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Published in:
Clinical and Diagnostic Laboratory Immunology

DOI:
10.1128/CDLI.9.6.1382-1384.2002

2002

Link to publication

Citation for published version (APA):
Ornstein, K., Ostberg, Y., Bunikis, J., Noppa, L., Berglund, J., Norrby, R., & Bergström, S. (2002). Differential immune response to the variable surface loop antigen of P66 of Borrelia burgdorferi sensu lato species in geographically diverse populations of lyme borreliosis patients. *Clinical and Diagnostic Laboratory Immunology*, *9*(6), 1382-1384. https://doi.org/10.1128/CDLI.9.6.1382-1384.2002

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Download date: 19. Dec. 2025

CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Nov. 2002, p. 1382–1384 Vol. 9, No. 6
1071-412X/02/$04.00+0 DOI: 10.1128/CDLI.9.6.1382–1384.2002
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Differential Immune Response to the Variable Surface Loop Antigen of P66 of *Borrelia burgdorferi* Sensu Lato Species in Geographically Diverse Populations of Lyme Borreliosis Patients

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Received 7 November 2001/Returned for modification 25 April 2002/Accepted 26 August 2002

We have studied the immune response to a variable surface-exposed loop region of the P66 outer membrane protein from *Borrelia burgdorferi* sensu lato by using an enzyme immunoassay. Lyme borreliosis populations found in North America and Sweden were preferentially more seroreactive to P66 from their respective regional species, namely, *B. burgdorferi* sensu stricto and *B. garinii* and *B. afzelii*, respectively.

Lyme borreliosis is the most common tick-borne infection in Europe and North America (7, 15). In two recent reviews, genospecies distribution was shown to be associated with geographic origin (11, 19). *Borrelia burgdorferi* sensu stricto is the only species found in North American Lyme disease, while three species, *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*, are associated with Lyme borreliosis in Europe (11, 19). In earlier studies, a correlation between the serological response among Lyme borreliosis patients living in different geographic regions to antigens from various *B. burgdorferi* sensu lato genospecies has been shown (2, 10, 13).

The P66 membrane protein has a surface-exposed loop region including a hypervariable immunogenic determinant that is polymorphic among *Borrelia* species (3, 4, 5). That this antigen is species specific was suggested by its reactivity in immunoblot assays with P66 protein derived from a *B. burgdorferi* sensu stricto strain from North American patients with Lyme disease (5). The aim of this investigation was to further characterize this antigenic site by determining the effect of sequence polymorphism on the ability of anti-P66 antibodies to detect P66 in Lyme borreliosis patients from Sweden and the United States.

The strains used were *B. burgdorferi* sensu stricto B31 (ATCC 35210) and *B. garinii* Ip90 (12) and Swedish strains *B. afzelii* LU81 and *B. garinii* LU59, LU116, LU118, LU170, LU185, L190, and LU222 (16).

The sequence diversity of the P66 loop of Lyme borreliosis strains recovered in Sweden was studied by performing partial *p66* gene sequence analysis by PCR and cycle sequencing. A primer pair targeting the P66 loop was chosen (5′-GAAATT TCAAGCTATGAAGAC-3′ and 5′-CTACATATGCTTCTG TTGAAATGG-3′). For subsequent recombinant enzyme immunoassays (EIAs), we generated peptides corresponding to the P66 loop region (rP66) of *B. burgdorferi* sensu stricto B31, *B. afzelii* LU81, and *B. garinii* Ip90 and LU59 as previously described (5). The following primer pairs were chosen in accordance with previously published sequences (4): 5′-TGGAT TAGGATCCATAACATCTATCGGTC-3′ and 5′-TTTCATT GCGAATTCAAATGTCAGATTAGG-3′ for *B. burgdorferi* sensu stricto, 5′-GGACTGGGTTGGATCCACATCTATCG GTC-3′ and (5′-TCATTGCGAATTCAAATGTTAAATTA G-3′ for *B. afzelii*, and 5′-GGACTAGGATGGATCCACATC TATCGGTC-3′ and (5′-TCATTGCGAATTCAAATGTTAG ATTAG-3′ for *B. garinii*. The endonuclease restriction site [1]) is underlined in each primer sequence.

