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Pneumococcal Predictive Proteins Selected by Microbial Genomic Approach Are Serotype Cross-Reactive and Bind to Host Extracellular Matrix Proteins

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Abstract *Streptococcus pneumoniae* is a colonizer of the human nasopharynx, which accounts for most of the community-acquired pneumonia cases and can cause non-invasive and invasive diseases. Current available vaccines are serotype-specific and the use of recombinant proteins associated with virulence is an alternative to compose vaccines and to overcome these problems. In a previous work, we describe the identification of proteins in *S. pneumoniae* by reverse vaccinology and the genetic diversity of these proteins in clinical isolates. It was possible to purify a half of 20 selected proteins in soluble form. The expression of these proteins on the pneumococcal cells surface was confirmed by flow cytometry. We demonstrated that some of these proteins were able to bind to extracellular matrix proteins and were recognized by sera from patients with pneumococcal meningitis infection caused by several pneumococcal serotypes. In this context, our results suggest that these proteins may play a role in pneumococcal pathogenesis and might be considered as potential vaccine candidates.

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Keywords *Streptococcus pneumoniae* · Surface-exposed pneumococcal proteins · Extracellular matrix proteins · Cross-reactivity · Vaccine

Introduction

Streptococcus pneumoniae is a common commensal colonizer of the human nasopharynx, especially in young children [1–3]. Transmission occurs through respiratory droplets [4] and colonization is mostly asymptomatic but can progress to respiratory or even systemic disease causing many invasive and non-invasive diseases like otitis media and pneumonia, as well as invasive pneumococcal diseases (IPD) like bacteremic pneumonia, meningitis, and sepsis, leading to significant rates of mortality and morbidity around the world [5–8].

The current conjugate vaccines, based on pneumococcal capsule, are effective against IPD; however, the vaccine coverage is limited to the vaccine-type strains and it is estimated that the coverage of these vaccines for serotypes causing invasive disease in Brazil is around 79–94% [9, 10]. As more than 90 pneumococcal serotypes have been described and a non-vaccine serotype replacement is a serious threat [11], the search for new vaccine candidates that would elicit protection against a broader range of pneumococcal strains is interesting. In light of this, much attention has been given on the development of protein-based vaccines using conserved common pneumococcal protein antigens, particularly surface-exposed proteins that are targets for the action of antibodies [12, 13].

Mechanisms and pneumococcal factors that enable host epithelial and tissue barriers to be breached during the progression from colonization to invasive infection are still poorly understood. In order to better understand the pathogenic processes of pneumococcus, some studies have been conducted and allowed the identification of proteins potentially involved in host-pathogen interactions [14–18]. It now appears clearly that cell-surface proteins participate in many stages of the colonization process.

Extracellular matrix (ECM) proteins are components secreted by different host cells that enable the adherence and invasion of *S. pneumoniae* expressing adhesins that target these factors. Several surface-exposed pneumococcal proteins have been shown to bind to different ECM proteins [19]. For example, PavB binds to fibronectin and plasminogen. RrgA (a tip protein of the pneumococcal pilus-1 structure) has been shown to bind to fibronectin, collagen I, and laminin [20] and PsaA to E-cadherin [21]. In vivo, the same proteins have been shown to facilitate nasopharyngeal colonization and/or the establishment of lower respiratory tract infection.

The proposal of identification and characterization of surface protein as potential vaccine candidates [22–24] is important not only to understand the interaction of pneumococci with the ECM proteins but also to observe the accessibility of the antibodies that is generated in an immune response against pneumococcal surface proteins in different serotypes [19].

In this context, we took advantage of reverse vaccinology in order to select predicted pneumococcal surface proteins, which are widely distributed and conserved among different *S. pneumoniae* strains [22]. In a previous work, a dataset has been constructed containing streptococci protein sequences that are present only in pneumococcus genus. A total of 151 selected pneumococcal proteins were submitted to in silico analysis in order to choose proteins with positive prediction for signal peptide, transmembrane region, lipobox motifs, and subcellular location. A total of 20 from the 151 analyzed proteins were selected because they showed positive prediction for at

least one of the features described above according to the bioinformatics software used. In the previous work [22], we demonstrated that selected proteins were highly conserved showing more than 96% of amino acid and nucleotide identity between the analyzed *S. pneumoniae* serotypes.

In the present work, we demonstrate by flow cytometry (FACS) the surface localization of these proteins, validating the predictive data obtained in silico. We also describe the accessibility of these antigens in ten different pneumococcal strains. Furthermore, in order to identify new host-pneumococcal interactions that may play a role in colonization and disease progress, we evaluated the ability of these proteins to interact with ECM as well as to be recognized by sera from patients with pneumococcal disease.

In this context, our results show that pneumococcal proteins selected by reverse vaccinology demonstrate some features that have important implications on pneumococcal vaccine design based on highly conserved surface proteins.

Materials and Methods

Bacteria Cell Lines and Growth

Escherichia coli TOP10 was used for routine plasmid cloning, and the recombinant proteins were expressed in *E. coli* BL21 Star (DE3) cell line (Life Technologies, Carlsbad, California, USA, both type of cells). *E. coli* cells were cultured in Luria Bertani (LB) broth or Terrific Broth (TB) containing 100 μg/ml ampicillin and were grown at 37 °C at 200 rpm or statically in LB plates. The *S. pneumoniae* strains were kindly provided by Dra. Joice Reis (CPqGM/Fiocruz/Salvador/Brazil] and Dra. Maria Cristina Brandileone (Adolfo Lutz Institute (IAL—Instituto Adolfo Lutz)/São Paulo/Brazil) (Table 1). *S. pneumoniae* serotype 5 strain 617/00 (here designated SPN5) was used as a source of genomic DNA for PCR amplification of the selected genes. The *S. pneumoniae* serotypes 1 strain 525/00, 4 strain 729, 5 strain 617/00, 6B strain 793, 7F strain 800, 9 V strain 724, 14 strain 722, 18C strain 845, 19F strain 720, and 23F strain 756 (designated in this study as SPN1, SPN4, SPN5, SPN6B, SPN7F, SPN9V, SPN14, SPN18C, SPN19F, and SPN23F, respectively) were used as a source for the surface protein accessibility studies. The pneumococci were routinely grown on trypticase soy agar (TSA) plates (Becton & Dickinson, Franklin Lakes, New Jersey, USA), supplemented with 5% sheep blood (blood agar), at 37 °C for 16–18 h.

Pneumococcal Chromosomal DNA Extraction

Frozen SPN5 was cultivated as describe above. Chromosomal DNA was extracted using the Wizard SV Genomic DNA Purification System (Promega, Fitchburg, Wisconsin, USA) according to the manufacturer's protocol.

