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Validity of Western Immunoblot Band Patterns in the Serodiagnosis of Lyme Borreliosis

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Serodiagnosis of Lyme disease is hampered by low specificity of the standard assays currently used. The Western immunoblot has therefore been proposed as a potential confirmatory test. For the present report, the method was evaluated by testing sera from patients with clinically defined early- and late-stage borreliosis. In early-stage borreliosis, the 41,000-molecular-weight flagellin protein (41K) of *Borrelia burgdorferi* was the major antigen detected by antibodies in sera, but the specificity of the reaction pattern was dependent on the intensity of the band. The evaluation of different interpretation rules based on a semiquantitative record of band intensities showed the highest specificity (96%) and a corresponding sensitivity of 78% if there was at least one distinct (optical density range, 0.2 to 0.4) immunoglobulin G and immunoglobulin M reaction with the 41K band. Blots of *B. burgdorferi* proteins were also probed with sera from patients who were diagnosed by clinical criteria as having stage III Lyme borreliosis and with a control group of sera from asymptomatic persons with positive antibody titers against *B. burgdorferi* in the standard assays. Reaction patterns were recorded densitometrically. Statistical analysis and graphical marker analysis revealed significant discriminating capacities and relatively high specificities, respectively, for the 94K, 30K, and 21K bands, whereas the 41K and 60K bands were not discriminative between the symptomatic and asymptomatic groups and were specific only at high intensity values. Different multiple-band rules were evaluated, revealing a low specificity for positivity definitions of the type "four or five bands present" if the rules were not confined to known major bands.

Lyme disease is a multisystem disorder which is caused by the tick-borne spirochete *Borrelia burgdorferi*. Multiple clinical manifestations have been attributed to the different stages of the disease, of which the most characteristic are erythema migrans in stage I, meningopolyneuritis in stage II, and arthritis or acrodermatitis in stage III borreliosis (23). Diagnosis of the disease is primarily a clinical one, although multiple differential diagnoses have to be considered. Therefore, serodiagnostic tests are widely used as a laboratory aid in diagnosis. The indirect immunofluorescence assay (IFA) and various enzyme immunoassays (EIA) which mostly use whole cells or sonic extracts of *B. burgdorferi* as antigens are accepted standard procedures in the serodiagnosis of Lyme borreliosis (1, 6, 20). However, the problems inherent to these tests are a cutoff-dependent lack of sensitivity in the diagnosis of early-stage borreliosis and insufficient specificity (2, 7). The latter is revealed by a high prevalence of positive antibody titers in average populations, which often do not correspond to clinical manifestations of Lyme borreliosis (29). Cross-reacting antibodies, induced by saprophytic spirochetes and other bacteria sharing common epitopes with major antigenic determinants of *B. burgdorferi*, may be responsible for these findings (12, 17, 21).

Various proposals have been made in order to overcome these problems. The use of physically purified fractions of the spirochetes or of 41,000-molecular-weight flagellin (41K)-enriched preparations as antigens was reported to improve the validity parameters of EIA (13, 19).

The humoral immune response to *B. burgdorferi* antigens has been analyzed by immunoblotting (5, 11, 26), and the Western immunoblot technique has been proposed as a confirmation test, but criteria for the interpretation of blots have so far not been developed. Recommendations to consider a Western blot result positive if at least two to five bands are detected (8, 11) are insufficient because multiple-band patterns can be caused by cross-reactive antibodies.

We present here a statistical evaluation of the diagnostic validity of Western blot band patterns in early- and late-stage Lyme borreliosis.

MATERIALS AND METHODS

In vitro cultivation of *B. burgdorferi*. Main experiments were performed with *B. burgdorferi* TN, which was kindly provided by V. Preac-Mursic, Munich, Federal Republic of Germany. The strain, which originated from a naturally infected ixodes tick, has previously been described (26, 28). Additionally, strains B31 (ATCC 35210) and N34 (kindly provided by R. Ackermann, Cologne, Federal Republic of Germany) were used. Strains were cultivated in modified BSK medium as described elsewhere (22).

Patients and sera. Patient and serum groups are listed in Table 1. A panel of sera from patients with early-stage disease (DI, $n = 23$) was obtained from 17 patients suffering from stage II neuroborreliosis (DIa) and 6 patients with erythema migrans (DIb). A total of 17 cases had positive serology by standard assays (EIA and IFA), and 6 were negative with these tests. In all cases of neuroborreliosis, the diagnosis was clinically apparent with regard to characteristic symptoms, a history of preceding tick bites or erythema migrans and, if cerebrospinal fluid (CSF) was available ($n = 9$, 8 positive by standard assays), concomitant lymphocytic pleocytosis in CSF. A control serum panel (NI, $n = 59$) which originated from patients suffering from other neurological diseases was used, irrespective of antibody titers against *B. burgdorferi*.

