Design, construction and evaluation of multi-epitope antigens for diagnosis of Lyme disease

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Abstract

Introduction and objective. Lyme disease (LD) is the most common vector-borne disease in the temperate zone of the Northern Hemisphere. Diagnosis of LD is mainly based on clinical symptoms supported with serology (detection of anti-*Borrelia* antibodies) and is often misdiagnosed in areas of endemicity.
Materials and method. In this study, the chimeric proteins (A/C-2, A/C-4 and A/C-7.1) consisting of B-cell epitopes of outer surface proteins OspA and OspC from *Borrelia* genospecies prevalent in Eastern Slovakia, were designed, over-expressed in *E. coli,* and used to detect specific anti-*Borrelia* antibodies in serologically characterized sera from patients with Lyme-like symptoms to evaluate their diagnostic potential.
Results. Results showed that chimeras vary in their immuno-reactivity when tested with human sera. Compared with the results obtained from a two-tier test, the application of recombinant multi-epitope chimeric proteins as diagnosis antigens, produced fair agreement in the case of A/C-2 ($0.20<\kappa<0.40$) and good agreement ($0.60<\kappa<0.80$) when A/C-7.1 was used as capture antigen. Chimera A/C-4 were excluded from further study due to loss of reactivity with OspA-specific antibodies.
Conclusions. The combination of specific B-cell epitopes from OspA and OspC proteins may improve the diagnostic accuracy of serologic assays, but further studies are required to address this hypothesis.

Key words

Lyme disease, epitope, chimera, ELISA, Slovakia

INTRODUCTION

Lyme disease (LD) is a multisystem and multistage infection caused by three major *Borrelia* genospecies. Diagnosis of LD is based on clinical symptoms and the two-tier detection of anti-*Borrelia* antibodies. First, an ELISA is performed, followed by a Western blot to confirm the results from the first serology, as well as to identify the infecting *Borrelia* species [1]. Unfortunately, the assays available commercially and assays developed in many laboratories worldwide have wide divergent sensitivity and specificity. Whole-cell antigens, purified antigens (flagellar components), whole-cell antigens combined with recombinant antigens and exclusively recombinant antigens (e.g. C6 peptide, P39) are being used in serodiagnosis of LD [2]. The vast majority of borrelial proteins are cross-reactive [3]. The complexity of the antigenic composition among the *Borrelia* genospecies and differential expression of proteins in host and vector (temporal and spatial antigenic variability) has posed challenges for the serodiagnosis of LD. Therefore, the optimal combination of the most specific epitopes could increase the discrimination abilities of serodiagnostic tests.

Among numerous antigens of *Borrelia,* the outer surface proteins OspA to OspG and the flagellin are important in diagnostics [4]. OspA protein has been detected in the serum from patients diagnosed with early LD [5]. Elevated levels of anti-OspA antibodies have also been shown in the sera of patients with severe arthritis and neurological symptoms (late LD) [6]. An important protective B-cell epitope of OspA, LA-2 epitope, was identified earlier by murine monoclonal antibody [7]. Besides OspA, outer surface protein OspC is a promising candidate for the construction of an LD diagnostic marker due to its high immunologic potential and its early expression during LD infection [8]. Six OspC B-cell epitopes were identified recently using phage display [9].

Although the genospecies of *B. burgdorferi* sensu lato complex express a generally similar repertoire of antigens, significant sequence variation both between and within species do occur [10]. This variation complicates the development of single immunodiagnostic assay for all major *Borrelia* genospecies [4]. The development of assays based on antigens specific for the genospecies observed in a defined geographic area represents a promising approach for improvement in the accuracy and reliability of immuno-assays [11]. In the presented study, chimeric proteins were produced, consisting of OspA and OspC B-cell epitopes for Eastern Slovakia specific genospecies and their diagnostic potential was evaluated and compared to the commercial two-tier test. Application of A/C-2 produced fair agreement ($0.20<\kappa<0.40$) and good agreement ($0.60<\kappa<0.80$) when A/C-7.1 was used as the capture antigen.

