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Interlaboratory and Intralaboratory Comparisons of Indirect Immunofluorescence Assays for Serodiagnosis of Lyme Disease

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A conventional indirect immunofluorescence assay (IFA) and an anticomplement indirect immunofluorescence assay (ACIF) for detecting serum antibodies to *Borrelia burgdorferi* in humans were evaluated during a prevalence survey in northern California. Sera obtained from 119 current or former residents of an area in which Lyme disease is endemic were split and tested by the IFA in two laboratories and the ACIF in a third. The seropositivity rate ranged from 15 to 20% with 88 to 93% agreement among laboratories. Interlaboratory agreement was statistically highly significant in each of the three pairwise comparisons and was positively associated with clinical manifestations of Lyme disease. Intralaboratory agreement ranged from 93 to 96% in two laboratories and was also statistically highly significant. Immunoblotting confirmed 100 of 101 of the nondiscrepant immunofluorescence test results and likewise was positively correlated with the degree of interlaboratory agreement. The ACIF was found to be a highly specific test (100% specificity) with a much lower cutoff titer (1:8) than the conventional IFA (determined to be 1:128 or 1:256 in two laboratories) for detecting antibodies to *B. burgdorferi*. It also appeared to be more sensitive (80 versus 68%) than the IFA as determined by comparative immunoblotting, though the absolute sensitivity of the ACIF for serodiagnosis of early Lyme disease has yet to be determined. Significant serologic cross-reactivity was demonstrated between *B. burgdorferi*, *Borrelia coriaceae*, and *Borrelia hermsii* by the IFA, which may confound spirochetal serosurveys in California where all three spirochetes are known to coexist.

A prospective epidemiologic study of a population at high risk for Lyme disease was initiated in a small community (population, ca. 150) in northern California in 1988. Part of that investigation, which is being conducted in the Ukiah area of Mendocino County, involves a serosurvey of study participants for antibodies to *Borrelia burgdorferi*. However, serious concerns have been expressed about the lack of standardization and the reliability of serologic tests that are being used routinely for diagnosis of Lyme disease (14, 24, 33; H. W. Wilkinson, H. Russell, and J. S. Sampson, Letter, J. Clin. Microbiol. **21**:291, 1985). The serodiagnosis of Lyme disease in the far western United States, especially in California, may be confounded further by the occurrence in that region of other species of tick-borne borreliae that cross-react serologically with *B. burgdorferi* (29). These include three species of relapsing fever spirochetes (*B. hermsii*, *B. parkeri*, and *B. turicatae*) and the recently described *B. coriaceae* (17, 18). Indeed, it has been suggested recently that a chronic neuroborreliosis syndrome that is caused by *B. burgdorferi* and other borreliae (29) may occur in California, since it is known that various neurologic sequelae may be caused by both Lyme disease and relapsing fever spirochetes (10, 25, 26, 29, 30, 34, 35).

Therefore, serum specimens obtained from subjects at entry into the northern California epidemiologic study were subjected to a three-way interlaboratory comparison; this was done to determine the agreement between and the reliability of the conventional indirect immunofluorescence assay (IFA) in the research laboratories of R.S.L. and J.E.M. and the anticomplement indirect immunofluorescence assay (ACIF) in the commercial reference laboratory of E.T.L. We selected indirect immunofluorescence for comparison because this assay is presently being used for serodiagnosis of Lyme disease in many commercial laboratories that test for Lyme disease in California.

The specific objectives of this study were to compare the results of the three indirect immunofluorescence assays for detecting antibodies to *B. burgdorferi* among members of the population at high risk for Lyme disease, to conduct an intralaboratory comparison with the same sera in the two laboratories using the conventional IFA, to determine the degree of serologic cross-reactivity of the B31 strain of *B. burgdorferi* from New York with a strain of *B. burgdorferi* from California as well as with *B. coriaceae* and *B. hermsii*, and to correlate the positive serologic test results with those obtained by Western immunoblot analysis and with clinical manifestations of the disease.

