

Original article**Identification of B-cell epitopes of *Borrelia burgdorferi* outer surface protein C by screening a phage-displayed gene fragment library**

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Running head: Identification of epitopes of OspC

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ABSTRACT

Outer surface protein C of *Borrelia* stimulates remarkable immune responses during early infection and is currently considered as a leading diagnostic and vaccine candidate. Sensitivity and specificity of serological test based on whole protein OspC is still an issue in the diagnosis of Lyme disease. The minimal B-cell epitopes are key in the development of reliable immunodiagnostic tool. Using OspC fragments displayed on phage particles (phage library) and anti-OspC antibodies isolated from sera of naturally infected patients we identified six OspC epitopes capable of distinguishing between LD patient and healthy control sera. Three epitopes are located at N-terminus (OspC E1 aa19-27, OspC E2 aa38-53, OspC E3 aa62-66) and three at C-terminal end (OspC E4 aa155-163, OspC E5 aa184-190 and OspC E6 aa201-207). The OspC E1, E4 and E6 showed high level of conservation among LD related borreliae. To our knowledge, epitopes OspC E2, E3 and E5 were identified for the first time in this study. The minimal B-cell epitopes would provide fundamental data for the development of multi-epitope based diagnostic tool for LD.

Key Words: B-cell epitope; gene fragment library; Lyme disease; outer surface protein C

ABBREVIATIONS

Amp	ampicillin
CFU	colony forming unit
CNBr	cyanogen bromide
EDTA	ethylenediaminetetraacetic acid
HRP	horseradish peroxidase
IPTG	isopropyl β -D-1 thiogalactopyranoside
LD	Lyme disease
MSA	Multiple sequence alignment
PBS	phosphate buffered saline
PBST	phosphate buffered saline supplemented with Tween20
Tet	tetracycline
TTBS	Tris buffered saline supplemented with Tween20

INTRODUCTION

Lyme disease is the most prevalent vector-borne disease in a temperate zone of the Northern hemisphere. It is a progressive multi-systemic disorder caused by members of *Borrelia burgdorferi* sensu lato complex. *Borrelia* is able to adapt to different host environments during its enzootic life cycle, which is associated with precise regulation of a variety of genes and expression of a wide repertoire of outer surface proteins (Osp) [1, 2]. Phase variation of two borrelial lipoproteins, OspA and OspC was described as one of the major host adaptation mechanisms [3]. Borrelial virulence factor OspC (~22kDa) is universal among *B. burgdorferi* sensu lato complex and plays an essential role in vector-host transmission [4]. It provides borreliae with protection from the innate immune system e.g. via binding tick salivary protein Salp15 and facilitates dissemination in the host by binding mammalian plasminogen. However, the precise function of OspC remains controversial [5, 6]. Despite of high amino acid sequence variability of OspC (even among strains collected from a single geographic area), its protein structure is highly conserved [7]. Till to date more than 30 *ospC* genotypes (phyletic groups differentiated by letters from A to U) have been described in the literature [8, 9]. Correlation between invasive potential of *Borrelia* and *ospC* genotype has been discussed previously. For example, four *ospC* genotypes A, B, I and K were shown to be associated with systemic disease in human [10, 11, 9, 12].

OspC is highly immunogenic and possess significant diagnostic value as the anti-OspC antibodies (borreliacidal antibodies) developed in high concentrations shortly after infection in human [13, 14] as well as in non-human models [15]. However, in many cases the serological test may fail in early stage of LD because of the

inadequately developed antibody response. Repeated blood collection (e.g. a week later) and serological testing based on conserved immunogenic epitopes (like in OspC) would improve the accuracy of serodiagnosis.

Epitope mapping is crucial to determine antibody binding sites important in the development of vaccines, diagnostics and immunotherapeutic compounds. Minimal epitopes, usually spanning minimum six consecutive amino acids, can be reliably mapped by gene-fragment libraries. Single round of panning is usually sufficient to unequivocally determine a linear epitope, which makes gene-fragment phage display libraries a trustworthy tool for mapping of linear epitopes [16]. In this study we present mapping of OspC epitopes with OspC gene fragment library and identification of minimal epitopes that could be used as a component of a LD serological tests with improved capabilities for the diagnosis of LD.

MATERIALS AND METHODS

Lyme disease positive serum samples

The human sera were collected from 89 patients positive for LD with at least one of the following symptoms: *erythema migrans* (early stage of LD), early neurologic or cardiac symptoms (early disseminated LD), radiculoneuropathy or arthritis (late stage of LD) attributed to this disease. Commercial *Borrelia* recombinant IgG ELISA (Biomedica Immunoassays, Bratislava, Slovakia) and in-house developed western blotting were used for confirmation of anti-*Borrelia* antibodies in patient sera and OspC positive sera were pooled. Serum samples from 5 healthy donors with no serological evidence of the LD were also included. Written informed consent was obtained from all donors.

