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# PspA family typing and PCR-based DNA fingerprinting with BOX A1R primer of pneumococci from the blood of patients in the USA with and without sickle cell disease

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## SUMMARY

Disease and mortality rates for *Streptococcus pneumoniae* infections are much higher in patients with sickle cell disease (SCD) than in age-matched patients without SCD. Pneumococcal surface protein A (PspA) has been proposed as a component in human vaccines against *S. pneumoniae* to provide greater breadth of coverage than can be obtained with the 7-valent conjugate vaccine. The cross-reactivity of PspA is associated with the 'PspA family' structure. In this study we examined strains of *S. pneumoniae* from patients with and without SCD to determine whether the strains infecting the hypersusceptible population of SCD patients were limited to the same two PspA families already known to comprise over 95% of strains infecting non-SCD patients. Each strain was also evaluated according to the presence or absence of specific PCR fragments based on repetitive BOX elements to screen for possible SCD-associated clonal structure. Strains from SCD and non-SCD patients were similarly dispersed among the most common BOX PCR groups and strains from both groups expressed a similar distribution of PspA variants. Thus, a PspA vaccine designed for the population at large should also be appropriate for patients with SCD.

## INTRODUCTION

*Streptococcus pneumoniae* is an important human pathogen causing life-threatening infections such as pneumonia, bacteraemia, and meningitis, as well as less severe, but highly prevalent infections such as otitis media and sinusitis [1, 2]. The age groups at highest risk for pneumococcal sepsis include the elderly, children less than 2 years of age, persons with poor or absent antibody responses to pneumococcal capsular polysaccharides, and those who lack good splenic function because of anatomic asplenia or sickle cell disease (SCD) [3, 4]. Because of their heightened susceptibility it is likely that SCD patients might be infected with strains of *S. pneumoniae* that may not be highly virulent in children without SCD. The risk of pneumococcal infection among children with SCD is greatly reduced by penicillin prophylaxis [3, 5]. However, the emergence of antimicrobial drug-resistant strains of pneumococci has threatened to reduce the efficacy of antibiotic prophylaxis and further increase the morbidity and mortality caused by this pathogen [6]. Highly effective pneumococcal vaccines are, therefore, important to this patient population.

*S. pneumoniae* produce a capsular polysaccharide, which is a major surface component [7]. Like other encapsulated bacteria, the pneumococcus relies on its

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polysaccharide surface coat, or capsule, to help it evade the host's phagocytic defences. Antibodies to the capsular polysaccharide are known to be protective [8] and hence this antigen has long been a focus of vaccine development. However, the purified capsular polysaccharide vaccine does not reliably induce protective antibody responses in children younger than 2 years – the age group that shows the highest incidence of invasive pneumococcal infection [4, 8]. A 7-valent polysaccharide–protein conjugate vaccine does appear to provide protection of immunologically normal children against pneumococcal bacteraemia [9]. Low or rapidly declining antibody levels after pneumococcal polysaccharide vaccinations have been noted among SCD patients [10] but this may be minimized, however, by the use of the conjugate vaccine which has been found to be immunogenic in SCD patients [11]. The poor antibody responses to capsular polysaccharide in SCD patients probably also contributes to their increased susceptibility to pneumococcal infection.

Other problems with capsular vaccines result from the fact that they do not contain all capsular types, and they fail to provide complete protection against otitis media and carriage [12]. Moreover, the present conjugate vaccine is too expensive for use in parts of the world with the most cases of SCD (between $200 and $300 per patient in the United States). Alternative approaches to pneumococcal vaccines are, therefore, being considered and immunization with non-capsular antigens that could induce protection against all serotypes may be an improvement. Pneumococcal surface protein A (PspA) has been shown to elicit antibodies in humans that were protective in an animal model [13]. PspA interferes with complement deposition on the pneumococcal surface [14] and is a potential vaccine but efficacy trials in humans have not yet been conducted.

Multiple variants of PspA have been detected. Based on the amino-acid sequences of the protection-eliciting region of the molecule, PspAs have been grouped into two major families which are subdivided into five clades [15]. Although all PspAs are somewhat cross-reactive, cross-reactions are strongest among members of the same PspA family [16] and as a result of genetic exchange in the environment these families are largely randomized among pneumococcal capsular serotypes. This serological heterogeneity indicates that a mixture of selected PspAs would be able to protect against infections with most pneumococci regardless of capsular type [17].

