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ORIGINAL ARTICLE

Determination of phenotypes and pneumococcal surface protein A family types of *Streptococcus pneumoniae* from Malaysian healthy children

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Susceptibility pattern

Background: There is limited information about pneumococcal carriage among healthy children in Malaysia. Therefore, this study was conducted to determine the prevalence rate, serotype distribution, susceptibility pattern, and pneumococcal surface protein A (PspA) family types of *Streptococcus pneumoniae* isolates in the nasal carriage of children 5 years old or younger in three day care centers in Kuala Lumpur, Malaysia.
Methods: Nasal swabs were collected from 195 healthy children, age 5 years or younger, from June to December 2010. *S pneumoniae* was identified by phenotypic and genotypic methods. The serotyping was performed using Pneumotest kit (Statens Serum Institut, Copenhagen, Denmark) and the susceptibility pattern was determined by using the E-test method (AB Biodisk, Solna, Sweden). PspA family typing was done using polymerase chain reaction.
Results: *S pneumoniae* was found in the nasal carriage of 35.4% of children (69 of 195) and penicillin resistance was found in 23.2% (16 of 69). Among the 69 isolates, multidrug-resistant *S pneumoniae* (MDRSP) was present in 20.3%. All 16 penicillin-resistant *S pneumoniae* (PRSP) isolates were resistant to erythromycin and 14 PRSPs (87.5%) were resistant to co-trimoxazole. The six most common serotypes were 6A, 23F, 19A, 6B, 19F, and 15C, which were found in 87% of all isolates. Of the 69 isolates, 24.6% belonged to PspA family 1, 71.0% to PspA family 2, and 4.3% to PspA family 3.
Conclusion: Twenty-eight of the isolates (40.6%) belonged to serotypes included in the pneumococcal polysaccharide vaccines (PCV) 7 and 10, whereas 48 (69.5%) were included in PCV13. The high rate of PRSP and MDRSP supports the need for continuing surveillance of

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pneumococcal carriage. The major PspA families were 1 and 2 (95.7%), thus making them suitable candidates for future vaccines.

Introduction

Streptococcus pneumoniae is a major agent for respiratory and invasive disease worldwide, particularly in children younger than 5 years, elderly persons, and immunocompromised individuals.[1] In many cases, pneumococcal diseases are preceded by nasopharyngeal colonization. Crowded day care centers facilitate the spread of pneumococcal diseases. Many studies have shown that the highest frequency of pneumococcal colonization is found in young children, and this group might be the most important vector for horizontal spread of pneumococcal strains within the community.[2] The efficacy of penicillins and other antibiotics has been dramatically reduced recently because of an increasing rate of resistance and rapid spread of multidrug-resistant strains.[3,4]

Currently, immunization is the ideal way to prevent pneumococcal disease. There are two types of licensed vaccines: pneumococcal polysaccharide vaccine (PPV) and pneumococcal conjugate vaccine (PCV). Although the 23-valent PPV is protective in children older than 2 years, this vaccine fails to protect children younger than 2 years because of its inability to elicit immunologic memory.[5] To induce immunogenicity, several PCVs such as PCV7, PCV10, and PCV13 are produced to provide protection, especially in children 2 years old and younger.[6] However, its cost limits the use of PCV in developing countries. Also, it has been shown that vaccine serotypes are now being replaced by nonvaccine serotypes as causes of disease.[7,8] To date, there are no data to support the premise that serotype replacement has occurred in Malaysia. Although the use of vaccine serotypes is a positive step toward prevention, efforts are under way to produce more effective vaccines. Pneumococcal virulence proteins, which are found in all disease-causing strains, are currently the focus of vaccine development.[9] Several virulence proteins, including pneumolysin (ply), autolysin (lytA), pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC), and neuraminidase A (NanA), are being studied.[9,10] PspA is among the most studied and highly promising virulence proteins because it is highly immunogenic and expressed virtually by all pneumococci.[11,12] PspA is composed of three structural domains: the N-terminal region, C-terminal choline binding region, and proline-rich region. Based on sequences of the N-terminal region, which is composed of repeated alpha-helices and is highly polymorphic, it can be grouped into three families and can be subdivided into six clades, with most pneumococcal strains belonging to family 1 and family 2.[13–15] The family can be determined by polymerase chain reaction (PCR) and the clades are identified by sequencing.[13] This study was conducted to determine the prevalence rate of *S pneumoniae,* serotype distribution, and susceptibility pattern of *S pneumoniae* isolated from healthy children in three day care centers in Malaysia. The distribution of PspA family type and its relationship with serotype distribution and penicillin susceptibility were also investigated.

