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Antibiotic susceptibility according to genotype of penicillin-binding protein and macrolide resistance genes, and serotype of *Streptococcus pneumoniae* isolates from community-acquired pneumonia in children

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***Objectives*: Antibiotic susceptibilities, genes mediating β-lactam and macrolide resistance, and serotypes were analysed for strains of *Streptococcus pneumoniae*.**

***Methods*: A total of 392 strains of *S. pneumoniae* were isolated from paediatric patients with community-acquired pneumonia between May 2002 and 2004. All strains were classified into six genotype patterns according to the mutations found in the *pbp1a*, *pbp2x* and *pbp2b* genes identified by PCR. These results are represented by adding 'g', indicating genotypic identification.**

***Results*: Thirty-nine per cent of the isolates showed mutations in either one or two PBP genes (gPISP, where PISP stands for penicillin-intermediate resistant *S. pneumoniae*) and 52.3% had mutations in three genes (gPRSP, where PRSP stands for penicillin-resistant *S. pneumoniae*). The majority of the strains had a macrolide resistance gene: *mef*(A), (30.6%); *erm*(B), (48.5%); or both *mef*(A) and *erm*(B), (7.7%). The most frequent serotypes of these strains were: 6B (23.2%), 23F (17.6%), 19F (17.3%), 14 (10.5%) and 6A (8.2%). Serotypes of the seven-valent conjugate vaccine covered 70.9% of all isolates, and 89.8% of gPRSP. Serotypes of the strains with cefotaxime MICs of ≥2 mg/L were almost all of a vaccine type.**

***Conclusions*: The results suggest that introduction of conjugate vaccines into infants and children is necessary for the prevention of pneumococcal infections in Japan.**

Keywords: *S. pneumoniae*, antibiotic resistance genes, PBPs, respiratory tract infections

Introduction

Streptococcus pneumoniae is a major pathogen associated with respiratory tract infections (RTIs), acute otitis media, septicaemia, and meningitis acquired in the community.

In Japan, penicillin non-susceptible *S. pneumoniae* has been increasing rapidly since around 1990. According to a nationwide surveillance study that was conducted between 1998 and 2000, the prevalence of penicillin-intermediate resistant *S. pneumoniae* (PISP) and penicillin-resistant *S. pneumoniae* (PRSP) was 34.4 and 49.0%, respectively.[1] In parallel, macrolide resistance increased to 70%.[2]

β-Lactam resistance in *S. pneumoniae* is mediated mainly by multiple genes encoding the PBP1A, PBP2X and PBP2B enzymes (where PBP stands for penicillin-binding protein), which are involved in peptidoglycan synthesis.[3] We have developed a rapid identification method using PCR to detect mutations in these genes.[4]

Conjugate vaccines have recently attracted attention for prevention of various pneumococcal infections[5,6] and large-scale clinical

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trials of the 7- or 11-valent vaccine were conducted in several countries. As a result, some countries have already approved the use of this vaccine in children.

In the present paper, we describe antibiotic susceptibility and identification of resistance genes for *S. pneumoniae* from paediatric patients with pneumonia, and estimate the coverage rates of 7- and 13-valent conjugate vaccines in Japan.

Materials and methods

Strains

The acute respiratory diseases (ARD) study was made possible by participation of paediatric physicians belonging to 10 medical institutions between May 2002 and 2004. A total of 392 strains of *S. pneumoniae* were isolated from clinical samples of infants and children (n = 1121) with community-acquired pneumonia (CAP). Identification of the isolates as *S. pneumoniae* was confirmed by PCR for the autolysin (*lytA*) gene in our laboratory (Kitasato Institute for Life Sciences, Kitasato University).

