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OspA Sequence Comparison and Protection Against *Borrelia burgdorferi* Infection in Gerbils by Recombinant OspA Protein

*A Karami [1], P Hindersson [2], N Høiby [3], S Morovvati [1]

[1] Research Center of Molecular Biology, Institute of Military Medicine, Bagiyatallah University of Medical Sciences, Tehran,Iran
[2] Dept. of Clinical Biochemistry, Bispebjerg Hospital,University of Copenhagen, Denmark.
[3]Dept. of Clinical Microbiology, National University Hospital, Rigshospitalet, Copenhagen, Denmark

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Abstract

Lyme borreliosis is a tick-born disease caused by the spirochete *Borrelia burgdorferi*. The aim of this research was molecular typing of different strains of *B. burgdorferi* and protective efficacy of outer surface protein in animal model. Seven strains of *B. burgdorferi* isolated from skin and CSF of Lyme disease patients were compared using PCR- RFLP as well as DNA and amino acid sequences. The gene coding for Outer Surface Protein A (OspA) was cloned and recombinant protein was expressed. Immunization experiment was conducted on gerbils with purified recombinant OspA protein. The OspA gene sequence of *B. afzelii* and *B. garinii* showed 83.76% similarity and calculated amino acid identity of 84 % with each other and 82.5% similarity to B31 strain. DK6 was 100% similar to Pbi, 99.6 % to Ptrob isolate and 88 % to B31 strain. Cloning and expression of OspA from DK6 strain in *E. coli* has shown lipoprotein with molecular weight of 32 kDa. Immuno blot of recombinant protein from DK6 with rabbit anti DK1 revealed strong reaction with OspA from DK6 strain. Immunization experiment in gerbils with three doses of rOspA protein revealed that all immunized animals either with OspA fusion protein alone or combined with adjuvant were protected against infection with DK1 or DK6 pathogenic strains.

Keywords: Borrelia burgdorferi, *Linear and circular plasmid, Outer surface protein A (OspA), Recombinant protein, Vaccination*

Introduction

Lyme borreliosis is a tick-born disease caused by the spirochete *Borrelia burgdorferi* (1, 2). Various typing systems based on molecular and immunological characteristics have divided *B. burgdorferi* into several different groups (3). In *B. burgdorferi* the genes encoding major outer membrane proteins OspA and OspB are located on a 49 kb linear plasmid (4). Immunochemical and biochemical studies of the OspA protein of *B. burgdorferi* have revealed differences in apparent molecular mass and reactivity with monoclonal antibodies (5). This heterogeneity has been shown to be more prominent among European isolates than North American isolates (6). Furthermore, in both the European and the North American *B. burgdorferi* isolates, the OspA-B protein shown more strain variability (7). OspA is protective antigen of this bacteria and induces a protective immunity against spirochete infection in mice (8). Recombinant OspA protein has been used as vaccine with or without adjuvant or adsorbed to alum and elicited a strong, dose-dependent immunoglobulin G response (9). Antibodies to OspA have been shown to be important in protective immunity against infection with homologous strain, but

***Corrsponding author:** Tel: +98 21 88039883, Fax: +98 21 88057023, E-mail: Alika95@nrcgeb.ac.ir

partial or not at all against those of different OspA genotype (10). Because of variability of OspA gene in European strains, it has been suggested that vaccine development against one strain are unlikely to provide immunity to organisms from others (11).

We have studied the presence and difference of OspA gene in skin and CSF isolates and cross protectivity of recombinant OspA protein produced in *E. coli* between two strains in animal model.

Materials and Methods

Strains and culture condition: Seven clinical strains of *B. burgdorferi*, DK1, DK2, DK3, DK4, DK5 and DK7 isolated from the skin of patients with erythema chronicum migrans and DK6 from the cerebrospinal fluid of a patient with neuroborreliosis were obtained from Statens Seruminstitut, Copenhagen, Denmark.

Genospecies was determined as described by Marconi et al. (12), and according to the OspA serotype classification determined by Wilske et al. (13) are shown in Table 1. *E. coli* Jm109 competent cell, Novo Blu and BL21 (DE3) was cultured in Luria-Bertani (LB) broth with appropriate antibiotics when necessary. *Borrelia* was cultured in BSKII medium as described (2). After final growth at 33 °C for 3 weeks bacteria were collected by centrifugation at 14000 rpm for 25 min at 4 °C, washed with 5 mM $mgCl_2$ before extraction of DNA.

