Impact of Strain Heterogeneity on Lyme Disease Serology in Europe: Comparison of Enzyme-Linked Immunosorbent Assays Using Different Species of *Borrelia burgdorferi* Sensu Lato

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For the standardization of serological tests for Lyme borreliosis (LB) in Europe, the influence of the heterogeneity of *Borrelia burgdorferi* sensu lato must be assessed in detail. For this study four immunoglobulin M (IgM) and IgG enzyme-linked immunosorbent assays (ELISAs) with octyl-β-D-glucopyranoside extracts of strains PKo (*Borrelia afzelii*), PBi (*Borrelia garinii*), and Pka2 and B31 (both *B. burgdorferi* sensu stricto) were compared. Strains PKo, PBi, and Pka2 at the passages used for antigen preparations abundantly expressed outer surface protein C (OspC), whereas strain B31 at the passage used for antigen preparation did not express OspC. Sera (all from Germany) from 222 patients with clinically defined LB of all stages, 133 blood donors, and 488 forest workers were tested. None of the forest workers had symptoms consistent with LB at the time that the samples were collected. For IgM tests, receiver operating characteristic curves demonstrated that discrimination between sera from patients and blood donors was best with strain PKo and worst with strain B31. The discriminatory abilities of the four IgG ELISAs were similar in a diagnostically reasonable specificity range (90 to 100%). More than 20% of the sera from forest workers reacted strongly in the PKo IgG ELISA (optical density value, >1.5; other assays, less than 8%). Western blots of the sera with the most discrepant ELISA results revealed almost exclusive reactivity with p17. This highly immunogenic antigen is only expressed by strain PKo. This observation might be important for the development of assays enabling discrimination between asymptomatic or previous infection and active disease.

Lyme borreliosis (LB) is a global tick-associated disease caused by infection with *Borrelia burgdorferi* sensu lato. The disorder develops in stages and has different manifestations. In Europe, three species pathogenic for humans (2) and at least eight serotypes of *B. burgdorferi* sensu lato (40, 44) demonstrating both inter- and intraspecies heterogeneity are known. Different species seem to show a preferential association with different clinical manifestations (1, 35, 44). The most frequent disorders in Eurasia are erythema migrans (EM), neuroborreliosis (NB), acrodermatitis chronica atrophicans (ACA), and arthritis (19, 32). In Europe, all three pathogenic species have been isolated from human biopsy specimens and cerebrospinal fluid (CSF) as well as from ticks (*Ixodes ricinus*), but the predominant species seem to be *Borrelia afzelii* and *Borrelia garinii*. *B. afzelii* has been found to be associated more frequently with skin lesions, whereas *B. garinii* is the predominant species found in patients with neurological disorders (8, 35, 40, 44). In North America only *B. burgdorferi* sensu stricto occurs (30, 44); arthritis occurs frequently, whereas ACA is almost unknown, and multiple EM lesions are more common in North America than in Europe (33).

The diagnosis of Lyme disease is based on the recognition of typical clinical signs and is supported by laboratory tests, especially if the clinical picture is not clear. Since culture is laborious and insensitive and PCR assays are still considered controversial, routine testing comprises mostly serological methods. However, serology also harbors several problems: The occurrence of cross-reacting antibodies may result in false-positive findings (5). Furthermore, patients may still be seronegative in the early stages of the infection and the humoral immune response can be diminished after the early onset of antibiotic treatment (37). Several strategies for increasing both sensitivity and specificity (i.e., the discriminatory ability of the test) have been developed, for instance, preabsorption of cross-reactive antibodies with *Treponema phagedenis* (49), the use of detergent extracts of *B. burgdorferi* sensu lato (3), and the use of purified flagella (16) or various recombinant antigens (7, 31, 41, 42).

Serological tests for Lyme disease have not been standardized so far, leading to considerable variations in test results among different laboratories. The heterogeneity of Lyme disease borreliae as well as different methods of antigen preparation and test performance may contribute to the problem.

In Europe, the extent of variation resulting from the use of different strains for antigenic preparations is still widely discussed (1, 23, 46, 48). Differences in the regional distributions of *Borrelia* species may further influence the preferential reactivities of sera from patients with LB (6, 26).

