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Invasive *Streptococcus pneumoniae* isolates from Argentinian children: serotypes, families of pneumococcal surface protein A (PspA) and genetic diversity

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SUMMARY

PspA is an antigenically variable virulence factor of *Streptococcus pneumoniae* that inhibits complement deposition and is a potential candidate for human vaccines. Of 64 published strains 96% are in PspA families 1 and 2; optimal protection is family-specific. Effective development of a PspA-containing vaccine requires more information about the PspA family of strains in parts of the world where the vaccine is most needed. In these studies we observed that of 149 isolates (of 19 capsular types) from Argentina, 54·4% were family 1, 41·6% were family 2 and 4·0% expressed both family 1 and family 2 PspAs. Box typing revealed the Argentinian strains to be from at least 10 clonally related groups.

INTRODUCTION

Streptococcus pneumoniae is a human pathogen of increasing clinical relevance causing important diseases such as meningitis, pneumonia, bacteraemia and otitis media. Disease rates are particularly high in young children, the elderly, and patients with immunosuppressive illness. Control of pneumococcal diseases is complicated by the increasing prevalence of penicillin-resistant and multidrug-resistant pneumococcal strains [1]. The 23-valent polysaccharide vaccine is highly effective in young adults, but prevents only 60% fatal pneumococcal bacteraemic pneumonia in the elderly [2, 3], and is unable to elicit protective antibody responses in children younger than 2 years of age [4]. Newer vaccines, in which smaller numbers of polysaccharides are chemically conjugated to non-pneumococcal proteins elicit improved immune responses in children, but these vaccines are expensive to produce. Their high cost may limit their use, especially in those parts of the world with the highest pneumococcal death rates [5]. Since the pneumococcal capsular types vary among different regions of the world, different formulations will be needed to obtain optimal effectiveness in different countries. A 7-valent conjugated polysaccharide vaccine was recently licensed for use with children in Argentina, but the distribution of prevalent types suggests that it might prevent only 66% of infections in children aged <2 years and only 56% of pneumococcal invasive infections in all children aged <6 years [6].

For these reasons, there is much interest in developing more cross-reactive pneumococcal protein

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vaccines. One of the best candidates is PspA, an antigenically variable, yet cross-reactive, surface virulence factor that interferes with complement-mediated clearance of pneumococci [7, 8] and that binds human lactoferrin [9]. Antibodies to PspA protect mice against lethal systemic infection and pulmonary infection [10]. This protection is often cross-protective for strains of different capsular serotype expressing distinct PspA molecule types because virtually all PspAs share significant short stretches of amino-acid identities in spite of the extraordinary degree of mosaicism exhibited by the *pspA* gene [11, 12]. Mucosal immunization with PspA can also prevent nasopharyngeal carriage and invasive disease of pneumococci in mice [13, 14].

Based on antibody cross-reactivity and sequence PspAs have been divided into three families. The families are divided into clades based on sequence differences [15]. PspA family 1 is composed of clades 1 and 2 and PspA family 2 is composed of clades 3, 4 and 5. The best protection in mice has been obtained when both the immunizing and challenge PspAs are of the same family [16]. Of 64 strains of pneumococci from the United States, Canada, Europe and Colombia, 97% expressed families 1 or 2. Only one of the 64 strains was found to express family 3, a family that cross-reacts poorly with families 1 and 2 [15, 17]. These data suggest that a vaccine designed to protect against strains of PspA families 1 and 2 would be the optimum. However, 64 strains are an insufficient base upon which to formulate a worldwide PspA-containing vaccine. More data are needed to ensure that there are not parts of the world that have very different PspA distributions and that might require a different or expanded PspA vaccine formulation.

Since 1993 the Pan American Health Organization has coordinated a surveillance network for *S. pneumoniae* in Latin America. From 1993 to 2000, with the participation of the Argentinian *Streptococcus pneumoniae* Working Group, 1293 invasive isolates were studied to determine capsular-type distribution and antimicrobial susceptibility. The aim of the present study was to evaluate the distribution of PspA families among Argentinian invasive isolates recovered from children aged <6 years. We selected a sample of 149 strains with the same serotype distribution as in the total collection in order to characterize their PspA family. The genetic relatedness of isolates of the major serotypes was also evaluated.

