Prevention, Association of State and Territorial Public Health Laboratory Directors Conference and those proposed by BBI Clinical Laboratories (BBICL; New Britain, CT). Our results indicated that the BBICL western blots were more sensitive than those of the CDC, MarDx (Carlsbad, CA), or Cambridge Biotech (Rockville, MD). However, use of the CDC criteria with the BBICL western blots increased specificity to 100% but reduced sensitivity to 74.3%. A sample table is provided as an example of the test results obtained with the BBI performance panel. Obviously, this work should be confirmed by other investigators.

In response to numerous reports on problems associated with Lyme disease testing [1–3], participants in the recent Centers for Disease Control and Prevention (CDC), Association of State and Territorial Public Health Laboratory Directors (ASTPHLD) Conference on the Serological Diagnosis of Lyme Disease [4] made several recommendations including:

(1) Lyme disease testing should be performed only in laboratories that have comprehensive quality assurance programs.

(2) Serum samples used to evaluate screening tests or western blots in proficiency testing should cover all stages of Lyme disease, and samples should be representative of the target population. Each sample should be from a single donor.

(3) A repository of serum specimens from patients with well-characterized *Borrelia burgdorferi* infections (early and late), other spirochetal infections, other infections and inflammatory disorders that have shown cross-reactivity in Lyme disease testing, and normal serum samples from areas of nonendemicity should be maintained by the CDC. Industry should provide resources to develop appropriate serum panels. These panels should be made available to research and development laboratories and to testing laboratories for validation studies. At least two such panels are currently available: one, which comprises a 45–47-member panel, is available from the CDC, and the other, which comprises a 15-member mixed titer panel, is available from Boston Biomedica (West Bridgewater, MA).

Materials and Methods

The CDC performance panel was used to evaluate the sensitivity and specificity of three western blot products (BBI Clinical Laboratories [BBICL; New Britain, CT], MarDx [Carlsbad, CA], and Cambridge Biotech [Rockville, MD]). In a separate evaluation, the CDC panel was also used to compare the BBICL western blot and the CDC western blot. The Boston Biomedica Lyme Disease Mixed Titer Performance Panel can also be used to validate new Lyme disease antibody tests and to compare the sensitivity and specificity of a newly adopted antibody test.

Each of the serum samples in the CDC panel has limited clinical classification, including presence/absence of erythema migrans (EM), culture results, and whether the patient was IgG/IgM reactive or seronegative. The western blot and ELISA results on this panel are not available to the purchaser until the testing has been performed and sent to the CDC for analysis; only then are the reference results released. Hence, use of the panel is blinded. While clinical characterization is provided, there are no data available on when the specimens were collected in reference to the appearance of EM or a culture positive for *B. burgdorferi.*

We compared three western blot kits for the detection of IgM and IgG antibodies to *B. burgdorferi.* They included the BBICL western blot kit, made by Biotech Research Laboratories (BBI), the MarDx kit, and the Cambridge Biotech kit.

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Table 1. Comparison of three western blot kits for detection of IgM antibody to *Borrelia burgdorferi* with use of the performance panel of the Centers for Disease Control and Prevention (CDC).

	Western blot product		
Result	BBICL	MarDx	Cambridge Biotech
Sensitivity (%)	90.0	78.9	64.3
Specificity (%)	86.4	100	68.2

NOTE. BBICL = BBI Clinical Laboratories (New Britain, CT).

Each assay was performed by using the procedure described in the manufacturer's product literature.
The BBICL criteria are:

IgM-significant bands—23, 39, 41, 83 kD*
Reactive—two of the following four bands (23, 39, 41, 83 kD) must be present.
Equivocal—one of the following bands (23, 31, 34, 37, 39, 41, 83 kD) must be present.
Nonreactive—no Lyme-specific bands are present.
IgG-significant bands—20, 23, 31, 34, 35, 39, 83 kD
Reactive—three of the following bands (20, 23, 31, 34, 35, 39, 83 kD) must be present.
Equivocal—one or two of the following bands (20, 23, 31, 34, 35, 39, 83 kD) must be present.
Nonreactive—no Lyme-specific bands are present.

The CDC/ASTPHLD criteria are:

IgM-significant bands
Reactive—two of the following three bands (23, 39, 41 kD) must be present.
Nonreactive—fewer than two bands are present.
IgG-significant bands
Reactive—five of the following bands (18, 21, 28, 30, 41, 45, 58, 66, 93 kD) must be present.
Nonreactive—fewer than five bands are present.

* The 83 and 93 kD bands are equivalent.

Interpretive criteria for the BBICL western blot included both the BBICL criteria and the CDC/ASTPHLD criteria [4]. The results obtained with use of the other two kits were interpreted on the basis of the CDC/ASTPHLD criteria. Each blot was read independently by two technologists and then validated by a director, all of whom were employees of BBICL. Both the technologists and the director were blinded as to the blot manufacturer, and the specimens were coded.

Results and Discussion

Table 1 shows the results of our comparison of the three western blot products for detecting IgM, based on the CDC/ASTPHLD performance panel criteria. The BBICL IgM western blot is more sensitive than either the MarDx or Cambridge Biotech IgM western blot and slightly less specific than the MarDx IgM western blot. The BBICL criteria for IgM western blot are virtually identical to the proposed CDC/ASTPHLD criteria, except for the inclusion of the 83/93-kD band and an indeterminate category. Thus, differences in performance of the kits are probably product related and not due to differences in interpretive criteria.

Table 2 shows the results of our comparison of the three western blot products for detecting IgG with use of the CDC performance panel. On the basis of the CDC/ASTPHLD criteria, the BBICL IgG western blot is more sensitive than and as specific as the other two IgG western blot products. However, if the BBICL criteria are applied, the sensitivity of the BBICL IgG western blot increases to 87%, but its specificity is reduced. The reduced sensitivities of all IgM western blot kits, particularly those of MarDx and Cambridge Biotech, could well reflect the time at which the specimens were drawn for the panel. If the patients donated $\geq$1 unit of plasma months after the initial acute episode of Lyme disease, then the IgM titer would be expected to be diminished.

The second study was also done in blinded fashion at BBICL. IgG and IgM western blots were performed on the 46-member CDC panel, and the results were sent to the CDC for analysis. A comparison of results obtained with BBICL or CDC western blots and clinical information are shown in figures 1 and 2. BBICL IgM western blots classified 28 of 30 patients with Lyme disease as IgM positive (figure 1). The diagnosis was missed in two patients (6%). The CDC reported apparently false-negative IgM western blots for 10 patients, all of whom were symptomatic. Of these 10 patients who were negative by the CDC IgM western blot and positive or equivocal by BBICL IgM western blot, the one patient who was positive by the BBICL IgM western blot had confirmed EM and was culture positive for *B. burgdorferi.* Of the nine patients who were negative by the CDC western blot and equivocal by the BBICL western blot, seven were positive for IgG antibodies to *B. burgdorferi* by western blot in both laboratories, and two were negative for IgG antibodies in both laboratories. All nine patients had confirmed EM and cultures positive for *B. burgdorferi.*

BBICL reported that 35 of 46 patients had either positive (19 patients) or equivocal (16 patients) IgG western blots (figure 2). Of these 35 patients with clinically defined Lyme disease, 23 were found to be seropositive with use of the CDC western blot. There were 12 false-negative results. One of the specimens negative for IgG by the CDC western blot but positive by the BBICL western blot was from a *B. burgdorferi*–infected patient whose IgM western blot was found to be positive in both laboratories. The 12 patients who were negative by the CDC/ASTPHLD criteria but had confirmed Lyme disease were equivocal by the BBICL criteria. Seven of these *B. burgdorferi*–infected patients had a positive IgM western blot in both laboratories.

Four patients had a history of tick bite, EM, and positive cultures. There were no clinical data for one patient. There were five patients who were positive by the CDC/ASTPHLD criteria and equivocal by the BBICL criteria. There were multi-

Table 2. Comparison of three western blot kits for the detection of IgG antibody to *Borrelia burgdorferi* with use of the performance panel of the Centers for Disease Control and Prevention (CDC), according to the criteria of the BBI Clinical Laboratories or the criteria of the CDC.

Result	BBICL (BBICL criteria)	BBICL (CDC criteria)	MarDx (CDC criteria)	Cambridge Biotech (CDC criteria)
Sensitivity (%)	87.2	74.3	47.0	43.6
Specificity (%)	60.0	100	100	100

ple bands on both western blots for all five of these patients, but there were not enough bands to fulfill the BBICL criteria for positivity. Thus, BBICL classified all (35) patients who met the CDC criteria for Lyme disease as either positive or equivocal, while the CDC western blot results indicated that 12 patients who met the CDC criteria for Lyme disease were negative. The case of one patient who was western blot–positive for IgM at the CDC and negative for IgM with use of the BBICL western blot is still unresolved. In addition, five specimens were equivocal with use of the BBICL IgG western blot but positive with use of the CDC IgG western blot.

The BBICL western blot appears to be more sensitive than the CDC western blot, the MarDx western blot, or the Cambridge Biotech western blot. Of course, the issue is whether a western blot should be more sensitive than specific, or vice versa. If the western blot is to be used solely for confirmation of the results of ELISA, then specificity may be more desirable than sensitivity. However, a specific western blot with low sensitivity may invalidate a sensitive and specific ELISA. A highly sensitive and specific western blot is desirable for a two-tiered test system. Of major significance is the fact that despite the CDC recommendation for two-tiered testing, many physicians who treat patients with Lyme disease do not believe that an ELISA is an appropriate screening test and consequently use the western blot as a primary test for Lyme disease or request that both ELISA and western blot be done.

If the western blot is to be used in this manner, then sensitivity may be preferred over specificity, particularly for patients with acute Lyme disease or for those with suspected persistent disease and symptoms that are not commonly observed. We recognize, however, that reduced specificity may further complicate the serodiagnosis of Lyme disease because of the potential for increased numbers of false-positive tests.

Workers at Boston Biomedica have assembled a set of 15 aliquots of frozen serum and plasma units with reactivity to *B. burgdorferi* ranging from negative to strongly positive when used with a variety of currently available test methodologies. Samples have been selected to demonstrate IgG and/or IgM reactivity. In addition, one negative plasma unit has been included as a nonreactive control. These specimens are undiluted aliquots from plasma and serum units collected from 1994 to 1995. The units were processed by sterile filtration. No preservatives were added. Clinical information on panel members was included when available. The purpose of this performance panel of naturally occurring serum and plasma samples is to enable manufacturers and diagnostic laboratories to evaluate their tests for detection of antibodies to *B. burgdorferi* with characterized samples and to provide comprehensive data for comparative analysis.

The tables provided in the Panel give results from both commercially available test kits and in-house procedures performed at BBICL, Boston Biomedica, and internationally recognized reference laboratories. Product numbers are indicated for identification of each method. Numeric results are the means of duplicate tests. Some results are expressed as signal-to-cutoff ratios to facilitate comparisons among kits; ratios of $\geqslant 1.0$ are

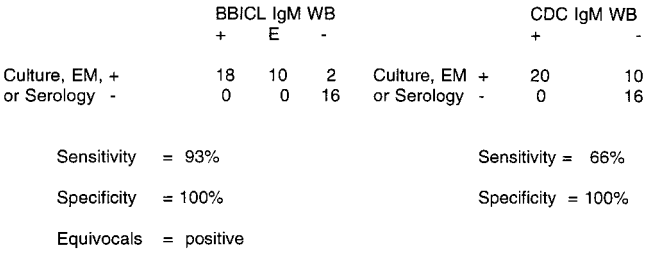

Figure 1. Comparison of the sensitivity and specificity of BBI Clinical Laboratory (New Britain, CT) and Centers for Disease Control and Prevention western blots for IgM antibody to *Borrelia burgdorferi* with that of clinical information (culture results, the presence of erythema migrans [EM], and serology) in the detection of *B. burgdorferi* infection.

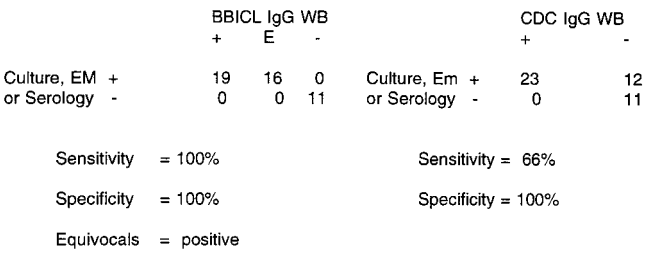

Figure 2. Comparison of the sensitivity and specificity of BBI Clinical Laboratory (New Britain, CT) and Centers for Disease Control and Prevention western blots for IgG antibody to *Borrelia burgdorferi* with that of clinical information (culture results, the presence of erythema migrans [EM], and serology) in the detection of *B. burgdorferi* infection.

MarDx IgM Western Blot
BBI

Panel ID #	-Significant IgM Bands- 23	39	41	Result*	-----Other IgM Bands---- 30	31	34	37	58	66
PTL202-01	23		41	POS						
PTL202-02	23		41	POS						
PTL202-03†		39	41	POS	30		34	37		
PTL202-04†				NEG						
PTL202-05	23		41	POS		31			58	66
PTL202-06				NEG						
PTL202-07	23		41	POS						
PTL202-08				NEG						
PTL202-09				NEG						
PTL202-10	23	39		POS			34			
PTL202-11				NEG						
PTL202-12				NEG						
PTL202-13				NEG						
PTL202-14	23		41	POS						
PTL202-15	23			NEG		31				
Run Date:	11/25/95									
Kit Lot #:	6098									
Exp. Date:	8/96									
Product #:	40-265M									

*MarDx IgM Western Blot Interpretation:
POS = Positive = 2 or more significant bands
NEG = Negative = Less than 2 significant bands

MarDx IgG Western Blot
BBI

Panel ID #	----------Significant IgG Bands---------- 18	23	28	30	39	41	45	58	66	93	Result**	-Other IgG Bands- 31	34	37	60
PTL202-01	18	23				41					NEG				
PTL202-02	18			30	39	41	45	58	66	93	POS				60
PTL202-03†	18			30	39		45	58		93	POS				60
PTL202-04†	18			30	39		45	58		93	POS				60
PTL202-05		23	28								NEG		34		60
PTL202-06	18	23	28	30	39		45	58		93	POS				60
PTL202-07		23				41	45				NEG				
PTL202-08											NEG	31			
PTL202-09											NEG				
PTL202-10	18	23		30	39	41		58		93	POS				60
PTL202-11	18	23	28	30	39	41	45	58	66	93	POS			37	60
PTL202-12	18	23	28	30	39		45	58		93	POS				60
PTL202-13	18	23	28	30	39	41		58		93	POS				60
PTL202-14	18	23				41					NEG				
PTL202-15	18	23		30	39	41		58	66	93	POS	31	34		60
Run Date:	11/24/95														
Kit Lot #:	6096														
Exp. Date:	8/96														
Product #:	40-2065G														

**MarDx IgG Western Blot Interpretation:
POS = Positive = 5 or more significant bands
NEG = Negative = Less than 5 significant bands

†Member #03 and 04 are from the same donor; member #04 was drawn approximately 5 months after member #03.

Figure 3. A representative sample of results for panel members of a BBI Performance Panel for a MarDx (Carlsbad, CA) western blot kit.

considered reactive. Results with use of indirect fluorescent antibody are endpoint dilutions.

There are no universally accepted criteria for western blot interpretation; therefore, the interpretation of the band pattern was based on the manufacturers' criteria for their kits and the in-house criteria (BBICL) for the in-house methods. Figure 3 shows a representative sample of the results provided with the panel, in this case western blot results for panel members of a MarDx western blot kit. This performance panel will be invaluable to both kit manufacturers and hospital laboratory personnel who wish to validate their diagnostic procedures for Lyme disease. While this Lyme disease panel is antibody based, PCR is becoming more widely used for the laboratory diagnosis of Lyme disease [5]. Molecular panels are now needed for the diagnosis of Lyme disease.

Conclusion

The results of our comparative testing of available western blot kits with use of a CDC performance panel indicated that BBICL western blots were more sensitive than those of competing manufacturers. However, application of the CDC/ASTPHLD interpretive criteria to the BBICL results increased specificity but reduced sensitivity. Sample data are also provided from a commercially available Lyme disease antibody performance panel. Use of such a panel should enable laboratory personnel to compare results with their currently used test kits to those obtained with a wide variety of kits and methods.

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Evaluation of Two Commercial Systems for Automated Processing, Reading, and Interpretation of Lyme Borreliosis Western Blots▽

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The diagnosis of Lyme borreliosis (LB) is commonly made by serologic testing with Western blot (WB) analysis serving as an important supplemental assay. Although specific, the interpretation of WBs for diagnosis of LB (i.e., Lyme WBs) is subjective, with considerable variability in results. In addition, the processing, reading, and interpretation of Lyme WBs are laborious and time-consuming procedures. With the need for rapid processing and more objective interpretation of Lyme WBs, we evaluated the performances of two automated interpretive systems, TrinBlot/BLOTrix (Trinity Biotech, Carlsbad, CA) and BeeBlot/ViraScan (Viramed Biotech AG, Munich, Germany), using 518 serum specimens submitted to our laboratory for Lyme WB analysis. The results of routine testing with visual interpretation were compared to those obtained by BLOTrix analysis of MarBlot immunoglobulin M (IgM) and IgG and by ViraScan analysis of ViraBlot and ViraStripe IgM and IgG assays. BLOTrix analysis demonstrated an agreement of 84.7% for IgM and 87.3% for IgG compared to visual reading and interpretation. ViraScan analysis of the ViraBlot assays demonstrated agreements of 85.7% for IgM and 94.2% for IgG, while ViraScan analysis of the ViraStripe IgM and IgG assays showed agreements of 87.1 and 93.1%, respectively. Testing by the automated systems yielded an average time savings of 64 min/run compared to processing, reading, and interpretation by our current procedure. Our findings demonstrated that automated processing and interpretive systems yield results comparable to those of visual interpretation, while reducing the subjectivity and time required for Lyme WB analysis.

Lyme disease is a multisystem, tick-borne disease caused by the spirochete *Borrelia burgdorferi*. In 2006, the Centers for Disease Control and Prevention (CDC) reported 19,931 cases of Lyme disease in the United States (7), confirming that the disease continues to represent a significant public health threat. The clinical manifestations of early localized disease range from nonspecific sequelae, including malaise, myalgia, and lymphadenopathy, to more characteristic findings, such as erythema migrans (EM). In the absence of appropriate therapy, disease progression may lead to significant complications, including rheumatologic, neurologic, or cardiac manifestations (15, 16).

The diagnosis of Lyme borreliosis (LB) can be made clinically when patients from regions where the disease is endemic present with EM (5, 8, 18). However, in patients without EM but with objective clinical findings suggestive of disseminated LB, serologic testing is an important diagnostic approach. Appropriate serologic testing should follow the two-tier algorithm recommended by the CDC (6), consisting of initial testing with a sensitive screening assay (e.g., enzyme immunoassay) with positive or equivocal specimens to be tested by Western blot (WB) analysis. Current CDC criteria for WB interpretation recommend that ≥2 bands on the immunoglobulin M (IgM) WB or ≥5 bands on the IgG WB be present for the immunoblot to be considered positive (6, 9). Although WB is considered to be highly specific, current testing protocols in most clinical laboratories rely on visual reading and interpretation of WB strips. These procedures require the laboratory technologist to visually compare band intensities on the patient strip to those of a weakly reactive control. This approach is labor-intensive, time-consuming, and subjective, allowing for potential intra- and interlaboratory variation in WB reading and interpretation. Previous studies analyzing the performance of LB serologic tests among testing laboratories have demonstrated significant variation in results, even for more objective methods, such as enzyme immunoassay (2, 3, 10). Therefore, given the inherent subjectivity in reading and interpreting WBs for diagnosis of LB (i.e., Lyme WBs), one would expect to observe significant variation in WB results, with potentially adverse effects on the laboratory diagnosis of Lyme disease and subsequent patient management decisions. Due to the need for more objective and consistent interpretation of Lyme WBs, we undertook a study to evaluate and compare two systems (TrinBlot/BLOTrix [Trinity BioTech, Carlsbad, CA] and BeeBlot/ViraScan [Viramed Biotech AG, Munich, Germany]) designed for automated processing, reading and interpretation of Lyme WBs. The goal of the present study was to determine whether automated systems yield comparable results to visual reading and interpretation while reducing the subjectivity and time required for Lyme WB analysis.

