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Significant Improvement of the Recombinant *Borrelia*-Specific Immunoglobulin G Immunoblot Test by Addition of VlsE and a DbpA Homologue Derived from *Borrelia garinii* for Diagnosis of Early Neuroborreliosis

Ulrike Schulte-Spechtel,[1] Gisela Lehnert,[1] Gaby Liegl,[1] Volker Fingerle,[1] Christiane Heimerl,[1] Barbara J. B. Johnson,[2] and Bettina Wilske[1]*

Max von Pettenkofer-Institut für Medizinische Mikrobiologie und Hygiene der Ludwig-Maximilians-Universität München, D-80336 Munich, Germany,[1] and Centers for Disease Control and Prevention, Division of Vector-Borne Infectious Diseases, Fort Collins, Colorado 80522[2]

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We investigated whether the recombinant *Borrelia* Western blot test previously described (B. Wilske, C. Habermann, V. Fingerle, B. Hillenbrand, S. Jauris-Heipke, G. Lehnert, I. Pradel, D. Rössler, and U. Schulte-Spechtel, Med. Microbiol. Immunol. 188:139–144, 1999) can be improved by the addition of VlsE and additional DbpA and OspC homologues. By using a panel of sera from 36 neuroborreliosis patients and 67 control patients, the diagnostic sensitivity of the recombinant immunoblot test was significantly increased (86.1% versus 52.7%) without loss of specificity and was higher (86.1% versus 63.8%) than that of the conventional whole-cell lysate immunoblot test (U. Hauser, G. Lehnert, R. Lobentanzer, and B. Wilske, J. Clin. Microbiol. 35:1433–1444, 1997). Improvement was mainly due to the presence of VlsE and DbpA.

Diagnosis of Lyme borreliosis primarily depends on clinical symptoms and on serological findings. Though serological tests are widely used, they are still poorly defined regarding sensitivity, specificity, and standardization. In both the United States and Europe, a two-step approach is recommended by the Centers for Disease Control and Prevention and the German Society for Hygiene and Microbiology, respectively. The first step is a sensitive enzyme-linked immunosorbent assay (ELISA). In cases resulting in a reactive first test, a Western immunoblot test is performed (2, 11, 20). This implies that the immunoblot test must be highly reliable, with high specificity. In immunoblot tests using whole-cell lysate (conventional blot tests), reliable identification of diagnostic bands is very difficult due to difficulties in distinguishing specific and nonspecific reactivities of antigens with similar molecular weights. In contrast, evaluation of blot tests using recombinant selected proteins is reliable and easy. However, up to now the conventional blot test has been superior to the recombinant test in sensitivity (18).

In a previous study, Wilske et al. described the use of the following recombinant antigens for serodiagnostic immunoblot tests: p83/100 derived from strain PKo (*Borrelia afzelii*); p39 (BmpA) and OspC from strains PKa2 (*B. burgdorferi* sensu stricto), PBi (*B. garinii*, OspA-type 4), and PKo; p41i (internal flagellin fragment) from PKo and PBi; p58 derived from PBi; and Osp17 from PKo (18). In the present study, we investigated whether the additional use of three further recombinantly expressed highly immunogenic proteins, decorin binding protein A (DbpA) derived from *B. garinii* strain PBr (OspA-type 3), VlsE from *B. burgdorferi* sensu stricto strain PKa2, and OspC from *B. garinii* strain 20047, can improve the previously described recombinant immunoglobulin G (IgG) immunoblot test. VlsE, a recently detected lipoprotein of *B. burgdorferi* sensu lato, was shown to undergo antigenic variation (21). However, ELISA studies with American Lyme disease patients and a limited panel of European patients indicated that VlsE is a highly sensitive diagnostic antigen with conserved immunogenic epitopes (12, 14). DbpA is a major in vivo-expressed lipoprotein of *B. burgdorferi* sensu lato with high sequence heterogeneity (15). Therefore, and since neuroborreliosis in Europe is associated with *B. garinii* in 60 to 70% of cases (17), we wanted to investigate whether the use of DbpA from a *B. garinii* strain in addition to DbpA from a *B. afzelii* strain (formerly Osp17); (18) can improve the sensitivity of the recombinant immunoblot test in patients with neuroborreliosis. We also asked whether the sensitivity of the blot test can be improved by the use of an additional *B. garinii* OspC besides the OspC from strain PBi, since *B. garinii* OspCs are rather heterogeneous (17). Furthermore, results from the new recombinant blot test were compared with results from the conventional whole-cell lysate immunoblot test (5). In this study, sera from patients with early neuroborreliosis (neuroborreliosis stage II) were investigated, since a considerable fraction of these samples have been negative in the previous tests.