MATERIALS AND METHODS

Bacterial isolates

A total of 149 invasive isolates recovered from children under 6 years of age were selected from the collection containing 1293 isolates, representing each year from 1993 to 2000. Each serotype was represented in the same proportion as it was present in the total sample. Antimicrobial susceptibility patterns were not considered in the selection of isolates. Capsular serotyping was performed by Quellung reaction, with antisera (12 pools of antisera, type sera and factor sera), produced by Statens Seruminstitut of Copenhagen, Denmark [18]. Based on minimum inhibitory concentrations (MIC), isolates were classified as susceptible to penicillin (MIC $\leqslant 0{\cdot}06\,\mu$g/ml) or with reduced penicillin susceptibility (MIC $\geqslant 0{\cdot}12\,\mu$g/ml).

DNA isolation

Pneumococcal strains were inoculated from agar plates containing 5% sheep blood into 15 ml cultures of Todd Hewitt Broth (Difco Laboratories, Detroit, MI, USA) and cells were harvested at the beginning of the stationary phase. Chromosomal DNA was isolated by a modification of the Promega genomic DNA procedure (Promega Corporation, Madison, WI, USA) which included suspension in TE–sucrose (10 mM Tris–HCl, 1 mM EDTA, 10% sucrose; pH 8·0) followed by treatment with lysozyme and mutanolysin (final concentration 1 mg/ml and 0·5 U/μl respectively) (Sigma-Aldrich, St. Louis, MO, USA).

PspA family typing

Identification of PspA family was performed by PCR with family-specific primers and by dot–blot immunoassay. DNA from each strain was used as template in two PCR reactions. The primers for family 1 reaction were LSM12 (5′-CCGGATCCAGCGTCGCTATCTTAGGGGCTGGTT-3′) [19] and SKH63 (5′-TTTCTGGCTCATC/TAACTGCTTTC-3′). LSM12 and SKH52 (5′-TGGGGGTGGAGTTTCTTCTTCATCT-3′) were used for PspA family 2 detection. Reactions were performed in an Eppendorf Mastercycler Personal (Brinkmann, Hamburg, Germany). The samples were denatured at 95 °C for 3 min and subjected to 30 cycles of 94 °C for 1 min; 62 °C for 1 min and 72 °C for 3 min. Samples were held at 72 °C for an additional 10 min prior to cooling to 4 °C. The amplicons were then analysed by

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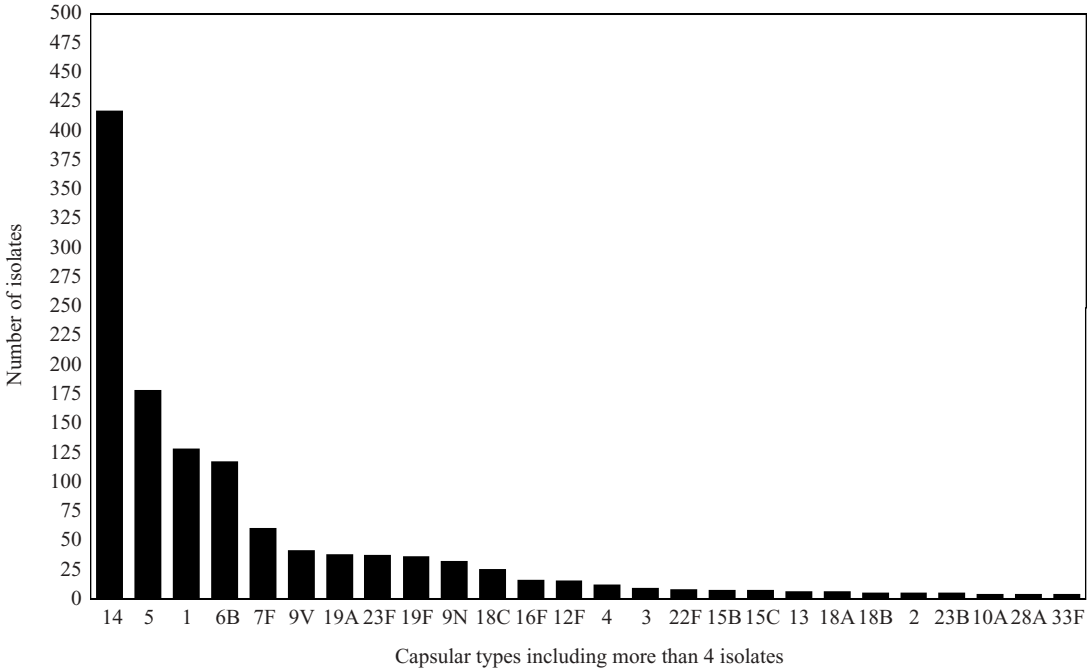