Purification of total antibodies

The IgG's from pooled patient sera were purified with hydrophobic interaction chromatography (MEP HyperCel, PALL Life Science, Vienna, Austria). Briefly, HyperCel sorbent was equilibrated with 50 mM Tris-HCl (pH 8.0) and incubated with 4 ml of serum for 1 hr at room temperature with gentle shaking. After three washings, bound antibodies were eluted under low pH conditions (50mM sodium acetate, pH 4.0). Eluate was dialyzed against PBS and stored in 50% glycerol in PBS.

Production of recombinant OspC and BmpA

Whole OspC protein coding region was PCR amplified with F 5'-CGCGGATCCATGACTTTATTTTTATTTATATCTTGTAATAAT and R 5'-TATGTCGACAGGTTTTTTTGGACTTTCTGC primers from genomic DNA of *Borrelia burgdorferi* sensu stricto (strain SKT-2, accession number AY597021.1). Amplicons were digested with *Bam*HI and *Sal*I restriction enzymes (Fermentas,

Finland) and ligated into the UA-mCherry-GFP expression vector (in house modified pQE30-UA vector, fuses GFP tag to overexpressed protein) by using QIAexpress-UA cloning kit (Qiagen, USA). *E. coli* SG13009 host cells were transformed with recombinant vector and subsequent induction of recombinant OspC protein was carried out according to manufacturer's instructions (Qiagen, USA). Recombinant His and GFP tagged OspC protein was purified using Ni-NTA agarose (Qiagen, USA) according to manufacturer's instructions. The presence of His-tag proteins was confirmed by SDS-PAGE and on western blot using anti-His antibody (Abcam, UK, 1:1,500).

Recombinant BmpA protein was produced exactly as described above (anti-BmpA antibodies were isolated and used as negative control in this study). The primers used to amplify BmpA gene were F 5'-TAAGGATCCAGTGGAATTCCTCAAGGTATCT and R 5'-CCGGTTCGACTTCTTTAAGAAATTTTTCATAACT.

Affinity selection of anti-OspC and anti-BmpA antibodies

Briefly, pre-swollen activated CNBr-Sepharose 4B beads (Pharmacia Fine Chemicals, Stockholm, Sweden) were mixed with purified His-tagged OspC or BmpA proteins dissolved in coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3). Sepharose beads were washed with 5 column volume of coupling buffer. Non-specific active sites on the beads were blocked with 1M Tris-HCl pH 8.0. Total antibodies purified above were diluted in an equal volume of PBS (pH 7.4) and incubated with beads for 3 hrs at room temperature. Unbound antibodies were removed by washing of the beads at least with 10 column volume of PBS. Bound Anti-OspC or anti-BmpA antibodies were eluted from the beads with 3.6 M MgCl₂. The pH of eluate was immediately adjusted to pH 7.5. A small aliquot of eluate was subjected to SDS-PAGE.

Evaluation of specificity of anti-OspC and anti-BmpA antibody for *Borrelia* strains

Whole cell proteins of *B. bavariensis*, *B. burgdorferi* sensu stricto (SKT-2) and *B. afzelii* (SKT-4) (10µg/well) were electrophoretically separated and transferred onto nitrocellulose membrane. Non-specific binding sites were blocked with TTBS (TBS [25 mM Tris, 150 mM NaCl, pH 7.2] with 0.05% Tween 20) containing 2% BSA overnight at 4°C. After washing with TTBS, membranes were incubated with purified anti-OspC antibody for 2 hrs. After five washings with TTBS, membrane was incubated with anti-human HRP secondary antibody (1:20000 in TTBS with 1% BSA). Interaction was visualized with ECL substrate (Pierce, Rockford, USA) and signal was captured on C-DiGit Blot scanner (LI-COR, Lincoln, USA).

Library construction and packaging

ospC gene of the *B. burgdorferi* sensu stricto (strain SKT-2, accession number AY597021.1) was PCR amplified with F 5'-CGCGGATCCATGACTTTATTTTTATTTATATCTTGTAATAAT and R 5'-TATGTCGACAGGTTTTTTTGGACTTTCTGC primers from genomic DNA. Ten micrograms of purified amplicons were treated with DNaseI (0.01U) (Fermentas, Finland) for 2 min at 37°C in reaction buffer with 10 mM MnCl₂, to obtain DNA fragments of the length <75bp. Reaction was stopped by heating at 65°C for 20 min. Fragments were gel purified, phosphorylated and ligated into the dephosphorylated phagemid vector pSEX81 (New England Biolabs, Frankfurt, Germany). The vector was digested with EcoRV and PvuII restriction enzymes (Fermentas). Phosphorylation of the fragments, dephosphorylation of digested vector and ligation with T4 ligase were performed exactly according to manufacturer's instructions (Fermentas). *E. coli* XL-1 Blue cells were transformed with ligated vector and transformants were selected on

