Pneumococcal surface protein A (PspA) family distribution among clinical isolates from adults over 50 years of age collected in seven countries

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The pneumococcal surface protein PspA, a cell-wall-associated surface protein, is a promising component for pneumococcal vaccines. In this study, the distribution of the PspA family was determined in a panel of invasive and clinically important pneumococcal isolates from adults over 50 years of age, collected between 1995 and 2002. One thousand eight hundred and forty-seven recent isolates from invasive pneumococcal disease were obtained from seven Western countries, together with clinical data. An ELISA-based serological method was standardized in order to determine the PspA family and clade distribution. Molecular tests were used when isolates were non-typable by ELISA (PspA family typing by PCR). Only 42 (2.3%) isolates were non-typable by ELISA and PspA family typing by PCR was performed. Finally, 3 isolates were considered as non-pneumococcal and 1844 were classified as follows: 749 (40.6%) were PspA family 1, 1078 (58.5%) were PspA family 2, 13 (0.7%) were PspA family 1 and 2 and 4 (0.2%) remained non-typable. The cross-reactivity of antibodies to PspAs of different clades was confirmed. In conclusion, inclusion of PspA family 1 and family 2 in future pneumococcal vaccines would ensure broad coverage of pneumococcal strains infecting people over 50 years of age.

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INTRODUCTION

Streptococcus pneumoniae is a major cause of morbidity and mortality worldwide. In adults, the 23-valent capsular polysaccharide (PS) vaccine offers a clinical protection rate of about 60% and confers serotype-specific protection (Jackson *et al.*, 2003). A second-generation PS-conjugate vaccine with 7-valent serotypes was designed (Eskola, 2000) in order to exploit T-cell-dependent properties such as immunogenicity in early infancy, stimulation of high levels of IgG isotype antibodies and enhanced immunological-memory response. This vaccine is now indicated for young children, in whom it has been shown to be effective; it may also slightly reduce the risk of pneumococcal disease in adults, through indirect effects (Whitney *et al.*, 2003).

The challenge of achieving full serotype coverage with PS-conjugate vaccines in adults, along with the potential reactogenicity of conjugate vaccines in older individuals,

have stimulated attempts to develop protein-based pneumococcal vaccines (Bogaert *et al.*, 2004). The most intensively studied candidate protein is pneumococcal surface protein A (PspA), a cell-wall-associated surface protein. Other potential protein antigens include pneumococcal surface adhesin A (PsaA) and pneumolysin. PspA, a structurally related choline-binding protein, binds human lactoferrin (Shaper *et al.*, 2004) and interferes with complement deposition on the bacterial surface (Ren *et al.*, 2004). PspA is relatively variable at the DNA and protein sequence levels, and two major alleles have been identified (PspA family 1 and family 2); each family is subdivided into several clades. PspA family 1 contains two clades (1 and 2), and PspA family 2 contains three clades (3, 4 and 5). A third family contains one clade (clade 6) but it currently includes only a few strains (S. K. Hollingshead, unpublished data).

This PspA classification has been made using the sequence of the complete α -helical portion of *pspA* genes from 24 unrelated isolates (Hollingshead *et al.*, 2000). The surface-exposed region of the α -helical portion, which includes protection-eliciting epitopes, encompasses the 100 aa residue *pspA* region that defines the clade, making it critical for

Abbreviations: IPD, invasive pneumococcal disease; NT, non-typable; PS, polysaccharide; PspA, pneumococcal surface protein A; UAB, University of Alabama at Birmingham.

the development of PspA as a vaccine component (Gor *et al.*, 2005; Roche *et al.*, 2003). To accommodate the variability, it has been postulated that a combination of three PspA antigens from PspA family 1 (clade 2) and PspA family 2 (clade 3 and clade 4) would elicit protection against the vast majority of *S. pneumoniae*.

In this study, we determined the PspA family distribution in a panel of invasive and clinically important pneumococcal isolates from people over 50 years of age, a risk group for severe pneumococcal disease. The isolates and clinical data were obtained from seven pneumococcal centres in Australia, Europe (France, Spain, Sweden, the UK) and North America (Canada, the USA).