Susceptibility testing

MICs of β-lactam and macrolide antibiotics were determined by an agar dilution method using Mueller–Hinton agar (MH; Difco Laboratories) supplemented with 5% defibrinized sheep blood. Antibiotics employed in this study were: penicillin and ampicillin (Meiji Seika Kaisha Ltd, Tokyo, Japan); cefotaxime and telithromycin (Aventis Pharma Ltd, Tokyo, Japan); and meropenem (Sumitomo Pharmaceuticals Co., Ltd, Osaka, Japan). *S. pneumoniae* ATCC 49619 was used as a quality control strain for susceptibility testing.

Serotyping

Serotypes of *S. pneumoniae* strains were determined by the Quellung reaction using antiserum purchased from the Statens Serum Institut (Copenhagen, Denmark).

Identification of antibiotic resistance genes by PCR

Oligonucleotide primers for detection of three PBP genes and macrolide resistance genes, *mef*(A) and *erm*(B), were used as previously described.[2]

Each reaction mixture contained (i) primers for detecting the *lytA* and *pbp1a* genes, (ii) primers for detecting the *pbp2x* and *pbp2b* genes, or (iii) primers for detecting the *mef*(A) and *erm*(B) genes. PCR conditions were also described previously.[4]

Sequencing

A total of 28 strains showing MICs of ≥4 mg/L for ampicillin or ≥2 mg/L for cefotaxime were termed tentatively high-resistant PRSP (H-PRSP), and the DNA region corresponding to the transpeptidase domain in each PBP gene was amplified. The nucleotide sequences were determined with an ABI PRISM 377 DNA sequencer.

PFGE analysis

PFGE analysis was carried out by a modification of the method described previously.[7]

For restriction endonuclease digestion, the plugs were incubated in restriction enzyme buffer with 20 U of *Apa*I at 37°C for 16 h. Electrophoresis was performed with a CHEF Mapper (Bio-Rad Laboratories, Hercules, CA, USA). Separation of fragments was carried out at 5.7 V/cm at 14°C for 18 h.

Results

All 392 strains were firstly classified into the following six genotypes by PCR for *pbp1a*, *pbp2x*, and *pbp2b* genes: (i) gPSSP with three normal genes (n = 33, 8.4%); (ii) gPISP (*pbp2x*) with abnormal *pbp2x* (n = 72, 18.4%); (iii) gPISP (*pbp2b*) with abnormal *pbp2b* (n = 2, 0.5%); (iv) gPISP (*pbp2x* + *2b*) with abnormal *pbp2x* and *pbp2b* (n = 26, 6.6%); (v) gPISP (*pbp1a* + *2x*) with abnormal *pbp1a* and *pbp2x* (n = 54, 13.8%); (vi) gPRSP with three abnormal PBP genes (n = 205, 52.3%).

The MIC ranges and MIC_{50}s of four β-lactam antibiotics against gPRSP were as follows: penicillin, 0.5–4 and 2 mg/L; ampicillin, 0.25–8 and 2 mg/L; cefotaxime, 0.25–8 and 0.5 mg/L; meropenem, 0.063–1 and 0.5 mg/L.

Although strains of gPISP (*pbp2x*) exhibited negligible resistance to ampicillin with a 2-fold increase over the MIC_{50} of gPSSP (0.031 mg/L), the MIC_{50} of cefotaxime was 16-fold higher than that of gPSSP (0.016 mg/L). MIC_{50}s of ampicillin and cefotaxime also varied in gPISP (*pbp2x* + *2b*) (0.5 and 0.25 mg/L) and gPISP (*pbp1a* + *2x*) (0.25 and 1 mg/L). Cefotaxime MICs against gPISP (*pbp1a* + *2x*) increased markedly, and were also affected by abnormal *pbp1a* genes. Susceptibilities to ampicillin were equally influenced by abnormal *pbp1a*, *pbp2x* and *pbp2b* genes.

Along with the above results, it was noteworthy that some strains showed an ampicillin MIC of ≥4 mg/L (n = 13) or a cefotaxime MIC of ≥2 mg/L (n = 15). Five PBP genes in these H-PRSP strains were analysed in detail, as described below.