MATERIALS AND METHODS

Serum specimens. Sera were obtained from 99 current and 20 former residents of a small rural community located in the foothills near Ukiah in Mendocino County, Calif. This population was chosen for study because supporting clinical data from a previous serosurvey conducted there revealed that Lyme disease is hyperendemic in the area (J. E. Madigan, unpublished data). In addition to a consent form, a questionnaire was distributed to each participant at entry to obtain information about various demographic factors, clinical manifestations of Lyme disease, and potential risk factors for the disease.

To establish the cutoff titers, 115 serum specimens from residents of the San Francisco, Calif., metropolitan area who had no known history of Lyme disease were used as test control sera. They included 68 serum specimens from individuals age and sex matched (34 males and 34 females) to 68 members of the high-risk population; 35 serum specimens

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from children between 6 and 24 months of age, when the risk of tick exposure is low; 6 serum specimens positive for rheumatoid factor; 5 serum specimens positive for antinuclear antibodies; and a single serum specimen from a syphilitic patient.

Sera from the high-risk population and the test control population were blind coded, split three ways, and tested in each laboratory. Hereinafter, the three laboratories are designated as A, B, and C. The sera were decoded upon completion of the serologic testing. The results from the test control sera were used to determine the cutoff titer only; they were not used in analyzing the epidemiologic data.

Antigens. The type strain of *B. burgdorferi* (B31), from Shelter Island, N.Y., was grown at each site and used as the primary antigen in all three laboratories. The spirochetes were grown in BSK-II medium (3), except that horse serum was substituted for rabbit serum in laboratory C. The cultures were grown for 5 to 7 days, and the cells were harvested by centrifugation (8,200 × *g*) for 30 min. The organisms were washed in either phosphate-buffered saline (PBS) or PBS–5 mM magnesium chloride one to three times and diluted to approximately 100 organisms per high dry field (magnification, ×400). In the conventional IFA, 10 to 20 μl of the suspensions was added to each well of Teflon-coated microscope slides, air dried, and fixed in either 100% methanol for 3 min or acetone for 10 min. To determine cross-reactivity, sera that reacted to the B31 strain in laboratory A were tested against three additional borreliae: *B. burgdorferi* CA5, isolated from an *Ixodes pacificus* tick from Sonoma County, Calif. (19), and the type strains of *B. coriaceae* (C053), isolated from the soft tick *Ornithodoros coriaceus* in Mendocino County, Calif. (17, 18), and *B. hermsii* (HS1), isolated from *Ornithodoros hermsi* in Spokane County, Wash. *B. burgdorferi* CA5 had been passaged six times and *B. coriaceae* and *B. hermsii* had been passaged numerous times in vitro over a period of several years before use in this study.

IFA and ACIF. In the conventional IFA, serial twofold dilutions of test sera were prepared from an initial dilution of 1:2 or 1:16 in PBS. Ten to fifteen microliters of each dilution, starting at 1:64, was put on each antigen-coated well, and the slides were incubated in a humidified chamber at 34 to 37°C for 30 min. The slides were then washed three times with or soaked in PBS (10 min) and air dried. Fluorescein isothiocyanate-labeled goat anti-human immunoglobulin polyvalent conjugate (ICN Immunobiologicals, Costa Mesa, Calif.) was applied to each well at a working dilution of 1:5,000 and incubated as described above, and the slides were washed with PBS and rinsed in distilled water. The slides were mounted with cover slips, and the wells were examined by fluorescence microscopy. High-titered sera from patients with confirmed Lyme disease and sera from persons with no prior exposure to *B. burgdorferi* were used as positive and negative controls.

For the ACIF, smears of strain B31 were fixed in acetone:methanol (1:1) for 1 min and incubated successively with serially diluted, heat-inactivated sera at an initial dilution of 1:8, guinea pig complement in PBS containing 0.5 mM $MgCl_2$ and 0.015 mM $CaCl_2$, and fluorescein isothiocyanate-labeled goat anti-guinea pig C3 (1:50) (Organon Teknika-Cappel, Malvern, Pa.). Each addition was incubated at 37°C for 30 min and then rinsed twice with PBS. For scoring purposes, only organisms exhibiting even, complete staining patterns were considered positive; uneven and broken staining patterns on >50% of the organisms were considered negative or nonspecific. In both the IFA and the ACIF, the dilution at which 50% or more of the organisms stained specifically was considered to be the endpoint titer. Sera were processed in each laboratory by the individual protocols of the participating researchers. In laboratories A and B, each serum was tested twice. In laboratory C, only sera that were reactive at ≥1:8 dilutions were retested for confirmation. Therefore, only the first two rounds of test results from laboratories A and B could be evaluated as part of the intralaboratory comparison. Significant discrepancies in endpoint titers between the two runs were resolved by a third round of testing.