2xTY agar containing 0.1% glucose, 100 µg/ml ampicillin and 10 µg/ml tetracycline. Integration of *ospC* fragments into the vector and their fusion with the gene encoding gp3 was examined in randomly picked colonies by PCR and direct sequencing using vector-specific primers (pSEX81 F 5'-ATGAAATACCTATTGCCTACGGCAG; pSEX81 R 5'-CTACAACGCCTGTAGCATTCCAC). After overnight incubation at 37°C bacterial colonies were counted and scrapped into the LB medium containing 50% glycerol. An aliquot containing 10^{12} cells representing each member of the library was used to inoculate 100 ml 2xTY medium containing 0.1% glucose, 100 µg/ml ampicillin and 10 µg/ml tetracycline. Mid-log phase culture (OD_{600} 0.6) was superinfected with Hyperphage M13K07ΔpIII (PROGEN Biotechnik, Heidelberg, Germany) at a multiplicity of infection 20 and incubated at 37°C for one hour without shaking. Bacteria were pelleted by centrifugation and resuspended in fresh 2xTY medium containing 100 µg/ml ampicillin and 10 µg/ml tetracyclin. The volume of the superinfected culture was 50% larger than that of the pre-infected culture. Infected bacteria were grown overnight at 30°C with shaking and cells were pelleted. Phage particles from the supernatant were concentrated by two rounds of PEG-precipitation. White pellet of the phage particles displaying OspC fragments was resuspended in buffer containing 10 mM TrisHCl, 20 mM NaCl and 2 mM EDTA (pH 7.5). Number of phage clones was calculated by colony forming units (CFU) on XL-1 Blue cells in 2xTY plates containing 50 µg/ml ampicillin and 10 µg/ml tetracyclin (2xTY/Amp/Tet plates) as described before [17].

Selection (panning) of phages interacting with anti-OspC antibody

Anti-OspC antibodies were captured on Ezview Red Protein G Affinity beads (Sigma Aldrich, Bratislava, Slovakia) according to manufacturer's instructions. Beads were

washed two times with sterile PBS and 200 μl (1×10^{11}) of the phages carrying OspC fragments were resuspended in buffer containing 10 mM TrisHCl, 20 mM NaCl and 2 mM EDTA (pH 7.5) were added and incubated for 1 h with gentle shaking. Beads were washed with PBS for at least 10 times. Bound phages were eluted in 500 μl of trypsin (10 $\mu\text{g/ml}$) in PBS for 30 min at 37°C with gentle shaking. Number of eluted phages were calculated by CFU on XL-1 Blue cells in 2xTY plates containing 50 $\mu\text{g/ml}$ ampicillin and 10 $\mu\text{g/ml}$ tetracyclin (2xTY/Amp/Tet plates) as described before [17]. Eluted phages were amplified in 20 ml of XL-1 Blue culture and superinfected with M13K07. Rescued phages were PEG/NaCl precipitated and panned against anti-OspC antibody as described above. After third round of biopanning, one tenth of the total number of *E. coli* colonies carrying phage clones were selected separately for small-scale phage rescue. The number of eluted phages from each *E. coli* clone was calculated by CFU.

Phage FLISA (fluorescent linked immunosorbent assay) with anti-OspC antibody

96-well microtiter plates (Corning Costar, Tewksbury, USA) were coated with anti-OspC antibody (10 $\mu\text{g/well}$) in coating buffer (15mM Na_2CO_3 , 35mM NaHCO_3) overnight at 4°C. Wells were blocked with Odyssey Blocking Buffer (LI-COR) for 2 hrs at room temperature followed by three washings with PBST. Approximately 10^{12} of phage particles released from each positive phage infected *E. coli* colony were added to each well and incubated for 1 hr at room temperature. After three washings, bound phage particles were detected with anti-M13 pIII monoclonal antibody (New England Biolabs) conjugated with CF770 and signals were captured at 800 nm using Odyssey infrared imaging system (LI-COR). M13K07 phage coated in the well served as a positive control. As negative control, anti-OspC antibody was replaced by anti-BmpA

IgG previously isolated from LD patient serum. Experiments were repeated three times and mean values \pm SD (Standard Deviation) of three independent repeats were calculated using Graph Pad prism 6 (GraphPad software La Jolla, USA). A one-way ANOVA was used to determine significance (RFU of clones C1 to C20 compared to FRU of negative control). Values were considered significant when $P < 0.01$.

Sequence analysis of immunoreactive phages

DNA from *E.coli* colonies carrying FLISA positive phage clones was isolated and OspC gene fragments inserts were amplified with vector specific primers (pSEX81 F and pSEX81 R). Amplicons were sequenced, translated *in silico* and amino acid sequence of each clone was aligned with OspC sequence used for library construction.

