

Chimeric Protein PSPF, a Potential Vaccine for Prevention *Streptococcus pneumoniae* Infection

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Abstract

A chimeric protein, designated PSPF (*Pneumococcus* Surface Proteins and Flagellin) was constructed from conservative and immunogenic fragments of *S. pneumoniae* surface proteins PspA, Spr1875, PsaA and the *Salmonella typhimurium* flagellin terminal domains FliC1, FliC2 functioning as adjuvant. PSPF was highly immunogenic in mice and induced protection from virulent *S. pneumoniae* strains. All the subtypes of *S. pneumoniae* under study were recognized by anti-PSPF sera. Thus, the chimeric recombinant PSPF protein is a promising vaccine candidate for immunization of humans against *S. pneumoniae*.

Keywords: Pneumonia; *Streptococcus pneumoniae*; Chimeric protein; Recombinant vaccine; TLR-5; Flagellin

Introduction

According to WHO data, *Streptococcus pneumoniae* is among the most common bacterial infections responsible for mortality among children in developing countries [1, 2]. *S. pneumoniae* is also the most often cause of bacterial pneumonia, meningitis, bacteremia, bronchitis, sinusitis and otitis media. In Europe and the US, the annual incidence of invasive pneumococcal disease ranges from 10 to 100 cases per 100000 of the population [1]. *S. pneumoniae* isolates are covered by a thick polysaccharide capsule, which provides protection against opsonization and phagocytosis. Pneumococcal capsule polysaccharides (PSs) are the first surface elements recognized by an immune system and characterized by high antigenic variability. At present 91 *S. pneumoniae* serotypes have been discovered and the number is constantly growing [3]. The increasing frequency of the pneumococcal isolates resistant to commonly used antibiotics complicates control of pneumococcal infections [4-6]. Current pneumococcal vaccines are all based on PSs antigens. Therefore, the effect of a polysaccharide-based vaccine is restricted to the PS serotypes included in the vaccine, and the coverage varies due to different geographical serotype distributions [7]. Another disadvantage of PS vaccines are based on the weak immunological memory [8]. In this respect, surface proteins of *S. pneumoniae* which are also exposed and may be reached by the opsonising antibodies are advantageous vaccine candidates due to the smaller degree of variability and higher immunogenicity.

The goal of the work was to study immunogenicity and preventive effect of chimeric protein, composed of the fragments of three surface proteins of *S. pneumoniae*. The choice of conservative and immunogenic fragments of surface proteins PsaA, PspA and Spr1875

to include in the chimeric vaccine was based on previous studies, which reported their ability to induce protective antibodies [9-13]. PsaA is a highly conservative lipoprotein expressed by several serotypes and essential for virulence of *S. pneumoniae* [9]. PspA (Pneumococcal surface protein A) is a surface protein known to contribute to *S. pneumoniae* virulence by binding human lactoferrin and by impeding complement activation in vivo and in vitro [10,11]. The surface protein Spr1875 (also known as LysM domain-containing protein) exhibits immunogenic properties and contributes to *S. pneumoniae* virulence due to its role in the development of pneumococcal meningitis [12,13].

Salmonella typhimurium flagellin FliC N- and C-terminal fragments known to interact with Toll-like receptor-5 (TLR-5) were included into the vaccine molecule as an activator of innate and adaptive immune responses [14,15]. All these components assembled in one molecule were expressed in *E.coli* system, isolated and tested for immunogenicity and the ability to provide the appearance of protective IgG.

Materials and Methods

Modeling the recombinant chimeric protein, artificial gene and expression plasmid

Amino acid sequence of chimeric protein based on PspA, Spr1875, PsaA and FliC fragments, separated with appropriate flexible bridges (called PSPF) was designed *in silico* according to the previously accumulated knowledge of the immunogenic domains and putative three dimensional structures. Nucleotide sequence of the chimeric protein was optimized for expression in *E. coli* (Figures 1, 2). Synthesis of nucleotide sequence of PSPF was performed by elongation of the overlapping oligonucleotides [16]. The synthetic chimeric gene was

cloned in pET24b+ vector (Invitrogen), producing the expression plasmid pPSPF, which was transformed into *E. coli* BL21 (Invitrogen). The gene encoding for chimeric protein devoid of *S. typhimurium* flagellin fragments (PSP) was inserted into pET25b+ giving expression plasmid pPSP, which was also transformed into *E. coli* BL21(DE3).