Sera from patients suffering from severe chronic arthritis

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TABLE 1. Characterization of serum panels used for immunoblot evaluation

Group	No. of serum specimens	Clinical manifestation
DI	23	Early-stage borreliosis (stage I or II)
DIa	17	Stage II neuroborreliosis
DIb	6	Erythema migrans
NI	59	Neurological diseases (control group)
DII	10	Lyme arthritis, diagnosis ensured by hard clinical criteria and typical history of previous manifestations of Lyme borreliosis
D?II	20	Chronic arthritis, positive antibody titers against *B. burgdorferi* by standard assays, but diagnosis not ensured by typical history
NII	9	Chronic arthritis of origin other than Lyme borreliosis but positive antibody titers against *B. burgdorferi* by standard assays
DIII	39	Positive antibody titers against *B. burgdorferi* by standard assays (patients from an area in which Lyme borreliosis is endemic; symptoms attributed to stage III Lyme borreliosis)
NIII	39	Positive antibody titers against *B. burgdorferi* by standard assays (asymptomatic patients from an area in which Lyme borreliosis is endemic)

were provided by J. Goronzij (rheumatological section, Department of Internal Medicine, University Hospital, Heidelberg, Federal Republic of Germany). Cases with a positive antibody titer against *B. burgdorferi* by the standard assays (IFA and EIA) were divided into three groups according to the strength of clinical criteria suggesting the diagnosis of Lyme borreliosis. Group DII patients (n = 10) had a typical history of previous manifestations of Lyme borreliosis. Additionally, they were all found to be positive in a T-cell stimulation assay with *B. burgdorferi* antigens (24). Other possible etiologies of their joint manifestations were excluded as far as possible. In group D?II patients (n = 20), Lyme borreliosis was considered as a differential diagnosis because other origins of the disease could not be confirmed, but the patients had no history of other manifestations that were strongly indicative for Lyme borreliosis. Group NII (n = 9) comprised patients with positive antibody titers against *B. burgdorferi*, whose clinical symptoms were definitely caused by diseases other than Lyme borreliosis.

A further panel of antibody-positive sera was collected from 78 patients of a clinical practice in an area in the Northern Badenian region of Germany in which Lyme borreliosis is endemic (tick infestation rate, approximately 20%). All patients were subjected to an accurate clinical examination followed by a 2-year postobservation period in order to record clinical symptoms (14). Sera were divided into two groups according to whether patients had (DIII, n = 39) or did not have (NIII, n = 39) symptoms of Lyme borreliosis. Typical symptoms were oligoarthritis (most frequent symptom), acrodermatitis, and lymphadenosis cutis benigna, in part accompanied by a history of previous erythema chronicum migrans. Symptoms were in all cases attributed to the symptom complex of stage III (late) Lyme borreliosis. Besides the restriction of group DII to cases of severe chronic arthritis, a major difference between groups DII and DIII was the strength of clinical criteria applied for the definition of cases. An unequivocal history of characteristic symptoms (erythema chronicum migrans and neuroborreliosis) was not required for the diagnosis of DIII patients. Sera used for the comparison of strains and correlation of blot results with antibody titers of standard assays were chosen according to the criteria described in Results.

SDS-PAGE and immunoblot. Spirochetes from 4- to 6-day-old log-phase cultures were harvested and washed with TEN buffer (5 mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 7.4) by three cycles of centrifugation at 10,000 × g for 30 min each cycle. The final pellet was resuspended in distilled water. Protein concentration was measured by the method of Bradford (3). Samples were dissolved in an equal volume of lysing buffer (0.01 M Tris hydrochloride, 10% glycerol, 2% sodium dodecyl sulfate [SDS], 0.5% β-mercaptoethanol, 0.1% bromphenol blue, pH 8), heated for 5 min at 95°C, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (12% acrylamide, 0.4% bisacrylamide) according to the method of Laemmli (16). Proteins were transferred onto nitrocellulose sheets (BA85; pore size, 450 nm; Schleicher & Schüll, Dassel, Federal Republic of Germany) by semidry graphite blotting. Transfer efficiency was controlled by staining the blots with 0.2% (wt/vol) Ponceau S dissolved in 3% (vol/vol) trichloroacetic acid. Nitrocellulose sheets were quenched overnight with blocking buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 2% bovine serum albumin, 0.25% gelatin, 0.05% Tween 20, pH 7.4) and stored dry at −70°C until use. The blotted membranes were probed with test serum samples diluted 1:100 in blocking buffer. After incubation (overnight at 4°C or for 3 h at room temperature), blots were washed with washing buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.05% Tween 20) and incubated for 1 h at room temperature with the appropriate horseradish peroxidase-labeled second antibody (MEDAC, Hamburg, Federal Republic of Germany) at a dilution of 1:500 in blocking buffer. After three cycles of washing, color was developed at room temperature by incubating the blots with substrate solution; 120 ml contained 60 mg of 4-chloro-1-naphthol (Sigma, Munich, Federal Republic of Germany), dissolved in 20 ml of ice-cold methanol, and 60 μl of hydrogen peroxide (30% stock solution) in Tris-buffered saline (pH 7.4). For serum immunoglobulin M (IgM) and CSF reactions, an alkaline phosphatase detection system was used. CSF samples were diluted 1:100 and alkaline phosphatase-conjugated second antibodies were diluted 1:1,000 in blotting buffer (phosphate-buffered saline [PBS] containing 0.05% [vol/vol] Tween 20 and 2% [wt/vol] bovine serum albumin), and blots were washed with PBS containing 0.05% Tween 20. Color was developed by use of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (final concentration, 150 μg/ml; stock solution, 15 mg/ml in dimethylformamide) and Nitro Blue Tetrazolium (300 μg/ml; stock solution, 30 mg/ml in dimethylformamide) freshly dissolved in sodium carbonate buffer (pH 9.6). For the detection of IgM antibodies, the IgG fraction was removed by preabsorption with rheumatoid factor sorbent (Mastsorb; Mast, Hamburg, Federal Republic of Germany) and sera were used at a final dilution of 1:200.