Production of OspC peptides and western blotting

XL-1 blue *E. coli* carrying FLISA positive phage clones representing each epitope (n = 6) were grown in 2xTY containing glucose, ampicillin and tetracycline at 37°C till OD₆₀₀ 0.5. Bacteria were pelleted for 10 min at 3,220 x g and resuspended in same volume of 2xTY supplemented with tetracycline, ampicillin and 50 μ M IPTG. Cultures were incubated at 30°C overnight at 250 rpm. Cells were pelleted, resuspended in periplasmic extraction buffer (100mM Tris-HCl, 10mM EDTA, pH 7.4) and incubated at 30°C overnight with constant agitation. The periplasmic extracts containing overexpressed OspC peptides fused with pIII protein from M13 filamentous phage were clarified by centrifugation at 15,000 x g for 45 min. Periplasmic extracts (10 μ g total protein) were separated with SDS-PAGE and transferred to Immobilon FL membrane. Recombinant OspC protein with GFP tag was also included in the experiment as a positive control. Membrane was blocked with Odyssey Blocking Solution (LI-COR) at 37°C for 1 h and incubated with pooled patient sera. After five washings with PBST

(PBS containing 0.1% Tween-20), membrane was incubated in IRDye 680RD detection reagent (Li-COR; diluted in 1:4,000) at 37°C for 1 h. Membrane was washed five times with PBST and once with PBS. Signals were captured at 700 nm using Odyssey infrared imaging system. As negative control, pool of sera from healthy donors was used.

Amino acid sequence alignment with orthologs from other *Borrelia* genospecies

ospC gene sequences of LD borreliae, *B. burgdorferi* sensu stricto (strain SKT-2 - GenBank accession number AY597021.1, T255 - X81524 and PKa-2 - X69589), *B. bavariensis* (strain PBi - CP000014.1) and *B. garinii* (strain PBr - CP001305.1) and *B. afzelii* (strain PKo - X62162 and ACA1 - L42892.1), were retrieved from GenBank repository and *in silico* translated. These sequences were aligned with epitopes using Genious software (Biomatters, San Francisco, USA).

RESULTS

Antigen affinity purification and characterization of anti-OspC antibodies

Thirteen serum samples from the patients in early stage LD and early dissemination LD were chosen for total antibody purification based upon the results of ELISA and western blot used to confirm anti-*Borrelia* antibodies (data not shown). Serum IgG were purified using the hydrophobic interaction chromatography. To ensure the purity of IgG, small aliquot was fractionated on SDS PAGE (Fig.1 SuppInfo Panel A, lane 1). Total IgG were further used to separate specific antibodies against produced recombinant OspC or BmpA (Fig.2 SuppInfo Panel A and B) by affinity chromatography. Specific antibodies were fractionated on polyacrylamide gel (Fig. 1 SuppInfo Panel A, lane 2 and lane 3) and tested to confirm their antigen binding ability with WB. Both anti-OspC and anti-BmpA antibodies showed specificity for the antigens from the tested *Borrelia* strains (Fig. 1 SuppInfo Panel B and Panel C).

Library construction and panning

In order to determine the amino acid sequences recognized by the anti-OspC antibody (OspC epitope mapping), a library of random fragments of the *ospC* gene was constructed using a phagemid vector pSEX81. The size of the *ospC* fragment display library was 6×10^9 and the length of fragments was maximum 75 bp (Fig. 3 SuppInfo). Thus, a chain of maximum 25 aa of OspC was fused to the major coat protein pIII and displayed on M13KO7 filamentous phage through two-gene phagemid system. A library was incubated with affinity purified anti-OspC antibody to select immunoreactive phage clones. Phage clones escaped from individual *E. coli* clone were used in FLISA to confirm their affinity to anti-OspC antibody. Out of twenty randomly picked clones, sixteen phages showed strong affinity to anti-OspC antibody ($P < 0.01$, Fig. 1). Whereas, binding ability of C1, C4, C19 and C20 was weak (statistically insignificant when compared to negative control $P > 0.01$, Fig. 1) and were not included in further analysis.

Epitope identification and characterization

The *ospC* gene fragments from XL-1 blue clones, except C1, C4, C19 and C20, were amplified with vector specific primers (Fig. 4 SuppInfo), sequenced, translated *in silico* and aligned with the amino acid sequence of OspC to map the epitopes. We found six immunoreactive regions on the OspC (Fig. 2 and Table 1). Three OspC fragments were aligned with a common stretch of 9 residues (CNNSGKDGN; OspC E1), two were aligned with a common stretch of 5 residues (VETLL; OspC E3). At the C-terminus of OspC, three fragments were overlapped with AKKAILKTH (OspC E4), four peptides had a common sequence of 7 residues LKAAKEM (OspC E5) and three peptides

overlapped with PVVAESP region (OspC E6). A single peptide was aligned with 16 amino acid stretch KPNLVEISKKITDSN (OspC E2) (Fig. 2 and Table 1).

Distinguishing between continuous and discontinuous epitopes is an important step in epitope mapping. It is accepted that discontinuous epitopes is unrecognized by antibodies in western blot due protein denaturation [17]. To determine whether the epitopes mapped by us are linear or conformational, epitopes were overexpressed and used in the western blot against pooled patient serum. The results confirmed that OspC peptides C10, C14, C2, C12 and C7 are the immunoreactive sites on OspC and may represent linear epitopes (Fig. 3). Absence of reactivity of C9 in western blot may suggest discontinuous character of this potential epitope.