MATERIALS AND METHOD

Design and cloning of recombinant chimeras. The OspC epitopes of *B. burgdorferi* SKT-2 (AY597037.1) were mapped with the help of phage display in our previous study [9]. Epitopes in other genospecies (*B. bavariensis* SKT-7.1 GU906888.1 and *B. afzelii* strain SKT-4 AY518771.1; both

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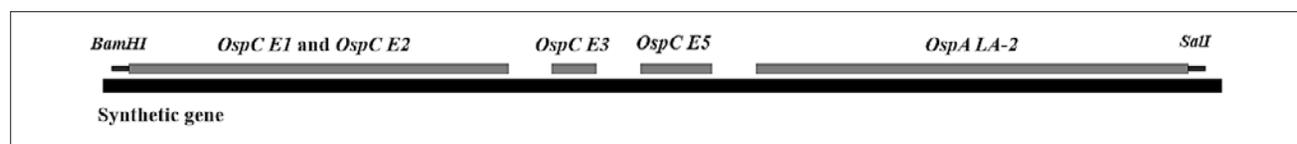
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Figure 1. Strategy used to design synthetic genes

strains isolated in Slovakia) were defined with multiple alignment of OspC sequences using SKT-2 as reference. The LA-2 epitope of OspA was identified by murine monoclonal antibody LA-2 [7]. This epitope was defined in OspA of borrelial strains specific for Slovakia (SKT-2 strain AY597021.1, *B. bavariensis* strain SKT-7.1 AY597026.1 and *B. afzelii* strain SKT-4 AY597023.1) with multiple alignment, using OspA from B31 as a reference sequence.

Synthetic genes (*a/c-2* – 381 bp, *a/c-7.1* – 414 bp and *a/c-4* – 384 bp) were constructed consisting of *BamH*I restriction site on 5′ end, followed by four OspC epitopes (OspC E1, OspC E2, OspC E3 and OspC E5) and OspA epitope LA-2. Between each epitope the flexible (GSGSG) linker was inserted. The epitopes OspC E1 and OspC E2 were present in tandem. At the 3' end of the synthetic gene the stop codon was followed by *Sal*I restriction site. Synthetic gene sequence was assembled *in silico* with the help of Geneious pro software (Biomatters, New Zealand) and custom synthesized (Life Technologies). The strategy used for designing of a chimeric gene is shown in Figure 1. After digestion with restriction enzymes, chimeric genes were gel purified and ligated into vector pQE-mCHERRY-GFP (in house modified vector). Recombinant vectors were transformed in *E. coli* (XL-1 blue) and transformants were selected by plating on kanamycin (25 µg/ml) and carbenicillin (50 µg/ml) containing LB plates (1% tryptone, 0.5% yeast extract, 1% NaCl and 1% agar). DNA from carbenicillin-resistant colonies was isolated, amplified with vector specific primers (sense CGCATCACCATCACCATCACG and antisense ACCAAAATTGGGACAACACCAGTG), and amplicons were characterized by sequencing.

Protein production and purification. A single colony for each chimera was grown in 50 ml of TB medium (1.5% peptone, 3% yeast extract, 1.25 % NaCl, 0.25 % $MgCl_2.6H_2O$ and 0.735% glycerol) supplemented with metal mix (0.17M KH_2PO_4 and 0.72M K_2HPO), carbenicillin (50 µg/ml), kanamycin (25 µg/ml) and 1% glucose at 37 °C with constant shaking 300 rpm to the OD_{600} 6.0. Cells were harvested by centrifugation (6,000 rpm, 10 min, 20 °C) and resuspended in 50 ml of TB medium without glucose. Recombinant protein expression was induced by adding IPTG (isopropyl-β-d-thiogalactopyranoside) to a final concentration of 1.0mM and cells were grown for an additional 12 hrs at 18 °C with constant shaking. Cells were harvested by centrifugation and resuspended in denaturative lysis buffer (50mM $NaH_2PO_4.2H_2O$, 300mM NaCl, 8M urea, pH 7.5) supplemented with DNase I (Fermentas) and ProteoBlock protease inhibitor cocktail (Fermentas). Repeated freeze-thaw cycles (5 cycles) and sonication on ice (5 pulses of 10 seconds, amplitude of 50 at frequency of 20 kHZ) were used to lyse cells. Supernatant containing chimeric proteins was cleared by repeated centrifugation at 14,000 rpm for 20 min. The chimeric proteins were purified under denaturative conditions using Ni-NTA agarose beads (Qiagen, USA) and eluted with 300mM immidazole in elution buffer (50mM $NaH_2PO_4.2H_2O$, 300mM NaCl, 8M urea, pH 7.5). The eluate

