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# Human antibody response towards the pneumococcal surface proteins PspA and PspC during invasive pneumococcal infection☆

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**Abstract**

IgG antibodies against pneumococcal surface protein A, family 1 (PspA1) and family 2 (PspA2), protein C (PspC), and protein Hic were investigated in 41 patients with invasive pneumococcal disease. Pre-existing antibody levels against the four pneumococcal proteins were not significantly different from those found in 40 patients with non-pneumococcal bacteremia or 80 healthy controls. However, during convalescense a strong immune response developed especially against PspA, and there was a high degree of cross-reactivity between PspA- and PspC-antibodies. Our findings on immunogenicity and cross-reactivity suggest that in a future pneumococcal protein based vaccine, only a limited number of proteins could be sufficient.


*Keywords:* *Streptococcus pneumoniae*; PspA; PspC; Antibody response

## 1. Introduction

*Streptococcus pneumoniae*, a ubiquitous human pathogen, is a major cause of invasive infections such as community-acquired pneumonia and meningitis, and is a common cause of otitis media, sinusitis and bronchitis. Risk groups are primarily young children, the elderly, and patients with immunodeficiencies. Invasive disease still carries significant mortality, and together with the emergence of pneumococcal strains with multiple antimicrobial resistances the need for preventive strategies against this common pathogen has been emphasized. The potential advantage of pneumococcal protein based vaccines compared to polysaccharide vaccines would be protection against multiple pneumococcal serotypes and induction of memory responses with a longer duration and immunogenicity in all ages. In animal models, several pneumococcal proteins have been shown to elicit a protective immune response [1].

Pneumococcal surface protein A (PspA) is a surface protein expressed in all strains of pneumococci. The molecule has an N-terminal α-helical domain exposed on the bacterial surface and a C-terminal choline-binding repeat region responsible for the attachment to the cell wall [2,3]. PspA sequences are variable, especially in the N-terminal domain, and have been classified into three families based on sequence homologies. Ninety-five percent of pneumococcal strains carry PspA of family 1 (PspA1) or family 2 (PspA2) [4], and there is a significant serological cross-reactivity between the different families. PspA interferes with complement function and has been shown to function as a virulence factor for pneumococcal infection [5].

Pneumococcal surface protein C (PspC) is strucurally related to PspA but has a distinct α-helical N-terminal region

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[6]. The protein is present in approximately 75% of all strains of *S. pneumoniae* and interferes with the complement system by the binding of factor H. Hic, the factor H-binding inhibitor of complement, is a distinct variant of PspC, which is found mainly in serotype 3 strains of pneumococci. Sequence homology to PspC proteins of other strains is most dominant in the N-terminal region [7,8].

In the present study, we investigated the immune response towards PspA1, PspA2, PspC, and Hic in patients with pneumonia and pneumococcal bacteremia, and correlated the levels of specific IgG antibodies to the presence of the respective surface proteins in strains isolated from the patients.

## 2. Materials and methods

### 2.1. Patients and sera

Serum samples were obtained from 41 patients with invasive infection with pneumococcal bacteremia (median age 51 years, age range 30–83 years, male/female ratio 22/19) and 40 patients with non-pneumococcal bacteremia (median age 63 years, age range 21–92 years, male/female ratio 13/27) that were hospitalized at the Clinic of Infectious Diseases, Lund University Hospital, Sweden, between 1992 and 2003. No patients with bronchial asthma, chronic obstructive pulmonary disease, previous pneumonia, malignancy or immunosuppressant therapy were included in the study. In the pneumococcal bacteremia group all patients had characteristic pneumonial infiltrates on chest X-rays together with positive blood cultures. The patients in the non-pneumococcal bacteremia group had growth in blood cultures of *Escherichia coli* ($n=28$), *Salmonella species* ($n=3$), *Staphylococcus aureus* ($n=3$), *Neisseria meningitidis* ($n=2$), *Campylobacter jejuni*, *Capnocytophaga canimorsus*, *Klebsiella pneumoniae* or *Streptococcus bovis* ($n=1$). Acute sera were obtained within 1–4 days after onset of infection (mean/median: 2.6/3 days) from the pneumococcal bacteremia group and after 1–5 days (mean/median: 2.3/3 days) from the non-pneumococcal bacteremia group. Convalescent sera were obtained from 22 patients with pneumococcal bacteremia >7 days after onset of infection. Serum samples from 80 healthy Swedish blood donors (median age 50 years, male/female ratio 40/40) were also collected.

