

Species-Specific Serodiagnosis of Lyme Arthritis and Neuroborreliosis Due to *Borrelia burgdorferi* Sensu Stricto, *B. afzelii*, and *B. garinii* by Using Decorin Binding Protein A

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The antigenic potential of decorin binding protein A (DbpA) was evaluated in serodiagnosis of human Lyme borreliosis (LB). The *dbpA* was cloned and sequenced from the three pathogenic *Borrelia* species common in Europe. Sequence analysis revealed high interspecies heterogeneity. The identity of the predicted amino acid sequences was 43 to 62% among *Borrelia burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*. The respective recombinant DbpAs (rDbpAs) were produced and tested as antigens by Western blotting and enzyme-linked immunosorbent assay (ELISA). One hundred percent of patients with neuroborreliosis (NB) and 93% of patients with Lyme arthritis (LA) reacted positively. Sera from the majority of patients reacted with one rDbpA only and had no or low cross-reactivity to other two variant proteins. In patients with culture-positive erythema migrans (EM), the sensitivity of rDbpA immunoglobulin G (IgG) or IgM ELISA was low. The DbpA seems to be a sensitive and specific antigen for the serodiagnosis of LA or NB, but not of EM, provided that variants from all three pathogenic borrelial species are included in the combined set of antigens.

Lyme borreliosis (LB) is a multiorgan infection caused by the spirochete *Borrelia burgdorferi* sensu lato. A subspecies, *B. burgdorferi* sensu stricto, causes human LB infections in the United States (38). In Europe, however, three different borrelial subspecies, *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*, are known etiologic agents of LB (1).

The diagnosis of LB is clinical, but laboratory tests, culture, PCR, and serologic assays (enzyme-linked immunosorbent assay [ELISA] and Western blotting [WB]), are frequently needed to confirm the diagnosis. Culturing *B. burgdorferi* from clinical samples other than erythema migrans lesions is difficult (43), and the PCR-based methods seem to be too insensitive for routine laboratory testing for LB (29, 37), probably because of the scarcity of bacteria in clinical samples (39). The mainstay of laboratory diagnosis for LB has been serologic assays of antibodies against *B. burgdorferi*, although their performance in different laboratories is highly variable (5). ELISA has been widely used as a screening test. In Europe, however, these assays using whole-cell lysates (WCL) or flagellin as antigens are not standardized, which limits their sensitivity and specificity (11). The Centers for Disease Control and Prevention in the United States suggested a two-test approach in which unequivocal results in the ELISA are confirmed by WB. In Europe especially, the clinical implications of this recommended strategy have remained unclear (3, 20, 34). WB does not seem to discriminate between true- and false-positive test results or between active and previous *B. burgdorferi* infections (11). One factor causing difficulties in serologic tests is the existence of

three different pathogenic species of *B. burgdorferi* sensu lato causing LB in Europe (23, 24). Among individual borrelial proteins from different species, sequence heterogeneity varies up to 40% (8, 21, 33, 35), and their use as antigens may affect the sensitivity of the assays (18–20).

In hopes of increasing the specificity of serodiagnosis, a number of borrelial recombinant proteins have been tested (an 83-kDa protein, flagellin, OspA, OspB, OspC, OspE, OspF, p22, BBK32, VlsE, and P39) (10, 17, 25–28, 31, 32, 35). So far, none of them has proved superior to the current routine serology.

Decorin binding protein A (DbpA), a borrelial outer surface protein, is one of the key proteins in *B. burgdorferi*. DbpA elicits a strong antibody response during experimental murine borreliosis and has been suggested as a potential vaccine protein (6, 9, 14, 15). DbpA has not, however, been tested in the serodiagnosis of human LB.

We therefore tested DbpA in the serodiagnosis of LB. *dbpA* was cloned and sequenced from the three European pathogenic borrelial species, *B. burgdorferi* sensu stricto, *B. afzelii* and *B. garinii*. The respective recombinant DbpAs (rDbpA) were thereafter evaluated as antigens in WB and in ELISA.

MATERIALS AND METHODS

Bacterial strains. Finnish borrelial strains were received from Matti Viljanen (National Public Health Institute, Turku, Finland). *B. burgdorferi* sensu stricto IA was isolated from cerebrospinal fluid of a Finnish patient with neuroborreliosis (NB), and *B. afzelii* A91 and *B. garinii* 40 were isolated from skin biopsy samples of Finnish patients with LB. *B. afzelii* A91 and *B. garinii* 40 are low-passage strains, and *B. burgdorferi* sensu stricto IA is a high-passage strain. The strains were genotyped by PCR and sequencing, the target DNA being a fragment from the flagellin gene of *B. burgdorferi* (24). *B. afzelii* strain SK1 was used in our in-house ELISA to detect antibodies against borrelial WCL proteins. *Borrelia* cells were cultivated in Barbour-Stoenner-Kelly (BSK-H) medium (Sigma, St.