Cloning and Sequencing

In a previous work [22], we demonstrated the use of reverse vaccinology to identify a set of proteins conserved in *S. pneumoniae*, as well as the selection process of proteins with extracellular region. These proteins would be possible targets for vaccine development. A

Table 1 Strains of *S. pneumoniae*, patient age, and site of isolation

Strain number	Year of isolation	Patient age	Serotype	Site of isolation	Kindly provided by	Origin
720	2007	5 years	19F	Cerebrospinal fluid	Fiocruz[a]	Salvador, Brazil
722	2007	1 year 4 months	14	Cerebrospinal fluid	Fiocruz	Salvador, Brazil
724	2007	12 years	9V	Cerebrospinal fluid	Fiocruz	Salvador, Brazil
729	2007	23 years	4	Cerebrospinal fluid	Fiocruz	Salvador, Brazil
756	2008	40 years	23F	Cerebrospinal fluid	Fiocruz	Salvador, Brazil
793	2009	0 year 11 months	6B	Cerebrospinal fluid	Fiocruz	Salvador, Brazil
800	2009	46 years	7F	Cerebrospinal fluid	Fiocruz	Salvador, Brazil
845	2010	2 years	18C	Cerebrospinal fluid	Fiocruz	Salvador, Brazil
525/00	NA[b]	NA	1	Pleural fluid	ALI[c]	Sao Paulo, Brazil
617/00	NA	NA	5	Cerebrospinal fluid	ALI	Sao Paulo, Brazil

[a] *Fiocruz* Fundação Osvaldo Cruz, Oswaldo Cruz Research Foundation

[b] *NA* not applicable

[c] *ALI* Adolfo Lutz Institute

set of 20 targets were cloned and expressed, but only 10 proteins were purified in a soluble form. Only the gene region coding for the extracellular portion of the selected proteins (Table 2) was amplified by PCR. The primers used are described in Table 3. The PCR amplicons were cloned into pET100/D-TOPO plasmid (Life Technologies, Carlsbad, California, USA) and the products used to transform *E. coli* TOP10. The recombinant clones were confirmed by agarose gel, PCR, digestion with *Xba*I and *Hin*dIII restriction enzymes (Life Technologies, Carlsbad, California, USA), and sequencing analysis. Nucleotide sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies, Carlsbad, California, USA) following the manufacturer's instructions, and the reactions were resolved on an ABI PRISM® XL3500 Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA). The electropherograms and the generated contigs were analyzed using SeqManII v5:05 (DNASTAR, Madison, Wsconsin, USA). The recombinant plasmids were used to transform *E. coli* BL21 Star (DE3), and the cells were stored at −70 °C in 25% glycerol.

Recombinant Protein Expression and Evaluation of the Solubility

Recombinant *E. coli* BL21 Star (DE3) was cultivated in TB medium supplemented with 100 μg/ml ampicillin, at 37 °C, 200 rpm, until reaching the exponential phase (OD_{600} 0.6–0.8). Protein expression was induced with 1 mM of isopropyl-β-D-1-thiogalactopyranoside (IPTG) and grown for 4 h at 28 °C or 37 °C in shaking. Both non-induced and induced pellets were evaluated by SDS-PAGE [25] to confirm the protein expression. For solubility analysis, the pellets were resuspended in lysis buffer (20 mM Tris–HCl /1 mM EDTA,

Table 2 Cloned genes: fragment size, description of the removed gene/protein region, expression and solubility

ORF number in SPN5	ORF in *S. pneumoniae*	Protein identification (Blastp)/name in the article	Construct (total protein size)/expressed protein (aa)	Expressed protein (Da)	Aa removed from de original protein size	Expression	Solubility
00962	Thioredoxin family protein [*Streptococcus pneumoniae* TIGR4] 818836:819,402	Thioredoxin family protein/TRF	189/167	22,700	22 aa initial corresponding to signal peptide	+	+
02009	Hypothetical protein SP_0191 [*Streptococcus pneumoniae* TIGR4] 1707542:1,708,111	Hypothetical protein/HP	190/171	23,100	19 aa initial corresponding to signal peptide	+	+
01065	Sensor histidine kinase, putative (*Streptococcus pneumoniae* SP3-BS71) 912439:913536	TCS11—sensor histidine kinase/ TCS11	366/222	28,800	149 aa initial corresponding to cytoplasmic and transmembrane regions of the protein	+	+
00333	ABC-type antimicrobial peptide transport system, permease component (*Streptococcus pneumoniae* Hungary19A-6) 285799:287787	ABC-type antimicrobial peptide transport system, permease component/ABC-PC	663/230	30,400	Cloning the gene portion comprising the 307–536 aa of the protein (the initial and the final portions of the proteins, comprising the cytoplasmic and the transmembrane regions, were removed)	+	+
01929	Amino acid ABC transporter, amino acid-binding protein, putative [*Streptococcus pneumoniae* SP14-BS69] 1645562:1,646,362	Glutamine ABC transporter substrate-binding protein/G-ABC	267/247	31,600	20 aa initial corresponding to signal peptide	+	+
01909	Transmembrane protein Vexp3 (*Streptococcus pneumoniae* TIGR4) 1624721:1626100	Vexp3/Vexp3	460/266	33,400	Cloning the gene portion comprising 36-301 aa of the protein (the initial and final portions of the protein, comprising the signal peptide, cytoplasmic, and transmembrane regions, were removed)	+	+

Table 2 (continued)

ORF number in SPN5	ORF in *S. pneumoniae*	Protein identification (Blastp)/name in the article	Construct (total protein size)/expressed protein (aa)	Expressed protein (Da)	Aa removed from de original protein size	Expression	Solubility
02072	Iron-compound ABC transporter, iron-compound-binding protein (*Streptococcus pneumoniae* Hungary19A-6) 1746756:1747721	PiuA/PiuA	322/297	36,300	25 aa initial corresponding to signal peptide	+	+
00265	psr protein (*Streptococcus pneumoniae* TIGR4) 234312:235586 forward	Psr/Psr	425/310	38,300	115 aa initial corresponding to transmembrane region of the protein	+	+
00080	ABC transporter, ATP-binding protein (*Streptococcus pneumoniae* D39) 70465:72024	ABC transporter, ATP-binding protein/ABC-ATP	520/519	63,600	ATG initial of the protein	+	+
02232	Spermidine synthase (*Streptococcus pneumoniae* SP3-BS71)	CbpE/CbpE	632/606	73,700	26 aa initial corresponding to signal peptide	+	+

[a] *ORF* open read frame

[b] *aa* amino acid

[c] *Da* Dalton

Table 3 Primers

Gene in SPN5 strain 617/00	Forward primers (5′–3′)	Reverse primers (5′–3′)	Primers used for
ABC-atp	CACCCACTATGAA CATTCTAGAAAGGG	TTATAAATCTTCTAAATT AACTAGCTTCAA	PCR and sequencing
psr	CACCTACCAAGATGC TAGCGCAAAGAAA	TTATCTCAAGCCCAT TTGAGCGAGCTT	PCR and sequencing
ABC-pc	CACCTTCAATTCCGCA GAATCCTTT	TTAGCCAAAGAGGAC ACTCATTTGAGC	PCR and sequencing
*tcs*11	CACCATTCGCCTTGT CGAGGATTTGAAAG	TTAGTTTCTCTCCTTAT AAGGTAGTCGAA	PCR and sequencing
*vexp*3	CACCATCAAGGGAG CTACTGCCAAGGCT	TTAGGCCATCTTGTACA TACCAGAGAT	PCR and sequencing
*cbp*E	CACCGAAAGTTCAGG AAATAAAATC	CTACTGTTCTATTCCG ATTTGTTT	PCR and sequencing
*cbp*e	AAAGTCAATGGCAA GAAAA and CAAGA TAAAGAGCAGTGG	TTTTCTTGCCATTGA CTTT and TACCACTG CTCTTTATCTT	Sequencing
trf	CACCAAGTCCGTGACT AGTGAACAC	CTAGGCTAATTCCTTCA AAGTTTGCA	PCR and sequencing
trf	ATTCTGGGCTTCTTG GTGTT	TTTCTTGCCCTTGTAATC	Sequencing
hp	CACCCAGAAAAAA GAAACTGGACCAGC	CTATTGTTCTGTCGCG CCATTTGC	PCR and sequencing
g-ABC	CACCGCTACACCCAA AGAGTCCAG	TTATTGTAACTGAGAT TGATCTGGGAGG	PCR and sequencing
*piu*A	CACCAACTCAAGCA CTAGTCAGACAG	TTATTTCAAAGCTTTTT GTATGTCTTC	PCR and sequencing
*piu*A	AATATCGAATCCTTAGCA	CACTTGCTAAGGAT TCGATATT	Sequencing

CACC sequence necessary on the forward primer for cloning the PCR product in the pET100-D/TOPO vector

pH 8.0), with or without 0.1% of triton X-100, and disrupted by sonication on ice using a Microson Ultrassonic Cell Disruptor (Misonix, Farmingdale, New York, EUA). Separation of soluble (supernatant) and insoluble (pellet) fractions were carried out by centrifugation of the sonicated lysate at 20,000×*g* for 5 min, and both the fractions were analyzed by SDS-PAGE.