In this study, enzyme-linked immunosorbent assays (ELISAs) with detergent extracts of different strains representing the three species pathogenic for humans were compared. Strains PKo (*B. afzelii*), PBi (*B. garinii*), and Pka2 (*B. burgdorferi* sensu stricto) at the passage used to obtain coating antigens abundantly expressed outer surface protein C (OspC), whereas strain B31 (*B. burgdorferi* sensu stricto) at the passage used to obtain coating antigen did not express OspC. In Western blot studies OspC has been shown to be one of the most immuno-
dominant antigens for the early immune response (12, 13, 17, 48). The influence of the expression of this lipoprotein on the results of ELISA was especially monitored by comparison of the test results for the otherwise closely related B. burgdorferi sensu stricto strains PKa2 and B31 (20, 29, 38, 43). To analyze the sensitivities and specificities of the four ELISAs, we probed sera from German patients with different clinical manifestations as well as sera from healthy blood donors and forest workers (as an example of a group highly exposed to ticks and at high risk of contracting infections with B. burgdorferi sensu lato).

MATERIALS AND METHODS

Sera. Sixty-six serum specimens from unslected, untreated patients with EM were obtained by a dermatologist during a previous multicenter therapy study (37). The median time period between the appearance of EM and collection of the serum samples was 3 weeks (range, 1 day to 31 weeks). Four patients with EM were diagnosed by a dermatologist during the month April through January. The NB group (n = 125) included 38 patients (designated NB I) from whom B. burgdorferi sensu lato was isolated from CSF and 87 other patients (designated NB II) with typical signs of EM, placed in duplicate on BSK II (Biolog, Manheim, Germany). The S. cerevisiae culture used for the calibrator OD was determined by an in-house ELISA with strain Pko (39). All serum specimens were obtained on the same days that the CSF specimens were obtained. The median time period between the appearance of EM and collection of the serum samples was 3 weeks in both NB groups (range, 1 day to 1 year). These sera were collected during the years 1984 through 1995 during the months May through January (72 serum specimens were obtained in August or September). The patients came from throughout Germany, but most were from the south. Thirty-one serum specimens were obtained from patients with ACA diagnosed by a dermatologist. Samples were collected during the years 1988 through 1995 with no seasonal dependence. The patients came from throughout Germany, but most were from the south. Sera (n = 485) from healthy forest workers from Bavaria (southern Germany) were collected during a former study (25) during the years 1984 and 1985. Sera from 133 blood donors were investigated for determination of cutoffs. Samples were obtained in December 1990 from a Munich blood bank. The whole of Bavaria is a region where LB is endemic. All sera were stored in aliquots at -20°C.

Preparation of antigens. Borrelial strains Pko (B. afzelii; Ospa serotype 2), PBi (B. garinii; Ospa serotype 4), PKa2 (B. burgdorferi sensu stricto; Ospa serotype 1), and B31 (B. burgdorferi sensu stricto; Ospa serotype 1) (44) were used for antigen preparations. Strains Pko, PBi, and PKa2 have been isolated from German patients at the Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, Munich; Pko was isolated from skin biopsies (EM), and PBi and PKa2 were isolated from CSF. Strain B31 was obtained from W. Burgdorfer (Hamilton, Mont.). Strains were grown in modified Kelly medium (28) at 33°C for 4 to 5 days and were harvested at a cell density of 10^7/ml. The protein concentration of the final suspension was adjusted by the Bradford (45) protein assay (BioRad, Munich, Germany). The protein concentration was stored at 20°C.

Low-passage strains (approximately 25 passages) Pko, PBi, and PKa2 abundantly expressed OspC, whereas the high-passage strain B31 did not. This was shown by Coomassie brilliant blue staining after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as well as by probing with the OspC-specific monoclonal antibody L22 1F8 (45). If OspC had been expressed by the passage of B31 used, it would have been recognized by L22 1F8 (43).