For macrolide resistance, the numbers of strains possessing *mef*(A), *erm*(B) or both genes were 120 (30.6%), 190 (48.5%) and 30 (7.7%), respectively. The number of susceptible strains without any resistance gene was as low as 52 strains (13.3%). The MICs of telithromycin for the strains with *erm*(B) or *mef*(A) were from 0.063 to 0.125 mg/L, except for four (1.0%) strains with MICs of 2–4 mg/L.

Capsular serotypes of tested strains are listed in Table 1 according to the genotypic patterns for PBP genes.

The most frequent serotypes were: 6B (23.2%), 23F (17.6%), 19F (17.3%) and 14 (10.5%). The disproportionate distributions of serotypes of gPSSP, gPISP and gPRSP were confirmed (χ^2 = 595.9100, P = 0.0000). Various serotypes were also noted in gPISP (*pbp2x*) strains, of which serotypes 6A, 6B and 3 were most prevalent. Serotypes 23A and 14 showed the greatest prevalence in gPISP (*pbp2x* + *2b*) and gPISP (*pbp1a* + *2x*) strains, respectively. Five serotypes (19F, 6B, 23F, 6A and 14) accounted for 96.1% of gPRSP, while there were only three strains each of serotypes 23A and 35.

Incidentally, 7- and 13-valent conjugate vaccines covered 70.9 and 84.9% of all strain serotypes tested, respectively. However, being confined to gPRSP, the coverage rates of 7- and 13-valent conjugate vaccines were as high as 89.8 and 96.1%, respectively.

DNA sequences of transpeptidase regions in *pbp1a*, *pbp1b*, *pbp2a*, *pbp2x* and *pbp2b* genes were determined for H-PRSP strains. The results were compared with the sequences of gPRSP and the R6 strain.

Table 2 shows the results obtained from 16 strains that had amino acid substitutions different from those found in the major gPRSP. Among strains with an ampicillin MIC of ≥4 mg/L, 10 amino acid substitutions near the conserved motif Lys^{614}-Thr^{615}-Gly^{616} (KTG) in the *pbp2b* gene, as described by Kosowska *et al.*[8] were observed in only one strain (ARD-963) with an MIC of

Table 1. Serotype distribution according to genotype patterns classified by PCR for *pbp* genes in *S. pneumoniae* isolates

Genotype	Serotype														Total
	1	3	4	6A	6B	9	11	14	15	18	19A	19F	23A	23F	other	NT	
gPSSP	2		2	1		2	5	1	2	1	6	1	1	1	8		33
gPISP (*pbp2x*)		8		15	14	4	1	7	3		5	1		5	9		72
gPISP (*pbp2b*)					1								1				2
gPISP (*pbp2x* + 2*b*)				1	6			2	1			1	11	4			26
gPISP (*pbp1a* + 2*x*)				2	12		2	27	6		2			2		1	54
gPRSP (*pbp1a* + 2*x* + 2*b*)				13	58			4				65	3	57	3	2	205
Total (%)	2 (0.5)	8 (2.0)	2 (0.5)	32 (8.2)	91 (23.2)	6 (1.5)	8 (2.0)	41 (10.5)	12 (3.1)	1 (0.3)	13 (3.3)	68 (17.3)	16 (4.1)	69 (17.6)	20 (5.1)	3 (0.7)	392 (100)

8 mg/L which were different from those found in other strains; substitutions of Ala^{591}→Ser, Gly^{596}→Pro, Asn^{605}→Asp, Leu^{608}→Thr, Ala^{618}→Gly, Asp^{624}→Gly, Gln^{627}→Glu, Thr^{629}→Asn, Ser^{639}→Thr and Asp^{640}→Glu.