Purification of the Recombinant Proteins

The *E. coli* BL21 Star (DE3), expressing the recombinant proteins, were grown and disrupted according to describe above. Proteins were purified from the soluble fraction of recombinant *E. coli* lysates by using immobilized metal ion affinity chromatography (IMAC) in a column containing immobilized nickel ions (GE Healthcare Life Sciences, Fairfield, Connecticut, EUA) and the fast protein liquid chromatography (FPLC) ÄKTA Purifier 10 or Akta Purifier 100 (GE Healthcare Life Sciences, Fairfield, Connecticut, EUA). Recombinant *E. coli* BL21 Star (DE3) cells resuspended in lysis buffer (Tris 20 mM, imidazole 20 mM pH 8.0) with inhibitor phenylmethylsulfonyl fluoride (PMSF) at the final concentration of 1 mM were disrupted by sonication on ice, and the soluble fraction was submitted to purification. The buffers used for purification were PBS pH 7.4 (buffer A) and PBS/1 M imidazole pH 7.4 (buffer B–elution buffer). Purifications were performed by washing with different imidazole

concentrations (20, 40, 60, and 80 mM), and the recombinant proteins were eluted by washing with PBS pH 7.4 containing 300 mM imidazole, except for CbpE protein that was eluted with 80 mM of imidazole. The HP and G-ABC proteins were eluted in a gradient from 180 to 230 mM and from 200 to 300 mM of imidazole, respectively. In Table 4, the purification steps employed for each recombinant protein were demonstrated. After purification, the recombinant proteins were dialyzed and quantified by the BCA method (Thermo Scientific Pierce, Waltham, Massachusetts, EUA) according to the manufacturer's recommendations. Densitometry was used to evaluate the recombinant protein purity (evaluated by SDS-PAGE) using a GS-800 calibrated densitometer (Bio-Rad, Hercules, California, USA) and quantified using the software QuantiOne 4.4. (Bio-Rad, Hercules, California, USA).

Production of Hyperimmune Mouse Sera against Pneumococcal Antigens

Hyperimmune mouse sera specific for ten purified proteins were generated by intramuscular immunization (i.m.) of BalbC/AN mice with each recombinant protein emulsified in Alhydrogel (Brenntag, Frederikssund, Frederiksborg, Denmark). Antigens were formulated in the ratio of 0.2 mg of each protein to 2 mg of aluminum hydroxide for a period of 16–18 h at 4 °C under gentle shaking. After the formulation, the material was diluted in PBS pH 7.4 and a dose of 100 μL, containing 10 μg of recombinant protein with 0.1 mg of adjuvant, was administrated to the animals. The immunization scheme was carried out as follows: three doses with 7 days of intervals followed by an intraperitoneal (i.p.) booster with the recombinant protein without adjuvant. Sera specific for the types 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23 capsular polysaccharides were generated immunizing the animals i.m. four times at 7-day

Table 4 Induction temperature for expression of recombinant proteins and the purification steps used to obtain each of them

Protein	Induction temperature	Lysis buffer (0.1% Triton X-100 added)	Purification steps
ABC-PC	37 °C	No	5 CV 20 mM; 2 CV 60 mM; *3 CV 300 mM*; 6 CV 1 M
Vexp3	37 °C	No	5 CV 20 mM; 4 CV 60 mM; *4 CV 300 mM*; 6 CV 1 M
TCS11	37 °C	No	5 CV 20 mM; 10 CV 40 mM; 3 CV 60 mM; *3 CV 300 mM*; 5 CV 1 M
Psr	37 °C	No	2 CV 20 mM; 2 CV 40 mM; 1 CV 80 mM; *3 CV 300 mM*; 3 CV 1 M
ABC-ATP	28 °C	Yes	5 CV 20 mM; 10 CV 40 mM; 3 CV 60 mM; *10 CV 300 mM*; 10 CV 1 M
TRF	37 °C	Yes	5 CV 20 mM; 5 CV 60 mM; 5 CV 80 mM; *6 CV 300 mM*; 5 CV 1 M
HP	37 °C	Yes	Gradient from 20 mM to1 M (*elution from 180 to 230 mM imidazole*)
G-ABC	37 °C	No	Gradient from 20 mM to 1 M (*elution from 200 to 300 mM imidazole*)
PiuA	37 °C	No	5 CV 20 mM; 5 CV 60 mM; 5 CV 80 mM; *6 CV 300 mM*; 5 CV 1 M
CbpE	28 °C	No	5 CV 20 mM; 5 CV 40 mM; *5 CV 80 mM*; 10 CV 1 M

Lysis buffer—Tris 20 mM/imidazole 20 mM/PMSF 1 mM pH 8.0 with or without 0.1% Triton X-100; buffer A—PBS pH 7.4; buffer B (elution buffer)—PBS/1 M imidazole pH 7.4

CV column volume

intervals with the 10-valent conjugate vaccine (PCV10Synflorix—GlaxoSmithKline Biologicals) obtained from Bio-Manguinhos/Fiocruz.

MAPIA and Western Blot

For the antigenic analysis against human sera, the MAPIA test was employed [26]. An amount of 200 ng of the recombinant proteins was printed on nitrocellulose membrane HiFlow 180 (Millipore, Milan, Lombardy, Italy) using the Automatic TLC Sampler 4 (CAMAG, Muttenz, Arlesheim, Switzerland). After overnight blocking (PBS pH7.4 with 4% of skim milk and 0.05% Tween 20), membranes were washed with PBS and incubated with primary antibodies (sera from patients with cerebrospinal fluid culture positive for different *S. pneumoniae* serotypes) diluted 1:200 (in PBS pH7.4 containing 0.25% bovine albumin fraction V and 0.05% Tween 20) for 2 h at room temperature. The sera were pre-adsorbed with 2.5% *v/v* of *E. coli* lysate for 30 min before being incubated with the membranes. After the incubation with the primary antibody, the membranes were washed with PBS pH7.4 containing Tween 20 0.05%. Then, conjugated anti-human IgG (γ chain-specific)-alkaline phosphatase antibody (Sigma-Aldrich, St. Louis, Missouri, EUA) was applied to the membranes, diluted 1:30,000 (in PBS pH7.4 containing 0.25% bovine albumin fraction V and 0.05% Tween 20) and incubated for 1 h at room temperature. The membranes were washed with PBS pH7.4 and revealed with Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega, Fitchburg, Wisconsin, USA).

Western blot reactions were performed to verify the antigenicity of the recombinant proteins against polyclonal antibodies generated in mice. For this technique, concentrations of 500 and 250 ng of the recombinant proteins were applied on polyacrylamide gel (SDS-PAGE) and subjected to electrophoresis. After that, the proteins were transferred from the gels to nitrocellulose membranes using the protocol described in "Mini Trans-Blot Electrophoretic Transfer cell" (Bio-Rad). The membranes were blocked, incubated with the primary and secondary antibodies, and washed like describe above but was used the polyclonal mouse serum at the dilutions of 1:100 to 1:51,200 and the anti-mouse IgG (whole molecule) alkaline phosphatase (Sigma-Aldrich, St. Louis, Missouri, EUA) diluted 1:30,000.