Serum samples were collected from two geographically separated Lyme borreliosis populations, i.e., 100 patients from southern Sweden (50 with neuroborreliosis, 25 with arthritis, and 25 with acrodermatitis) and 38 patients from North America (23 with erythema migrans and 15 with disseminated Lyme disease [kindly provided by Martin Schäfer, Centers for Disease Control and Prevention, Fort Collins, Colo.]). Diagnoses were based on established case definitions (6). Serum samples from healthy Swedish blood donors (n = 100) collected in a tick-free area in Sweden were used to define the cutoff value for seropositivity in the EIAs.

The rP66 peptides were used as antigens in the EIAs. Preparation of the microtiter plates and the EIA protocol were performed as previously described (13). A protein concentration of 5 μg/ml was used. Serum samples from patients were diluted 1:200. Each plate contained a positive control. Samples were run in duplicate. An EIA index was calculated for each sample by subtracting the background activity (estimated by using an antigen-free well) from the mean optical density value and thereafter divided by the positive control. The cutoff values were calculated as the ≥95th percentile of the blood donor EIA index. The seroreactivity in the Lyme borreliosis groups was compared by using the Mann-Whitney rank sum test. The proportions of seropositive samples were compared by using McNemar's chi-square test (paired proportions) and the contingency tables chi-square test (independent proportions). A P value of <0.05 was considered statistically significant. The

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TABLE 1. P66 EIA seropositivity in Lyme borreliosis patients from the United States and Sweden

P66 EIA	No. (%) seropositive		*P* value
	U.S. patients (n = 15)	Swedish patients (n = 100)	
B. burgdorferi sensu stricto B31	6 (40.0)	1 (1.0)	<0.001
B. afzelii LU81	1 (6.7)	11 (11.0)[a]	>0.05
B. garinii Ip90	1 (6.7)	19 (19.0)[a]	>0.05
B. garinii LU59	0 (0)[a]	10 (10.0)[a]	>0.05

[a] The difference is statistically significant, within the same patient group (McNemar's test, $P < 0.05$), compared to the positivity rate in the B31 P66 EIA.

Primer of Biostatistics, version 3.01 was used for the statistical analyses (8a).

The deduced amino acid sequences corresponding to the P66 loop region of the Swedish Lyme borreliosis strains identified *B. afzelii* LU81 as identical to *B. afzelii* ACA1 (accession no. X87726). Two *B. garinii* sequence variants were identified. Strains LU59 and LU116 grouped in a previously unknown P66 loop sequence type, whereas the sequences from the other *B. garinii* strains were identical to that of a *B. garinii* laboratory strain from a tick isolate from Lithuania (5). None of the locally isolated *B. garinii* strains were identical to Ip90 (accession no. X87727).

The rP66 peptides employed in the EIAs were all recognized by polyclonal antibodies to P66 by Western blot analysis and sera from Swedish and American Lyme borreliosis patients.

The reactivity to rP66 was defined as a late immune response, since the American patient group with disseminated disease reacted more strongly to the *B. burgdorferi* sensu stricto antigen, with six positives (40.0%), than did the erythema migrans group, with one positive (4.3%) (chi-square, 5.490; P = 0.019). Therefore, only patients with disseminated disease were included in the statistical comparison.

The species-specific nature of the immune response to the P66 loop was evidenced by the higher seroreactivity of the North American group in the B31 P66 EIA ($P < 0.001$). Meanwhile, the EIA index values obtained from the LU81 and LU59 P66 EIAs were significantly higher in the Swedish group (P = 0.05 and <0.001, respectively).

Comparisons of the seropositivity rates obtained between and within the two patient groups in the different P66 EIAs are shown in Table 1.

The B31 P66 EIA produced significantly more positive results in the North American group, with six positive samples (40%), than in the Swedish group, which contained only one positive sample (1%) ($P < 0.001$). The seropositivity rate of the Swedish group in the EIAs based on *B. afzelii* and *B. garinii* was higher, although not significantly so, than that of the North American group. The cross-reactivity of the Swedish Lyme borreliosis group in the *B. afzelii* and *B. garinii* EIAs (46%) was extensive, and occasional cross-reactivity was also observed in the North American group.