ELISAs. Microtiter plates were coated with whole-cell octyl-β-D-glucopyranoside (OGP) extracts of the respective antigen preparations (27). The concentrations of the coating antigens were optimized for the best matching of the different strains by using a representative panel of human sera (11 individual serum samples from German patients and blood donors; 5 were IgG positive). The protein concentration (0.5 to 3 μg/ml) was not identical for each strain, since coating was optimized for each strain by antigen titration to obtain the best correlation of signals. Plates were coated identically for both IgG and IgM tests, and all assays were performed with the same batch of plates. Assays were performed separately for IgG and IgM, according to the manufacturer’s instructions for the commercial Pko ELISA, by using a Behring ELISA processor III (Enzymost Borreliosis; Behring Diagnostics, Marburg, Germany). The sample diluent contained ultrasonicated cell lysates of T. phagedenis (as an example of a group highly exposed to ticks and at high risk of contracting infections with B. burgdorferi sensu stricto) (43). The influence of the expression of this lipoprotein on the results of ELISAs was especially monitored by comparison of the test results for the otherwise closely related B. burgdorferi sensu stricto strains PKa2 and B31 (20, 29, 38, 43). To analyze the sensitivities and specificities of the four ELISAs, we probed sera from German patients with different clinical manifestations as well as sera from healthy blood donors and forest workers (as an example of a group highly exposed to ticks and at high risk of contracting infections with B. burgdorferi sensu lato).

Preparation of antigens. As demonstrated in Fig. 1, strains Pko, PBi, and PKa2 abundantly expressed OspC at the passages used, whereas strain B31 completely lacked this antigen at the passage used. OGP extracts of these whole-cell lysates were used as antigenic coatings. The OspC bands of strains Pko, PBi, and PKa2 are indicated on the left. Strain B31 did not express OspC. Numbers on the right indicate the molecular masses of the marker proteins (in kilodaltons).

RESULTS

Preparation of antigens. As demonstrated in Fig. 1, strains Pko, PBi, and PKa2 abundantly expressed OspC at the passages used, whereas strain B31 completely lacked this antigen at the passage used. OGP extracts of these whole-cell lysates were used as antigenic coatings.
Definition of cutoff values. Sera (n = 133) from blood donors were tested, and the OD values of all sera were sorted separately for each test for the determination of percentiles (Fig. 2). The 10 most strongly reacting serum samples in the IgG ELISAs and the 18 most strongly reacting serum samples in the IgM ELISAs were subsequently tested by WB with whole-cell lysates of the respective strains. Most sera were either positive or showed reactions with unspecific bands (for IgG WB with PKo, 6 positive and 3 unspecific serum samples; for IgG WB with PBi, 5 positive and 4 unspecific serum samples; for IgG WB with PKa2, 6 positive and 2 unspecific serum samples; for IgM WB with PKo, 6 positive and 10 unspecific serum samples; for IgM WB with PBi, 7 positive and 8 unspecific serum samples; for IgM WB with PKa2, 5 positive and 9 unspecific serum samples). Between the 92nd and 100th percentiles the OD values of the IgG ELISAs increased considerably, whereas in the IgM ELISAs a gradual increase was observed. The OD values of the 92nd and the 95th percentiles were defined as cutoffs for the IgG and the IgM assays, respectively (Table 1). For simplification, no borderline or retest ranges were defined in this study.

TABLE 1. Cutoff values for different ELISAs

<table>
<thead>
<tr>
<th>Ig class</th>
<th>Cutoff value (OD) for ELISA with following strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PKo</td>
</tr>
<tr>
<td>IgG</td>
<td>0.127</td>
</tr>
<tr>
<td>IgM</td>
<td>0.322</td>
</tr>
</tbody>
</table>
The discriminatory abilities (between patients and controls) of the four ELISAs were analyzed by ROC curves (Fig. 3). By this method, the properties of a diagnostic assay can be analyzed without reference to an individual (arbitrary) cutoff. For IgG tests the largest ROC areas resulted for the B31 ELISA, but in the specificity range of between 90 and 100%, which is conclusive for diagnostic purposes, the differences in the sensitivities of the four assays strongly depended on the specificity level. For example, at 92% specificity, the highest sensitivity was achieved with the PKo ELISA (PKo ELISA, 68%; Pbi ELISA, 65%; PKa2 ELISA, 57%; B31 ELISA, 63%), whereas at 97% specificity, the PKo ELISA was the least sensitive (PKo ELISA, 39%; Pbi ELISA, 46%; PKa2 ELISA, 50%; B31 ELISA, 53%). For IgM tests the ROC area of the PKo ELISA was the largest and the ROC area of the B31 ELISA was the smallest, meaning that the PKo test gave the best discrimination and the B31 test gave the worst.

