

Determination of pneumococcal serotypes/genotypes in nasopharyngeal secretions of otitis media children by multiplex PCR

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Abstract The appropriate clinical applications of pneumococcal polysaccharide vaccines against recent increases in antimicrobial resistant *Streptococcus pneumoniae* (*S. pneumoniae*) urgently require accurate analytical methodologies for determining and characterizing the serotypes. The results of current immunological determinations of serotypes with anti-capsular polysaccharide-specific sera are difficult to interpret in terms of quelling changes of the pneumococci. In this study, we applied the multiplex PCR technique for the rapid identification of pneumococci and simultaneous rapid determinations of their serotypes and genotypes that directly correlated with antimicrobial susceptibilities from nasopharyngeal secretions (NPS). Serogroups 6, 19F and 23F were the predominant capsular types of *S. pneumoniae* in the NPS samples. Strains of serotypes 19F and 23F frequently had mutations in *pbp1a*, *pbp2x* and *pbp2b* and expressed *ermB* and *mefA*; they also were mostly resistant to both penicillin G (PCG) and clarithromycin (CAM). Two NPS samples contained the strain of serotype 19F together with the strain of serotype 23F, although only the strain of serotype 19F was identified by a conventional bacterial culture. Pneumococci were identified in six NPS samples and their serotypes determined by the multiplex PCR, while a conventional bacterial culture failed to identify the pathogens. Our findings suggest that PCR-based serotyping and genotyping can provide an accurate and rapid distribution of pneumococcal

serotypes and antimicrobial resistance. The relatively minor populations in the nasopharynx may be determined using molecular techniques.

Keywords Acute otitis media · Genotype · Nasopharyngeal secretion · Serotype · *Streptococcus pneumoniae*

Introduction

Streptococcus pneumoniae is a leading causative pathogen responsible for acute otitis media (AOM) that frequently colonizes the nasopharynx [3, 6]. This pathogen has long been susceptible to penicillin, and AOM caused by pneumococci are easily improved by oral antimicrobial therapy. However, recent dramatic increases of antimicrobial resistance in *S. pneumoniae* are making the treatments of AOM with oral antibiotics more difficult [5, 8, 9]. There are urgent demands to prevent pneumococcal AOM through vaccinations. Nasopharyngeal colonization with causative pathogens is one of the more important risk factors for developing AOM. Consequently, reducing the frequency of nasopharyngeal carriage of *S. pneumoniae* is an important step towards preventing the development of AOM. However, there is less evidence for preventing AOM by vaccines [19]. A newly developed 10-valent vaccine conjugated with *H. influenzae* protein D shows more efficacy for reducing nasopharyngeal carriage [13]. Nevertheless, recent reports have shown a decrease in the carriage of vaccine serotypes and a parallel increase in non-vaccine serotypes following vaccination [1, 14, 20].

In order to be able to carry out a comprehensive evaluation of vaccine efficacies, it is first necessary to understand the prevalence of vaccine serotypes as well as the antimicrobial resistances of pneumococci associated

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with AOM. The determination of serotypes by immunological methods requires the isolation of pneumococci, is time consuming and expensive, and the results are difficult to interpret. In an earlier publication, we reported the successful applications of the multiplex PCR approach for determining pneumococcal serotypes [2]. We also showed that there was a correlation between the gene mutation and the minimal inhibitory concentration (MIC) to beta-lactam antibiotics. However, in that study, the gene mutation was investigated using isolated pneumococci [6]. To date, there has been no study which simultaneously evaluates the pneumococcal serotypes and gene types present in nasopharyngeal secretions (NPS). We hypothesized that the PCR technique could be used for identifying serotyping, and genotyping pneumococci present in NPS, thereby also enabling us to determine whether more than one serotype can co-exist there.

The aims of this study were (1) to simultaneously identify and determine serotypes of *S. pneumoniae* in the NPS of acute otitis media (AOM) patients, (2) to determine the genotypes of penicillin-binding proteins (PBPs) and macrolide-resistant traits in NPS of AOM patients and, in addition, (3) to examine whether one or more serotypes of *S. pneumoniae* may co-exist as nasopharyngeal flora in patients.