MATERIALS AND METHODS

Serum specimens. A total of 518 consecutive, unique serum specimens submitted to our reference laboratory for routine LB serologic testing between June and September 2007 were included in the study. The specimens were submitted without accompanying clinical information. The study protocol was reviewed and approved by the institutional review board of the Mayo Clinic.

In addition, two Lyme WB performance panels consisting of 55 clinically characterized and laboratory-characterized serum specimens were purchased from the CDC and Boston Biomedica, Inc. (BBI; West Bridgewater, MA).

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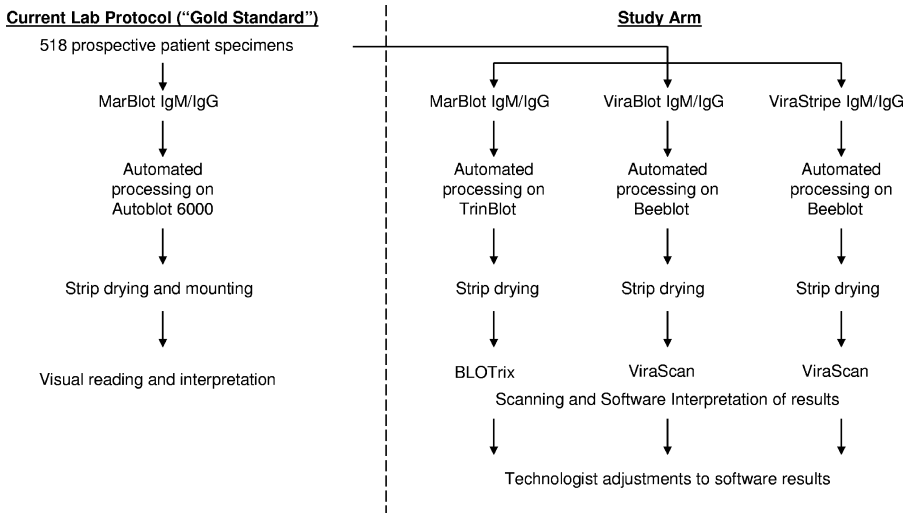

FIG. 1. Study design.

Study design. Each specimen was processed by our current procedure, performed according to the manufacturer's instructions for processing MarBlot IgG and IgM strips (MarDx Diagnostics, Carlsbad, CA) using the Autoblot 6000 instrument (MedTec, Inc., Hillsborough, NC). The strips were visually interpreted, and the results were recorded manually. Each specimen was also tested by MarBlot IgG and IgM strips using the automated TrinBlot processor (Bee Robotics, Caernarfon Gwynedd, United Kingdom) with subsequent scanning and analysis by the BLOTrix interpretive software. In addition, each specimen was tested by the ViraBlot and ViraStripe IgG and IgM strips (Viramed Biotech AG) on the automated BeeBlot processor (Bee Robotics) with subsequent analysis by the ViraScan interpretive software. A laboratory technologist blinded to the results of visual interpretation in the laboratory then reviewed the BLOTrix and ViraScan software interpretive results for each specimen (Fig. 1). The laboratory technologist, when reviewing the software interpretive results for each strip, (i) ensured that the software had analyzed only bands demonstrating uniform intensity across the entire width of the strip, (ii) checked that any background intensity (non-band intensity) had been accounted for, (iii) verified that the software had properly aligned test bands on the patient strip with bands on the serum band locator control strip, and (iv) ensured that the densitometric read was focused on the center of each test band.

WB assays. MarDx MarBlot IgM and IgG assays (Fig. 2) utilize antigens of *B. burgdorferi* strain B31 for Western blot analysis. The antigens are separated by electrophoresis through a sodium dodecyl sulfate-polyacrylamide gel. The resolved antigens are then transferred to a nitrocellulose membrane. Similarly, the ViraBlot and ViraStripe IgM and IgG assays use strain *B. burgdorferi* B31 as the source of antigen for Western blot analysis. ViraBlot IgM and IgG assays (Fig. 2) are manufactured by separation of antigens by gel electrophoresis, with subsequent transfer to nitrocellulose. In contrast, the ViraStripe IgM and IgG assays (Fig. 2) are generated by "printing" highly purified antigens at a defined location with standardized concentrations on a nitrocellulose membrane. The ViraStripe IgM and IgG assays are currently prototype Lyme WB strips, while the MarBlot and ViraBlot assays are both Food and Drug Administration cleared.

WB strip processing, reading, and interpretation. For each specimen tested by our current Lyme WB procedure, 80 μl of the serum band locator, 20 μl of the weakly reactive and negative controls, and 20 μl of patient serum were added to the appropriate channels of the Autoblot 6000 instrument. After being incubated and washed, strips were air dried and mounted for visual reading and interpretation. Each test band was visually compared to the 41-kDa band on the weakly reactive control strip and was considered present if the intensity was equal to or greater than that of the weakly reactive control. The IgM assay was considered positive if two of the following three bands were present: 23, 39, and 41 kDa. The IgG assay was considered positive if five of the following ten bands were present: 18, 23, 28, 30, 39, 41, 45, 58, 66, and 93 kDa.

Each specimen was also tested by the ViraBlot and ViraStripe IgM and IgG assays, performed according to the manufacturer's instructions on the BeeBlot automated processor. In brief, strips were added to the incubation tray and allowed to presoak in 1.5 ml of wash buffer for 5 min. Next, 20 μl of patient serum or 100 μl of control was added to the appropriate channel of the incubation tray. After being incubated and washed, strips were air dried in the incubation tray, scanned by using the ViraCam scanner (Viramed Biotech, AG), and analyzed by the ViraScan analysis software version 2.01. Based on densitometric analysis as described by Nishizuka et al. (12), 8-bit, grayscale images at a resolution of 220 dots per inch were stored in bitmap format for subsequent analysis by ViraScan. With the aid of predefined band-locator images, ViraScan locates and measures the immunospecific banding patterns on each patient strip. Each band is then analyzed for band location and maximum intensity. After a subtraction of background intensity (the average non-band intensity), the software assigns a numerical value to each band. The software then divides the numeric value of each test band by the numeric value assigned to a separate calibrator control band to determine a relative intensity. For the present study, a test band was considered present if the relative intensity met or exceeded the following manufacturer's recommended cutoff settings: ViraBlot IgG, 75%; ViraBlot IgM, 70%; ViraStripe IgG, 85%; and ViraStripe IgM, 60%.

Each specimen was also tested by the MarDx MarBlot IgM and IgG assays, performed according to the manufacturer's instructions using a TrinBlot automated processor. After automated processing, the strips were air dried in the incubation tray, scanned, and analyzed by the BLOTrix analysis software version 2.6. Similar to ViraScan, BLOTrix software utilizes densitometric analysis to compare the intensity of each test band to that of a separate calibrator control band and calculate a relative percent intensity. For the present study, a test band was considered present if the calculated relative intensity met or exceeded the following manufacturer's recommended cutoff settings: TrinBlot IgG, 90%; and TrinBlot IgM, 90%.

Statistics. Statistical analyses were performed by using statistical analysis software (SAS Institute Inc, Cary, NC). In addition to the percent agreement, kappa coefficients were determined as a secondary measure of agreement. Result agreements by kappa values are categorized as near perfect (0.81 to 1.0), substantial (0.61 to 0.80), moderate (0.41 to 0.60), fair (0.21 to 0.40), slight (0 to 0.20), or poor (<0) (11).

Workflow analysis. The average assay time for testing by our current procedure was calculated by timing three separate runs (40 specimens/run) from the addition of strips to the incubation tray through the manual recording of results by the technologist. The average assay time for testing by the automated systems was calculated by timing three separate runs (40 specimens/run) from the addition of strips to the incubation tray through the review of software results by the laboratory technologist.

RESULTS

Agreement between automated and visual interpretation. To assess agreement, the qualitative results (positive or negative based on CDC criteria) were compared after the testing of 518 consecutive serum specimens. Among the 518 specimens, 74 (14.3%) were positive for IgG, and 191 (36.9%) were positive

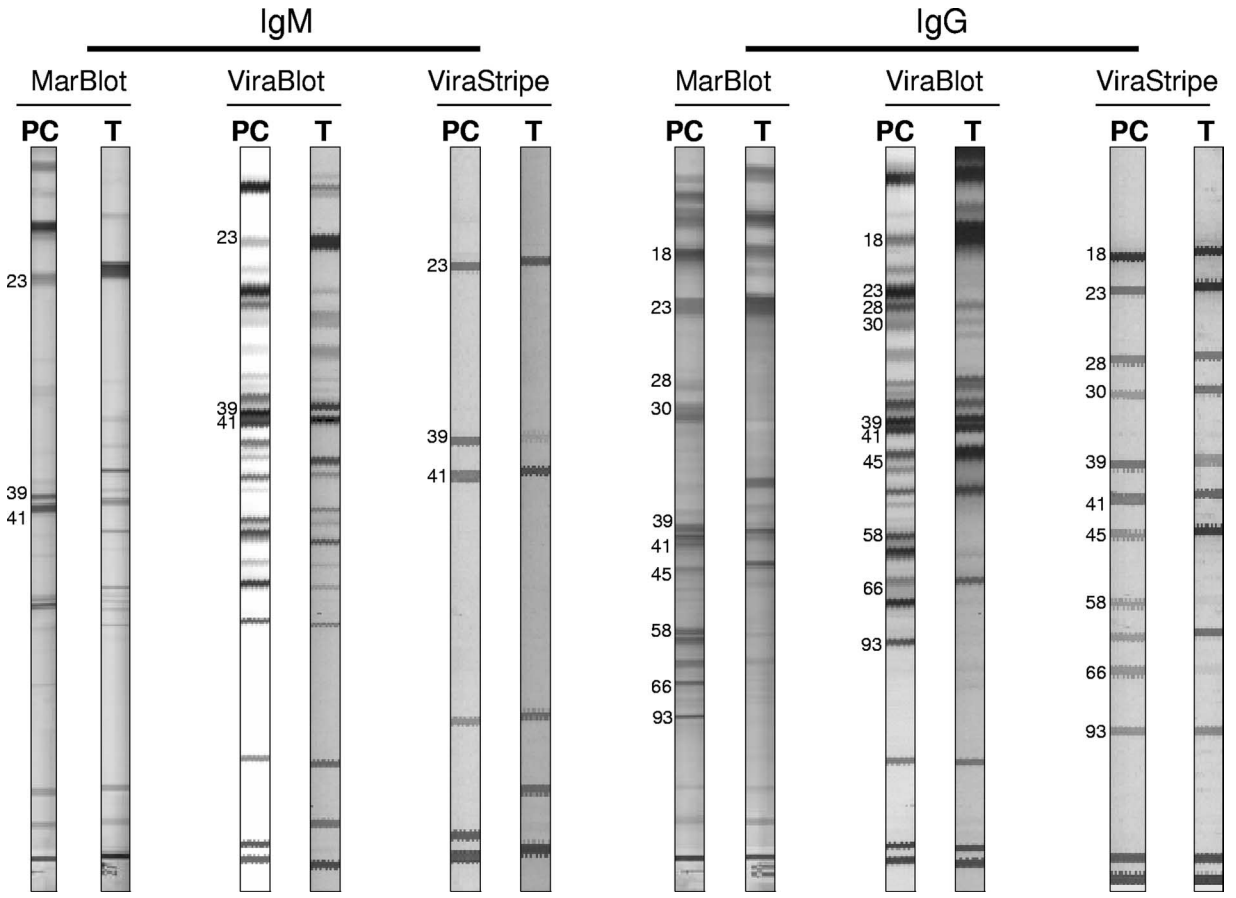

FIG. 2. Comparison of WB strips for a single patient specimen. The same patient specimen was tested by the MarBlot, ViraBlot, and ViraStripe assays. Strips were scanned by their respective systems, and the images were captured in tag image file format (TIFF). The migration positions of bands used in the CDC interpretation criteria are indicated by molecular mass (in kilodaltons). PC, positive control; T, test (patient) sample.

for IgM by our current procedure. BLOTrix analysis of the MarBlot assays demonstrated an agreement of 84.7% (439/518) for IgM and 87.3% (452/518) for IgG compared to results obtained by routine testing (Table 1). ViraScan analysis of the ViraBlot IgM and IgG assays showed 85.7% (444/518) and 94.2% (488/518) agreement, respectively, while analysis of the ViraStripe assays demonstrated 87.1% agreement (451/518) for IgM and 93.1% agreement (482/518) for IgG (Table 1). A technologist blinded to the results of routine testing then reviewed the BLOTrix and ViraScan interpretive results for each

TABLE 1. Agreement of Lyme WB strip results obtained by visual interpretation, automated software, and technologist-adjusted software interpretation among prospective serum specimens (n = 518)[a]

Assay[b]	Software alone		Technologist adjusted		K[d]
	% Agreement (95% CI)	K[c]	% Agreement (95% CI)	K[c]	
MarBlot					
IgM	84.7 (80.3–88.6)	0.69	87.1 (82.6–90.9)	0.73	0.86
IgG	87.3 (84.0–90.0)	0.57	92.3 (89.0–95.0)	0.72	0.77
ViraBlot					
IgM	85.7 (81.3–89.6)	0.70	85.9 (81.5–89.8)	0.71	0.97
IgG	94.2 (90.9–96.9)	0.75	94.8 (90.5–96.5)	0.75	0.90
ViraStripe					
IgM	87.1 (82.6–90.9)	0.72	86.7 (82.3–90.6)	0.71	0.97
IgG	93.1 (89.7–95.7)	0.72	91.7 (88.4–94.4)	0.69	0.89

[a] Percent agreement refers to percent agreement with visual interpretation of MarDx MarBlot strips.
[b] MarBlot results were analyzed by using BLOTrix software application; ViraBlot and ViraStripe results were analyzed by using the ViraScan software application.
[c] Kappa coefficient for comparison with visual interpretation.
[d] Kappa coefficient, software-alone interpretation versus technologist-adjusted software interpretation.

TABLE 2. Agreement of Lyme WB strip results obtained by visual interpretation and technologist-adjusted software interpretation among the BBI and CDC serum performance panels

Panel	% Agreement (95% CI)[a]			
	MarBlot*	MarBlot†	ViraBlot	ViraStripe
BBI				
IgM	73.3 (46.0–91.1)	73.3 (46.0–91.1)	93.3 (62.0–100.0)	80.0 (51.3–96.4)
IgG	86.7 (57.0–100.0)	86.7 (57.0–100.0)	86.7 (57.0–100.0)	80.0 (51.7–96.1)
CDC				
IgM	NA	80.0 (62.6–92.1)	67.5 (51.2–80.7)	82.5 (64.9–94.4)
IgG	NA	90.0 (71.8–100.0)	92.5 (74.1–100.0)	90.0 (71.8–100.0)

[a] The percent agreement with reference WB results provided by BBI or the CDC. *, tested by the current procedure with visual reading and interpretation; †, analyzed by using the BLOTrix software application. ViraBlot and ViraStripe results were analyzed by using the ViraScan software application. NA, not applicable (inadequate specimen volume to be tested by current lab procedure with visual interpretation).

specimen. The technologist-adjusted BLOTrix and ViraScan results showed substantial agreement ($0.61 < \kappa < 0.80$) with results obtained by routine testing with visual interpretation (Table 1).

In addition to the analysis of 518 consecutive serum specimens, two performance panels (BBI and CDC) consisting of 55 serum specimens were tested by the automated systems. BBI serum specimens were also tested by our current Lyme WB procedure. Agreement was assessed by comparing the results of testing to the reference WB results provided with the performance panels (Table 2). Interestingly, the technologist-adjusted ViraScan results of the ViraBlot and ViraStripe IgM assays showed closer agreement (93.3 and 80%, respectively) with the reference BBI WB results than did the results obtained by routine testing (73.3%) (Table 2).

Sensitivity and specificity of automated systems. After the testing of 518 prospective specimens, the results were analyzed by using the visual interpretation of the MarDx assays as the "gold standard." Although the automated systems demonstrated comparable performances overall, BLOTrix analysis showed higher sensitivities for IgM and IgG (93.3 and 81.1%, respectively) than did ViraScan analysis of the ViraBlot (86.1 and 74.3%, respectively) or ViraStripe (78.9 and 77.0%, respectively) strips (Table 3). In contrast, ViraScan analysis of the ViraStripe IgM and IgG strips showed the highest specificity (92.0 and 95.7%, respectively) compared to visual interpretation of the MarDx MarBlot assays (Table 3).

Adjusted sensitivities and specificities were then calculated after visual review of the automated software results by a laboratory technologist (Table 3). A total of 37/518 (7.1%) MarBlot IgM and 40/518 (7.7%) MarBlot IgG results were adjusted after visual review of the BLOTrix analyses. The majority of the changes (56/77 = 72.7%) made to the BLOTrix interpretations were from positive to negative. These adjustments yielded a marginal increase in specificity for the MarBlot assays (Table 3). In contrast, a total of 8/518 (1.5%) ViraBlot IgM, 12/518 (2.3%) ViraBlot IgG, 8/518 (1.5%) ViraStripe IgM, and 15/518 (2.9%) ViraStripe IgG results were adjusted after review of the ViraScan analyses. The majority of changes (35/43 [81.4%]) made to the ViraScan interpretations were from negative to positive, resulting in the adjusted sensitivities and specificities outlined in Table 3.

The clinical sensitivity and specificity of the automated systems were further evaluated by using the 40-member CDC serum performance panel. Specimens were categorized by clinical diagnosis according to detailed histories included with the panel, and WB results were analyzed by comparison to the clinical findings. Reference CDC WB results showed a sensitivity of 51.4% (18/35) for IgM and 48.6% (17/35) for IgG in patients with confirmed or probable Lyme disease (Table 4).

TABLE 3. Sensitivity and specificity of automated software or technologist-adjusted software interpretation in prospective serum specimens ($n = 518$)[a]

Assay[b]	Sensitivity (95% CI)		Specificity (95% CI)		No. of technologist adjustments (% of total tests)
	Software alone	Technologist adjusted	Software alone	Technologist adjusted	
MarBlot					
IgM	93.3 (88.9–96.0)	92.8 (88.3–95.7)	79.6 (75.8–82.8)	83.6 (79.2–87.3)	37 (7.1)
IgG	81.1 (70.7–88.4)	86.5 (76.9–92.5)	88.3 (85.0–91.0)	93.2 (90.5–95.2)	40 (7.7)
ViraBlot					
IgM	86.1 (82.3–88.5)	86.1 (82.3–88.5)	85.5 (81.2–88.9)	85.8 (82.4–88.4)	8 (1.5)
IgG	74.3 (66.3–82.9)	81.1 (70.7–88.4)	97.5 (95.6–98.6)	96.0 (93.7–97.4)	12 (2.3)
ViraStripe					
IgM	78.9 (72.6–84.0)	77.8 (73.7–80.9)	92.0 (88.5–94.5)	92.0 (89.1–93.9)	8 (1.5)
IgG	77.0 (66.3–85.1)	81.1 (70.7–88.4)	95.7 (93.4–97.2)	93.5 (90.8–95.4)	15 (2.9)

[a] Visual interpretation of MarDx MarBlot strips served as the gold standard for sensitivity and specificity results.
[b] MarBlot results were analyzed by using the BLOTrix software application; ViraBlot and ViraStripe results were analyzed by using the ViraScan software application.