Table 1: *B. burgdorferi* strains, origin and passage number

Strain	Passage number	Source[a]	OspA serotype[b]	Genotype[b]
DK1	11	Skin, (EM)	1	*B. afzelii*
DK2	11	Skin, (ACA)	2	*B. afzelii*
DK3	11	Skin, (ACA)	2	*B. afzelii*
DK4	10	Skin, (EM)	2	*B. afzelii*
DK5	12	Skin, (ACA)	2	*B. afzelii*
DK6	12	CSF, (LMR)	4	*B. garinii*
DK7	30	Skin, (ACA)	1	*B. burgdorferi* senso stricto

[a]EM, erythema migrans; ACA, acrodermatitis chronica atrophicans; CSF, cerebrospinal fluid; LMR, lymphocytic meningoradiculitis.[b] Determined by comparison with the OspA typing system of Wilske et al (13).

DNA extraction The bacterial pellet was suspended in 300 µl of STE buffer and DNA was extracted by alkalyin lysis method (14).

PCR amplification of OspA gene OspA, and OspA-B primers from the conserved region of OspA were designed (15) PR1, 5′-CATATGAAAAAATATTTATTGG-3′ and PR2, 5′-TTGCGAAATTTTATTCCTAGG-3′, PR3, 5′-GGGGGTTTACTTATATAT-TTTTAAAGC-3' were used. PCR was performed as standard method. Amplification was carried out at 94°C for 1min, 45 °C for 1 min and 72 ºC for 2.5 min for 35 cycles (DNA Thermal Cycler, Perkin Elmer Cetus, Norwalk, CO, USA). Ten µl of the PCR reactions were analyzed by agarose gel electrophoresis (0.8%) (Sea-Kem FMC).

Cloning and sequencing of OspA gene The amplified fragment of OspA from cerebrospinal fluid strain DK6 and skin isolate DK1 was blunted with the Klenow polymerase (Boehringer Mannheim) and digested with SmaI and HincII for deletion of BamHI site and cloned to dephosphorylated pUC18 vector and transformed to JM109 competent cell. The resulted recombinant plasmids were selected on LB agar plates containing (IPTG, XGAL, and sigma) and 0.2 mg/ml ampicillin). The plasmids were extracted using the boiling mini-prep method.

Plasmid extraction and sequencing of OspA gene Selected colonies were grown in LB broth containing 0.2 mg/ml ampicillin. The

plasmids were extracted using the boiling mini-prep method and sequenced by dedoxynucleotide chain termination method (16).

Expression of OspA protein from DK6 strain Plasmid containing the OspA gene of DK6 strain was digested with BamHI and NdeI and cloned to the pET16b expression vector and transformed to BL21 (DE3) competent *E. coli*. Cell were cultured in LB agar containing 0.2 mg/ml carbencillin and expression of recombinant OspA protein was carried as standard methods. For purification of recombinant OspA protein metal chelating column chromatography (Ni^{2+} charged His-Bind Resin column) were used.

***Preparation of antibody against* B. burgdorferi** Antigen was prepared from cultured bacteria and used for immunization. Three white female rabbits were injected 4 times with 2 weeks interval with 0.5 ml of protein. One week after the last injection blood were taken from rabbits. Antibody was absorbed with sonicated *E. coli* Bl21 (DE3) antigen.

Western Blotting Purified recombinant OspA protein were transferred to nitrocellulose membrane and analyzed by western blot.

Vaccination with recombinant OspA Forty male gerbils of the strain *Meriones unguiculatus,* weight, 50 to 70 g, were obtained from Shamrock United Kingdom and divided to six groups. Group one (G1): ten gerbils for vaccination with OspA fusion protein alone and challenge with DK1 strain. Group two (G2): ten gerbils for vaccination with OspA protein alone and challenge with strain DK6. Group three (G3): five gerbils for vaccination with OspA in complete Freund's adjuvant and challenge with DK1 strain. Group four (G 4): five gerbils for vaccination with OspA in complete freund's adjuvant for challenge with DK6 strain. Group five (G5): five control gerbils injected with PBS alone and group six (G6): five control gerbils injected with adjuvant alone. Gerbils were vaccinated intraperitonealy by 0.2 ml fusion OspA protein (10ug), alone in PBS or in 200 ul complete freund's adjuvant (Statens Seruminstitute, Copenhagen,Denmark).Three boosts containing same amount of vaccines were administered on days 14 and 28 following the initial injection.