Quantitative and semiquantitative analyses of bands. All blots that were quantitatively evaluated were prepared under the same conditions, with the same lots of blotted membranes, second antibodies, and buffers. Control sera with broad reactivity patterns were integrated in the assays in order to ensure reproducibility. Optical densities (OD) of bands were measured by use of a reflection video densitom-

eter (Bio-Rad, Munich, Federal Republic of Germany). For semiquantitative analysis, band intensities were scored as ++ (strong; OD, >0.4; average, 0.45), + (distinct; OD range, 0.2 to 0.4; average, 0.3) or (+) (weak; OD range, 0.05 to 0.2; average, 0.1). For qualitative analysis, a decision (yes/no) was made concerning the presence of an individual band (OD, >0).

Statistical analysis. χ^2 (band present [yes/no]—qualitative evaluation) and Wilcoxon tests (for the continuous part of the distribution, e.g., OD values of >0—quantitative evaluation) were used to explore whether the distributions of the OD values of different bands were significantly different between the D and N groups (which is necessary but not sufficient for diagnostic purposes). For each possible cutoff value c for the OD, the proportion of patients with ODs of $<c$ in the DIII sample and the proportion of patients with ODs of $>c$ in the NIII sample were calculated as estimates for the specificity and the sensitivity, respectively, of the different bands dichotomized at OD level c. Receiver operating characteristic (ROC) curves were obtained by plotting sensitivity against specificity for various cutoff values c. The straight line connecting the 1/0 and the 0/1 points represents the ROC curve of a completely noninformative diagnostic test (random assignment of individuals to diagnoses). The distance between this curve and the ROC curve of a marker is a measure for the discriminating capacity of this marker. The point with maximal sensitivity (and minimal specificity) corresponds to the mere qualitative use of the band. Since a confirmatory test requires high specificity, the cutoff OD was increased until specificity was just above 90%, and the resulting sensitivity was used to compare different bands. In a third step, different bands were combined by modified "believe-the-positive" rules (positive diagnosis whenever both bands are present and one band exceeds the respective critical OD level). In this analysis, the couple of critical OD levels for the two bands was chosen to obtain maximum sensitivity under the restriction of specificity in the given sample.

TABLE 2. Correlation of Western blot band patterns with the results of the standard IFA

M_r (10[3])	Characterization[a]	No. (%) of serum samples detecting the band	
		IFA titer positive (>256 [n = 31])	IFA titer negative (<256 [n = 23])
94		16 (51.6)	3 (13)
73		10 (32.2)	4 (17.3)
60		11 (35)	7 (30.4)
43		12 (38.7)	7 (30.4)
41	Flagellin	25 (80.6)	18 (78.2)
39		13 (41.9)	5 (21.7)
34	OSP B	3 (9.6)	2 (9.6)
31	OSP A	2 (6.4)	0
30		18 (58)	6 (26)
23		9 (29)	1 (4.3)
21		6 (19.3)	3 (13)
13		12 (38.7)	7 (30.4)

[a] OSP, Outer surface protein.

RESULTS

Occurrence of bands in correlation to antibody titers. Major bands that were regularly detected by sera are listed in Table 2, and representative immunoblots are shown in Fig. 1. The 94K protein was the component with highest molecular mass among all bands regularly detected in the blots. The 41K band represents the flagellin protein, as was confirmed by staining with a specific monoclonal antibody (kindly provided by M. D. Kramer), and the 31K and 34K bands represent the outer surface proteins A and B. A 40K protein was running at the leading front of the 41K protein. This band could not be demarcated visually in the blots from a distinct 41K band and therefore was not considered in the evaluation. A small band which was regularly detected below the 41K protein had an apparent M_r of 39,000, and a small band running just below the leading front of outer