TABLE 4. Clinical sensitivity and specificity of technologist-adjusted software interpretation among the CDC serum performance panel (n = 40)

Clinical diagnosis	No. of patients	No. (%) positive[a] by:							
		CDC reference WB*		MarBlot (BLOTrix)†		ViraBlot (ViraScan)†		ViraStripe (ViraScan)†	
		IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
Confirmed Lyme disease[b]	28	16 (57.1)	10 (35.7)	12 (42.9)	6 (21.4)	27 (96.4)	8 (28.6)	10 (35.7)	11 (39.3)
Probable Lyme disease[c]	7	2 (28.6)	7 (100.0)	2 (28.6)	7 (100.0)	3 (42.9)	6 (85.7)	1 (14.3)	6 (85.7)
Subtotal	35	18	17	14	13	30	14	11	17
Healthy donor	5	0 (0)	0 (0)	0 (0)	0 (0)	1 (20.0)	0 (0)	0 (0)	0 (0)
Clinical sensitivity[d] (%)		51.4	48.6	40.0	37.1	85.7	40.0	31.4	48.6
Clinical specificity[e] (%)		100.0	100.0	100.0	100.0	80.0	100.0	100.0	100.0

[a] *, MarDx MarBlot (visual interpretation at the CDC); †, results obtained after technologist-adjusted software interpretation.
[b] Physician-documented EM and/or positive culture for *Borrelia burgdorferi*.
[c] Positive LB serology and meeting CDC case definition.
[d] The subtotal number compared to the total number of confirmed and probable Lyme disease cases (n = 35).
[e] The number of negative healthy donor results compared to the total number of healthy donors (n = 5).

The technologist-adjusted BLOTrix results showed a sensitivity of 40.0% (14/35) for IgM and a sensitivity of 37.1% (13/35) for IgG. In contrast, ViraScan analysis of the ViraBlot IgM and IgG strips demonstrated sensitivities of 85.7% (30/35) and 40.0% (14/35), respectively, while the ViraStripe assays showed sensitivities of 31.4% (11/35) for IgM and 48.6% (17/35) for IgG. Each of the systems demonstrated 100% specificity for IgM and IgG with the exception of the ViraBlot IgM assay, which showed a specificity of 80% (4/5) (Table 4).

DISCUSSION

It is estimated that over 2.5 million LB serology tests are performed annually in the United States (1, 18). In 2006, our laboratory at the Mayo Clinic performed 75,478 LB serology tests, with 37,338 (49.5%) of these being done by Lyme WBs. These numbers indicate that LB serology continues to play an important role in the diagnosis of the disease. Furthermore, these data emphasize the need to improve the efficiency of Lyme WB processing, reading, and interpretation due to the significant time and effort required by laboratory personnel.

A significant limitation of current Lyme WB testing is the subjectivity involved in the visual reading and interpretation of test strips. Preliminary studies in our laboratory have demonstrated considerable variation in Lyme WB results when strips are visually read by different laboratory technologists (M. Binnicker, unpublished data). This variation in WB results may contribute to inaccurate diagnoses, resulting in various consequences to patients, as described in past studies (4, 13, 14, 17). Therefore, an important need of clinical laboratories is to enhance the objectivity and consistency of Lyme WB interpretation.

Our findings show substantial agreement between the results of automated and visual interpretation of Lyme WBs. Both systems we evaluated demonstrated comparable results, excellent reproducibility (data not shown), and similar features and total average assay times (Table 5). We should emphasize that both systems are designed to aid in band identification and result interpretation and yet require a laboratory technologist to review and verify results prior to reporting. In our experience, the ViraScan software application was more intuitive to operate and required fewer result modifications by the reviewing laboratory technologist (Table 3). This difference may be due, in part, to the specific manufacturer's recommended cutoff settings used in our evaluation. Clinical laboratories should perform their own thorough evaluation prior to implementing an automated system, since the appropriate cutoff settings may differ between regions where Lyme disease is and is not endemic.

The present study has several additional limitations. First, the number of clinically characterized specimens tested was limited and, therefore, no firm conclusions can be made regarding the accuracy of the automated interpretive systems. Future studies should test a large panel of clinically defined and laboratory-defined specimens in order to more accurately determine the clinical sensitivity and specificity. Second, the data presented here compare the qualitative interpretations (positive versus negative) using the current CDC criteria and do not focus on a direct comparison of individual bands. However, we observed very good correlation, overall, for the detection of specific bands between automated and visual interpretation. Interestingly, correlation seemed to be lowest for the 41- and 58-kDa bands (data not shown), and this may be due, in part, to their migration proximity to the 39- and 60-kDa

TABLE 5. Comparison of features between automated systems

System feature	TrinBlot/BLOTrix	BeeBlot/ViraScan
Software application	BLOTrix	ViraScan
Total assay time/run (h)[a]	2.62	2.65[b]
Maximum run size[c]	50	50
Scanning resolution (dpi)[d]	104	220
IgG and IgM in single run	No	Yes
Electronic interface with LIS[e]	Yes	Yes
Serum and conjugate control bands on assay strip	Yes	Yes

[a] This result represents the average time of three separate runs (adding strips to the incubation tray through technologist review and verification of software results; 40 specimens/run). The total assay time for the current protocol using MarBlot strips (adding strips to the incubation tray through visual reading and interpretation; 40 specimens/run) was 3.7 h.
[b] The total assay time for either ViraBlot or ViraStripe.
[c] The maximum run size for the current protocol (MarBlot strips on AutoBlot 6000) = 60 strips.
[d] dpi, dots per inch.
[e] LIS, Laboratory Information System.

antigens, respectively. These antigens are often difficult to distinguish by visual interpretation and may also require a more detailed verification following the automated software interpretation. A third limitation of the present study is that the same laboratory technologist reviewed and verified the software results for each specimen. Although this approach was used for consistency, the decision to manually adjust software results may vary between technologists. In order to minimize subjectivity and result variability, it will be essential for testing laboratories to establish specific criteria to guide and regulate the modification of software results. A point of interest for future studies will be to determine whether automated systems decrease inter- and intralaboratory result variability in comparison to visual reading and interpretation.

In summary, automated systems showed results comparable to those obtained by routine testing, while demonstrating several advantages. First, the average turnaround time was reduced by 64 min/run, translating into a time savings of approximately 1,000 h/year for clinical laboratories testing 37,000 to 40,000 specimens by WBs. Second, automated systems yield an approximate savings of 0.3 full-time equivalent in comparison to routine testing with visual interpretation. Additional benefits include the ability to electronically store data, share results with clinicians, and interface results with the Laboratory Information System. Finally, automated systems allow for a more objective interpretation of test strips, which may prove to significantly enhance the consistency of Lyme WB results.

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RESEARCH LETTER

Detection of *Borrelia burgdorferi sensu lato* DNA by PCR in serum of patients with clinical symptoms of Lyme borreliosis

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Abstract

Lyme borreliosis is a disease caused by spirochaetes belonging to the genospecies complex *Borrelia burgdorferi sensu lato* (*s.l.*) transmitted by *Ixodes* ticks. At present, serology remains the main diagnostic tool for laboratory diagnosis of Lyme borreliosis. Recently, the PCR technique has been applied for diagnosis of *B. burgdorferi s.l.*, but, until now, a reliable, easy-to-perform and sensitive method has not been described. Here we present a new PCR-based method for the detection of both *B. burgdorferi s.l.* and *Borrelia* genospecies DNAs in serum samples collected from patients showing Lyme disease symptoms. Of 265 serum samples of patients included in this study, 7.5% were positive, 1.9% was borderline and 90.6% were negative for antibodies against *B. burgdorferi* by enzyme-linked immunosorbent assay and Western blotting. The *B. burgdorferi s.l.* 16S rRNA gene was detected by PCR in all serum-positive and in two borderline samples. None of the serum-negative samples nor serum samples collected from healthy subjects gave positive PCR reactions. Of PCR-positive serum samples, 50% gave a positive reaction for *Borrelia afzelii*, 18% for *Borrelia garinii* and 23% for two *Borrelia* species. Two samples (9%) were not identified to species level. The new protocol could be considered to be reliable as neither false-positive nor false-negative reactions were recorded, and to be sensitive as it detects DNA from one bacterial cell.

Introduction

Lyme borreliosis is an infectious disease caused by spirochaetes belonging to the genospecies complex *Borrelia burgdorferi sensu lato* (*s.l.*) transmitted by *Ixodes* ticks.

Three genospecies, *Borrelia afzelii*, *Borrelia garinii* and *B. burgdorferi sensu stricto* (*s.s.*), are widely distributed in Europe causing human borreliosis (van Dam *et al.*, 1993). Each species is correlated with distinct clinical manifestations: *B. garinii* is predominantly associated with neurological symptoms, *B. afzelii* with late skin manifestations and *B. burgdorferi s.s.* with arthritis. Other *Borrelia* genospecies, such as *Borrelia valaisiana*, have been isolated from ticks in Europe and are involved in human Lyme borreliosis (Escuredo *et al.*, 2000). In addition, *Borrelia spielmanii* has been isolated from a patient affected by erythema migrans (Wang *et al.*, 1999) and *Borrelia lusitaniae* has been isolated from a patient for the first time in 2004 in Europe (Collares-Pereira *et al.*, 2004).

At present, borreliosis is diagnosed mainly on the basis of clinical symptoms and serological tests. These tests, based on demonstration in human serum of anti-*B. burgdorferi s.l.* IgG and IgM antibodies, are usually carried out by enzyme-linked immunosorbent assay (ELISA) and Western blot tests (Aguero-Rosenfeld *et al.*, 2005). Unfortunately, serological tests have a poor reliability for the identification of different *Borrelia* genospecies, such as *B. afzelii*, *B. garinii* and *B. burgdorferi s.s.* Misdiagnosis (both false-positive and false-negative) is frequent due to technical reasons (Niscigorska *et al.*, 2003).

The PCR technique has been applied for the diagnosis of *B. burgdorferi s.l.* in human serum samples (Schmidt, 1997). Although the recovery of *B. burgdorferi s.l.* DNA in clinical samples represents an appealing alternative tool, the low number of spirochetes in blood, plasma and serum could represent a serious problem for PCR reliability (Aguero-Rosenfeld *et al.*, 2005).

In order to develop a reliable PCR-based method, widely applicable to the identification of various *Borrelia*

genospecies, several protocols have been attempted and DNA target sequences have been used (Portnoi *et al*., 2006). However, until now, an easy-to-perform and sensitive method has not been described.

Here we present a new PCR protocol able to detect both *B. burgdorferi s.l.* and *Borrelia* genospecies DNAs in serum samples collected from patients showing symptoms of Lyme disease. In particular, the new protocol demonstrated both high sensitivity, giving a PCR-positive reaction with *Borrelia* DNA extracted from human serum samples containing 1 bacterium mL^{-1}, and 100% specificity, as neither false-negative nor false-positive reactions were detected.

Materials and methods

Bacterial isolates and culture conditions

Borrelia burgdorferi B31, *B. afzelii* and *B. garinii*, kindly provided by the Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy, were used in this study. All strains were cultured in BSKII medium (Sigma Chemical Co., St. Louis, MO) at 34 °C. Growth was checked by dark-field microscopy.

Patient samples

A total of 265 human serum samples were collected during January 2005 and April 2007 at the Policlinico Umberto I Hospital, Sapienza University of Rome, from patients presenting clinical manifestations of Lyme borreliosis, including early manifestations (erythema migrans, fever, malaise, fatigue, skin rash, arthralgia, myalgia) or later manifestations of Lyme disease (severe arthritic, neurologic and cardiac manifestations). Twenty serum samples collected from healthy subjects were used as negative controls. All serum samples were stored at −20 °C until use.

Serological tests

The presence of specific IgM and/or IgG antibodies against *B. burgdorferi s.l.* was determined by ELISA (recomWell IgM/IgG, Mikrogen, Neuried, Germany) and Western blot (ViraBlot IgM/IgG, Viramed Biotech AG) tests. Recombinant proteins specific for *B. burgdorferi s.l.* were used as positive control.

ELISA tests were considered negative, positive or borderline when values were lower than 20, higher than 24 or between 20 and 24 U mL^{-1}, respectively, as suggested by the manufacturer.

Detection of *B. burgdorferi s.l.* DNA by PCR

Borrelia DNA was extracted from human serum samples according to the following experimental protocol (Protocol A): 100 μL of human serum samples was incubated in the presence of 200 μL ammonium hydroxide (0.7 M) at 100 °C for 5 min in a 1.5-mL tube, followed by 10 min at 100 °C with the tube open. *Borrelia* DNA precipitation was obtained by adding 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate. The samples were centrifuged at 12 000 ***g*** for 15 min and DNA rinsed with 70% ethanol. After centrifugation, DNA samples were air dried, suspended in 100 μL Tris-EDTA (TE) buffer and stored at −70 °C until use. In comparative experiments *Borrelia* DNA was extracted from human serum samples following the protocol of Guy & Stanek (1991) (Protocol B). PCRs were performed using 16S rRNA gene PCR primers described by Marconi & Garon (1992) and Liebisch *et al*. (1998). In particular, the primer sets LD, BB, BG, BA and BV were specific for *B. burgdorferi s.l.*, *B. burgdorferi s.s.*, *B. garinii*, *B. afzelii* and *B. valaisiana*, respectively (Table 1). PCR reactions were performed in a reaction volume of 25 μL containing 12.5 pmol of appropriate primer set and 1 μL of bacterial DNA extracted from human serum samples as described above. Companion PCR experiments were carried out using

Table 1. PCR primer sets used in this study

Primer set	Sequence (5′–3′)	Amplimer (bp)	Annealing temperature (°C)	Reference
LD	ATGCACACTTGGTGTTAACTA GACTTATCACCGGCAGTCTTA	357	50	Marconi & Garon (1992)
BB	GGGATGTAGCAATACATTC ATATAGTTTCCAACATAG	574	50	Marconi & Garon (1992), erratum (1993)
BG	GGGATGTAGCAATACATCT ATATAGTTTCCAACATAGT	574	44	Marconi & Garon (1992)
BA	GCATGCAAGTCAAACGGA ATATAGTTTCCAACATAGC	591	46	Marconi & Garon (1992), erratum (1993)
BV	GCAAGTCAAACGGGATGTAGT GTATTTTATGCATAGACTTATATG	549	49	Liebisch *et al*. (1998)

LD, *Borrelia burgdorferi sensu lato*; BB, *Borrelia burgdorferi sensu stricto*; BG, *Borrelia garinii*; BA, *Borrelia afzelii*; BV, *Borrelia valaisiana*.

as template DNA extracted from *Borrelia* strains, i.e. *B. burgdorferi* B31, *B. afzelii*, *B. garinii*. *Borrelia* strains were cultured in BSK-II medium for 3 days at 34 °C. After incubation, bacterial cells were treated for DNA extraction according to the protocol outlined above (Rosa & Schwan, 1989).

PCR amplifications were performed using a Perkin-Elmer Cetus thermocycler by denaturing the template DNA for 1 min at 94 °C, with extension for 1.5 min at 72 °C for 35 cycles. Annealing temperatures ranged from 44 to 50 °C for the different primer sets as detailed in Table 1. Amplification products were visualized after electrophoresis with 10 μL of the PCR reaction volume in 1.0% agarose gels in TAE buffer [40 mM Tris-acetate, 2 mM EDTA (pH 8.5)] containing ethidium bromide at 0.5 pg mL^{-1}.

In order to confirm the PCR-based species attribution, amplimers obtained with species-specific primer sets were digested with HindIII and SacII restriction enzymes.

To determine PCR sensitivity, serum samples from two healthy subjects were pooled and divided into 1-mL aliquots. Each aliquot was infected by 10-fold serial dilutions of *B. burgdorferi* B31 live cells. DNA was extracted from 100 μL infected serum samples following the methods described above (protocols A and B) and PCRs were performed using the LD (*B. burgdorferi s.l.*) primer set.

Results

To confirm PCR reliability, preliminary experiments were carried out using DNA samples extracted from reference *Borrelia* strains. As expected, PCRs performed using extracted DNA from cultured strains and LD and genospecies-specific primer sets were positive (data not shown).

To test PCR sensitivity, *Borrelia* DNA was extracted from serial dilutions of *B. burgdorferi* B31 culture in serum samples following both protocols A and B. PCRs were performed using the BB primer set (Table 2). Using DNA extracted following Protocol A, PCR reactions were positive on serum samples infected with 10^5, 10^4 and 10^3 CFU mL^{-1}; following Protocol B, PCR was positive on serum sample infected with 10^5 UFC mL^{-1}. As PCRs were performed using 1 μl of extracted DNA, the sensitivity was equal to 1 and 100 bacterial cells for protocols A and B, respectively.

In order to investigate the possibility of increasing the sensitivity of Protocol A, a further set of experiments were carried out. The amount of serum to be treated was raised to 200 μL and the volume of TE to suspend *Borrelia* DNA was reduced to 25 μL. The results did not indicate a significant increase in PCR sensitivity (data not shown).

Of the 265 patient samples included, 20 (7.5%) were positive, five (1.9%) were borderline and 240 (90.6%) were negative for antibodies against *B. burgdorferi* by ELISA and Western blotting.

Twenty seropositive, five borderline and nine seronegative samples, as well as 20 serum samples collected from healthy subjects were analyzed for the presence of *B. burgdorferi s.l.* DNA by PCR (Table 3). *Borrelia burgdorferi s.l.* DNA was found in all 20 seropositive and in two of the five borderline serum samples. All nine seronegative as well all 20 serum samples from healthy subjects gave negative PCRs.

PCR reactions with species-specific primers for *B. burgdorferi s.s.*, *B. afzelii*, *B. garinii* and *B. valaisiana* were carried out on the 22 *B. burgdorferi s.l.* PCR-positive serum samples. Fifteen samples (68%) contained DNA from a single *Borrelia* genospecies, and five samples (23%) showed the presence of DNA from two different genospecies. The genospecies attribution could not be made for two serum samples (9%) positive for *B. burgdorferi s.l.* as PCRs performed with genospecies-specific primers were negative (Fig. 1). None of the serum sample was positive for *B. burgdorferi s.s.*

In order to confirm the PCR-based species attribution, amplimers obtained with species-specific primer sets were digested with HindIII and SacII restriction enzymes. Results showed restriction fragments consistent with species-specific 16S rRNA gene sequences (data not shown).

The distribution of *Borrelia* genospecies in relation to clinical manifestations is shown in Table 4. Of particular note, 16 patients had early clinical manifestations and six late clinical manifestations.

Table 2. PCR sensitivity of *Borrelia burgdorferi* B31 DNA detection in human serum samples

B. burgdorferi B31 (CFU mL^{-1} of serum)	Serum sample treated for DNA extraction (μL)	DNA used as PCR template (μL)	BB primer set PCR	
			Protocol A	Protocol B
100 000	100	1	+	+
10 000	100	1	+	−
1000	100	1	+	−
100	100	1	−	−
10	100	1	−	−
1	100	1	−	−
None	100	1	−	−

The 16S rRNA gene BB primer set specific for *B. burgdorferi s.s.* was employed (see Table 1).