METHODS

Study design and isolates. The primary objective was to evaluate the distribution of PspA in a panel of isolates from adults over 50 years of age. The main test used to examine this was an ELISA-based serological test, using rabbit antisera to PspA clades 2, 3 and 4 produced by Sanofi Pasteur. The prediction was that these three antisera pools would collectively detect at least 95% of all *S. pneumoniae* isolates from patients with invasive pneumococcal disease (IPD) in this age group. Differential reactivity patterns with the sera would allow classification of all isolates detected as to PspA family. A secondary objective was to characterize the cross-reactivity of antibodies to PspAs of different clades, based on their differential reactivity patterns with the three rabbit antisera pools.

Pneumococcal isolates were deemed eligible for inclusion if they were: (1) from a patient aged over 50 years; (2) isolated between 1995 and 2002; (3) accompanied by information on demographic characteristics (age group, sex), clinical features (principal clinical site of infection, outcome), capsular serotype and antibiotic susceptibility; (4) obtained from a normally sterile body fluid (blood, cerebrospinal fluid, pleural fluid, peritoneal fluid or other). The percentage of IPD isolates collected in each centre represented the size of the population over 50 years of age from its country (for instance, 50% of the isolates came from the USA) and the distribution of IPD isolates was divided into two age groups as follows: 50–64 (30%) and ≥65 years (70%). The isolates were stratified in this way for two reasons. First, 65 years is the age usually used as the entry to the category known as elderly adult. Second, 65 is the age recommended for delivery of the 23-valent pneumococcal vaccine.

Only one pneumococcal isolate per patient was included in the study, and a unique code was generated for each isolate before shipment, to guarantee patient anonymity. This code was also used to export demographic and clinical data with a standardized electronic questionnaire developed with Excel for Windows (Microsoft). Clinical data were sent to Sanofi Pasteur, merged into a single database, and checked for coherence before analysis. All isolates were shipped frozen to the testing laboratory at the University of Alabama at Birmingham (UAB). In addition, upon arrival at the test site, 10% of randomly selected isolates were subjected to comparison of PspA family classification by ELISA and molecular tests.

Preparation of pneumococcal lysates. Isolates were tested for optochin sensitivity and for bile solubility using standard methods. Optochin-resistant isolates that also failed the bile solubility test were discarded. To prepare the lysates, a 30 ml culture of bacteria was grown in Todd-Hewitt broth (Difco BD) supplemented with 0.5% yeast extract, until OD₄₂₀ 0.5–0.6 was reached. A lysis buffer (5 ml), containing 0.01% sodium lauryl sulfate, 4.4% sodium citrate and

0.1% deoxycholic acid, was added to the 30 ml culture. The resulting lysates were incubated for 1 h at room temperature, subcultured to ensure bacterial death, and stored at –80 °C until needed.

ELISA method. An ELISA method was used to test the isolates' ability to bind to antibodies raised in rabbits immunized with one of three recombinant PspA antigens, namely rPspA-Rx1 (aa 1–315) from family 1 clade 2, rPspA-EF3296 (aa 1–479) from family 2 clade 3 and rPspA-EF5668 (aa 1–370) from family 2 clade 4. Three pools of rabbit antisera, each containing serum from four rabbits immunized with the same antigen, were provided by Sanofi Pasteur. The pooled antisera were diluted in PBS containing 2% BSA and Tween 20 (0.05%) by addition of 0.25, 0.4 and 0.05 µl ml^{–1} for clade 2, 3 and 4 antisera, respectively. These dilutions were chosen to normalize the reactivity of each antiserum with the relevant immunization strain.