HATTS. All sera that were positive for antibodies to B31 in any of the three laboratories were tested by a hemagglutination treponemal test for syphilis (HATTS) (Difco Laboratories, Detroit, Mich.) according to the instructions of the manufacturer. The sensitivity and specificity of the HATTS are comparable to those of the fluorescent treponemal antibody-absorption test for the serodiagnosis of syphilis (12, 38).

Western blot analysis. To determine the sensitivity of the indirect immunofluorescence assays, all 119 serum specimens from the high-risk population were assayed for antibodies to *B. burgdorferi* by immunoblotting. One hundred microliters of B31 extract, prepared by previously described methods (4, 5, 19), was electrophoresed with prestained low-molecular-weight standards on a 10-cm minigel at 200 V for 1.5 h. The proteins were transferred onto nitrocellulose paper in a Bio-Rad transphor cell (Bio-Rad Laboratories, Richmond, Calif.) overnight at room temperature at 30 mA. Each blot was allowed to equilibrate for 30 min in Tris-buffered saline with 1% Tween 20 as a blocking agent. The blots were incubated with undiluted human sera in a Miniblotter 28 (Immunetics, Cambridge, Mass.) for 1 h, washed with 300 ml of Tris-buffered saline for 15 to 30 s, removed, and reincubated for 30 min with goat anti-human immunoglobulin G-alkaline phosphatase conjugate diluted 1:3,000 (Bio-Rad). Next, they were developed in BCIP (5-bromo-4-chloro-3-indolylphosphate)-Nitro Blue Tetrazolium alkaline phosphatase color development solution (Bio-Rad) after they had been washed three times for 10 min each time in Tris-buffered saline. After development, the blots were washed twice in distilled water, dried between two pieces of filter paper, and stored in darkness. A serum specimen was considered to be positive for *B. burgdorferi* by immunoblotting if antibodies reactive to polypeptides having relative mobilities of ca. 31 to 32, 34 to 35, and 40 kilodaltons (kDa) were present.

Statistical analysis. After all sera had been scored either positive or negative in each laboratory, the κ statistic was used to characterize the agreement among all three laboratories and within laboratories A and B only (11). This statistic is adjusted for chance agreement and varies from −1.0 (perfect disagreement) to +1.0 (perfect agreement).

RESULTS

Control sera. The cutoff titers were determined from the results obtained with the 115 negative control serum specimens. In each laboratory, the cutoff titer was the lowest serum titer below which 99% of the control sera would be considered negative. With this criterion, the minimum test specificity of 99% could be preset within each laboratory and thus standardized among laboratories. In laboratory A, 114 of 115 of control serum specimens were reactive at titers of ≤1:64 with the remaining serum reactive at a titer of 1:128. In laboratory B, 114 of 115 serum specimens were reactive at ≤1:128 and 1 was reactive at 1:256. In laboratory C, all 115

TABLE 1. Interlaboratory comparison of IFA and ACIF titers to *B. burgdorferi*

Laboratory and IFA test result	No. (%) of tests with indicated result[a]			
	ACIF (laboratory C)		IFA (laboratory B)	
	+	−	+	−
A				
+	15 (12.6)	9 (7.6)	14 (11.8)	10 (8.4)
−	5 (4.2)	90 (75.6)	4 (3.4)	91 (76.5)
B				
+	15 (12.6)	3 (2.5)		
−	5 (4.2)	96 (80.7)		

[a] Statistical measures were as follows. For IFA (laboratory A) and ACIF: percent agreement = 88.2, $\kappa = 0.610$, and $P = 10^{-6}$. For IFA (laboratory A) and IFA (laboratory B): percent agreement = 88.3, $\kappa = 0.597$, and $P = 10^{-6}$. For IFA (laboratory B) and ACIF: percent agreement = 93.3, $\kappa = 0.750$, and $P = 10^{-6}$.