Fig. 1. Distribution of capsular-type of 1293 invasive isolates of *Streptococcus pneumoniae*.

agarose gel electrophoresis. The amplified PCR products were approximately 1000 bp for family 1 and 1200 bp for family 2.

Dot–blot immunoassay was carried out as described previously using pools of rabbit immune sera so as to include anti-PspA clades 1 and 2 for family 1 detection and anti-PspA clades 3 and 4 for family 2 detection [17]. In both assays control strains of families 1 and 2 were R36A [20] and BG11703 respectively [15].

BOX–PCR strain typing

A 30 μl PCR reaction mixture was prepared; the reaction mixture contained 5 μl DNA (100–200 ng/μl), 60 pmol BOX A1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′) [21], 200 μM each dNTPs and 2 U *Taq* polymerase (Promega Corporation) in its appropriate buffer. The mixture was heated at 95 °C for 7 min, 40 °C for 5 min and 65 °C for 5 min, followed by 30 cycles, each consisting of denaturation at 95 °C for 1 min, annealing at 40 °C for 2 min and extension at 65 °C for 2 min. A final extension step was performed at 65 °C for 16 min. Nine microlitres of each PCR product were subjected to electrophoresis in a 1·6% agarose gel at 50 V for 5·5 h in standard Tris–borate EDTA electrophoresis buffer and stained with 0·5 μg/ml ethidium bromide. To ensure the reproducibility of the method within our laboratory a 100-bp DNA ladder (Gibco–BRL, Life Technologies, NY, USA) and three standard isolates were run on each gel. Gel-banding patterns were digitalized and analysed. Comparison of the fingerprints produced was performed by the UPGMA clustering method, applying the Jaccard coefficient.

RESULTS

In the surveillance period the capsular types of 1293 *S. pneumoniae* recovered from normally sterile sites of Argentinian children aged under 6 years were determined at the Instituto Nacional de Enfermedades Infecciosas – ANLIS 'Dr C. G. Malbrán'. The distribution of capsular types is presented in Figure 1. There were 149 isolates selected for further study. PspAs of all 149 isolates were characterized as belonging to the two major PspA families. Eighty-one (54·4%) were family 1 PspA, 62 (41·6%) were family 2, and 6 isolates (4·0%) gave positive reactions for both families 1 and 2 by PCR and dot–blot immunoassay (see Table). The PspA PCR and the dot–blot assay correlated in 100% of cases. The distribution of both family 1 and family 2 *pspA* alleles among the capsular serotypes of the 149 strains was examined (see Table). Although the distribution of PspA families within capsular types was non-random, of the 10 capsular types represented by two or more strains, 7 contained both family 1- and family 2-expressing strains.

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Table. *PspA families of 149 strains selected among the most frequently isolated serotypes*

Serotype	No. of isolates	Family 1	Family 2	Families 1 and 2
14 S*	26	22	3	1
14 R*	23	1	22	—
5	21	21	—	—
1	18	18	—	—
7F	13	—	12	1
6B	11	4	6	1
9V	9	3	6	—
6A	8	2	4	2
23F	8	2	5	1
19F	2	1	1	—
3	2	2	—	—
19A	1	1	—	—
9N	1	—	1	—
18C	1	—	1	—
16F	1	1	—	—
15C	1	1	—	—
12F	1	1	—	—
4	1	—	1	—
19A	1	1	—	—
Total	149	81	62	6

* 14 S, Serotype-14 isolates with susceptibility to penicillin; 14 R, serotype-14 isolates with decreased susceptibility to penicillin.