ELISA plates (Maxisorb; Nunc) were coated with *S. pneumoniae* lysate supernatant diluted 1:5 in 0.01 M PBS and incubated at 4 °C for 16–24 h. The lysate solutions contained between 200 and 250 µg ml^{–1} total protein. The plates were then washed three times with PBS containing 0.05% Tween 20 (PBS-T). Coated plates were blocked with PBS-T containing 2% BSA for 1 h at room temperature, then incubated with rabbit antisera for 90 min at room temperature. Each of the three rabbit antisera pools was diluted and tested in triplicate. Two negative controls were also tested in triplicate: (1) wells not coated with lysate, but tested with rabbit antisera, (2) wells coated with lysate, but tested with non-immune rabbit serum. After incubation with antisera, the plates were washed three times with PBS-T and then incubated with alkaline phosphatase-conjugated purified goat immunoglobulin to rabbit antiserum IgG (Southern Biotechnology Associates) to detect bound antibodies. After a final wash, the plates were developed with *p*-nitrophenyl phosphate substrate (Sigma), and the A₄₀₅ value determined. Reactivity with each rabbit antiserum was expressed as the ratio between the mean of triplicate wells and the mean of negative control wells coated with non-immune rabbit serum. All test samples with ratios above 2 were considered 'scoreable'.

As each pneumococcal lysate could react with one, two or all three of the antisera, they were recorded as reactive with clades 2, 3, 4, 2 and 3, 2 and 4, 3 and 4 or 2, 3 and 4. Most lysates reacted differently with the three antisera. The final clade classification of each lysate was based on the antiserum with the strongest reactivity. Finally, the results are reported here as PspA family 1, PspA family 2, PspA family 1 and 2 or non-typable (NT) by ELISA. Isolates classified as PspA family 1 and 2 or NT by ELISA were subjected to PCR amplification of the *pspA* gene.

DNA purification procedure. A 50 ml culture of bacteria was grown as described above and the bacterial cells collected by centrifugation. Cells were resuspended in 0.35 ml Tris-EDTA buffer (containing 10 mM Tris/HCl, 1 mM EDTA, pH 8.0) plus 3.5 µl 10% SDS solution and heated for 15 min at 65 °C. Potassium acetate pH 5.0 (70.7 µl) 5 M solution was added and the incubation continued at 65 °C for another 15 min. The mixture was frozen at –20 °C for 30 min and centrifuged at 4 °C at 12 000 r.p.m. for 35 min. The supernatant was transferred to a new tube and 707 µl cold 95% ethanol was added, the tube was inverted for 30 s and centrifuged for 5 min at 4 °C. The pellets were further washed with 707 µl 70% ethanol twice more, allowed to dry on the bench overnight and were then resuspended in 50 µl Tris-EDTA buffer.

Molecular tests. Molecular tests consisted of PspA family typing by PCR. This test was applied to a random 10% of the isolates and to all isolates classified as PspA family 1 and 2 or NT by ELISA. Genomic DNA was extracted from them and the samples were stored at 4 °C. The recommended concentration of bacterial DNA is 1–10 ng per PCR reaction mix, corresponding to 1 µl DNA solution from these extractions.

Genomic DNA from each pneumococcal isolate was used as the template for a series of PspA family-specific PCR reactions. The oligonucleotide primers used were LSM12 (5'-CCGGATCCAGCGT-CGCTATCTTAGGGGCTGGTT-3') (Swiatlo *et al.*, 1997) and SKH63 (5'-TTTCTGGCTCATYAACTGCTTTC-3') for PspA family 1, and LSM12 and SKH52 (5'-TGGGGGTGGAGTTTCTTCTTCATCT-3') for PspA family 2. PCRs were carried out in a standard mix of 25 µl containing 2.5 mM MgCl₂, 200 µM (each) deoxynucleoside triphosphate, 50 pmol each primer and 2.5 units *Taq* DNA polymerase. The following cycling conditions were used: 95 °C (3 min), then 30 cycles of 95 °C (1 min), 62 °C (1 min) and 72 °C (3 min), and finally, 72 °C (10 min), using an Eppendorf Gradient Master cycler. The expected sizes of the amplified PCR products were approximately 1000 and 1200 bp for PspA family 1 and 2, respectively.

The PCR products (3 µl) were loaded on 0.8% agarose gels and electrophoresed at 80 V for 1 h. The gels were stained with 0.5 g ethidium bromide ml⁻¹ to visualize the DNA. Controls for PspA family 1 (strain Rx1) and PspA family 2 (strain EF3296) were run in each set of reactions. PCR products were initially run at the calculated optimal annealing temperature of 62 °C for the PspA family 1 and PspA family 2 primer pairs (see above). PCR products were recorded as PspA family 1, PspA family 2, PspA family 1 and 2, or not amplified.