Table 3. PCR detection of *Borrelia burgdorferi s.l.* DNA in human serum samples

Subjects	Serological tests	No. tested	PCR	
			Positive	Negative
With clinical manifestations	Positive*	20	20	0
	Negative	10	0	10
	Borderline	5	2	3
Healthy subjects	Negative	20	0	20
Positive control		1	1	0

*Positive, $>$ 24 U mL^{-1}; negative, $<$ 20 U mL^{-1}; borderline, between 20 and $\geq$24 U mL^{-1}.

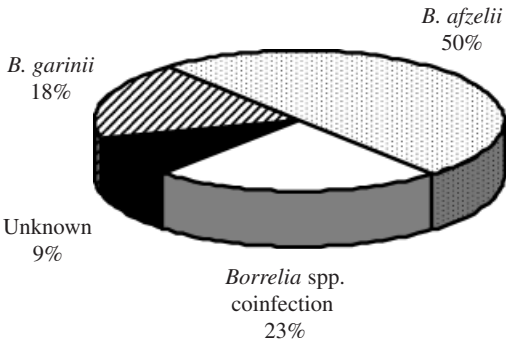

Fig. 1. *Borrelia burgdoferi* DNA genospecies recovered in human serum samples.

Table 4. Distribution of *Borrelia* genospecies in human serum samples in relation to clinical manifestations

Genospecies	Clinical manifestation		
	Early	Late	Total
B. afzelii	7	4	11
B. garinii	4	–	4
B. afzelii and *B. garinii*	3	–	3
B. afzelii and *B. valaisiana*	–	1	1
B. garinii and *B. valaisiana*	1	–	1
Unknown	1	1	2
Total	16	6	22

Discussion

The aim of the present study was to improve the PCR-based protocol for identification of *B. burgdorferi s.l.* from human serum samples and to compare the diagnostic value of PCR with that of serology. At present, serology remains the main diagnostic tool for laboratory diagnosis of Lyme borreliosis. However, in the early phase of infection, the level of antibodies against *B. burgdorferi* is low. False-negative results occur primarily during the first weeks of infection (Hofmann, 1996). Culturing *B. burgdorferi* from body fluids is difficult because of the long incubation period, the high cost of culture medium and small proportion of positive results (Aguero-Rosenfeld *et al.*, 2005). Therefore, the need for a reliable diagnostic method is evident (Hofmann, 1996). As PCR has been considered as a sensitive tool for identification of fastidious microorganisms that are difficult to culture, attempts have been made to apply this technique to detect *Borrelia* DNA in human serum (Niscigorska *et al.*, 2003).

Here we present a simple, easy-to-perform, and reliable PCR protocol for the identification of *Borrelia* from human serum samples. The protocol involves a simple method to extract *Borrelia* DNA from human serum samples. Several PCR methods do indeeds yield acceptable results when *Borrelia* DNA is extracted from skin biopsy of patients with Erythema chronicum migrans (ECM) or synovial fluid in patients with Lyme arthritis (Aguero-Rosenfeld *et al.*, 2005).

In the present study, the extracted DNAs were used to perform PCRs using different primer sets to identify *Borrelia* to genospecies level. We have chosen to employ the previously described primer sets that amplify the 16S rRNA gene. It is well known that these primer sets are specific for *B. burgdorferi s.l.* and *B. burgdorferi* genospecies (Liebisch *et al.*, 1998; Marconi & Garon, 1992). Our protocol has been shown to be more sensitive than a previously published method, giving a positive PCR reaction using DNA from 1 as compared with 10^3 bacterial cells (Portnoi *et al.*, 2006). Sensitivity similar to that for our protocol has been reported by Joss *et al.* (2008), who described a real-time PCR method to detect *Borrelia* from serum samples. However, it is well known that real-time PCR is costly, laborious to perform and, moreover, requires the use of sophisticated apparatus.

The protocol proposed shows 100% reliability: no false-positive or false-negative reactions were recorded. In fact, our protocol recognized as positive only those serum samples positive by ELISA and Western blotting and two serum samples from subjects with early symptoms that give borderline reactions in serological tests. This result confirms the poor reliability of serological tests in early *Borrelia* infections (Riesbeck & Hammas, 2007). Moreover, the protocol we have described allowed us to identify at species level *Borrelia* DNA in human serum samples. To confirm the reliability of the proposed method, we have also analyzed the PCR amplimers obtained by digesting them with restriction enzymes. As expected, DNA fragments obtained by restriction enzymes were consistent with PCR amplification and confirmed the PCR-based *Borrelia* identification.

In the present study, 7.5% of the patients with suspected Lyme borreliosis had antibodies against *B. burgdorferi* in blood serum. *Borrelia burgdorferi* DNA was detected by PCR in all serum samples from seropositive patients (100%) and from two (22%) seronegative subjects. Of note, 72.7% of patients with positive PCR showed symptoms referable to early borreliosis, while only 27.3% of PCR-positive patients showed late borreliosis. Moreover, two borderline patients manifesting early symptoms as erythema migrans were PCR positive. These results are in agreement with those of Guy & Stanek (1991), underlining that the PCR method can contribute to the reliability of diagnosis during early stages of infection.

In the present study, the frequency of recovery of *Borrelia* DNA was equal to 8.3% (22 positive of 265 serum samples). Variable frequency of recovery values have been reported in the past, ranging from 76 to 3.8% (Oksi *et al.*, 1999, 2001; Kondrusik *et al.*, 2004; Chmielewska-Badora *et al.*, 2006). These discrepancies are probably due to the frequency of infection and to the different protocols used in performing

PCR reactions, underlining the need to develop a reliable PCR protocol.

Regarding genospecies identification, *B. afzelii* was the most frequent species (50%, 11 of 22 serum samples) while *B. burgdorferi s.s.* DNA was not recovered. Interestingly, 23% of the samples were positive for two different *Borrelia* species. Several studies indicate that different *Borrelia* species may be associated with specific reservoir hosts and with distinct clinical manifestations of Lyme disease (van Dam *et al.*, 1993; Balmelli & Piffaretti, 1995). Therefore, *Borrelia* identification at genospecies level in patients with Lyme borreliosis appears to be of epidemiological, pathogenetic and diagnostic importance.

Mixed infections with two or more *Borrelia* species are frequent in ticks (Santino *et al.*, 1998; Santino *et al.*, 2003) and have been reported also in human patients (Ruzic-Sablijic *et al.*, 2005). The present study indicates that human patients with Lyme borreliosis can simultaneously harbor different *B. burgdorferi s.l.* strains and that these infections are mainly double infections with the presence of *B. garinii* and *B. afzelii* or *B. valaisiana*.

In conclusion, the protocol described here could be usefully employed in PCR-based *Borrelia* identification from human serum samples.

Acknowledgements

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Seronegativity in Lyme borreliosis and Other Spirochetal Infections

16 September 2003

"If false results are to be feared, it is the false negative result which holds the greatest peril for the patient."

Gestational Lyme borreliosis. Implications for the fetus. MacDonald AB. *Rheum Dis Clin North Am*, 15(4):657-77. 1989.

	Author	*Year*	*Title*	*Journal*

Borrelia burgdorferi

1. **Dejmkova H; Hulinska D; Tegzova D; Pavelka K; Gatterova J; Vavrik P.** 2002 **Seronegative Lyme arthritis caused by Borrelia garinii.** Clinical Rheumatology, 21(4):330-4

[From the abstract:] "A case of a female patient suffering from Lyme arthritis (LA) without elevated antibody levels to Borrelia burgdorferi sensu lato is reported. Seronegative Lyme arthritis was diagnosed based on the classic clinical manifestations and DNA-detected Borrelia garinii in blood and synovial fluid of the patient, after all other possible causes of the disease had been ruled out. The disease was resistant to the first treatment with antibacterial agents. Six months after the therapy, arthritis still persisted and DNA of Borrelia garinii was repeatedly detected in the synovial fluid and the tissue of the patient. At the same time, antigens or parts of spirochaetes were detected by electron microscopy in the synovial fluid, the tissue and the blood of the patient. The patient was then repeatedly treated by antibiotics and synovectomy has been performed."

2. **Tylewska-Wierzbanowska S; Chmielewski T;** 2002 **Limiation of serologic testing for Lyme borreliosis: evaluation of ELISA and western blot in comparison with PCR and culture methods.** Wien Klin Wochenschr, 114(13-14):601-5

[From the abstract:] "No correlation was found between levels of specific B. burgdorferi antibodies detected with a recombinant antigen ELISA and the number of protein fractions developed with these antibodies by immunoblot. Moreover, Lyme borreliosis patients who have live spirochetes in body fluids have low or negative levels of borrelial antibodies in their sera. This indicates that an efficient diagnosis of Lyme borreliosis has to be based on a combination of various techniques such as serology, PCR and culture, not solely on serology." [Testing was performed on samples from 90 patients.]

3. **Breier F; Khanakah G; Stanek G; Kunz G; Aberer E; Schmidt B; Tappeiner G.** 2001 **Isolation and polymerase chain reaction typing of Borrelia afzelii from a skin lesion in a seronegative patient with generalized ulcerating bullous lichen sclerosus et atrophicus.** Br J Dermatol, 144(2):387-392

[From the abstract:] "Spirochaetes were isolated from skin cultures obtained from enlarging LSA lesions. These spirochaetes were identified as Borrelia afzelii by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and polymerase chain reaction (PCR) analyses. However, serology for B. burgdorferi sensu lato was repeatedly negative."

4. **Brunner M.** 2001 **New method for detection of Borrelia burgdorferi antigen complexed to antibody in seronegative Lyme disease.** J Immunol Methods, 249(1-2):185-190

[From the abstract:] "...serologic tests for early Lyme disease can be falsely negative due to lack of sensitivity of ELISAs and Western blots. Most routine antibody tests are designed to detect free antibodies, and in early, active disease, circulating antibodies may not be free in serum but sequestered in complexes with the antigens which originally triggered their production. This difficulty may be overcome by first isolating immune complexes (IC) from the serum and using this fraction for testing. Free Borrelia-specific antibodies can then be liberated from the immune complexes which may enhance test sensitivity in patients with active disease. We developed a technique that captures the antibody component of IC on immunobeads, and subsequently releases the antigen component of IC. Immunoblotting with monoclonal antibody detected at least one antigen to be OspA, thus definitively demonstrating a Borrelia-specific antigen in circulating IC in early Lyme disease. This test is also useful in demonstrating Bb antigen in otherwise seronegative Lyme disease patients."

	Author	Year	Title	Journal
5.	**Wang P; Hilton E.**	2001	**Contribution of HLA alleles in the regulation of antibody production in Lyme disease.**	Front Biosci, 6:B10-B16

"Of eighteen seronegative LD patients, 14 were OspA PCR positive on mononuclear cells and 5 were positive on CSF. ...The presence of certain HLA alleles with seronegativity to disease has been reported in malaria (10), HIV (16,17), rheumatoid arthritis (RA) (18) and spondylarthropathies (SpA) (19). ...Our results provide evidence of a correlation between certain HLA genotypes and the ability to mount an antibody response to Bb. In this study, 9 of 22 (40.9%) seropositive LD patients and only 1 out of 18 (5.6%) seronegative LD patients had HLA-DR7 alleles. ...

Our study provides evidence that HLA alleles are involved in antibody responsiveness or non-responsiveness to Bb infection. A low frequency of HLA-DR7 alleles and HLA-DR6 alleles and a high frequency of HLA-DR1 alleles may contribute to non-responsiveness of antibody production in LD patients. Thus, genetic predisposition may be a critical factor in the regulation of the host immune response and the diagnosis and prognosis of Lyme disease."

6.	**Grignolo MC; Buffrini L; Monteforte P; Rovetta G.**	2001	**Reliability of a polymerase chain reaction (PCR) technique in the diagnosis of Lyme borreliosis.**	Minerva Med, 92(1):29-33

[From the abstract:] "50% of the PCR positive results, obtained with serum and cerebrospinal fluid samples corresponded to patients who were true positives at clinical examination but negatives at serologic tests. 62.5% of urine samples positive results belonged to tp patients who had negative serologic and serum PCR RESULTS. CONCLUSIONS: The obtained results suggested a good reliability of positive results obtained with the PCR technique used in this study and allowed the false negatives of serologic tests to be detected, more specifically when urine samples were used."

7.	**Klempner MS; Schmid CH; Hu L; Steere AC; Johnson G; McCloud B; Weinstein A.**	2001	**Intralaboratory reliability of serologic and urine testing for Lyme disease.**	American Journal of Medicine, 110(3):217-19

"In the 21 patients with Lyme disease, the results of the initial western blot analysis were positive in 14 cases and negative in 7. ... Repeat testing of the 7 seronegative samples showed fewer than 5 reactive bands in all samples."

8.	**Honegr K; Hulinska D; Dostal V; Gebousky P; Hankova E; et al.**	2001	**[Persistence of Borrelia burgdorferi sensu lato in patients with Lyme borreliosis].**	Epidemiol Mikrobiol Imunol, 50(1):10-6

[From the abstract:] "In 18 patients with Lyme borreliosis the authors proved the persistence of Borrelia burgdorferi sensu lato by detection of the causal agent by immune electron microscopy or of its DNA by PCR in plasma or cerebrospinal fluid after an interval of 4-68 months. ...Examination of antibodies by the ELISA method was negative in 7 of 18 patients during the first examination and in 12 of 18 during the second examination. In all negative examinations the specific antibodies were assessed by the Western blot or ELISA method after liberation from the immunocomplexes."

9.	**Paul A.**	2001	**[Arthritis, headache, facial paralysis. Despite negative laboratory tests Borrelia can still be the cause.]**	MMW Fortschr Med, 143(6):17

10.	**Pleyer U; Priem S; Bergmann L; Burmester G; Hartmann C; Krause A.**	2001	**Detection of Borrelia burgdorferi DNA in urine of patients with ocular Lyme borreliosis.**	Br J Ophthalmol, 85(5):552-5

[From the abstract:] "RESULTS: Only four of six uveitis patients suspected for Lyme borreliosis were ELISA positive, while all six subjects showed a positive western blot. B burgdorferi PCR was positive in all of these six patients. Whereas two of the 30 controls had a positive Lyme serology, B burgdorferi DNA was not detectable by PCR in any sample from these patients. CONCLUSIONS: PCR for the detection of B burgdorferi DNA in urine of uveitis patients is a valuable tool to support the diagnosis of ocular Lyme borreliosis. Moreover, these patients often show a weak humoral immune response which may more sensitively be detected by immunoblotting."

11.	**Eldoen G; Vik IS; Vik E; Midgard R.**	2001	**[Lyme neuroborreliosis in More and Romsdal].**	Tidsskrift for Den Norske Laegeforening, 121(17):2008-11.

[From the abstract:] "Fourteen of 25 (56%) patients had positive Borrelia burgdorferi-IgM and IgG titres in cerebrospinal fluid despite negative tests in serum."

Author	*Year*	*Title*	*Journal*
12. **Brunner M; Sigal LH.**	2000	**Immune complexes from serum of patients with Lyme disease contain Borrelia burgdorferi antigen and antigen-specific antibodies: potential use for improved testing.**	Journal of Infectious Diseases, 182(2):534-9

[From the abstract:] "We report sequestration of specific IgM anti-Borrelia burgdorferi (Bb) and Bb antigens within immune complexes (ICs) isolated from serum of patients with Lyme disease (LD). ...Immunoblot demonstrated that ICs contained antibodies against specific Bb proteins, whereas reactivity was absent or significantly lessened in unprocessed serum."

13. **Kaiser R.** 2000 **False-negative serology in patients with neuroborreliosis and the value of employing of different borrelial strains in serological assays.** J Med Microbiol, 49(10):911-5.

[Abstract:] "The risk of obtaining false-negative results in serological assays in serum and CSF specimens with only one strain of Borrelia burgdorferi sensu lato as antigen was investigated in 79 patients with neuroborreliosis with specimens obtained at initial presentation. Serum antibodies were assessed by immunoblotting; the criteria of Hauser et al. were used to evaluate the test. The intrathecal synthesis of borrelial-specific IgM and IgG antibodies was examined by enzyme immunoassay (EIA). Strains of B. burgdorferi sensu stricto (BbZ160), B. garinii (Bbii50) and B. afzelii (PKO) served as sources of antigen in both assays. All patients produced either a positive IgM or IgG test in serum with at least one strain of B. burgdorferi sensu lato. Reactivity of IgM or IgG antibodies, or both, with antigens of all three strains was demonstrated in 67 (85%) of 79 sera. The correlation of results of immunoblotting with different strains was significantly better for IgG (85%) than for IgM antibodies (54%). The variability of positive IgM reactions in 18 specimens was mainly due to the fact that the antibodies were directed to the relevantvariable outer-surface protein C (p23). Intrathecal synthesis of IgG antibodies was demonstrated in 58 patients (81%) of 72 and of IgM antibodies in 25 of 58 patients. No patient had isolated intrathecal synthesis of IgM antibodies. The majority of CSF samples (56 of 58) were assessed as IgG antibody-positive, independent of the borrelial strain used as antigen in EIA, whereas only 10 of 25 IgM antibody-positive CSF specimens reacted with all three strains. All patients in the study had intrathecal antibody synthesis demonstrable at 6-week follow-up. From this study it is concluded that there is a small, but real, risk of false-negative serological findings at the time of initial clinical presentation in patients with typical symptoms of neuroborreliosis. In these patients a negative serological result with one strain should prompt the repetition of the test with other strains of B. burgdorferi sensu lato."

14. **Kmety E.** 2000 **[Dynamics of antibodies in Borrelia burgdorferi sensu lato infections.]** Bratisl Lek Listy, 101(1):5-7

[From the abstract:] "...During 1994-1998 at least two serum samples were submitted for serological testing from more than 1200 patients. An immunofluorescence test was performed paralelly [sic] with two pools of antigen (B. bg.s.s. + B. afzelii, and two serological different strains of B. garinii, all of local origin). In 92-96% of patients no change of antibody level was found in repeated tests, about 20% of them being negative (< 1:512).

...Only in 9 cases a rise of the titer appeared during 3 weeks after the first negative sample, at contrary in 7 cases no rise of the titer was seen in that time. 2 patients were still after 1 month, 3 after 3 months and 1 even after 7 months (patient with a positive CSF culture) serologically negative."

15. **Wilke M; Eiffert H; Christen HJ; Hanefeld F.** 2000 **Primarily chronic and cerebrovascular course of Lyme neuroborreliosis: case reports and literature review.** Arch Dis Child, 83(1):67-71.

"In this context, even the complete absence of specific antibodies has been observed; in a girl diagnosed as having focal vasculitis through CNS biopsy, the presence of B burgdorferi in CSF was confirmed by polymerase chain reaction. No specific antibodies were detectable. In three other children, B. burgdorferi could be cultured from CSF in the absence of specific antibodies in CSF or blood."

16. **Sheets JT; Rossi CA; Kearney BJ; Moore GE.** 2000 **Evaluation of a commercial enzyme-linked immunosorbent assay for detection of Borrelia burgdorferi exposure in dogs.** J Am Vet Med Assoc, 216(9):1418-22

"The commercial ELISA kit evaluated in this study appeared to lack adequate sensitivity for detecting all potential cases of borreliosis in dogs."