Artificial protein structure prediction

The sequence of the designed artificial protein PSPF was virtually cut into domains and the 3D structures of these domains were found in public databases or *ab initio*. The final refinement of the overall 3D structure was performed employing I-Trasser algorithm [17]. The reliability of the structure prediction by I-Trasser is 70%. Prediction of intrinsic disorder and disorder-based binding sites of the designed artificial protein PSPF and its parent proteins were evaluated by several predictors of the PONDR family, such as PONDR[®] VLXT [18], PONDR[®] VSL2 [19], PONDR[®] VL3 [20], and PONDR[®] FIT [21]. Disorder-based binding sites in the PSPF protein were identified by the ANCHOR algorithm [22].

Chimeric proteins expression and purification

Recombinant *E. coli* with plasmids encoding for PSPF and PSP were grown in PYP5052 medium containing 0.2% lactose according to Studier's method [23]. The proteins PSPF and PSP were extracted from the *E. coli* cells by cell lysis in 20 mM Tris-HCl pH 7.5, 5 mM EDTA, 1 mM PMSF and sonication (7 rounds of 30 sec pulses with 30 sec pauses, ultrasound frequency 22 kHz). Bacterial lysates were centrifuged and the pellets were suspended in 1M urea. After the next centrifugation the pellets were suspended in 2M urea and centrifuged again. The pellets were dissolved in 20 mM Na-phosphate buffer, with 0.5M NaCl, 6M guanidine-HCl, pH 8.0 and subjected to Ni-NTA (Invitrogen) purification chromatography according to producer's protocol. After final dialysis against 20 mM tris-HCl pH 7.5, 5 mM EDTA and 1 mM PMSF the obtained products contained the chimeric proteins PSPF and PSP with purity of 97% and concentration 1 mg/ml with LPS concentration not exceeding 0.016 EU/mg evaluated by Endosafe LAL assay (Charles River).

Number	Collection number	Serotype
1	ATCC BAA-1667	6
2	ATCC [®] 6319 [™]	19
3	442	19F
4	68	6ABC
5	16	9VA
6	133	19A
7	73	3
8	1734	34
9	88	8
10	78	14
11	835	9LN

Table 1: *Streptococcus pneumoniae* strains used in the study.

S. pneumoniae strains

S. pneumoniae clinical isolates were obtained from the collection of the Research Institute of Pediatric Infections (St. Petersburg, Russia) and two strains were purchased from ATCC (ATCC[®] 6319[™] type 19, ATCC BAA-1667-type 6) (Table 1). Pneumococci were cultured in anaerobic conditions at 37°C for 18 hours in THB medium with 20% horse serum (Difco). Schaedler agar with sheep red blood cells was used as a solid medium.

Animals

The study was performed using BALB/c inbred mice (female, 8 weeks old, 18-20 g) obtained from «Pushino» nursery. All animals were housed in illuminated and temperature controlled conditions. Mice were offered standard commercial feed and water *ad libitum* and treated according to the Guide for the Care and Use of laboratory animals of the Institute of Experimental Medicine.

The experimental design

The animal study of vaccine described in Tables 2-3. In short, three groups of animals were injected with three different dosages of vaccine. Two groups used as controls: the reference vaccine group and a group of mice received placebo.

Immunogen	Groups	The first immunization dose	The second immunization dose	The mode of <i>S. pneumoniae</i> infection	Number of animals
PSPF adjuvant +	1	5 µg/mouse	2,5 µg/mouse	intraperitoneally	10
				intranasally	10
	2	10 µg/mouse	5 µg/mouse	intraperitoneally	10
				intranasally	10
	3	20 µg/mouse	10 µg/mouse	intraperitoneally	10
				intranasally	10
Adjuvant only	4	No PSPF (control group)		intraperitoneally	10
				intranasally	10
Reference vaccine	5	1 vial/8 mice	1 vial /16 mice	intraperitoneally	10
				intranasally	10

Table 2: Scheme of immunization.

Reference vaccine

Human pneumococcal conjugate vaccine (Prevnar 7) containing 2 µg of each polysaccharide of 4, 6B, 9V, 14,18C, 19F, 23F serotypes and 20 micrograms of carrier protein CRM197 per dose was used as reference vaccine.