Materials and methods

Study design

A cross-sectional descriptive study was carried out. Samples were obtained from children age 5 years and younger who attended three day care centers in Kuala Lumpur, Malaysia from June to December 2010. This study was approved by the Universiti Putra Malaysia (UPM) Medical Research Ethics Committee.

Study population

All healthy children 5 years old and younger who attended three day care centers were eligible to participate in this study; those who were recently hospitalized or receiving antibiotic treatment were excluded. Written informed consent was obtained from the parent or guardian.

Sample collection

A nasal sample from both sides of the anterior nares was obtained from each child using a cotton-tipped swab. A nasal swab was used because it is much easier to perform without sacrificing the sensitivity of detection of pneumococcal carriage.[16] The swabs were placed into Amies transport media and transferred to the laboratory at room temperature. Within 3 to 4 hours of sample collection, the swabs were cultured onto sheep blood agar and incubated overnight at 35 °C in 5% CO_2.

Identification of isolates

Pneumococcal isolates were identified based on colonial morphology, Gram staining, catalase test, bile solubility, and susceptibility to ethylhydrocupreine hydrochloride (optochin).[17] The identity of *S pneumoniae* was also confirmed using PCR for detection of *ply* and *lytA* genes.

Serotyping

Serotyping was performed by latex agglutination using Pneumotest kit commercial antisera[18] (Statens Serum Institut, Copenhagen, Denmark).

Antibiotic susceptibility testing

Susceptibility of pneumococcal isolates to penicillin, erythromycin, co-trimoxazole, cefotaxime, ceftriaxone, and amoxicillin/clavulanic acid was determined based on the minimum inhibitory concentration (MIC) using the E-test method (AB Biodisk, Solna, Sweden). The test was performed using the methodology recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines.[19] *S pneumoniae* ATCC 49619 was used as the control strain. The MIC results were compared to CLSI interpretive criteria and interpreted as susceptible (S), intermediate (I), or resistant (R). Multidrug-resistant *S pneumoniae* (MDRSP) is defined as resistance to more than two different classes of antibiotics.[20]

DNA extraction and purification

DNA was extracted using the GeneAll® Exgene™ kit and purification was done using the GeneAll® Expin™ PCR SV Protocol Handbook (GeneAll Biotechnology Co. Ltd, Seoul, Korea) in accordance with the instructions provided by the manufacturer.

PCR detection of pneumolysin (ply) and autolysin (lytA) genes and PspA family typing

The presence of *ply* gene, *lytA* gene, and PspA family typing were determined by using the primers listed in Table 1.[12–14,21,22]

Amplification was carried out in a total volume of 25 μL containing 0.5 μM each primer, 30 μM each dNTP, 1.5 mM $MgCl_2$, 1 U *Taq* DNA polymerase, and 1-10 ng DNA template. *S pneumoniae* ATCC 49619 was used as a positive control sample. A negative control sample containing the same reaction mixture except the DNA template was included in every experiment. All PCR amplifications were perfomed using a thermal cycler (BioRad MyCyclerTM Themalcycler, USA). Initial denaturation was at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 for 30 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 30 seconds. Finally, an additional extension was set for 10 minutes at 72 °C. The amplification conditions for the *ply* and *lytA* gene fragments were similar except for the annealing temperature (Table 1). Three μL of amplicon of each PCR product was electrophoresed on a 1.2% agarose gel for 1 hour at 85 V, visualized and photographed under UV illumination with a 100-bp DNA ladder as a molecular weight marker. Representatives of the 348-bp product of *ply* gene and 319 bp of *lytA* gene were sequenced at First Base Laboratories (Seri Kembangan, Malaysia), and compared in the GenBank database for gene confirmation.