The high susceptibility of SCD patients to pneumococcal infection might be due to the fact that these patients are frequently infected with strains of *S. pneumoniae* that are not as virulent as the strains from non-SCD patients. Thus, it is possible that the subset of strains infecting SCD patients might not express the same PspAs as strains infecting non-SCD patients. If this were the case, a PspA vaccine for the population as a whole might not cover the majority of infections in SCD patients. To examine this possibility we have determined the PspA family of pneumococcal isolates from SCD patients with invasive infection and compared them with isolates from matched control patients. Differences in the genetic background of the strains from both groups were probed by PCR typing of conserved repetitive DNA sequences (BOX) within the pneumococcal genome [18].

## MATERIALS AND METHODS

### Bacterial isolates

Pneumococcal isolates from patients with and without SCD, were selected from a collection of the Centers for Disease Control and Prevention's (CDC) Pneumococcal Sentinel Surveillance System [19]. For this surveillance system, which was established primarily for the study of pneumococcal vaccine effectiveness, participating hospitals submitted all pneumococcal isolates from normally sterile body sites and provided basic patient demographic and clinical data on a standardized form. Isolates were confirmed to be *S. pneumoniae* by sensitivity to optochin and bile solubility and stored on defibrinated blood at $-70\,^{\circ}$C. Blood isolates from patients with SCD were randomly selected from cases reported from 1985 to 1996. For each isolate from a SCD patient, we attempted to identify two control blood isolates from non-SCD patients. One control isolate was matched for patient age by category (age $<2$, 2–5, 6–17, or $\geqslant 18$ years), for date of specimen collection (within 1 year), and for state of patient residence (control group 1). The second isolate was matched for age category, date of specimen collection, and capsular serotype (control group 2). Isolates in the two control groups were matched for different properties because the diversity of the control strains and their origins were too great to create a single group matched for age, date of specimen collection, capsular type, and state of residence.

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### Capsular typing

Serotyping and genetic typing of isolates were performed blind with regard to the source of the isolates. Isolates were serotyped upon receipt at the CDC by the Quellung reaction using type-specific capsular antisera prepared at CDC. The serotype was confirmed after retrieval and shipment to the University of Alabama at Birmingham Laboratory by slide agglutination with Danish group and typing sera (Statens Seruminstitut, Copenhagen, Denmark) [20]. The Quellung reaction was used to subtype group 6 organisms with 6A and 6B typing sera prepared at the University of Alabama [21].

### DNA extraction

Pneumococci from overnight blood agar plates were grown for 5 h in 10 ml of Todd–Hewitt broth (Difco, Franklin Lakes, NJ, USA) supplemented with 0·5% yeast extract. The cells were lysed by the addition of 350 $\mu$l of 10 mM Tris (pH 8) and 1 mM EDTA buffer, 35 $\mu$l 10% SDS and the protein pellet was extracted with 70·7 $\mu$l of 5 M potassium acetate and 707 $\mu$l ethanol to precipitate the DNA [22].

### BOX-PCR

BOX-PCR was performed using the primer BOXA1R, a subunit primer of *boxA* – an interspersed repetitive DNA sequence distributed widely among Gram-positive bacteria (DNA International, Inc., Lake Oswego, OR, USA) (sequence: 5′-CTACGGCAAG-GCGACGCTGACG-3′), Ready-To-Go (Pharmacia Biotech, Piscataway, NJ, USA) RAPD analysis beads (containing AmpliTaq), and DNA. The PCR programme was as follows: initial denaturation at 95 °C for 7 min, 30 cycles each of denaturation at 90 °C for 30 s, primer annealing at 52 °C for 1 min, extension at 65 °C for 8 min, and a single cycle extension at 65 °C for 16 min.

### Gel electrophoresis and pattern analysis

The amplified products were separated by size on a 1% agarose gel run in 89 mM Tris–borate, 2 mM EDTA buffer (pH 8) at 120 V for 3·25 h. The gel was stained with ethidium bromide (1 $\mu$g/ml) and viewed with UV light [23]. The molecular weights of individual bands were calculated by reference to a 1 kb DNA ladder (Life Technologies, Carlsbad, CA, USA) with a tolerance of 2% in band positions. Banding patterns were inspected visually and strains that differed from each other by more than three bands were placed in different BOX groups. Each strain was compared for capsule type relative to BOX group.