	Author	*Year*	*Title*	*Journal*
17.	**Wang P; Gartenhaus R; Sood SK; DeVoti J; Singer C; et al.**	2000	**Detection of Borrelia DNA in circulating monocytes as evidence of persistent Lyme disease.**	J Spirochetal and Tick-borne Diseases, 7(1):16-19
			[Abstract:] "We report the detection of Borrelia burgdorferi DNA in circulating monocytes in a 31-year-old female who presented with a flu-like syndrome followed by neurological abnormalities after a trip to Southampton, Long Island, New York. ELISA and Western blot were negative. Lymphocyte proliferation assay to Borrelia burgdorferi was positive. Borrelia burgdorferi DNA was detected in circulating monocytes using a nested polymerase chain reaction (PCR). Treatment with parenteral ceftriaxone resulted in clinical improvement and repeat PCR on monocytes was negative. The use of detecting DNA by PCR from circulating monocytes may be useful in evaluating seronegative patients with a high suspicion of Lyme disease."	
18.	**Brown SL; Hansen SL; Langone JJ.**	1999	**Role of serology in the diagnosis of Lyme disease. (FDA Medical Bulletin)**	JAMA, 282(1): 62-65
			"*The Food and Drug Administration (FDA) is concerned about the potential for misdiagnosis of Lyme disease based on the results of commonly marketed tests for detecting antibodies to Borrelia burgdorferi, the organism that causes Lyme disease. It is important that clinicians understand that a positive test result does not necessarily indicate current infection with B. burgdorferi, and a patient with active Lyme disease may have a negative test result.*	
			The tests should be used only to support a clinical diagnosis of Lyme disease and should never be the primary basis for making diagnostic or treatment decisions."	
19.	**Bertrand E; Szpak GM; Pilkowska E; Habib N; et al.**	1999	**Central nervous system infection caused by Borrelia burgdorferi. Clinico-pathological correlation of three post-mortem cases.**	Folia Neuropathol, 37(1):43-51
			"Case 1: ...Specific borrelia IgM and IgG value in serum and CSF were normal (<250). However, on microscopical examination the spirochete B. burgdorferi was demonstrated in serum and CSF. The bacteria were cultured both from blood and from CSF, in CSF they were also identified by PCR."	
20.	**Mikkila H, Karma A, Viljanen M, Seppala I.**	1999	**[The laboratory diagnosis of ocular Lyme borreliosis.]**	Graefes Arch Clin Exp Ophthalmol, 237(3):225-30
			"Seven patients, including two with negative ELISA, had a positive immunoblot. Seven of the 13 patients in whom PCR was examined during clinically active disease had a positive PCR result. Immunoblot analysis gave a negative result from the sera of five PCR-positive patients. CONCLUSIONS: For efficient diagnosis of ocular Lyme borreliosis, immunoblot analysis and PCR should be used in addition to ELISA."	
21.	**Oksi J; Marjamaki M; Nikoskelainen J; Viljanen MK.**	1999	**Borrelia burgdorferi detected by culture and PCR in clinical relapse of disseminated Lyme borreliosis.**	Annals of Medicine, 31(3):225-32
			"Three of the 13 patients had only IgM antibodies against B. burgdorferi, and one culture-positive patient was seronegative despite the disseminated stage of the disease. The reason for the lack of IgG antibodies, or of both IgM and IgG antibodies, was not restriction of the infection to privileged sites, as all these patients had a multiorgan disease. We have previously shown that patients with late LB with live spirochetes or borrelial DNA in their body fluids may have low or negative serum borrelia antibody levels."	
22.	**Hudson BJ; Stewart M; Lennox VA; et al.**	1998	**Culture-positive Lyme borreliosis.**	Med J Aust, 168(10):500-2
			[From the abstract:] "We report a case of Lyme borreliosis. Culture of skin biopsy was positive for Borrelia garinii, despite repeated prior treatment with antibiotics.'	
			"The results of conventional serological and histopathological tests were negative, despite an illness duration of at least two years."	

	Author	*Year*	*Title*	*Journal*
23.	**McCaulley, Mark E., M.D.**	1998	**Guidelines for the clinical diagnosis of Lyme disease.**	Annals of Internal Medicine, 129(5): 422-423

[Letter to the Editor:] "The position paper on laboratory diagnosis of Lyme disease is based on a widely accepted paradigm that is inconsistent with a growing body of medical literature. According to this paradigm, cases of Lyme disease are overwhelmingly seropositive and are unlikely to be associated with persistent symptoms after presumed adequate therapy. In addition, any patients remaining persistently symptomatic are presumed to no longer have Lyme disease at all but rather to have such conditions as fibromyalgia, depression, or the chronic fatigue syndrome and, as a result, to be unlikely to respond to additional antibiotic therapy. Such presumptions are inconsistent with an increasing number of reports.

A 1994 article reports the increased frequency of multiple symptoms in previously treated patients with Lyme disease compared with controls. Antibodies on ELISA were found in less than half of the patients with Lyme disease. Re-treatment was associated with improvement in half of re-treated patients. Had the guidelines been followed in a clinical evaluation of these or similar patients, Lyme disease would have been diagnosed in few of them.

In a 1996 report, Borrelia burgdorferi plasmid DNA was detectable by polymerase chain reaction assay only in a subset of patients with Lyme disease who were seronegative. Many case reports have described patients with Lyme disease who remain antigen positive and symptomatic despite intensive antibiotic treatment.

I suggest the acceptance of a new paradigm that incorporates the above information. Physicians involved in the treatment of Lyme disease should consider that 1) Patients with Lyme disease, especially those in late stages of the disease, are frequently seronegative; 2) the persistence of symptoms, which may be vague, is common and may respond to additional antibiotic therapy; and 3) there is much to be learned about the optimal treatment of Lyme disease at any stage."

24.	**Petrovic M; Vogelaers D; Van Renterghem L; Carton D; De Reuck J; Afschrift M.**	1998	**Lyme borreliosis - a review of the late stages and treatment of four cases.**	Acta Clinica Belgica, 53(3):178-83

[From the abstract:] "Difficulties in diagnosis of late stages of Lyme disease include low sensitivity of serological testing and late inclusion of Lyme disease in the differential diagnosis. Longer treatment modalities may have to be considered in order to improve clinical outcome of late disease stages...The different clinical cases illustrate several aspects of late borreliosis: false negative serology due to narrow antigen composition of the used ELISA format, the need for prolonged antibiotic treatment in chronic or recurrent forms and typical presentations of late Lyme disease, such as lymphocytic meningo-encephalitis and polyradiculoneuritis."

25.	**American Academy of Neurology 49th Annual Meeting April 12-19.**	1997	**Lyme encephalopathy may surface despite antibiotic treatment.**	http://www.medscape.com/CPG/ClinReviews/1997/v07.n06/c0706.cnu/c0706.cnu.html#Lyme

"Of the 8 patients with CNS infection, only 2 were seropositive on both the ELISA and Western blot tests. Four had indeterminate ELISA results and a negative Western blot, and 2 had negative results on both the ELISA and the Western blot. Neither of the 2 seropositive patients had received antibiotics during the first month of infection for early localized or disseminated disease," said the Boston researchers. Of the 6 seronegative patients with CNS infection, however, 5 (84%) had received a recommended course of oral or intravenous antibiotics during the first month of infection.'"

26.	**Branigan P; Rao J; Rao J; Gerard H; Hudson A; Williams W; Arayssi T; Pando J; Bayer M; Rothfuss S; Clayburne G; Sieck M; Schumacher HR.**	1997	**PCR evidence for Borrelia burgdorferi DNA in synovium in absence of positive serology.**	American College of Rheumatology, Vol 40(9), Suppl:S270

"PCR evidence for Borrelia has been identified in synovial biopsies of patients with clinical pictures that had not initially suggested Lyme disease. All 6 PCR-positive] patients were negative for antibodies to Borrelia and some were PCR positive in synovium despite previous treatment with antibiotics."

	Author	*Year*	*Title*	*Journal*
27.	**Donta ST.**	1997	**Tetracycline therapy for chronic Lyme disease.**	Clin Infect Dis, Jul;25 Suppl 1:S52-6
			"Treatment outcomes for seronegative patients (20% of all patients) were similar to those for seropositive patients. Western immunoblotting showed reactions to one or more Borrelia burgdorferi-specific proteins for 65% of the patients for whom enzyme-linked immunosorbent assays were negative."	
28.	**Hauser U; Wilske B.**	1997	**Enzyme-linked immunosorbent assays with recombinant internal flagellin fragments derived from different species of Borrelia burgdorferi sensu lato for the serodiagnosis of of Lyme.**	Medical Microbiology & Immunology. 186(2-3):145-51
			[From the abstract:] "The serodiagnosis of early Lyme neuroborreliosis is hampered by false negative results and one of the reasons could be the heterogeneity of strains of Borrelia burgdorferi sensu lato."	
29.	**Pradella SP; Krause A; Muller A.**	1997	**Acute Borrelia infection. Unilateral papillitis as isolated clinical manifestation.**	Ophthalmologe, Aug;94(8):591-4
			[From the abstract:] "Seronegative values in subjects strongly suspected of having Lyme disease do not necessarily exclude the diagnosis of Lyme disease."	
30.	**Schumacher HR.**	1997	**PCR evidence for Borrelia burgdorferi DNA in synovium in absence of positive serology.**	Abstract ACR 61st National Scientific Meeting November 8-12
31.	**Aberer E; Kersten A; Klade H; Poitschek C; Jurecka W.**	1996	**Heterogeneity of Borrelia burgdorferi in the skin.**	American Journal of Dermatopathology, 18(6):571-9
			"Neuralgias arising 6 months after ECM in spite of antibiotic therapy were evident in a seronegative patient who showed perineural rod-like borrelia structures."	
			"The morphological forms of borreliae seen in biopsies were correlated with clinical findings. Seropositive patients showed clumped and agglutinated borreliae in tissue, whereas seronegative patients exhibited borreliae colony formation (n=2). ...the behavior of borreliae within collagen fibers is strongly influenced by immune recognition by the patient. Borrelia may escape immune surveillance by colony formation and masking within collagen, resulting in seronegativity."	
32.	**Breier P; Klade H; Stanek G; Poitschek C; Kirnbauer R; Dorda W; Aberer E.**	1996	**Lymphoproliferative responses to Borrelia burgdorferi in circumscribed scleroderma.**	Br J Dermatol, 134(2):285-91
			"These findings show that the pattern of Bb-specific immune responses is more complex than previously thought, and underscore the importance of lymphocyte function assays in evaluating the diagnosis of potential Bb infection in seronegative patients."	

	Author	Year	Title	Journal
33.	**Huppertz HI; Mosbauer S; Busch DH; Karch H.**	1996	**Lymphoproliferative responses to Borrelia burgdorferi in the diagnosis of Lyme arthritis in children and adolescents.**	Eur J Pediatr, 155(4):297-302
			"In one patient with seronegative LA [Lyme arthritis] specific lymphocyte proliferation and polymerase chain reaction for borrelial fla sequences in urine were positive."	
34.	**Luft BJ.**	1996	**Chronic Lyme disease: an evolving syndrome.**	9th Annual International Scientific Conference on Lyme Disease & Other Tick-Borne Disorders, Boston, MA, April 19-20
			[From the abstract:] "In the case of the ticks, environmental factors such as temperature, humidity and source of blood meal may alter the major outer surface proteins (Osp) of the spirochete within the tick vector. ...Humans with chronic arthritis are more likely to show an immune response to Osp A." *[Seronegativity:] "Chronic Lyme disease patients may be seropositive or seronegative with or without a documented history of Lyme disease."* *[Diagnosis:] "Since Lyme disease is a clinical diagnosis, research must continue to improve diagnostic assays using recombinant proteins which are more sensitive and specific than the whole organism sonicate used for both ELISA and Western blots."*	
35.	**Luft BJ; Dattwyler RJ; Johnson RC; Luger SW; Bosler EM; Rahn DW; et al.**	1996	**Azithromycin compared with amoxicillin in the treatment of erythema migrans. A double-blind, randomized, controlled trial.**	Annals of Internal Medicine, 124(9):785-91
			"Fifty-seven percent of patients who had relapse were seronegative at the time of relapse."	
36.	**Mouritsen CL; Wittwer CT; Litwin CM; Yang L; Weis JJ; Martins TB; Jaskowski TD; Hill HR.**	1996	**Polymerase chain reaction detection of Lyme disease: correlation with clinical manifestations and serologic responses.**	American Journal of Clinical Pathology. 105(5):647-54
			[From the abstract:] "...nine serum samples and one synovial fluid from patients with definite clinical features of Lyme disease were found to be negative by EIA and Western blot analysis for IgG and IgM antibody, but contained B burgdorferi DNA, as detected by PCR. Polymerase chain reaction analysis of serum and synovial fluid may be of significant diagnostic value in Lyme disease, especially in the absence of a serologic response in early, partially treated and seronegative chronic disease....This is the first study to report an association between PCR positivity and the absence of a serologic response to Lyme borreliosis."	
37.	**Mursic VP; Wanner G; Reinhardt S; Wilske B; et al.**	1996	**Formation and cultivation of Borrelia burgdorferi spheroplast L-form variants.**	Infection, 24(3):218-26
			This study investigated In vitro morphological variants of B. burgdorferi, in an effort to explain the clinical persistence of active Lyme borreliosis despite antibiotic therapy. The authors suggest that these atypical forms may allow Borrelia to survive antibiotic treatment. *"Penicillin G was the most effective inducer of SL-forms [spheroplast-L-forms). The reversion of this form to the helical parental forms was mostly achieved by cultivation of isolated SL-colonies in penicillin G-free medium. The atypical forms isolated from patients treated with antibiotics show similar features. The same effect is probably obtained with all other ß-lactam antibiotics."* *"With regard to the polyphasic course of Lyme borreliosis, these forms without cell walls can be a possible reason why Borrelia survive in the organism for a long time (probably with all beta-lactam antibiotics) [corrected] and the cell-wall-dependent antibody titers disappear and emerge after reversion."*	
38.	**Preac Mursic V; Marget W; Busch U; Pleterski Rigler D; Hagl S.**	1996	**Kill kinetics of Borrelia burgdorferi and bacterial findings in relation to the treatment of Lyme borreliosis.**	Infection, 24(1):9-16
			"The patients had clinical disease with or without diagnostic antibody titers to B. burgdorferi."	

	Author	Year	Title	Journal
39.	**Pachner A.**	1995	**Early disseminated Lyme disease.**	Am J Med, 98 (suppl 4A):4A-30S-51S – Discussion.

"The correlation between a positive Western blot and Lyme arthritis is probably the best of almost any Western blot and any Lyme disease manifestation. With neurologic disease, I have had a lot of patients who don't have a positive Western blot; they just have not developed a peripheral antibody response, for whatever reason."

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40. **Coyle PK; Schutzer SE; Deng Z; Krupp LB; Belman MD; Benach JL; Luft BJ.** 1995 **Detection of Borrelia burgdorferi-specific antigen in antibody negative cerebrospinal fluid in neurologic Lyme disease.** Neurology, 45:2010-2014

[From the abstract:] " RESULTS: Of the 35 of 83 (42%) patients who were positive for OspA antigen in their CSF, 15 (43%) were antigen positive despite being antibody-negative in CSF. Seven of these 15 (47%) had otherwise normal routine CSF analyses. Six of these 15 (40%) patients met strict CDC surveillance criteria for Lyme disease: four (27%) patients had seroconversion coincident with new neurologic problems; and three (20%) with characteristic syndromes for Lyme disease were seronegative, but had complexed antibody to B. burgdorferi. The final two patients (13%) were seropositive and had unexplained neurologic problems not characteristic of Lyme disease. CONCLUSIONS: B. burgdorferi antigen can be detected in CSF that is otherwise normal by conventional methodology, and can be present without positive CSF antibody. Since CSF antigen implies intrathecal seeding of the infection, the diagnosis of neurologic infection by B. burgdorferi should not be excluded solely on the basis of normal routine CSF or negative CSF antibody analyses."

[From the article:] "Prompt and precise diagnosis is difficult because basic microbiologic tests such as culture and staining have not been useful, on a broad scale, to document the presence of the spirochete in a body fluid. Instead, detection of specific antibodies to B burgdorferi in blood and CSF is commonly used to support or refute a clinical suspicion of infection. Many of the commercially available assays have been plagued by lack of sensitivity, specificity, and reproducibility. Furthermore, the absence of free antibodies to B burgdorferi components has been documented in well-characterized erythema-migrans-positive cases of Lyme disease, including those with prominent neurologic involvement."

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41. **Karma A; Seppala I; Mikkila H; Kaakkola S; Viljanen M; Tarkkanen A.** 1995 **Diagnosis and clinical characteristics of ocular Lyme borreliosis.** American Journal of Ophthalmology, 119(2):127-35

[From the abstract:] "Results of ELISA disclosed that five patients [out of ten] were seropositive, two patients showed borderline reactivity, and three patients were seronegative. Four of the five patients with borderline or negative results by ELISA had a positive result by western blot analysis. ... CONCLUSIONS: Late-phase ocular Lyme borreliosis is probably underdiagnosed because of weak seropositivity or seronegativity in ELISA assays."

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42. **Lawrence C; Lipton RB; Lowy FD; Coyle PK.** 1995 **Seronegative chronic relapsing neuroborreliosis.** European Neurology, 35(2):113-7

[From the abstract:] This article reports a Lyme disease patient "who experienced repeated neurologic relapses despite aggressive antibiotic therapy." The patient was seronegative. "Although the patient never had detectable free antibodies to B. burgdorferi in serum or spinal fluid, the CSF was positive on multiple occasions for complexed anti-B. burgdorferi antibodies, B. burgdorferi nucleic acids and free antigen."

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43. **Millner M.** 1995 **Neurologic manifestations of Lyme borreliosis in children.** Wiener Medizinische Wochenschrift,145(7-8):178-82

"Our own observations in children which suffered from an acute neuroborreliosis (NB) showed the following:... Indeed, there is a seronegative NB also in children."

	Author	*Year*	*Title*	*Journal*
44.	**Oksi J; Uksila J; Marjamaki M; Nikoskelainen J; Viljanen MK.**	1995	**Antibodies against whole sonicated Borrelia burgdorferi spirochetes, 41-Kilodalton flagellin, and P39 protein in patients with PCR- or culture-proven late Lyme borreliosis.**	Journal of Clinical Microbiology, 33(9):2260-4
			[From the abstract:] "These results show that antibodies to B. burgdorferi may be present in low levels or even absent in patients with culture- or PCR-proven late LB [Lyme borreliosis]. Therefore, in addition to serological testing, the use of PCR and cultivation is recommended in the diagnosis of LB."	
45.	**Skripnikova IA; Anan'eva LP; Barskova VG; Ushakova MA.**	1995	**The humoral immunological response of patients with Lyme disease.**	Ter Arkh, 67(11):53-6
			"Both acute and chronic borreliosis can be seropositive or seronegative."	
46.	**Schubert HD; Greenebaum E; Neu HC.**	1994	**Cytologically proven seronegative Lyme choroiditis and vitritis.**	Retina, 14(1):39-42
			[From the abstract:] "RESULTS: Intravitreal spirochetes consistent with Borrelia burgdorferi were found in this seronegative patient. CONCLUSION: Vitreous specimens of patients with choroiditis and vitritis of unknown cause should be examined cytologically, particularly when serologic results do not corroborate the clinical findings of Lyme disease."	
47.	**Sigal LH.**	1994	**The polymerase chain reaction assay for Borrelia burgdorferi in the diagnosis of Lyme disease.**	Annals of Internal Medicine, 120(6):520-521
			"Polymerase chain reaction may be more sensitive than antibody detection techniques in human Lyme neuroborreliosis [17,19] and the murine experimental model [22] and clearly is more sensitive than current culture techniques. Our experience suggests that a few patients may be positive by PCR despite negative immunologic assay results in inflammatory fluid and blood (Sigal LH and Liebling M. Unpublished observation)."	
48.	**Bojic I; Mijuskovic P; Dokic M; Nozic D; Lako B; et al.**	1993	**Clinical characteristics of Lyme disease.**	Vojnosanit Pregl, 50(4):359-64
			[From the abstract:] "Clinical characteristics of Lyme disease were analysed in 22 patients. Erythema migrans was found in 20 (91%), arthralgia in 18 (81%), neuralgia in 8 (36%), encephalitis in 3 (13%), carditis in 2 (9%) and arthritis in 2 (9%) patients. The positive antibody titer was found in 14 (63%) patients.	
49.	**Coyle PK.**	1993	**Antigen detection and cerebrospinal fluid studies.**	In "Lyme Disease," ed. P. Coyle, p.143
			"...spirochetes show a peculiar feature compared to other bacterial neurologic infections: the organisms can be present in CSF without inducing inflammatory changes. This is well-documented for neurosyphilis, leptospirosis, and relapsing fever, and appears to be occasionally true for Lyme disease as well. In Europe, B. burgdorferi has been cultured from otherwise normal CSF."	
50.	**Häupl T; Hahn G; Rittig M; Krause A; Schoerner C; Schonherr U; et al.**	1993	**Persistence of Borrelia burgdorferi in ligamentous tissue from a patient with chronic Lyme borreliosis.**	Arthritis & Rheumatism, 36(11):1621-6
			[From the abstract:] "The initially significant immune system activation was followed by a loss of the specific humoral immune response and a decrease in the cellular immune response to B burgdorferi over the course of the disease." [From the article:] "Interestingly, the cellular immune responses were also directed against the surface protein OspA during each recurrence of clinical symptoms, even though anti-OspA antibodies were not detectable by immunoblot."	