was dialyzed against PBS (137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4.12H_2O$, 1.47mM KH_2PO_4, pH 7.4) using 3.5 kDa MWCO dialysis membrane (Spectrumlabs). The protein concentration was determined by the Bradford method. The integrity and purity of chimeric proteins were confirmed by SDS-PAGE. The exact molecular mass of proteins was identified on MALDI–TOF MS as described by Mlynarcik et al. [12].

Affinity purification of anti-OspA and anti-OspC antibodies. The antigen-specific antibodies were affinity purified, as described previously [9]. Briefly, pre-swollen CNBr-Sepharose 4B beads (Pharmacia Fine Chemicals) were mixed with purified his-tagged OspA or OspC proteins of SKT-2, SKT-4 and SKT-7.1 (previously produced in the author's laboratory [9, 13]). Total IgGs (previously purified in the author's laboratory [9]) were incubated with beads for 3 hrs at room temperature, and antigen-specific antibodies eluted from the beads with 3.6M $MgCl_2$. A small aliquot of eluate was subjected to SDS-PAGE to confirm the purity of isolated antibodies.

Immunospot with antigen-specific antibodies. Chimeric proteins (1 µg total protein) were spotted on the Immobilone FL membrane. The membrane was blocked with Odyssey Blocking Solution (LI-COR) at 37 °C for 1 h, and incubated with purified antigen-specific antibodies (anti-OspA or anti-OspC antibody). After five washings with PBS-T (PBS containing 0.1% Tween 20), the membrane was incubated in IRDye 680RD detection reagent (LI-COR; 1:4,000 diluted in Odyssey Blocking Solution) at 37 °C for 1 h. After washing with PBS-T and PBS, signals were captured at 700 nm using an Odyssey CLx near-infrared fluorescence imaging system (LI-COR). As negative control, a pool of sera from healthy donors was used. For input control, chimeric antigens were incubated with monoclonal Anti-6X His IgG CF™770 antibody (Sigma Aldrich) diluted 1:5,000 in Odyssey Blocking Solution for 1 hr. Recombinant OspA and OspC observed on the membrane was used as a positive control.

Collection of human serum samples and screening with commercial two-tier tests. The total of 169 serum samples from patients with Lyme-like symptoms (skin rash, meningitis, arthritis) were collected from the P. J. Safarik University Hospital in Kosice. The collected sera were pre-screened for the presence/absence of *Borrelia*-specific antibodies by BORRELIA IgM ELISA and BORRELIA IgG ELISA (Biomedica Immunoassays) before screening with chimera-based ELISA

Lyme disease BORRELIA IgM ELISA and BORRELIA IgG ELISA (Biomedica Immunoassays) were used for the detection of anti-*Borrelia* antibodies. The tests are polyvalent and were performed according to the manufacturer's instructions. In the case of IgM ELISA, OspC of *B. afzelii* PKo and *B. garinii* 20047 (p21), inner part of flagelin of *B. bavariensis* PBi (p41/I) and fusion proteins of VlsE from different *Borrelia*

genospecies were used as antigens. In the case of IgG ELISA, OspC of *B. burgdorferi* sensu stricto B31 and *B. garinii* 20047, p18 and p100 of *B. afzelii* PKo and fusion proteins of VlsE from different *Borrelia* genospecies were used as antigens. Values between two and three standard deviations were considered equivocal and counted as positive. Immunoblot *Borrelia* ViraStripe® (Viramed Biotech, Germany) was used for the detection of anti-*Borrelia* IgMs and IgGs in ELISA-positive sera. The test strips carried purified antigens (p83, p58, p43, p30, p21, p14, DbpA, Osp17 and OspC for igG and p41, p39, OspC and Osp17 for IgM detection) from *B. afzelii* (Pko), *B. burgdorferi* sensu stricto and recombinant VlsE. Immunoblots were performed and interpreted according to the manufacturer's instructions.