NT isolates. Isolates that were not initially amplified by PCR were retested with the same cycling pattern at an annealing temperature of 58 °C, or, if this also failed, 55 °C. These lower annealing temperatures

can compensate for sequence divergence in the primer region in some pneumococcal isolates. A test for the rarely seen PspA family 3 was also run, using primers SKH41 (5'-CGCACAGACTTAACAG-ATGAAC-3') and SKH42 (5'-CTTGTCATCAACTTCATCC-3'). Isolates negative in all molecular tests (less than 0.5% of the total) were classified as NT. These remaining isolates were tested for the presence of the pneumolysin gene by PCR using primers *ply1* (5'-ATTTCTGTAAACAGCTACCAACGA-3') and *ply2* (5'-GAATTC-CCTGTCTTTTCAAAGTC-3'), and for the presence of a *pspA* gene by PCR using primers LSM12 (as described above) and SKH02 (5'-CCACATACCGTTTCTTGTTCAGCC-3') (Hollingshead *et al.*, 2000).

All statistics were run using SAS software (version 8 for Windows; SAS Institute).

RESULTS AND DISCUSSION

The final analysis included 1847 IPD isolates and the distribution of the isolates by country was as follows: 100 (5.4%) from Australia, 148 (8.0%) from Canada, 215 (11.6%) from France, 150 (8.1%) from Spain, 67 (3.6%) from Sweden, 237 (12.8%) from the UK and 930 (50.5%) from the USA. Table 1 shows the distribution of isolates by country, age group, sex, clinical syndrome, outcome and

Table 1. Clinical and demographic properties of isolates by country

	No. of isolates							Total	
	Australia	Canada	France	Spain	Sweden	UK	USA	<i>n</i>	%
IPD cases	100	148	215	150	67	237	930	1847	—
Age group									
50–64 years	29	45	50	65	36	70	435	730	39.5
≥65 years	71	103	165	85	31	167	495	1117	60.5
Sex*									
Male	59	79	107	95	36	102	470	948	51.3
Female	41	69	105	55	31	135	460	896	48.5
Syndrome									
Pneumonia	25	120	55	142	56	167	650	1215	65.8
Bacteraemia†	22	16	133	0	3	18	227	419	22.7
Meningitis	2	6	20	6	3	32	—	69	3.7
Peritonitis	0	0	3	1	0	0	—	4	0.2
Other	22	6	4	1	5	0	53	91	4.9
Unknown	29	—	—	—	—	20	—	49	2.7
Outcome									
Survived	—	115	15	—	61	—	755	946	51.2
Died	—	33	10	—	6	19	156	224	12.1
Unknown	100	—	190	150	—	218	19	677	36.7
Penicillin‡									
Susceptible	83	131	5	112	65	218	702	1316	71.3
Intermediate	11	11	101	21	1	12	89	246	13.3
Resistant	6	3	105	14	1	7	129	265	14.3
Unknown	—	3	4	3	—	—	10	20	1.1

*Missing data *n*=3.

†Bacteraemia without focus.

‡Isolates were classified as susceptible when the MIC was ≤0.06 mg l⁻¹, intermediate when 0.12 ≤ MIC ≤ 1 mg l⁻¹ and resistant to penicillin when MIC was ≥ 2 mg l⁻¹.