Challenge On day 42 post vaccination, gerbils received intraperitoenal inoculation containing approximately $1\times10^{8\ 1}$- 10^{10} of live bacteria (DK1 or DK6) in 200 ul of BSKII medium, caged separately to prevent contact transmission. Control animals were challenged only by the DK1 strain. Five weeks later, the gerbils were sacrificed and the blood and spleen tissues were aseptically removed. Blood (50 ul) and the portion of spleen was inoculated without further processing into 10 ml of BSK II medium con-taining the antibiotic phosphomycin (100 ug/ ml) and Rifampicin (50 ug/ml) and grown at 33 ºC for 3 weeks. Spirochete growth was determined by dark-field microscopy. DNA from gerbil tissues were tested by PCR.

Extraction of DNA from gerbil's tissues and PCR Total DNA from gerbil's tissue (spleen) was extracted by standard method (16) and used for DNA amplification. The OspA specific primer pairs (above) were used for PCR amplification of DNA extracted from gerbil's tissue, in the same condition above, but using higher concentration of primers (0.8 mM). Purified DNA from DK1 strain of *B. burgdorferi* was used as a positive control.

Sequence analysis Simultaneous alignment for DNA and protein sequences and phylogenetic tree construction were performed by using Genetic Computer Group Sequence Analysis program and PHYLIP (17).

Results

PCR amplification of DK1 to DK7 with OspA specific primers has produced the OspA fragment of the expected size (Fig. 1). The OspA sequence of the DK6 isolate is 83 % similar to DK1 and 88 % to North American type strain B31. Hyper variable region in DK1 and DK6

strains are between amino acids 12 and 150 with 23 amino acid differences. The alignment and of deduced amino acid sequences shows variable region from amino acids 53 to 271 and several hyper variable sequences. The OspA sequense of DK6 strain isolated from CSF of patient with neuroborreliosis is 100% similar to previously published OspA sequence of other European strain PBi, isolated from the CSF of patient in Germany and 99% similar to strain PTrob isolated from skin in Germany. OspA gene from DK1 strain has 86.5% similarity to PBi, 83% to B31, 77 % to 19857 (isolated from kidney of rabbit in USA) and 94% similar in amino acid level to skin isolate, PKo from Germany. OspA sequence of DK29, skin isolate from Denmark has 82 and 90% similarity to DK1 and DK6 respectively. Another Hyper variable region of variation in DK1 and PKo is located between amino acid No 130 and 150.

The conserved region of 130 amino acid at C terminus only with one amino acid No 95 is substituted from serine to glycine. Another identical region is located from amino acid number 150 to 270. Conserved OspA proteins are only among strain belonging to the same species. Phylogenetic tree analysis of OspA deduced amino acid sequences from Danish strain with other European and North American strains that complete sequence of OspA are available from databanks, reveals the presence of three *Borrelia* genospecies. Comparing deduced amino acid sequences of OspA protein from 24 *B. burgdorferi* isolated in different geographical areas showed that they have 128 identical amino acids (46.3%), 87 amino acids with only one difference (31%) and 61 amino acids with difference in more than one amino acids (22%). Phylogenetic tree analysis of OspA deduced amino acid sequences from DK1, DK6 and DK29 with other strains revealed the presence of three Borrelia genospecies (Fig. 2). All North American strains are in one cluster, *B. burgdorferi* sensu stricto. Lack of signal peptide region of CA1, CA3 and CA8 strains effected tree structure in one cluster but along with North American strains. All European strains divided to three genospecies of *B. burgdorferi, B. garinii* and *B. afzeli.* One strain from Japan *B. Japonica* remains in different cluster suggesting that this strain is different genospecies.

Cloning and expression of OspA gene from DK6 strain in *E. coli* produces 32 kDa lipoprotein. Western blot of OspA fusion protein with polyclonal antibody against DK1 strain confirms the expression of lipoprotein in *E. coli* (Fig. 3).