### 2.2. Bacterial isolates

Serotyping of the pneumococcal strains was performed at the Department of Clinical Microbiology, Lund University Hospital, using the quellung reaction [9] with antisera from Statens Seruminstitut, Copenhagen, Denmark.

### 2.3. PCR analysis

To analyze the presence of *psp* genes in different pneumococcal isolates, PCR template DNA was prepared by boiling bacteria for 5 min in sterile water. Cell debris was removed by centrifugation and 1 μl of the boiled lysate was used for PCR amplifications. The primer pspA-F (5′-CCGGATCCAGCGTCGCTATCTTAGGGGCTGGTT-3′) is located in the highly conserved 5′-end of the *pspA* gene, while the primers pspA-R1 (5′-TTTCTGGCTCAT(CT)AACTGCTTTC-3′) and pspA-R2 (5′-TGGGGGTGGAGTTTCTTTCTTCTTCATCT-3′) correspond to diverging regions and distinguish between *pspA* families 1 and 2, respectively. Similarly, the primer pspC-F (5′-CGACGAATAGCTGAAGAGG-3′) is located in a conserved region upstream of the *pspC* locus, while the primers pspC-R (5′-CCACATACCGTTTTCTTGTTTCCAGCC-3′) and hic-R (5′-TAGATCC(AT)GT(AT)GATGGCAA-3′) correspond to regions that discriminate between *pspC* and the less common *hic*-like alleles. Each isolate was analysed with four primer pair combinations, thus determining the type of gene found in the *pspA* and *pspC* loci. PCR products were analyzed by agarose (0.8%) gel electrophoresis.

### 2.4. Proteins

PspA family 1 (aa 1–303), PspA family 2 (aa 1–411) and PspC (aa 1–445) proteins were from recombinant cloned fragments in pET20b (Novagen Inc.) as previously described [5,6]. The three recombinant proteins were purified by nickel affinity chromatography according to the Novagen system. A fragment of Hic, covering aa 1–223 of the mature Hic protein, was expressed as a GST fusion protein as previously described [7].

### 2.5. ELISA methods

ELISA experiments were performed as previously described [10]. After coating microtiter plates (Maxisorb, NUNC) with different pneumococcal antigens (1 μg/ml), a fixed dilution (1/400) of serum samples from patients and from healthy individuals were added to the wells. Bound antibodies were detected by a horseradish peroxide-conjugated antibody against human IgG diluted 1/3000 (Bio-Rad). After the substrate reaction the OD at 415 nm was determined. A blank without serum was included in quadruplicate on each plate and these OD values were subtracted from the values obtained with the serum samples. An ELISA index was calculated by dividing the mean OD value for each serum sample by the mean OD value for a standard positive serum sample run in quadruplicate on each plate.

### 2.6. Statistical evaluation

The two-sample $z$-test, assuming unequal variances, was used for comparing antibody levels in different groups. $p$ values $<0.05$ were regarded as statistically significant.