	Author	*Year*	*Title*	*Journal*
51.	**Hulinska D; Krausova M; Janovska D; et al.**	1993	**Electron microscopy and the polymerase chain reaction of spirochetes from the blood of patients with Lyme disease.**	Central European Journal of Public Health. 1(2):81-5
		[From the abstract:] "Results of studies using direct antigen detection suggest that seronegative Lyme borreliosis is not rare and support the hypothesis that Borrelia antigens can persist in humans."		
52.	**Kazakoff MA; Sinusas K; Macchia C.**	1993	**Liver function test abnormalities in early Lyme disease.**	Arch Fam Med, 2(4):409-13
		[From the abstract:] "PATIENTS: Thirty-seven female and 36 male patients with erythema migrans who had not yet been treated with antimicrobial agents. ... Only seven patients (9%) had a positive titer in response to the enzyme-linked immunosorbent assay for Lyme disease."		
53.	**Liegner KB; Shapiro JR; Ramsay D; Halperin AJ; Hogrefe W; Kong L.**	1993	**Recurrent erythema migrans despite extended antibiotic treatment with minocycline in a patient with persisting Borrelia burgdorferi infection.**	Journal of the American Academy of Dermatology, 28(2 Pt 2):312-4
		[Abstract:] "Erythema migrans recurred in a patient 6 months after a course of treatment with minocycline for Lyme disease. Polymerase chain reaction on heparinized peripheral blood at that time demonstrated the presence of Borrelia burgdorferi-specific DNA. The patient was seronegative by Lyme enzyme-linked immunosorbent assay but showed suspicious bands on Western blot. Findings of a Warthin-Starry stain of a skin biopsy specimen of the eruption revealed a Borrelia-compatible structure. Reinfection was not believed to have occurred. Further treatment with minocycline led to resolution of the erythema migrans."		
54.	**Schutzer SE.**	1993	**Seronegative Lyme disease.**	In "Lyme Disease," ed. P. Coyle, p.192
		"The number and percentage of seronegative Lyme disease cases remain controversial. At some academic centers the estimate is 5%, and in certain private settings the number may be higher. There is little question that seronegative Lyme disease can exist."		
55.	**Sigal LH.**	1993	**Lyme disease: testing and treatment. Who should be tested and treated for Lyme disease**	Rheum Dis Clin North Am, 19(1):79-93
		[From the abstract:] "LD is not a diagnosis that can be made on the basis of serologic testing. By this is meant that vague symptoms plus a positive serologic test do not assure that the patient has LD. On the other hand, a patient with ECM or other manifestations of LD may still be seronegative."		
56.	**Steere AC.**	1993	**Seronegative Lyme disease.**	JAMA, (270):1369.
		"The number and percentage of seronegative Lyme disease cases remain controversial. At some academic centers the estimate is 5%, and in certain private settings the number may be higher. There is little question that seronegative Lyme disease can exist."		
57.	**Preac-Mursic V; Pfister HW; Spiegel H; Burk R; Wilske B; el al.**	1993	**First isolation of Borrelia burgdorferi from an iris biopsy.**	J Clin Neuroophthalmology, 13(3):155-61; discussion 162.
		[From the abstract:] "Antibiotic therapy may abrogate the antibody response to the infection as shown by our results. Patients may have subclinical or clinical disease without diagnostic antibody titers. Persistence of B. burgdorferi cannot be excluded when the serum is negative for antibodies against it."		
58.	**Oksi J; Viljanen MK; Kalimo H; Peltonen R; Marttia R; Salomaa P; et al.**	1993	**Fatal encephalitis caused by concomitant infection with tick-borne encephalitis virus and Borrelia burgdorferi.**	Clinical Infectious Diseases, 16(3):392-6
		"Serology for Borrelia was negative. Autopsy revealed necrotizing encephalitis and myelitis with involvement of the dorsal root ganglion. With use of polymerase chain reaction tests, segments of two separate genes of B. burgdorferi were amplified from the patient's CSF."		

	Author	Year	Title	Journal
59.	**Keller TL; Halperin JJ; Whitman M.**	1992	**PCR detection of Borrelia burgdorferi DNA in cerebrospinal fluid of Lyme neuroborreliosis patients.**	Neurology, 42(1):32-42
			"PCR detected B burgdorferi DNA in the CSF of seven patients whose blood serology ...failed to demonstrate prior exposure to the organism. The failure of existing diagnostic methods to detect patients exhibiting well-recognized manifestations of Lyme disease has been described. This has generally been attributed to abrogation of the host immune response by noncurative antimicrobial treatment. This explanation seems unlikely in at least four of the seven patients who had no recollection of having received antibiotics. "	
60.	**Banyas GT.**	1992	**Difficulties with Lyme serology.**	J Am Optom Assoc, 63(2):135-9
			[From the abstract:] "A major problem has been seronegativity in persons possessing the disease (false negatives). At present, seronegativity in persons strongly suspected of having Lyme disease does not necessarily exclude the diagnosis of Lyme disease. The clinician must recognize this in patients who may have Lyme disease or a recurrence of the disease."	
61.	**Dinerman H; Steere AC.**	1992	**Lyme disease associated with fibromyalgia.**	Annals of Internal Medicine, 117:281-5
			"The small percentage of patients who are seronegative by enzyme-linked immunosorbent assay (ELISA) later in the illness usually have positive Western blots or cellular immune responses to borrelial antigens (9,10)."	
62.	**Fraser DD; Kong LI; Miller FW.**	1992	**Molecular detection of persistent Borrelia burgdorferi in a man with dermatomyositis.**	Clinical & Experimental Rheumatology, 10(4):387-90.
			[From the abstract:] "Antibiotic therapy was reinstituted after Borrelia burgdorferi was detected in the patient's peripheral blood leukocytes by the polymerase chain reaction (PCR). All serologic, T-cell stimulation, and western blot analyses, however, were negative... In addition, this case emphasizes the potential clinical utility of PCR technology in evaluating the persistent sero-negative Lyme disease which may occur in immunocompromised individuals."	
63.	**Keller TL; Halperin JJ; Whitman M.**	1992	**PCR detection of Borrelia burgdorferi DNA in cerebrospinal fluid of Lyme neuroborreliosis patients.**	Neurology, 42(1):32-42
			[Abstract:] "We used the polymerase chain reaction (PCR), a method useful in the detection of Borrelia burgdorferi in vitro, to evaluate CSF in patients thought to have neuroborreliosis. Nested pairs of oligonucleotide primers were designed to recognize the C-terminal region of B burgdorferi OspA. CSF samples were obtained from (1) patients with immunologic evidence of systemic B burgdorferi infection and clinical manifestations suggestive of CNS dysfunction, (2) seronegative patients with clinical disorders consistent with Lyme borreliosis, and (3) patient and contamination controls; all were analyzed in a blinded fashion. PCR detected B burgdorferi OspA DNA in CSF of (1) 10 of 11 patients with Lyme encephalopathy, (2) 28 of 37 patients with inflammatory CNS disease, (3) seven of seven seronegative patients with Lyme-compatible disorders, and (4) zero of 23 patient controls. Zero of 83 additional contamination controls were PCR-positive"	
64.	**Faller J; Thompson F; Hamilton W.**	1991	**PCR detection of Borrelia burgdorferi DNA in cerebrospinal fluid of Lyme neuroborreliosis patients.**	Foot & Ankle, 11(4):236-238.
			"Patient 8 had several negative ELISA assays. She then had a lymphocyte reactivity test for cell mediated immune (CMI) response to Borrelia burgdorferi antigen. Her peripheral blood lymphocytes were markedly responsive to the spirochete, with an index of 46 (18 is three standard deviations above the controls.)"	
65.	**Reik L, Jr.**	1991	**Lyme Disease and the Nervous System.**	New York: Thieme Medical Publishers, Inc.
			"In some cases, specific serum antibody is present but sequestered in immune complexes, and therefore not measurable by routine ELISA." *"...test results for serum antibodies are not always positive when neurologic abnormalities develop, especially in stage 2."*	
66.	**Havlik J; Rohacova H; Hulinska D; et al.**	1991	**Seronegative Lyme borreliosis.**	The 5th European Congress of Clinical Microbiol. and Infect. Diseases, Sept. 8-11, Oslo, Norway:1385.
67.	**Nields JA; Kueton JF.**	1991	**Tullio phenomenon and seronegative Lyme borreliosis.**	Lancet, 338(8759):128-9
68.	**Steere AC.**	1991	**Rheumatology research in the 90s.**	Rheumatology News

	Author	*Year*	*Title*	*Journal*
69.	**Nadelman RB; Pavia CS; Magnarelle LA; Wormser GP.**	1990	**Isolation of Borrelia burgdorferi from the blood of seven patients with Lyme disease.**	American Journal of Medicine, 88:21-6
			"The absence of significant antibody titers to B. burgdorferi is not uncommon in Lyme disease, especially in early disease. ...Although it was initially believed that patients with neurologic Lyme disease generally have antibodies to B. burgdorferi, this may not always be the case. ...We would advise that in an endemic area, the differential diagnosis of nonspecific muscle and joint aches without rash should include Lyme disease--even in the absence of antibodies to B. burgdorferi."	
70.	**Schutzer SE; Coyle PK; Belman AL; Golightly MG; Drulle J.**	1990	**Sequestration of antibody to Borrelia burgdorferi in immune complexes in seronegative Lyme disease.**	Lancet, 335(8685):312-5
			[From the abstract:] "Apparent B burgdorferi seronegativity in serum immune complexes may thus be due to sequestration of antibody in immune complexes."	
71.	**Bianchi G; Rovetta G; Monteforte P; Fumarola D; et al.**	1990	**Articular involvement in European patients with Lyme disease. A report of 32 Italian patients.**	British Journal of Rheumatology, 29(3):178-80
			[From the abstract:] "In addition, interpreting serological tests for antibodies against B. burgdorferi and the real prevalence of arthritis in LD [Lyme disease] is complicated by the possible existence of seronegative LD and by the effect of early antibiotic treatment."	
72.	**Dieterle L; Kubina FG; Staudacher T; Budingen HJ.**	1989	**Neuro-borreliosis or intervertebral disk prolapse?**	Dtsch Med Wochenschr, 114(42):1602-6
			[From the abstract "Between September 1986 and November 1988, 17 patients were hospitalized and treated for neuro-borreliosis. ...Three of 14 patients had no IgG antibodies against Borrelia, either in serum or cerebrospinal fluid at the initial examination, two had positive titres in serum only.	
73.	**Guy EC; Turner AM.**	1989	**Seronegative neuroborreliosis.**	Lancet, 1:441
			"We wish to report a case with a clinical diagnosis of acute Lyme neuroborreliosis for whom negative serology was reported by a Lyme disease referral centre using ELISA. ... *Western blot analysis of sera revealed both IgM and IgG binding to several B burgdorferi proteins, compatible with acute Lyme disease. Antibody binding to a larger number of B burgdorferi proteins was found when the CSF was tested similarly. Our observation of a more diverse antibody response in CSF compared with serum is consistent with the findings of a previous study which showed 44% of patients with neurological manifestations of Lyme disease to have positive CSF antibody titres but negative serum titres when measured by ELISA. Analysis of CSF for the detection of IgM and IgG binding to B burgdorferi is essential if seronegative cases of acute Lyme neuroborreliosis are to be identified."*	
74.	**MacDonald AB.**	1989	**Gestational Lyme borreliosis. Implications for the fetus.**	Rheum Dis Clin North Am, 15(4):657-77
			"From a biologic perspective, most of the fatal cases of LB [Lyme borreliosis] in pregnancy were reactive either in titers in the borderline region or were completely nonreactive in serologic tests. The tendency toward seronegativity in pregnancy makes maternal serology a less satisfactory discriminator of maternal infection and useless as a practical tool to predict the actual state of the fetus..."	
75.	**Trock DH; Craft JE; Rahn DW.**	1989	**Clinical manifestations of Lyme disease in the United States.**	Connecticut Medicine, Vol 53, No. 6
			"...cases of seronegative Lyme disease have been reported..."	

	Author	*Year*	*Title*	*Journal*
76.	**Dattwyler RJ; Volkman DJ; Luft BJ.**	1989	**Immunologic aspects of Lyme borreliosis.**	Rev Infect Dis, II(Suppl 6):S1494-98.
			"Three separate groups of investigators have reported individuals who lacked diagnostic levels of specific antibody in their serum, yet had neurologic involvement and diagnostic levels of antibody in their CSF. We have confirmed this finding in our laboratory."	
77.	**Preac-Mursic V; Weber K; Pfister HW; Wilske B; Gross B; Baumann A; Prokop J.**	1989	**Survival of Borrelia burgdorferi in antibiotically treated patients with Lyme borreliosis.**	Infection, 17(6):355-9
			[From the abstract:] "We conclude that early stage of the disease as well as chronic Lyme disease with persistence of B. burgdorferi after antibiotic therapy cannot be excluded when the serum is negative for antibodies against B. burgdorferi."	
			[Seronegativity:] "As shown, negative antibody-titers do not provide evidence for successful therapy; antibody-titers may become negative despite persistence of B. burgdorferi." (p.358)	
78.	**Berger BW; MacDonald AB; Benach JL.**	1988	**Use of an autologous antigen in the serologic testing of patients with erythema migrans of Lyme disease.**	Journal of the American Academy of Dermatology, 18(6):1243-6
			[Abstract:] "We attempted to detect an early rise in antibody titers to Borrelia burgdorferi in the serum of patients with erythema migrans of Lyme disease by utilizing B. burgdorferi isolates obtained from patients' own skin lesions instead of the B31 reference strain. B. burgdorferi was isolated from nine of 23 skin biopsy specimens submitted for culture. Elevated antibody titers were not detected in any of the 23 acute serum samples by immunofluorescence assay. The antigens derived from patient isolates were no more effective than the reference strain in detecting antibodies in patients with early Lyme disease."	
79.	**Dattwyler RJ; Volkman DJ; Luft BJ; Halperin JJ; Thomas J; Golightly MG.**	1988	**Seronegative Lyme disease. Dissociation of specific T- and B-lymphocyte responses to Borrelia burgdorferi.**	New England Journal of Medicine, 1;319(22):1441-6
			[From the abstract:] "Although these patients had clinically active disease, none had diagnostic levels of antibodies to B. burgdorferi on either a standard enzyme-*linked immunosorbent assay or immunoflourescence assy. ...We conclude that the presence of chronic Lyme disease cannot be excluded by the absence of antibodies against B. burgdorferi and that a specific T-cell blastogenic response to B. burgdorferi is evidence of infection in seronegative patients with clinical indications of chronic Lyme disease."*	
80.	**Pachner AR.**	1988	**Borrelia burgdorferi in the nervous system: the new "great imitator".**	Annals of the New York Academy of Sciences, 539:56-64
			"The antibody response in serum in CNS Lyme disease seems to be related to the presence of other manifestations; patients who have had both arthritis and CNS disease have quite high titers, while those with only CNS disease sometimes do not."	
81.	**Lavoie PE; Lattner BP; Duray PH; Barbour AG; Johnson HC.**	1987	**Culture positive seronegative transplacental Lyme borreliosis infant mortality.**	Arthritis Rheum, Vol 30 No 4, 3(Suppl):S50
			"We report a culture positive neonatal death occurring in California, a low endemic region. ...Bb was grown from a frontal cerebral cortex inoculation. The spirochete appeared similar to the original Long Island tick isolate. Silver stain of brain & heart was confirmatory of tissue infection. The mother had been having migratory arthralgias and malaise since experiencing horse fly & mosquito bites while camping on the Maine coast in 1971. The family was seronegative for LB by ELISA at Yale. Cardiolipin antibodies were also not found."	
82.	**MacDonald AB.**	1987	**Lyme disease. A neuro-ophthalmologic view.**	Journal of Clinical Neuro-Ophthalmology, 7(4):185-90
			[From the abstract:] "Potential pitfalls in the diagnosis of Lyme disease with an emphasis on false negative serology and currently available diagnostic modalities are presented."	

	Author	Year	Title	Journal
83.	**Hederstedt B; Hovmark A; Stiernstedt G; Asbring E.**	1986	**[Borrelia-diagnos aktuell aret om visar serologisk undersokning av 1985 ars fall.]**	Lakartidningen, 83:3987-89

[According to Guy & Turner, 1989, this study found that 44% of patients with neurological Lyme disease had positive CSF antibody titres but negative serum titres when measured using ELISA testing.]

84.	**Schmidt R; Kabatzki J; Hartung S; Ackermann R.**	1985	**[Erythema migrans borreliosis in the Federal Republic of Germany. Epidemiology and clinical aspects.]**	Deutsche Medizinische Wochenschrift, 110(47):1803-7

[Abstract:] "A positive antibody titre against Ixodes-ricinus-Borrelia (burgdorferi), using indirect immunofluorescence or ELISA, could be detected in serum and (or) liquor of 935 (32%) out of a total of 2955 patients between January 1984 and July 1985. In 289 of these cases the typical clinical manifestations were lacking whereas a characteristic disease picture enabled a diagnosis to be made in 171 patients with negative or borderline antibody titres. The 1106 cases of infection observed covered all regions of the country. A typical clinical syndrome was seen in 817 (74%) of these. Most common were erythema chronicum migrans (n = 458) and meningopolyneuritis Garin-Bujadoux-Bannwarth (n = 404); in 42% of the cases meningopolyneuritis was preceded by an erythema. Arthritis (n = 63), acrodermatitis chronica atrophicans (n = 72), carditis (n = 13) and lymphadenosis benigna cutis (n = 5) were much less common. Chronic Borrelian encephalomyelitis (n = 45) appeared surprisingly often (n = 45). The fact that in 73% of cases the various syndromes appeared alone, were double in 24% and combined only in 3%, illustrates the polymorphic nature of this disease."

Other Spirochetes

85.	**Stephan C; Hunfeld KP; Schonberg A; et al.**	2000	**[Leptospirosis after a staff outing].**	Dtsch Med Wochenschr, 125(20):623-627.

"[From the abstract:] "The symptoms are non-specific and, moreover, in some cases the laboratory tests are negative, so that clinical diagnosis remains crucial."