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TABLE 1. Primers used for PCR amplification of *dbpA*

No.	Primer ^a	Location	Source
<i>B. burgdorferi</i>			
sensu stricto			
1	5'-ATA TTG AAA ATG GTG GAG AG-3'	-172-153	B31 (AF069269)
2	5'-CCG GAT CCG GAC TAA CAG GAG CAA CAA AAA TAA G-3'	76-95	<i>B. burgdorferi</i> sensu stricto IA (AF441834)
3	5'-CAG ATG GAT TTG GTT GGG TAT TGT TTT TA-3'	628-600	B31
4	5'-CCG GTA CCG AGA TGG ATT TGG TTG GGT ATT GTT-3'	628-604	<i>B. burgdorferi</i> sensu stricto IA
<i>B. garinii</i>			
5	5'-GTC AAT TCT TGG CTA TGT TTG-3'	-356-336	Ip90 (AF069258)
6	5'-TAA ACA CAG CTG AAA GAT TG-3'	-245-226	Ip90
7	5'-GTT AGC CTG TTA GTA GCA TG-3'	46-65	<i>B. garinii</i> 40 (AF441832)
8	5'-CCG GAT CCG GCT TAA CAG GAG AAA CTA-3'	67-85	<i>B. garinii</i> 40
9	5'-CAT GCT ACT AAC AGG CTA AC-3'	65-46	<i>B. garinii</i> 40
10	5'-ACT GTT CCT GTC ATT TTT TG-3'	407-388	Ip90
11	5'-CCG GTA CCT TAT GTA GTA GCA GCA GTG-3'	561-543	<i>B. garinii</i> 40
12	5'-ATA AAA ATG TTG TTT ATT ATG TAG-3'	578-554	Ip90
<i>B. afzelii</i>			
13	5'-ATG ATT AAA TAT AAT AAA ATT ATA C-3'	1-25	BO23 (AF069267)
14	5'-CTA GCC TGT TAG CAG CAT GT-3'	44-63	BO23
15	5'-TGT AGT TTA ACA GGA AAA GC-3'	61-80	BO23
16	5'-CCG GAT CCA GTT TAA CAG GAA AAG CTA G-3'	64-83	<i>B. afzelii</i> A91 (AF441833)
17	5'-GCA ACA GAA GAG GAA ACT AT-3'	199-218	<i>B. afzelii</i> A91
18	5'-ATA GTT TCC TCT TCT GTT GC-3'	218-199	<i>B. afzelii</i> A91
19	5'-TTA TTT TTG ATT TTT AGT TTG TT-3'	513-491	BO23
20	5'-CCG GTA CCT TAT TTT TGA TTT TTA GTT TGT T-3'	513-491	<i>B. afzelii</i> A91
21	5'-ATA AAA ATG TTG TTT ATT TTT G-3'	529-505	BO23, B31 ^b

^a Restriction enzyme sites of *Bam*HI and *Kpn*I in expression primers are underlined.

^b 3' end from BO23.

Louis, Mo.) at 33°C in 5% CO₂. The *Escherichia coli* host cell strains used for cloning and expression of recombinant proteins were INFαF (Invitrogen, Leek, The Netherlands) and M15 (Qiagen, Hilden, Germany), respectively.

DNA purification. Borrelial genomic DNA was purified with a Dneasy tissue kit (Qiagen). Purified DNA was used in PCR and cloning experiments. Plasmid DNA was purified with a QIAprep-spin plasmid kit (Qiagen).

PCR and DNA sequencing. A PCR-based approach was employed to amplify and sequence the *dbpA* alleles from three different isolates of *B. burgdorferi* sensu lato, *B. burgdorferi* sensu stricto, *B. afzelii* A91, and *B. garinii* 40. Primers for *dbpA* PCR amplification were designed on the basis of published *dbpA* sequences (Table 1). Several primer pairs were designed and tested to ensure that the entire coding sequence of the *dbpA* was obtained. To eliminate any errors possibly made by *Taq* polymerase, the two strands of each *dbpA* were sequenced independently at least twice. Expression primers for each strain encoding the mature portion of the DbpA protein after cysteine at the site of posttranslational acylation were chosen from the analyzed sequences. Approximately 1 ng of template DNA was used under standard PCR conditions: 30 cycles of 94°C denaturing for 1 min, 50°C annealing for 1 min, and 72°C extension for 1 min 30 s with AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, Conn.). The PCR amplified full-length or partial *dbpA* was cloned to the pCR 2.1-TOPO vector (Invitrogen) for sequencing. DNA sequencing was performed at the Core Facility of the Haartman Institute, University of Helsinki, with a DyePrimer (T7, M13Rev) cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.). Sequencing reactions were run and analyzed by the automated sequencing apparatus model 373A (Applied Biosystems Inc.). DNA and protein sequences were analyzed with Lasergene software (DNASTAR, Inc., Madison, Wis.).

Cloning and expression of DbpA. For expression of recombinant DbpA (rDbpA), six-His-tagged protein constructs were generated. The forward and reverse primers included a *Bam*HI site and a *Kpn*I site, respectively. The PCR-amplified DNA encoding the mature portion of DbpA was cloned into pCR 2.1-TOPO plasmid (Invitrogen). The recombinant plasmid was purified and digested with *Bam*HI and *Kpn*I restriction enzymes. The cleaved *dbpA* was then ligated to a similarly digested pQE-30 expression plasmid (Qiagen) and transformed into *E. coli* M15 host cells. The transformation mixture was plated onto Luria-Bertani plates containing 100 µg of ampicillin per ml. A primary culture for expression of the DbpA construct was started by inoculating a single colony from a fresh

