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□ Original Article □

# Pneumococcal Surface Protein A of *Streptococcus pneumoniae* Isolates from Koreans

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**Purpose :** Pneumococcal protein vaccine based on pneumococcal surface protein A (PspA) is in development with the potential to offer a broad range of protection against different strains. PspA elicits protection in mice against fatal sepsis as well as carriage and lung infection. This study was performed to investigate the frequency of PspA families among *Streptococcus pneumoniae* recovered from Korean children and adults.

**Methods :** A total of 89 pneumococcal isolates was included in the study. They were capsule serotyped by the slide agglutination assay with commercial antisera. PspA families were determined with polymerase chain reaction using the pair of primers for family 1 and family 2.

**Results :** Seventeen pneumococcal serotypes were found in a total of 89 isolates. PspA typing was able to ascertain 79 of the 89 isolates (88.8 percent). Among these, 20 (22.5 percent) isolates were family 1 PspA, 59 (66.3 percent) were family 2. Moreover, because 9 (10.1 percent) isolates were of positive reactions for both, families 1 and 2 primers, the potential coverage of PspA vaccine was 98.9 percent. PspA families were not associated with age group, source of isolates, or penicillin susceptibility. However, the relative distribution of family 1 isolates to family 2 isolates was significantly different over capsular serotypes.

**Conclusion :** The finding that 98.9 percent of Korean isolates belonging to PspA families 1 and 2 support the hypothesis that a human PspA vaccine covering a few PspA families could be broadly effective. The monitoring of the PspA families derived from large population-based isolates will be necessary in the context of vaccine development. **(Korean J Pediatr 2005;48:1206-1211)**

**Key Words :** *Streptococcus pneumoniae*, Pneumococcal surface protein A

## Introduction

Pneumococcal vaccines have been developed using various combinations of pneumococcal capsular polysaccharide (PS) as immunogen since *Streptococcus pneumoniae* is an important pathogen for young children and older adults worldwide[1)]. Moreover the increased frequency of isolation of multidrug-resistant strains of *S. pneumoniae* accentuates the need for an effective vaccine[2)]. An available 23-valent PS vaccine is not effective in young children and has low efficacy among the elderly[1, 3)]. A recently introduced heptavalent conjugate vaccine is effective among young children, but its serotype coverage is limited[4)].

An alternative to plain PS or conjugated vaccine could be the use of protective protein antigens that are expressed in the majority of pneumococcal strains, including pneumolysin[5)], pneumococcal surface adhesin A[6)], and pneumococcal surface protein A (PspA)[7)]. Protein-based pneumococcal vaccines offer several advantages. These advantages include the possibility of developing broadly cross-protective vaccines by including proteins that are present on all clinically significant strains of *S. pneumonia*e and the more amenable manufacturing process for producing protein-based vaccines than a PS-protein conjugated vaccine.

The PspA pneumococcal vaccine is currently under development since PspA is a well-known pathogenic factor found on the surface of *S. pneumonia*e[7)]. In *in vivo* studies, the human antibodies to PspA enhanced the clearance of bacteria from blood and were found to be protective primarily against sepsis[8-10)]. PspA has been shown to inhibit

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the activation of complement 3 (C3) at the pneumococcal surface through binding factor H and destroying C3 convertase by dissociating Bb from C3b[11]. This PspA's inhibition of complement activation at the pneumococcal surface may reduce the opsonizing action of the anti-capsular antibody and anti-PspA antibodies may have an indirect action on this process.

Information about the basic protein structural domains of PspA originally came from the DNA sequences of the PspA in Rx1 and EF5668 pneumococcal strains[12]. From the alignment of PspA sequences of 24 strains, the sequence differences in a centrally located clade-defining region (CDR) were used to group PspA proteins into six clades[13]. The CDR is roughly the same as that shown to elicit cross-protective responses[13]. The six clades have also been grouped into three families[13].