The route of administration

Chimeric proteins PSPF and PSP and reference vaccine were administered subcutaneously. Each injection of chimeric proteins contained 0.1 ml of the adjuvant Imject[®] Alum (Thermo Scientific) and 0.1 ml of the protein solution in PBS buffer. A placebo group of mice

were injected with 0.1 ml of PBS and 0.1 ml of adjuvant. A reference vaccine group immunized with Prevnar 7 in proportion 1 human dose per 8 mice.

Day	Action
Day 0	The first vaccination: groups 1-3, 5 Adjuvant injection in control group: group 4
Day 21	Taking the blood from mice to identify specific antibodies (IgG) titer
Day 22	The second vaccination (1/2 of the first vaccination dose): groups 1-3, 5 Adjuvant injection in control group: group 4
Day 35	Taking the blood from mice to identify specific antibodies (IgG) titer
Day 42	Taking the blood from mice to identify specific antibodies (IgG) titer
Day 49	Taking the blood from mice to identify specific antibodies (IgG) titer
Day 50	Intranasal infection with <i>S.pneumoniae</i> strain 73 serotype 3
Day 53	Intraperitoneal infection with <i>S.pneumoniae</i> strain 442 serotype 19F
Days 54-63	Monitoring of the animals survival

Table 3: Schedule of immunization.

Serum IgG titer detection

Blood was collected from submaxillary vein. Specific IgG levels were determined by ELISA in 96-well ELISA plates (Nunc) coated overnight at 4°C with the PSP or PSPF proteins. The diluted sample (100 µl) was added to duplicate wells and incubated 1 h at 37°C. HRP-labeled goat anti-mouse IgG antibodies (Sigma) diluted in blocking buffer were added (100 µl/well). After incubation at room temperature for 1 hr, plates were developed with TMB substrate (BD Bioscience) according to the manufacturer's instructions. ELISA endpoint titers were defined as inverse value of the highest dilution that yielded an OD value above 2 times the mean of negative control wells.

Interaction of PSPF- specific sera with different *S. pneumoniae* serotypes

The study was performed using dot -blot assay on nitrocellulose membranes. Daily cultures of *S. pneumoniae* were washed in PBS and three microliters of each bacterial suspension were applied to nitrocellulose membranes and dried. The membrane was incubated in blocking buffer (5% dry milk dissolved in PBS pH 7.4). After the incubation, membrane was treated with mice sera containing PSPF-specific antibodies with titer 1:400000 diluted 1000 times in blocking buffer. Membrane was placed in a conjugate solution (anti-mouse IgG (Fc-specific)-peroxidase). Color was developed in ready to use TMB Liquid Substrate System for Membranes (Sigma) for 3-5 minutes.

Estimation of *S. pneumoniae* lethal doses

Bacterial aliquots of *S. pneumoniae* were inoculated into mice by intraperitoneal or intranasal administration. Survival level was monitored for 10 days. The minimum bacterial doses which caused the

death of the 50 or 100% of the animals were evaluated as LD₅₀ or LD₁₀₀.

Protective efficacy of recombinant vaccine

Ten *S. pneumoniae* strains belonging to ten different serotypes were analyzed (Table 1). Only two of these, strain 442 (serotype 19F) and strain 73 (serotype 3) were highly virulent for the mice and were used to investigate the vaccination effectiveness in lethal models of pneumococcal infection. All bacterial strains were tested for recognitions by PSPF-specific IgG antibodies in dot-blot ELISA.

Vaccinated and controlled mice were infected with virulent strains by intraperitoneal or intranasal administration on the 50th day after the first immunization. Intraperitoneal infection was induced with LD₁₀₀ of the strain 442 (serotype 19F). One infective dose contained 3×10^2 CFU of the pathogen. Intranasal infection was induced with LD₅₀ of the strain 73 (serotype 3). One infectious dose contained 1×10^7 CFU of the pathogen. Each experimental group included 8-10 mice.

Statistics and Data Handling

Data analysis included the estimation of the medium values and the standard deviation ($M \pm \sigma$). Statistical relevance was estimated employing t-test by Student or U-test by Mann-Whitney. Differences were considered significant at $p < 0.05$.