**Sensitivities for different study groups.** Subsequently, the sensitivities of the four ELISAs were determined separately for all study groups (Table 2). Whenever possible, differences between ELISAs were analyzed by McNemar's χ² test.

For NB I, similar sensitivities were obtained by the PKo, Pbi, and PKa2 ELISAs, but the sensitivities of the B31 ELISAs were lower. However, since no more than 38 samples were available for this group (a positive culture of CSF was required), these differences were insignificant. By testing for both IgG and IgM, 73.7% (B31 ELISA) to 84.2% (PKa2 ELISA) of the sera were found to be positive. For NB II, the PKo tests were the most sensitive, followed by the Pbi, PKa2, and B31 tests, in decreasing order.

The best strain for the detection of antibodies in patients with EM lesions was PKo. The Pbi and PKa2 ELISAs showed similar results, while the results of the B31 IgG ELISA were comparable to those of the PKo IgG assay, but the B31 IgM ELISA was the least sensitive of the four IgM tests. By testing for both IgG and IgM, 62.1% (PBi and PKa2 ELISAs) to 75.8% (PKo ELISA) of the sera were positive. All sera from patients with ACA were reactive in all four IgG ELISAs. IgM antibodies could be detected in 9.7% (B31 ELISA) to 48.4% (PKo ELISA) of the patients with ACA.

The sera from the forest workers were more frequently reactive in IgG ELISAs with PKo or PKa2 than in IgG ELISAs with Pbi or B31. IgM tests were positive for 1.1% (PKo ELISA) to 12.0% (PKo ELISA) of the sera. A total of 41.5% (B31 ELISA) to 46.9% (PKo ELISA) of the sera were positive by evaluation by both IgG and IgM tests.

**Correlation of OD values of different ELISAs.** To demonstrate correlations of ELISA results, OD values from different assays were plotted against each other (Fig. 4 and 5). In IgG tests (Fig. 4) with sera from patients with NB the highest correlations occurred between the PKo, Pbi, and PKa2 ELISAs (all Spearman rank correlation coefficients, \( r > 0.96 \)). The correlation coefficients of the plots between ELISAs with each of the last three strains and the ELISA with strain B31 were between 0.84 and 0.87. For the groups of forest workers as well as patients with skin manifestations (EM and ACA), results of tests with strains Pbi, PKa2, and B31 correlated better (\( r > 0.95 \) for all correlations) than the PKo ELISAs with any of these other assays (\( r < 0.96 \)). This scattering resulted from the fact that several sera from these groups were considerably more reactive with PKo than with the other strains.

In IgM tests (Fig. 5) with sera from all patients with LB (both neurological and skin manifestations), the best correlations were found between PKo, Pbi, and PKa2 ELISAs (\( r > 0.96 \)). The scattering was wider in the plots of the data for the B31 ELISA against the data for the ELISAs with each of the other strains (\( r < 0.74 \)). In tests with the sera from the forest workers, \( r < 0.61 \) (PKo ELISA versus B31 ELISA) and 0.80 (PBi ELISA versus PKa2 ELISA).
TABLE 2. Sensitivities of the different ELISAs