Cultivation and sources of strains PKa2, PBr, and 20047 as used in this study have been described previously (19). Cloning of the *vlsE* gene from strain PKa2 was performed using primer F4120 (5′-CGGGATCCAAGTTGCTGATAAGGACGACC C-3′) containing a *Bam*HI restriction site and primer R4121 (5′-CGGAAGCTTCAATCATGAGGGCATAGTCGTGTC

* Corresponding author. Mailing address: Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, Ludwig-Maximilians-Universität München, Pettenkoferstr. 9a, D-80336, Munich, Germany. Phone: 49 89-51605231. Fax: 49 89-51604757. E-mail: Bettina.Wilske@mvp-bak.med.uni-muenchen.de.

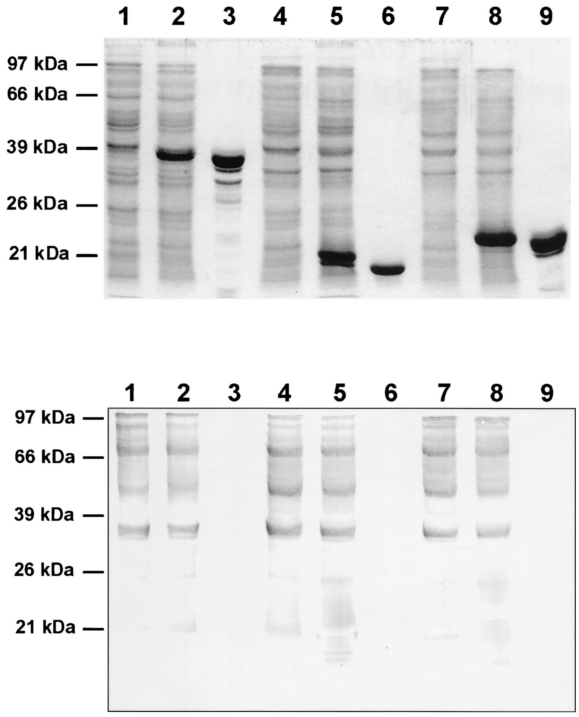

FIG. 1. Purification of recombinant antigens. (Top panel) SDS-PAGE results for VlsE (lane 1, uninduced recombinant *E. coli* whole-cell lysate; lane 2, induced recombinant *E. coli*; lane 3, purified protein), DbpA (lane 4, uninduced recombinant *E. coli* whole-cell lysate; lane 5, induced recombinant *E. coli*; lane 6, purified protein), and OspC (lane 7, uninduced recombinant *E. coli* whole-cell lysate; lane 8, induced recombinant *E. coli*; lane 9, purified protein). (Bottom panel) Immunoblot test results with immune serum against *E. coli*. Antigens used were as described for the top panel.