Vaccination of gerbils with three doses of 10 ug, purified OspA recombinant fusion protein of DK6 strain injected intraperitoneally alone or in complete freund's adjuvant strongly protects animals against infection with 10^{8-10} organisms from virulent strain of DK1 or DK6. Nine of ten control animals injected with DK1 and DK6 strain were found to be infected and blood and tissue culture were positive. PCR of DNA extracted from the spleen of all animals by OspA specific primer showed nine positive in ten control animals (Fig. 4). All blood and tissue culture in immunized animal either by OspA fusion protein alone or in adjuvant were negative. PCR detection of *B. burgdorferi* in DNA extracted from tissue of immunized animals was negative.

Table 2: Protection of gerbils vaccinated with rOspA from DK6 alone or in adjuvant against challenge with DK1 and DK6 strains. * Complete frund's adjuvant

Vaccine	Challenge	Culture + / totall	PCR + / totall
rOspA Alone	DK1	0/10	0/10
rOspA Alone	DK6	0/10	0/10
rOspA In adjuvant*	DK1	0/5	0/5
rOspA In adjuvant*	DK6	0/5	0/5
Controls	DK1	5/5	5/5
Not Vaccinated	DK6	4/5	4/5

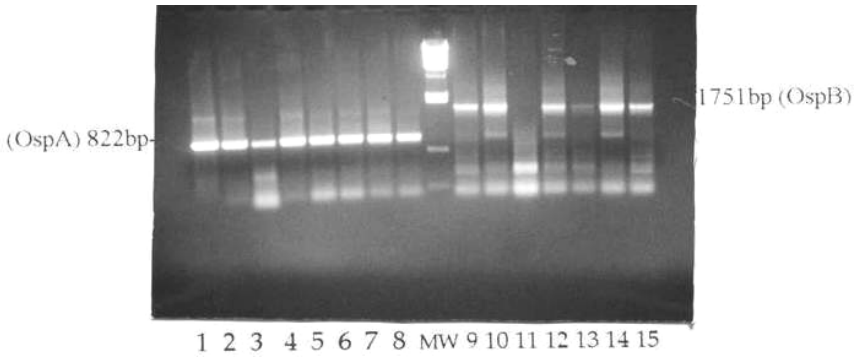

Fig. 1: PCR amplification of total plasmid extracted from seven strains of *B. burgdorferi* with OspA and ospA-B specific primers . All strains give the exact fragment size of 822 bp. From left to right lane 1-7 are OspA amplification from DK1 - DK7. Lane 8 is control PCR from DNA extracted from B31 strain. Lane 9-15 are PCR amplification of the OspA-B operon from the same strains. DK1 (1,9), DK2 (2,10), DK3 (3,11), DK4 (4,12), DK5 (5,13), DK6, (6,14), DK7 (7,15). MW (Hind III fragments of lambda DNA: 23130, 9416, 6557, 4361, 2322, 2027, 564 bp)