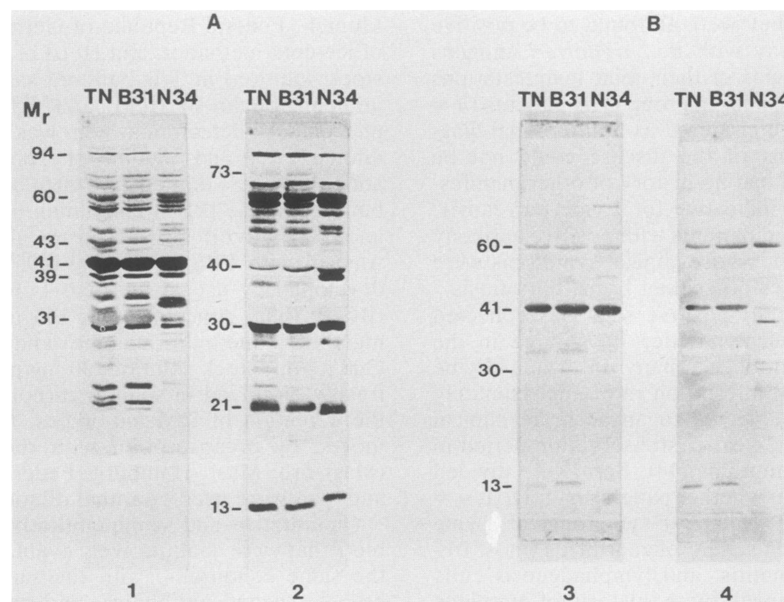

FIG. 1. Representative immunoblots of *B. burgdorferi* TN, B31, and N34 with sera from patients with stage III (A), stage II (B3), and stage I (B4) Lyme borreliosis, the last one showing a different reaction with the 41K protein of strain N34. Molecular weights of major bands are indicated (in thousands) on the left.

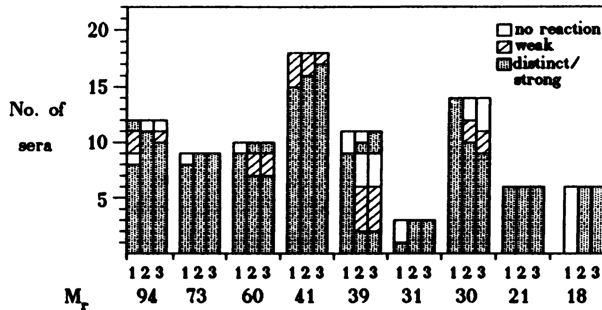

FIG. 2. Comparison of immunoblot reaction patterns of 22 serum specimens with three different strains of *B. burgdorferi*. 1, Strain N34 (European, human isolate); 2, strain B31 (American, tick isolate); 3, strain TN (European, tick isolate). Band intensities are indicated semiquantitatively; molecular weights are indicated in thousands.

surface protein A was designated the 30K band. In the low-M_r range, proteins with M_rs of 23,000, 21,000, 18,000, and 13,000 were regularly detected. A total of 54 serum specimens were chosen according to their IFA titers: 31 of them had an antibody titer below the cutoff titer (1:256), and 23 had an antibody titer above the cutoff titer. The qualitative representation of bands in both groups is shown in Table 2. Significant differences between the two groups were found for the 94K, 30K, and 23K bands (χ^2 test, $P < 0.05$).

Heterogeneity among strains. Blots of three different strains were probed with 22 serum specimens which were found to be antibody positive by standard assays, and the reaction patterns were compared (Fig. 2). Most of the reactions were homologous with all three strains, although differences in the strengths of bands were observed. The European strain N34 did not reveal an 18K band. Two of twelve serum specimens detecting a 94K band in the blot reacted only with two of the three strains, lacking reaction with either the N34 or B31 strain. Three of fourteen serum specimens showed a discordant reaction with the 30K band, and five of eleven showed a discordant reaction with the 39K band. The 18 serum specimens recognizing a 41K band reacted with all three strains, but strain-dependent differences of the band intensities were seen in 3 serum specimens.

Validity of bands in the serodiagnosis of stage I and II borreliosis. Only a limited number of the major antigenic proteins of *B. burgdorferi* were strongly recognized in the blots of the sera of group DIa and corresponding CSFs (Fig. 3). An obvious feature was a strong 41K band in most of the sera and CSFs. The 94K protein, however, was strongly detected in only three cases. All of them already had a prolonged course of the disease (>6-week duration at the time of diagnosis). Any of the above-mentioned major bands were detectable in some of the blots (less than 50% of the cases), but they were always accompanied by a 41K band, which was in most of these cases the strongest among the bands. With regard to the time elapsed from the onset of the disease, the 41K and 21K bands were detected first, followed by the 60K and other bands (data not shown). The 94K band was the latest band appearing, being not detectable before the 6th to 12th week after the presumed onset of infection. The diagnostic sensitivities and specificities of different interpretation criteria for the 21K (which was chosen because of its favorable marker capacities for Lyme borreliosis, as demonstrated below) and 41K bands in blots of sera from patients with early-stage borreliosis (group DI) are shown in Table 3. Specificity and sensitivity values were calculated by means of control panel NI and panel DI, respectively. It turned out that the mere qualitative presence (OD of >0) of a 41K band had only low specificity for the diagnosis of Lyme borreliosis. A specificity above 90% was obtained only for each of the following rules: presence of a 21K band, a strong IgG and/or IgM 41K band (OD above 0.4), and cooccurrence of a distinct (OD of >0.2) 41K IgG and IgM band. The last criterion proved to achieve the highest sensitivity (78%) at this specificity level. The criterion of a "predominant reaction" with the flagellin, meaning an IgG and/or IgM band that is at least distinct and at the same time is the strongest band in the blot, achieved a specificity of only 86% and a sensitivity of 78%. The specificity of the IgM reaction with the 41K protein was not higher than the specificity of the IgG reaction. CSF bands were found to be highly specific, since none of 40 control CSFs revealed any bands (data not shown). CSF IgM reaction was even more sensitive than the IgG reaction, and the band pattern was not confined to the 41K band, although this was the predominant band in all cases.