serum specimens were reactive at <1:8. Hence, the cutoff titers were set at 1:128, 1:256, and 1:8 for laboratories A, B, and C, respectively, to achieve the desired test specificity of ≥99%. Notably, only one of the three highest-titered serum specimens in laboratory A also was reactive in laboratory B, and none was from patients with autoimmune diseases. Moreover, none of the four serum specimens producing titers ≥1:128 in laboratory A or B was positive by immunoblotting.

Sera from a high-risk population. With the above cutoff titers, the seropositivity rate detected for the population residing in the area in which Lyme disease is hyperendemic varied from 15 to 20% among laboratories. The resultant pairwise interlaboratory agreement ranged from 88 to 93% and was statistically highly significant in each of the three pairwise comparisons. The lowest agreement occurred between the two IFA procedures and between the IFA in laboratory A and the ACIF; the highest agreement was between the IFA in laboratory B and the ACIF (Table 1). The absolute interlaboratory agreement was 85%, since 87 serum specimens were negative and 14 were positive in all three laboratories. Of the remaining 18 serum specimens, 16 were positive in one laboratory and 2 were positive in two laboratories. The run-to-run agreement varied from 93% in laboratory A ($\kappa = 0.80$) to 96% in laboratory B ($\kappa = 0.84$) and was statistically highly significant in both laboratories ($P < 0.000001$). Titers for positive sera ranged from 1:128 to 1:1,024 in laboratory A, 1:256 to 1:4,096 in laboratory B, and 1:16 to 1:1,024 in laboratory C.

Immunoblot results. While the overall agreement among laboratories was highly significant, there were discrepancies. To resolve interlaboratory disagreements and to validate interlaboratory agreements, immunoblotting was performed on all 119 test serum samples. Of the 32 serum specimens positive by the IFA, the ACIF, or both, 24 (which included all 14 nondiscrepant sera and 10 of 18 discrepant sera) were confirmed as positive by Western blot analysis (Table 2). The percentages of the 24 serum specimens confirmed by Western blot analysis that were IFA or ACIF positive in laboratory A, B, or C were 71, 71, and 83%, respectively. Of the 87 serum samples that were negative in all three laboratories, 1 was positive by immunoblot analysis while 3 were indeterminate. The latter sera were reactive primarily to the 40-kDa polypeptide and did not meet the criteria for positivity. Twenty-one of the 25 Western blot-positive serum specimens were strongly reactive and 4 were weakly reactive to outer surface polypeptides specific for *B. burgdorferi*, i.e., those having relative mobilities of ca. 31 to 32 and 34 to 35 kDa. Also, three HATTS-positive serum specimens that were positive in all three laboratories by the IFA and the ACIF for *B. burgdorferi* also were Western blot positive against the B31 antigen.

TABLE 2. Interlaboratory comparison of IFA, ACIF, and Western blotting

Western blot result	No. of tests with indicated result					
	IFA (laboratory A)		IFA (laboratory B)		ACIF (laboratory C)	
	+	−	+	−	+	−
+	17	8	17	8	20	5
−	7	87	1	93	0	94

Clinical correlates. Questionnaires were filled out by 93 of the 99 participating current residents and by 10 of 20 former residents whose sera were assayed for antibodies to *B. burgdorferi*. As summarized in Table 3, 79% of the subjects whose sera were reactive in all three laboratories reportedly had one or more clinical manifestations consistent with a diagnosis of Lyme disease, and, in fact, 85% of them had been diagnosed with the disease before entry into the study by their physicians. Among the 15 subjects whose sera were reactive in just one laboratory, only 33% had signs or symptoms suggestive of Lyme disease and only 29% had been diagnosed previously with the disease.