The classifications of PspA family were determined by using primers for family 1 (LSM12/SKH63), family 2 (LSM12/SKH52), and family 3 (SKH41/SKH42) as listed in Table 1. PCRs were carried out in a standard mixture of 25 μL containing 0.5 μM each primer, 30 μM each dNTP, 1.5 mM $MgCl_2$, 1 U *Taq* DNA polymerase, and 1-10 ng DNA template. Cycling conditions were as follows: 95 °C (3 minutes); 30 cycles of 95 °C (1 minute), 62 °C (1 minute), 72 °C (3 minutes), and 72 °C for 10 minutes. PCR amplicon products were electrophoresed on a 1.0% agarose gel (40 minutes) at 90 V, visualized and photographed under UV illumination with a 1-kb DNA ladder as a molecular weight marker. PCR product for family 1 is approximately 1000 bp and 1200 bp for family 2. LSM12/SKH2 primer was used to amplify any PspA family from all strains. The results were recorded as nontypeable when the PspA family was not determined after three attempts.[12–14] The PCR products from each of the three families were purified and sequenced (First Base Laboratories, Seri Kembangan, Malaysia) using SKH2 primer. A homology search was performed using Basic Local Allignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast). The PspA clades were analyzed by comparing the homology nucleotide sequences found from isolates in this study with those sequences of the clade-defining region of invasive references sequences retrieved from GenBank.[13]

Data interpretation

Data analysis such as serotype distribution, antimicrobial susceptibility pattern, and other variables were performed by SPSS software (IBM SPSS, New York USA). Differences in proportion were compared using the Chi-square test of independence and Fisher exact test. A p value < 0.050 was considered statistically significant.

Table 1 Oligonucleotide primers used in this study

Primer	Primer sequence (5′ - 3′)	Annealing temperature	Reference
Pneumolysin *(ply)*	Forward ATTTCTGTAACAGCTACCAACGA Reverse GAATTCCCTGTCTTTTCAAAGTC	55 °C	21
Autolysin *(lytA)*	Forward CAACCGTACAGAATGAAGCGG Reverse TTATTCGTGCAATACTCGTGCG	53 °C	22
LSM12 SKH63	CCGGATCCAGCGTCGCTATCTTAGGGGCTGGTT TTTCTGGCTCATC/TAACTGCTTTC	62 °C	(13,14)
LSM12 SKH52	CCGGATCCAGCGTCGCTATCTTAGGGGCTGGTT TGGGGGTGGAGTTTCTTCTTCATCT	62 °C	(13,14)
SKH41 SKH42	CGCACAGACTTAACAGATGAAC CTTGTCCATCAACTTCATCC	62 °C	(12,14)
LSM12 SKH2	CCGGATCCAGCGTCGCTATCTTAGGGGCTGGTT CCACATACCGTTTTCTTGTTTCCAGCC	58 °C	(12,13)

Results

Of the 210 children attending the three day care centers, only 195 were included in the study (response rate was 93%). Of these, 108 (55.4%) were boys and 87 (44.6%) were girls. A total of 69 strains (35.4%) of pneumococci were isolated from the nasal swabs of 195 healthy children. Of these, 67 strains showed 11 different serotypes and 2 strains (2.9%) were nontypeable (Table 2). The top six serotypes were 6A, 23F, 19A, 6B, 19F, and 15C, constituting 87% of all strains. Also, 40.6% of the serotypes are found in PCV7 and PCV10 and the coverage rates increased to 69.5% if PCV13 is taken into account.

Among the 69 isolates, 16 (23.2%) were penicillin-resistant *S pneumoniae* (PRSP), 5 (5.8%) were penicillin-intermediate *S pneumoniae* (PISP), and 48 (69.6%) were penicillin-susceptible *S pneumoniae* (PSSP). For erythromycin, 23 strains (33.3%) were resistant and 46 (66.7%) were susceptible. Resistance to co-trimoxazole was 31.9%, whereas 62.3% were susceptible. All PRSPs were resistant to erythromycin and 14 PRSPs (87.5%) were resistant to co-trimoxazole. Overall, 14 strains (20.3%) were MDRSP by being resistant to more than two classes of antibiotic. The most common type of PRSPs and MDRSPs was serotype 6A (14.5%), followed by 23F (4.3%) and nontypeable NT (1.5%). All pneumococcal isolates were susceptible to amoxicillin/clavulanic acid, ceftriaxone, and cefotaxime.