Flow Cytometry (FACS)

FACS analyses were carried out as previously [27–29]. Pneumococcal cultures were diluted to OD_{600} 0.3 (~10^8 CFU/mL) and incubated with sera from immunized and control mice (dilution of 1:100) for 1 h at 37 °C, followed by washing with PBS pH 7.4. The secondary antibody incubation (conjugated anti-IgG + anti-IgA + anti-IgM FITC) (Sigma-Aldrich, St. Louis, Missouri, EUA) was diluted 1:250, and the cells were incubated for 1 h at 37 °C. The cells were washed and fixed with 1% formaldehyde. The analysis was performed by using the flow cytometer BD (Becton & Dickinson, Franklin Lakes, New Jersey, USA), and the acquisition protocol was performed using the program CellQuest and analysis was conducted using the FlowJo (Tree Star Inc., Ashland, Oregon, USA). At least 15,000 events were acquired in the gate for each sample analyzed.

Enzyme-Linked Immunosorbent Assay

Nunc-Immuno™ MicroWell™ 96 well Nunc MaxiSorp (Sigma-Aldrich, St. Louis, Missouri, EUA) was coated with 100 ng of each extracellular matrix (collagen IV, laminin, fibronectin

and fibrinogen) (Sigma-Aldrich, St. Louis, Missouri, EUA), diluted in an awareness buffer (carbonate buffer), and incubated overnight at 4 °C. The plates were washed with PBS pH 7.4 containing 0.05% Tween 20 (PBST) and blocked with PBS pH7.4 containing BSA 5% (PBSB) for 2 h at 37 °C. After blocking, the plates were washed with PBST and incubated with 100 ng of each of the recombinant pneumococcal proteins (diluted in PBSB) for 1 h at 37 °C. The plates were washed and incubated with the primary antibody (monoclonal anti-HisTag-IgG) (Sigma-Aldrich, St. Louis, Missouri, EUA) diluted 1:3000 in PBSB. Afterward, the plates were washed and the reaction was incubated with the secondary peroxidase-labeled antibody (anti-mouse IgG whole molecule) (Sigma-Aldrich, St. Louis, Missouri, EUA) diluted 1:30,000 in PBSB. Both antibodies were incubated for 1 h at 37 °C. The plates were washed and the reaction was revealed with SureBlue TMB Microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland, USA) for 10 min and terminated by the addition of 2 N H_2SO_4. The plates were read in a Versa Max Tunable Microplate Reader (Molecular Devices, Sunnyvale, California, USA) at OD_{450}.

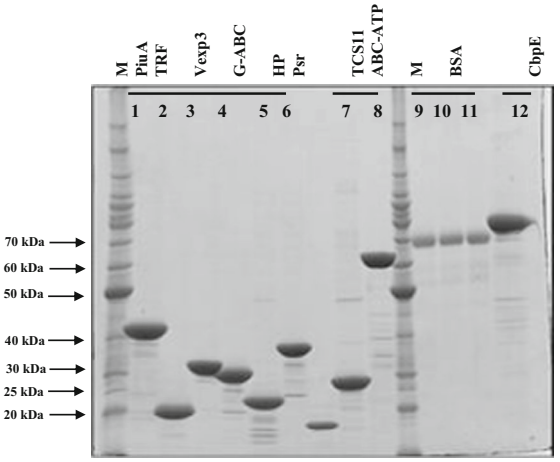
Results

Cloning, Expression, and Purification of Pneumococcal Recombinant Proteins

The ten pneumococcal target proteins evaluated in this study were selected using reverse vaccinology [22]. Briefly, a database was created using PERL language and conserved pneumococcal proteins were selected from it [22]. Then, protein sequences were submitted to in silico analysis (using bioinformatics software) for prediction of presence: signal peptide, transmembrane regions, lipobox motifs, and subcellular location. After these analyses, we selected targets that were positive for at least one of the analyzed features describe above and the coding gene region for the extracellular portion of the selected proteins was amplified at PCR. Half of the 20 selected targets (10 proteins), which were cloned and expressed, were purified in soluble form (Table 2). After the purification process (Table 4), ABC-ATP, TRF, and PiuA proteins presented precipitation. To overcome this issue, Triton X-100 (0.05%) was used during the lysis and purification. The ten purified and dialyzed proteins were analyzed in relation to the purity by SDS-PAGE in a gradient from 8 to 17% and they presented around 77 to 97% of purity (Fig. 1).

MAPIA and Western Blot

We also evaluated the ability of recombinant antigens of pneumococcus to be recognized by sera from patients presenting pneumococcal meningitis. Twenty-four sera from patients with pneumococcal meningitis (caused by different pneumococcal serotypes) were tested. We also included six sera obtained from children who did not have pneumococcal disease at the time of collection; these sera were considered as “control sera” (Fig. 2). As positive control for the test, we included protein A and the PsaA protein, a known sero-reactive protein widely distributed and conserved among pneumococci [30]. The *psa*A gene was previously cloned and the protein expressed and purified by our group [31, 32]. Three negative sera (A, C, and E) were found to weakly react against PiuA, CbpE, and PsaA proteins, while the other three



Number	Protein	kDa	Purity (Homogeneity in SDS-PAGE)
1	PiuA	36.3	85%
2	TRF	22.7	97%
3	Vexp3	33.4	97%
4	G-ABC	31.6	81%
5	HP	23.1	77%
6	Psr	38.3	91%
7	TCS11	28.8	80%
8	ABC-ATP	63.6	86%
9	CbpE	73.7	80%

Fig. 1 SDS-PAGE demonstrating the purity/homogeneity of the recombinant proteins. The recombinant proteins from the *S. pneumoniae* serotype 5 strain 617/00 were expressed and purified from *E. coli* lysates by immobilized metal affinity chromatography (IMAC). The proteins (30 μg per lane and 5 μg for BSA) were subjected to SDS-PAGE (gradient from 8 to 17%) and detected by direct staining with Coomassie brilliant blue. Apparent molecular size markers (in kilodaltons) are indicated. The gel was scanned in the GS-800 calibrated densitometer (Bio-Rad, Hercules, CA, USA) and statistical analyses were performed with the Quantity one 4.6.8 program

analyzed sera (B, D, and F) presented only anti-PsaA antibody. On the other hand, the majority of sera obtained from patients with pneumococcal meningitis strongly reacted with PsaA (23 of 24 sera), PiuA (17 of 24), and CbpE (23 of 24) proteins. Data suggest that during pneumococcal meningitis infection, those proteins lead a high specific antibody immune response. Nevertheless, Vexp3, ABC-ATP, HP, TRF, TCS11, ABC-PC, and Psr proteins were weakly recognized by a smaller number of sera (11 sera of 24 to ABC-ATP, 10 sera to HP, 9 sera to TCS11, 7 sera to Vexp3 and TRF, 2 sera to Psr, and 1 serum to ABC-PC), showing a lower reactivity of patients' sera against those proteins. Finally, G-ABC protein was not recognized by any patients' sera, possibly indicating its low expression during clinical disorder or low immunogenicity.