Cross-reactivity between *B. burgdorferi* B31 antigen and other spirochetal antigens. Twenty-four serum specimens that were positive by IFA in laboratory A were retested with *B. burgdorferi* CA5, *B. coriaceae* C053, and *B. hermsii* HS1 (Table 4). There was considerable cross-reactivity with all three antigens. In the comparison involving the two *B. burgdorferi* strains, B31 and CA5, 11 of 24 serum specimens had two- to eightfold higher titers to B31, 10 had identical titers to both strains and 3 had twofold higher titers to CA5. When B31 was compared with *B. coriaceae* C053, 17 serum specimens had two- to greater than eightfold higher titers to B31, 6 had identical titers to both, and 1 had a twofold higher titer to C053. Similarly, when B31 was compared with *B. hermsii* HS1, 21 serum specimens yielded two- to greater than eightfold higher titers to B31, 2 had identical titers to both, and 1 had a higher titer to HS1. If a cutoff titer of 1:128 had been selected for CA5, *B. coriaceae*, and *B. hermsii*, then 5 of the 24 serum specimens would have been negative for CA5, 9 would have been negative for C053, and 11 would have been negative for HS1 in comparison with B31.

DISCUSSION

In a previously reported interlaboratory comparison in Minnesota involving four laboratories, the seropositivity rate obtained by participants for identical samples varied widely from 0 to 35% (14). In that report, a single procedure (IFA) was used with a uniform cutoff titer of 1:256. On the basis of the significantly different results obtained, the authors concluded that a lack of standardization in serologic testing can lead to significant variation in results as well as to difficulties in interpreting the full clinical spectrum of Lyme disease.

Similarly, serum specimens from 132 outdoor workers in New Jersey were tested for antibodies to Lyme disease in as many as four independent laboratories to evaluate inter- and

TABLE 3. Clinical manifestations of Lyme disease correlated with seroreactivity in three laboratories

No. of laboratories in which sera were reactive (no. of subjects)	No. (%) with manifestations of Lyme disease[a]					
	Erythema migrans	Arthritis or arthralgia	Cardiac	Neurologic	None	Diagnosed previously with Lyme disease
3 (14)	4 (29)	9 (75)	1 (8)	5 (36)	3 (21)	11 (85)
2 (2)	2 (100)	2 (100)	0 (0)	1 (100)	0 (0)	0 (0)
1 (15)	2 (13)	4 (27)	1 (7)	2 (14)	10 (67)	4 (29)
Total	8 (26)	15 (52)	2 (7)	8 (27)	13 (42)	15 (52)

[a] Not all subjects responded to every question concerning clinical manifestations, and therefore the percentages tabulated here are based on slightly reduced sample sizes in several instances.

intralaboratory agreement (33). Two laboratories performed IFA and two performed enzyme-linked immunosorbent assays, and each laboratory had a different cutoff to determine seropositivity. Interlaboratory agreement, as measured with the κ statistic, was consistently low to moderate (κ values ranged from 0.45 to 0.53), as was intralaboratory agreement in a single commercial laboratory. The authors concluded that the lack of interlaboratory agreement indicates that the methods, antigens, and reagents used for the serodiagnosis of Lyme disease should be standardized statewide.

In contrast to the reports described above, we found that there was a statistically highly significant agreement within each of two laboratories as well as among all three laboratories (Table 1). In the design of the experiment, the selection of the procedure to be used in each laboratory was discretionary, and the reagents were prepared individually by the participants. However, each participant agreed to use *B. burgdorferi* B31 antigen as the primary antigen, to evaluate the test and control panels in a blind fashion, to determine the reproducibility of each subject's results with repeat testing, and, finally, to determine the sensitivity and specificity of the procedure used in his or her laboratory against a reference immunoblotting procedure performed in laboratory A. It was anticipated that the cutoff titer established for each laboratory would be affected by differences in the procedures used, how reagents were prepared, which microscopes were available, and the reading standards. It was decided that absolute standardization for those four factors would not be feasible or necessary. Rather than adopting a standardized cutoff titer, the participating laboratories decided that the cutoff titer should be established within each laboratory to minimize the effects of potential interprocedural and interlaboratory differences. This was accomplished effectively by setting a test specificity of 99% as a criterion on the basis of results obtained from testing 115 negative control serum specimens.