All 69 isolates in this study showed amplification in the PspA family typing. PCR products ranged from 1000 bp for family 1 and 1200-1400 bp for family 2. Of these, 24.6% belonged to PspA family 1 (15 isolates clade 1, 2 isolates clade 2), 71.0% were PspA family 2 (26 isolates clade 3, 10 isolates clade 4, and 13 isolates clade 5) and 4.3% were family 3 (3 isolates clade 6). With regard to PspA clade distribution, clade 3 (37.7%) was predominant, followed by clade 1 (21.7%), clade 5 (18.8%), clade 4 (14.5%), clade 6 (4.3%), and clade 2 (2.9%).

As shown in Table 3, serotype 6A, 23F, 19A, and 6B, which were predominant isolates, were grouped as either family 1 or family 2. PspA family 2 was found in serotypes 19F, 15C, 11A, 18C, 23A, and 20 whereas PspA family 1 was found in serotypes 6A, 23F, 19A, and 6B. Chi-square and Fisher exact analysis in all possible orientations of the variables showed no significant correlations except between PspA family typing and serotypes 23F ($p = 0.028$) and 6B ($p = 0.010$), respectively.

PCR detection of pneumolysin (*ply*) gene and autolysin (*lytA*) gene on all strains gave positive results for pneumococci (Figs. 1 and 2) which then was confirmed by sequencing.

There was no significant difference in PspA family typing by age group, sex, and penicillin susceptibility ($p > 0.050$).

There was no significant difference in PspA family typing by capsular serotypes ($p > 0.050$) except for serotypes 23F and 6B ($p < 0.050$).

Discussion

The colonization rate of 35.4% in the current study is higher than the 10-11% rate reported among children in earlier studies in Malaysia.[23,24] High pneumococcal carriage rates in children have been recorded in other countries such as Indonesia (48%),[2] Vietnam (44%),[25] and Taiwan (41%).[26] Pneumococcal carriage can be the main source for horizontal spread of pneumococcal infection within the

Table 2 Antimicrobial susceptibility patterns and serotype distribution of *S pneumoniae* among carriage isolates from three day care centers in Kuala Lumpur, Malaysia

Capsular serotypes	Antimicrobial agent[a]										
	Nonsusceptible strain[b]										Susceptible strain[b]
	P	SXT	E	AMC	CTX	CRO	P,E	E, SXT	P, SXT	P,E, SXT [c]	P,E,SXT,AMC,CRO,CTX
6A (29%)	—	—	1	—	—	—	1	1	—	10	7
23F (18.8%)	—	1	—	—	—	—	1	—	—	3	8
19A (11.6%)	—	1	—	—	—	—	—	—	—	—	7
6B (10.1%)	—	—	1	—	-	—	—	1	—	—	4
19F (8.7%)	—	1	1	—	—	—	—	2	—	—	2
15C (8.7%)	—	—	—	—	—	—	—	—	—	—	6
11A (2.9%)	—	—	—	—	—	—	—	—	—	—	2
18C (2.9%)	—	—	—	—	—	—	—	—	—	—	2
23A (1.4%)	—	—	—	—	—	—	—	—	—	—	1
23B (1.4%)	—	—	—	—	—	—	—	—	—	—	1
20 (1.4%)	—	—	—	—	—	—	—	—	—	—	1
Nontypeable(NT) (2.9%)	—	—	—	—	—	—	—	—	—	1	1