| Study group       | Total no. of serum samples | Ig class(es) evaluated | % Sera positive in ELISA with the following strain: | P value  
|-------------------|-----------------------------|-----------------------|---------------------------------------------------|----------
|                   |                             |                       | Pko | PBi | PKa2 | B31 | Pko versus PBi | Pko versus PKa2 | PBi versus PKa2 | Pko versus B31 | PBi versus B31 | PKa2 versus B31 |
| NB I              | 38                          | IgG                    | 55.3 | 55.3 | 57.9 | 42.1 | ND             | >0.05            | >0.05            | >0.05            | >0.05            | >0.05            |
|                   |                             | IgM                    | 68.4 | 68.4 | 71.1 | 52.6 | >0.05         | >0.05            | >0.05            | ND              | ND              | ND              |
|                   |                             | IgG and IgM            | 78.9 | 81.6 | 84.2 | 73.7 | ND             | ND              | ND              | ND              | ND              | ND              |
| NB II             | 87                          | IgG                    | 80.5 | 79.3 | 70.1 | 75.9 | >0.05         | <0.05            | <0.05            | ND              | ND              | ND              |
|                   |                             | IgM                    | 87.4 | 83.9 | 78.1 | 48.3 | >0.05         | <0.05            | <0.05            | ND              | ND              | ND              |
|                   |                             | IgG and IgM            | 97.7 | 95.4 | 93.1 | 83.9 | ND             | ND              | ND              | ND              | ND              | <0.05            |
| Patients with EM  | 66                          | IgG                    | 45.5 | 37.9 | 34.8 | 47.0 | >0.05         | <0.05            | <0.05            | ND              | ND              | ND              |
|                   |                             | IgM                    | 63.6 | 42.4 | 45.4 | 39.4 | ND             | <0.01            | <0.05            | <0.05            | <0.05            | ND              |
|                   |                             | IgG and IgM            | 75.8 | 62.1 | 62.1 | 65.2 | <0.05         | <0.05            | <0.05            | <0.05            | <0.05            | <0.05            |
| Patients with ACA | 31                          | IgG                    | 100.0 | 100.0 | 100.0 | 100.0 | ND             | ND              | ND              | ND              | ND              | ND              |
|                   |                             | IgM                    | 48.4 | 35.5 | 29.0 | 9.7  | >0.05         | ND              | ND              | ND              | ND              | ND              |
|                   |                             | IgG and IgM            | 100.0 | 100.0 | 100.0 | 100.0 | ND             | ND              | ND              | ND              | ND              | ND              |
| Forest workers    | 458                         | IgG                    | 44.8 | 40.0 | 44.8 | 41.0 | <0.001        | >0.05            | <0.01            | <0.05            | <0.05            | >0.05            |
|                   |                             | IgM                    | 12.0 | 6.6  | 7.4  | 1.1  | <0.001        | <0.001           | <0.05            | <0.001           | <0.001           | <0.001           |
|                   |                             | IgG and IgM            | 46.9 | 42.4 | 47.4 | 41.5 | <0.01         | >0.05            | <0.01            | <0.05            | <0.05            | <0.01            |

a Samples with OD values exceeding the cutoff values listed in Table 1.
b P values were determined by McNemar’s x^2 test.
ND, not done. Performance of McNemar’s x^2 test is not possible if one or both of the parameters test A positive and test B negative or test B positive and test A negative are equal to zero.
c At least one test (IgG or IgM) was positive.

WB of selected sera. Several sera which had shown discrepant reactivities in ELISAs with different antigens were further examined by WB to identify the individual antigens which were recognized. Since most serum samples did not show such discrepant reactivities, this selection does not represent the respective study groups, with the exception of some of the sera from forest workers and patients with ACA. Examples of some results are presented in Fig. 6. Serum samples A (from a patient with NB) and B (from a patient with EM) showed preferential IgM reactivity with OspC from PBi. Serum sample C (from a patient with NB) reacted with p41 of PKa2 and PBi but not with p41 of Pko, whereas serum sample D (also from a patient with NB) reacted almost only with p17 of Pko (both serum sample C and serum sample D were tested by WB for IgG). Serum samples E and F were obtained from a patient who had had an ACA 5 years previously (serum sample E) and a forest worker (serum sample F). They both showed remarkable preferential IgG reactivity with Pko, and the most immunodominant protein was p17. An additional 22 serum samples from forest workers, which were selected due to their predominat IgG reactivities with Pko, were also strongly reactive with p17 by WB.