ELISA with chimeric proteins. Solutions of purified chimeric proteins in 140mM sodium carbonate, pH 9.0, were used to coat the wells of flat-bottomed plates (Nunc). Aliquote 50 µl of a solution with concentration 1.0 µg/ml was added to each well, and the microwell plate incubated at 4°C overnight. The antigen solution was removed from the wells, the plate was washed three times with 140 mM sodium carbonate (pH 9.0) and 200 µl of blocking solution (2% bovine serum albumin fraction V in 140mM sodium carbonate, pH 9.0) was added. After 1 hr of incubation at 37°C, the plates were washed three times with PBS-T (0.05% Tween 20, pH 7.5). Serum samples were diluted 1:100 in 2% fetal bovine serum (Gibco) in PBS, and 50 µl of each sample was added to the ELISA plate microwells. After 1 hr of incubation at 37°C, the wells were washed with PBS-T and Protein A/G conjugated with HRP (Pierce; diluted 1:10,000 in PBS, pH 7.4) was added. Following incubation for 1 h and washing, 100 µl of HRP680 assay buffer (LI-COR) was added to each well. After incubation, stop solution (LI-COR) was added to each well. The absorbance was read at 700 nm on an Odyssey CLx near-infrared fluorescence imaging system (LI-COR). As negative controls, five serum samples from healthy individuals from non-endemic area were used. The same negative controls were included in each plate.

Data analysis. The cutoff values of ELISA were calculated as the mean absorbance of the true negative sera plus three times standard deviations. Sensitivity and specificity were calculated according the following formulas:

$$\% \text{ Sensitivity} = [\text{True positive}/(\text{True positive} + \text{False negative})] \times 100;$$
$$\% \text{ Specificity} = [\text{True negatives}/(\text{False positives} + \text{True negatives})] \times 100.$$

The % positive predictive value (PPV) is defined as PPV = [True positive/(True positive + False positive)] × 100; The % negative predictive value (NPV) is defined as NPV = [True negative/(True negative + False negative)] × 100.

For measurement of inter-assay agreement between conventional two-tier test and chimera based ELISA, Cohen´s kappa was used.

RESULTS

Chimeric proteins. Borrelial outer surface proteins OspA and OspC were the focus of the presented strudy Borrelial outer surface proteins OspA and OspC were the focus of the presented study due to their diagnostic potential. On the basis of the selected epitopes, we synthesized the chimeric genes (*a/c-2*, *a/c-4* and *a/c-7.1*) selected epitopes (OspC E1, OspC E2, OspC E3 and OspC E5 from OspC and LA-2 epitope from OspA) we designed, synthesized the chimeric genes (*a/c-2*, *a/c-4* and *a/c-7.1*). Genes were ligated into expression vector and electrotransferred into *E. coli*. The transformants were selected in the presence of carbenicillin. The chimeric genes from selected *E. coli* clones were amplified with vector specific primers and analyzed by sequencing.