[a] Antimicrobial susceptibility pattern was determined by the E-test and interpreted according to CLSI guidelines; penicillin (P)- MIC of $\leq$ 0.06 μg/mL: susceptible, MIC of $\geq$ 0.12 to 1.0 μg/mL: intermediate and MIC of $\geq$ 2.0 μg/mL: resistant; erythromycin (E)-MIC of $\leq$ 0.25 μg/mL: susceptible, MIC of 0.5 μg/mL: intermediate and MIC of $\geq$ 1 μg/mL: resistant; co-trimoxazole (SXT)- MIC of $\leq$ 0.5 μg/mL:susceptible; MIC of 1 to 2 μg/mL: intermediate and MIC of $\geq$ 4 μg/mL: resistant; amoxicillin/clavulanic acid (AMC)- MIC of $\leq$ 2.0 μg/mL: susceptible; MIC of 4 μg/mL: intermediate and MIC of $\geq$ 8 μg/mL: resistant; cefotaxime (CTX) and ceftriaxone (CRO)-MIC of $\leq$ 1.0 μg/mL: susceptible; MIC of 2 μg/mL: intermediate and MIC of $\geq$ 4 μg/mL: resistant.

[b] No of nonsusceptible (resistant or intermediate) and susceptible strain.

[c] Multidrug-resistant strains.

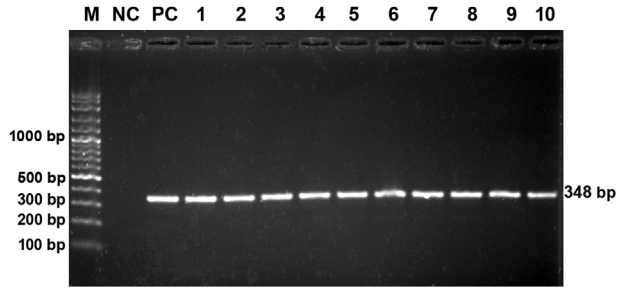

Figure 1. Presence of pneumolysin gene by amplification of 348-bp *ply* gene. First lane is Molecular Weight Marker (M) for 100 bp. NC = negative control; PC = positive control. Lanes 4 to 13: *S pneumoniae* strains 1-10.

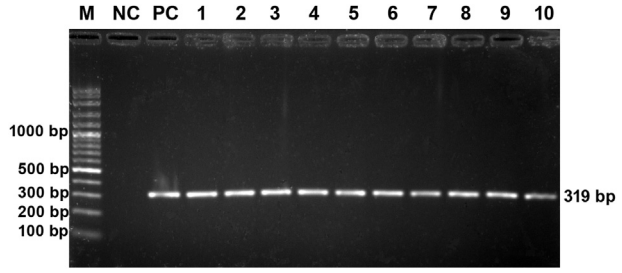

Figure 2. Presence of autolysin gene by amplification of 319 bp *lytA* gene. The first lane is Molecular Weight Marker (M) for 100 bp. NC = negative control; PC = positive control. Lanes 4 to 13: *S pneumoniae* strains 1-10.

community. In healthy children, risk factors such as crowding as seen in day care centers may play an important role in colonization and spread of pneumococcal strains.

Serotype distribution of pneumococcal carriage in this study showed findings similar to those in other Asian countries.[24] Six serotypes (6A, 23F, 19A, 6B, 19F, and 15C) are dominant (87%). These serotypes are also the cause of certain diseases in Malaysian children.[27] Vaccine coverage of PCV7 (40.6%), PCV10 (40.6%), and PCV13 (69.5%) among carriage isolates in this study showed findings almost similar to those from the study done by Yasin et al[27] where among the invasive isolates in children younger than 2 years, 44%, 56%, and 78% belonged to serotypes included in PCV7, PCV10, and PCV13, respectively. In Malaysia, PCV7 was introduced in 2005, followed by PCV10 in 2009 and PCV13 in 2010.[27] However, this conjugate vaccine is not given to all Malaysian children because it is not yet included in the National Immunisation Program (NIP).

The use of penicillin and other antibiotics had reduced the mortality and morbidity from pneumococcal infections worldwide. However, in recent years, *S pneumoniae* has demonstrated increasing resistance to commonly used antibiotics, especially penicillin. These problems are more frequently found in children than in adults.[28] The high rate of resistance to penicillin and other antimicrobial agents in *S pneumoniae* in this study is consistent with that in other countries in the Asian-Pacific region.[24] We also found that the PRSP rate among carriage strain was higher (23.2%) when compared with the rate in a previous study done in Malaysia (13.3%).[24] The increased difficulty in treating pneumococcal infections makes prevention through vaccination even more important.[15] Although the currently licensed PPV23 and PCV7 have proved to be effective, the immunogenicity is age dependent. Nevertheless, a pneumococcal conjugate vaccine such as PCV7 confers protection against a limited number of serotypes, and many developing countries cannot afford to purchase it because of its cost.