In this study, the frequency of PspA family 1 and family 2 proteins was investigated among pneumococcal isolates from invasive or noninvasive diseases and from carriage without disease. These results are the first report about PspA families of pneumococci isolated in Korea and will be valuable to learn the diversity of PspA in Korean isolates.

## Materials and Methods

### 1. Sources of *S. pneumoniae* isolates

A total of 89 pneumococcal isolates were included. Carriage isolates (n=50) were from the oropharynx of healthy children ≤5 years old from five different day care centers in Seoul. Invasive isolates (n=10) were from blood (n=4) and cerebrospinal fluid (n=6). The remaining isolates (n=29) were from various sites of patients with less serious diseases, such as tracheal aspirate (n=1), sputum (n=18), sinus aspirate (n=1), ear discharge (n=6), eye discharge (n=2) and stool (n=1) (Table 1). The age of the patients ranged from children 6 to 108 months old (n=15), and adults 18 to 64 years old (n=24).

**Table 1.** Sources of *S. pneumoniae* Isolates

| Sources of *S. pneumoniae* | | Number | % |
|---|---|---|---|
| Carriage | oropharynx | 50 | 56.2 |
| Invasive disease | blood | 4 | 4.5 |
| | cerebrospinal fluid | 6 | 6.7 |
| Noninvasive disease | tracheal aspirate | 1 | 1.1 |
| | sputum | 18 | 20.2 |
| | sinus aspirate | 1 | 1.1 |
| | ear discharge | 6 | 6.7 |
| | eye discharge | 2 | 2.2 |
| | stool | 1 | 1.1 |
| Total | | 89 | 100 |

The date of isolation was from 1996 to 1998.

All isolates were confirmed to be *S. pneumoniae* by standard procedures such as alpha-hemolysis, Gram stain, optochin test, and bile solubility. Cultures were stored at -70℃ in a solution of Todd-Hewitt broth (Difco, Detroit, MI) supplemented with yeast extract plus 10% glycerol.

### 2. Methods

#### 1) Penicillin susceptibility test

Susceptibility testing to penicillin was carried out by the disc diffusion method. Penicillin resistance in *S. pneumoniae* isolates was screened using 1 μg oxacillin discs (BBL Microbiology System, Cockeysville, MD, USA) and strains showing ≥20 mm inhibition zones were assessed as susceptible. Strains exhibiting smaller inhibition zones around the 1 μg oxacillin discs were identified as penicillin non-susceptible.

#### 2) Slide agglutination assay for capsular serotypes of pneumococcal isolates

All isolates were capsule serotyped by the slide agglutination assay with commercial antisera (Statens Serum institut, Copenhagen, Denmark).

#### 3) PspA polymerase chain reaction (PCR) for PspA families of pneumococcal isolates

PspA families of pneumococcal isolates were determined with PCR.

Pneumococci were cultured for 6 hr in 10 mL Todd-Hewitt broth (Difco) supplemented with 1% yeast extract, 1% glucose, and 22 μg/mL glutamic acid. After centrifugation, the cells were resuspended in 0.1 mL TE (10 mM Tris HCL, 1 mM EDTA, pH 8.0), and lysozyme (10 μL at 50 mg/mL) was added. The cells were incubated for 30 min at 37℃, and 0.5 mL GES (5 M guanidine thiocyanate, 0.1 M EDTA, pH 8.0, and 0.5% sarkosyl) was added. After incubation at room temperature for 10 min, 0.25 mL (7.5 M) ammonium acetate was added, and placed on ice for 10 min. Chloroform/isoamyl alcohol (24 : 1) was added. After centrifugation, 0.7 mL of aqueous phase was recovered. The DNA was precipitated with ethanol and resuspended in 50-100 μL of TE.