To be effective within the constraints of an immunization, an epitope must be highly conserved. Variation in immunodominant epitopes/antigens used in immunization often fail to generate cross-reactive antibodies. Multiple sequence alignment (MSA) of OspC from 7 European *Borrelia* genospecies is presented in Figure 4. MSA showed conservation of CNNSG motif within OspC E1, while four residues KDGN (aa 24-27) were hypervariable. OspC E2, the longest epitope found in our study, was relatively conserved (consensus linear pattern KGPNL and ISKKITDSN). In contrast, epitope OspC E3 and E5 were the most variable epitopes. Sequence variability of OspC E3 was observed even within the same genospecies. OspC E6, the C-terminal epitope, was the most conserved among all immunoreactive regions found in the study. The consensus linear pattern PVVAESP was found in all analyzed OspCs, except in PBr (Fig. 4).

DISCUSSION

A plethora of outer surface proteins of *B. burgdorferi* have been shown to induce a protective immune response in animal models [18]. Previously, it was reported that *Borrelia* rapidly upregulates expression of OspC within the first hours of tick attachment on the host [3]. OspC is required for survival of *Borrelia* during the early stages of borreliacidal infection. It is hypothesized that OspC binds a mammalian-derived ligands presented in the blood, extracellular fluid or tissues to evade host immune response [19, 7, 20]. Borreliacidal anti-OspC antibodies are formed shortly after infection. High concentration of borreliacidal antibodies specific for a region near N-terminus (the first helix) have been demonstrated in sera from patients with early LD [21]. In another report, it has been shown that borreliacidal OspC antibodies are predominantly IgM type with specificity for epitope(s) within the 50 amino acids residues located at C-terminus [22].

Hitherto, full length or truncated proteins have been used as antigens in serodiagnosis of LD, however, non-specificity remains a major issue [23, 24]. Epitope-based diagnostics, whereby cross-reactive epitopes can be excluded, are an attractive alternative to currently available immunoassays. Considering the limitations of serodiagnostic tests based on whole OspC or its truncated forms, a phage display was employed to define the minimal antibody binding regions (minimal epitopes) on OspC that have potential to improve capabilities of serodiagnostic assays. Using *ospC* fragment library and purified anti-OspC antibodies, we mapped potential immunoreactive minimal epitopes: ¹⁹CNNSGKDG^{N27} OspC E1; ³⁸KGPNLVEISKKITDS^{N53} OspC E2; ⁶²VETLL⁶⁶ OspC E3, ¹⁵⁶AKKAILK¹⁶³ OspC epi 4; ¹⁸⁴LLKAAKEM¹⁹¹ OspC E5 and ²⁰²PVVAES²⁰⁷ OspC E6. Of the six identified epitopes, OspC E1 located at the N-terminus and OspC E4 and OspC E6 both located at

C-terminus, were highly conserved among the different OspC orthologs (Fig. 4) and capable of distinguishing between LD patient and healthy control sera (Fig. 3).

Till to date, few works were focused to identify epitopes on OspC using truncated proteins [25-28]. These studies have attempted to identify both conformational and linear epitopes. Two epitopes located at N- terminus (aa 20-35 and aa 35-55) of OspC recognized by monoclonal antibodies have been determined [28]. Furthermore, N-terminal peptide ¹¹MTLFLFISCNNSGKDGNTSA³⁰ was suggested as an epitope in serodiagnostic assay for diagnosis of early LD [25]. In our study we found that only nine amino acid stretch ¹⁹CNNSGKDG²⁷ (OspC E1) are sufficient for binding of anti-OspC antibody. Truncation of amino acid stretch (11 to 30 aa) might improve the specificity of epitope based serodiagnostic assay. MSA revealed high sequence similarity within OspA orthologs (Fig. 4). It is noteworthy that, cysteine at 19th aa position in OspC E1 (¹⁹CNNSGKDG²⁷) is conserved and has been shown to be essential for dimerization via formation of an intermolecular disulfide bridge resulting in higher OspC dimer-IgM affinity compared to monomer [29]. Epitope OspC E2 (³⁸KGPNLVEISKKITDSN⁵³) mapped in our study corresponds with the antibody binding domain (aa35-55) identified previously with OspC-specific monoclonal antibodies [28]. This epitope appears to be conformational because its immunoreactivity was lost when linearized on SDS-PAGE and then incubated with anti-OspC antibody in western blotting (Fig. 3). Epitope OspC E3 (⁶²VETLL⁶⁶) was found to be located in ligand binding domain LBD1, which binds a mammalian-derived ligands [19, 7, 20].