Table. *Frequency of serotype, PspA family, and BOX PCR type for* S. pneumoniae *isolates from blood of patients with sickle cell disease and controls*

| | Sickle cell disease *n* (%) | Control group 1[a] *n* (%) | Control group 2[b] *n* (%) |
|---|---|---|---|
| Serotype | | | |
| 19F | 6 (23·1) | 1 (7·7) | 6 (25·0) |
| 23F | 5 (19·2) | 3 (15·0) | 5 (20·8) |
| 14 | 4 (15·4) | 4 (20·0) | 4 (16·7) |
| 6B | 3 (11·5) | 3 (15·0) | 3 (12·5) |
| Other | 8 (31·0) | 9 (45·0) | 6 (25) |
| Serotype in 23-valent vaccine | 22 (84·6) | 16 (80·0) | 21 (87·5) |
| Serotype in 7-valent conjugate vaccine | 21 (80·8) | 13 (65·0) | 20 (83·3) |
| PspA family | | | |
| 1 | 12 (46·2) | 10 (50·0) | 12 (50·0) |
| 2 | 14 (53·8) | 10 (50·0) | 10 (41·7) |
| 1 and 2 | 0 | 0 | 1 (4·2) |
| Non-typable | 0 | 0 | 1 (4·2) |
| Box PCR | | | |
| A | 4 (15·4) | 2 (10·0) | 4 (16·7) |
| B | 5 (19·2) | 5 (25·0) | 5 (20·8) |
| C | 5 (19·2) | 5 (25·0) | 4 (16·7) |
| D | 2 (7·7) | 1 (5·0) | 5 (20·8) |
| E | 1 (3·8) | 1 (5·0) | 1 (4·2) |
| F | 2 (7·7) | 2 (10·0) | 4 (16·7) |
| G | 1 (3·8) | 0 | 0 |
| H | 2 (7·7) | 0 | 0 |
| I | 0 | 0 | 1 (4·2) |
| J | 4 (15·4) | 0 | 0 |
| K | 0 | 2 (10·0) | 0 |
| L | 0 | 1 (5·0) | 0 |
| M | 0 | 1 (5·0) | 0 |

[a] Matched for patient age, date of specimen collection, and state of patient residence.
[b] Matched patient age, date of specimen collection, and isolate serotype.

### PspA family typing

PCR was carried out on genomic DNA extracted as above. The primer LSM12 for family 1 was: 5′-CCGGATCCAGCGTCGCTATCTTAGGGGCTG-GTT-3′ and SKH63, 5′-TTTCTGGCTCATC/TAA-CTGCTTTC-3′. For family 2 the SKH63 primer was replaced with SKH52, 5′-TGGGGGTGGAGTT-TCTTCTTCATCT-3′. The following PCR conditions

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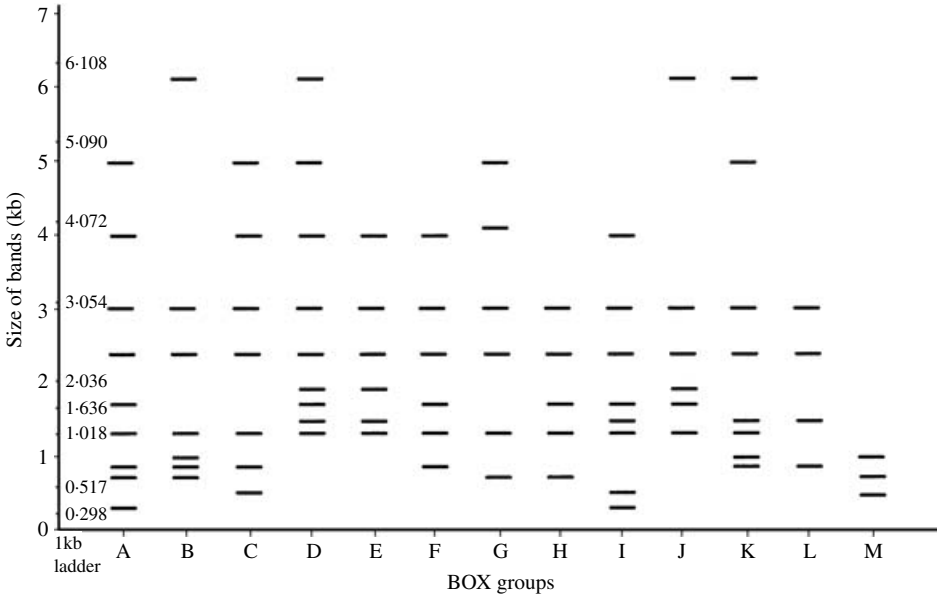


**Fig.** Patterns of PCR DNA bands for BOX groups A–M.

were used: 30 cycles of 95 °C (3 min), 95 °C (1 min), 62 °C (1 min), 72 °C (3 min) and 72 °C (10 min) [15] in a PTC 150 Thermocycler (MJ Research, Waltham, MA, USA). Primer LSM12 is in the promoter region of *pspA* and primers SKH2, SKH52, and SKH63 are all C-terminal to the $\alpha$-helical region of *pspA* [15, 16]. Agarose gel electrophoresis was carried out at 80 V for 1·5 h and the gel was stained with ethidium bromide (0·5 $\mu$g/ml). The amplicon sizes for PspA families 1 and 2 were 1000 bp and 1200 bp respectively and isolates were assigned to a family according to the presence of the specific amplicon. Strains BG9739 (clade 1) and AC122 (clade 3) were used as PspA family controls 1 and 2 respectively.