The protein yield of the recombinant chimeras was approximately 60 mg for both A/C-2 and A/C-4 and 200 mg
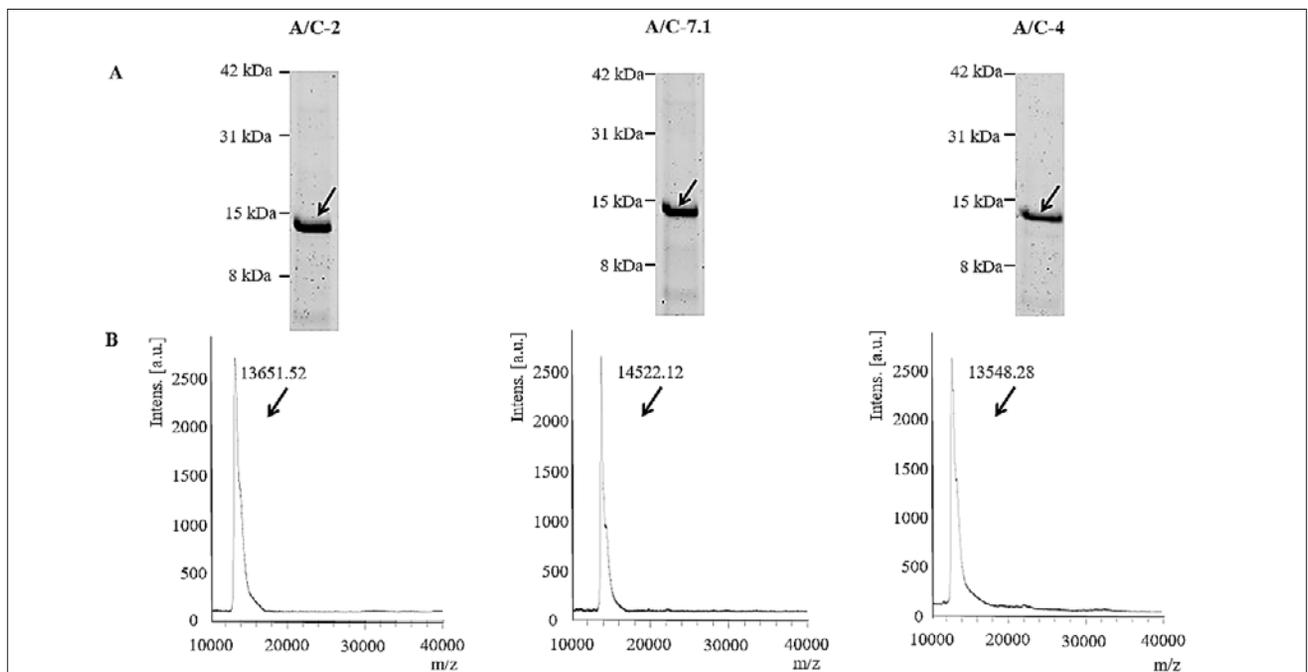

Figure 2. Production of recombinant chimeric proteins. Presence of the recombinant A/C-2 ~13.6 kDa, A/C-7.1 ~14.4 kDa and A/C-4 ~13.5 kDa and molecular weight was confirmed by SDS-PAGE (Panel A) and mass spectrometry (Panel B)

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Table 1. Epitopes selected for design of chimeric proteins

Chimera	Protein	Epitope	Sequence
A/C-2	OspC	E1 + E2	CNNSG**KDANTSAITA**DES**V**KGPNLT**E**ISKKITDSN
		E3	VE**T**LL
		E5	L**L**KAA**KEM**
	OspA	LA-2	**SSA**ATKKT**AA**W**N**S**G**TSTLT**I**TVNSKKT**KD**LVFTK**EN**TITVQQYDS**N**GT**K**
A/C-4	OspC	E1 + E2	CNNSG**KGGDSASTNPA**DES**A**KGPNLT**E**ISKKITDSN
		E3	VE**T**LL
		E5	L**L**KAA**QVA**
	OspA	LA-2	**TTQ**ATKKT**GA**W**D**S**K**TSTLT**I**SVNSKKT**TQ**LVFTK**QD**TITVQKYDS**A**GT**N**
A/C-7.1	OspC	E1 + E2	CNNSG**GDSASTNP**DES**A**KGPNLT**V**ISKKITDSN
		E3	VE**A**LL
		E5	L**S**KAA**QAA**
			L**S**KAA**KEA**
	OspA	LA-2	**STQ**ATKKT**GK**W**D**S**N**TSTLT**I**SVNSKKT**KN**IVFTK**ED**TITVQKYDS**A**GT**N**

Variable amino acids shown in bold

for A/C-7.1 per liter of medium. Purity of chimeric proteins was verified by SDS-PAGE (Fig. 2, Panel A). The exact molecular weights of his-tagged chimeras were confirmed on the MALDI–TOF, which were in accordance with the masses predicted *in silico* (13.6 kDa for A/C-2, 14.4 kDa for A/C-7.1 and 13.5 kDa for A/C-4) (Fig. 2, Panel B).