Table 2. ELISA determination of PspA family based on antisera to PspA clades

	No.	%
PspA family 1		
Clade-like 2	740	40.1
PspA family 2		
Clade-like 3	757	41.0
Clade-like 4	294	15.9
Clade-like 3 and 4	2	0.1
Total PspA family 2	1053	57.0
PspA family 1 and 2		
Clade-like 2 and 3	8	0.3
Clade-like 2 and 4	1	0.05
Clade-like 2 and 3 and 4	3	0.25
Total PspA family 1 and 2	12	0.6
Non-typable	42	2.3
Total	1847	100.0

penicillin susceptibility. Sites of isolation were as follows: 1734 (93.9%) from blood, 91 (4.9%) from cerebrospinal fluid, 14 (0.8%) from pleural fluid, 4 (0.2%) from peritoneal fluid and 4 (0.2%) from other normally sterile sites. Pneumococcal vaccine status was available for only 233 patients (12.6%), of whom 23 (1.2%) had been vaccinated with a 23-valent PS vaccine. A total of 53 pneumococcal capsular serotypes were identified among the 1656 IPD isolates for which this information was available (data missing in 191 cases). The most frequent capsular serotypes in IPD cases were as follows: 14 (16.7%), 6A (11.5%), 9V (10.2%), 23F (9.2%), 4 (8.1%) and 6B (7.9%). The percentage of serotypes included in the 23-valent PS was 89.7% (1485/1656) and in the 7-valent vaccine was 58.6% (971/1656).

ELISA showed that 97.7% (1805/1847) of isolates from IPD expressed PspA families 1, 2, or 1 and 2 (Table 2). In addition, 1431/1847 (77.5%) of the isolates were ELISA reactive with 2 or 3 antisera. Only 374/1847 (20.3%) samples reacted with a single antiserum. The remaining 42/1847 (2.3%)

isolates were ELISA NT. The isolates reacting with two or three antisera formed four groups: 2 and 3 ($n=162$), 2 and 4 ($n=291$), 3 and 4 ($n=487$), and 2 and 3 and 4 ($n=491$). Among them, 14 lysates had similar reactivity with 2 or 3 antisera and were classified as clade-like 2 and 3 ($n=8$), clade-like 2 and 4 ($n=1$), clade-like 3 and 4 ($n=2$), or clade-like 2 and 3 and 4 ($n=3$) as shown in Table 2.

The 42 pneumococcal isolates that were NT by ELISA were subjected to molecular tests. Of the isolates, 35 (83.3%) had *pspA* families 1, 2, or 1 and 2 by molecular methods, while 7 isolates (16.7%) appeared to lack a *pspA* gene of family 1 or 2 (0.4% of the 1847 isolates tested). Among the seven NT isolates, three (one from Spain and two from the USA) were optochin resistant and lacked the pneumolysin gene, although they were soluble in deoxycholate. These three isolates were considered either atypical or non-pneumococcal species. The other four (one from France, one from Spain and two from the USA) NT isolates were typical pneumococci in that they were optochin sensitive, bile soluble and contained a pneumolysin gene. This group contained two isolates with evidence of a family 3 *pspA*-like gene, while two isolates remained negative in all relevant tests. Overall, 99.8% (1840/1844) of pneumococcal isolates were classified as follows: 747 (40.5%) PspA family 1, 1081 (58.8%) PspA family 2, and 12 (0.7%) PspA family 1 and 2 (Table 3). In addition, all but 1 of the 12 isolates classified as PspA family 1 and 2 by ELISA kept this classification after PCR; the remaining 1 isolate was classified as PspA family 2 after PCR. PspA family 2 predominated in all countries except Australia and the UK. The asymmetry of PspA family 1 and family 2 (25.1 and 73%, respectively) was strongest for French isolates. This was possibly due to the selection of isolates with diminished penicillin susceptibility in France (E. Varon, Centre de Référence du Pneumocoque, Hôpital Européen Georges Pompidou, Paris, France, personal communication). Patient age and clinical manifestations appeared to have no effect on the PspA family distribution (data not shown).

