MAJOR ARTICLE

Effect of *Borrelia burgdorferi* Genotype on the Sensitivity of C6 and 2-Tier Testing in North American Patients with Culture-Confirmed Lyme Disease

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Background. A potential concern with any serologic test to detect antibodies to *Borrelia burgdorferi* is whether the epitopes incorporated in the test provide sufficient cross-reactivity to detect infection with all of the pathogenic strains of the species. This is a particular concern for the C6 test, which is based on reactivity to a single peptide.

Methods. C6 testing and 2-tier testing were performed on acute-phase serum samples obtained from >158 patients with erythema migrans for whom the genotype of the borrelial isolate was defined on the basis of an analysis of the 16S-23S ribosomal DNA spacer region and/or on the genetic variation of the outer surface protein C gene *(ospC)*. The sonicated whole cell–based enzyme-linked immunosorbent assay, the immunoblots used in the 2-tier testing, and the C6 assay all used antigens from *B. burgdorferi* sensu stricto strain B31.

Results. The sensitivity of C6 testing (69.5%) was greater than that of 2-tier testing (38.9%) ($P < .001$); the difference in sensitivity, however, was statistically significant only for patients infected with 2 of the 3 ribosomal spacer type–defined genotypes. The lower sensitivity of 2-tier testing was attributable to the low sensitivity of the immunoblot tests, rather than the first-tier enzyme-linked immunosorbent assay. There was also a trend for the sensitivity of 2-tier testing to vary according to the *ospC* genotype for the 14 genotypes represented in the study ($P = .07$); this relationship was not observed with C6 testing.

Conclusions. Lack of sensitivity of the C6 test because of strain diversity seems less likely to be a limitation of this serologic test, compared with 2-tier testing in North American patients with early Lyme disease.

Detection of antibody to C6, a 26–amino acid peptide that reproduces the sequence of the sixth invariable region (IR6) within the central domain of the VlsE protein of *Borrelia burgdorferi* sensu lato, is currently used for the serologic diagnosis of Lyme disease [1–4]. A potential concern with a test based on a single peptide is the possibility of sequence variation and a consequent lack of antigenic cross-reactivity among pathogenic strains of *B. burgdorferi* sensu lato, leading to reduced diagnostic sensitivity in some infected patients.

B. burgdorferi sensu stricto (hereafter referred to as *B. burgdorferi*) isolates can be categorized into 3 distinct ribosomal spacer restriction fragment length polymorphism genotypes (RSTs) on the basis of analysis of the 16S-23S ribosomal DNA (rDNA) spacer region [5, 6]. North American isolates of *B. burgdorferi* can also be differentiated into at least 16 major genetic groups on the basis of variability in the genetic sequence encoding outer surface protein C (OspC) [7, 8]. Studies employing these methods have demonstrated that pathogenicity is dependent at least in part on the genotype of *B. burgdorferi* causing the infection; for example, evidence indicates that RST1 and RST2 are more likely to be associated with hematogenous dissemination in humans than is RST3 [6].

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Although serologic testing of patients with erythema migrans is not routinely recommended for the management of these patients, serum samples from such patients are often used to validate or compare serologic assays, because this is the most common manifestation of Lyme disease [9], and it is the only manifestation for which the diagnosis can frequently be confirmed by the microbiologic gold standard of recovering *B. burgdorferi* on culture [10]. In a previous study [11], we demonstrated the comparable sensitivity of a C6 assay in 79 patients from North America with erythema migrans, irrespective of the RST genotype that caused the infection. The current study expands on the previous study by increasing the number of patient serum samples evaluated and by also considering the *ospC* genotype of the infecting strain of *B. burgdorferi*. In addition, the effect of the genotype of *B. burgdorferi* on the sensitivity of 2-tier testing was evaluated.

MATERIALS AND METHODS

Serologic assays. The C6 Lyme ELISA kit (Immunetics), a test kit approved by the US Food and Drug Administration, was used according to the manufacturer's instructions. In place of the original cutoff formula that was based on a serum sample positive for Lyme disease, a simplified cutoff formula based on a negative calibrator serum sample was employed. The assay cutoff value was determined by adding 0.3 to the absorbance value of the calibrator serum sample. The Lyme index value for each patient sample was calculated by dividing the absorbance of the sample at 450–650 nm by the cutoff value. This modification yielded statistically equivalent sensitivity and specificity to what had been demonstrated with the original cutoff formula; the original cutoff formula was chosen to yield a specificity of 99.6% (95% CI, 97.9–100%) in a group of blood donors from endemic and nonendemic areas and a sensitivity of 85.8% in a combined group of patients with early- and late-stage Lyme disease (A.L., unpublished data).