transformant plate into 50 ml of Luria-Bertani broth containing 100 µg of ampicillin per ml. The culture was incubated at 37°C with shaking overnight. After 1:50 dilution, 1,500 ml of Luria-Bertani broth containing 100 µg of ampicillin per ml was incubated at 37°C for 3 h (until growth reached the mid-log phase; the optical density at 600 nm [OD₆₀₀] was ca. 0.6). Isopropyl-β-D-thiogalactoside was added to a final concentration of 0.7 mM, and the mixture was incubated for a further 3 h. The cells were centrifuged at 8,000 rpm in a superspeed centrifuge, Sorvall RC-5B Plus; DuPont Company, Wilmington, Del.) for 10 min, washed with phosphate-buffered saline (PBS), and sonicated in PBS with a Soniprep 150 sonicator (Sanyo, Japan) for 5 min, and centrifuged at 13,000 rpm. The sonicate supernatant containing the rDbpA protein was applied to a Chelating Sepharose Fast Flow column (Pharmacia, Sweden) containing Ni²⁺ ions. rDbpA was eluted from the column by increasing the amount of imidazole. The expression and purity of the rDbpA was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoblotting. rDbpAs originating from *B. burgdorferi* sensu stricto IA, *B. afzelii* A91, or *B. garinii* 40 (referred to here as rDbpA_{Bb1a}, rDbpA_{BaA91}, and rDbpA_{Bg40}, respectively) were fractionated in SDS-PAGE (12.5% polyacrylamide) and transferred to a nitrocellulose membrane (Bio-Rad; 0.2-µm pore size) by semidry transfer with 40 mM glycine-50 mM Tris (pH 9.0)-0.375% (wt/vol) SDS-20% (vol/vol) methanol buffer. Twelve micrograms of each rDbpA was used for one 7-cm-wide nitrocellulose membrane. The nitrocellulose membranes were cut to approximately 2-mm strips which were soaked in 0.1% Tween 20-0.9% NaCl. Serum samples were diluted in 0.1% Tween 20, 0.9% NaCl, 0.1 g of fat-free bovine milk powder per liter (Valio, Helsinki, Finland). Samples were incubated at a 1:100 dilution for 2 h. After four buffer rinses for a total of 20 min, the blots were incubated with alkaline phosphatase-conjugated rabbit anti-human immunoglobulin G (IgG) (Jackson Immuno Research Laboratories, Inc., West Grove, Pa.) at 1:5,000 for 2 h. The secondary antibody for immunoblots with plasma from mice infected with *B. garinii* was alkaline phosphatase-conjugated rabbit anti-mouse IgG (Orion, Espoo, Finland). After washing, the bands were visualized with 5-bromo-4-chloro-3-indolylphosphate-nitro blue tetrazolium (Sigma Chemical Co.). The reaction was terminated 10 to 15 min later by washing with distilled water.

ELISA. For ELISAs measuring anti-DbpA antibodies, the wells in a microtiter plate were coated with 100 µl (2 µg/ml) of variant recombinant DbpA proteins

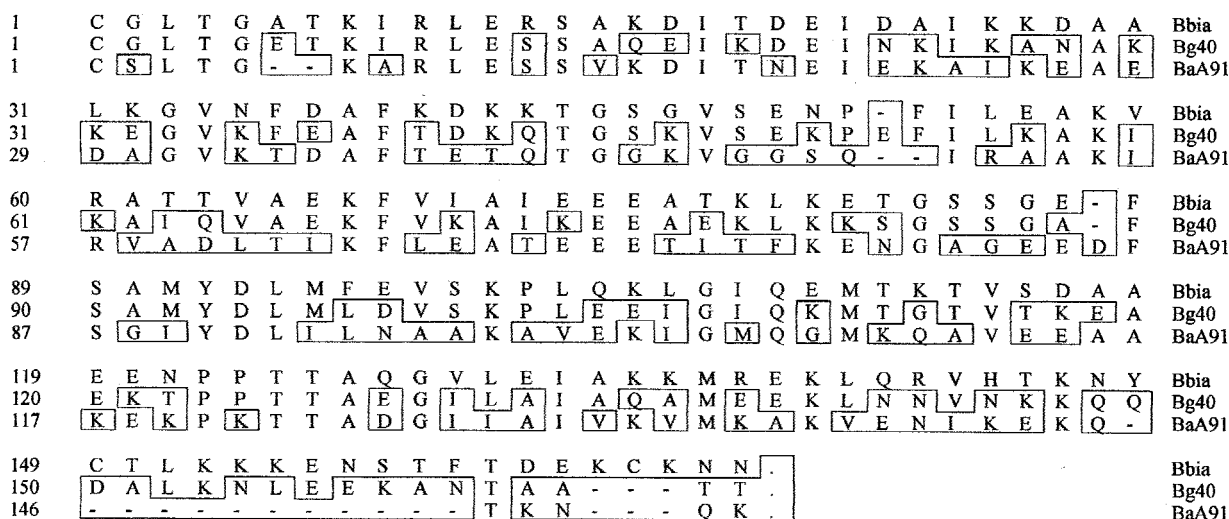


FIG. 1. Alignment of predicted amino acid sequences of mature DbpA of the Finnish isolates *B. burgdorferi sensu stricto* IA (Bbia), *B. garinii* 40 (Bg40), and *B. afzelii* A91 (BaA91). Residues different from those of *B. burgdorferi sensu stricto* IA are boxed.

overnight. After washing, 100 µl of diluted serum samples was added to the wells, and these mixtures were incubated overnight. Serum samples were diluted 1:100 or 1:10 in 5 mg of bovine serum albumin (BSA) per ml in 155 mM NaCl-0.04% Tween 20 buffer. The 1:10 dilution was used with serum samples from erythema migrans (EM) patients. After being washed, the wells were incubated with alkaline phosphatase-conjugated rabbit anti-human IgG or IgM (Jackson Immuno Research Laboratories, Inc.) at 1:5,000 in BSA-NaCl-Tween for 2 h. The reactions were visualized with 4-nitrophenylphosphate (Boehringer Mannheim GmbH, Mannheim, Germany) at 1 mg/ml in diethanolamine buffer pH 10.0. The OD measurements were done after 15 to 30 min at a wavelength of 405 nm with a Multiscan photometer (Thermo Labsystems, Helsinki, Finland).