Validity of band patterns in stage III Lyme borreliosis. The validity of the Western blot in the serodiagnosis of stage III

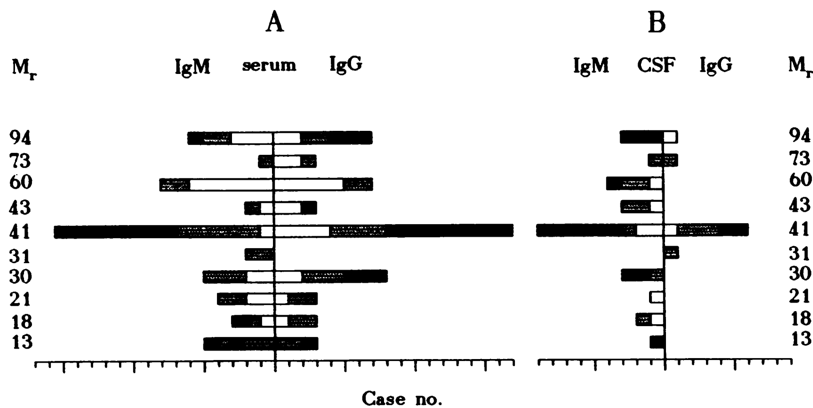

FIG. 3. Frequency and strength of the major bands of *B. burgdorferi* detected in sera (A) and CSFs (B) of patients with stage II neuroborreliosis (panel DIa). Filled bars indicate strong bands, dotted bars indicate distinct bands, and empty bars indicate weak bands. M_rs are given in thousands. Case no., Number of specimens exhibiting the indicated bands.

TABLE 3. Sensitivities and specificities of different interpretation criteria in the evaluation of Western blots of sera for the diagnosis of stage I and II borreliosis[a]

M_r (10^3)	Ig class	Band intensity recorded: Qualitatively	Band intensity recorded: Semiquantitatively	Sensitivity (%)	Specificity (%)
21	G/M	Y		43	93
41	G	Y		100	55
41	G		+/++	82	86
41	G		++	52	98
41	G/M	Pred.		78	86
41	M	Y		91	64
41	M		+/++	91	86
41	M		++	60	96
41	G+M		+/++	78	96
41	G+M		++	30	100
41	G/M		++	52	94

[a] Interpretation criteria were the intensities of the bands which were recorded semiquantitatively (+, OD range of 0.2 to 0.4; ++, OD of >0.4) or qualitatively (band present [yes/no];Y [yes], OD of >0; Pred. [predominant], the band is present and also is the strongest band on the blot). G/M means that the criteria must be met by the IgG- or IgM-specific blot; G+M means that the IgG- and the IgM-specific bands must meet the criteria. Sensitivities and specificities were calculated by means of the serum panels DI and NI.

borreliosis was evaluated at the level of a confirmatory test, meaning that standard assays that preceded gave positive results. Representative immunoblots from groups DII and NII are shown in Fig. 4. The qualitative evaluation of band patterns revealed significant differences (P <0.05) for the 94K and 21K bands (Fig. 5A and Table 4). The presence of a 41K band, however, was not suitable to discriminate between the groups. The 60K band was found in 30% of the sera of panel NII and therefore had low discriminative capacity, as did most of the other bands. The outer surface proteins A and B were not detected by any of the sera. The quantitative evaluation (Fig. 5B) showed marked differences of the distribution of the OD values of most of the major bands among groups DII, DII?b, and NII, indicating that even bands which bear unspecific epitopes are strongly recognized in late-stage borreliosis. The 94K band was found to possess the highest discriminative capacity, with average OD values being seven to eight times higher in the group of Lyme arthritis patients (DII) than in the group of patients with arthritis of other origin (NII). The D?II serum panel took an intermediate place between DII and NII with regard to the qualitative and quantitative evaluations, indicating the presence of true clinical cases in this group that can be diagnosed by Western blot. Similar analysis was performed with sample III, comprising symptomatic (DIII) and asymptomatic (NIII) antibody-positive patients from an area in which Lyme borreliosis is endemic and a high prevalence of positive antibody titers is found (Table 4). Again, a relatively low discriminative value of most of the bands with regard to the clinical diagnosis of Lyme borreliosis was observed. Only the 94K and 21K bands showed significant differences in frequency between the symptomatic and asymptomatic groups (χ^2 test), and the 73K, 31K, and 18K bands showed a weak trend. No correlation was observed for the 41K band. In the quantitative evaluation, the 94K and 30K bands were found to possess potential discriminative capacities for higher OD values (higher specificity), as suggested by a highly significant Wilcoxon test result. The OD of the 21K band, however, did not discriminate between those blots of the two groups in which the band was present, indicating that the OD range covered by this band was rather narrow. ROC analysis for the 94K, 73K, 60K, 41K, 30K, and 21K proteins (Fig. 6 and 7) confirmed the findings that the ROC curves obtained for the 41K and 60K bands were only slightly above the curves for a random assignment, whereas the ROC curves obtained for the 94K, 30K, and 21K bands indicated a considerably higher discriminating capacity. Acceptable specificities could be obtained only at high intensity values of these bands, with the exception of the 21K band, which had only low sensitivity but was highly specific even if its intensity was only weak. The sensitivity at a specificity