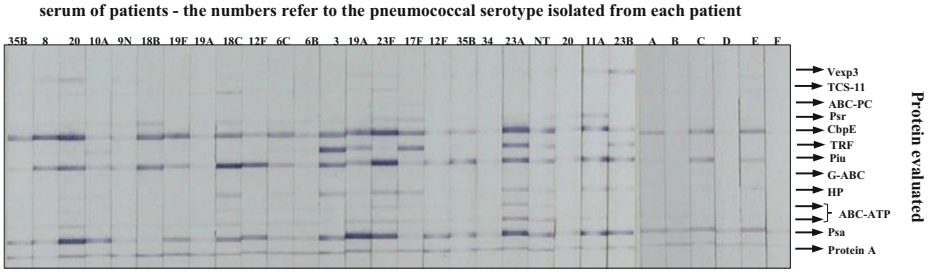


Fig. 2 MAPIA—analysis of the antigenicity of recombinant pneumococcal proteins using sera from patients with pneumococcal meningitis. The identification of recombinant proteins is shown on the right side of the figure, and the identification of the pneumococcal serotypes is shown at the top. *NT* not tippable, *A to F* "control sera". A total amount of 200 ng of each protein was used. The sera (primary antibody) was used at 1:200 dilution, and the secondary antibody was used at 1:30,000 dilution. The membranes were revealed with alkaline phosphatase system

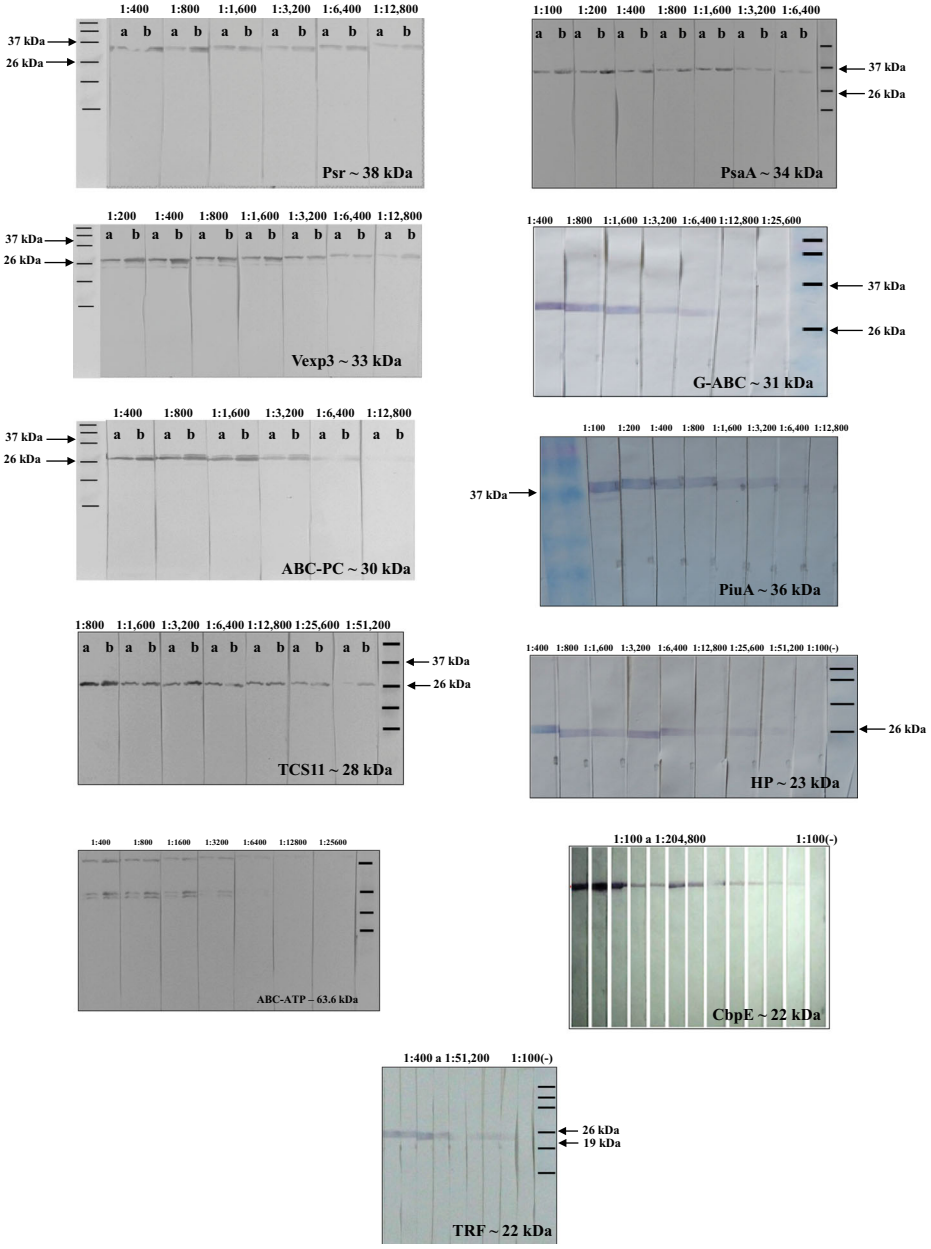


Fig. 3 Western blot: recombinant pneumococcal proteins in concentrations of 500 and 250 ng (indicated by letters *a* and *b*). Five hundred nanograms for G-ABC, PiuA, HP, TRF, and CbpE proteins was used. The polyclonal mice sera were used at the dilutions of 1:100 to 1: 51,200 (from left to right), except for protein CbpE for which we tested until the dilution of 1:204,000. The conjugated secondary antibody was used at the dilution of 1: 30,000 and used the alkaline phosphatase-based developer system

In relation to western blot (Fig. 3), we can verify that all proteins are specifically recognized by the polyclonal mice sera.