Immunoreactivity of chimeric antigens with antigen-specific antibodies. To demonstrate that epitopes are accessible to paratopes of specific antibodies, the chimeric proteins were tested for reactivity with anti-OspA and anti-OspC antibodies. First, the antigen-specific antibodies were purified from total IgGs against recombinant OspC or OspA by affinity chromatography. The A/C-2 and A/C-7.1 chimera showed reactivity on immunoblot with both antigen-specific antibodies, and were subjected to further serological testing (Fig. 3, lines 1 and 3). The chimeric proteins did not show cross-reactivity when incubated with healthy donor sera (Fig. 3, line 2). The chimeric protein A/C-4 lacked immuno-reactivity with anti-OspA antibody and was not subjected to further studies.

Screening of human serum samples by commercial assays. Based on the results of commercial assays, 116 serum samples
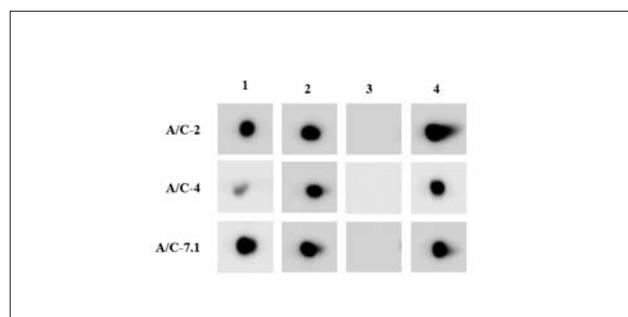

Figure 3. Immuno-reactivity of chimeric protein with anti-OspA and anti-OspC antibody. Overexpressed His-tagged chimeric proteins were separated on SDS-PAGE, transferred on membrane and incubated with anti-OspA antibody (line 1) and anti-OspC antibody (line 2) and IRDye 680RD detection reagent. Chimeric protein incubated with healthy donor sera served as negative control (line 3). Membranes incubated with monoclonal Anti-6X His IgG, CF™770 antibody served as an input control (line 4).

were classified into Panel 1 (LD serum samples; 37 sera positive for IgM and 79 sera positive for IgG) and 53 serum samples were classified into Panel 2 (non-LD serum samples; negative for IgM and IgG). Panel 2 represents potentially cross-reactive sera from patients with Lyme-like symptoms (skin rash, meningitis, arthritis) (Tab. 2).

Evaluation of chimera-based ELISAs using pre-screened human sera. Next, the purified chimeric proteins A/C-2 and A/C-7.1 were evaluated as a diagnostic reagent to detect anti-*Borrelia* antibodies using this well-characterized serum panels. The chimeric proteins A/C-2 and A/C-7.1 showed sensitivity of 80.17% and 91.37%, respectively, when tested with serologically characterized serum panels. The 23 serum samples from Panel 1 (116 serologically positive sera) were diagnosed as negative by the A/C-2 ELISA and ten serum

Table 2. Serum samples used in study and results of immunoassays

Clinical symptoms	Positive ELISA results/number of sera tested		
	Two-tier test	A/C-2	A/C-7.1
Skin rash	22/34	25/34	23/34
Meningitis	60/93	66/93	66/93
Arthritis	34/42	27/42	31/42
Total	**116/169**	**118/169**	**120/169**

Table 3. Comparison of commercial two-tier test with chimera-based ELISA

Chimera ELISA		Panel 1	Panel 2	Total	Sens.	Spec.	PPV	NPV	Cohen´s κ
A/C-2	Positive	93	25	118	80.17%	52.83%	78.81%	54.90%	0.3334
	Negative	23	28	51					
A/C-7.1	Positive	106	14	120	91.37%	73.58%	88.33%	26.41%	0.6632
	Negative	10	39	49					

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samples were negative by the A/C-7.1 ELISA. Conversely, when testing the chimera-based ELISA on sera from serologically negative patients with Lyme-like symptoms (Panel 2), both chimeras showed immune-reactivity. The 25 serum samples from Panel 2 showed immuno-reactivity in A/C-2 ELISA, and 14 serum samples were negative when A/C-7.1 was used as a capture antigen.