Recent research is focusing on protein-based vaccine candidates such as PspA. PspA inhibits fixation of complement C3 on the pneumococcal cell surface, thus protecting the pneumococcus from clearance by the host complement

Table 3 Frequency of PspA family typing and PspA clade of *S pneumoniae* isolates

Variables		Family 1 (n = 17)		Family 2 (n = 49)			Family 3 (n = 3)
Clade		Clade 1	Clade 2	Clade 3	Clade 4	Clade 5	Clade 6
Age	$\leq$ 2 y	3	0	9	5	3	0
	$>$ 2-5 y	12	2	17	5	10	3
Sex	Male	7	2	20	6	6	1
	Female	8	0	6	4	7	2
Penicillin susceptibility	Susceptible (n = 48)	13	2	10	10	12	1
	Nonsusceptible (n = 21)	2	0	16	0	1	2
Capsular serotypes	6A (29%)	4	0	12	2	2	0
	23F (18.8%)	7	0	4	0	2	0
	19A (11.6%)	1	0	0	0	7	0
	6B (10.1%)	3	2	1	0	1	0
	19F (8.7%)	0	0	4	1	0	1
	15C (8.7%)	0	0	0	5	1	0
	11A (2.9%)	0	0	2	0	0	0
	18C (2.9%)	0	0	2	0	0	0
	23A (1.4%)	0	0	0	1	0	0
	23B (1.4%)	0	0	0	0	0	1
	20 (1.4%)	0	0	1	0	0	0
	Nontypeable (2.9%)	0	0	0	1	0	1

system. PspA also binds to the iron- bound form lactoferrin, thus providing protection against apolactoferrin, which is bactericidal against the pneumococcus.[19,29] In this study, all the isolates showed amplification of PspA family typing. The predominant PspA family 2 among pneumococcal carriage in this study showed findings similar to those in other countries such as Brazil, Korea, and Spain.[7,30,31] However, the clade distribution of PspA was slightly different in healthy Brazilian children,[7] in whom clade 4 was predominant, followed by clades 1, 3, 2, and 5. In our study we observed that clade 3 was predominant, followed by clades 1, 5, 4, 6, and 2.

PspA is said to be cross-protective and has been shown to protect mice against pneumonia, fatal sepsis, and carriage.[32–35] PspA also has been shown to induce both systemic and mucosal antibody response against experimental pneumococcal carriage in humans.[36,37] Baril et al[38] demonstrated that PspA was efficient at eliciting T cell and humoral immune responses. A study has shown that fusion proteins containing family 1 and family 2 PspA fragments can induce broader protection against pneumococcus in mice.[39] In our study, there was no correlation between PspA families and sex, age, and penicillin susceptibility, thus making PspA a suitable vaccine candidate. However, PspA families showed a significant result in relation to some capsular serotypes as was seen in serotype 23F and 6B. There was no suitable explanation in this situation. To obtain more conclusive data, further investigation of PspA family distribution among nasal carriages and clinical isolates in this country is required. In this study, 95.7% of pneumococcal isolates expressed either PspA family 1 or 2. This finding supports a mixture of PspA family 1 and family 2 could be considered for development of future vaccines. To our knowledge, this is the first study relating PspA family types in Malaysia. By studying and exploring the pattern of distribution based on PspA typing, it would provide useful information for the suitability of this protein antigen as a vaccine candidate against pneumococcal population. Other protein candidates such as pneumolysin, PsaA, CbpA, neuraminidase, and autolysin have been suggested as potential candidates, but PspA, PsaA, and *ply* have been the leading vaccine candidates.[10] Combinations of PspA with other proteins should be considered in future studies for better protection (Table 3).

Conflict of interest

All authors report no conflicts of interest relevant to this article.

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