CATACA-3′) with a *Hin*dIII restriction site (the *Bam*HI and *Hin*dIII restriction sequences, respectively, are underlined). The amplified fragment (1,227 bp without a leader sequence) was ligated into *Bam*HI- and *Hin*dIII-treated pQE30 vector, which contains a sequence encoding an N-terminal His_6 tag. The recombinant plasmid was transformed into *Escherichia coli* SURE (Stratagene, Amsterdam, The Netherlands). Using the sequence of the *dbpA* gene of *B. garinii* PBr (GenBank accession no. AF069281) (15), we constructed a plus-strand primer, FdbpA-A1 (5′-GAGGGATCCATCATGGGCTTAA CAGGAGAAACTAA-3′) (the recognition sequence for *Bam*HI is underlined), and a minus-strand primer, RdbpA-B1 (5′-AAACTGCAGTTAATGGTGATGGTGATGGTGTGT AGTAGTAGCAGTTTTGGC-3′) (the recognition sequence for *Pst*I is underlined; amplification using the sequence indicated with double underlining resulted in a C-terminal His_6 tag). The resulting amplification product was 495 bp in length. The fragment was ligated into *Bam*HI-*Pst*I-treated pUHE21 vector, and the recombinant plasmid was transformed into *E. coli* XL1-Blue. Using standard *ospC* primers from our laboratory as described previously (9), the *ospC* gene from strain 20047 was amplified without a leader sequence. The expression of *dbpA*, *vlsE*, and *ospC* in recombinant *E. coli* XL1-Blue and SURE was induced by the addition of isopropyl-β-D-thiogalactopyranoside. VlsE and DbpA were purified using an FPLC system (Pharmacia Biotech, Freiburg, Germany). Recombinant proteins containing a His_6 tag (DbpA and VlsE) were subjected to affinity chromatography on a $NiSO_4$-loaded IMAC column (Fractogel EMD Chelat; Merck, Darmstadt, Germany) as described previously (10, 16). Recombinant OspC of strain 20047 was purified first by anion exchange chromatography (DEAE-Sepharose) and then by cation exchange (Fractogel SO_3).

E. coli clones were obtained which effectively expressed DbpA, VlsE, and OspC from strains PBr, PKa2, and 20047, respectively (Fig. 1). At this stage of the study, the expression of the VlsE clone was controlled using an anti-VlsE-positive serum from an American patient (laboratory of B.J.). A clone expressing VlsE from *B. burgdorferi* sensu stricto strain B31 generated in the same laboratory (1) served as a positive control. The serum recognized VlsEs from the recombinant clones; *E. coli* without a *vlsE* insert gave a negative result (data not shown). Purified proteins of DbpA (PBr), VlsE (PKa2), and OspC (20047) clones were obtained by chromatography without contamination of other proteins (Fig. 1). Even in the immunoblot test with a high-titered immune serum against *E. coli*, no contaminating proteins were detected (Fig. 1). Purified PKa2 VlsE was reactive with a rabbit immune serum raised against the recombinant PKa2 VlsE (Fig. 2), and purified OspC was reactive with the OspC-specific monoclonal antibody L22 C11 (17) (data not shown). Purified DbpAs from