The C6 peptide used as the antigen in the C6 Lyme ELISA kit is derived from the *B. burgdorferi* B31 strain sequence, which differs from the originally described IP90 *Borrelia garinii* sequence by 4 amino acids. The kit is formatted as an indirect ELISA in which both IgG and IgM antibodies to the C6 peptide are detected by an enzyme conjugate. Two-tier serologic testing was performed using an IgG-IgM ELISA kit (Wampole Laboratories), followed by Lyme IgG and IgM immunoblot kits (MarDx/Trinity Biotech). The 2-tier ELISA and immunoblot kits were approved by the US Food and Drug Administration for in vitro diagnostic use, and testing was performed according to the manufacturers' instructions. Both the ELISA and immunoblot kits used a sonicated whole-cell preparation of *B. burgdorferi* B31 as the source of antigen. A positive result by 2-tier testing required a positive or equivocal ELISA result and either a positive IgM or IgG immunoblot result, interpreted according to recommended criteria [12].

Serum specimens from patients with Lyme disease. Serum specimens that had been stored at −80°C were tested using the C6 and 2-tier methods as part of a performance comparison. The serum samples were originally obtained from patients with erythema migrans whose clinical diagnosis was confirmed by the growth of *B. burgdorferi* from culture of a skin biopsy or blood sample, with use of methods reported elsewhere [6]. Serum specimens were obtained before antibiotic treatment was initiated. The patients who provided the serum samples were subjects in approved research studies at New York Medical College (Valhalla). The patients resided in Westchester County, New York, or in the immediately surrounding geographic areas. Information on duration of illness and number of erythema migrans skin lesions was obtained from study records.

Genotyping methods. *B. burgdorferi* DNA was isolated from low-passage (1–5 passages) cultures with use of a nucleic acid extraction kit (IsoQuick; Orca Research). A 941–base pair fragment of the 16S-23S rDNA intergenic spacer was amplified using PCR with primers PA and P95 as described elsewhere [5]. PCR-based restriction fragment length polymorphism analyses of the 16S-23S rDNA intergenic spacer region were performed using the restriction enzyme *Tru*1I (Fermentas) [13]. A 522–base pair region of *ospC* was amplified using seminested PCR with external primers OC6 (+) and OC623 (−) and internal primers OC6 (+) (fluorescein) and OC602 (−) [14, 15]. Amplicons were then probed with *ospC*-type–specific probes by use of reverse line blot [14, 15]. *ospC* amplicons that did not hybridize with any *ospC*-type–specific probes were reamplified and sequenced in both directions (Genewiz) by use of either primer sets *ospC*-N and *ospC*-C [14] or OC6 (+) and OC623 (−). Isolates that produced ambiguous sequence results were cloned using limiting dilution, and sequence analyses were performed on 2 clones from each isolate. Some of the isolates in the current study have been reported in the context of other investigations regarding the pathogenicity of particular genotypes of *B. burgdorferi* [6, 7, 16, 17].

Mice and infections. Six-week-old C3H/HeN mice (Charles River Laboratories) were used for the experiments. All mice were bled prior to inoculation to obtain baseline serum specimens. Two *B. burgdorferi* isolates were inoculated—namely, the *ospC* type K, RST2 isolate from the sole patient with a C6-negative serology and positive 2-tier test result and an isolate (*ospC* type N, RST2) recovered from a patient who was seropositive according to the C6 ELISA result. Four mice per isolate were needle-inoculated subcutaneously with 1×10^5 organisms in 0.5 mL of Hank's Balanced Salt Solution (Gibco/Invitrogen). Two weeks after inoculation, 2-mm ear biopsies were obtained from each ear of the mice and were placed

Table 1. Comparison of the sensitivity of C6 testing and 2-tier testing among patients according to the ribosomal spacer type (RST) of the strain of *Borrelia* isolated from the patients (excludes mixed infections).