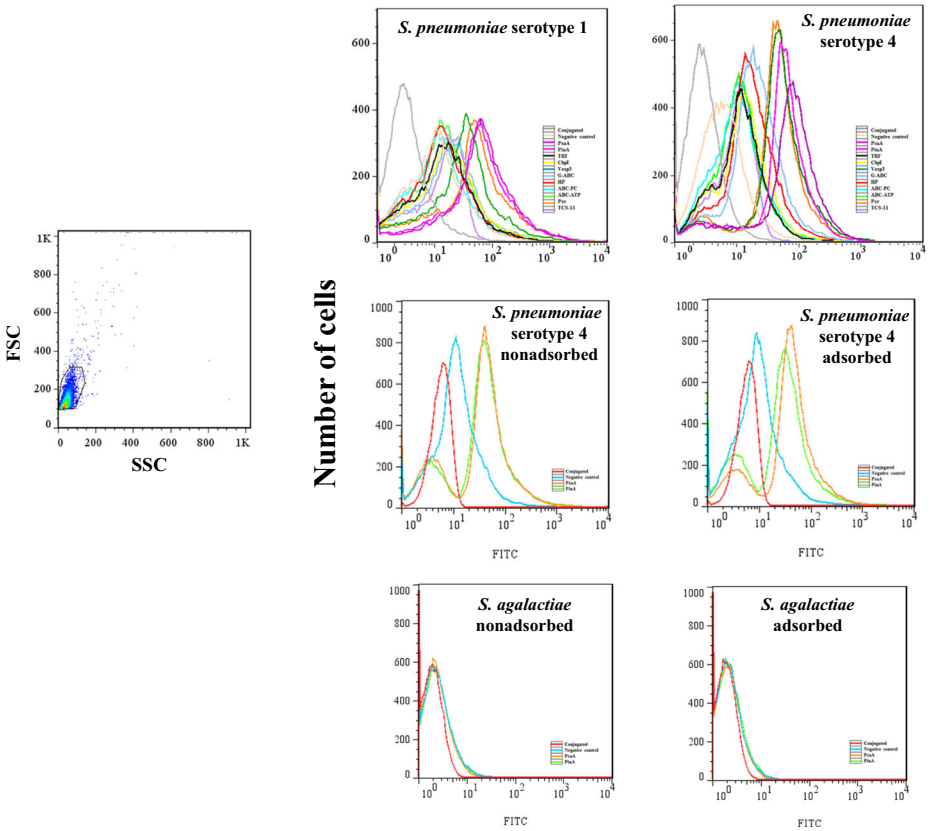


Fig. 4 Flow cytometry (FACS) was used for detection and evaluation of protein expression in the cell surface of pneumococci. The polyclonal anti-PsaA was used as a positive control and as negative control, pneumococci marked with serum from immunized mice (pre-immune serum—negative control) or pneumococci marked with the secondary antibody FITC-conjugated (conjugated) was used. Primary polyclonal antibodies were used at a 1:100 dilution and the secondary antibody FITC-conjugated at 1:250 dilution. The graph on the left refers to the dot plot and shows the profile of size × granularity (SSC × FSC) of the pneumococci cells demonstrating the gate selected for the evaluation. The two graphs found at the top, with overlap, show the expression of the ten selected proteins in the bacterial cell surface of the *S. pneumoniae* serotypes 1 and 4 (as an example, every serotype available was able to express the selected proteins on the cell surface). In addition, other controls were added to the assay: the pneumococci serotype 4 was marked with anti-PsaA and anti-PiuA polyclonal antibodies, adsorbed and not adsorbed with *E. coli* lysate, in order to demonstrate that the displayed labeling was specific to proteins under study. We also include in our analysis the marking of *S. agalactiae* to demonstrate the generation of non-cross-reactivity of polyclonal antibodies

Surface Expression of Antigens in Intact *S. pneumoniae*

These analysis showed the functionality of the polyclonal anti-sera, produced by mice immunization with recombinant pneumococcal proteins, to be able to recognize epitopes on the native proteins expressed in vitro by SPN5 and also displayed cross-reactivity to different evaluated pneumococcal serotypes (Figs. 4 and 5). On the obtained histograms (Fig. 4) were shown that all predicted and selected surface proteins (ABC-ATP, Vexp3, G-ABC, HP, PiuA, CbpE, Psr, TCS11, ABC-PC, TRF, and PsaA—positive control) were expressed on the cell surface of the ten pneumococcal serotypes (1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F)

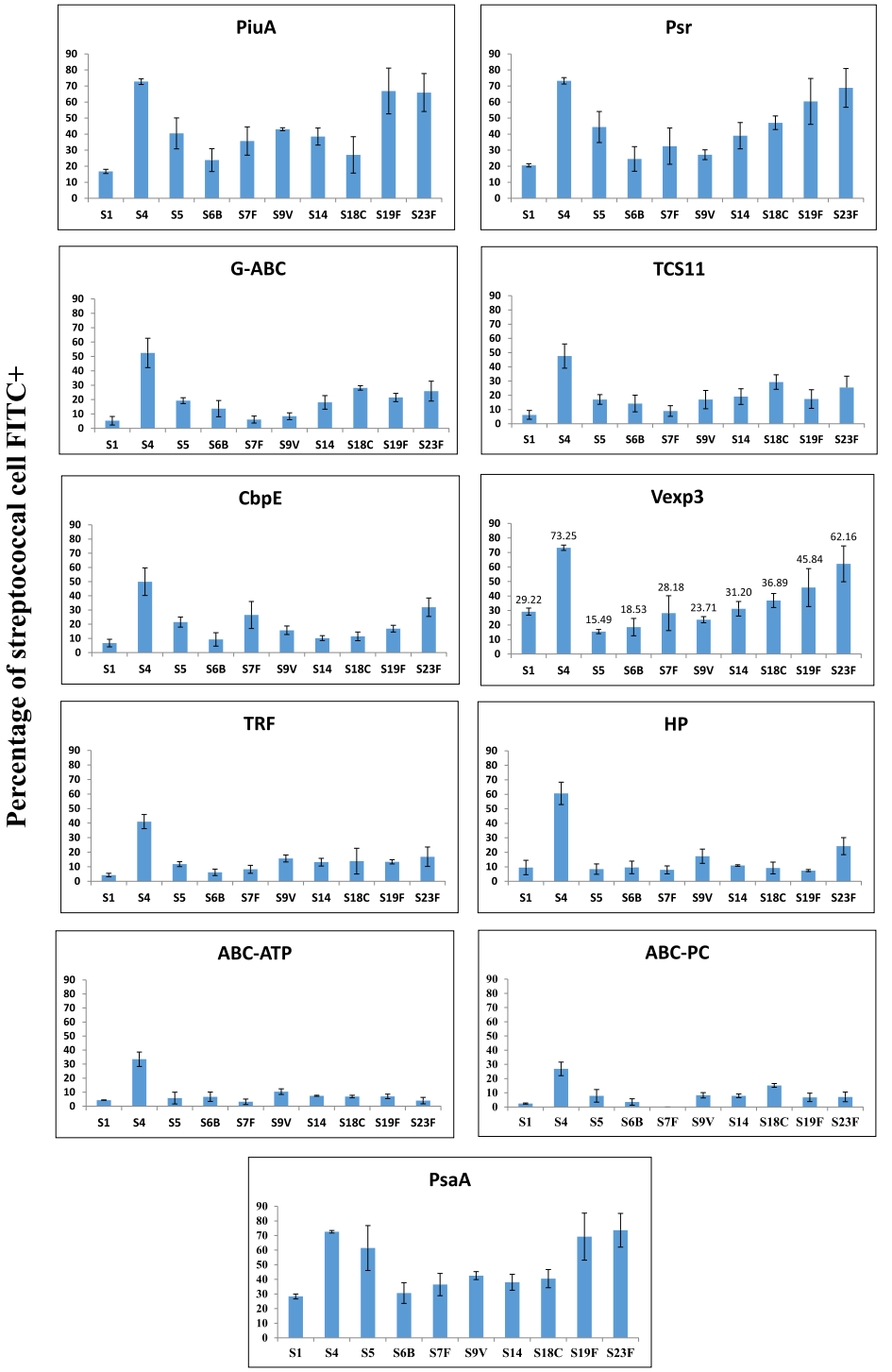

PiuA
Psr
G-ABC
TCS11
CbpE
Vexp3
TRF
HP
ABC-ATP
ABC-PC
PsaA
S1 S4 S5 S6B S7F S9V S14 S18C S19F S23F
29.22
73.25
15.49
18.53
28.18
23.71
31.20
36.89
45.84
62.16
Percentage of streptococcal cell FITC+
Pneumococcal serotypes evaluated

Fig. 5 Percentage of cells expressing the pneumococcal proteins on the cell surface of different pneumococcal serotypes. The polyclonal anti-PsaA was used as a positive control for the labeling of the PsaA protein on the bacterial cell surface. As a negative control, we used pneumococci marked with the secondary antibody FITC-conjugated and serum from unimmunized mice (conjugated and negative control, respectively). The values shown on the graphs are those resulting from subtracting the percentage of cells FITC+, for each protein marked/evaluated, of the percentage of cells stained non-specifically using the pre-immune serum (negative control). The experiments were performed among three to six times and the error bar is shown

analyzed in this study, except for S7F (serotype 7F) which did not react with the anti-ABC-PC serum. In this work, we demonstrate the histogram graphics for the protein expression on the cell surface of the pneumococcal serotypes 1 and 4. The graphics were representatives for the data obtained for the other pneumococcal serotypes. As we can observe, the protein expression ranged among pneumococcal serotypes (Fig. 5). For example, the proteins PsaA, PiuA, Psr, and Vexp3 were highly accessible to antibodies or were highly expressed on pneumococcal cell surface, whereas other proteins like as TRF, ABC-ATP, ABC-PC, and HP were not.