Elevated specific IgG concentrations in patients with clinical manifestations and asymptomatic infections. The significance of OD values in different ranges of the four IgG ELISAs is demonstrated in Table 3. The percentages of sera with OD values greater than three arbitrarily defined levels (ODs of >0.5, >1.5, and >2.5) were determined for the patients with clinically defined LB as well as for the forest workers. The Pko ELISA showed no significant differences between these two groups (P > 0.05). However, in assays with all other strains, significantly higher percentages of sera from patients with LB were strongly reactive in comparison to the sera from forest workers (P < 0.01 for most comparisons).

DISCUSSION

Definition of cutoff values. A basic precondition for the comparison of sensitivities of different tests is the definition of cutoff values. The 133 blood donors whom we tested for this purpose came from southern Bavaria, where Lyme disease occurs relatively frequently (19, 25) (incidence and prevalence are not known exactly, but on average, 12.6% [15] of the ticks in this area have been shown to be infected with B. burgdorferi sensu lato). The sera were obtained from a blood bank, and therefore, exclusion of samples from persons with a history of LB was not possible. Considering the remarkably higher IgG reactivities of a few of these serum specimens (Fig. 2), which were mostly confirmed by WB, it was assumed that these sera might represent samples from persons with prior infections. Thus, the cutoff OD values for the IgG ELISAs were defined by the 92nd percentile and not by the 95th percentile, as usual. Furthermore, cutoff values must provide a realistic basis for the comparison of the four assays, and the OD values between the 92nd and the 100th percentiles seemed to be much more strongly influenced by chance (Fig. 2). Since IgM reactivity reflects early disease, which presumably occurs comparably less strongly influenced by chance (Fig. 2). Since IgM reactivity reflects early disease, which presumably occurs comparably...
FIG. 4. Plots of OD values of the different IgG ELISAs against each other. Continuous lines, cutoff OD values for the respective assays. $r$ values, Spearman rank correlation coefficients.
FIG. 5. Plots of OD values of the different IgM ELISAs against each other. Continuous lines, cutoff OD values for the respective assays. $r$ values, Spearman rank correlation coefficients.
proteins are indicated on the right. The most important immunodominant molecular mass marker. The numbers on the left indicate the molecular masses PKo, 2.479; PBi, 0.327). Lanes 1, PKa2; lanes 2, PKo; lanes 3, PBi; lanes M, patient who had had ACA 5 years previously; IgG (PKa2, 0.296; B31, 0.092; PKo, 0.849; PBi, 0.135). (E) Serum from a patient with EM; IgM (PKa2, 0.325; B31, 0.437; PKo, 0.709; PBi, 0.898). (C) Serum from a patient with NB; IgG (PKa2, 0.153; B31, 0.062; PKo, 0.849; PBi, 0.135). (E) Serum from a patient who had had ACA 5 years previously; IgG (PKa2, 0.296; B31, 0.092; PKo, 2.687; PBi, 0.659). (F) Serum from a forest worker; IgG (PKa2, 0.323; B31, 0.213; PKo, 2.479; PBi, 0.327). Lanes 1, PKa2; lanes 2, PKo; lanes 3, PBi; lanes M, molecular mass marker. The numbers on the left indicate the molecular masses of the marker proteins (in kilodaltons). The most important immunodominant proteins are indicated on the right.

ROC curves. However, because all other methods are arbitrary as well, we retained it.

**ROC curves.** The ROC curves for IgG tests (Fig. 3A) indicate that the best overall discrimination between sera from patients with LB and controls was achieved with B31 from the passage at which it lacked OspC. OspC is an early antigen which can also be recognized by unspecific antibodies at a very low level (13). Perhaps this could explain the lower background.

### Table 3. Significance of elevated titers in sera from patients with LB versus sera from healthy forest workers by different IgG ELISAs