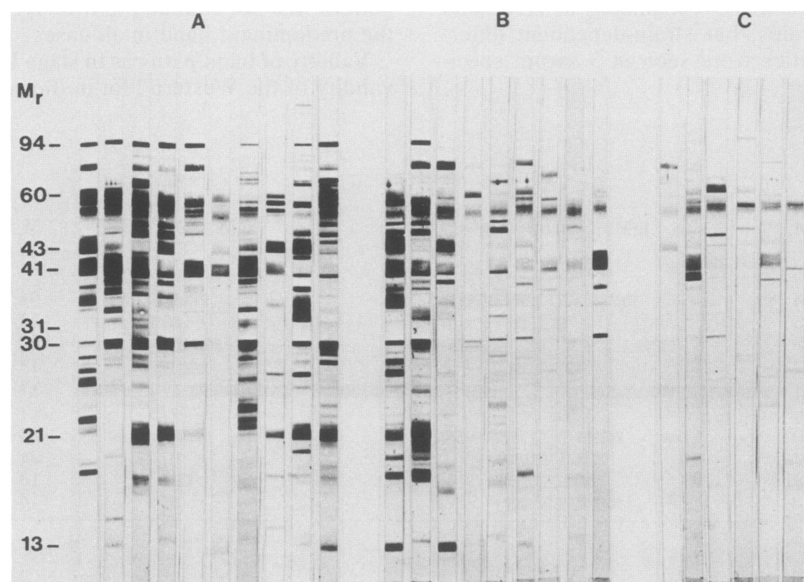

FIG. 4. Representative immunoblots of *B. burgdorferi* TN with sera from groups DII (A), D?II (B), and NII (C). Molecular weights of major bands are indicated (in thousands) on the left.

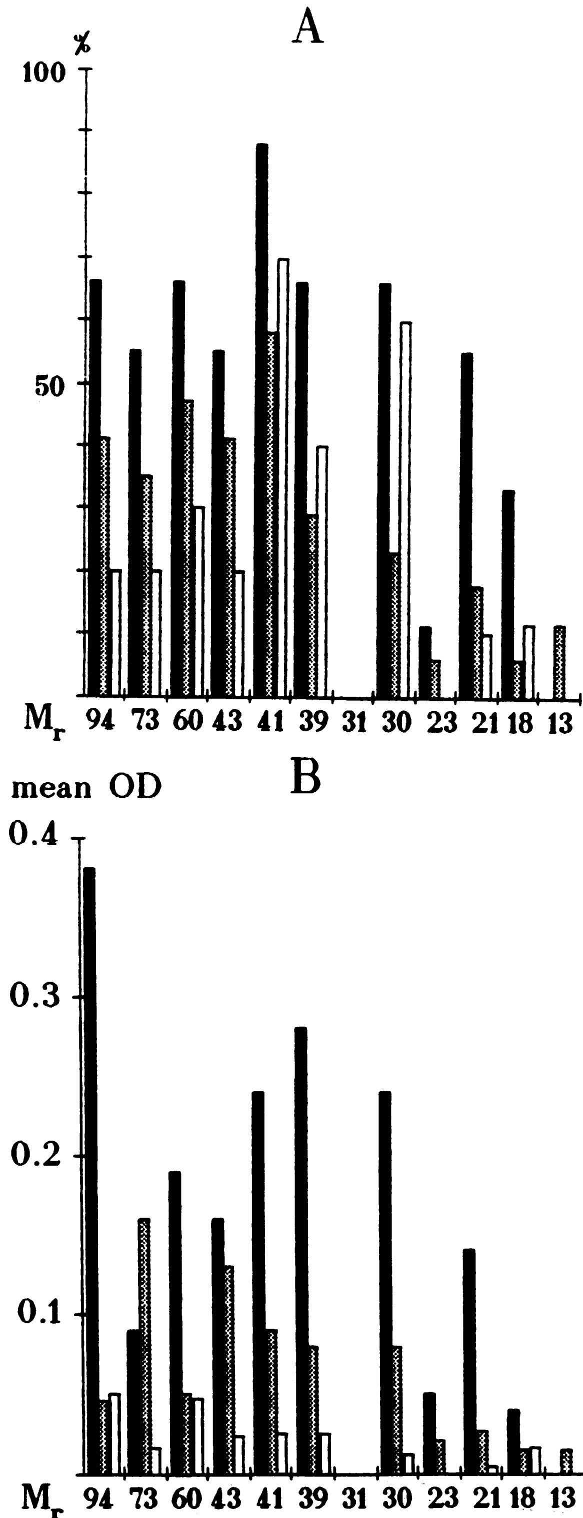

FIG. 5. Qualitative (A) and quantitative (B) evaluation of Western blot band patterns of patients positive for antibodies to *B. burgdorferi* by standard assays. Patients with clinically proven Lyme arthritis (DII, filled bars) were compared with patients with arthritis of other origin (NII, empty bars) and patients with arthritis of unknown origin, possibly attributable to Lyme disease by clinical criteria (D?II, dotted bars). The relative frequencies of occurrence of different bands are shown in panel A; the average intensities of the bands (OD values of 0 included) are shown in panel B.