## 3. Results

### 3.1. Bacterial isolates

Eleven different serotypes were found; type 1 ($n = 7$), type 3 ($n = 3$), type 4 ($n = 4$), type 6 ($n = 1$), type 7 ($n = 7$), type 8 ($n = 3$), type 9 ($n = 6$), type 12 ($n = 3$), type 14 ($n = 5$), type 22 ($n = 1$), and type 23 ($n = 1$). Thus, types 1, 7 or 9 comprised 50% of the isolates. PCR analysis of 35 of the 41 pneumococcal isolates revealed that 20 (57%) isolates harboured the *pspA*1 gene and 15 (43%) the *pspA*2 gene. Twenty-six (74%) isolates had the *pspC* gene and four (11%) the *hic* gene. In five isolates, no genes encoding PspC or Hic were found by the PCR assays used.

### 3.2. Serologic testing

Detectable IgG antibody levels against PspA1, PspA2, PspC and Hic were found in all serum samples analyzed from patients with pneumococcal bacteremia, non-pneumococcal bacteremia and from healthy blood donors. There were no significant differences ($p > 0.05$) in antibody levels against PspA1, PspA2, PspC, or Hic when comparing the acute sera from pneumococcal bacteremia patients with acute sera from non-pneumococcal bacteremia patients or sera from healthy controls, respectively (Fig. 1). Demographic data were comparable between the groups.

When analyzing immune response during convalescence in the pneumococcal bacteremia group 19 paired serum samples were available with corresponding pneumococcal strains (Table 1). The day-to-day variation of each ELISA was 9.4% for PspA1, 7.5% for PspA2, 4.0% for PspC, and 9.8% for Hic. An increase of ELISA index between acute and convalescent serum of >25% was therefore judged as significant, and an increase of 25–250% was expressed as (+). An increase of 250–500% was assigned (++), and an increase of >500% as (+++). All 19 patients developed a significant titer rise against PspA1, although nine of the 19 isolates lacked the *pspA*1 gene when analyzed by PCR. Eighteen patients showed an antibody response against PspA2, although ten isolates lacked the *pspA*2 gene. Thus, most patients were capable of responses that were cross-reactive for both PspA major families. Eleven patients responded serologically to PspC, whereof two corresponding isolates were PCR-negative for the *pspC* gene, and 15 patients developed a significant antibody response to Hic although all but two patients were infected with *hic* PCR-negative strains. Again, there was an apparent cross-reactivity of the response to the PspC/Hic family of proteins.

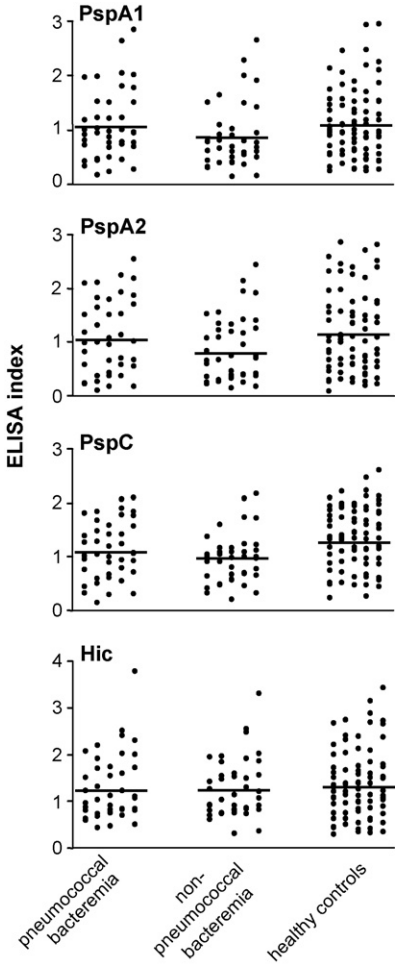


Fig. 1. ELISA index values against PspA1, PspA2, PspC, and Hic in acute serum from patients with pneumococcal bacteremia and non-pneumococcal bacteremia, and in healthy controls. Mean values are shown as horizontal lines. The $p$ values when comparing the pneumococcal bacteriemia and the non-pneumococcal bacteriemia group in a two-sample $z$-test were 0.11 (PspA1), 0.10 (PspA2), 0.13 (PspC), and 0.91 (Hic). When comparing the pneumococcal bacteriemia group with healthy controls the $p$ values were 0.77 (PspA1), 0.41 (PspA2), 0.48 (PspC), and 0.64 (Hic).