Samples. For WB, human serum samples were collected from well-defined, clinically typical LB patients (10 with neuroborreliosis [NB], and 10 with Lyme arthritis [LA]). Clinical diagnosis of all patients was confirmed in ELISA by demonstrating antibodies in serum against WCL from *B. afzelii* strain SK1 (in-house ELISA) and flagella (DAKO, Glostrup, Denmark), as described earlier (36). Serum samples from five patients with syphilis, five rheumatoid factor (RF)-positive serum samples, five Epstein-Barr virus (EBV) IgM-positive serum samples, and five serum samples from healthy blood donors were used as controls.

For ELISA analyses, we collected human serum samples from 14 patients with NB, 15 patients with LA, and 23 patients with culture- or PCR-positive EM (*B. afzelii*, *n* = 17; *B. garinii*, *n* = 4, and genotyping not feasible, *n* = 2). In patients with NB and LA, the diagnosis was confirmed serologically, as in samples for WB. From the EM patients, serum samples were taken at diagnosis and 1 to 3 months posttreatment. Serum samples from patients with syphilis, systemic lupus erythematosus (SLE), RF positivity, antistreptolysin (ASO) positivity, or EBV infection and from healthy blood donors were used as controls.

Plasma samples from mice infected with *B. garinii* strain Å218 were obtained from Matti Viljanen (National Public Health Institute, Turku, Finland). The infection was verified by culturing ear pinnae from each mouse at each time point when groups of five mice were sacrificed. Plasma from individual infected mice was pooled at time points 2, 4, 8, and 16 weeks postinfection.

Nucleotide sequence accession number. The nucleotide sequences for the *dbpA* were submitted to GenBank under the following accession numbers: (*B. afzelii* A91, XXX; *B. garinii* 40, YYY; and *B. burgdorferi sensu stricto* IA, ZZZ). Published *dbpA* sequences from *B. burgdorferi sensu lato* strains BO23 (AF069267), ACA1 (AF069278), PGau (AF069270), U01 (AF069284), Ip90 (AF069258), VSBP (AF069272), PBr (AF069281), JEM4 (AF079362), 297 (U75866), LP4 (AF069271), MC1 (AF079361), HB19 (AF069254), B31 (AF069269), and N40 (AF069252) were obtained from GenBank.

RESULTS

Sequence analysis of DbpA of the Finnish borrelial isolates.

The deduced mature portions of DbpA_{BaA91}, DbpA_{Bg40}, and DbpA_{Bbia} contained 150, 165, and 167 residues, respectively (Fig. 1). Differences in the amino acid sequences were distributed along the entire sequence, but deletions in DbpA_{BaA91} were near the carboxy terminus. The calculated molecular masses of the predicted mature proteins DbpA_{BaA91}, DbpA_{Bg40}, and DbpA_{Bbia} (without lipid acylation) were 16.2, 18.0, and 18.5 kDa, respectively. Protein analysis revealed only slight differences in the frequency of charged, polar, and hydrophobic amino acid composition (data not shown), yet the balance between acidic and basic amino acids differed, yielding a calculated pI for DbpA_{BaA91} of 5.69, but pIs of 8.75 and 8.20 for DbpA_{Bg40} and DbpA_{Bbia}, respectively. Comparison of the deduced mature DbpA amino acid sequences of DbpA_{BaA91} and DbpA_{Bbia}, DbpA_{BaA91} and DbpA_{Bg40}, and DbpA_{Bg40} and DbpA_{Bbia} showed 43, 45.3, and 62% identity, respectively (Fig. 2).

***B. afzelii* sequence analyses.** The amino acid sequence of DbpA_{BaA91} was compared with the published human *B. afzelii* DbpA sequences from the BO23 (AF069267), ACA1 (AF069278), PGau (AF069270), and U01 (AF069284) strains (Fig. 2). The percentages of deduced mature amino acid sequence identity among them ranged from 45 to 100%. DbpA_{BaA91} protein was identical to that in strain ACA1. The DbpA sequences in strains PGau and U01 showed the highest divergence among the *B. afzelii* strains: 45.9 and 45.3% identity to DbpA_{BaA91}, respectively. The DbpA_{BaA91} sequence was also compared with four published outer surface protein 17 (Osp17) sequences from European *B. afzelii* strains (22). The identity between DbpA_{BaA91} and the four Osp17 sequences ranged from 88.2 to 100%. The differences in DbpA and Osp17 sequences of *B. afzelii* strains were evenly spread along the protein.

