# 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

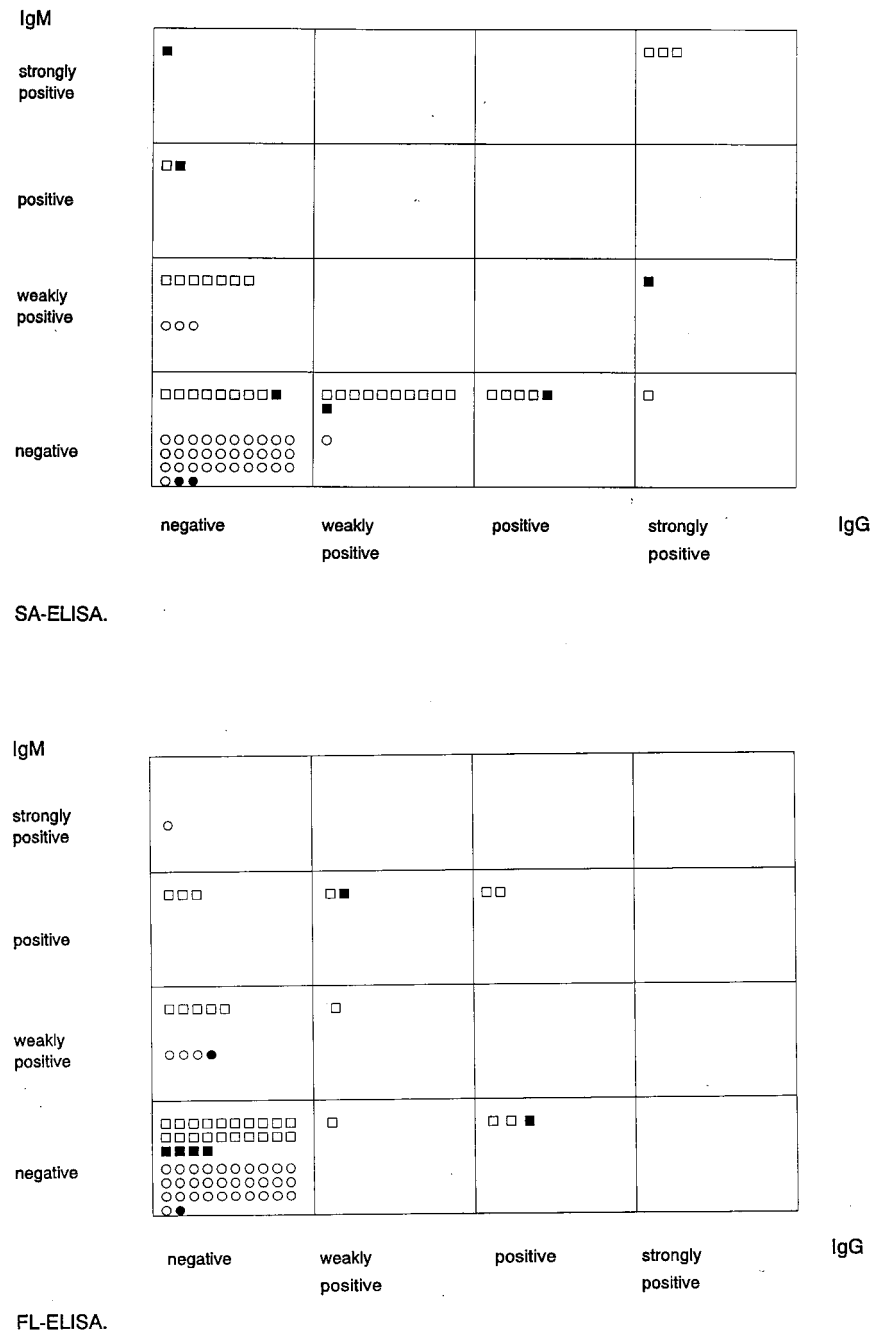| Group | No. of results | | | | | |
|---|---|---|---|---|---|---|
| | SA-ELISA (IgM, IgG, or both) | | FL-ELISA (IgM, IgG, or both) | | P39-ELISA | |
| | Positive | Negative | Positive | Negative | Positive | Negative |
| Patients | | | | | | |
| Culture positive (n = 12) | 11 | 1 | 6 | 6 | 5 | 7 |
| 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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