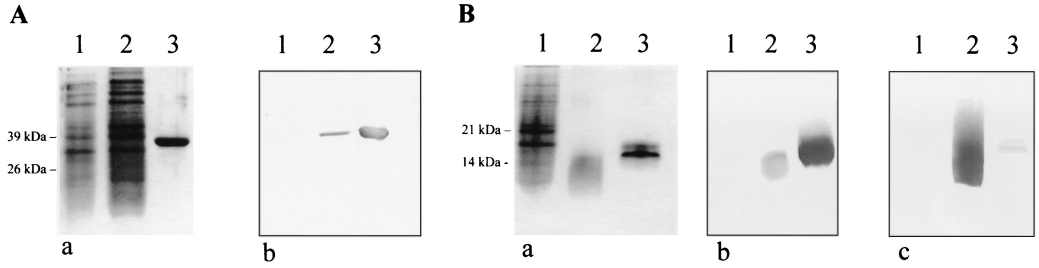

FIG. 2. Reactivity of recombinant proteins with rabbit immune sera. (A) Reactivity of recombinant VlsE with homologous rabbit immune serum. (a) SDS-PAGE results for *E. coli* whole-cell lysate without *vlsE* insert (lane 1), induced recombinant *E. coli* (lane 2), and purified protein (lane 3). (b) Immunoblot test results with immune serum against recombinant VlsE. Antigens used were as described for panel a. (B) Reactivity of recombinant DbpA from strains PBr and PKo with homologous and heterologous immune sera, respectively. (a) SDS-PAGE results for *E. coli* whole-cell lysate (lane 1), purified DbpA strain PBr (lane 2), and purified DbpA strain PKo (lane 3). (b) Immunoblot test results with immune serum against DbpA strain PKo. Antigens used were as described for panel a. (c) Immunoblot test results with immune serum against DbpA strain PBr. Antigens used were as described for panel a.

TABLE 1. Comparison of the newer recombinant immunoblot test with the older recombinant test and the whole-cell lysate immunoblot test

Group	Total no. of serum samples	No. (%) of positive serum samples by the:		
		Newer recombinant immunoblot test	Older recombinant immunoblot test	Whole-cell lysate immunoblot test
Neuroborreliosis stage II	36	31 (86.1)	19 (52.7)	23 (63.8)
Control[a]	67	0	0	2

[a] The control group consisted of 49 healthy persons, 8 patients with syphilis, and 10 patients positive for rheumatoid factor.

strains PBr and PKo were reactive with rabbit immune sera against DbpAs from strains PBr and PKo, respectively. Reactivities of the homologous sera were very strong, whereas reactivities were very weak with the heterologous sera (Fig. 2). PBr DbpA was not reactive with monoclonal antibody L17 G2, whereas PKo DbpA was strongly reactive (data not shown), as previously described (10).

In this study we used sera from a clinically well-defined serum panel previously investigated in the studies of Hauser et al. (5) and Wilske et al. (18). The sera used were from the following groups of patients: (i) 36 neuroborreliosis patients (stage II) with positive intrathecal *Borrelia*-specific antibody production and/or positive IgM ELISA results (confirmed by immunoblot testing) and (ii) negative-control patients, whose sera comprised 49 serum samples from blood donors, 8 serum samples positive for rheumatoid factor, and 10 serum samples from syphilis patients. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and conventional and recombinant immunoblot tests were performed as previously described (5, 18). A concentration of 12.5% was used for all gels. Human and anti-*E. coli* rabbit serum samples were diluted 1:200, and bound IgG antibodies were detected with horseradish peroxidase-labeled anti-human IgG purchased from Dakopatts (Copenhagen, Denmark). Goat immune serum against rabbit Igs (Dakopatts) was used for detection of rabbit antibodies against VlsE and DbpA (10). For the whole-cell lysate immunoblot test, the antigen was *B. afzelii* isolate PKo. This strain expresses the immunodominant antigens OspC and DbpA in culture (5). Immunoblot strips for use with the older recombinant blot test contained the following recombinant