***B. garinii* sequence analyses.** The DbpA_{Bg40} sequence was compared with published *B. garinii* DbpA sequences from the Ip90 (AF069258), VSBP (AF069272), PBr (AF069281), and JEM4 (AF079362) strains (Fig. 2). The identity ranged from

	<u><i>B. afzelii</i></u>								<u><i>B. garinii</i></u>				<u><i>B. burgdorferi sensu stricto</i></u>								
	<u>BaA91</u>	ACA1	BO23	PGau	U01	Osp17/PKo	Osp17/PGau	Osp17/PLe	Osp17/PBo	<u>Bg40</u>	VSBP	PBr	JEM4	Ip90	<u>Bbia</u>	B31	297	LP4	MC1	HB19	N40
<u>BaA91</u>	- 100	85.4	45.9	45.3	91.4	92.8	100	88.2	<u>45.3</u>	46.6	46.6	42.6	45.3	<u>43.0</u>	43.0	40.7	41.3	41.7	40.4	41.1	
ACA1		- 85.4	45.9	45.3	91.4	92.8	100	88.2	45.3	46.6	46.6	42.6	45.3	43.0	43.0	40.7	41.3	41.7	40.4	41.1	
BO23			- 44.9	42.9	86.8	84.1	85.4	88.1	41.5	42.9	42.9	39.5	42.9	43.3	43.3	40.9	41.6	41.3	40.0	40.7	
PGau				- 66.9	50.0	48.0	45.9	49.3	67.5	64.4	64.4	65.0	67.5	57.7	57.7	56.8	55.6	58.3	56.4	55.8	
U01					- 45.9	45.9	45.3	46.6	88.0	83.7	83.7	84.3	99.4	60.1	60.1	61.1	61.1	62.0	61.3	61.3	
Osp17/PKo						- 92.1	91.4	96.7	45.9	46.6	46.6	43.9	45.9	47.0	47.0	44.7	45.3	45.7	43.0	43.7	
Osp17/PGau							- 92.8	88.8	46.6	45.9	45.9	44.6	45.9	47.0	47.0	44.0	44.7	45.0	42.4	43.0	
Osp17/PLe								- 88.2	45.3	46.6	46.6	42.6	45.3	43.0	43.0	40.7	41.3	41.7	40.4	41.1	
Osp17/PBo									- 46.6	47.3	47.3	44.6	46.6	46.4	46.4	44.0	44.7	44.4	41.7	42.4	
<u>Bg40</u>										- 90.4	89.8	85.5	88.6	<u>62.0</u>	62.0	63.6	62.3	63.2	62.6	62.0	
VSBP											- 99.4	81.9	84.3	57.7	57.7	58.6	57.4	58.3	57.7	57.7	
PBr												- 82.5	84.3	57.1	57.1	58.6	57.4	58.3	57.1	57.1	
JEM4													- 84.9	58.9	58.9	60.5	59.3	61.3	60.7	58.9	
Ip90														- 60.7	60.7	61.7	61.7	62.6	62.0	62.0	
<u>Bbia</u>															- 100	87.9	86.7	88.0	80.5	76.3	
B31																- 87.9	86.7	88.0	80.5	76.3	
297																	- 98.8	98.2	70.9	68.5	
LP4																		- 97.0	69.7	67.3	
MC1																			- 71.1	68.7	
HB19																				- 95.9	
N40																					-

FIG. 2. Identities of deduced amino acid sequences of DbpA among *B. burgdorferi sensu lato* isolates. The percent identities were calculated without the sequence encoding the leader peptide by Multiple sequence alignment, according to the Jotun Hein method, with Lasergene software. Underlined numbers represent identities of DbpA between the Finnish borrelial isolates. BaA91, *B. afzelii* A91; Bg40, *B. garinii* 40; Bbia, *B. burgdorferi sensu stricto* IA.

85.5 to 90.4%. The differences in the DbpA sequences were located mainly near the carboxy terminus.

***B. burgdorferi sensu stricto* sequence analyses.** The DbpA_{Bbia} sequence was compared with published DbpA sequences of human *B. burgdorferi sensu stricto* from the 297 (U75866), LP4 (AF069271), MC1 (AF079361), and HB19 (AF069254) strains and with two sequences from the tick isolates B31 (AF069269) and N40 (AF069252) (Fig. 2). The deduced mature amino acid sequence identity ranged from 76.3 to 100%. The *dbpA* sequence in *B. burgdorferi sensu stricto* IA differs by only one nucleotide (position 100) from the *dbpA* sequence of strain B31, which does not change the translated amino acid sequence.

Immunoblot reactivity of LB patient samples. Serum samples from 16 of the 20 LB patients were positive in immunoblots with rDbpA_{BaA91}, rDbpA_{Bg40}, and rDbpA_{Bbia} as antigens (Table 2). Nine of the 10 patients with NB reacted positively; 4 samples showed immunoreactivity with rDbpA_{BaA91}, 5 with rDbpA_{Bg40}, and 2 with rDbpA_{Bbia}. Two samples recognized two rDbpAs each—one rDbpA_{BaA91} and rDbpA_{Bg40} and the other rDbpA_{BaA91} and rDbpA_{Bbia}. Seven of the 10 samples from patients with LA were positive; 5 with rDbpA_{BaA91}, 3 with rDbpA_{Bg40}, and 2 with rDbpA_{Bbia} antigens. One sample showed antibodies against all three rDbpAs, and one had antibodies to rDbpA_{BaA91} and rDbpA_{Bg40}. Four sera that reacted

TABLE 2. IgG WB reactivity against recombinant DbpA proteins from *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii* of LB patients and controls

Patient/control group	No. of positive WBs/no. of samples			
	Total	rDbpA		
		<i>B. afzelii</i> A91	<i>B. garinii</i> 40	<i>B. burgdorferi</i> sensu stricto IA
NB ^a	9/10	4/10	5/10	2/10
LA ^b	7/10	5/10	3/10	2/10
Syphilis	0/5	0/5	0/5	0/5
Positive for RF	0/5	0/5	0/5	0/5
IgM positive for EBV	0/5	0/5	0/5	0/5
Blood donors	0/5	0/5	0/5	0/5

^a Two samples reacted with two DbpAs.