Within the Swedish group, the seropositivity rate was significant lower in the B31 P66 EIA, with 1 positive sample (1.0%), than in the LU81 P66 EIA, with 11 positive samples (11.0%); the Ip90 P66 EIA, with 19 positive samples (19.0%); and the LU59 P66 EIA, with 10 positive samples (10.0%) (P = <0.001, 0.004, and 0.004, respectively). In the North American group, the seropositivity rate was significant higher in the B31 P66 EIA than in the LU59 P66 EIA but not in the LU81 and Ip90 P66 EIAs. Six North American samples were positive in the B31 P66 EIA (40%), one sample was also positive in the LU81 and Ip90 P66 EIAs (6.7%), and no sample was positive in the LU59 P66 EIA (0%) (P = 0.131, 0.131, and 0.041, respectively).

Our sequence analysis of the *p66* gene from Swedish *B. afzelii* and *B. garinii* human isolates confirms the variable species-specific sequence epitope-containing region of the P66 loop. The species-specific nature of the immune response to *B. burgdorferi* sensu stricto P66 was supported by the preferential immunoglobulin G antibody response among the North American patients in the EIA that used the *Borrelia* species causing Lyme disease in North America. In contrast, the two species known to be present in Sweden, *B. afzelii* and *B. garinii*, reacted more often in the Swedish patient group. The two latter genospecies have been isolated from skin and cerebrospinal fluid from Swedish patients (16). *B. burgdorferi* sensu stricto has been identified in cerebrospinal fluid by PCR in Sweden (17). In our present seroepidemiological study, the two geographically separated Lyme borreliosis populations exhibited preferential reactivity to their respective regional *Borrelia* species.

The cross-reactivity observed among the Swedish samples in the assays based on *B. afzelii* and *B. garinii* could be caused by multiple *Borrelia* infections or peptide similarities in the epitope. Our finding indicates that the P66 loop antigen of *B. afzelii* and *B. garinii*, but not *B. burgdorferi* sensu stricto, might be sufficiently cross-reactive for detection by antibodies to P66 from patients with Lyme borreliosis. P66 has been identified as one of five proteins with discriminative potential in immunoblots for late disease testing in Switzerland (18) but has not been recognized as an important diagnostic immunoblot marker in Europe (9). The general utility of P66 as a diagnostic tool in Europe needs to be further evaluated.

The location of immunogenic epitopes in the P66 protein has been discussed in two different studies (3, 14). Both studies propose that the C-terminal portion, including the loop region, is immunoglobulin G immunoreactive. Ntchobo et al. have proposed multiple antibody epitopes throughout *B. burgdorferi* P66 protein (14). Retention of the native conformation of P66 (Oms66) was found to be essential for eliciting bactericidal P66 antibodies by Exner et al. (8). Further analysis of the immune response to P66-specific peptides could provide additional information needed to localize the species-specific immunoreactive epitopes of the P66 membrane protein.

In summary, our findings confirm the species specificity of the P66 loop protein and that the immune response to the *B. burgdorferi* sensu stricto P66 loop is species specific. In addition, the findings suggest that the loop antigen of P66 of *B. garinii* and *B. afzelii* is somewhat cross-reactive. This study supports the notion that *B. afzelii* and *B. garinii* are the major causative agents of Lyme borreliosis in Sweden.

Nucleotide sequence accession numbers. The sequences determined in this study were deposited in the GenBank database and assigned accession numbers AY090472 (LU59), AY090473 (LU81), AY90474 (LU116), AY090475 (LU118), AY090476 (LU170), AY090477 (LU185), AY090478 (LU190), and AY090479 (LU222).

We thank Maria Alice Alves for excellent laboratory support and Michael Mecklenburg for reviewing the manuscript.

This work was supported by the Swedish Medical Research Council (grants 07922 and 04723), the Royal Physiographic Society in Lund, and the Medical Faculty of Lund University.

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