Of 15 strains with a cefotaxime MIC of ≥2 mg/L, 13 strains possessed amino acid substitutions in the *pbp2x* gene that changed Ser^{337}-The^{338}-Met^{339}-Lys^{340} (STMK) to Ser-Ala-Phe-Lys (SAFK). In all of the tested strains, no unknown amino acid substitutions were observed in the *pbp1a*, *pbp1b* and *pbp2a* genes.

Figure 1 shows the PFGE patterns of 16 H-PRSP strains listed in Table 2. PFGE was performed according to each serotype of these strains. Serotype 19F ($n = 6$) was most common, followed by two strains each of 14, 23F, 6A, 6B and non-typeable. Comparisons based on a dendrogram indicated a high similarity among strains of serotypes 19F and 14, but apparent differences in all other pairs of serotype strains.

Discussion

Resistance to various chemotherapeutical agents in *S. pneumoniae*, a leading cause of respiratory infections, has become a major worldwide problem. In Japan, according to the results based on our PCR methods, the causative gPISP and gPRSP in meningitis reached 37.3 and 41.5%, respectively in 2002.[4] Nearly 70% of these isolates also possessed *mef*(A) or *erm*(B) genes mediating macrolide resistance. Several reasons for the rapid increase in resistance were considered. One reason was that almost all of the oral cephalosporin antibiotics prescribed to outpatients obtain low serum concentrations and tissue distribution. Basically, circumstances differ from those in Europe and the US, where amoxicillin/clavulanic acid, which obtains high serum concentrations, is recommended as the first choice oral antibiotic for paediatric outpatients with RTIs. It follows that these differences in the prescribing patterns are reflected in the PBP gene alterations in PRSP and PISP. Non-susceptible strains with an abnormal *pbp2x* gene, resulting in intermediate-resistance to cephalosporin antibiotics, are isolated at a high rate in Japan, while strains with an abnormal *pbp2b* are more prevalent in the US.[9]

The relatively high population density in Japan is a secondary factor, which results in easy transmission of resistance among young children.

On a global basis, with the goal of preventing pneumococcal infections in children, large-scale clinical trials of 7-valent conjugate vaccine have been conducted in a number of countries, and vaccination programmes have already become compulsory in some countries.[5] However, the *S. pneumoniae* serotype distributions are known to vary between developing and developed countries, and with patient's age and variety of infection.

In Japan, a clinical trial of a 7-valent conjugate vaccine for children has been in progress since 2004. No recent epidemiological data regarding the serotypes in RTIs, which are fundamental for vaccine introduction, are available except the data of the Nationwide Surveillance for Bacterial Meningitis.

As described in the results, the proportion of gPRSP was as high as 52.3%, while the proportion of gPISP was confirmed as 39.3%, and the majority of them with vaccine serotypes. The 7-valent conjugate vaccine covered 70.9% of all the isolates, but 89.8%, if limited to gPRSP including H-PRSP.

Several data have been reported regarding a change in serotype after vaccination, and the increase in types 3 and 6A is of particular

Table 2. Serotype, antibiotic susceptibility and amino acid substitutions for three *pbp* genes in *S. pneumoniae* showing high MICs of ampicillin and cefotaxime