<table>
<thead>
<tr>
<th>Antigen</th>
<th>OD value</th>
<th>% of sera with OD values greater than the indicated level (n)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Patients with LB</td>
<td>Forest workers</td>
</tr>
<tr>
<td>PKo</td>
<td>&gt;2.5</td>
<td>9.5</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>&gt;1.5</td>
<td>17.6</td>
<td>20.3</td>
</tr>
<tr>
<td></td>
<td>&gt;0.5</td>
<td>38.7</td>
<td>31.7</td>
</tr>
<tr>
<td>PBi</td>
<td>&gt;2.5</td>
<td>5.0</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>&gt;1.5</td>
<td>15.3</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>&gt;0.5</td>
<td>32.0</td>
<td>21.6</td>
</tr>
<tr>
<td>PKa2</td>
<td>&gt;2.5</td>
<td>5.9</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>&gt;1.5</td>
<td>15.3</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>&gt;0.5</td>
<td>33.3</td>
<td>22.3</td>
</tr>
<tr>
<td>B31</td>
<td>&gt;2.5</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>&gt;1.5</td>
<td>10.5</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>&gt;0.5</td>
<td>24.5</td>
<td>16.4</td>
</tr>
</tbody>
</table>

* Differences between patients with LB and forest workers were analyzed by Fisher’s exact test.

Sensitivities for individual study groups. For the sera from patients with EM and the NB II group, the best results were obtained with PKo. For the sera from patients with EM, this can be explained by the predominance of *B. afzelii* strains in skin lesions (8, 9, 44, 47). The sera from the NB II study group were selected on the basis of the criterion of a specific IgG CSF/serum index of >2.0, and this index was determined by a PKo-based ELISA. Therefore, the high sensitivity of the PKo ELISA in the current study could result from this selection. On the other hand, for sera from the NB I group (selection criterion, isolate from CSF), the sensitivities of the PKo, Pbi, and PKa2 ELISAs were very similar, with the PKa2 ELISA showing a slightly higher sensitivity. However, in other studies sera from patients with neurological disorders were preferentially reactive with *B. garinii* (1, 26, 42), and strains of this species have been isolated most frequently from CSF (8, 40, 44). All sera from patients with ACA were positive in all IgG ELISAs, suggesting that the source of antigen is not critical for the detection of LB in sera from patients with late-stage LB. For the group of forest workers, a relatively high frequency of asymptomatic and previous infections can be assumed. At least 40% of their sera were positive by all IgG ELISAs (compared to 8% for the group of blood donors).

**Correlation of OD values of different ELISAs.** For several sera from patients with ACA as well as from forest workers, a markedly stronger IgG reactivity with PKo was found (in comparison to those obtained with the other strains). Therefore, the correlations between PKo and all other strains were lower than the correlations among these other strains (Fig. 4). However, the good correlation of Pbi or PKa2 versus PKo for the NB groups may indicate that the strain discrepancy observed in forest workers and patients with ACA is not biased by the coating conditions of the ELISA solid phase. WB of several selected serum specimens with discrepant reactivities revealed that this preference of PKo was mostly caused by strong reactivity with p17 (Fig. 6E to F). This is a highly immunogenic protein of strain PKo (17, 46) which is apparently not expressed by the other three strains. For sera from patients with EM lesions, frequent IgG reactivity with several proteins unique for PKo was demonstrated previously (17) (other *B. afzelii* strains have not been tested so far). For the NB groups, the somewhat lower correlations between B31 and each of the other three strains could be explained by the lack of OspC in reactivity of the B31 IgG ELISA. However, the greatest distances between the four ROC curves were observed at specificity levels lower than 90%, which are not useful for diagnostic purposes. Regarding the section between 90 and 100% specificity, the curves are considerably more similar. Comparison of individual specificity levels, however (for example, 92 and 97%), leads to large variations in the differences in sensitivities between different tests. This clearly demonstrates the consequences of using arbitrarily defined cutoff levels.

For IgM ELISAs, the best discrimination was achieved with PKo and the worst was achieved with B31 (Fig. 3B). From Fig. 2 it is evident that 97% of the sera from blood donors had remarkably low OD values in the IgM PKo ELISA. On the other hand, by regarding the high degrees of correlation of the OD values from different IgM assays (Fig. 5), it seems as if the high sensitivity obtained with PKo might be caused by the relatively low cutoff. However, since all assays with the four antigen preparations were run in parallel and batches of plates were not changed throughout the study, a bias seems to be unlikely. The low discriminatory ability of the B31 IgM test can be explained by the lack of OspC, which is one of the most immunodominant proteins for the early immune response in patients with LB (12, 13, 17, 48).
B31. Thus, sera primarily recognizing OspC showed less intense reactivities with B31. However, a few individual serum specimens also showed discrepant ELISA reactivities caused by the discrepant recognition of other antigens (for example, Fig. 6C and D).