Materials and methods

Nasopharyngeal secretions

A total of 60 NPS were collected from pediatric patients (12–60 months old) with AOM at the clinics of Otolaryngology – Head and Neck Surgery, Wakayama Medical University Hospital. The NPS samples were collected by suction using a fine, flexible plastic catheter (no 5, French) and syringe. Informed consent was obtained from the parents or guardians of the patients prior to the collection of samples, in accordance with the guidelines of the institutional review board of Wakayama Medical University.

Bacterial culture

A portion of each of the 60 NPS samples was cultured on 5% sheep blood agar plates and chocolate agar plate (Nippon Becton Dickinson, Tokyo, Japan) for 48 h at 37°C in a humidified atmosphere supplied with 5% CO₂. *S. pneumoniae* was identified on the basis of alpha-hemolysis and colony morphology, Gram-stained smear, optochin disk sensitivity and bile solubility. Determinations of the MICs to penicillin G (PCG) and clarithromycin (CAM) were performed using CLSI methods [12], and the serotypes were determined using a standard laboratory method. Briefly, a bacterial suspension was mixed with group-

specific or type-specific antisera (The Statens Serum Institute, Copenhagen, Denmark) [15]. The quellung and agglutination were assessed by phase-contrast microscopy.

Preparation of genomic DNA

Total genomic DNA was purified from both NPS and *S. pneumoniae* isolates. Prior to purification of the genomic DNA from the NPS, the samples were diluted three times with sterilized saline and then centrifuged to remove inhibitory substances. The NPS pellets and a single colony of pneumococci were then digested with a lysis solution [1 M Tris, pH 8.9, 4.5 (v/v) nonident P-40, 4.5 (v/v) Tween 20, 10 mg/ml Proteinase K] for 1 h at 60°C. Following centrifugation, the supernatant was mixed with 3 M sodium acetate and the total genomic DNA was precipitated by ethanol.

PCR-based genotyping

Seven sets of oligonucleotide primers were used to amplify *bbp* genes (*bbp1a*, *bbp2x*, *bbp2b*), macrolide-resistant genes (*mefA* and *ermB*), the pneumococcal common autolysin gene (*lytA*) and the pneumolysin gene (*ply*) (Table 1) [16–18]. Each primer mixture contained multiplex PCR5, multiplex PCR6, multiplex PCR7 and PCR8 primers for *lytA* and *bbp1a*, *bbp2x* and *bbp2b*, *mefE* and *ermB*, and *ply*, respectively. The PCR reaction mixtures consisted of 1 µl of bacterial lysate or purified DNA from NPs, 8 µl 25 mM of dNTP mixture, 2.5 U of Tth DNA polymerase (Takara Biomedicals, Kyoto, Japan), 10 µl 10×PCR buffer, pH 8.3 and 60 ng of primer mixtures (multiplex PCR5, multiplex PCR6, multiplex PCR7 and PCR8) in 100 µl of solution. The reaction mixture was then subjected to 30 cycles (20 s at 94°C, 20 s at 55°C and 15 s at 72°C) of amplification in the programmable thermal cycler (Gene Amp PCR System 9700; Perkin-Elmer, Norwalk, Conn.). Amplified DNA fragments were analyzed using 3% agarose gel electrophoresis (Fig. 1).

PCR-based serotyping

The oligonucleotide primers were used to amplify capsular serotypes 1, 3, 4, 14, 19A, 19F, 23F and serogroups 6, 18, 19 and 23 for multiplex PCR-based serotyping (Table 1) [4, 10]. The PCR reaction mixtures consisted of 1 µl of bacterial lysate or purified DNA, 0.5 µl 10 mM of dNTP mixture, 0.5 µl *Taq* DNA polymerase, 2.5 µl 10×PCR buffer, 4 µl 25 mM MgCl₂, 5.0 µl Q-solution (Qiagen, Hilden, Germany), 1.25 µl 1 M KCl and 0.25 µl of each primer sets (multiplex PCR1, multiplex PCR2, multiplex PCR3, multiplex PCR4) in 25 µl of solution. For serotyping, the reaction mixture was subjected to denaturation at 94°C for 10 min, followed by 32 cycles of amplification