The survival curves of mice were compared for significance using the log-rank test. Differences were considered significant at $p < 0.05$

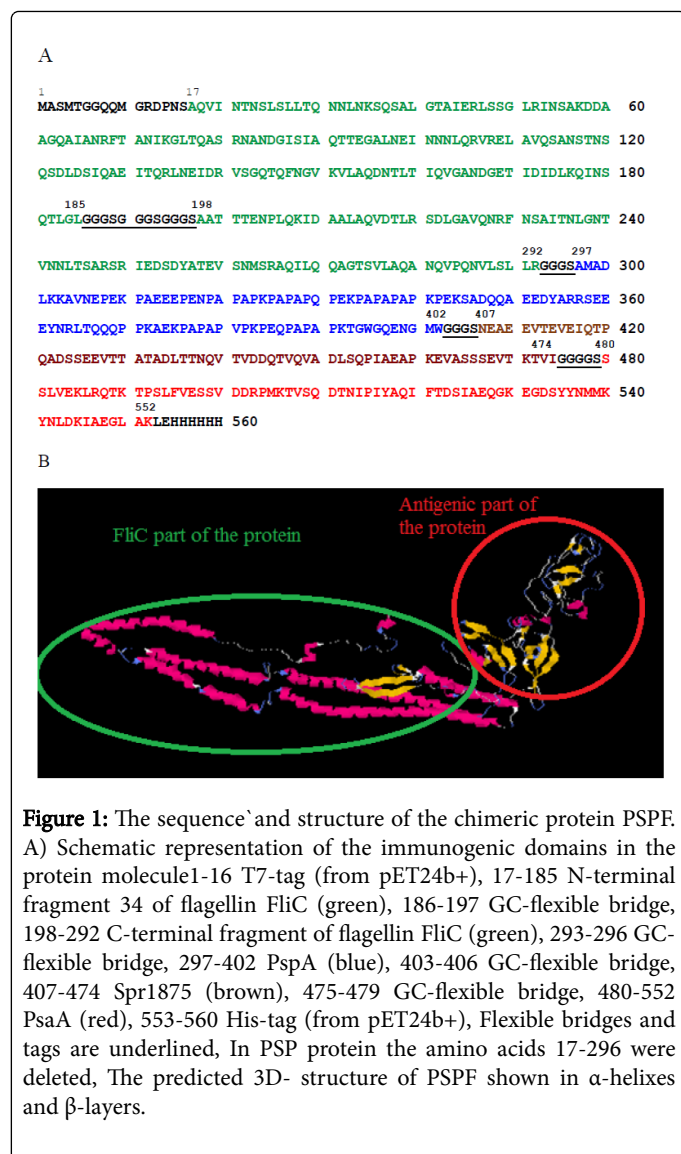
Results

Molecular design of artificial chimeric protein PSPF

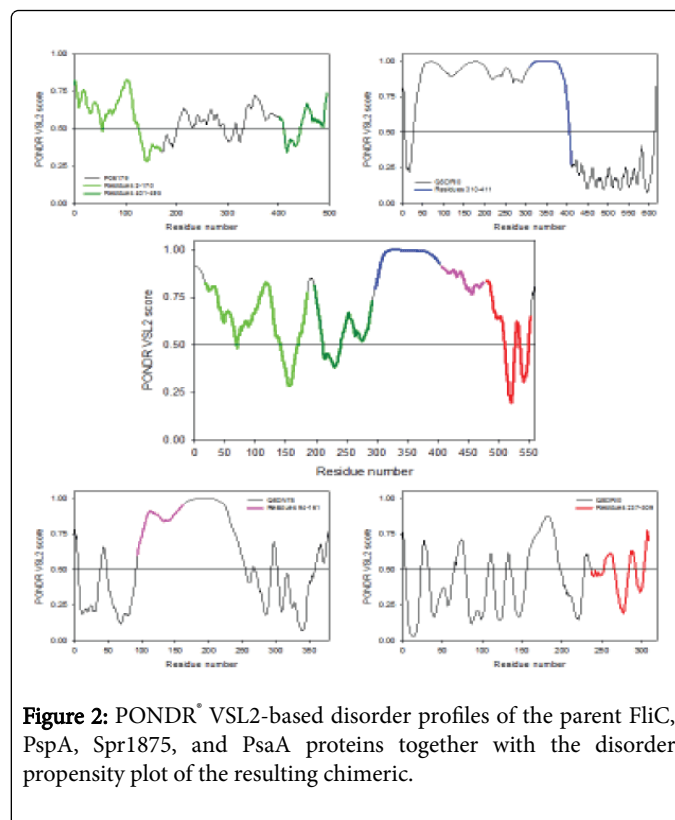
Developed chimeric recombinant protein PSPF contains the fragments of *S. pneumoniae* proteins PspA, PsaA, Spr1875 and *S. typhimurium* flagellin FliC. PspA is a surface protein showing high heterogeneity between pneumococcal strains [24]. In *S. pneumoniae* PspA varies in size from 439 to 649 amino acids and contains three major domains: the N-terminal 288 amino acid functional helical domain, 82 amino acid flexible proline-rich segments, and the C-terminal choline-binding domain responsible for cell wall binding. Protective Mabs against PspA predominately recognize the fragment between 192-260 amino acids of PspA [25,26]. Based on these data the conservative fragment of PspA corresponding to this region was included into chimeric protein (Figure 1).