Since OspC is one of the most immunodominant antigens in the early immune response, lower correlations of B31 with the other strains were especially marked in IgM ELISAs (Fig. 5). B. burgdorferi sensu stricto B31 tends to lose OspC after many passages, contributing to its poor IgM sensitivity in all stages of LB, while strain PKa2 expressing OspC shows a considerably higher IgM sensitivity. This view is consistent with a statement of U.S. health authorities that high-passage isolates of B31 lacking OspC are not recommended for use in serological tests for LB (10).

For two serum samples selected due to discrepant reactivities in IgM ELISAs, preferential reactivity with OspC of PBi could be demonstrated by WB (Fig. 6A and B). In a study by Mathiesen et al. (24), the OspC of a B. garinii strain was also more sensitive in WB than the OspCs of a B. burgdorferi sensu stricto strain and a B. afzelii strain.

In the current study, the highest correlations in both IgG and IgM ELISAs were observed between PBi and PKa2 in all study groups. Although these two strains belong to different species and some well-characterized proteins are rather heterogeneous (20, 29, 38, 43), immune reactivity with other presumably highly conserved antigens may be compensatory.

Clinical manifestations versus asymptomatic infection; what do elevated specific IgG concentrations mean? In general, specific IgG concentrations increase with the duration of LB (18, 34) (at least during the first months, if no antibiotic treatment is performed). Usually, in late stages, high antibody concentrations are observed. Unfortunately, however, especially in late LB, antibody activity can persist over years even if successful treatment is performed (14, 36). Thus, only limited information about the activity can be achieved by monitoring the specific IgG concentrations over the course of the disease. As already mentioned, many sera from the healthy forest workers had stronger IgG reactivities with PKo than with the other strains tested. This observation led to the assumption that assays with strains other than PKo might be more informative with regard to the activity of the disease. Therefore, the percentage of sera with OD values greater than a few exemplarily chosen levels were determined for all patients with LB as well as for the forest workers (Table 3). Significantly more sera from patients with LB than from forest workers reached OD values greater than these levels in tests with PBi, PKa2, and B31. With PKo, no significant differences were detected. Thus, it could be shown that highly elevated antibody concentrations are more likely to be associated with clinical manifestations of disease than with asymptomatic or passed infection if strains other than PKo are used. However, with respect to this evaluation it should be mentioned that the prevalences of clinical manifestations of LB in all patients with LB (n = 222) selected for this study does not represent the actual prevalences of manifestations of LB in the general population (e.g., EM is more common than NB).

A combination of a PKo assay and a test based on PBi or PKa2 might be most informative. For example, one assay could be used for screening and the other could be used for confirmation. The combination of strong PKo reactivity and weak non-PKo reactivity might be consistent with ACA or asymptomatic infection. If these results were obtained, for example, for a patient who is suffering from an atypical neurological disorder, B. burgdorferi sensu lato presumably might not be causative. However, these are unproven suggestions, and interpretation needs to be specified in detail. This might be possible only in specialized laboratories and would require intensive communication between physicians in the laboratories and in hospitals or offices.

Conclusion. In routine testing results can vary considerably among different laboratories. Besides differences in antigen preparation and test performance, this variation may result from the use of different species of B. burgdorferi sensu lato. By generalizing the results of the current study, it might be assumed that these variations occur mainly between B. afzelii-based tests on the one hand and assays with B. burgdorferi sensu stricto or B. garinii on the other hand. However, regional differences may further influence test results. Furthermore, variations in the level of expression or a complete lack of expression of immunodominant antigens such as OspC may be critical. Since the OGP extract of PKo was the most sensitive in this study but extracts of the other strains were better able to differentiate between active disease and asymptomatic infection, we suggest that a combination of a test with a B. afzelii strain and another test with a B. garinii strain (high sensitivity with sera from patients with NB) might be most informative. If WB with PKo were used, mere detection of a p17 band without other significant bands might be grounds for suspicion of previous LB. Further investigations are necessary to elucidate this assumption.

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