^b Two samples reacted with two or three DbpAs.

with two or three rDbpAs showed a strong reaction with one protein, but also some immunoreactivity against the other rDbpAs. All of the control samples were negative against the three rDbpAs (Table 2).

Immunoblot reactivity in mouse samples. When rDbpA_{Bg40} was used as the antigen in the immunoblot assay, weak reactions were observed with the infected mouse plasma 4 weeks postinfection with *B. garinii*. At 8 and 16 weeks postinfection, the pooled plasma reacted positively with rDbpA_{Bg40} (Fig. 3). The infected mouse plasma did not react with rDbpA_{BaA91} or rDbpA_{Bbia} at any time point (data not shown). Plasma from sham-infected mice was immunoblot negative (Fig. 3).

ELISA. In IgG ELISA, 14 of 14 samples (100%) from patients with NB were positive. Seven of 14 (50%), 7 of 14 (50%), and 6 of 14 (43%) were positive when rDbpA_{BaA91}, rDbpA_{Bg40}, and rDbpA_{Bbia}, respectively, were used as antigens (Fig. 4). Fourteen of the 15 samples (93%) from patients with LA were positive; 12 of 15 (80%), 3 of 15 (20%), and 9 of 15 (60%) were positive with rDbpA_{BaA91}, rDbpA_{Bg40}, and rDbpA_{Bbia}, respectively (Fig. 4). The majority of immunoreactivity was against rDbpA from *B. afzelii* and *B. garinii*. Of the 26 positive samples with either rDbpA_{BaA91} or rDbpA_{Bg40}, 23 reacted with one antigen only (Fig. 5). Only three samples “cross-reacted”, and their OD values with the two antigens were equally high. One patient only with LA had a high antibody response against

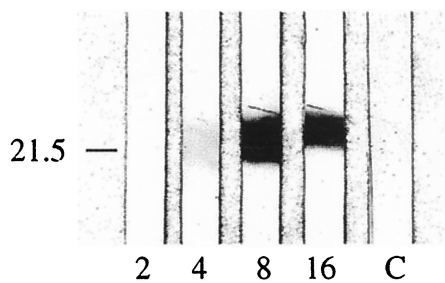


FIG. 3. Recombinant DbpA IgG immunoblot with plasma from mice infected with *B. garinii*. Pooled samples were tested against rDbpA_{Bg40} at 2, 4, 8, and 16 weeks postinfection. Control plasma (C) is from sham-infected mice. The value to the left denotes the location of the 21.5-kDa molecular mass standard. The image was produced with an Agfa Arcus II Desktop Scanner and Adobe Photoshop 5.0 and Adobe PageMaker 6.0 software.

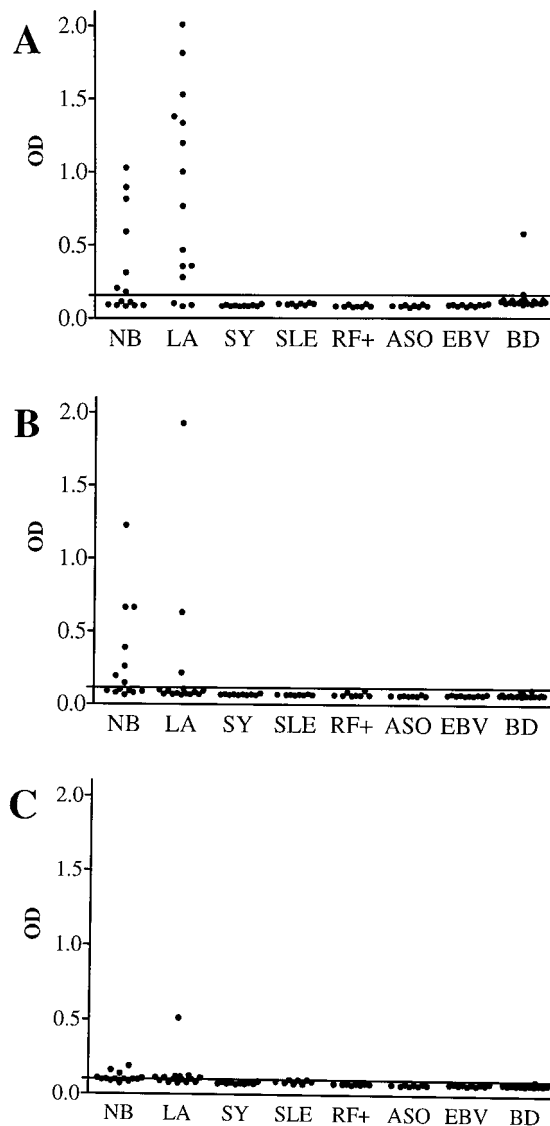


FIG. 4. IgG ELISA OD values with recombinant DbpA as an antigen from *B. afzelii* A91 (BaA91 [A]), *B. garinii* 40 (Bg40 [B]), and *B. burgdorferi* sensu stricto IA (Bbia [C]) with serum samples from NB and LA patients. Control samples were obtained from patients with syphilis (SY), systemic lupus erythematosus (SLE), EBV infection, positive for RF (RF+), or positive for antistreptolysin antibody (ASO) and from healthy blood donors (BD). The cutoff level (mean + 3 standard deviations of healthy blood donor samples) is indicated by a line.