PCR was carried out on genomic DNA. The oligonucleotide primers for family 1 were LSM12, 5′CCGGATCCAGC GTCG-

CTATCTTAGGGGCTGGTT3′ and SKH63, 5′TTTCTGGCTCAT(C and T)AACTGCTTTC3′ (at position 12, C and T in a 1 : 1 ratio) and for family 2 were LSM12, 5′CCGGATCCAGCGTCGCTATCTTAGGGGCTGGTT3′ and SKH52, 5′TGGGGGTGGAGTTTCTTCTTCATCT3′[14]. The following PCR conditions were used : an initial 95℃ (3 min), 30 cycles of 95℃ (1 min), 62℃ (1 min) and 72℃ (3 min), followed by 72℃ (10 min) in a PTC 150 Thermocycler (MJ Research, Watertown, MA, USA). PCR products were initially run at an annealing temperature of 62℃. The amplified PCR products were approximately 1,000 bp for family 1 and 1,200 bp for family 2. The PCR product was run on an agarose gel at 80 volts for 1.5 hr, and the gel was stained with 0.5 μg/mL ethidium bromide. Molecular weight standard for gel electrophoresis was the 1.0-kb ladder DNA (Promega, Madison, WI, USA). Strains BG9739 (clade 1) and AC122 (clade 3) were used as controls for family 1 and 2 tests, respectively [13]. The PspA family of these strains has been confirmed by DNA sequence of their pspA genes[13].

### 3. Data analysis

Chi-square test (2) was used to compare differences between proportions. *P* values less than 0.05 were considered statistically significant.

## Results

### 1. Serotypes of pneumococcal isolates

Seventeen pneumococcal serotypes were found in a total of 89 isolates. Seventy-seven percent of the disease isolates belonged to only five serotypes, namely 19F, 23F, 6A, 14 and 15F, in descending order of frequency (Table 2). Eighty-six percent of the carriage isolates belonged to six serotypes, namely 19F, 6A, 23F, 3, 29 and 14, in descending order of frequency (Table 2). The serotype distribution was similar between disease and carriage isolates. For example, serotype 19F was the most common in disease and carriage isolates. Serotypes 23F and 6A were the next frequent serotypes in disease isolates and 6A and 3 were those in carriage isolates. Serotype 3 and 29 were more common in carriage isolates than disease isolates. However, this difference was not statistically significant ($P>0.05$) between disease and carriage isolates or between isolates from invasive disease and those from noninvasive disease.

### 2. PspA families of pneumococcal isolates

PspA typing by PCR, using the pair of primers for family 1 and family 2, was able to ascertain 79 of the 89 isolates (88.8%). Among these, 20 (22.5%) isolates were family 1 PspA, 59 (66.3%) were family 2. The PspA family could not be defined in 10 isolates, because of positive reactions for both, family 1 and family 2 primers in 9 (10.1%) isolates, and negative reactions for both primers in one (1.1%) isolates (nontypeable) (Table 3). Isolate Pn-19,

**Table 2.** Serotypes of *S. pneumoniae* Isolates

| Serotypes | Diseases isolates | | Carriage isolates | | Total | |
|---|---|---|---|---|---|---|
| | number | % | number | % | number | % |
| 19F | 14( 3/11)* | 35.9 | 12 | 24.0 | 26 | 29.2 |
| 23F | 9( 1/ 8)* | 23.1 | 6 | 12.0 | 15 | 16.9 |
| 6A | 3( 1/ 2)* | 7.7 | 9 | 18.0 | 12 | 13.5 |
| 14 | 2( 1/ 1)* | 5.1 | 3 | 6.0 | 5 | 5.6 |
| 15F | 2( 1/ 1)* | 5.1 | 0 | 0 | 2 | 2.2 |
| 5 | 1( 1/ 0)* | 2.6 | 0 | 0.0 | 1 | 1.1 |
| 1 | 1( 1/ 0)* | 2.6 | 0 | 0.0 | 1 | 1.1 |
| 3 | 1( 0/ 1)* | 2.6 | 8 | 16.0 | 9 | 10.1 |
| 29 | 1( 0/ 1)* | 2.6 | 5 | 10.0 | 6 | 6.7 |
| 9N | 1( 0/ 1)* | 2.6 | 0 | 0.0 | 1 | 1.1 |
| 11F | 1( 0/ 1)* | 2.6 | 0 | 0.0 | 1 | 1.1 |
| 23A | 1( 0/ 1)* | 2.6 | 0 | 0.0 | 1 | 1.1 |
| 10A | 0 | 0 | 2 | 4.0 | 2 | 2.2 |
| 13 | 0 | 0 | 1 | 2.0 | 1 | 1.1 |
| 16F | 0 | 0 | 1 | 2.0 | 1 | 1.1 |
| 22F | 0 | 0 | 1 | 2.0 | 1 | 1.1 |
| 31 | 0 | 0 | 1 | 2.0 | 1 | 1.1 |
| nontypeable | 2( 1/ 1)* | 5.1 | 1 | 2.0 | 3 | 3.4 |
| Total | 39(10/29)* | 100 | 50 | 100 | 89 | 100 |