DISCUSSION

Because of a lack of pathognomonic symptoms, many cases of LD go unreported in areas of endemicity [4]. Accurate and reliable diagnostic assays are essential for planning the treatment as well as to exclude the large majority of uninfected patients with 'Lyme-like' symptoms. The development of universal assays for immunoserologic evaluation of LD is complicated by the presence of three *B. burgdorferi* sensu lato genospecies in Europe, and significant antigenic variation within each genospecies. Employment of B-cell epitopes from the strains specific for defined geographic areas has the potential to improve the performance of serological diagnosis [4]. Several studies have shown that synthetic peptides (B-cell epitopes) can be used for the serological diagnosis of viral and bacterial as well as parasitic diseases [14, 15]. To test this hypothesis, in the current study, chimeric proteins were generated containing B-cell epitopes of OspA and OspC of SKT-2 (*B. burgdorferi*), SKT-7.1 (*B. bavariensis*) and SKT-4 (*B. afzelii*) strains endemic in Eastern Slovakia, and their performance was tested in chimera-based assays (chimeras were used as capture antigens) with human serum samples.

The outer surface proteins OspA and OspC selected to construct multi-epitope chimeric proteins are highly immunogenic. The expression of OspA was shown to be downregulated during transmission to mammalian host and thus it is thought that the immune system is not exposed to significant OspA expression after infection [16]. However, weak but detectable antibody response induced by OspA was detected early after infection in mammal hosts [17, 18]. Disruption of borrelial plasma membrane triggered by the innate immune system can explain the detection of OspA in the serum from patients diagnosed with early LD, but remain at low levels or bound in immune complexes [5]. Schutzer et al. showed that IgM is the predominant isotype of OspA specific antibodies [18]. To our knowledge, this is the first time that OspA was used as antigen in diagnostic immunoassay.

The presented results show that chimeric proteins A/C-2 and A/C-7.1 vary in their immuno-reactivity when tested with sera from patients suspected of LD. Recombinant protein A/C-2 and A/C-7.1 detected anti-*Borrelia* antibodies in 118 respectively 120 sera from total number of collected sera. Recombinant chimeric protein A/C-2 was more sensitive in patients with skin rash, and produced less positivity when tested with arthritis sera, which may reduce repeated drug use for false-positive patients. Compared with the results obtained from commercial immunoassays, the application of recombinant multi-epitope chimeric proteins from strains specific for Slovakia as diagnosis antigens, produced fair agreement in the case of A/C-2 ($0.20 < \kappa < 0.40$), and good agreement ($0.60 < \kappa < 0.80$) when A/C-7.1 was used as the capture antigen. The 70 sera were tested positive in all assays.

The infectious and immunopathological conditions (viral infections, bacterial endocarditis, rheumatoid arthritis, and systemic lupus erythematosus) can compromise the accuracy of diagnostic assays [19]. Significant differences in the sensitivity and specificity of assays used to detect anti-*Borrelia* antibodies can be found when the results from different laboratories are compared [20, 21]. It was shown that results can be influenced by the choice of diagnostic assays (different antigens used for serological testing) and ELISA–immunoblot combination in the two-tier testing system [21]. Employment of single antibody binding regions (epitopes) of immunogenic proteins could remove cross-reactive epitopes, and has the potential to achieve higher specificity of diagnostic immunoassays.

The combination of multiple specific B-cell epitopes from antigenic borrelial proteins may increase the sensitivity and specificity of serologic assays. The chimeric proteins showed good sensitivity in diagnosis of LD infection and have the potential to improve the diagnosis of LD. In this study, it was also noted that the inclusion of the LA-2 epitope of OspA protein in the recombinant multi-epitope antigens may have enhanced the diagnostic accuracy of the serologic assays; however, further studies are required to address this hypothesis.

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