The C-terminal decapeptide PVVAESPKKP (commercially available as PepC10) is used to detect anti-OspC IgM in patient's sera in serodiagnostic tests [26, 30]. In the study presented by Arnaboldi and colleagues, the peptide PepC10 detected antibody in only 48 of 98 serum samples (49.0%) and generated false-positive results

among negative healthy and diseased controls [25]. This region adopts highly conserved polyproline II-like helix motif (PKKP), which is common to various surface-exposed structures involved in protein-protein interactions [26]. OspC E6 epitope found in our study lacks PXXP motif, which suggests that polyproline II-like helical structure is not crucial for antibody recognition and that only six residues ²⁰²PVVAES²⁰⁷ (OspC E6 epitope) are sufficient for detection of anti-OspC antibodies.

Despite the strong conservation of OspC N- and C-terminal domains, the antibody responses to the OspC types were found to be type specific, which suggests that the immunodominant linear OspC epitopes reside in the variable domains (i.e., type-specific domains) of the protein [27]. The region spanning residues 136 to 150 (termed as loop 5 epitope) and 168 to 210 (termed alpha 5 epitope) were identified as anti-OspC antibody binding sites responsible for high type specificity of antibody response to OspC. The loop 5 is surface exposed in both the mono- and dimeric models of OspC [7]. OspC E4 ¹⁵⁶AKKAILK¹⁶³ and OspC E5 ¹⁸⁴LLKAAKEM¹⁹¹ epitopes mapped in our study reside in these regions. The sequences of the loop 5 and alpha 5 domain are highly variable at the intertype level, with the exception of the last 20 residues of alpha 5 [27]. The OspC E5 represents the variable part of alpha 5 epitope. *In silico* analysis of the linear sequence and 3-dimensional structure of OspC pointed the fifth C-terminal helix (residue 179 to 188) as a major determinant of type-specific antibody binding site [31]. The five residues of OspC E5 (residue 184-191) may constitute a minimal region determining antibody specificity.

In this study we mapped minimal potential epitopes, reacting with antibodies from naturally infected LD patients. Use of these minimal B-cell epitopes could overcome the issues associated with cross-reactivity and non-specificity frequently observed in the serodiagnosis of LD and may help to develop epitope-based vaccines.

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DISCLOSURE

The authors have no conflict of interest to declare.

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FIGURE LEGENDS

Fig. 1. Phage FLISA with anti-OspC antibody. Reactivity of individual phage clones to anti-OspC and anti-BmpA IgGs is presented. IgGs were immobilized on microtitre well (solid columns) and incubated with each phage clone. Phages bound to IgGs were detected with anti-M13 pIII monoclonal antibody conjugated with CF770. M13K07 phage coated in the well served as a positive control (+ control). Negative control – anti-OspC antibody was replaced by anti-BmpA antibody (empty columns). C1 to C20 indicate phage clones. # no statistically significant difference, $p > 0.01$, was observed when relative fluorescence unit (RFU, calculated by Odyssey infrared imaging system) for C1, C4, C19 and C20 was compared with RFU of negative control. Other clones showed significant affinity to anti-OspC antibody ($P < 0.01$).

Fig. 2. Alignment of OspC epitopes. Epitope regions (OspC E1 to E6) within the amino acid sequence of OspC are depicted here. C with number presents each phage clone.

Fig. 3. Immunoreactivity of OspC peptides. Overexpressed OspC peptides fused with pIII protein from M13 filamentous phage (65 kDa) were separated on SDS-PAGE, transferred on membrane and incubated with LD patient sera (panel A) or with pool of sera from healthy individuals (negative control, panel B). GFP tagged OspC served as positive control.

Fig. 4. Multiple sequence alignment of OspC protein to highlight sequence variability within the OspC E1 to E6 regions. *B. burgdorferi sensu stricto* – strain SKT-2 (Slovakia), strain PKa-2 (Germany) and strain T255 (Germany), *B. bavariensis* – strain PBi (Germany), *B. garinii*, - strain PBr (Germany), *B. afzelii* – strain ACA1 (Sweden), strain PKo (Germany).

SUPPORTING INFORMATION

LEGENDS

Fig. 1 SuppInfo. Isolation of total IgG from LD patients and affinity purification of anti-OspC and anti-BmpA antibodies. Panel A – Total IgG isolated from LD patient sera with MEP Hypercel affinity column (lane 1) and then affinity purified with CNBr sepharose beads coupled with recombinant OspC (lane 2) or BmpA (lane 3). Antibodies were fractionated on native-SDS-PAGE. Arrows depict affinity purified antibodies. Panels B and C– Specificity of affinity purified antibodies. Whole cell proteins of *B. bavariensis* PBi (lane 1), *B. burgdorferi* sensu stricto SKT-2 (lane 2) and *B. afzelii* SKT-4 (lane 3) separated with SDS-PAGE, transferred on membrane and incubated with affinity purified antibodies. Bound antibodies were detected with anti-human HRP conjugated antibody.