TABLE 4. Statistical comparison (*P* values) of groups DII and NII as well as DIII and NIII with respect to the qualitative presence of bands (χ^2 test) and the OD values (Wilcoxon test, for sera with ODs of >0), respectively

M_r (10^3)	*P* value		
	χ^2 test		Wilcoxon test (DIII vs NIII)
	DII vs NII	DIII vs NIII	
94	0.04	0.02	0.0014
73	0.1	0.09	0.91
60	0.11	0.86	0.48
43	0.1	0.88	0.52
41	0.3	0.59	0.23
39	0.24	0.72	0.08
31		0.14	
30	0.76	0.1	0.015
23	0.27	0.3	0.49
21	0.03	0.001	0.97
18	0.5	0.1	0.9

level of >90% could be increased above the maximum value found for a single band by combination of the 94K and 30K bands (Table 5). A combination that is frequently observed in diagnostic work is the cooccurrence of weak 41K and 60K bands. This band pattern was found to be unspecific if observed at low intensity.

The evaluation of individual bands or combinations of two bands does not completely reflect the complexity of band patterns observed in the blots. In most cases, configurations of single bands that are considered specific are accompanied by multiple other bands. Table 6 shows the number of bands that were detected in blots which were considered diagnostic on the basis of a distinct 94K band. Multiple-band rules for the interpretation of blots were evaluated with serum samples DIII and NIII (Table 7). Rules of the type "existence of at least four or five bands" (irrespective of their molecular weights) were not suitable to achieve sufficient sensitivity and specificity. A four-band rule, requiring the 94K, 73K, 30K, and 21K bands, revealed the highest specificity, but sensitivity was only 28% in this panel.

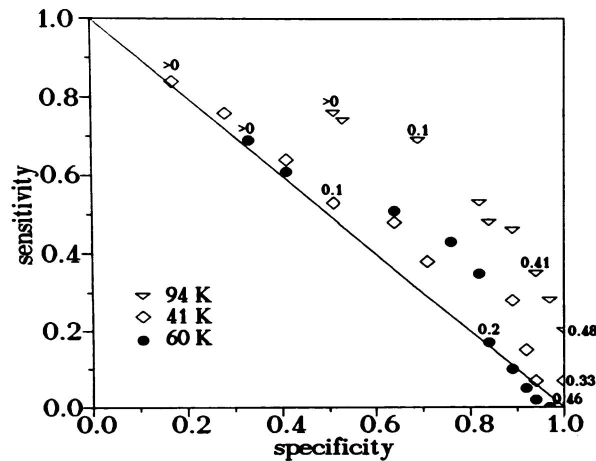

FIG. 6. ROC plots for the 94K, 41K, and 60K bands. Exemplary cutoff intensity values are indicated above or beside the band concerned.

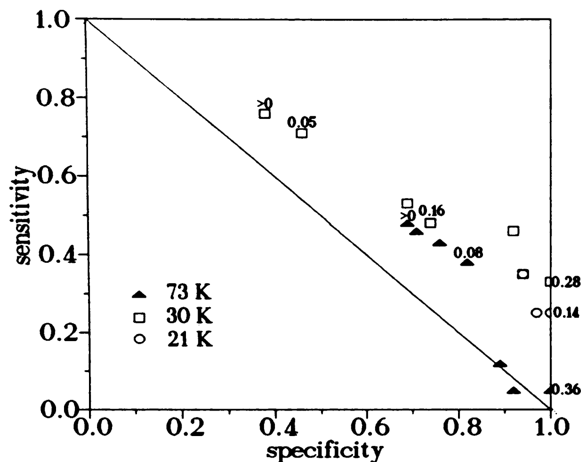

FIG. 7. ROC plots for the 73K, 30K, and 21K bands. Exemplary cutoff intensity values are indicated.

DISCUSSION

Western blot has been proposed as a confirmation test in the serodiagnosis of Lyme borreliosis, and a commercial blot test (Whittaker, Biomérieux) has been available for routine diagnostic use for a few months. Although the selective band pattern found in early-stage borreliosis and the fully expanded band pattern seen in characteristic cases of stage III Lyme borreliosis have been described previously (5, 11, 27), an evaluation of the multiple-band patterns that can be observed with regard to their diagnostic validity was lacking. Doubts have arisen regarding the specificity of the blots, since multiple-band patterns are often detected in sera from asymptomatic patients or sera that do not give positive reactions in the standard assays. Densitometric reading of blots was used in this work to develop rules for the interpretation of Western blots and to investigate the role of defined proteins of *B. burgdorferi* as diagnostic marker antigens in different stages of the disease. The semiquantitative interpretation criteria derived from the densitometric data are also useful in laboratories without the technical equipment necessary for densitometry. In early-stage borreliosis, a predominant reaction with the 41K (flagellin) and/or 21K band was observed in most cases, as was also reported by others (11, 26). However, it turned out that the specificity of this reaction pattern was largely dependent on the intensity of the 41K band. Acceptable specificity was obtained only if a strong 41K band was required for IgG blots or IgM blots or both. Even the detection of a distinct 41K band in the IgG blot of sera had no more than 86% specificity. High specificity, however, was found for the CSF band patterns, with IgM reaction being more sensitive than IgG reactions. The data are consistent with the findings of Grodzicki and Steere (11) and Karlsson and coworkers (15), who described a high incidence of unspecific binding to different antigens of *B. burgdorferi*, probably due to cross-reactive antibodies induced by other bacteria (e.g., oral treponemes). But these researchers did not develop definitions for the positivity of blots on the basis of a quantitative or semiquantitative evaluation.