Fragment containing amino acids from of the A-chain of the surface protein PsaA (480-552 in the protein as shown in Figure 1) was included into chimeric protein because this fragment contains epitopes that are immunogenic and protective in mice [27].

Spr1875 fragment of amino acids from corresponding to the amino acids from 94 to 162 in the original protein contains the epitopes immunogenic in mice and devoid of presumptive immunosuppressive regions located in the C-terminal part of the molecule was also included into chimeric protein [13]. All these fragments of *S. pneumoniae* proteins and two immunostimulatory *S.typhimurium* flagellin FliC N- and C-terminal fragments, responsible for TLR5 signaling [28,29] were connected with appropriate GS-flexible bridges. The total length of PSPF is 560 amino acids (Figure 1A).



PONDR[®] VSL2-based disorder profiles of the parent FliC, PspA, Spr1875, and PsaA proteins together with the disorder propensity plot of the resulting chimeric PSPF protein. Figure 2 clearly shows that similar to the parent proteins, the hybrid PSPF protein is expected to be mostly disordered. Curiously, distributions of the disorder propensities in the FliC, PspA, Spr1875, and PsaA regions incorporated in this protein differ significantly from distribution of their disorder predispositions within the corresponding parent proteins, indicating that disorder propensity is content-dependent.



PSPF protein

The majority of the PONDR curves (Figure 3A) are located above the 0.5 threshold, thereby indicating that the PSPF protein is expected to be highly disordered, since scores above 0.5 are considered to correspond to the disordered residues/regions. Often, intrinsically disordered proteins and regions are involved in protein-protein interactions and molecular recognitions there are numerous reports emphasizing that these proteins are able to undergo at least partial disorder-to-order transitions upon binding, which is crucial for recognition, regulation, and signaling [18,32]. The results of the analysis employing ANCHOR algorithm shown in Figure 3B indicate that this protein is expected to have at least 9 disorder-based binding sites (residues 20-29, 65-73, 211-216, 260-306, 339-382, 388-459, 473-483, 514-523, and 536-547). These regions have significant potential to be binding sites for an appropriate but typically unidentified partner protein.

Effect of FliC portion of the vaccine on immunogenicity

To compare the immunogenicity of PSPF and PSP, the mice were immunized with both proteins in the doses of 20 μ g/mouse for prime

and 10 µg/mouse for boost immunizations three weeks later. Figure 4A shows, that the presence of flagellin domain in PSPF increased specific antibody titers by 1.0-1.5 orders of magnitude at all the time points tested. Thus we could suppose that this effect was achieved by the immune-stimulatory activity of the flagellin domain included in PSPF.