It is important to highlight that for this study, we did not use any denaturing or permeabilizer agent in the buffers, so the detected proteins refer to those expressed and exposed ones on the surface of the pneumococcal cell. A total of three to six independent labeling experiments were performed on different days. As negative controls, we used pneumococcal cells not marked, in order to eliminate any event of auto-fluorescence; we also used labeled cells only with the secondary antibody conjugate in the absence of a primary antibody, to eliminate non-specific cell markers and pre-immune serum (serum from not immunized mice) and also to eliminate non-specific markings. As positive control markers, we used anti-PsaA antibodies and polyclonal anti-10-valent vaccine. It is described that the PsaA protein is expressed on the bacterial cell surface [33] while the 10-valent anti-sera is able to recognize polysaccharides in the bacterial cell surface of the ten pneumococcal serotypes used in this study (data not shown for marking polysaccharides). In addition, other controls were added to the assay: the pneumococcal serotypes 4 and 9V were marked with anti-PsaA and anti-PiuA polyclonal antibodies, adsorbed and not adsorbed with *E. coli* lysate in order to demonstrate that the displayed labeling was specific to proteins under study. We also include in our analysis the marking of *S. agalactiae* to demonstrate the non-cross-reactivity of generated polyclonal antibodies.

ELISA to Study the Interaction of Recombinant Proteins with the Extracellular Matrix Proteins

In order to identify new host-pneumococcal interactions that may play a role in colonization and disease progress, the ability of these proteins in interacting with EMC—laminin, fibrinogen, collagen IV, and fibronectin—was evaluated by ELISA assay (Fig. 6). A portion of the repetitive domains of Leptospiral immunoglobulin-like (LigB) was used as a positive control. This protein-comprising amino acids 582-943 of *Leptospira interrogans* serovar Lai, which has previously been cloned and expressed in our laboratory and had its ability to interact with extracellular matrix proteins also described [34–36]. As a negative control, a fragment of the LigB protein-comprising amino acids 131-649 of *L interrogans* serovar Pomona was used, which has no interaction with these matrix proteins [35].

We did not observe non-specific reactions between mouse anti-IgG antibody, linked to horseradish peroxidase, with pneumococcal proteins, and the average of the readings was obtained at Abs_{450} between 0.05 and 0.1; these averages were considered “the white.” To

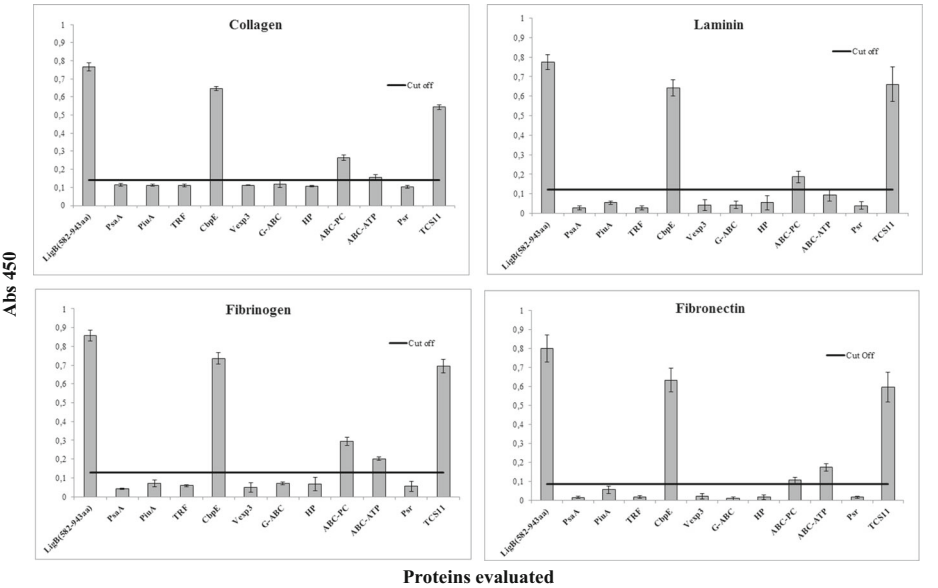


Fig. 6 Analysis of the interaction of recombinant pneumococcal proteins with ECM proteins by ELISA. Pneumococcal protein evaluation is indicated on the "x" axis and the reading of the Abs450 on the "y" axis. The ECM proteins tested are indicated at the top of the graphics. The experiments were performed in triplicate and the standard deviation bar is indicated. The *black line* indicates the "cutoff" value

calculate the "cut off," we subtract the average readings (at Abs_{450}) of the negative controls from the average readings obtained for white and added to this value twice the standard deviation.

The ELISA assay showed a strong interaction of CbpE, TCS11, and LigB (control) proteins with all tested matrix proteins, followed by ABC-PC and ABC-ATP proteins with weak interaction. No pneumococcal proteins analyzed showed a positive reaction/binding with the extracellular matrix proteins evaluated in this study.

Discussion

S. pneumoniae is the main causative agent of respiratory infections leading yearly to the death of about 1.6 million people. Due to the significant virulence developed by this human pathogen, the need of new drugs and vaccines is pressing. Surface-exposed proteins, which play multiple roles in the virulence process, are considered potential target candidates, and their therapeutical validation requires functional characterization [12].

In this study, we demonstrate that the predictive data obtained in silico (selection of pneumococcal surface proteins using reverse vaccinology) [22] were confirmed by FACS, demonstrating the exposure of all selected proteins on the pneumococcal cell surface. In addition, the expression level of these proteins ranged among the ten pneumococcal serotypes analyzed. This phenomenon may be a result of differential expression of the gene products during bacterial growth or partial binding inhibition of the antibodies caused by other surface molecules, such as the capsule. Since the pneumococcal adhesion is highly dependent on the ability of bacterial proteins to interact with ligands on the host cell surface, the presence of

capsule may negatively affect these interactions [37–39] and explain the different expressions of these select proteins among vaccine serotypes used in this study.

In [39], PspA, CbpA, and PsrP proteins showed different levels of accessibility to antibodies, and this accessibility depends on the presence or absence of capsule on the bacterial surface. Furthermore, the higher and lower accessibility of the antibody is related to capsular type. In this same study [39], it was demonstrated that the antibodies against some pneumococcal proteins of the pneumococcal serotypes 6A, 7F, and 23F showed reduced accessibility as compared to serotype 4, which demonstrated the highest accessibility. Furthermore, they demonstrated that the presence of capsule blocked the accessibility to antibodies when it is compared to encapsulated and non-capsulated strains. Our data were in accordance with previous report showing a high impact of capsule type in the ability of immobilized antibody against PspA, CbpA, and PsrP proteins to capture pneumococci [37]. Capsule types 6B, 7F, and 23F, for instance, permitted less accessibility than did capsule type 4; therefore, the biochemical properties of these capsule types alter their exposure on pneumococcal surface [39].

This differential expression and/or recognition of proteins expressed on the surface of pneumococci can also be explained through the hypothesis of the occurrence of a differential expression of proteins in pneumococcal serotypes, as demonstrated in the study [40]. However, to assert which event is occurring, in vitro differential expression of the evaluated pneumococcal proteins or change in the accessibility of these proteins to antibodies, additional studies such as RT-PCR and evaluation of capsule production must be performed. However, in our work, the objective was only to verify if the selected proteins were really expressed on the surface of pneumococci (like indicated by the in silico analyses) or not.

Additionally, anti-sera raised against recombinant proteins and obtained from SPN5 were able to recognize the corresponding proteins in all pneumococcal serotypes, indicating that these proteins contain conserved regions among serotypes [22]. Furthermore, searching for peptides containing conserved epitopes is an important feature of new vaccine design.