Overall, little association between the capsular serotype and the PspA family was found. In a few cases, isolates of a given

Table 3. Final PspA family classification after molecular tests

	PspA family 1	PspA family 2	PspA family 1 and 2	Non-typable	Total
Australia	54	46	—	—	100
Canada	55	91	2	—	148
France	52	159	3	1	215
Spain	57	90	1	2	150
Sweden	28	39	—	—	67
UK	120	117	—	—	237
USA	381	539	6	4	930
Total	747 (40.4%)	1081 (58.5%)	12 (0.7%)	7* (0.4%)	1847

*Two isolates were PspA family 3, two appeared to be typical pneumococci and three were considered either atypical pneumococci or from another species.

capsular serotype belonged mainly to a single PspA family (serotype 3 and family 2, serotype 9V and family 2, serotype 22F and family 1, etc.), while other capsular serotypes contained both PspA families (serotypes 14, 19A, 23F, etc.). The serotypes for which there was an apparent association tended to be serotypes for which there are few circulating clones (Brueggemann *et al.*, 2003). Most serotypes that have large numbers of distinct clones showed no association. However, the presence of highly prevalent antibiotic-resistant clones might cause a sampling bias, leading to an apparent association for those types.

One hundred and ninety three IPD isolates were randomly selected at UAB; the ELISA and molecular results for these were 98.0 % concordant (Table 4). No significant difference was found in the distribution of PspA family 1 and family 2 with the two methods (McNemar's test using exact inference, two-sided, $P=0.5$). In addition, all isolates belonging to PspA family 1 or 2 were confirmed to belong to the relevant family by molecular methods.

PspA represents a promising component for pneumococcal protein vaccines and it was essential to characterize the variability of PspA family distribution in clinical isolates from people aged over 50 years. A broadly protective PspA-based vaccine composed of three PspA antigens from family 1 (clade 2) and family 2 (clades 3 and 4) must accommodate this variability, and the variability should be monitored over time to account for population fluctuations that might impact upon its distribution. This is supported by the observation that human immunization with a clade 2 recombinant PspA (from strain Rx1) elicits increased binding of post-immune (versus pre-immune) sera to 37 pneumococcal isolates expressing PspAs of clades 1, 2, 3, 4 and 5 (Briles *et al.*, 2000a; Nabors *et al.*, 2000), and by the characterization of the antibody responses to IPDs in older adults (Baril *et al.*, 2004).

We showed that the ELISA technique developed for this study is a convenient and powerful tool for screening large numbers of pneumococcal isolates for their PspA family. Moreover, ELISA assays are also less costly and time-consuming than molecular tests. Using this assay, we confirmed that the vast majority of isolates from adults over

50 years of age with pneumococcal disease express PspA. In addition, two-thirds of isolates had PspA proteins that reacted with more than one of the three antiserum pools used. This indicates that most PspAs have shared epitopes, but the basis of this cross-reactivity is unclear. However, it is worthy of note that the shared epitopes often crossed the border between family 1 and family 2 PspAs. It could be hypothesized that recombination creates mosaic *pspA* genes with shared characteristics. We conclude that the high rate of cross-reactivity observed here supports the view that recombination among *pspA* genes is frequent.

Moreover, the molecular tests, applied to 10 % of randomly selected isolates, showed that the ELISA method correctly classified 98 % of isolates. The remaining 2 % of isolates were classified as family 1, 2, or 1 and 2 PspA by molecular methods, bringing to 100 % the total proportion of isolates with PspAs belonging to these two families. Thus, although pneumococcal isolates from older individuals showed a broad spectrum of capsular serotypes, there was no evidence that these isolates had significantly divergent PspAs beyond those previously identified (Beall *et al.*, 2000; Brandileone *et al.*, 2004; Mollerach *et al.*, 2004; Vela Coral *et al.*, 2001). We showed that isolates that were NT by ELISA (42/1847) but typable by molecular methods may show differences in PspA expression. Molecular analysis indicated the presence of a *pspA* gene, but it may have been inactive. A frameshifted *pspA* gene expressing a truncated PspA protein was reported in a nasopharyngeal isolate used in an experimental model of human colonization (McCool *et al.*, 2002).

We also applied the ELISA method to 242 sputum isolates reported as being probable cases of pneumococcal pneumonia by the centres. We found that the proportion of ELISA NT isolates (21/242) was significantly higher among sputum isolates than IPD isolates (8.7 % vs 2.3 %, Pearson χ^2 test, $P < 10^{-4}$). This might indicate a lower expression of PspA molecules on the surface of pneumococci that cause airway disease than on those that are invasive isolates. However, sputum isolates may also be carriage strains, which may be less likely to express *pspA* than invasive strains. This would be consistent with what has been observed in mouse models of infection (Briles *et al.*, 2000b; Ogunniyi *et al.*, 2000; Roche *et al.*, 2003).