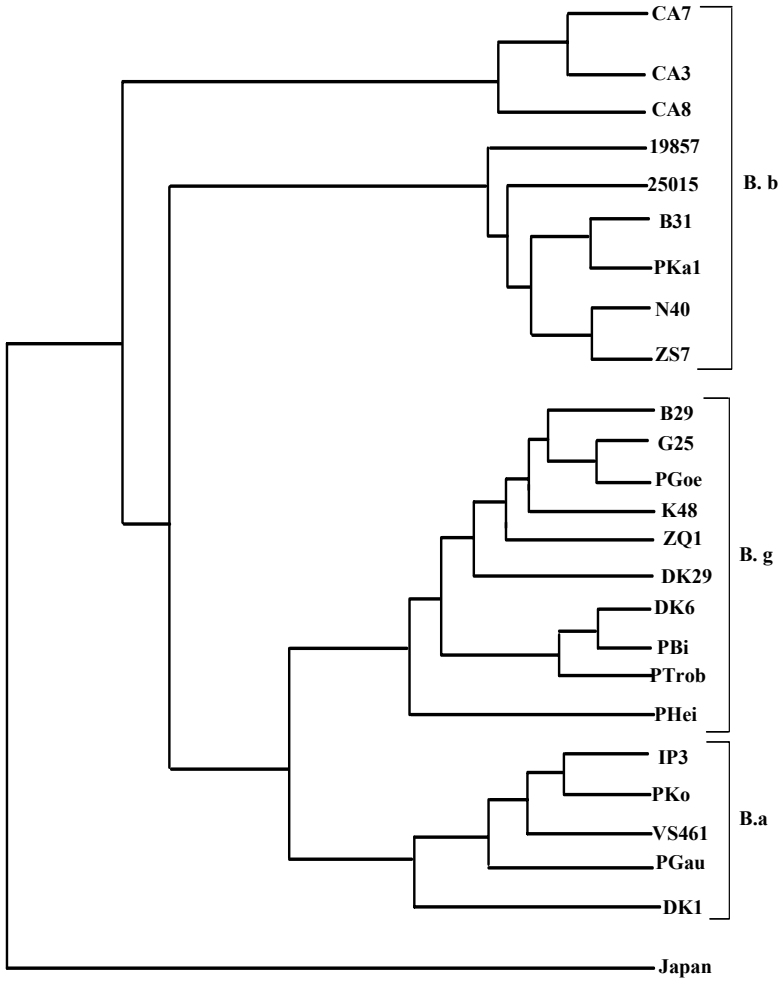

Fig. 2: Phylogenetic tree analysis of 25 OspA protein sequences derived from individual *B. burgdorferi* isolates. The deduced OspA amino acid sequences were aligned by using the tree alignment and phylogenetic tree construction program phylip.

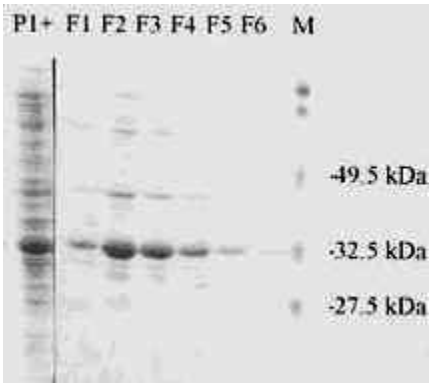

Fig. 3: Western blott of total protein extracted from DK1 and DK6 strains and purified expressed OspA protein from *E. coli* with polclonal antibody generated against DK1 strain F, F1, F2-F6 are fractions of rOspA during purification process, P1+ is toal protein from *E. coli* containing pET16b-OspA plasmid after induction with IPTG, (M) standard protein molecular weights are 106, 80, 49.500, 32.500, 27.500 and 18.500 kDa.

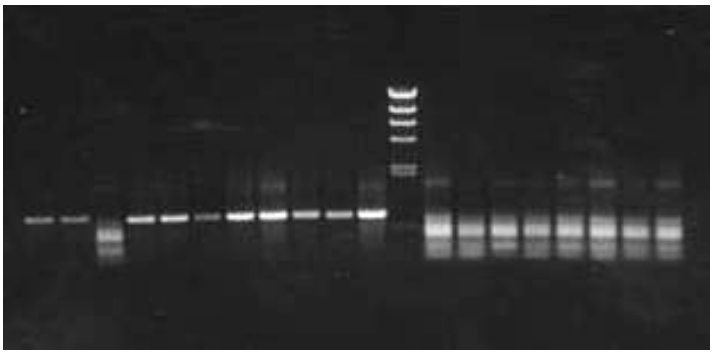

Fig. 4: PCR amplification for OspA gene of DNA extracted from tissues of controls and vaccinated gerbils. Lanes 1-10 : amplified OspA gene from control gerbils (not vaccinated) only gerbil No3 dos not produced OspA fragment. Lane 11 is a positive control with DNA extracted from the DK1 strain. Lanes 13-20, PCR of DNA extracted from gerbils vaccinated with rOspA and challenged with the DK1 strain. Lane 12 is lambda HindIII digested molecular weight: 23130, 9416, 6557, 4361, 2322, 2027, 564 bp)

Discussion

OspA and OspB are major Outer surface protein of *B. burgdorferi* (18) and gene that encodes these lipoproteins is located in linear plasmid with different size from 49 to 56 kb in different strains. In *B. burgdorferi* 49 kb, *Borrelia garinii* 55 kb and in *B. afzelii* has 56 kb size (19). In DK1 strain OspA gene is located on a linear plasmid with 50 kb size. PCR of seven strain tested by pair of OspA specific primer shows that all of strain have OspA gene. Restriction map of OspA gene resulted from PCR shows similar pattern in DK1, 2, 3, 4 and DK5 but DK 6 and DK7 has missed HincII restriction site. Sequence of OspA gene from skin and CSF isolates of patients in Denmark shoed variation in the OspA genes. CSF isolate, DK6 has perfect sequence match with PBi, a CSF isolate and 99% similarity to skin isolate, PTrob both from Germany but DK1 from skin of Lyme disease patient has only 86% similarity to European strains. Deduced amino acid

sequence from DK1, DK6, B31, PBi and PKo shows perfect mach in first 52 amino acids.