RST	No. of patients	No. (%) of patients with a positive test result		*P*
		C6 test	2-tier test	
RST1	46	32 (69.6)	25 (54.3)	.20
RST2	81	54 (66.7)	30 (37.0)	<.001
RST3	40	30 (75.0)	10 (25.0)	<.001
All	167	116 (69.5)	65 (38.9)	<.001

in 5 mL BSK-H (Sigma) at 34°C. Blood was also collected at this time (day 14 after inoculation). Four weeks after inoculation (day 28), mice were euthanized and blood was collected. The ears, hearts, bladders, and tibiotarsal joints were removed and placed in saline. A section (~2 mm) of each organ was removed and placed in BSK-H for the culture of spirochetes. Cultures were inspected after 12 and 23 days for the presence of spirochetes.

C6 ELISA for mice. The preinfection, day-14, and day-28 serum samples from each mouse were tested using the C6 peptide ELISA, as described elsewhere [18]. Serum was diluted 1:200 for the assays, and samples were tested in triplicate.

Statistical methods. Differences were assessed using the Fisher's exact test or a χ^2 analysis [19]. *P* values were estimated using a Monte Carlo simulation with 100,000 replicates for cases in which the marginal values summed <5, which made the χ^2 distribution inappropriate [20]. A *P* value of <.05 was considered to be significant in this study.

RESULTS

One hundred eighty-one acute-phase serum specimens from our collection were tested to compare the C6 assay with 2-tier testing. Of these, 167 had been obtained from patients with culture-confirmed erythema migrans for whom the corresponding 167 borrelial isolates were characterized genotypically according to RST genotype; in these patients, there was no evidence of a mixed infection with >1 RST genotype. One hundred fifty-eight of the borrelial isolates were also characterized according to *ospC* genotype, and these isolates had no evidence of a mixed infection with >1 *ospC* genotype. Data regarding C6 test results and corresponding borrelial isolates were reported elsewhere for 39 of these serum samples [11]. The overall sensitivity of C6 testing (69.5%) of the 167 serum samples exceeded that of 2-tier testing (38.9%) ($P < .001$). The sensitivity of C6 testing exceeded that of 2-tier testing among patients specifically infected with genotypes RST2 and RST3 ($P < .001$) but not RST1 ($P = .20$) (table 1). The sensitivity of C6 testing did not vary statistically significantly in relationship to either the RST ($P = .65$) or *ospC* genotype ($P = .78$) of the specific strain of *B. burgdorferi* that caused the infection. In contrast, the sensitivity of 2-tier testing varied significantly in relation to RST genotype ($P < .02$) (table 1). Two-tier testing was more than twice as sensitive among RST1-infected patients than among patients infected with an RST3 isolate ($P = .008$). There was also a trend for the sensitivity of 2-tier testing to vary according to the *ospC* genotype for the 14 genotypes represented in the study ($P = .07$) (table 2). Only 1 patient, who was infected with a strain of *B. burgdorferi* characterized as *ospC* type K and RST2, had a negative C6 assay result and a positive 2-tier test result. When a group of 4 C3H/HeN mice were inoculated subcutaneously with 1×10^5 spirochetes of this isolate, cultures of ear, heart, bladder, and tibiotarsal joint fragments that were collected at the time of euthanasia (week 4 after infection) were positive for *B. burgdorferi* in 2 of the 4 mice. More importantly, serum from all 4 mice contained statistically significant levels (greater than the mean plus 3 times the SD of preinfection values) of antibodies to C6. A previously characterized *B. burgdorferi* isolate known to have elicited a C6 antibody response in an infected patient also elicited a significant C6 antibody response in the mouse experiment. Thus, these data indicate that the *B. burgdorferi* isolate that yielded a negative C6 response in an infected patient was able to express the VlsE protein and elicit a C6 antibody response in mice.