rDbpA from *B. burgdorferi* sensu stricto (Fig. 4C). This sample also reacted with the other rDbpAs, but these antibody responses were weak positives. Two samples from patients with NB were positive only for rDbpA_{Bbia}. The majority of the 15 positive reactions against rDbpA_{Bbia} were close to the cutoff level (Fig. 4C).

ELISA of EM patient samples. We also tested serum samples from culture- or PCR-positive EM patients for anti-DbpA antibodies. In IgM ELISA with rDbpA_{BaA91} or rDbpA_{Bg40} as an antigen, 1 or 2 of the 23 samples (4 to 9%) at diagnosis and 1 or 2 (4 to 9%) at the convalescence phase, respectively, were positive (data not shown). In IgG ELISA, 3 or 1 of the 23 samples

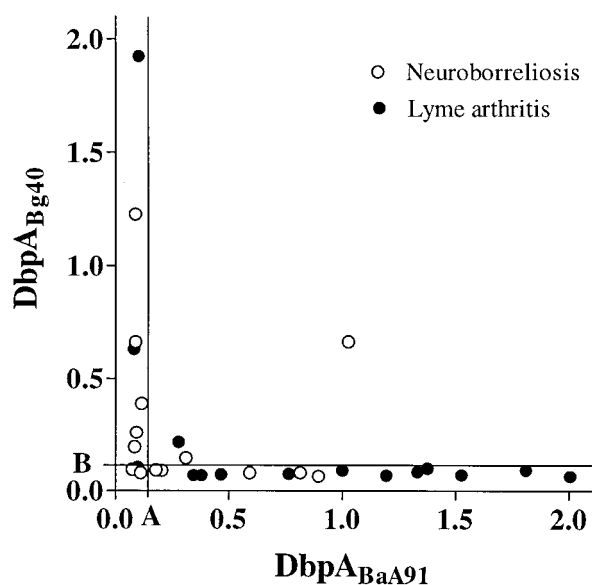


FIG. 5. Correlation between OD values in IgG ELISA with recombinant DbpA from *B. afzelii* A91 (rDbpA_{BaA91}) and *B. garinii* 40 (rDbpA_{Bg40}). The cutoff level (mean + 3 standard deviations of healthy blood donor values) for rDbpA_{BaA91} is indicated by line A, and that for rDbpA_{Bg40} is indicated by line B. Open circles indicate patients with NB, and solid circles indicate patients with LA.

(13 to 4%) at diagnosis and 4 or 0 (17 to 0%) at the convalescence phase were positive when rDbpA_{BaA91} or rDbpA_{Bg40}, respectively, was used as an antigen (data not shown).

DISCUSSION

We analyzed and compared sequences of DbpA from three European isolates of *B. burgdorferi* sensu lato, *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*. Compatibly with a previous study (33), we observed high interspecies heterogeneity. Our hypothesis was that the heterogeneity of the amino acid sequences might have major implications for the usefulness of a given antigen in the serodiagnosis of LB. If the antigenic epitopes in the variant proteins were different, the sensitivity of an immunoassay would be low with a single variant antigen only. In the present study, we demonstrate that inclusion of DbpA variants from three pathogenic species of *B. burgdorferi* as antigens significantly increased the sensitivity of the Western blotting and ELISAs, compared with the use of a single DbpA antigen.

For reliable serodiagnosis of LB, a confirmatory immunoblotting after the ELISA has been advocated. In Europe especially, the use of different species and strains of *B. burgdorferi* sensu lato as sources of antigens leads to inconsistent results because of variations in the expression of the immunogenic proteins (18, 34). Furthermore, immunoblotting not only is a tedious procedure in the laboratory routine, but also is prone to subjective interpretations of band intensities. A recent European multicenter study formulated a panel of seven immunoblotting rules to be adopted in relation to the characteristics of LB in local areas (34). In another European study, exclusion of EBV and cytomegalovirus infections by appropriate serology gave better predictive power than confirmation with im-

munoblotting after the ELISA (11). Beyond any doubt, this emphasizes the need for novel methods that would be standardized at least with regard to performance, relevance, techniques, and antigen preparation. Use of recombinant antigens is an option. In the present study, rDbpAs from *B. burgdorferi* sensu lato isolates seemed to be sensitive and specific antigens in the WB and ELISAs for disseminated LB. Among well-defined patients with LA or NB, up to 93 to 100% of samples reacted positively with rDbpAs. WB results and quantification of immunoreactivities by ELISA provided evidence for the species specificity of serological responses. In the majority of cases with positive reactions against more than one DbpA variant proteins, the immunoreactivity was superior against one DbpA antigen. Moreover, the absence of immunoreactivity in non-Lyme sera indicates specificity of DbpA antigens.