Table 4. Concordance between the PspA family classifications obtained by ELISA and molecular tests for the randomly selected isolates ($n=193$)

ELISA	Molecular test		
	PspA family 1 ($n=76$)	PspA family 2 ($n=115$)	PspA family 1 and 2 ($n=2$)
PspA family 1	75	—	1
PspA family 2	1	114	1
PspA family 1 and 2	—	1	—
Non-typable	—	—	—

The lack of association between the capsular serotype and the PspA family may or may not reflect the relative diversity of clonal lineages of these serotypes; those with a predominant PspA family may be predominantly of a related lineage, while those with mixed PspA families may represent a number of diverse lineages. If, on the other hand, clonal diversity is similar for the serotypes with marked PspA family preference, then the tendency for some serotypes to inherit a particular PspA family could reflect a subtle interaction between PspA of a certain family and some capsule types. As both PspA and other surface proteins contribute to deficient complement deposition on the pneumococcal surface, this possibility is logical, but it remains to be tested. Moreover, PspAs of family 1 and family 2 appear to have similar functions, based on experiments in which PspAs were switched relative to the strain background prior to functional tests (Ren *et al.*, 2003). Similarly, no relation between penicillin susceptibility and the PspA family classification was found here. Isolates demonstrating a degree of penicillin resistance (intermediate or resistant) represented 28 % of PspA family 1 isolates and 30 % of PspA family 2 isolates from IPD cases. As discussed above, within particular resistant clones there may be a tendency to inherit a particular PspA family. Finally, the distribution of the isolates between PspA family 1 and family 2 in the present study did not differ substantially from that originally reported (Hollingshead *et al.*, 2000) or in more recent studies from Latin American countries (Brandileone *et al.*, 2004; Mollerach *et al.*, 2004; Vela Coral *et al.*, 2001).

Our results expand and confirm previous information from human studies and animal models on the extent of cross-reactivity of antibodies to PspA from different clades within PspA families. The use of molecular testing methods allowed us to classify 80 % of the isolates that were non-typable by ELISA. The results of this study support the inclusion of PspA family 1 and family 2 molecules in a protein-based pneumococcal vaccine in order to ensure broad coverage of pneumococcal strains infecting adults over 50 years of age. Similar analyses of PspA and of other pneumococcal proteins considered as potential vaccine candidates should be performed for isolates from other geographic regions or age groups.

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REFERENCES

- Baril, L., Briles, D. E., Crozier, P., King, J., Punar, M., Hollingshead, S. K. & McCormick, J. B. (2004). Characterization of antibodies to PspA and PsaA in adults over 50 years of age with invasive pneumococcal disease. *Vaccine* **23**, 789–793.
- Beall, B., Gherardi, G., Facklam, R. R. & Hollingshead, S. K. (2000). Pneumococcal *pspA* sequence types of prevalent multiresistant pneumococcal strains in the United States and of internationally disseminated clones. *J Clin Microbiol* **38**, 3663–3669.
- Bogaert, D., Hermans, P. W., Adrian, P. V., Rumke, H. C. & de Groot, R. (2004). Pneumococcal vaccines: an update on current strategies. *Vaccine* **22**, 2209–2220.
- Brandileone, M. C., Andrade, A. L., Teles, E. M., Zanella, R. C., Yara, T. I., Di Fabio, J. L. & Hollingshead, S. K. (2004). Typing of pneumococcal surface protein A (PspA) in *Streptococcus pneumoniae* isolated during epidemiological surveillance in Brazil: towards novel pneumococcal protein vaccines. *Vaccine* **22**, 3890–3896.
- Briles, D. E., Hollingshead, S. K., King, J., Swift, A., Braun, P. A., Park, M. K., Ferguson, L. M., Nahm, M. H. & Nabors, G. S. (2000a). Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. *J Infect Dis* **182**, 1694–1701.
- Briles, D. E., Ades, E., Paton, J. C., Sampson, J. S., Carlone, G. M., Huebner, R. C., Virolainen, A., Swiatlo, E. & Hollingshead, S. K. (2000b). Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. *Infect Immun* **68**, 769–800.
- Brueggemann, A. B., Griffiths, D. T., Meats, E., Peto, T., Crook, D. W. & Spratt, B. G. (2003). Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. *J Infect Dis* **187**, 1424–1432.
- Eskola, J. (2000). Immunogenicity of pneumococcal conjugate vaccines. *Pediatr Infect Dis J* **19**, 388–393.
- Gor, D. O., Ding, X., Briles, D. E., Jacobs, M. R. & Greenspan, N. S. (2005). Relationship between surface accessibility for PpmA, PsaA, and PspA and antibody-mediated immunity to systemic infection by *Streptococcus pneumoniae*. *Infect Immun* **73**, 1304–1312.
- Hollingshead, S. K., Becker, R. & Briles, D. E. (2000). Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. *Infect Immun* **68**, 5889–5900.
- Jackson, L. A., Neuzil, K. M., Yu, O. & 7 other authors, for the Vaccine Safety Datalink (2003). Effectiveness of pneumococcal polysaccharide vaccine in older adults. *N Engl J Med* **348**, 1747–1755.