						Amino acid substitution								
		MIC (mg/L)				PBP1A			PBP2X			PBP2B		
Strain no.	Serotype	PEN	AMP	CTX	MEM	STMK	SRNVP	KTG	STMK	SSNVGM	LKSG	SVVK	SSNT	KTGTA
Major gPRSP		0.5–2	1–4	0.5–1	0.125–0.5	-A--	----T	---	-A--	------	V---	----	---A	-----
ARD-963[a]	19F	2	8	0.5	1	-S--	----T	---	-A--	------	V---	----	---A	----G
ARD-691	19F	2	4	4	0.25	-S--	----T	---	-AF-	------	V---	----	---A	-----
ARD-850	19F	2	2	2	0.25	-S--	----T	---	-AF-	------	V---	----	---A	-----
ARD-2337	19F	2	2	4	0.25	-S--	----T	---	-AF-	------	V---	----	---A	-----
ARD-2098	19F	2	4	2	0.5	-S--	----T	---	-AF-	-----T	V---	----	---A	-----
ARD-1346	19F	2	4	4	0.5	-S--	----T	---	-AF-	-----T	V---	----	---A	-----
ARD-470	14	1	0.5	2	0.063	-A--	----T	---	-AF-	-----T	V---	----	---A	-----
ARD-986	14	1	1	4	0.063	-A--	----T	---	-AF-	-----T	V---	----	---A	-----
ARD-1152	23F	2	4	8	0.5	-S--	----T	---	-AF-	-----T	V---	----	---A	-----
ARD-1268	23F	2	4	2	0.5	-S--	----T	---	-A--	------	V---	----	---A	-----
ARD-213	6A	2	4	4	0.5	-S--	----T	---	-AF-	------	V---	----	---A	-----
ARD-1850	6A	2	2	2	0.25	-S--	----T	---	-AF-	-----T	V---	----	---A	-----
ARD-15	6B	2	2	2	0.25	-S--	----T	---	-AF-	-----T	V---	----	---A	-----
ARD-724	6B	1	1	2	0.125	-A--	----T	---	-AF-	-----T	V---	----	---A	-----
ARD-241	NT	2	2	2	0.25	-S--	----T	---	-AF-	------	V---	----	---A	-----
ARD-1334	NT	1	0.25	2	0.063	-S--	----T	---	-G--	------	V---	----	----	-----

PEN, penicillin; AMP, ampicillin; CTX, cefotaxime; MEM, meropenem.
[a]Ten amino acid substitutions near the conserved motif KTG in *pbp2b* gene were identified.

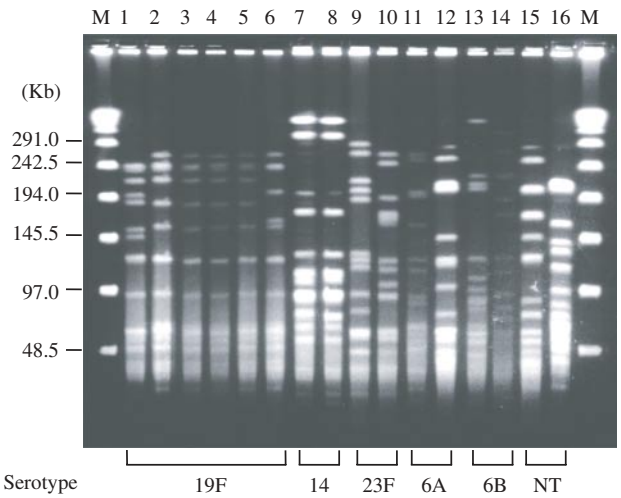

Figure 1. PFGE patterns of chromosomal DNA from H-PRSP digested with *Apa*1 restriction enzyme. These strains show ampicillin MICs ≥4 mg/L, or cefotaxime MICs ≥2 mg/L and possess several additional amino acid substitutions in *pbp2x* ($n = 15$) or *pbp2b* ($n = 1$) genes compared with the common PRSPs. Lanes: M, λ ladder molecular size marker; 1, ARD-963; 2, ARD-691; 3, ARD-850; 4, ARD-2337; 5, ARD-2098; 6, ARD-1346; 7, ARD-470; 8, ARD-986; 9, ARD-1152; 10, ARD-1268; 11, ARD-213; 12, ARD-1850; 13, ARD-15; 14, ARD-724; 15, ARD-241; 16, ARD-1334.

concern.[10] A rapid change to a 13-valent conjugate vaccine is required.

Finally, to ensure the effectiveness of the vaccination, it would be ideal to add serotypes 10 and 22, which are common in severe infections in adults, to serotypes 6A, 3 and 23A.

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