Because it is known that seropositivity is more likely in patients with a single erythema migrans skin lesion of longer duration or when multiple skin lesions are present [21], we

Table 2. Comparison of the sensitivity of C6 testing and 2-tier testing in patients according to the *ospC* genotype of the strain of *Borrelia* isolated from the patients (excludes mixed infections).

ospC type	No. of patients	No. (%) of patients with a positive test result	
		C6 test	2-tier test
A	26	17 (65.4)	14 (53.8)
B	18	13 (72.2)	9 (50.0)
C	1	1 (100.0)	0 (0.0)
D	2	2 (100.0)	0 (0.0)
E	4	2 (50.0)	0 (0.0)
F	6	4 (66.7)	0 (0.0)
G	9	7 (77.8)	4 (44.4)
H	8	6 (75.0)	4 (50.0)
I	10	9 (90.0)	3 (30.0)
J	0	0 (0.0)	0 (0.0)
K	50	34 (68.0)	20 (40.0)
M	4	3 (75.0)	0 (0.0)
N	11	5 (45.5)	1 (9.1)
O	1	1 (100.0)	0 (0.0)
T	0	0 (0.0)	0 (0.0)
U	8	4 (50.0)	3 (37.5)
All	158	108 (68.4)	58 (36.7)

investigated whether skin lesions of shorter duration or single erythema migrans lesions were overrepresented in patients infected with certain RST genotypes (table 3). The results do not provide evidence that the insensitivity of 2-tier testing in RST2- and RST3-infected patients is attributable to an overrepresentation of short-duration single erythema migrans skin lesions in patients infected with these genotypes. Although not a statistically significant difference, there were fewer patients with multiple erythema migrans skin lesions in the group of RST3 infected patients. However, this cannot explain the low frequency of seropositivity determined by the 2-tier test in RST3-infected patients, because the frequency of positive 2-tier test results in the subgroup of patients with multiple skin lesions varied across genotypes in a manner similar to that of the overall group. Among the patients with multiple erythema migrans skin lesions, 13 (76.5%) of 17 RST1-infected patients had positive 2-tier test results, compared with 3 (37.5%) of 8 RST3-infected patients ($P = .09$).

DISCUSSION

We observed that the sensitivity of C6 testing (69.5%) on acute-phase sera was significantly greater than that of 2-tier testing (38.9%) among 167 patients with culture-confirmed erythema migrans ($P < .001$). These findings are consistent with those of a previous study in which C6 testing was demonstrated to be twice as sensitive as 2-tier testing in patients with culture-confirmed erythema migrans (16 [42.1%] of 38 vs. 8 [21.1%] of 38; $P = .08$) [2]. However, the difference in sensitivity in the present study was significant only for patients specifically infected with genotypes RST2 and RST3 ($P < .001$) but not for patients with infection caused by the most invasive genotype, RST1 ($P = .20$). The sensitivity of 2-tier testing varied according to RST genotype ($P < .02$), with RST1-infected patients more than twice as likely as RST3-infected patients to be seropositive (54.3% vs. 25.0%, $P = .008$). There was also a trend for the sensitivity of 2-tier testing to vary according to the *ospC* genotype for the 14 genotypes represented in the study ($P = .07$), whereas no relationship was observed among different genotypes delineated by either typing system and C6 antibody reactivity. The relative insensitivity of 2-tier testing for RST2- and RST3-infected patients was not attributable to an overrepresentation of short-duration single erythema migrans skin lesions among patients infected with these genotypes, a circumstance that has been reported to predispose patients to low rates of seroreactivity [21].

No association was observed between RST genotype and seropositivity in our previous study [6], but in that study, only single-tier ELISA testing was performed, without immunoblot testing. In agreement with that earlier investigation, the present study also failed to demonstrate any relationship between RST genotype and the results of the whole cell–based ELISA that was used as our first-tier assay ($P = .98$) (data not shown). It is possible that the lower sensitivity of 2-tier testing among patients infected with RST2 or RST3 genotypes of *B. burgdorferi* is the result of antigenic diversity of these strains. The antigens in the 2-tier assay that was used in this study were derived from strain B31, which is an RST1 isolate [16]. The genomic sequences of RST1 isolates are highly homologous and substantially divergent from those of RST2 and RST3 strains [22, 23]. Such genotypic diversity would not be expected to affect the performance of a whole cell–based ELISA, because reactivity is the sum of reactivities to dozens of antigens, but the diversity might impact the performance of an immunoblot, in which variation in a small number of particular antigens could be pertinent to satisfying interpretive criteria for seropositivity. Because the number and intensity of bands on immunoblot would be expected to increase over the course of time in untreated patients [24, 25], this concern would likely be relevant only during early infection.