- McCool, T. L., Cate, T. R., Moy, G. & Weiser, J. N. (2002). The immune response to pneumococcal proteins during experimental human carriage. *J Exp Med* **195**, 359–365.
- Mollerach, M., Regueira, M., Bonofiglio, L., Callejo, R., Pace, J., Di Fabio, J. L., Hollingshead, S., Briles, D. & the *Streptococcus pneumoniae* Working Group (2004). Invasive *Streptococcus pneumoniae* isolates from Argentinian children: serotypes, families of pneumococcal surface protein A (PspA) and genetic diversity. *Epidemiol Infect* **132**, 177–184.
- Nabors, G. S., Braun, P. A., Herrmann, D. J. & 8 other authors (2000). Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. *Vaccine* **18**, 1743–1754.
- Ogunniyi, A. D., Folland, R. L., Briles, D. E., Hollingshead, S. K. & Paton, J. C. (2000). Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. *Infect Immun* **68**, 3028–3033.
- Ren, B., Szalai, A. J., Thomas, O., Hollingshead, S. K. & Briles, D. E. (2003). Both family 1 and family 2 PspA proteins can inhibit complement deposition and confer virulence to a capsular serotype 3 strain of *Streptococcus pneumoniae*. *Infect Immun* **71**, 75–85.
- Ren, B., Szalai, A. J., Hollingshead, S. K. & Briles, D. E. (2004). Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. *Infect Immun* **72**, 114–122.
- Roche, H., Ren, B., McDaniel, L. S., Hakansson, A. & Briles, D. E. (2003). Relative roles of genetic background and variation in PspA in the ability of antibodies to PspA to protect against capsular type 3 and 4 strains of *Streptococcus pneumoniae*. *Infect Immun* **71**, 4498–4505.
- Shaper, M., Hollingshead, S. K., Benjamin, W. H., Jr & Briles, D. E. (2004). PspA protects *Streptococcus pneumoniae* from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin. *Infect Immun* **72**, 5031–5040.
- Swiatlo, E., Brooks-Walter, A., Briles, D. E. & McDaniel, L. S. (1997). Oligonucleotides identify conserved and variable regions of *pspA* and *pspA*-like sequences of *Streptococcus pneumoniae*. *Gene* **188**, 279–284.
- Vela Coral, M. C., Fonseca, N., Castaneda, E., Di Fabio, J. L., Hollingshead, S. K. & Briles, D. E. (2001). Pneumococcal surface protein A of invasive *Streptococcus pneumoniae* isolates from Colombian children. *Emerg Infect Dis* **7**, 832–836.
- Whitney, C. G., Farley, M. M., Hadler, J. & 10 other authors, for the Active Bacterial Core Surveillance of the Emerging Infections Program Network (2003). Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. *N Engl J Med* **348**, 1737–1746.