Fig. 2 SuppInfo. Production of recombinant OspC and BmpA. Protein purity and absence of protein degradation of the recombinant OspC ~ 50 kDa (21.72 kDa of OspC fused to His and GFP tag of approx. 29 kDa) in panel A, and BmpA ~ 67 kDa (36.97 kDa of BmpA fused to His and GFP tag of approx. 29 kDa) in panel B were confirmed by SDS-PAGE (lanes 1) and western blot (lanes 2).

Fig. 3 SuppInfo. Agarose gel electrophoresis of *ospC* gene fragments. *ospC* gene of *B. burgdorferi* sensu stricto (strain SKT-2) was amplified (lane 1) and fragmented using Dnase I (lane 2). Fragments <75 bp were gel purified (dashed rectangle).

Fig. 4 SuppInfo. Agarose gel electrophoresis of *ospC* fragments amplified with vector specific primers.

Table 1. Summary of minimal epitopes identified in the study

Epitope	Minimal epitope (amino acid sequence)	Position in sequence*	Total length (amino acids)
OspC epi 1	CNNSGKDG	19-27	9
OspC epi 2	KGPNLVEISKKITDSN	38-53	16
OspC epi 3	VETLL	62-66	5
OspC epi 4	AKKAILKTH	155-163	9
OspC epi 5	LKAAKEM	184-190	7
OspC epi 6	PVVAESP	201-207	7

*Numbering is based on the amino acid sequence of *OspC* from Uniprot WP_010890595.1.