86.	**Jacobs R.**	1999	**Infectious Diseases: Spirochetal. Syphilis.**	Current Medical Diagnosis & Treatment 1999. 38th Edition. Appleton & Lange. Stamford, CT. Ed. Tierney LM Jr; McPhee SJ, Papadakis MA.

"The VDRL titer is usually high (>1:32) in secondary syphilis and tends to be lower (< 1:4) or even negative in late forms of syphilis."

87.	**Vecsei AK, Vecsei PV, Dangl-Erlach E,**	1999	**[Congenital syphilis: late diagnosis in spite of screening].**	Wien Klin Wochenschr, 111(10):410-3

[Abstract:] "We report the case of an infant in whom congenital syphilis was diagnosed at the age of 5 weeks. The case is remarkable because of (a) the negative venereal disease laboratory test from the cord blood, (b) the incidental diagnosis of the disease in the fifth week of life, (c) pneumonia alba being one of the symptoms, (d) the occurrence of a mild Jarisch-Herxheimer reaction after initiation of penicillin therapy and (e) the successful treatment of infection related anaemia with recombinant human erythropoietin."

	Author	*Year*	*Title*	*Journal*
88.	**Uribe CS; Garcia FA.**	1998	**[Neurosyphilis and the prozone effect].**	Rev Neurol, 27(160):970-2
	[Abstract:] "INTRODUCTION: Neurosyphilis (NS) is an entity which still frequently presents to our Neurology Department. The prozone phenomenon occurs in approximately 2% of all cases of late primary syphilis or secondary syphilis; we have found no cases described of prozone and neurosyphilis occurring together. CLINICAL CASE: We present the unusual case of a 44 year old patient with NS and dementia PGP (progressive general paralysis). Initially serum VDRL was negative, but in CSF reacted at dilutions of 1:32. When serum VDRL was repeated using dilutions, it was reactive 1:128 and serum FTA was also reactive. The patient was treated with i.v. crystalline penicillin, after which his condition improved. CONCLUSIONS: We wish to draw attention to the possibility that patients with a dementia syndrome and negative serum VDRL may have the prozone phenomenon, and the laboratory should therefore be asked to do serial dilutions."		
89.	**Tikjob G; Russel M; Petersen CS; Gerstoft J; Kobayasi T.**	1991	**Seronegative secondary syphilis in a patient with AIDS: identification of Treponema pallidum in biopsy specimen.**	Journal of the American Academy of Dermatology, 24(3):506-8
90.	**Berkowitz K; Baxi L; Fox HE.**	1990	**False-negative syphilis screening: the prozone phenomenon, nonimmune hydrops, and diagnosis of syphilis during pregnancy.**	Am J Obstet Gynecol, 163(3):975-7
	[From the abstract:] "Recently we encountered four cases of false-negative syphilis serologic results in women who gave birth to infants with congenital syphilis. The false-negative results were caused by the prozone phenomenon. The prozone phenomenon, seen during primary and secondary syphilis, occurs because a higher than optimal amount of antibody in the tested sera prevents the flocculation reaction typifying a positive result in reagin tests. Serum dilution is necessary to make the correct diagnosis. We recommend that for any pregnant woman with apparently negative syphilis serologic results in whom fetal compromise of unknown etiology exists, particularly nonimmune hydrops, nontreponemal testing should be repeated using serum dilutions to prevent a missed diagnosis of syphilis. We further recommend serum dilution as a routine procedure for all pregnant women in areas of high syphilis prevalence."		
91.	**Borisenko KK; Vinokurov IN; Toporovskii LM.**	1989	**[Characteristics of the course of latent forms of syphilis].**	Vestn Dermatol Venerol, (11):25-9
	[Abstract:] "Seven patients with latent syphilis are described, in whom the routine serologic tests (RST) were negative during the first examination and over the course of therapy, and the specific tests (T. pallidum immobilization and immunofluorescence) were repeatedly positive before therapy. Early latent seropositive recurrent forms of syphilis were detected in the majority of these patients' sexual partners. The patients were not administered antisyphilis therapy before. The diagnosis of latent seronegative early syphilis negative in the RST is epidemiologically significant, for it helps timely carry out the necessary treatment and prophylaxis measures to prevent the disease dissemination."		
92.	**Ovcinnikov NM.**	1981	**[Important problems in the serodiagnosis of syphilis.].**	Vestn Dermatol Venerol, 8:22-26
	[According to Mattman L., 1993: "It is thought [by Ovcinnikov] that false negative serological tests for syphilis may be explained because cystic and granule stages of the treponeme have not stimulated antibody reactive with the spirochetal stage."]			
93.	**Il'in II. Pakhomova LV.**	1981	**[Seronegative forms of latent syphilis].**	Vestnik Dermatologii i Venerologii. (1):66-9

	Author	*Year*	*Title*	*Journal*
94.	**Sparling PF.**	1971	**Diagnosis and treatment of syphilis.**	New England Journal of Medicine, 284: 642-653

Some infected patients had negative or equivocal serologic tests for syphilis. “These studies [reviewed] emphasize the fact that late syphilis can occur even if all serologic tests are negative.”

Includes a review of recent [as of 1971] evidence indicating that penicillin treatment is not always curative in patients with late syphilis. "Penicillin therapy of neurosyphilis has not been as effective [as in early syphilis]. Several studies have reported relapses... Clinical progression of symptomatic neurosyphilis is relatively common despite antibiotics." (p.650)

95.	**Ch'ien L. Hathaway BM. Israel CW.**	1970	**Seronegative dementia paralytica: report of a case.**	Journal of Neurology, Neurosurgery & Psychiatry. 33(3):376-80
96.	**Hallock J; Tunnessen WW.**	1968	**Congenital syphilis in an infant of a seronegative mother.**	Obstet Gynecol, 32(3):336-8
97.	**Roitburd MF.**	1968	**[2 cases of seronegativity in secondary syphilis].**	Vestn Dermatol Venerol, 42(8):82-3
98.	**Smith JL.**	1968	**Spirochetes in late seronegative syphilis, despite penicillin therapy.**	Med Times, 96(6):611-23
99.	**Smith JL; Israel CW.**	1967	**Spirochetes in the aqueous humor in seronegative ocular syphilis. Persistence after penicillin therapy.**	Archives of Ophthalmology, 44:474-477

"The purpose of this communication is to report the presence of spirochetes in the aqueous humor both before and after the intramuscular administration of 9,200,000 units of long-acting penicillin for seronegative ocular syphilis. The treponemes were found by the flourescein antibody technique, in which the spirochetes stained with flourescein tagged anti-Treponema pallidum globulin when viewed with ultraviolet microscopy. ...

Spirochetes have now been found in aqueous humor, cerebrospinal fluid, liver, and lymph nodes in several patients with late seronegative syphilis."

100.	**Smith JL; Israel CW.**	1967	**The presence of spirochetes in late seronegative syphilis.**	JAMA, 199(13):980-984

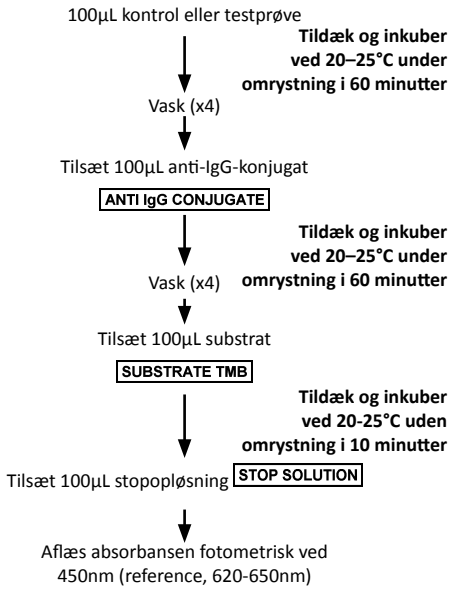
[Abstract:] "Late seronegative syphilis refers to clinical signs of ocular or neurosyphilis in a patient whose routine blood (reagin) test is nonreactive, but in whom a specific treponemal test is reactive. This report documents the presence of spirochetes in aqueous humor, cerebrospinal fluid, and at liver biopsy in such patients. Spirochetes have been found in the aqueous humor with no biomicroscopic abnormality and in cerebrospinal fluids which had normal cell counts, protein levels, and reagin and colloidal gold test results. Identification of the organisms depends on use of the flourescein antibody technique, in that spirochetes stain with flourescein-tagged anti-Treponema pallidum globulin on ultraviolet microscopy."

[From the article:] "The finding of motile spirochetes in the aqueous humor of animal eyes which showed no clinical signs of inflammation at the time of paracentesis led to the initial clinical studies reported here. It must be emphasized that several patients were found to have negative findings in darkfield examinations of aqueous humor and cerebrospinal fluid, in which later study with the fluorescent antibody technique revealed morphologically typical spirochetes which stained with anti-T pallidum globulin. Darkfield examination of CSF requires that the fluid be centrifuged and examined within ten minutes after lumbar puncture in order to see motile treponemes."

Author	*Year*	*Title*	*Journal*
101. **Smith JL; Singer JA; Moore MB, Jr; Yobs AR.**	1965	**Sero-negative ocular and neuro-syphilis.**	American Journal of Ophthalmology, 59:753-762
102. **Taylor WH; Smith JL; Singer JA.**	1965	**Experimental seronegative syphilis.**	American Journal of Ophthalmology, 60:1093-1098
103. **McCord.**	1929	**Study of two hundred autopsies made on syphilitic foetuses.**	Am J Obst & Gynec, 18:597

brug

Fortynd serumprøverne 1/200 ved tilsætning af 10µL serum til 2mL prøvediluent.



11. KVALITETSKONTROL OG FORTOLKNING AF TESTRESULTATERNE

11.1. PRØVEDILUENT

OD-værdien af brønden med prøvediluent skal være mindre end 0,100, men større end 0,000 (dobbelt bølgelængde). Hvis værdien er over 0,100, kan utilstrækkelig vask eller kontaminering af substratet være årsagen. Hvis værdien er under 0,000, bør pladeaflæseren nulstilles mod luft på ny, og mikrobrøndene aflæses igen.

Hvis kvalitetskontrolkravene ikke er opfyldt, er testresultaterne ugyldige, og analysen skal gentages.

11.2. IgG-CUT-OFF-KONTROL OG POSITIV IgG-KONTROL

Beregn middel-OD-værdierne for de 3-IgG-Cut-Off-kontrolmikrobrønde ($OD_{IgG\ Cut\text{-}Off}$) og for de 2 positive IgG-kontrolmikrobrønde ($OD_{IgGPositiv}$). De enkelte OD-værdier bør ikke afvige mere end 25% fra middel-OD-værdien. Hvis en af OD-værdierne for IgG-Cut-Off-kontrollen afviger mere end 25% fra middel-OD-værdien, bør den udelukkes fra beregningen, og middelværdien skal beregnes igen.

Forskellen mellem OD-værdien for Cut-Off-kontrol og positiv IgG-kontrol skal være mindst 0,500. Hvis forskellen er mindre end 0,500, kan det skyldes utilstrækkelig vask, utilstrækkelig omrystning under inkuberingerne eller for lav omgivende temperatur navnlig under inkubation med substratet.

Hvis kvalitetskontrolkravene ikke er opfyldt, er testresultaterne ugyldige, og analysen skal gentages.

11.3. PATIENTPRØVER

Beregn middel-OD-værdien for hver patientprøve ($OD_{prøve}$). De enkelte OD-værdier bør ikke afvige mere end 25% fra middelværdien. Alle sådanne prøver bør analyseres igen. Hvis imidlertid begge testmikrobrønde viser et negativt resultat, kan en forskel på mere end 25% være acceptabelt uden ny analyse, da lave OD-værdier måles med mindre præcision.

11.4. FORTOLKNING AF RESULTATER

Testen IDEIA Borrelia burgdorferi IgG omfatter en cut-off-kontrol indeholdende en bestemt mængde af IgG-antistoffet Borrelia burgdorferi. Det niveau af antistoffet, som påvises over cut-off, er i høj grad indikativ for aktiv Borrelia burgdorferi-infektion. IDEIA Borrelia burgdorferi IgG-kittet kan påvise antistoffet ved et niveau under cut-off, og antistoffet kan være tilstede i form af latent antistof fra tidligere infektion eller et meget lavt niveau af antistof straks efter en nylig infektion. Det lave antistofniveau skal fortolkes forsigtigt og det anbefales at der for at være sikker på betydningen af det lave antistofniveau følges op med prøvetagning hos patienterne efter mindst 2 uger for at identificere forandring i antistofniveauet, der bedre kan angive betydningen af antistoffet.

11.4.1 Kvalitativ fortolkning

Grænsen for påvisning ($OD_{IgG\ DETECTION}$) for IgG-antistof vedr. Borrelia burgdorferi beregnes som $OD_{Cut\text{-}Off}$ x 0,5.

Det niveau, over hvilket antistoffet ventes at være tilstede på grund af aktiv infektion, svarer til $OD_{IgG\ Cut\text{-}Off}$.

Prøve-OD mellem de to niveauer angiver tilstedeværelsen af et lavt antistofniveau, som skal fortolkes forsigtigt. Fortolkes som følger:

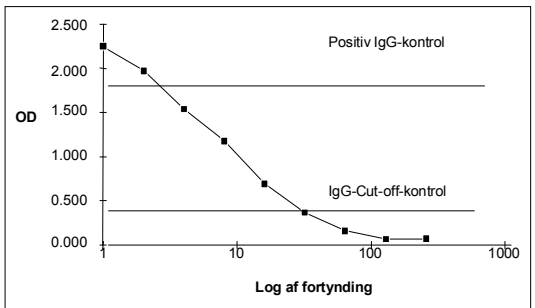
$OD_{prøve} < OD_{IgG\text{-}påvisning}$	Negativ for IgG-antistoffer mod B. burgdorferi:
$OD_{prøve} > OD_{IgG\text{-}påvisning}$ and $< OD_{IgG\text{-}cut\text{-}off}$	Antistof tilstede.

Der anbefales opfølgende prøvetagning efter to uger for at bekræfte patientstatus

$OD_{prøve} > OD_{IgG\text{-}cut\text{-}off}$	Positiv for aktiv produktion af IgG-antistof til B. burgdorferi

Semikvantitativ fortolkning (arbitrære enheder)

r området af OD-værdier mellem $OD_{IgG\ Cut\text{-}Off}$ and $OD_{IgG\ Positiv}$ D-værdierne direkte til logaritmen af arbitrære enheder af specifikt antistof i prøven. Dette er illustreret i figur 1, der viser resultater fra et IgG anti-B. burgdorferi-positivt serum fortyndet serielt i negativt serum.



Figur 1 Resultater af en serie 2-gangefortyndinger af et serum positivt for IgG-antistoffer mod B. burgdorferi i negativt serum. Endvidere er OD-værdierne for IgG-Cut-Off-kontrollen og den positive IgG-kontrol vist.

Niveauet af specifikke antistoffer i IgG-Cut-Off-kontrollen er defineret som 1 ($U_{IgG\ Cut\text{-}Off}$ = 1 enhed). Niveauet af specifikke antistoffer i den positive IgG–kontrol er justeret til 8 x $U_{IgG\ Cut\text{-}Off}$ ($U_{IgG\ Positiv}$ = 8 enheder).

For en prøve kan de arbitrære enheder af specifikke antistoffer (Uprøve) beregnes ved at benytte formlen:

$$U_{prøve}=10^{a},\ a = \frac{DO_{prøve} - DO_{IgG\ Cut\text{-}off}}{DO_{IgG\ Positiv} - DO_{IgG\ Cut\text{-}off}} \times 0{,}9^{*}$$

$$^{*}\log U_{IgG\ Positiv} - \log U_{IgG\ Cut\text{-}off} = \log 8 - \log 1 = 0{,}9$$

Tvivlsommer resultater

Grænsen for påvisning af IgG-antistof svarer til 0,9 enheder. Det niveau over hvilket, at antistoffet ventes at være tilstede på grund af aktiv infektion, svarer til 1,1 enhed. Prøver med mindre end 0,9 enhed specifikt antistof fortolkes som negative for IgG-antistoffer mod B. burgdorferi. Prøver med mellem 0,9 og 1.1 enheder angiver tilstedeværelsen af lavt antistofniveau, som skal fortolkes med forsigtighed, og der anbefales opfølgende prøvetagning efter to uger for at bekræfte patientstatus. Prøver med 1,1 eller flere enheder specifikt antistof fortolkes som positive for IgG-antistoffer mod B. burgdorferi.

For prøver med OD-værdier over $OD_{IgG\ Positiv}$ bør de rapporterede enheder af specifikke antistoffer mod B. burgdorferi være højere end 8.

En ændring af en patients specifikke antistofniveau kan betragtes som signifikant, når de arbitrære enheder i en efterfølgende prøve er enten fordoblet eller halveret. Dette er udelukkende vejledende.

11.4.3 Kommentarer til resultatfortolkninger

Negative resultater

Et negativt resultat udelukker ikke eksponering mod B. burgdorferi. Hvis der stadig er mistanke om Lyme borreliosis, bør der udtages en yderligere prøve på et senere tidspunkt.

Positive resultater

Et positivt resultat indikerer nylig eksponering mod B. burgdorferi.

Tvivlsomme resultater

Alle resultater inden for ±20% af OD-IgG-Cut-off bør betragtes som tvivlsomme og fortolkes med forsigtighed. Det anbefales at gentage analysen af sådanne prøver.

Et tvivlsomt resultat bør føre til, at der inden for 2 uger udtages en yderligere prøve til analyse. Hvis begge (eller yderligere senere) prøver giver tvivlsomme resultater, kan patienten betragtes at være IgG-negativ.

12. TESTENS BEGRÆNSNINGER

12.1. Et negativt resultat udelukker ikke muligheden for B. burgdorferi-infektion hos patienten. Manglende påvisning af B. burgdorferi kan være resultat af sådanne faktorer som udtagning af prøven på et forkert tidspunkt inden fremkomsten af detekterbare antistoffer, forkert prøveudtagning eller forkert håndtering af prøven. Tidlig antibiotikabehandling kan undertrykke antistofreaktionen, og nogle patienter producerer muligvis ikke antistoffer i et detekterbart niveau.

12.2. Et positivt resultat indikerer en tidligere immunologisk eksponering og er ikke et bevis på aktiv infektion.

12.3. Alle positive resultater skal fortolkes i forbindelse med patientrelateret klinisk information og epidemiologiske data og retfærdiggør ikke behandling af en patient. Muligheden for eksponering mod flåtbid bør altid tages i betragtning.

12.4. Testens resultat bliver påvirket, hvis reagenserne modificeres eller opbevares under forhold, som afviger fra de i afsnit 5.2. anførte.

13. FORVENTEDE VÆRDIER

Niveauet af IgG-Cut-Off-kontrollen bør justeres til en specificitet på 98% for normale sera. Antistofreaktionen mod B. burgdorferi-flageller afhænger af infektionens kliniske manifestation og sygdommens varighed. Ved nogle af de mest almindelige kliniske manifestationer af infektion med B. burgdorferi blev den diagnostiske sensitivitet af en indirekte IgG-analyse anvendende oprensede, native B. burgdorferi-flageller som testantigen rapporteret som følger: [6,8]

Klinisk manifestation	Diagnostisk sensitivitet
Erythema migrans	36%
Lymfocytisk meningoradiculitis	77%
Acrodermatitis chronica atrophicans	100%

14. SÆRLIGE EFFEKTIVITETSKARAKTERISTIKA

14.1. SPECIFICITET

Specificiteten af IDEIA Borrelia burgdorferi, IgG blev evalueret på et uafhængigt rutinediagnostiklaboratorium i Sverige. Studiet blev udført på et panel af serumprøver udtaget fra formodentligt raske bloddonorer, der boede i et område med endemisk Lyme borreliosis.