Unfortunately, the low sensitivity of IgM and IgG serology for EM limits the utility of rDbpA as a diagnostic antigen in early stages of LB. This has been the experience with several other recombinant borrelial antigens (27, 28). Obviously, the immunological properties and/or differences in expression of the DbpA molecule account for the delayed antibody response during the course of Lyme disease. Also, it is unclear at the moment whether early antibiotic treatment of EM patients would have contributed to our inability to detect anti-DbpA antibodies when using convalescent-phase sera.

To our knowledge, only one study has evaluated immune responses to rDbpA in the serodiagnosis of human LB (7). In Italian patients, immune responses to rDbpA from *B. burgdorferi* sensu stricto 297 were detected in 13% of patients with EM and 35% of those with disseminated LB (NB and LA). In children with suspected LB, by using rDbpA originating from *B. burgdorferi* sensu stricto N40, we observed the immunoreactivity to be at approximately the same level (unpublished observations). The proportion of samples reacting with rDbpA from *B. burgdorferi* sensu stricto in the present study is in accord with the previous results. These findings are probably due to heterogeneity of the proteins resulting in differences in the epitope specificity of the antibodies. In the present study, the predominance of immunoreactivity to rDbpA from *B. afzelii* and *B. garinii* is compatible with the greater prevalence of these two genospecies compared to that of *B. burgdorferi* sensu stricto in Europe (24, 41).

The immunoreactivity to DbpA in experimental murine borreliosis in this study supports the species specificity of LB serology. The antibody response in infected mice corresponds to that in earlier studies, in which detectable antibody levels were seen at 2 to 4 weeks postinfection (9, 14, 15). Feng et al. (9) observed some cross-reactivity of antibodies against heterologous DbpA. They reported that plasma from mice infected with the *B. afzelii* PKo strain or *B. garinii* PBi strain reacted against rDbpA from genetically distant *B. burgdorferi* sensu stricto N40, yet at significantly lower levels than those against plasma from mice infected with homologous *B. burgdorferi* sensu stricto N40. These data suggest some degree of cross-reactivity among isolates of antigenically divergent *B. burgdorferi* sensu lato. In fact, this is in line with the observed differences in the immune responses to the DbpA variants in the present study.

A recent study described a novel immunodominant outer surface protein of *B. afzelii*, Osp17 (22); sequence similarities

with DbpA suggested a relationship between this protein and DbpA. In that study, *osp17* genes could not be amplified from *B. garinii* or *B. burgdorferi* sensu stricto strains, indicating heterogeneity between the sequences. Interestingly, on comparison of the four published Osp17 sequences with the DbpA sequence from the *B. afzelii* strain in the present study (DbpA_{BaA91}), the identity of the sequences was 88 to 100%. In contrast, the homology of the Osp17 sequences and DbpA sequences from *B. garinii* and *B. burgdorferi* sensu stricto was approximately 45%, corresponding to that of DbpA_{BaA91} and DbpA from *B. garinii* and *B. burgdorferi* sensu stricto. Taken together, the close homology between DbpA and Osp17 from the *B. afzelii* strains suggests that these proteins represent species-specific variants of the same (ancestral) protein. Even more definitely, on the basis of genetic and biochemical evidence, Ulbrandt et al. (40) very recently concluded that Osp17 and DbpA from *B. afzelii* strain Pko are the same protein. The Osp17 antigen from this particular borrelial strain has been proposed as a component in a new recombinant serodiagnostic immunoblot (42), with seropositivity of 24 to 36% in patients with early disseminated borreliosis and up to 85% in patients with late disease (30, 42). It is obvious that inclusion of rDbpAs (Osp17) derived from different strains would increase the sensitivity of the recombinant blot.

The high sequence heterogeneity of DbpA among the three borrelial subspecies raises interesting considerations regarding the potential differences in the interaction between this protein and the host immune system. The DbpA of *B. burgdorferi* has been suggested to have a biological function (i.e., decorin binding activity) (12, 13), during the mammalian phase of LB. This ability is believed to be important for promoting colonization by the spirochetes when they penetrate into the skin via a tick bite and adhere to the collagen-associated proteoglycan decorin (15). Evaluation of the decorin binding activity of our DbpA constructs was beyond the scope of this study. However, the critical lysine residues responsible for this activity (4) were present in all three Finnish human isolates of *B. burgdorferi* sensu lato examined (data not shown). The heterogeneity of DbpA proteins may also have implications for the vaccine development of this antigen (6, 15, 16). Although a recent study suggests that conformational epitopes are needed to elicit protective antibodies against homologous and heterologous strains (40), the present findings on the species specificity of serologic responses to DbpA imply that development of a wide-spectrum vaccine may be more complicated than anticipated.

In conclusion, DbpA seems to be a sensitive and specific antigen for the serodiagnosis of LA or NB, provided that variants from all pathogenic borrelial species are included in the antigen set. This approach may reduce the number of false-negative results. The present findings also imply that DbpA antigens might be used for the species-specific serodiagnosis of LA and NB. These characteristics could be useful at least for epidemiologic purposes, as well as, possibly, prognostic or therapeutic purposes. It has been shown that the three pathogenic borrelial subspecies are preferentially associated with distinct clinical pictures (41). Moreover, a recent study suggested that even at the subspecies level, certain strains, on the basis of genetic diversity of *ospC* genes, might cause more invasive or severe disease than other strains (2). Given the

well-known difficulties in detecting borreliae from clinical samples, either by culture or PCR, it can be speculated that knowledge of the causative borrelial subspecies might be beneficial when evaluating the course of the disease and intensity of the antibiotic therapy.

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