TABLE 4. Prevalence of homologous or heterologous indirect immunofluorescent antibodies to strains of *Borrelia* spp.[a]

Specimen no.	Reciprocal titration endpoints for antigen of strain:			
	B31	CA5	C053	HS1
2	512	64	<64	<64
8	256	64	<64	<64
9	256	256	64	<64
14	256	512	256	512
30	1,024	256	512	256
32	1,024	512	512	512
39	128	128	64	<64
40	128	64	64	<64
45	512	512	256	128
53	128	64	128	<64
65	512	512	512	256
67	256	512	512	256
70	512	512	512	256
71	512	512	<64	128
75	512	512	512	512
77	256	512	128	128
79	256	128	128	64
92	512	512	512	64
94	128	128	64	64
95	1,024	512	512	512
100	512	128	128	128
101	512	256	256	256
110	128	<64	64	64
113	128	128	64	64

[a] Prevalence of antibodies to *B. burgdorferi* CA5 and *B. coriaceae* C053 from California and the type strain (HS1) of *B. hermsii* among 24 human serum samples that reacted with the type strain (B31) of *B. burgdorferi* from New York at titers ≥1:128 in laboratory A.

Immunoblotting confirmed 99% of the nondiscrepant results and correlated well with the agreement between laboratories. In 100 of 101 instances in which a serum was positive (n = 14) or negative (n = 87) in all three laboratories, it was confirmed by Western blot analysis. Conversely, only 56% of discrepant sera were confirmed by Western blot analysis. Of the two immunofluorescence procedures, the ACIF appeared to be more sensitive and specific. Relative to the immunoblotting assay, the sensitivity and specificity were 80 and 100% for the ACIF, 68 and 99% for the IFA in laboratory B, and 68 and 93% for the IFA in laboratory A.

Interlaboratory agreement in seroreactivity was positively associated with clinical manifestations of Lyme disease (Table 3). Subjects whose sera were reactive in all three laboratories were more than twice as likely to have reported one or more manifestations (i.e., arthritic, cardiac, dermatologic, or neurologic manifestations) associated with Lyme disease and three times as likely to have been diagnosed with the disease before entry into the study than subjects whose sera were reactive in a single laboratory. Three (21%) of fourteen subjects whose sera were reactive in all three laboratories reported that they had not experienced manifestations of Lyme disease. One of them had been diagnosed with and treated for Lyme disease on the basis of an earlier positive serologic test result, whereas the two remaining subjects are being monitored prospectively to determine whether they will develop the disease. Overall, subclinical infections occurred in 10 (42%) of 24 subjects whose sera were positive by both the IFA (i.e., in one or more laboratories) and immunoblotting and who completed a questionnaire. Among the 93 current residents of the test population who participated in this study, 9 (9.7%) had subclinical infections. Likewise, 6.8 to 9.0% of residents of Fire Island,

N.Y., Great Island, Mass., and Middletown, Conn., had asymptomatic infection with *B. burgdorferi* (7, 13, 37).

Serologic cross-reactivity between *B. burgdorferi* and other spirochetes, particularly borreliae and treponemes, has been demonstrated previously (2, 8, 16, 21, 22, 27, 28, 31, 39). Sera from patients with a variety of other clinical conditions (e.g., autoimmune disorders, human immunodeficiency virus infection, infectious mononucleosis, multiple sclerosis, rheumatoid arthritis, or Rocky Mountain spotted fever) also cross-react with *B. burgdorferi* in serologic tests (20, 21, 23, 27, 28, 36). We detected considerable serologic cross-reactivity between *B. burgdorferi* and antigens prepared from each of two other tick-borne borreliae (*B. coriaceae* and *B. hermsii*) that occur naturally in California (Table 4). Cross-reactivity between *B. burgdorferi* and the relapsing fever spirochete *B. hermsii* has been recorded for both human and coyote sera by others (6, 21, 22, 27), but cross-reactivity between *B. burgdorferi* and *B. coriaceae* has not been reported previously. In California, *B. hermsii* occurs predominantly in mountainous regions above 5,000 ft (1,524 m) (40), which do not include coastal counties such as Mendocino, the source of the sera evaluated in the present investigation. In contrast, *B. coriaceae* has been isolated only in Mendocino County from its soft tick vector, *O. coriaceus* (18). Although *B. coriaceae* is not known to cause human infection, *O. coriaceus* bites humans occasionally and reactions to its bite may produce systemic reactions in some individuals (9, 15). When the 24 IFA-positive sera in laboratory A were retested with *B. burgdorferi* (two strains), *B. coriaceae*, and *B. hermsii* as sources of antigens, many of the sera reacted equally well to two or more of these antigens. It is impossible to ascertain from the antibody titers alone which species of the genus *Borrelia* was the actual infecting organism. In our study, the degree of cross-reactivity was greatest between *B. burgdorferi* and *B. coriaceae* and least between the former and *B. hermsii*.