Table 1 Primers used in this study

Primers	Target gene	Sequences	PCR products (bp)
Multiplex PCR 1	Serogroup 6	5'-TATAGATCCGATACGACGTAAC-3' 5'-ATACCAATTACACCAAAGTCTG-3'	200
	Serogroup 19	5'-CTAATGAGCCTAAACGTCTCT-3' 5'-TTGACTGCACCAAGTACACT-3'	222
	Serogroup 18	5'-GCATCTGTACAGTGTGCTAATTGGATTGAAG-3' 5'-CTTTAACATCTGACTTTTTCTGTTCCTCCAAC-3'	478
	Serogroup 23	5'-GATGCAAGAAATGTCGGTA-3' 5'-TCTGCCTCATTGTTCTCC-3'	126
Multiplex PCR 2	Serotype 1	5'-GTCGTTATGAGAAGGTGGA-3' 5'-TGACCAAATAGAACCTGATG-3'	108
	Serotype 19F	5'-GTTCAACGACTAGGACGC-3' 5'-TAGGCACCAATGTTTCACT-3'	130
	Serotype 3	5'-ATGTGGATTTCGAGAGTG-3' 5'-GATTACGCTCAGGGTCAA-3'	152
Multiplex PCR 3	Serotype 14	5'-AACCGACAAAAACAATAAG-3' 5'-AACCGACAAAAACAATAAG-3'	220
	Serotype 23F	5'-TGGTAGTGACAGCAACGA-3' 5'-CAAAGGCTAATTCAGCATC-3'	177
	Serotype 4	5'-CTGTTACTTGTTCTGGACTCTCGTTAATTGG-3' 5'-GCCCACTCCTGTTAAAATCCTACCCGCATTG-3'	430
Multiplex PCR 4	Serotype 19A	5'-GTTAGTCCTGTTTATAGATTTATTTGGTGATGT-3' 5'-GAGCAGTCAATAAGATGAGACGATAGTTAG-3'	478
	<i>pbp1a</i>	5'-AAACAAGGTCGGACTCAACC-3' 5'-ATATACATTGGTTTATAGTAAGTT-3'	430
Multiplex PCR 5	<i>lytA</i>	5'-TGAAGCGGATTTACTGTC-3' 5'-GCTAAACTCCCTG TATCAAGCG-3'	273
	<i>pbp2x</i>	5'-CCAGGTTCCACTATGAAAGTG-3' 5'-ATCCCAACGTTACTTGAGTGT-3'	292
	<i>pbp2b</i>	5'-CCTATATGGTCCAAACAGCCT-3' 5'-GGTCAATTCTGTCGCAGTA-3'	147
Multiplex PCR 6	<i>mefA</i>	5'-CTGTATGGAGCTACCTGCTGG-3' 5'-CCCAGCTTAGGTATACGTAC-3'	402
	<i>ermB</i>	5'-CGTACCTTGGATATTCACCG-3' 5'-GTAAACAGTTGACGATATTCTCG-3'	224
	<i>ply</i>	5'-ATTTCTGTAACAGCTACCAACGA-3' 5'-GAATTCCTGTCTTTCAAAGTC-3'	348