## 4. Discussion

To our knowledge, the present study is the first report on the immune response against both PspA families and against PspC and Hic in adults with invasive pneumococcal infection. We tested whether it was possible to detect a difference in pre-existing antibody titers to proteins from the PspA and the PspC/Hic families among patients who succumbed to pneumococcal bacteremia. At mean 2.6 days after onset of clinical symptoms of infection, we were unable to demonstrate any quantitative difference in the level of antibody as compared with controls. There is, however, a possibility that antibody production could have begun prior to the start of symptoms of the subsequent bacteremia, such as at the time of pneumococcal colonization. In a recent study of experimental human pneumococcal carriage, only PspA and PspC were immunogenic in colonized subjects, as determined by a statistically significant rise in the serum IgG titer [11]. This contrasted, however, with the lack of a significant titer rise against pneumococcal capsular polysaccharide upon this challenge. Although we could not detect reduced titers for susceptibles in acute-phase sera, it is, however, still possible that qualitative differences of the antibodies could exist.

Table 1
Immune response during convalescence against PspA1, PspA2, PspC and Hic in relation to the presence of the corresponding genes in the respective pneumococcal isolate from 19 patients

| Patient | Serotype | Gene content | | | | ELISA index increase | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *pspA1* | *pspA2* | *pspC* | *hic* | PspA1 | PspA2 | PspC | Hic |
| 1 | 23 | + | − | + | − | +++ | + | +++ | + |
| 2 | 1 | + | − | + | − | ++ | + | − | − |
| 3 | 1 | + | − | + | − | + | + | + | + |
| 4 | 1 | + | − | + | − | +++ | ++ | ++ | +++ |
| 5 | 1 | + | − | + | − | ++ | ++ | + | ++ |
| 6 | 1 | + | − | + | − | ++ | +++ | +++ | ++ |
| 7 | 1 | + | − | + | − | ++ | ++ | ++ | ++ |
| 8 | 1 | + | − | + | − | + | + | − | − |
| 9 | 3 | + | − | − | + | ++ | ++ | + | + |
| 10 | 8 | + | − | − | − | ++ | + | − | + |
| 11 | 7 | − | + | + | − | + | + | + | + |
| 12 | 4 | − | + | + | − | + | + | ++ | + |
| 13 | 7 | − | + | + | − | + | − | − | + |
| 14 | 7 | − | + | + | − | + | + | − | − |
| 15 | 4 | − | + | + | − | +++ | +++ | ++ | + |
| 16 | 14 | − | + | + | − | ++ | ++ | − | + |
| 17 | 14 | − | + | + | − | + | + | − | + |
| 18 | 3 | − | + | − | + | + | ++ | − | − |
| 19 | 14 | − | + | − | − | ++ | + | + | + |

+ corresponds to an increase of the ELISA index of 25–250% during convalescence; ++ corresponds to an increase of 250–500%; +++ refers to an increase of ELISA index of >500%.

Data on the immune response to pneumococcal proteins in severe human clinical infections are scarce. In children, PspA antibody levels seemed to correlate with invasive pneumococcal disease [12], but the situation in adults is more complex as they have probably experienced multiple exposures. In two previous studies of invasive pneumococcal infection in adults, pre-existing anti-PspA [13] or anti-PspA1 and anti-PspA2 [14] antibody levels were not different from those of healthy controls, which is in agreement with our results. However, as onset of infection was defined as day of first isolation of the pneumococcus [13] or time of hospital admission [14] and no information was given on time of onset of clinical symtoms, an early boosted immune response was not excluded. Also, the careful medical history obtained from our pneumococcal or non-pneumococcal bacteremic patients should with high probability exclude previous invasive pneumococcal infection or pneumonia of any etiology, and also exclude any immunosuppression likely to affect the immune response against pneumococci.