Specificitet	
Forventet	**Evaluering***
98%	98.5%

*Tvivlsommer resultater er fortolket som negative.

14.2. DIAGNOSTISK SENSITIVITET

Den diagnostiske sensitivitet af IDEIA Borrelia burgdorferi, IgG blev evalueret på et uafhængigt rutinediagnostiklaboratorium i Sverige. Studiet blev udført på tre paneler af serumprøver fra patienter med nogle af de mest almindelige kliniske manifestationer af infektion med B. burgdorferi: 45 serumprøver fra patienter med erythema migrans, 38 serumprøver fra patienter med lymfocytisk meningoradiculitis og 20 serumprøver fra patienter med acrodermatitis chronica atrophicans. Resultaterne er sammenlignet med tidligere rapporterede forventede værdier[6,8].

	Diagnostisk sensitivitet	
Klinisk manifestation	**Forventet**	**Evaluering***
Erythema migrans	36%	38%
Lymfocytisk meningoradiculitis	77%	79%
Acrodermatitis chronica atrophicans	100%	100%

*Tvivlsommer resultater er fortolket som negative.

14.3. KRYDSREAKTIVITET

Sera fra patienter med syfilis og inflammatoriske sygdomme (positive for rheumatoid faktor (RF)) blev testet med IDEIA Borrelia burgdorferi IgG med følgende resultater:

Patientsera	Antal sera	Antal positive*
Syfilis	25	0
FR	18	1

*Tvivlsommer resultater er fortolket som negative.

15. REFERENCER

1. Burgdorferi W, Barbour AG, Hayes SF, Benach JL, Grunwaldt E, Davis JP. (1982) Lyme disease - a tick-borne spirochetosis? Science 216: 1317-9.

2. Steere AC, Grodzicki RL, Kornblatt AN, Craft JE, Barbour AG, Burgdorferi W, et al. (1983) The spirochetal etiology of Lyme disease. N Engl J Med 308: 733-40.

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4. Craft JE, Duncan KF, Shimamoto GT, Steere AC. (1986) Antigens of Borrelia burgdorferi recognized during Lyme disease. Appearance of a new immunoglobulin M response and expansion of the immunoglobulin G response late in the illness. J Clin Invest 78: 934-9.

5. 5.Zöller L, Burkard S, Schäfer H. (1991) Validity of Western immunoblot band patterns in the serodiagnosis of Lyme borreliosis. J Clin Microbiol 29: 174-82.

6. Hansen K, Pii K, Lebech A-M. (1991) Improved immunoglobulin M serodiagnosis in Lyme borreliosis by using a µ-capture enzyme-linked immunosorbent assay with biotinylated Borrelia burgdorferi flagella. J Clin Microbiol 29: 166-73.

7. Hansen K, Hindersson P, Pedersen NS. (1988) Measurement of antibodies to the Borrelia burgdorferi flagellum improves serodiagnosis in Lyme disease.J Clin Microbiol 26: 338-46.

8. 8.Karlsson M. (1990) Western immunoblot and flagellum enzyme-linked immunosorbent assay for serodiagnosis of Lyme borreliosis. J Clin Microbiol 28: 2148-50.

Retur til dok liste

X7841 revideret oktober 2011

OXOID Limited,
Wade Road, Basingstoke,
Hampshire, RG24 8PW,
Verenigd Koninkrijk

Ved alle henvendelser, kontakt venligst Deres lokale Oxoid filial eller forhandler

12. QUALITY CONTROL

LIAISON® controls should be run in singlicate to monitor the assay performance. Quality control must be performed by running LIAISON® Borrelia IgG and Borrelia IgG Liquor controls
(a) at least once per day of use,
(b) whenever a new reagent integral is used,
(c) whenever the kit is calibrated,
(d) whenever a new lot of Starter Reagents is used,
(e) to assess adequacy of performance of the open integral beyond four weeks, or in agreement with guidelines or requirements of local regulations or accredited organizations.

Control values must lie within the expected ranges: whenever one or both controls lie outside the expected ranges, calibration should be repeated and controls retested. If control values obtained after successful calibration lie repeatedly outside the predefined ranges, the test should be repeated using an unopened control vial. If control values lie outside the expected ranges, patient results must not be reported.

The performance of other controls should be evaluated for compatibility with this assay before they are used. Appropriate value ranges should then be established for quality control materials used.

13. INTERPRETATION OF RESULTS IN SERUM OR PLASMA

13.1. Borrelia IgG test (serum or plasma samples)

The analyzer automatically calculates *Borrelia burgdorferi* IgG antibody concentrations expressed as arbitrary units (AU/mL) and grades the results. For details, refer to the analyzer operator's manual.
Calibrators and controls may give different RLU or dose results on LIAISON® and LIAISON® XL, but patient results are equivalent.

Assay range. 5 to 240 AU/mL *Borrelia burgdorferi* IgG.
Samples containing antibody levels above the assay range may be prediluted by the Dilute function of the instrument and retested (the recommended dilution factor is 1:10). The results will then be automatically multiplied by the dilution factor to obtain the antibody levels of the neat specimens. The specimen diluent excess available in the reagent integral allows up to 10 sample predilutions to be performed.

Sample results should be interpreted as follows:
Samples with *Borrelia burgdorferi* IgG concentrations below 10 AU/mL should be graded *negative*.
Samples with *Borrelia burgdorferi* IgG concentrations ranging between 10 and 15 AU/mL should be graded *equivocal*. *Equivocal samples must be retested in order to confirm the initial result. Samples which are positive at the second test should be considered positive. Samples which are negative at the second test should be considered negative. A second sample should be collected and tested no less than one week later when the result is repeatedly equivocal.*
Samples with *Borrelia burgdorferi* IgG concentrations equal to or above 15 AU/mL should be graded *positive*.

13.2. Interpretation of results for serum or plasma samples

A negative result for IgM and/or IgG antibodies to *Borrelia burgdorferi* generally indicates that the patient has not been infected, but does not always rule out acute borreliosis, because the infection may be in its very early stage and the patient may be still unable to synthesize *Borrelia burgdorferi* specific antibodies, or the antibodies may be present in undetectable levels. Specific IgM antibodies are more easily detected in the early stages of infection; in later stages they progressively decline. It should be underlined that the test scores negative during the first weeks after infection. If clinical exposure to *Borrelia burgdorferi* is suspected despite a negative or equivocal finding, a second sample should be collected and tested for IgM and IgG later during the course of infection.

A positive result for IgM and/or IgG antibodies to *Borrelia burgdorferi* generally indicates exposure to the pathogen (acute or past infection). A single specimen, however, can only help estimate the serological status of the individual. An isolated positive IgM result is observed relatively often in the early stages of the disease, but rarely in the later stages. An isolated positive IgG result may indicate either active Lyme disease or past infection with persisting antibodies. The following table summarizes the different immunological pictures. Results were obtained using LIAISON® Borrelia assays.

Borrelia burgdorferi IgM result	*Borrelia burgdorferi* IgG result	Interpretation
negative	negative	No evidence of infection. In case of clinical uncertainty (presence of tick bite or neurological symptoms), the patients should be followed up during time.
positive	negative	Probable infection at an early stage.
negative	positive	Probable infection at any stage.
positive	positive	Probable acute infection.

14. INTERPRETATION OF RESULTS IN CEREBROSPINAL FLUID

14.1. Borrelia IgG test (cerebrospinal fluid samples)

The analyzer automatically calculates *Borrelia burgdorferi* IgG antibody concentrations expressed as arbitrary units (AU/mL) and grades the results. For details, refer to the analyzer operator's manual.
Calibrators and controls may give different RLU or dose results on LIAISON® and LIAISON® XL, but patient results are equivalent.

Assay range. 0.2 to 240 AU/mL *Borrelia burgdorferi* IgG.
Samples containing antibody levels above the assay range may be prediluted by the Dilute function of the instrument and retested. The recommended dilution factor is 1:10; when the diluted samples still score above the assay range, the test should be repeated after prediluting the samples 1:100. The results will then be automatically multiplied by the dilution factor to obtain the antibody levels of the neat specimens. The specimen diluent excess available in the reagent integral allows up to 10 sample predilutions to be performed.

Sample results should be interpreted as follows:
Samples with *Borrelia burgdorferi* IgG concentrations below 4.5 AU/mL should be graded *negative*.
Samples with *Borrelia burgdorferi* IgG concentrations ranging between 4.5 and 5.5 AU/mL should be graded *equivocal*. *Equivocal samples must be retested in order to confirm the initial result. Samples which are positive at the second test should be considered positive. Samples which are negative at the second test should be considered negative. A second sample should be collected and tested no less than one week later when the result is repeatedly equivocal.*
Samples with *Borrelia burgdorferi* IgG concentrations equal to or above 5.5 AU/mL should be graded *positive*.

14.2. Interpretation of results for cerebrospinal fluid samples

A negative result for IgG antibodies to *Borrelia burgdorferi* indicates unlikely intrathecal synthesis of *Borrelia burgdorferi* antibodies. If neuroborreliosis is strongly suspected despite a negative finding, further diagnostic investigation is suggested.

A positive result for IgG antibodies to *Borrelia burgdorferi* suggests possible intrathecal synthesis of *Borrelia burgdorferi* antibodies: neuroborreliosis is therefore suspected.
Positive results may be observed in patients with extremely high levels of circulating *Borrelia burgdorferi* antibodies as well as in patients positive for *Borrelia burgdorferi* serum antibodies associated with high albumin concentrations in cerebrospinal fluid. The latter finding suggests a possible damage to the blood/cerebrospinal fluid barrier.

Intrathecal presence of specific *Borrelia burgdorferi* antibodies should be evaluated taking into due consideration basic cerebrospinal fluid variables, such as increased cell count, total IgG concentration, total protein concentration. For more reliable quantification of intrathecal immunoglobulin synthesis, a hyperbolic function should be referred to for discriminating between brain- and blood-derived protein fractions present in cerebrospinal fluid (according to H. Tumani, G. Nölker, H. Reiber, 1995). The cerebrospinal fluid to serum volume ratio used in the LIAISON® Borrelia IgG test is 10.

15. LIMITATIONS OF THE PROCEDURE

Assay performance characteristics have not been established when any LIAISON® Borrelia test is used in conjunction with other manufacturers' assays for detection of specific *Borrelia burgdorferi* serological markers. Under these conditions, users are responsible for establishing their own performance characteristics.

A skillful technique and strict adherence to the instructions are necessary to obtain reliable results.

Bacterial contamination or heat inactivation of the specimens may affect the test results.

Test results are reported quantitatively as positive or negative for the presence of *Borrelia burgdorferi* IgG. However, diagnosis of infectious diseases should not be established on the basis of a single test result, but should be determined in conjunction with clinical findings and other diagnostic procedures as well as in association with medical judgement.

Antibiotic therapy during the early stages of the disease often prevents development of antibody response.

Integrals may not be exchanged between analyzer types (LIAISON® and LIAISON® XL). Once an integral has been introduced to a particular analyzer type, it must always be used on that analyzer until it has been exhausted. Due to traceability issues resulting from the above statement, patient follow-ups may not be concluded between analyzer types. These must be accomplished on one particular analyzer type (either LIAISON® or LIAISON® XL).

Assay Procedure

Bring all assay reagents to room temperature (20 – 25°C) before beginning the assay. All steps are performed at room temperature (20 – 25°C).

1. Record the sample identity for each well on the provided record sheet to determine the number of strips necessary to perform the assay. Five wells will be needed for controls and calibrators. One well will be needed for each sample.
2. Remove the microplate frame containing the microplate strips from the foil pouch. Remove unneeded microplate strips from the frame and reseal unused strips in the foil pouch with desiccant. Microplate frame should be retained at the end of the assay to be used with the remaining microplate strips.
3. Add 100_L of diluted Positive Control to one microwell and 100_L of diluted Negative Control to another microwell.
4. Add 100_L of diluted Calibrator to each of three microwells.
5. Add 100_L of each diluted patient sample to microwells.
6. Incubate for 30 minutes.
7. Aspirate wells. If using manual or semi-automated washing manifold, wash three times as follows. Dispense approximately 150_L (half of well volume) of 1X Wash Buffer into each well, then aspirate. Refill wells with approximately 300_L of 1X Wash Buffer (full volume of well) and aspirate a second time. Refill wells with 300_L of 1X Wash Buffer and aspirate a third time. Make sure that all wells have been aspirated after the third (final) wash step. (If an automated plate washer is used, wash four times with each wash consisting of 300-350_L of 1X Wash Buffer. After the final wash for both manual and automated washing, tap the plate on absorbent towels to remove all residual liquid.)
8. Dispense 100_L of Conjugate into each well.
9. Incubate for 20 minutes.
10. Aspirate wells. Perform four wash steps with 1X Wash Buffer as in step 8 above.
11. After the final aspiration, invert, shake out and blot the plate against absorbent towels to remove all residual liquid.
12. Dispense 100_L of TMB ELISA Substrate into each well.
13. Incubate for 4 minutes. Please note: Optimal assay performance requires precise timing of the TMB ELISA Substrate incubation step.
14. Dispense 100_L Stop Solution into each well in the same order as the TMB ELISA Substrate was dispensed in the previous step. Tap the plate gently to mix contents of wells. Read absorbance values within 5 minutes.
15. Read Absorbance at 450nm with a reference filter of 650nm using an ELISA plate reader. If the reader is not equipped with a 650nm filter, the use of an alternate filter between 590 – 650nm will provide equivalent results.

Quality Control

1. Control values must be within the following ranges in order for the assay to be considered valid:
2. Negative Control A_{450} must be <0.18
3. Each Calibrator A_{450} must be between 0.400 and 2.00
4. Positive Control A_{450} must be >1.2
5. If any control A_{450} value is not within the above ranges, the assay should be repeated.

Calculations

1. Calculate the mean value for the three Calibrator Controls. If any Calibrator absorbance value differs by more than 0.1 absorbance units from the mean, discard the data point that is farthest from the mean. Recalculate the mean from the two remaining data points. If the Calibrator absorbance values still differ by more than 0.1 absorbance units from the mean, the assay is invalid and must be re-run. The mean Calibrator absorbance value must be between 0.4 and 2.0 absorbance units.
2. Calculate the assay cutoff value by dividing the mean Calibrator value by 2.150 (Correction Coefficient).
3. Calculate the Lyme Index value (LI) for each patient sample by dividing the A_{450} of the sample by the cutoff value.

Interpretation of Results

Lyme Index	Interpretation
≤ 0.90	Negative result. No antibody to *B. burgdorferi* detected in the present assay. This result does not exclude the possibility of *B. burgdorferi* infection, and where early Lyme disease is suspected, a second sample should be drawn 2 – 4 weeks later and re-tested.
0.91 – 1.09	Equivocal result. The imprecision inherent in any method implies a lower degree of confidence in the interpretation of samples with A_{450} values very close to the calculated cutoff value. For this reason an equivocal category has been designated. Equivocal samples should be tested with a supplemental assay such as a standardized Western Blot test in accordance with CDC/ASTPHLD recommendations.
≥ 1.10	Positive result. Antibody to *B. burgdorferi* detected in the present assay. All positive results should be supplemented by re-testing the corresponding serum samples on a standardized Western Blot test in accordance with CDC/ASTPHLD recommendations[12].

The cutoff is determined for each assay run by dividing the mean calibrator value by the correction coefficient. In this way, the cutoff is intended to compensate for run-to-run assay variations, which might otherwise affect sensitivity and specificity. The calibrator has been designed to yield an absorbance value in the linear portion of the C6 ELISA dose-response curve. The correction coefficient was determined by analysis of C6 ELISA results for 131 normal donors and 108 well-characterized Lyme disease patients. In assay runs using two separate kit lots, the correction coefficient (2.15) was determined as the value which yielded a cutoff which minimized the number of false positive and of false negative results.

Limitations

1. A negative result does not exclude the possibility of infection with *B. burgdorferi*. Patients in early stages of Lyme disease and those who have been treated with antibiotics may not exhibit detectable antibody titers. Patients with clinical history, signs or symptoms suggestive of Lyme disease should be re-tested in 2-4 weeks in the event that the initial test result is negative.
2. A positive result is not definitive evidence of infection with *B. burgdorferi*. It is possible that other disease conditions may produce artifactual reactivity in the assay. All equivocal or positive results should be supplemented by re-testing the corresponding serum samples on a standardized Western Blot test in accordance with CDC/ASTPHLD recommendations[12].

1 – 9 = positivt

≥ 10 = stærkt positivt

Intratekal syntese angives som et antistof indeks.

Svartid

Analysen udføres mindst 1 gang ugentligt, og svar afgives umiddelbart herefter.

Tolkning og reference-værdier/interval

Diagnostik af de forskellige former for Borrelia infektion kan erfaringsmæssigt volde problemer, fordi der ofte er en del differentialdiagnostiske muligheder.

Antistoffer i serum: Kun ca. halvdelen af patienter med ubehandlet erythema migrans (1. stadium) har antistoffer når de opsøger en læge. En del af disse patienter forbliver seronegative.

Ca. 70 % af patienterne med ubehandlet neuroborreliose (2. stadium) er seropositive efter 2 ugers sygdomsvarighed. 100 % er seropositive efter 8 uger.

IgG resultatet er altid positivt ved 2. og 3. stadie manifestationer med sygdomsvarighed > 3 måneder. Ved acrodermatitis chronica atrophicans findes sædvanligvis meget høje IgG antistoffer og ingen IgM antistoffer.

Testen for serum antistoffer er for IgG og IgM indstillet således, at henholdsvis 95 % og 98 % af raske kontrolpersoner giver et negativt resultat.

Efter rekommanderet behandling og klinisk remission kan IgG syntese fortsætte i lang tid (år). Syntese af IgM ophører oftest i løbet af 6 måneder, men kan vare noget længere uden kliniske tegn på terapisvigt.

Ved gentagne reinfektioner produceres der efterhånden ikke IgM antistoffer. Mangel på IgM udelukker ikke aktiv infektion.

Intratekal antistofsyntese: Syntesen af antistoffer mod Borrelia begynder i løbet af 2 uger hos ca. 80 % af patienterne med ubehandlet neuroborreliose og kan altid påvises ved sygdomsvarighed over 6 - 8 uger. Efter rekommanderet behandling og klinisk remission kan IgG syntese fortsætte i lang tid (år). Syntese af IgM ophører oftest i løbet af 6 måneder, men kan vare noget længere uden kliniske tegn på terapisvigt.

Bemærkning

Falsk positiv IgM i serum kan ses ved bl.a. mononukleose og autoimmune sygdomme.

De anvendte testmetoder måler antistoffer mod de tre kendte europæiske genospecies af *Borrelia burgdorferi* sensu lato, der er årsag til human sygdom (*B. afzelii, B. garinii, B. burgdorferi* senso stricto).

Syfilis antistoffer kan give falsk positive fund.

Relaterede undersøgelser

Borrelia burgdorferi group (DNA) (R-nr. 1083)

Undersøgelsens princip

IgG: indirekte ELISA, IgM: μ-capture ELISA. Som antigen anvendes oprenset nativt flagel protein fra *B. afzelii* strain DK1.

Intratekal syntese af antistoffer: Samtidigt udtagen spinalvæske- og serumprøve undersøges ved en særlig capture ELISA for indhold af IgG og IgM Borrelia antistof med flagel fra *B. afzelii* strain DK1 som antigen. Ud fra mængderne i spinalvæske og serum beregnes separat for IgG og IgM et