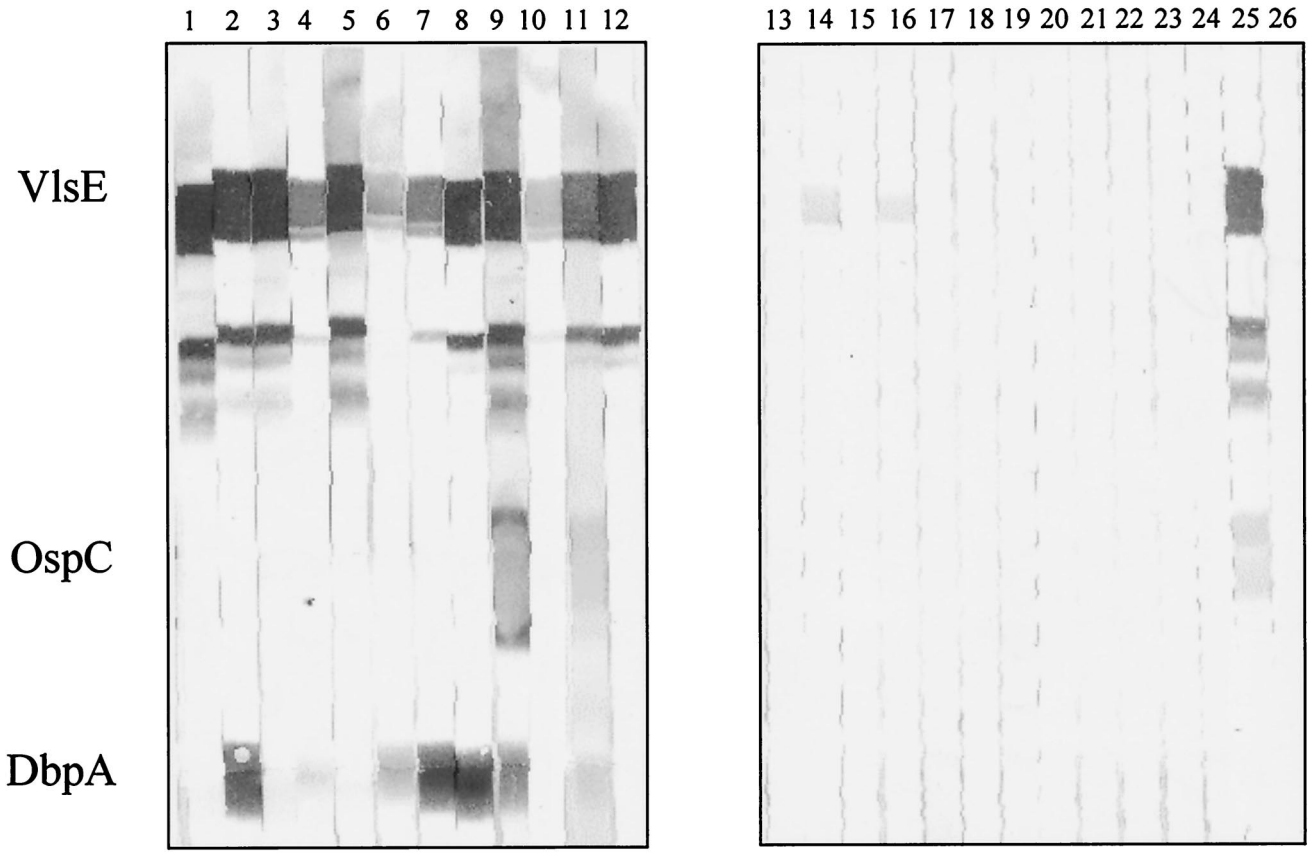

FIG. 3. Newer recombinant immunoblot test. Test results for reactivity of VlsE, OspC (strain 20047), and DbpA (strain PBr) with sera from patients with neuroborreliosis stage II (lanes 1 to 12) are shown. Lanes 13 to 24, negative-control sera; lane 25, positive control; lane 26, negative control. Note that the minor bands between VlsE and OspC represent degradation products of VlsE.

TABLE 2. Immunoreactivity of VIsE and Osp17 (DbpA) in the recombinant immunoblot test[a]

Group	Total no. of serum samples	No. (%) of serum samples with positive results for immunoreactivity of:			
		VIsE	Osp17 (DbpA) with:		
			PKo	PBr	PKo and/or PBr
Neuroborreliosis	36	30 (83.3)	14 (38.8)	16 (44.4)	25 (69.4)
Control	67	4 (6.0)	2 (3.0)	2 (3.0)	4 (6.0)

[a] In tests of reactivity of OspC (20047), only three neuroborreliosis sera gave positive results; all controls gave negative results.