Interestingly, during infection a strong immune response developed in our patients especially against PspA1 and PspA2, but also in high percentage against PspC and Hic. All patients in our study survived, and one might speculate whether a less pronounced immune response would be seen among non-survivors. Furthermore, a boosted response to a respiratory infection might prevent further invasion in the future, but to elucidate this, a study of recurrent invasive pneumococcal infection would be required.

As PspA seemed to be more immunogenic than PspC and Hic among our patients, this could be in accordance with previous findings that PspC is expressed more strongly by pneumococci in the nasopharynx than by pneumococci in the blood [15]. There are some conflicting data as to whether PspC can serve as a virulence factor in animal models of bacteremia [8,16]. The induction of PspC antibody might suggest that some PspC is present during invasive infection or it might reflect a response to prior colonization.

McCool et al. noted a significant strain-to-strain cross-reactivity in the IgG response elicited against PspA and PspC proteins upon colonization [11]. Another previous study on 14 patients with invasive pneumococcal infection demonstrated antibodies that cross-reacted between the families 1 and 2 of PspA [14]. Our study design allowed for the measurement of family-specific sera within PspA or PspC groups by choosing to include antigens for each of the major PspA families and by including both PspC and Hic. Responses were considered with respect to the PspA/C gene content of the isolate causing the bacteremia. All antigens tested in ELISA consisted of $NH_2$-terminal portions alone of PspA and PspC proteins and thus, lacked any of the conserved cell wall binding domains that PspA shares with PspC. In an all versus all comparison of the four proteins used in this study, there is some sequence identity between the two PspA fragments, and between the PspC and Hic fragments (Fig. 2). A very limited degree of identity was also observed between the two protein families. However, for any of the paired sequences, an optimal alignment necessitated introduction of numerous gaps, and sequence identity is limited to short stretches of contiguous amino acids (10–30 amino acids). Nevertheless, we detected a response to each family of PspA and to both PspC and Hic in most patients despite the fact that each isolate only exhibited one PspA family and either PspC or Hic. This indicated substantial cross-reactivity between PspA families 1 and 2 antibodies, as well as between PspC and Hic antibodies. If

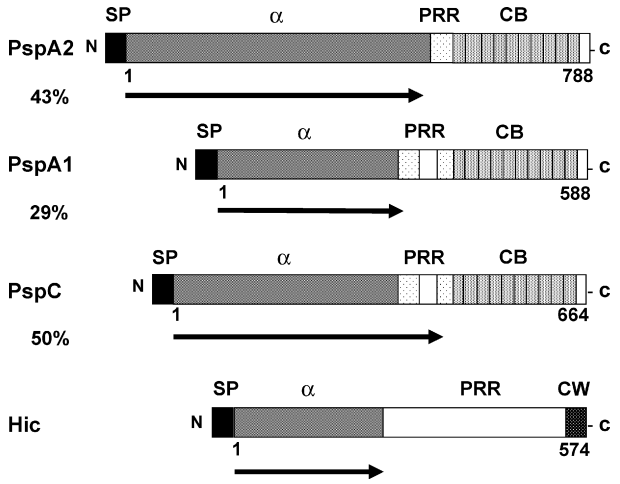


Fig. 2. Schematic drawing of the pneumococcal surface proteins used in this study. Functional/structural modules are indicated as follows: signal peptide (SP), α-helical region (α), proline-rich region (PRR), choline-binding motif (CB), and cell wall-attachment motif (CW). The recombinantly expressed fragments are indicated by horizontal arrows. Percentages show amino acid identity between the fragments using the Smith–Waterman algorithm, and proteins are ordered with closest neighbours.

any of these proteins would be part of a future pneumococcal vaccine our data indicate that, contrary to capsular polysaccharides, only a limited number of pneumococcal proteins could be sufficient.

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