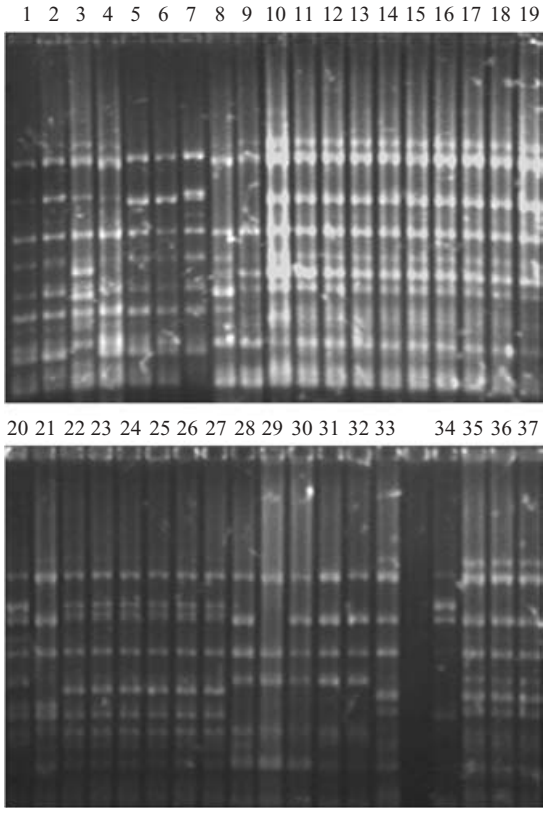

Fig. 2. Representative BOX–PCR DNA fingerprinting for *S. pneumoniae*. Lanes 1–37: isolates 2220 (type A_1), 2028 (type A_3), 2095 (type A_5), 1100 (type A_6), 3043 (type A_7), 2191 (type A_7), 2192 (type B), 3072 (type C_1), 1071 (type C_2), 2144 (type C_3), 770 (type C_3), 3103 (type C_3), 2211 (type C_3), 1110 (type C_3), 4057 (type C_3), 4012 (type C_3), 3065 (type C_3), 4169 (type C_3), 1053 (type C_4), 4004 (type E_2), 1171 (type E_3), 3047 (type G_5), 1174 (type G_5), 1225 (type G_5), 3166 (type G_5), 3047 (type G_5), 3016 (type G_5), 2008 (type H_1), 2089 (type H_2), 2101 (type H_3), 3110 (type H_4), 2011 (type H_6), 3093 (type I), 2219 (type J_2), 770 (type C_3), 3103 (type C_3), 2211 (type C_3).

The 49 capsular-type 14 isolates were analysed separately according to their penicillin susceptibility. PspA family 1 was present mainly in penicillin-susceptible capsular-type-14 isolates, while strains with reduced penicillin susceptibility were primarily PspA family 2. All 39 capsular-type-1 and -5 isolates were PspA family 1, whereas all 7F isolates were PspA family 2. These results suggested that significant numbers of strains within each capsular type could be clonally related, thus reducing the randomness of PspA family. To further explore this possibility BOX–PCR types were determined for the 62 isolates belonging to the five most common capsular types (selected examples are shown in Fig. 2). These 62 strains were divided into 10 BOX–PCR types at the 85% homology level (Fig. 3). These 10 major patterns were coded with capital letters (A–J), and different patterns (profiles) within each were considered to represent subtypes (capital letters with numerical subscripts).

In some cases there was an association between capsular typing and the clustering based on Box typing, but there were significant exceptions. Twelve strains of capsular serotype 14 showed a unique Box profile designated C_3. Fifteen isolates of capsular-type 5 exhibited five different Box profiles (G_1–G_5) with homology of approximately 94%, and 10 of these isolates shared profile G_5. Capsular-type 7F strains exhibited six different Box profiles among seven isolates with homologies of 85·5% or more. Strains of serotype 9V, serotype 1 and penicillin-susceptible serotype 14 were each distributed among several different BOX–PCR types.