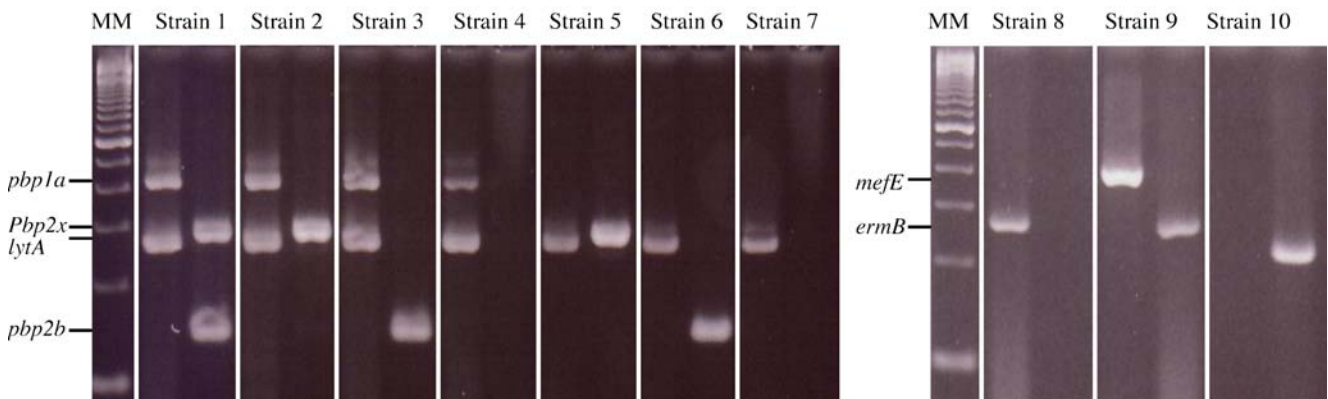


Fig. 1 PCR-based genotypes of seven clinical isolates of *Streptococcus pneumoniae*. MW Molecular-weight marker (100 bp). Strain:1 No mutation in *pbp* genes, 2 mutation in *pbp2b*, 3 mutation in *pbp2x*, 4 mutations in *pbp2b* and *pbp2x*, 5 mutations in *pbp1a* and *pbp2b*, 6 mutations in *pbp1a* and *pbp2x*, 7 mutations in *pbp1a*, *pbp2x* and *pbp2b*, 8 *ermB*-positive, 9 *ermB*- and *mefE*-positive, 10 *mefE*-positive

consisting of denaturation at 94°C for 30 s, annealing at 61°C for 30 s and extension at 72°C for 1 min and a further extension at 72°C for 5 min for serotyping. Strains of serotype 4 (ATCC BAA-334) and 19F (ATCC 49619) obtained from the American Type Culture Collection (ATCC, Manassas, Va.) were used for quality control in every reaction (Fig. 2).

Results

Identification of *S. pneumoniae* in NPS

S. pneumoniae were identified in 30 (50.0%) and 36 (60.0%) of the 60 NPS samples tested by conventional culture and PCR analysis, respectively. Not only were culturable *S. pneumoniae* identifiable by conventional bacterial culture methodology and PCR analysis, but even samples containing pneumococci that were unidentifiable by culture techniques showed amplifications of both *lytA* and *ply* with PCR analysis.

Based on the CLSI criteria, the pneumococcal isolates consisted of 12 penicillin-sensitive *S. pneumoniae* (PSSP; 40.0%), 11 penicillin-intermediate *S. pneumoniae* (PISP) (36.7%) and seven penicillin-resistant *S. pneumoniae* (PRSP; 23.3%). They were also classified into six (20.0%), one (3.33%) and 12 (76.67%) strains that showed sensitivity, intermediate resistance and resistance to CAM, respectively. Based on the serology, the isolates were classified into serotypes 3 (1; 3.3%), 14 (3; 10.0%), 19F (5; 16.7%), 23F (6; 20.0%), 6B (10; 33.3%), 6A (1; 3.3%), 15 (1; 3.3%), 9V (1; 3.3%) and non-11 (2; 6.7%) (Table 2). All serotype 23F strains and three (60.0%) 19F strains were resistant to both PCG and CAM.

PCR-based genotypes

Among 36 NPS samples, 19 (52.8%) samples contained strains with mutations in the three PBP genes (Table 2), eight (22.2%) contained either strains with mutations in *pbp1a* and *pbp2x* (four samples; 11.1%) or in *pbp2x* and *pbp2b* (four samples; 11.1%), eight (22.2%) contained either strains with a mutation in *pbp1a* (one sample; 2.8%), in *pbp2b* (one sample; 2.8%) and in *pbp2x* (six samples; 16.7%) and only one (2.8%) contained the strain without mutations in the three PBP genes.

Susceptibilities to PCG of strains with mutations in three PBP genes, in two types of PBP genes, in one type of PBP gene and without mutations in the PBP genes were 0.12–2, ≤0.06–1, ≤0.06 and ≤0.06 µg/ml, respectively. Twenty-six (72.2%) samples contained strains with macrolide-resistant genes: 15 (44.4%) samples with strains possessing *mefE*, 13 (19.4%) strains possessing *ermB* and three (8.3%) strains possessing both macrolide-resistant genes, respectively. Ten (27.8%) samples contained strains having neither type of macrolide-resistant gene. Susceptibilities to CAM of strains with the *mefE* gene, *ermB* gene and both were 1–4, >64 and >64 µg/ml, respectively.