*Numbers in parenthesis mean number of invasive isolates/ number of noninvasive isolates
There was no significant difference in the serotype distribution between disease isolates and carriage isolates ($P>0.05$). There is no significant difference in the serotype distribution between invasive disease isolates and noninvasive disease isolates ($P>0.05$)

**Table 3.** PspA Families of *S. pneumoniae* Isolates

| PspA Family | Diseases isolates | | Carriage isolates | | Total | |
|---|---|---|---|---|---|---|
| | number | % | number | % | number | % |
| 1 | 7( 4/ 3)* | 17.9 | 13 | 26.0 | 20 | 22.5 |
| 2 | 30( 5/25)* | 76.9 | 29 | 58.0 | 59 | 66.3 |
| 1+2 | 1( 1/ 0)* | 2.6 | 8 | 16.0 | 9 | 10.1 |
| nontypable | 1( 0/ 1)* | 2.6 | 0 | 0 | 1 | 1.1 |
| Total | 39(10/29)* | 100 | 50 | 100 | 89 | 100 |

*Numbers in parenthesis mean number of invasive isolates/ number of noninvasive isolates

**Table 4.** Frequencies of PspA Families by Variables of *S. pneumoniae* Isolates

| Variables | | Pneumococcal PspA | | | | Total (n=89) |
|---|---|---|---|---|---|---|
| | | Family 1 | Family 2 | Family 1+2 | Nontypeable | |
| Age group | Children | 16 | 41 | 8 | 0 | 65 |
| | Adults | 4 | 18 | 1 | 1 | 24 |
| Origin of isolates | Oropharynx | 13 | 29 | 8 | 0 | 50 |
| | Invasive disease | 4 | 5 | 1 | 0 | 10 |
| | Noninvasive disease | 3 | 25 | 0 | 1 | 29 |
| Penicillin susceptibility | Susceptible | 5 | 9 | 2 | 0 | 16 |
| | Non-susceptible | 15 | 50 | 7 | 1 | 73 |
| Capsular serotypes | 3 | 1 | 7 | 1 | 0 | 9 |
| | 6A | 6 | 2 | 3 | 1 | 12 |
| | 14 | 4 | 0 | 1 | 0 | 5 |
| | 19F | 1 | 25 | 0 | 0 | 26 |
| | 23F | 0 | 14 | 1 | 0 | 15 |
| | 29 | 1 | 5 | 0 | 0 | 6 |
| | Others* | 7 | 6 | 3 | 0 | 16 |

*1, 5, 9N, 10A, 11F, 13, 15F, 16F, 22F, 23A, 31, and nontypeable
There was no significant difference in the distribution of PspA families by age groups, origin of isolates or penicillin susceptibility ($P>0.05$)
There is significant difference in the distribution of PspA families by capsular serotypes ($P<0.01$)

which was capsular serotype 6A from tracheal aspirate, was the only one (1.1%) of the 89 that was neither family 1 or family 2 (Table 3).