The pneumococcal capsule is one of the major determinants of organism invasiveness, resulting in high morbidity and mortality rates [2]. Clinical trials with pneumococcal capsular conjugate vaccines in children have demonstrated a significant reduction of carriages of vaccine serotypes in the nasopharynx. This benefit, however, was accompanied by an increase of non-vaccine serotype colonization [41, 42, 3, 12, 43]. Thus, protein antigens, such as those identified here, may overcome the problem of capsular serotype replacement. They might also act synergistically with capsular conjugate vaccines providing protection against both mucosal and invasive disease, particularly if they are expressed during nasopharyngeal colonization.

The MAPIA identified different patterns of antigenicity of pneumococcal recombinant proteins against sera from patients with meningitis. It is important to note that not all proteins evaluated in this study were expressed in their original size; in some cases, signal peptide and transmembrane regions were removed. The removal of these portions may interfere with protein conformation and lead to a lower antigenicity of the recombinant proteins. Furthermore, regulation of pneumococcal gene expression is a complex process. Pneumococcus can upregulate or downregulate its own protein expression depending on in vitro or in vivo growth conditions, according to clinical presentation of the disease or even during the biofilm formation [40, 44, 45]. Our data do indicate that they are expressed in vivo, although the presence of an immune response against these antigens is not by itself predictive of protection. They are immunogenic during an infection in the host and are serologically cross-reactive among all pneumococcal serotypes tested (the recombinant proteins obtained from the

serotype 5 and the sera tested are from the patients infected with different pneumococcal serotypes).

The sera obtained from healthy children ("control sera") recognized some of the proteins tested, demonstrating that healthy individuals may have antibodies of anti-pneumococcal proteins. This is somewhat expected since this bacteria is a colonizer of the human nasopharynx and in fact, several studies have reported the presence of anti-pneumococcal protein antibodies in healthy children and adults [46–49]. However, we can see that few proteins were recognized by these sera of children that were not ill at the time of the collection. However, the proteins that were recognized by the children's sera showed signal intensity, in the Western blotting, lower than that displayed by sera from infected patients.

While considerable work has been done to examine the effect of capsule type on complement deposition and the serotype on the ability of pneumococci to colonize the nasopharynx and survive in bloodstream [50–53], we know little about the impact of capsule type on bacterial adhesion function. Since pneumonia is the most common serious disease caused by *S. pneumoniae* and the host-cell adhesion is a key step in the pathogenesis of the invasive disease [37, 52, 20, 54], it is also important to study the interaction of the pneumococcal protein with the host extracellular matrix proteins. Several studies have used ECM proteins for characterizing pneumococcal proteins, including studies with PfbA protein [55], α-enolase [56], PspC [57], Hic [58], and CbpE [59, 19].

Previous studies have demonstrated the interaction of CbpE protein with fibronectin, fibrinogen, laminin, and collagen IV with some differences between the results. In [19], it is described that CbpE does not present an interaction with collagen and fibronectin while in [59], a weak interaction of the CbpE with this ECM (collagen and fibronectin) is described. In our study, we observed a high interaction of CbpE with both ECMs described above. Despite the high protein identity of CbpE between the different serotypes, we thought that the differences of interaction between CbpE and ECM might be due to differences in the virulence among *S. pneumoniae* serotypes and strains used in the studies or because of the different methodologies used to verify this interaction.

In our study, we found that the TCS11 protein interacted with the ECM proteins examined in a similar manner to that observed for CbpE, while the ABC-PC and ABC-ATP proteins interacted weakly with the ECM proteins. Considering the high interest in finding surface proteins involved on adherence that would elicit protection against a broader range of pneumococcal strains, it is interesting to note that the identified recombinant proteins, expressed on the cell surface of vaccine serotypes, interacted with EMC proteins. The proteins ABC-PC and ABC-ATP presented weaker interaction with ECM proteins when compared with the interaction demonstrated by the CbpE and TCS11 proteins; perhaps they can also play a role in the accession process of pneumococcus to host cells. However, we did not find data in the literature describing the role of these proteins in the interaction with ECM proteins and in the bacterial adhesion process. In addition, so far, from what we know, these proteins are not described in the studies encompassing the pneumococcal surface proteins.

TCS11, in the literature, described that this regulation is an important part of the bacterium's response to vancomycin stress [60]. In [61], it is related that the exposure to cigarette smoke condensate resulted in the significant upregulation of the genes encoding the two components of the three-regulatory system TCS11 (consisting of the sensor kinase - hk - and its cognate

response regulator - rr), in the setting of increased biofilm formation. These effects of cigarette smoke on the pneumococcus may contribute to colonization of the airways by this microbial pathogen. Our data demonstrate that TCS11 binds to some ECM proteins and contributes with the observation made by [61].

Thus, the results of this study suggest that the proteins CbpE, TCS11, ABC-PC, and ABC-ATP may have some role in adherence of the pneumococcus to host cells, once they were able to bind to ECM proteins. But on the other hand, these proteins were not strongly recognized by sera of analyzed patients, unlike the CbpE, PiuA and TRF proteins. This reinforces the complex nature of the interaction between the agent and the host. In the case of pneumococcal disease, we know that this involves the variability on the expression of the polysaccharide capsule (related directly on the adhesion and on the infection process), the differential expression of surface proteins (affecting the adhesion of bacteria to eukaryotic host cells), and the variability on the expression of pneumococcal proteins (depending on whether the bacteria is colonizing the host or already fixed some immunological barriers and causing infection and what type of disease—pneumonia, sepsis, or meningitis).

In this context, we believe that not only one or two proteins of a pathogen can inhibit the role of the infectious agent, especially considering the high capability of adaptation of the *S. pneumoniae*. Thus, it should be considered that a set of antigens is important in host-pathogen interaction, mainly when we thought in recombinant vaccine.

Conclusions

In conclusion, the data reveal that predicted pneumococcal proteins, previously selected by microbial genomic approach, are exposed on the cell surface of the pneumococcus, as demonstrated by FACS analysis. Furthermore, these selected proteins show a high degree of conservation between the pneumococcal serotypes; in our analysis, we demonstrate this cross-reactive serotype by FACS and MAPIA. In FACS, we can see that the mice polyclonal antibody (serum from mice immunized with recombinant proteins obtained from serotype 5) was able to recognize the proteins in the cell surface of ten different pneumococcal serotypes. In MAPIA, sera from patients infected with different pneumococcal serotypes recognized the recombinant proteins. We also demonstrate that some proteins presented in our study were able to bind to host extracellular matrix proteins; this observation may indicate some contribution of these proteins in the colonization of the airways by this microbial pathogen. These observed features have important implications for a pneumococcal vaccine based on recombinant proteins and suggest that these antigens can be potential targets for future studies as candidates for a vaccine. But, some limitations of this preliminary study preclude the establishment of a definitive relationship between these observations and the true effectiveness in induction of a protection in animal model.

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Compliance with Ethical Standards

Ethics Statement The study protocol was approved by Institutional Review Board (IOR00002090/IRB000026120) of the Gonçalo Moniz Research Center (CPqGM- Centro de Pesquisa Gonçalo Moniz) of the Oswaldo Cruz Foundation (Fiocruz—Fundação Oswaldo Cruz) and by the Brazilian National Ethical Committee on Human Subject (CONEP—Comissão Nacional de Ética em Pesquisa) (protocol 25000.045797/1005-18). The participants involved in the project provided written informed consent. The protocol and procedures presented in this project are in accordance with the Helsinki Declaration of 1975, as revised in 2000. Regarding the use of animals, the study protocol was approved by the Ethics Committee on Animal Use (CEUA—Comitê de Ética no Uso de Animais) of the Oswaldo Cruz Foundation protocol number P-7/10-4 titled "Development of Brazilian vaccine against *Streptococcus pneumoniae*."

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