proteins: p83/100 derived from strain PKo (*B. afzelii*); p39 (BmpA) and OspC from strains PKa2 (*B. burgdorferi* sensu stricto), PBi (*B. garinii*, OspA type 4), and PKo; p41i (internal flagellin fragment) from PKo and PBi; p58 derived from PBi; and DbpA from PKo (18). For the newer immunoblot test, an additional strip was produced containing the following antigens: DbpA (strain PBr), OspC (strain 20047) and VlsE (strain PKa2). The newer immunoblot test also included use of the antigens from the older immunoblot test. Thus, all of the relevant proteins—those from the older test and the additional new proteins—were evaluated for the newer blot test. For statistical analysis, Fisher's exact test for dichotomous variables was performed.

Results of the tests are shown in Table 1. Figure 3 shows examples of immunoreactivity for 12 sera from patients with neuroborreliosis and 12 control sera with the new antigens. Neuroborreliosis serum no. 9 was reactive with all three new recombinant proteins. Note the minor bands below the VlsE band; these bands apparently represent degradation products of VlsE (also seen in the Coomassie staining results shown in Fig. 1) and not *E. coli* contaminants, because they were detected only with strongly reacting VlsE antibody-positive sera and not with the immune serum against *E. coli* or VlsE antibody-negative sera. For all immunoblot tests, a two-band criterion (at least two reactive bands) was used to document a positive result. Use of a one-band criterion would result in very low specificity. In the control group, 10 of 67 samples had at least one positive band (resulting in a specificity of only 85%) but none had two positive bands. Overall, we found an increase in sensitivity (from 52.7 to 86.1%) when the three new proteins were added. The difference is significant ($P = 0.004$). The new recombinant blot test was also more sensitive than blot tests which used whole-cell lysate antigens (86.1% versus 63.8%). However, this difference was not significant ($P = 0.055$). The recombinant VlsE was the most sensitive antigen (83.3%), followed by DbpA from PBr (44.4%) (Table 2). Interestingly, the combination of different DbpAs was highly effective, since the two proteins showed low levels of cross-reactivity. At least one of the DbpAs was reactive in 69.4% of the neuroborreliosis sera. OspC from strain 20047 was reactive with only 3 (8.3%) of the 36 sera (Table 2).

In contrast to the United States, where causative strains are very homogeneous, the heterogeneity of the borreliae is a specific problem in Europe. This is especially true for the diagnosis of early neuroborreliosis (stage II), in which causative strains are very heterogeneous (4, 17) and, in contrast to late disease (5), the immune response recognizes only few antigens. We were able to show that both VlsE and the new DbpA homologue from strain PBr are important antigens for improvement of the diagnostic sensitivity of the previous recombinant blot test. The two different DbpAs complement each other, since only a few serum samples were reactive with both proteins. This shows that DbpA has type-specific epitopes which are diagnostically relevant. This has also been shown recently in two other studies (3, 6). Notably, VlsE is expressed by the borreliae in the mammalian host or upon contact with mammal cells but not in the unfed tick (7, 8, 21). VlsE was detected from cultured borreliae only with highly sensitive detection methods (e.g., chemiluminescence) but not in blot tests which used horseradish peroxidase conjugates (unpublished data). As shown previously (10), many strains do not express DbpA (Osp17) in culture. The low or variable level of expression of preferentially in vivo-expressed proteins in culture is a big problem for conventional immunoblot tests using lysates from in vitro-cultured borreliae. With the recombinant immunoblot test presented here, we now have a sensitive and easy-to-standardize confirmation test as recommended for the two-step approach for serodiagnosis of Lyme disease in Europe. It is an open question whether the sensitivity of the IgG immunoblot test can be improved further. It might be interesting to test the antigenicity of additional VlsE proteins derived from *B. garinii* or *B. afzelii* to investigate whether epitopes restricted to single species or types are relevant for serodiagnosis. This might be relevant, since the immunodominant C-terminal region is conserved among *B. burgdorferi* sensu lato species to only a limited extent (13).

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