Table 4 shows the frequencies of the PspA families according to the study variables. There was no significant difference in the distribution of PspA families by age groups, origin of isolates or penicillin susceptibility ($P>0.05$).

However, the relative distribution of family 1 isolates to family 2 isolates was significantly different over capsular serotypes ($P<0.01$). The distribution of serotypes among the PspA families showed that serotypes 3 (77.8%), 19F (96.1%), 23F (93.3%) and 29 (83.3%) isolates expressed higher rates of family 2 protein compared to family 1, whereas, serotype 6A (50%) and 14 (80%) isolates expressed family protein more frequently. Family 1 PspA were found in other serotypes such as 1, 3, 5, 10A, 13, 15F, 16F, 19F, 29, and 31, and family 2 were found in other serotypes such as 6A, 9N, 11F, 15F, 22F and 23A.

## Discussion

In children under two years of age, the 23-valent PS vaccine is not helpful due to its poor immunogenicity in this age group[3]. The newly produced, 7-valent protein conjugated vaccine has shown high efficacy against invasive diseases in childhood, however, this vaccine elicits protection only against the 7 serotypes included in it and its cross reactive serotypes[4]. Moreover, because of its expensive price[15] the vaccine has been accessible only in the private sector to a small part of the population in many countries.

Another approach for improvement of pneumococcal vaccines has been the consideration of proteins common to all isolates of *S. pneumoniae*, which could provide a non-serotype-related vaccine[5, 6, 9]. Among these proteins, the pneumococcal surface protein A (PspA) has been considered as a potential antigen.

PspA, a surface protein and virulence factor found on all isolates of *S. pneumoniae*[7] has been examined for the development of a protein-based vaccine[13]. The basic structure of PspA has a central portion named as the CDR, which is used to group PspA proteins into six clades[13]. These six clades are grouped into three PspA families. PspA family 1 (clades 1 and 2) and family 2 (clades 3, 4, and 5) are the major families, while PspA family 3 is less commonly found and includes a single clade 6[13]. These PspA families can be identified by PspA polyclonal antisera[16] and by PspA family-specific primers in the PCR[17]. In this study, the PspA family type was defined by the PCR results. However, for one isolate not typeable by PCR in this study, the dot-blotting serological assay was also unable to type.

PspA is highly immunogenic[7]. Antibody responses to

PspA in mice or healthy adults have shown cross-reactivity against heterologous PspA proteins[13, 18]. Furthermore, immunization with a single PspA is protective in mice against fatal infection caused by different capsular types [8-10]. Mucosal immunization with PspA can also elicit immunity to carriage[18]. Since PspAs of different families have restricted cross-reactivity, however, a PspA vaccine should include members of the major PspA families[13] and a combination of family 1 and family 2 proteins has been proposed for the vaccine formulation[13].

It is necessary, therefore, to identify the PspA family prevalence among isolates to evaluate the appropriateness of PspA for use in vaccines in one country. This study was intended to know the distribution of PspA families among pneumococcal isolates and the potential PspA protein-based vaccine coverage rate in Korea.

Since all isolates but one belonged to PspA families 1 or 2 in this study, family 1 and 2 were the major PspA families identified in Korea. One isolate was not identified as either family 1 or family 2, and it could be speculated that this isolate might express the PspA family 3, which was not assessed in this study.

The distribution of the Korean isolates between PspA families 1 and 2 did not differ substantially from that observed for isolates from North America, Europe, and South America[13, 17]. Therefore, a vaccine formulation including these two families might cover isolates from Korea. The use of PspA as an alternative vaccine or as a carrier antigen to PS may offer a broader range of protection against a great number of strains, overcoming the concern about the restricted capsular serotypes in the conjugate vaccines[19, 20].

In the present study, PspA families were not associated to any age group. This result supports the use of PspA as a vaccine component to prevent pneumococcal infection during the whole span of life. Moreover, PspA families were not associated with site of isolates or penicillin susceptibility.