In the single case in which the C6 assay result was negative and the 2-tier test result was positive, the infecting strain of *B. burgdorferi* retained the C6 moiety, based on a mouse infection model with the strain. The existence of infectious strains of *B. burgdorferi* that either do not carry the *vlsE* gene or do not express the VlsE protein in nature is unlikely, when the recent demonstration of limited persistence of *vlsE*-deficient mutants of *B. burgdorferi* in mice is considered [26].

A limitation of our study is that the patients were infected in a single location in New York State or in the immediately surrounding geographic areas; thus, the results may not be generalizable to infections acquired in other locations. Another limitation may be related to the choice of the MarDx/Trinity Biotech immunoblot kits as the second-tier test. This may have contributed to the reduced sensitivity observed in this study,

Table 3. Comparison across ribosomal spacer types (RST) of the duration of single erythema migrans (EM) skin lesions and of the frequency of multiple EM skin lesions.

	Proportion (%) of patients			
EM characteristic	RST1	RST2	RST3	*P*
Single EM with duration of 1–7 days	11/29 (37.9)	21/52 (40.4)	13/32 (40.1)	.97
Single EM with duration of 1–14 days	21/29 (72.4)	39/52 (75.0)	25/32 (78.1)	.87
Multiple EM lesions	17/46 (37.0)	29/81 (35.8)	8/40 (20.0)	.16

because 1 comparative study has shown that the IgM kit is less sensitive than some other immunoblot assays [27].

The IR6 peptide in some VlsE proteins of strains of other species of *B. burgdorferi* sensu lato, such as *Borrelia afzelii* and *B. garinii,* may vary by up to 5 amino acids, compared with the IR6 region of the VlsE protein of *B. burgdorferi* B31 [3]. In North America, only strains of *B. burgdorferi* are known to cause Lyme disease, and variations in the IR6 region among North American isolates of *B. burgdorferi* have not been directly examined with a large number of isolates. Lack of sensitivity of the commercially available C6 test attributable to strain diversity, although a theoretical concern, has not yet been demonstrated and, on the basis of our findings in New York State, seems unlikely to be a limitation of this serologic test.