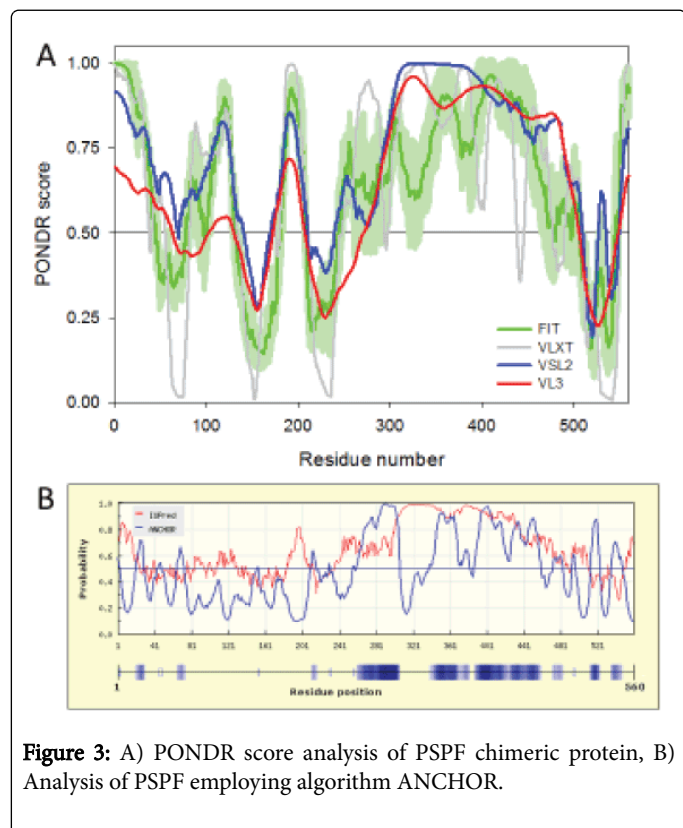


Figure 3: A) PONDR score analysis of PSPF chimeric protein, B) Analysis of PSPF employing algorithm ANCHOR.

Immunogenic properties of the PSPF applied in different doses

After the immunization, the titers of anti-PSPF antibodies in mice sera were determined by regimen indicated in Tables 2 and 3. Stimulation of the immune response in all the experimental groups were detected on the 14th day after the booster vaccination (35th day of the experiment), but the mean dose (10 µg/mouse) provided a statistically significant increase of the humoral immune response level as compared with the other experimental groups. However, starting from day 42 of the vaccination, this group showed the most pronounced downward trend in the quantity of circulating antibodies and after 28 days from the second vaccination (day 49 of the experiment), the level of specific circulating antibodies in all experimental groups was virtually identical. Specific IgG continued to circulate at the same level in all the groups for the following 20 days (Figure 4 B).

Study of the PSPF-Driven Protective Effects

Intraperitoneal infection

After the intraperitoneal infection, all the mice from control group (placebo) died on the second day of experiment. Death rates of immunized mice differed among the groups. The best protection was observed in the group of mice immunized with the lowest dose of the

vaccine (Figure 5A). Mice vaccinated twice with recombinant vaccine stimulated the development of a specific immune response, which provided 25-40% protection against lethal intraperitoneal infection. In the group of mice immunized with the reference vaccine, the mortality rate was close to the control value-88%. Thus, the level of protection provided by the recombinant pneumococcal vaccine was higher compared to the reference vaccine.