PCR-based serotypes

The multiplex PCR was able to determine the serotypes/serogroups of 36 NPS samples; in contrast, conventional methodology determined the serotype/serogroups in only 30 samples (Table 2); these were serotypes 3 (1; 2.8%), 14 (3; 8.3%), 19F (7; 19.4%), 23F (10; 27.8%) and 6 (11; 30.6%) as well as seven ‘others’ (19.4%). With the exception of the typeable pneumococci, all of the serotypes identified by multiplex PCR showed a similar quellung reaction with antisera. Four untypeable samples by multiplex PCR were classified into serogroups 15 (one), 9V (one) and non-11 (two) types by serological determination. In this study we compared the ratio of accurate determination of serotypes by both procedures. The correct determination of the serotypes when both procedures showed same results was determining. If one procedure failed to identify serotypes determined by the other procedure, the cases were defined as false determinations. The multiplex PCR determined the pneumococcal serotypes in 35 (97.2%) of the 36 NPS samples, while a conventional serological method determined the serotypes in 29 (80.5%) of the 36 NPS samples ($p < 0.05$). Two NPS samples were culture-negative but contained pneumococcal genomes. One sample (no. 8) showed serogroup 23 by multiplex PCR, but showed serotype 6B by serological determination. Two different serotypes were determined concurrently in two NPS by multiplex PCR. The positive and negative predictive value of PCR based on the serotyping of NPS

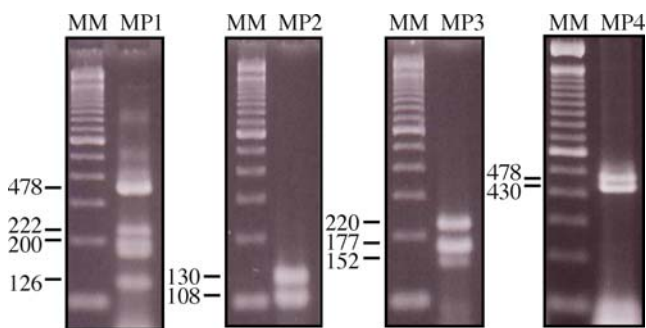


Fig. 2 A multiplex of PCR-based serotypes. *MM* Molecular-weight marker. *MP1* Serogroup 6, serogroup 19, serogroup 18 and serogroup 23, *MP2* serotype 1 and serotype 19F, *MP3* serotype 3, serotype 14 and serotype 23F, *MP4* serotype 4 and serotype 19A

Table 2 Results of the multiplex PCR and conventional culture analyses in terms of detecting, genotyping and serotyping strains of *S. pneumoniae* in pneumococcal-positive nasopharyngeal secretions