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References

1. Liang FT, Alvarez AL, Gu Y, Nowling JM, Ramamoorthy R, Philipp MT. An immunodominant conserved region within the variable domain of VlsE, the variable surface antigen of *Borrelia burgdorferi.* J Immunol **1999**; 163:5566–73.
2. Bacon RM, Biggerstaff BJ, Schriefer ME, et al. Serodiagnosis of Lyme disease by kinetic enzyme-linked immunosorbent assay using recombinant VlsE1 or peptide antigens of *Borrelia burgdorferi* compared with 2-tiered testing using whole-cell lysates. J Infect Dis **2003**; 187:1187–99.
3. Liang FT, Steere AC, Marques AR, Johnson BJ, Miller JN, Philipp MT. Sensitive and specific serodiagnosis of Lyme disease by enzyme-linked immunosorbent assay with a peptide based on an immunodominant conserved region of *Borrelia burgdorferi* VlsE. J Clin Microbiol **1999**; 37:3990–6.
4. Tjernberg I, Kruger G, Eliasson I. C6 peptide ELISA test in the serodiagnosis of Lyme borreliosis in Sweden. Eur J Clin Microbiol Infect Dis **2007**; 26:37–42.
5. Liveris D, Gazumyan A, Schwartz I. Molecular typing of *Borrelia burgdorferi* sensu lato by PCR-restriction fragment length polymorphism analysis. J Clin Microbiol **1995**; 33:589–95.
6. Wormser GP, Liveris D, Nowakowski J, et al. Association of specific subtypes of *Borrelia burgdorferi* with hematogenous dissemination in early Lyme disease. J Infect Dis **1999**; 180:720–5.
7. Seinost G, Dykhuizen DE, Dattwyler RJ, et al. Four clones of *Borrelia burgdorferi* sensu stricto cause invasive infection in humans. Infect Immun **1999**; 67:3518–24.
8. Wang IN, Dykhuizen DE, Qiu W, Dunn JJ, Bosler EM, Luft BJ. Genetic diversity of ospC in a local population of *Borrelia burgdorferi* sensu stricto. Genetics **1999**; 151:15–30.
9. Wormser GP, Dattwyler RJ, Shapiro ED, et al. The clinical assessment, treatment, and prevention of Lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. Clin Infect Dis **2006**; 43:1089–134.
10. Aguero-Rosenfeld ME, Wang G, Schwartz I, Wormser GP. Diagnosis of Lyme borreliosis. Clin Microbiol Rev **2005**; 18:484–509.
11. Embers ME, Wormser GP, Schwartz I, Martin DS, Philipp MT. *Borrelia burgdorferi* spirochetes that harbor only a portion of the lp28-1 plasmid elicit antibody responses detectable with the C6 test for Lyme disease. Clin Vaccine Immunol **2007**; 14:90–3.
12. Centers for Disease Control and Prevention. Recommendations for test performance and interpretation from the Second National Conference on Serologic Diagnosis of Lyme Disease. MMWR Morb Mortal Wkly Rep **1995**; 44:590–1.
13. Liveris D, Varde S, Iyer R, et al. Genetic diversity of *Borrelia burgdorferi* in Lyme disease patients as determined by culture versus direct PCR with clinical specimens. J Clin Microbiol **1999**; 37:565–9.
14. Brisson D, Dykhuizen DE. *ospC* diversity in *Borrelia burgdorferi*: different hosts are different niches. Genetics **2004**; 168:713–22.
15. Qiu WG, Dykhuizen DE, Acosta MS, Luft BJ. Geographic uniformity of the Lyme disease spirochete *(Borrelia burgdorferi)* and its shared history with tick vector *(Ixodes scapularis)* in the Northeastern United States. Genetics **2002**; 160:833–49.
16. Wang G, Ojaimi C, Wu H, et al. Disease severity in a murine model of Lyme borreliosis is associated with the genotype of the infecting *Borrelia burgdorferi* sensu stricto strain. J Infect Dis **2002**; 186:782–91.
17. Dykhuizen DE, Brisson D, Sandigursky S, et al. The propensity of different *Borrelia burgdorferi* sensu stricto genotypes to cause disseminated infections in humans. Am J Trop Med Hyg **2008**; 78:806–10.
18. Liang FT, Bowers LC, Philipp MT. C-terminal invariable domain of VlsE is immunodominant but its antigenicity is scarcely conserved among strains of Lyme disease spirochetes. Infect Immun **2001**; 69: 3224–31 (erratum: Infect Immun **2001**; 69:5216).
19. Sokal RR, Rohlf FJ. Biometry. New York: WH Freeman, **1995**.
20. Hope ACA. A simplified Monte Carlo significance test procedure. J R Stat Soc Ser B **1968**; 30:582–98.
21. Aguero-Rosenfeld ME, Nowakowski J, Bittker S, Cooper D, Nadelman RB, Wormser GP. Evolution of the serologic response to *Borrelia burgdorferi* in treated patients with culture-confirmed erythema migrans. J Clin Microbiol **1996**; 34:1–9.
22. Hanincova K, Liveris D, Sandigursky S, Wormser GP, Schwartz I. *Borrelia burgdorferi* sensu stricto is clonal in patients with early Lyme borreliosis. Appl Environ Microbiol **2008**; 74:5008–14.
23. Terekhova D, Iyer R, Wormser GP, Schwartz, I. Comparative genome analysis reveals substantial variation among clinical isolates of *Borrelia burgdorferi* with different pathogenic properties. J Bacteriol **2006**; 188: 6124–34.
24. Craft JE, Fischer DK, Shimamoto GT, Steere AC. 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 **1986**; 78:934–9.
25. Philipp MT, Lobet Y, Bohm RP Jr, et al. The outer surface protein A (OspA) vaccine against Lyme disease: efficacy in the rhesus monkey. Vaccine **1997**; 15:1872–87.
26. Bankhead T, Chaconas G. The role of VlsE antigenic variation in the Lyme disease spirochete: persistence through a mechanism that differs from other pathogens. Mol Microbiol **2007**; 65:1547–58.
27. Mogilvansky E, Chang Loa C, Adelson ME, Mordechai E, Tilton RC. Comparison of Western immunoblotting and the C6 Lyme antibody test for laboratory detection of Lyme disease. Clin Diagn Lab Immunol **2004**; 11:924–9.