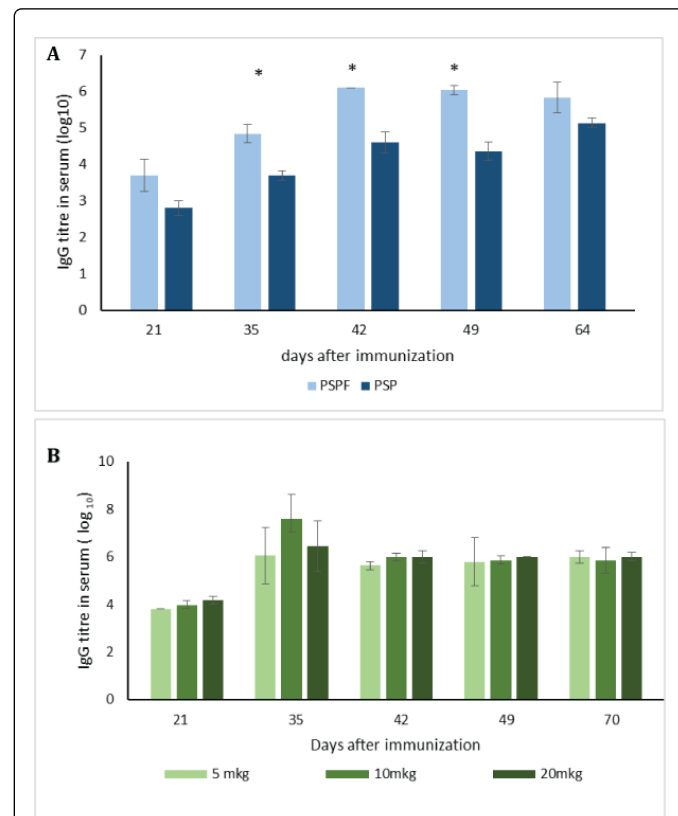


Figure 4: Immunogenicity of chimeric proteins in BALB/c mice. A) Stimulation of immune response by PSPF or PSP with or without Salmonella flagellin fragments. Mice (n=10) were immunized subcutaneously with the chimeric proteins PSPF and PSP in doses 20 µg and three weeks later with 10 µg. Specific anti-PSP IgG titers on days 35, 42, 49 and 70 were assessed. Results are shown as mean ± SD. *Differences between the groups immunized by PSPF or PSP were statistically significant on 35, 42 and 49 days after immunization. B) Immunogenicity of PSPF protein in different dosage. Mice (n=10) were immunized subcutaneously with the chimeric protein in doses of 5, 10 and 20 µg and three weeks later with one-half of each dose (boost). Specific IgG titers on days 21, 35, 42, 49 and 70 were assessed. Results are shown as mean ± SD.

Intranasal infection

Death of the animals after intranasal infection in control group started on the second day and on the third day in the groups immunized with PSPF. The mortality of animals immunized with PSPF was 40% after 10 days of monitoring. Death rate of animals in control group was 63% (Figure 5B). This experiment was repeated three times with different concentrations of vaccine protein. In all the cases the

difference was statistically significant. The protective level did not depend on the dose of the vaccine.

Thus, the PSPF immunization showed protective effect against *S.pneumoniae* intranasal infection.

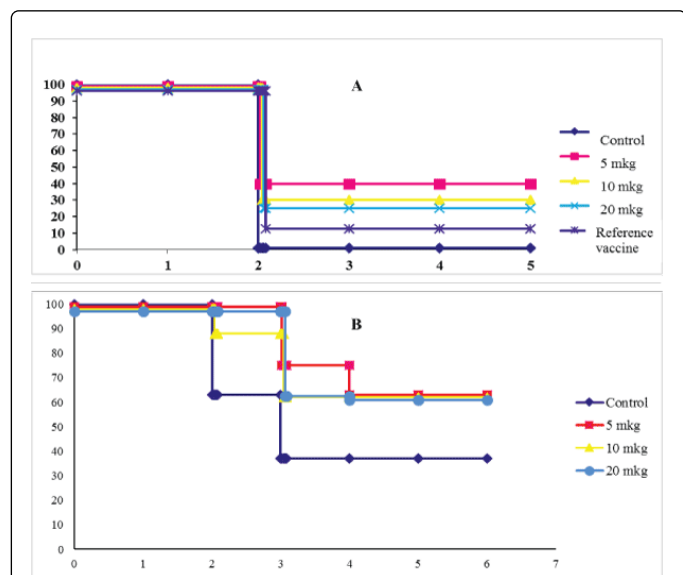


Figure 5: Surviving of animals in different groups after infection. A. Surviving of animals in different groups after peritoneal infection. The X axis shows the time after infection (days), on an axis Y - survival rate of animals (%). Infection was induced with 3×10^2 CFU/mouse *S. pneumoniae* strain 442 (type 19F), B) Surviving of animals in different groups after intranasal infection. The X axis shows the time after infection (days), on an axis Y - survival rate of individuals (%). Infection was induced with 1×10^7 CFU/mouse *S. pneumoniae* strain 73 (type 3). Each experimental group included 10 mice.

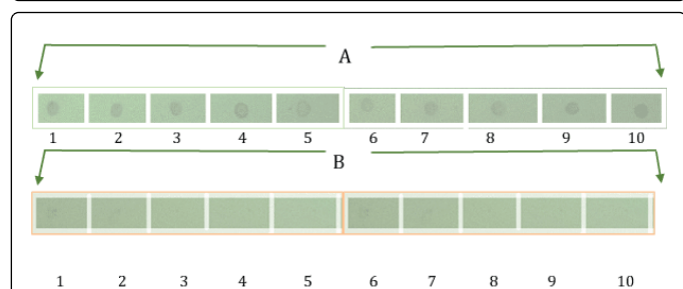


Figure 6: Dot-blotting analysis of *Streptococci pneumoniae* strains. A) anti-PSPF antisera; B) Control sera. *S. pneumoniae* serotypes under study: 1-6, 2-19, 3-19F, 4-6ABC, 6-9VA, 6-19A, 7-3, 8-34, 9-14, 10-9L.

Dot-Blot Analysis of Streptococci pneumoniae Strains

Interaction of PSPF-specific sera with *S. pneumoniae* strains listed in Table 1 was studied employing the dot-blot method. The sera recognized all *S. pneumoniae* strains under study belonging to different serotypes in contrast to control sera taken from non-immunized animals (Figure 6).