DISCUSSION

Surveillance for *S. pneumoniae* has become increasingly important because of the worldwide distribution

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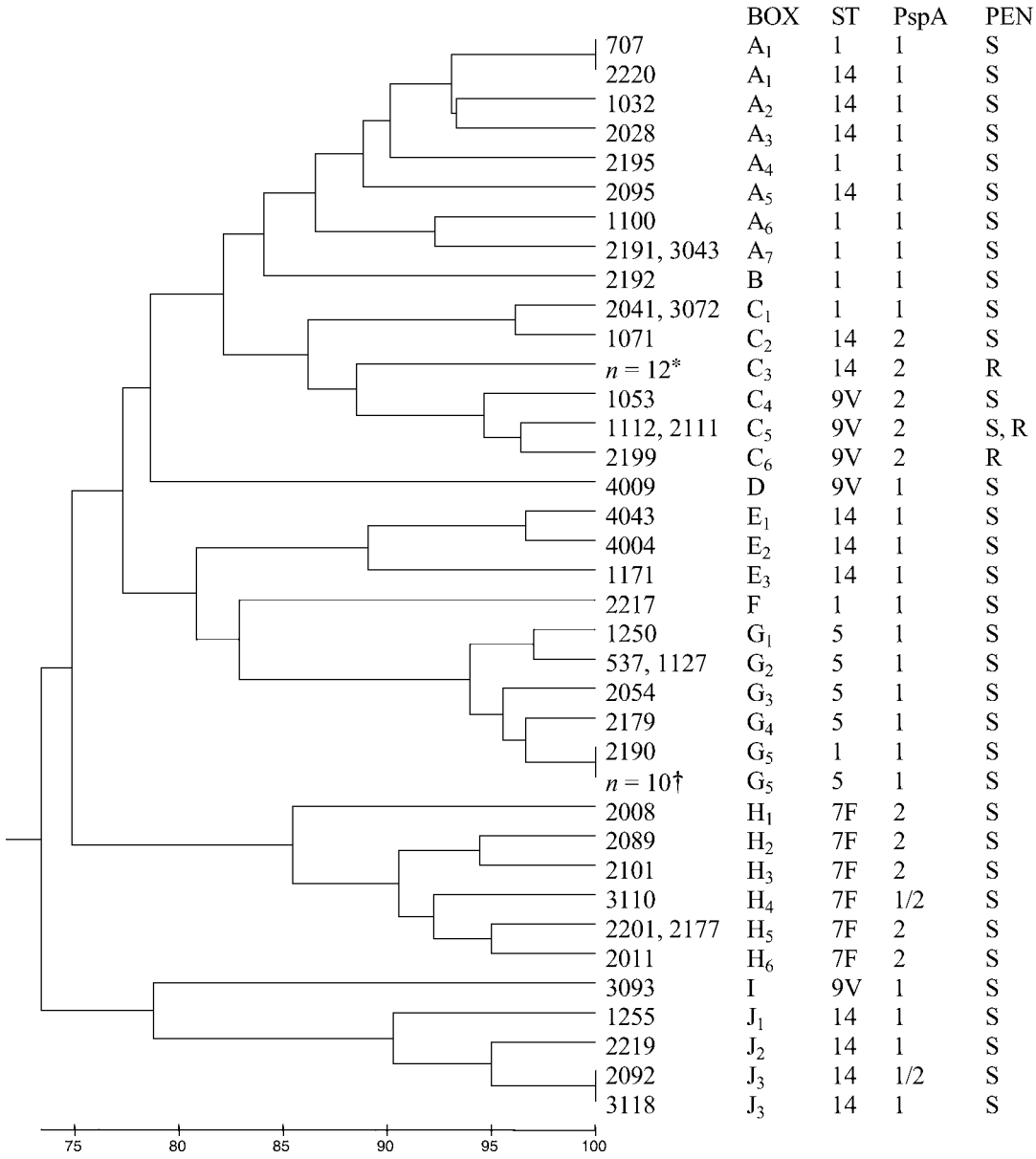