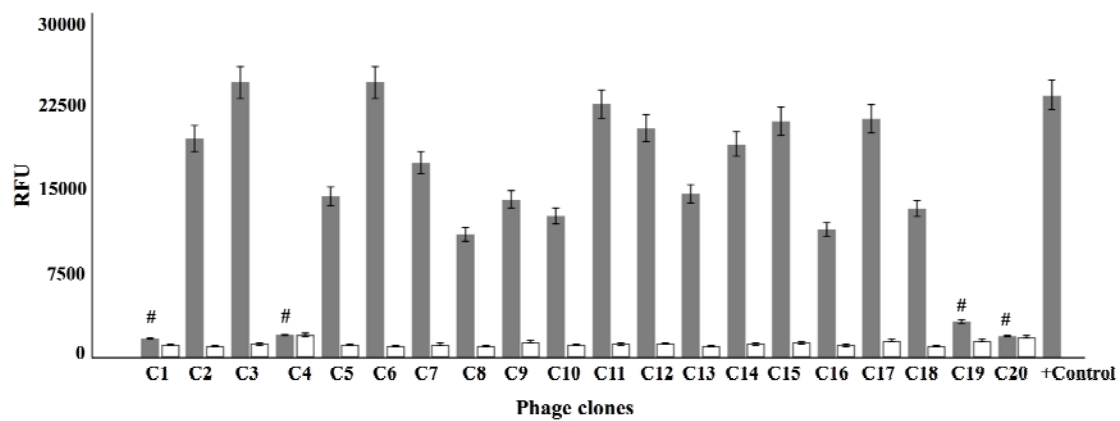


Figure 1

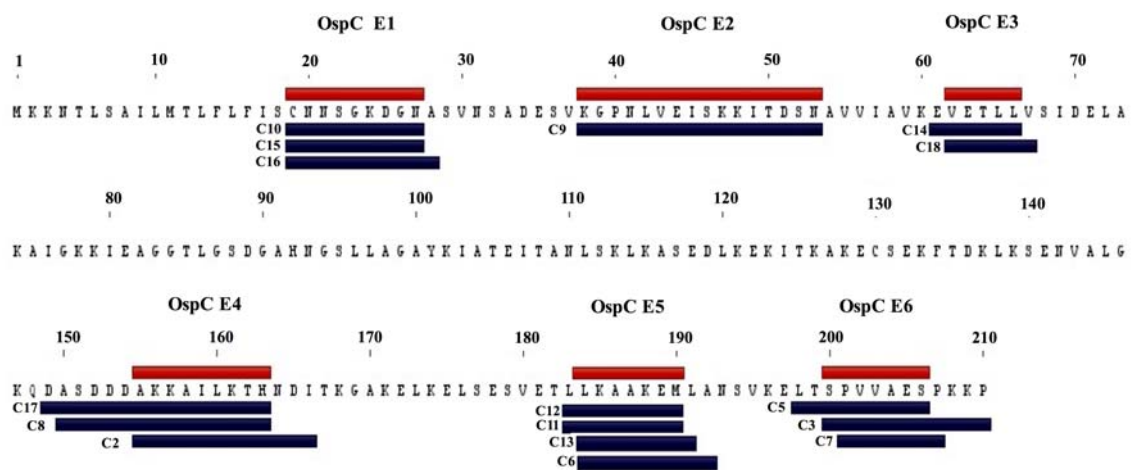


Figure 2

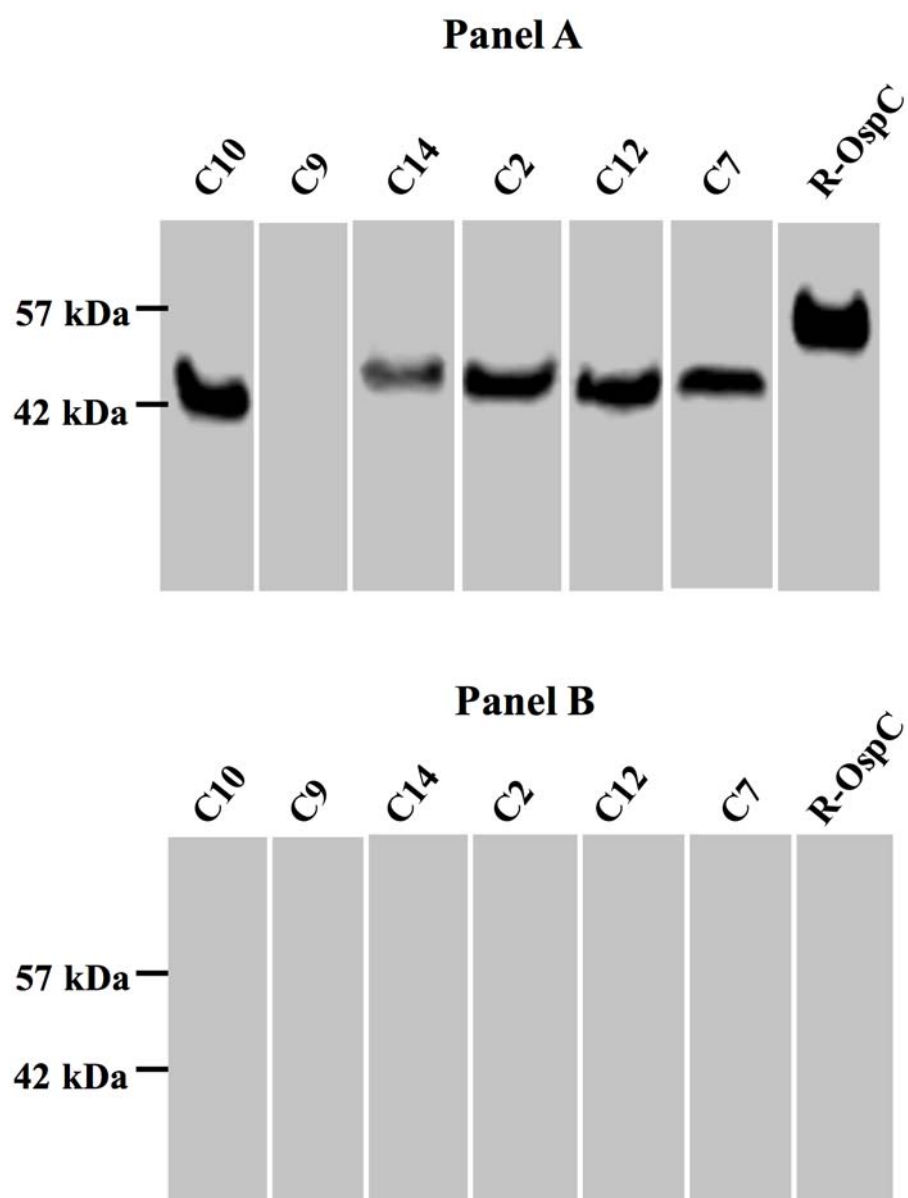


Figure 3

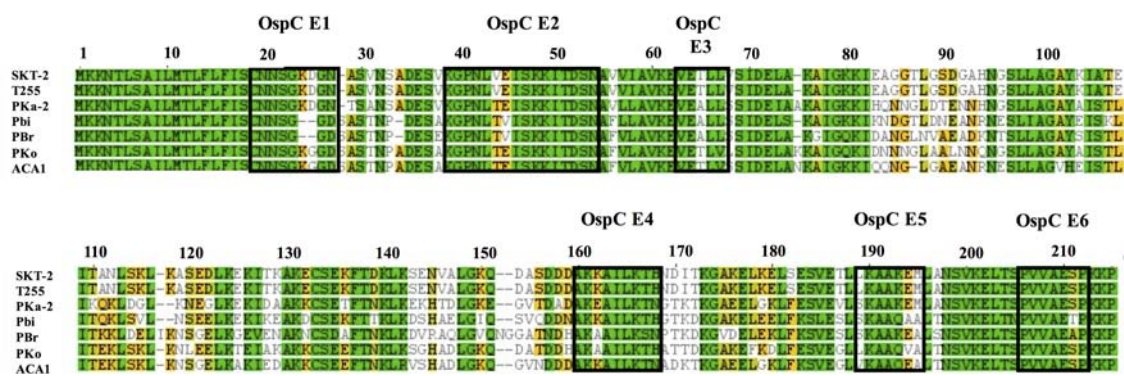


Figure 4