Discussion

At present several vaccines against *S. pneumoniae* infections were introduced in the public health systems. FDA approved two different types of vaccines: pneumococcal conjugate vaccines and pneumococcal 23-valent polysaccharide vaccine [33]. Polysaccharide vaccines implemented on the market have been proved both efficient and safe but they have several very important disadvantages. As there are over 90 PS serotypes and serotypes distribution differs in geographical areas, it is difficult to create one universal vaccine based only on polysaccharides. Polysaccharides are T-cell independent antigens, inducing short-term immunity without immune memory. Pneumococcal conjugated vaccines due to the carrier protein CRM197 are better recognized by T-cells and induce long-lasting immunity [34]. However, though the CRM197 is over 100 times less toxic than diphtheria toxin, it still retains some toxicity, especially in high doses [35].

The above considerations advocate for development of the protein vaccines against *S. pneumoniae*. Several proteins have been determined on the cell surface including PsaA, PspA, PcsB, StkP, Spr1875 and many others [36]. Many of them have been proved immunogenic and were able to induce protection against *S.pneumoniae* in the animal models. It is also determined that vaccines based on the epitopes from several proteins provide superior protection relatively to monovalent protein vaccines. The approach based on several epitopes in one chimeric protein allows avoiding the problem of the strains antigenic variability like in case of using PspA based vaccines, which are impeded by the high degree of genetic mosaicism and epitope variations [24,25]. On the other hand, implementation of the number of separate proteins and adjuvants in vaccine combination makes the vaccine a lot more difficult for standardization and it increases the cost. Major advantage of chimeric protein vaccine beside the cost effectiveness is their simplicity of production and its control, relatively high degree of conservation and T – dependent type of immune memory response [37-39]. These considerations resulted in construction and experimental testing of a new recombinant chimeric immunogenic protein PSPF comprising fragments of pneumococcal conservative proteins PspA, Spr1875, PsaA and flagellin domains (FliC). The molecule was analyzed *in silico* by several computer engines and several constructs have been generated employing DNA synthesis techniques with the following cloning and expression in the *E.coli* vectors. *Salmonella typhimurium* flagellin FliC N- and C-terminal fragments were included as "internal adjuvant" due to their ability to interact with Toll-like receptor-5 (TLR-5). Conserved N- and C- termini of flagellin cooperatively interact to generate a TLR-5 binding site and the middle highly immunogenic and pyrogenic part of the molecule can be deleted without the loss of TLR-5 stimulating activity [28,29].

Including the FliC-domains in PSPF significantly increased the level of the humeral immune response relatively to PSP, which does not possess this region.

Double subcutaneous immunization of mice with PSPF induce development of immune response typical for T-dependent antigens characterized with significant booster effects after the second immunization. We were not able to see the difference after immunizing with three doses of PSPF (20, 10 or 5 µg/dose) and it suggests that PSPF can be applied with less doses and provide little immunologic burden on the immunized individuals.

The ability of the strains under study to bind PSPF-specific antibodies has been tested *in vitro* employing dot-blot assay. All the strains belonging to the major serotypes were able to precipitate chimeric molecule – specific IgG equally. The ability of the anti-PSPF IgG to react with all *S. pneumoniae* strains under study reflects the broad specificity of the post vaccination immunity.

Protective features of antibodies against PSPF were studied on two *S. pneumoniae* strains, expressing significant virulence in mice. It was discovered that the immunized animals were significantly better protected relatively the control group of animals and group of mice vaccinated by reference vaccine Pevnar. Protection was determined both after nasal or intraperitoneal immunization.

Conclusion

This study has shown the efficiency of new recombinant chimeric protein PSPF which included the PsaA, PspA and Spr1875 epitopes assembled in one molecule as vaccine against *S. pneumoniae* infection in a mouse model. The PSPF immunization induced specific immune response against the range of *S. pneumoniae* serotypes and dramatically decreased the mortality after intraperitoneal and intranasal infection. The PSPF immunization showed stronger immune response than chimeric protein PSP without FliC portion and better protective effect in comparison with the reference conjugated pneumococcal vaccine. Results of protection did not depend on the dosage of vaccine, which correlated with similar dynamics of IgG production. Most likely lot smaller doses of PSPF could provide the protection. Thus, the new chimeric protein PSPF can be considered a promising vaccine candidate against *S. pneumoniae*.

Acknowledgments

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