The main problem of the diagnosis of stage III Lyme borreliosis is the lack of unequivocal symptoms and the low specificity of the standard assays used for antibody detection. The Western blot as a potential confirmation test should be able to differentiate between true-positive and false-positive results and therefore needs a high specificity. Only patients with positive IFA or EIA were included in both diseased and control groups, which corresponds to the function of the Western blot as a confirmatory test. The specificity of the total diagnostic procedure (standard assay with subsequent Western blot in the case of positive findings) is likely to be higher, whereas the global sensitivity may be lower. Because clinical symptoms are not always a satisfactory external criterion for the diagnosis of Lyme borreliosis, there may actually have been a number of false vclinical classifications in group DIII. This is a source of underestimation of the sensitivities calculated for the different band patterns. On the other hand, specificity may have been underestimated by neglecting the fact that characteristic band patterns can be found in latent (asympto-

TABLE 5. Diagnostic sensitivities of individual bands and exemplary combinations of two bands at specificity levels of >90%

M_r(s) (10^3) of band(s)	Sensitivity (%)	OD required to obtain specificity above 90%	OD maximum
21	35	>0	0.34
30	41	0.23	0.67
41	15	0.24	0.5
60	5	0.34	0.48
73	10	0.28	0.46
94	43	0.32	0.64
94 + 21	35	>0 (each band)	
30 + 21	35	>0 (each band)	
94 + 30	53	>0.32 (94K) or >0.23 (30K) (both bands present)[a]	
94 + 41	38	>0.24 (94K) or >0.26 (41K) (both bands present)[a]	
60 + 41	12	>0.41 (60K) or >0.22 (41K) (both bands present)[a]	

[a] Modified believe-the-positive rule.

TABLE 6. Number of major bands accompanying a 94K band in 50 blots

No. of serum specimens	No. of bands
3	1
4	2
7	3
9	4
12	5
9	6
3	7
3	8

TABLE 7. Evaluation of discrimination rules for Western blots in stage III Lyme borreliosis

Bands required	Sensitivity (%)	Specificity (%)
At least 4	79	35
At least 5	66	61
94K, 73K, 30K, 21K[a]	23	97
94K, 73K, 41K, 30K[a]	38	84
94K, 73K, 60K, 41K, 30K[a]	28	84

[a] As major bands (believe-the-negative rule).

matic) or past borreliosis. A major finding was that a high-M_r protein of *B. burgdorferi*, the 94K protein, was the best marker for stage III Lyme borreliosis, as proved by ROC analysis. Probably this protein is identical to a 100K protein of *B. burgdorferi* which has been mentioned as a main antigenic determinant in late-stage borreliosis by Wilske et al. (25). Other bands well correlated with the clinical diagnosis were the 30K and 21K bands, whereas a reaction with flagellin was specific only at high intensity values. The same was found for bands with M_rs of 60,000 and 73,000. These findings are consistent with the known cross-reactivity of these proteins. The 41K band was found to share homologous amino acid sequences with other spirochetes and even flagellum proteins of other bacterial families (9, 17). The 60K protein was recently identified as a common protein, sharing epitopes with a wide range of bacteria (12), and some proteins in the M_r range from 66,000 to 73,000 were found to possess homologies to a known heat shock protein (18). No cross-reactive antibodies or only weakly cross-reactive antibodies, however, could be induced against the 100K (94K) protein in rabbits upon immunization with other spirochetes (4). The outer surface proteins were only rarely detected by antibodies in the sera and consequently could not contribute to the sensitivity of the blots. The 39K protein was not found to significantly discriminate between the symptomatic and asymptomatic groups. This band is sometimes hardly distinguishable from other antigens with similar molecular weights that appear on the blots, and it shows considerable antigenic variation among strains. An 83K protein was described by others (5, 10) as a major antigen in the late stage of Lyme borreliosis. It was the component with the highest molecular mass regularly detected in their blots and seems to have an antigenic function similar to that of the 94K protein in our blots. Although we could not identify an 83K protein as a major antigen in our blots, the possibility cannot be excluded that both reports refer to the same antigen which may have been assigned different molecular weights because of preparative or analytical differences. Multiple-band patterns of undefined bands were found to possess only low specificity. However, different defined multiple-band rules which showed relatively high specificities were set up.

In conclusion, with clearly defined interpretation criteria, Western blot is considered a useful method for the confirmation of positive results obtained by standard assays and their differentation with regard to the clinical stage of the disease.

The possible role of the 94K protein of *B. burgdorferi* as a marker antigen in stage III Lyme borreliosis suggests its usefulness as an antigen in future selective or single-antigen tests for antibody detection.

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