Phylogenetic tree analysis of OspA protein with North American and European isolates shows close relationship of Danish strains with German isolates. The skin isolate PKo and CSF isolates PBi from Germany are in same group with DK1 and DK6 isolates. OspA protein sequence of DK29 another Danish skin isolate studied before (20) has 82 and 90% similarity to DK1 and DK6. This strain has higher similarity to strains isolated from tick, with 97.8 and 97.5% similarity to B29 and ZQ1 isolated in Germany, 99% to G25, in Sweden and 98% to strain K48 from Czechoslovakia. It has been shown before by OspC (another outer surface protein of *B. burgdorferi*) sequence comparison of DK1, DK6, DK7, DK26 and DK27 isolated from patients in Denmark that they resemble three OspC phenotypes correlated with three genospecies of *B. burgdorferi* (21). Another study by RFLP and monoclonal antibody serotyping of Danish strains, DK1-7, DK27 and DK29 has shown presence of three genospecies *B. afzelii* (DK1-5), *B. garinii* (DK6, DK27, DK29) and *B. burgdorferi* senso stricto (DK7) (3). Polyclonal antibody generated against total protein of DK1 strains gives strong reaction with purified OspA fusion protein of DK6 strain expressed in *E. coli*. Protection of mice immunized with OspA against heterogeneous borrelia has been studied and it has shown that mice vaccinated with outer surface protein A (OspA) from *B. burgdorferi* strain N40 are protected from challenge with an intradermal syringe inoculums of *B. burgdorferi* strains N40, B31, CD16 and 297, isolated from the cere-brospinal fluid of a patient with neuroborreliosis, but not against challenge with inoculum of 10^4 strain 25015, isolated from a tick (22). The deduced OspA-25015 protein sequence differs from OspA-N40 at 40 of 273 amino acids. The results extend the usefulness of OspA as a vaccine candidate, (23, 24) but indicate that OspA can vary among strains of *B. burgdorferi*. (25). Studies have shown that OspA protein derived from North American strain is not able to protect against *B. burgdorferi* isolated from European region (18). In our experiment we studied the protection of gerbils with recombinant OspA fusion protein derived from CSF isolate of *B. burgdorferi*, with 41 amino acid difference from OspA amino acid sequence of DK1 strain, against the virulent strain isolated from skin (DK1) and CSF (DK6) and we find strong protective activity against both strains. Previously it has been shown that low passage DK1 strain was infectious for gerbils of the strain *Meriones unguiculatus* (26). Our Infectivity test before this experiment in same animal has shown that low passage DK1 and DK6 were infectious in $1x10^{6-7}$ organisms. High passage number of this strain was non virulent (not shown). It is clear from this results that OspA fusion protein from CSF isolate alone and without adjuvant has the imunogenic potential to induce strong immunity against even high doses (10^{8-10}) of DK1 strain. Analysis of OspA protein sequences from DK1 and DK6 strains for prediction of antigenic determinants according to Wolf et al. 1988 (27), based on the probable secondary structure feature, regional backbone flexibility as well as parameters relating to surface accessibility. It is notable that, despite the fact that the homology of the amino acid sequence of strains DK1 and DK6 is only 86% we obtained very similar pattern for the antigenic index values. Cross immunity among types of *B. burgdorferi* has been demonstrated that cross reactive epitops are present in OspA from different isolates of *B. burgdorferi* (28). Comparing amino acid similarity and identity of OspA protein from DK6 strain with other *B. burgdorferi* suggests possible cross protectivity between this strain and other European strain.

In conclusion, OspA-B specific PCR primer could be used as diagnostic method for different *B. burgdorferi* genospecies and that rOspA provides strong protection against virulent isolates of *Borrelia burgdorferi.*

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