However, PspA families were associated with capsular serotypes. Family 1 and 2 PspAs were present among isolates of 17 different capsular serotypes in this study. Family 1 was associated with capsular serotypes 6A and 14; and family 2 was associated with capsular serotypes 3, 19F, 23F and 29. In one study, a remarkable prevalence of family 2 isolates associated with serotype 14 and penicillin non-susceptible isolates was observed[17]. In this study, a prevalence of family 2 isolates associated with serotype 3 and penicillin susceptible isolates was observed. All isolates of serotype 3 but one were family 2 and penicillin susceptible. But this finding was not observed in other serotypes associated with family 2 such as 19F, 23F and 29. Therefore, there is no suitable explanation for this PspA family associated with capsular serotypes.

So far, there are few explanations in the field of the immunology of the anti-PspA antibodies. Since PspA has been shown to inhibit the activation of C3 at the pneumococcal surface, these PspA′s inhibition of complement activation may reduce the opsonizing action of anti-capsular antibody and anti-PspA antibodies may have indirect action on the opsonization process. In other words, PspA inactivates the complement fixed on the bacteria and that anti-PspA antibody may reduce PspA′s ability to inactivate complement.

This study provided information on the prevalence of PspA family types in pneumococcal isolates in Korea. That 98.9% of Korean isolates belong to PspA families 1 and 2 supports the hypothesis that a human PspA vaccine covering a few PspA families could be broadly effective. PspA-specific vaccine, if efficacious, may represent a cost-effective and broadly cross-protective vaccine of *S. pneumoniae*. The monitoring of the PspA families derived from large population-based isolates will be necessary in the context of vaccine development.

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## 한 글 요 약

### 한국인에서 *Streptococcus pneumoniae* 분리주의 폐구균 표면 단백 A

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**김 경 효**

**목 적** : 현재 연구 개발되고 있는 PspA에 근거한 폐구균 단백 백신은 기존의 백신과 달리 여러 종류의 혈청형에 대해 동시

에 광범위한 방어 면역을 유도할 가능성이 있다. 동물 실험에서 PspA는 폐구균 보균과 호흡기 감염 뿐 아니라 패혈증에 대해서도 방어 면역을 형성함이 보고되었다. 본 연구는 한국 소아와 어른에게서 분리된 폐구균에서 PspA 백신에 포함된 PspA family인 family 1과 family 2의 분포를 알기 위해 시행되었다.

**방 법** : 총 89주의 폐구균을 대상으로 항혈청을 이용하여 슬라이드 응집 방법으로 폐구균 피막의 혈청형을 분류하였다. 또한 PCR을 이용하여 family 1과 family 2의 시발체를 사용하여 분리된 폐구균의 PspA family를 정하였다.

**결 과** : 총 89주의 폐구균 분리주에서 17 종류의 폐구균 혈청형이 분류되었다. PspA 종류는 79주(88.8%)에서 결정되었는데 20주(22.5%)가 family 1, 59주(66.3%)가 family 2이었다. 아홉주(10.1%)에서 family 1과 2에 모두 양성 반응을 보였으므로 family 1과 2 단백을 포함하는 PspA 백신의 가능한 방어 범위는 98.9%이었다. 각 PspA family들은 균이 분리된 사람의 나이, 분리된 곳, 혹은 페니실린에 대한 감수성 등과 의미 있는 관련성이 없었으나 피막 혈청형에 따라서는 의미 있는 차이를 보였다.

**결 론** : 본 연구에서 분리된 폐구균 분리주의 98.9%가 PspA family 1과 2에 속하였으므로 이를 포함한 백신은 폐구균에 대해 혈청형과 관련 없이 좀 더 광범위한 효과를 보일 것으로 추정된다. 향후 좀더 다양하고 많은 폐구균 분리주를 대상으로 PspA family의 연구가 이루어진다면 PspA 백신의 개발과 적용에 많은 도움이 될 것으로 생각된다.

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