### Data analysis

Mantel–Haenszel summary $\chi^2$ values for matched data were calculated using Epi-Info 6.0 (CDC) to compare the frequencies of serotype, PspA family, and BOX PCR type for isolates from patients with SCD and the two control groups. Statistical analyses were performed using the $2 \times 2$ contingency tables and Fisher's exact test to compare capsular serotype, PspA family, and BOX PCR type for SCD patients and patients in both control groups.

## RESULTS AND DISCUSSION

### Data collection

Twenty-six isolates of *S. pneumoniae* were identified from patients with SCD (Table). Their ages ranged from 15 months to 43 years (median 3 years). Twenty isolates were included in control group 1 (matched for patient age, specimen collection date, and state of residence) and 24 isolates in control group 2 (matched for patient age, specimen collection date, and serotype). One control group was matched for capsular type because different capsular types tend to be associated with slightly different distributions of PspA families [16, 24]. The other control group was not matched for capsular type to allow a more random sample of control strains.

All patients with SCD were African American. Race/ethnicity was reported for 17 (85%) of group 1 controls and 14 (70%) of group 2 controls; 64% of group 1 and 20% of group 2 were African American ($P < 0{\cdot}05$ for both control groups). Fifty-three per cent of SCD patients were female compared to 31% in controls. This difference was not statistically significant.

### Capsular type, PspA family and BOX type

The distribution of capsular serotype, PspA family, and BOX PCR type for SCD patients and patients in both control groups was similar (Table); Mantel–Haenzel summary $\chi^2$ ($P$ value $> 0{\cdot}05$ for all comparisons). The degree of diversity in BOX PCR type was similar to that observed among strains of pneumococci in a prior study [18]. However, the BOX- type pattern designations here (Fig.) do not coincide with the type patterns defined in the original study [18].

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The group assignments of each isolate were compared to matched controls. For control group 1, only 1 out of 20 isolates from SCD patients matched their control from a non-SCD child in BOX type. This was not surprising as group 1 controls were not matched for capsular serotype. BOX types have been shown to have a strong association with capsular serotype and strains of differing serotypes are known to generally differ in genetic background (BOX type) [21, 25, 26]. However, for control group 2, the BOX type of SCD and control isolates matched on 17 out of 24 occasions. This finding also agrees with what is known about pneumococcal clones in that some serotypes are represented by very few clonal backgrounds, while other serotypes are very clonally diverse [27]. Furthermore, the matches in BOX type suggests that SCD children are infected with strains similar to their counterparts without SCD. The most heterogeneity in BOX profile appeared in serotype 19F. The high diversity of 19F strains has been noted in previous studies [26, 28].

Of the 70 isolates from patients with SCD and controls, 16 (22·9%) had serotypes not represented in the 7-valent pneumococcal conjugate vaccine. These were serotypes 6A and 13, three isolates each; serotypes 9A, 12F, and 38, two isolates each; and one isolate each of serotypes 1, 8, 16, and 33F. Nine of these 16 isolates were PspA family 1, six were PspA family 2, and one isolate was PspA families 1 and 2. In previous studies we have found that 99% of strains have at least one *pspA* gene and between 2% and 5% of strains have two *pspA* genes and many of these elaborate both PspA families [20, 29]. The distribution of capsular types among strains from SCD patients indicates that the capsule-conjugate would be no less appropriate for children with SCD as for children in general (Table).

All isolates from SCD patients were assigned to PspA families 1 and 2. There was no statistically significant difference between the distribution of SCD and non-SCD isolates into the two PspA families (Table) and their distribution was very similar to that observed in other collections of antibiotic-susceptible and antibiotic-resistant strains from the United States [15, 30], Colombia [16], and Argentina [24]. Isolates within these families have been previously shown to be serologically cross-reactive and cross-protective [31, 32] and hence a vaccine that covered the PspA families infecting normal children would also cover SCD patients.

We conclude that despite the increased risk of invasive pneumococcal disease for persons with SCD, infections in these patients are caused by the predominant circulating strains of *S. pneumoniae*, rather than with a specific subset of strains. Surveillance for invasive disease in the general population should predict the prevalence of capsular serotypes and PspA types among patients with SCD and facilitate formulation of vaccines to protect these high-risk patients against life-threatening pneumococcal infection.

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