Sample no.	<i>S. pneumoniae</i> isolates										Nasopharyngeal secretions									
	Culture		Serotypes		PCR-based genotypes					PCR			PCR-based serotypes			PCR-based genotypes				
					MLR genes	MIC	PCG	CAM	<i>ply</i>	<i>lytA</i>										
1	+	+	3	-	-	≤0.06	≤0.06	+	+	+	+	3	-	+	+	-	-	-	-	-
2	+	+	6A	+	-	0.5	1	+	+	+	+	G6	+	+	+	+	+	+	+	-
3	+	+	6B	-	-	≤0.06	0.5	+	+	+	+	G6	-	-	-	-	-	-	-	-
4	+	+	6B	-	-	1	0.25	+	+	+	+	G6	-	-	-	-	-	-	-	-
5	+	+	6B	-	-	≤0.06	1	+	+	+	+	G6	-	-	-	-	-	-	-	-
6	+	+	6B	+	+	≤0.06	1	+	+	+	+	G6	+	+	+	+	+	+	+	-
7	+	+	6B	+	-	0.12	0.25	+	+	+	+	G6	+	+	+	+	+	+	+	-
8	+	+	6B	+	-	0.5	0.25	+	+	+	+	G23	+	+	+	+	+	+	+	-
9	+	+	6B	+	+	2	>64	+	+	+	+	G6	+	+	+	+	+	+	+	-
10	+	+	6B	+	-	0.12	2	+	+	+	+	G6	+	+	+	+	+	+	+	-
11	+	+	6B	+	-	≤0.06	2	+	+	+	+	G6	+	+	+	+	+	+	+	-
12	+	+	6B	+	+	≤0.06	>64	+	+	+	+	G6	+	+	+	+	+	+	+	+
13	+	+	9V	+	+	≤0.06	>64	+	+	+	+	others	+	+	+	+	+	+	+	+
14	+	+	14	+	+	0.12	>64	+	+	+	+	14	-	-	-	-	-	-	-	-
15	+	+	14	+	-	0.5	1	+	+	+	+	14	-	-	-	-	-	-	-	-
16	+	+	14	+	+	0.25	4	+	+	+	+	14	-	-	-	-	-	-	-	-
17	+	+	G15	-	+	≤0.06	>64	+	+	+	+	others	-	-	-	-	-	-	-	-
18	+	+	19F	+	+	≤0.06	>64	+	+	+	+	19F	+	+	+	+	+	+	+	+
19	+	+	19F	+	-	≤0.06	≤0.06	+	+	+	+	19F	+	+	+	+	+	+	+	+
20	+	+	19F	+	+	2	2	+	+	+	+	19F	+	+	+	+	+	+	+	+
21	+	+	19F	+	-	2	2	+	+	+	+	19F, 23F	+	+	+	+	+	+	+	+
22	+	+	19F	+	-	1	2	+	+	+	+	19F, 23F	+	+	+	+	+	+	+	+
23	+	+	23F	+	+	1	>64	+	+	+	+	23F	+	+	+	+	+	+	+	+
24	+	+	23F	+	-	2	>64	+	+	+	+	23F	+	+	+	+	+	+	+	+
25	+	+	23F	+	+	2	>64	+	+	+	+	23F	+	+	+	+	+	+	+	+
26	+	+	23F	+	+	2	>64	+	+	+	+	23F	+	+	+	+	+	+	+	+
27	+	+	23F	+	+	1	>64	+	+	+	+	23F	+	+	+	+	+	+	+	+
28	+	+	23F	+	+	2	>64	+	+	+	+	23F	+	+	+	+	+	+	+	+
29	+	+	Non-11	+	+	≤0.06	>64	+	+	+	+	others	+	+	+	+	+	+	+	+
30	+	+	Non-11	-	-	≤0.06	≤0.06	+	+	+	+	others	-	-	-	-	-	-	-	-
31	-	-	NA	NA	NA	NA	NA	+	+	+	+	G6	+	+	+	+	+	+	+	+
32	-	-	NA	NA	NA	NA	NA	+	+	+	+	19F	-	-	-	-	-	-	-	-
33	-	-	NA	NA	NA	NA	NA	+	+	+	+	19F	-	-	-	-	-	-	-	-
34	-	-	NA	NA	NA	NA	NA	+	+	+	+	23F	-	-	-	-	-	-	-	-
35	-	-	NA	NA	NA	NA	NA	+	+	+	+	others	+	+	+	+	+	+	+	+
36	-	-	NA	NA	NA	NA	NA	+	+	+	+	others	-	-	-	-	-	-	-	-

+, Pneumococcus-positive or mutation of *pbp* gene; -, pneumococcus-negative or no mutation of *pbp* gene; NA, not applicable; Non11, other than the 11 valents conjugated pneumococcal vaccine serotypes; others, other than capsular serotype 1, 3, 4, 14, 19A, 19F, 23F and serogroup 6, 18, 19 and 23; PCG, penicillin G; CAM, clarithromycin

compared with the serological method was 96.3% (26/27 samples) and 100%, respectively.

The multiplex PCR was able to determine the serotypes/serogroups of four NPS samples in which pneumococci were not identified by a conventional bacterial culture and in which it had not been possible to determine pneumococcal serotypes/serogroups.