Fig. 3. Dendrogram representing groups of related *S. pneumoniae* strains. The relatedness among the isolates was estimated based on the proportions of shared bands. ST, capsular type; PEN, penicillin; S, susceptible; R, decreased susceptibility. * Isolates: 770, 1110, 1227, 1277, 2144, 2211, 3065, 3103, 4012, 4057, 4169, 4171. † Isolates: 1174, 1225, 2143, 2162, 2179, 2182, 3016, 3047, 3074, 3166.

of penicillin-resistant and multi-resistant pneumococci in the last 15 years. The need for current information on capsular types causing disease in infants is of great importance in view of the availability of new pneumococcal protein–polysaccharide conjugate vaccines. Among protein vaccine candidates, PspA has undergone a phase I clinical trial that showed it to be safe and highly immunogenic in humans [22]. Human antibodies elicited by family 1 PspA were shown to provide passive protection of mice from fatal infection with *S. pneumoniae* expressing either family 1 or family 2 [10]. A more recent report has compared the results obtained by immunization with family 1 *vs.* family 2 PspAs and found that the best protection was generally obtained when the PspA family of the challenge strain matched that of the immunogen [16].

Our present report provides the first insight into the diversity of PspA within strains circulating in Argentina, and the first data on strain distribution in South America in a non-tropical country. All the isolates examined were invasive, and PspA family

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analysis showed that all strains expressed family 1 PspA, family 2 PspA, or both. This observation indicates that a PspA vaccine containing only PspAs of families 1 and 2 should be able to cover the bulk of the strains in this region. There was a very strong association of either PspA families 1 or 2 with many of the capsular types, which was suggestive of the presence of clonally related groups of pneumococci among the 149 Argentinean isolates.

A complete characterization of the diversity of pneumococcal isolates requires molecular techniques in addition to serotyping. In this study we selected BOX–PCR because it is a quick molecular method that is suitable for investigation of genetic relatedness of pneumococcal strains and provides results whose interpretation is relatively unambiguous [23, 24]. The Pneumococcal Molecular Epidemiology Network has included this molecular technique in the guidelines for the recognition of pneumococcal clones [25]. The relationship between PspA type and genetic diversity was previously explored by Muñoz et al. [26]; it was demonstrated that isolates assigned to a certain clone by multilocus enzyme electrophoresis and penicillin-binding protein fingerprinting expressed a single characteristic antigenic profile for PspA.

The genetic homogeneity of serotype-14 isolates with decreased susceptibility to penicillin was demonstrated by BOX–PCR analysis and these isolates were largely PspA family 2. The clonality of penicillin-resistant *S. pneumoniae* has previously been observed by others including Rossi et al. [27] who determined the frequent occurrence of the international Spanish/French clone among *S. pneumoniae* resistant to penicillin.

All 15 capsular-type 5 isolates belonged to the same clonal type and were very closely related; 94% homology or greater was obtained for all of them and profile G_5 was shared by 10 isolates. This is consistent with the observation that *S. pneumoniae* serotype-5 strains from Latin America are generally closely related genetically [28]. Capsular-type-1 isolates showed a different behaviour as 5 of the 10 studied isolates belonged to the Box-type A, while the others were distributed among four different types. PspA family 1 was very strongly associated with serotypes 1 and 5. All of the 39 capsular-type-1 and -5 isolates were found to express family 1 PspA (see Table). Recently Poirat et al. [29] conducted a study to examine clonal distribution of invasive serotypes 1 and 5 among children in Southern Israel and concluded that each serotype showed only one clonal type. Serotypes 5 and 1 are highly relevant to the paediatric population in South America, but are not very relevant to the North American paediatric population and are not included in the 7-valent vaccine.

In comparison with some of the other groups of isolates, the capsular-type 9V isolates and the capsular-type-14 penicillin-sensitive isolates were not clustered by BOX–PCR type (homologies as low as 74%) indicating a higher diversity in the chromosomal background of these two groups.

The present study provides epidemiological information about the PspA family distribution and genetic diversity of Argentinian isolates of *S. pneumoniae* and informs of the potential coverage of a PspA-based vaccine. Ongoing surveillance programmes for invasive pneumococcal disease can be used to monitor the appropriateness of existing vaccine formulations and to provide valuable information on which to base the formulation and application of new vaccines that are currently under development.

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