Cross-reactivity between *B. burgdorferi* and *Treponema pallidum* was not found to be a major problem in our study. Only 3 of 32 *B. burgdorferi*-positive serum specimens also reacted in the HATTS, two with titers of 1:16 and one with a titer of 1:32. Western blot analysis revealed that the immunoglobulins present in these three serum specimens were indeed directed against *B. burgdorferi*-specific antigen bands (i.e., 31- and 34-kDa polypeptides). Further, illnesses experienced by two of the individuals with *B. burgdorferi*- and HATTS-positive sera met a standardized case definition for Lyme disease. Although serologic cross-reactivity between *B. burgdorferi* and *T. pallidum* has been reported, antibodies to *B. burgdorferi* usually do not react in the microhemagglutination test for *T. pallidum* antibody, the rapid plasma reagin test, the Venereal Disease Research Laboratory assay, or as demonstrated here, in the HATTS (8, 16, 31).

In addition to interspecies cross-reactivity, we also examined intraspecies differences, i.e., between *B. burgdorferi* B31 and CA5. Previous reports indicated no significant serologic differences between *B. burgdorferi* isolates from the United States and Europe by IFA (1, 2, 31, 39). Russell et al. (31), for example, tested 12 serum specimens from residents of Minnesota and New Jersey with five isolates of *B. burgdorferi* (two from humans and three from ticks) and found that all five isolates produced similar results within a twofold dilution factor. In contrast, 5 of 24 (21%) serum specimens in the present study differed significantly in their production of antibodies against B31 and CA5 (Table 4). These differences may be due to a disparity in the in vitro passage level between the 2 cultures. Rapid antigenic changes and loss of one of the major outer surface proteins (OspB) in *B. burgdorferi* have been observed following its short-term (10 to 15 passages) in vitro cultivation in BSK-II medium, and this phenomenon must be considered when the results of spirochetal serosurveys are being interpreted (32). In addition, significant variations even exist in the major outer surface proteins (OspA and OspB) and other proteins (21 to 25 kDa) in fresh tick-derived isolates of *B. burgdorferi* from northern California (5, 19). Isolate CA5 possessed abundant proteins with relative mobilities of ca. 21.5 and 24 kDa that were not present in several other fresh isolates from ticks or in the B31 strain used as a control in this study (19).

This is the first report describing the use and validity of the ACIF for detecting antibodies to *B. burgdorferi*. The fact that only true antigen-antibody complexes (versus nonspecific antibody binding or Fc receptor-antibody reactions) can be detected with guinea pig complement and fluorescein isothiocyanate-labeled anti-guinea pig complement C3 component in this assay significantly reduces the nonspecific reactivity that may occur in the conventional IFA. Unlike the IFA, which usually exhibits gradual loss of intensity with decreasing antibody concentration, the staining observed in the ACIF has very sharp, reproducible endpoints that facilitate quantitation and interpretation. Therefore, a cutoff titer of only 1:8 was found to confer a test specificity of 100% with no apparent reduction in sensitivity relative to the IFA as determined by comparative immunoblotting. These findings establish that the ACIF is a useful serodiagnostic tool for Lyme disease. However, its absolute sensitivity for detecting antibodies in individuals experiencing the early stages of the disease remains to be determined.

In conclusion, a three-way interlaboratory comparison using independently developed procedures showed that significant agreement can be obtained in the serodiagnosis of Lyme disease provided that participating laboratories adhere to a uniform standard for test specificity.

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