Discussion

In this era of antimicrobial resistant *S. pneumoniae* and vaccine development against this pathogen, it is important to be able to evaluate the characteristics of the pneumococci colonizing the nasopharynx [3, 5, 6]. However, the current serological determinations of capsular serotypes are difficult to interpret in terms of the quellung reactions of pneumococcal cells with anti-polysaccharide sera [2]. Consequently, attempts have made to determine the accurate pneumococcal characteristics by molecular biological procedures. Earlier studies in our laboratory determined that the application of PCR-based serotyping and genotyping to pneumococcal isolates facilitates the identification of pneumococcal isolates [2] and that the multiplex PCR serotyping approach is able to determine particular types of capsular polysaccharides accurately. PCR-based genotyping also revealed that the frequencies of mutations in *pbp* and the expressions of *mefA* and *ermB* are closely related with susceptibilities to β -lactams and macrolides [6]. However, most of the studies applied PCR to pneumococcal isolates – and not directly to clinical samples [4, 10]. In the present study, we applied the multiplex PCR technique directly to NPS specimens and were able to identify and determine the characteristics of 11 pneumococcal serotypes and serogroups simultaneously. Those identified consisted of most of the serotypes used for current pneumococcal vaccines. We also applied PCR-based genotyping to the *pbp* genes and macrolide resistance genes.

Serogroups 6, 19F or 23F were the predominant capsular types in this study. Approximately 52.8% of the NPS samples contained pneumococci having mutations in the three *pbp* genes and could be classified as PRSP according to the CLSI criteria. Approximately 72.2% of the NPS samples contained pneumococci expressing either type of macrolide-resistant trait and were resistant to CAM. Most of these strains with mutations in the three *pbp* genes belonged to serogroups 6, 19F or 23F. The multiple PCR analysis was able to determine the pneumococci and the pneumococcal serotypes and serogroups isolated from the NPS samples more accurately than conventional culture and immunological methodology. In six samples, pneumococcal genomes were identified by the multiplex PCR, while a conventional culture method failed to identify pneumococci. In two cases, strains of serotypes 19F and 23F were

simultaneously identified in the NPS, although only one strain of serotype 19F was identified by a conventional culture method. Recent reports on vaccine efficacies against nasopharyngeal colonization with pneumococci have shown that the pneumococci in the nasopharynx can change from vaccine serotypes to non-vaccine serotypes following vaccination [1, 14, 20]. The underlying factors determining these changes are still unclear, although one hypothesis is a replacement of pneumococcal strains in the nasopharynx. Nasopharyngeal pneumococcal flora consist of several different strains, with minor pneumococcal populations present in the nasopharynx. Current conventional serological methodology requires the isolation of the pneumococcal strains. Huebner *et al.* suggested that if the less common serotype represents only 5% of the total pneumococcal population, 59 colonies from each specimen would need to be serotyped to have a 95% probability of picking the second pneumococcal type [7]. In addition, non-PCR methods can be prone to mis-interpretation. In the present study, a strain was determined to be serogroup 23 by the multiplex PCR, while based on the serology, the strain was serotype 6B. It is somewhat difficult to interpret the quellung changes and the serological determination sometimes failed to determine the accurate prevalence of pneumococci in the nasopharynx.

In this study, we have analyzed a limited number of serotypes by the multiplex PCR techniques because of the unavailability of the sequences of other pneumococcal serotypes. However, multiplex PCR-based serotyping can provide an accurate and rapid distribution of pneumococcal serotypes, including minor populations, among the *S. pneumoniae* populations of the nasopharynx. The multiplex PCR method does not need expertise to interpret the results and can be used to run many samples at one time. Moreover, multiplex PCR-based serotyping and genotyping can replace conventional microbiological methods that are used to identify and determine *S. pneumoniae*, pneumococcal capsular serotype, penicillin susceptibility and macrolide resistance traits. A follow-up study involving the quantitative evaluation by real-time PCR is necessary, and continuous monitoring of pneumococcal serotypes is essential since it has been shown that the incidence of types responsible for AOM can change over time [11]. We have shown that the direct application of PCR to the NPS enables a feasible analysis of minority strains in a pneumococcal population and confirmed this by studies on the carriage of multiple pneumococcal capsular types.

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