

## Antimicrobial susceptibility and serotype distribution of *Streptococcus pneumoniae* isolated from patients with community-acquired pneumonia and molecular analysis of multidrug-resistant serotype 19F and 23F strains in Japan

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### SUMMARY

A nationwide study was undertaken to determine the susceptibility to penicillin and serotypes of *Streptococcus pneumoniae* in Japan. *S. pneumoniae* was isolated from 114 adult patients with community-acquired pneumonia over 22 months at 20 hospitals and medical centres in different regions in Japan. All but five isolates were from sputum. Forty-eight isolates (42.1%) were susceptible, 40 (35.1%) showed intermediate resistance (MIC, 0.12–1.0 µg/ml) and 26 (22.8%) were resistant (MIC, ≥2.0 µg/ml) to penicillin G. All isolates were susceptible to ceftriaxone (breakpoint 1 µg/ml), imipenem (4 µg/ml) and vancomycin (4 µg/ml). Most were resistant to erythromycin, clarithromycin and azithromycin; only two were resistant to levofloxacin. Differences were found in the distribution of serotypes among isolates showing susceptibility to penicillin (predominant types 3, 6B, and 19F), intermediate resistance (6B, 14, 19F, and 23F) and full resistance (19F and 23F). PFGE typing showed that 14 of the 25 strains of serotype 19F had a single DNA profile, pattern A, a pattern closely similar to that of the Taiwan multidrug-resistant 19F clone. Twelve pattern A strains were not susceptible to penicillin but carried the macrolide resistance gene *mef(A)*. The DNA profiles of the 15 strains of 23F were also heterogeneous but six were highly similar (pattern b) yet distinct from the Spanish multidrug-resistant 23F clone although possibly related to the Taiwan multidrug-resistant 23F clone. The pattern b strains were not susceptible to penicillin and also harboured either *mef(A)* or *erm(B)*. Our results indicate that multidrug-resistant pneumococci are spreading rapidly in Japan. Efforts to prevent the spread of the pandemic multidrug-resistant serotypes should be intensified.

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## INTRODUCTION

*Streptococcus pneumoniae* is the major cause of community-acquired pneumonia (CAP) as well as otitis media, sinusitis, septicaemia, and meningitis [1]. Although susceptible to penicillin in the past, penicillin-resistant and multidrug-resistant pneumococci are now widespread in the world [1, 2]. In Japan, *S. pneumoniae* is the most common cause of CAP [3], and the proportion of multidrug-resistant strains is increasing at a dramatic rate [4, 5]. Nevertheless, there are few reports on the distribution of various serotypes and the genetic relatedness of pneumococci in this country [6]. In this study, we describe the results of a nationwide survey of the antimicrobial susceptibilities and the distribution of pneumococcal serotypes isolated from patients with CAP. We also examined the genetic relatedness of the predominant multidrug-resistant serotype 19F and 23F strains by pulsed-field gel electrophoresis (PFGE) and compared their DNA profiles with selected pandemic reference strains.

## METHODS

### Bacterial strains

A total of 114 isolates of pneumococci were recovered from 114 adult patients (mean age 67.4, range 20–99 years) presenting with CAP between November 2001 and August 2003 at 20 hospitals and medical centres located in different regions of Japan. Pneumonia was diagnosed if there was an appearance of a new abnormal shadow and likely infiltration on a chest roentgenogram and if at least two of the following clinical and laboratory findings were present: fever (temperature >37.8 °C), cough, production of purulent sputum, dyspnoea, and leukocytosis (WBC count >10 000/μl). The sources of the isolates were sputum (*n*=109), blood (*n*=3), pleural effusion (*n*=1) and bronchoalveolar lavage (*n*=1). Culture plates were incubated overnight in a 5% CO<sub>2</sub> atmosphere, and optochin sensitivity and bile solubility tests were performed to confirm *S. pneumoniae*.

### Antimicrobial susceptibility test

MICs were determined by an agar dilution method according to the guidelines of the National Committee for Clinical Laboratory Standards [7]. All isolates were tested for susceptibility to the following nine antibiotics: penicillin G (Meiji Seika Kaisha,

Tokyo, Japan), ceftriaxone (Chugai Pharmaceutical Co., Tokyo, Japan), cefditoren (Meiji Seika Kaisha), imipenem (Banyu Pharmaceutical Co., Tokyo, Japan), erythromycin (Dainippon Pharmaceutical Co., Osaka, Japan), clarithromycin (Taisho Pharmaceutical Co., Tokyo, Japan), azithromycin (Pfizer Japan Inc., Tokyo, Japan), levofloxacin (Daiichi Pharmaceutical Co., Tokyo, Japan), and vancomycin (Shionogi Co., Osaka, Japan).

### Serotyping

Isolates were serotyped by the capsular swelling (quellung reaction) observed microscopically after suspension in pneumococcal typing antisera (Statens Serum Institut, Copenhagen, Denmark).

### PCR

PCR was performed to detect alterations in penicillin-binding protein genes *pbp1a*, *pbp2x*, and *pbp2b* and macrolide resistance genes *mef(A)* and *erm(B)* by using a commercially available test kit (Wakunaga Pharmaceutical Co., Hiroshima, Japan) with primers modified as reported previously [8]. Briefly, a single colony from the blood agar medium was suspended in a microtube containing 30 μl of a lysis solution. The tube was placed in a thermal cycler, and bacterial cells were lysed at 60 °C for 10 min followed by 94 °C for 5 min. Next, 2 μl lysate was placed in a PCR tube containing 25 μl reaction mixture (1 ml reaction mixture contained 60 ng of a primer for each of the target genes, 80 μl of 10 mM dinucleoside triphosphate, 40 U *Tth* DNA polymerase, and 100 μl of 10 × PCR buffer). The PCR conditions were 94 °C for 20 s, 52 °C for 20 s, and 72 °C for 15 s for 30 cycles total.

### PFGE

PFGE was performed on strains of serotype 19F, 23F. The Spanish multidrug-resistant serotype 23F clone (ATCC 700669), the Taiwan multidrug-resistant serotype 19F clone (ATCC 700905) and the Taiwan multidrug-resistant serotype 23F clone (ATCC 700906) were used as reference standards [9]. Strains were grown overnight in brain heart infusion broth at 35 °C, and PFGE of *Sma*I chromosomal digests was performed as described previously [10]. DNA banding patterns were interpreted according to the criteria of Tenover *et al.* [11] with a greater than three bands

Table 1. Distribution of MICs for various antibiotics for 114 isolates of pneumococci in Japan

Antibiotic	No. of isolates for which MIC ( $\mu\text{g/ml}$ )															
	$\leq 0.004$	0.008	0.016	0.032	0.063	0.125	0.25	0.5	1	2	4	8	16	32	64	$\geq 128$
Penicillin G		1	3	26	18	11	9	5	15	25	1					
Ceftriaxone			4	6	1	22	25	23	33							
Cefditoren		1	3	9	4	26	18	40	10	3						
Imipenem	9	36	15	6	5	14	28		1							
Erythromycin				3	10	7	2	6	13	10	3	3	1			56
Clarithromycin			1	6	11	7		8	12	9	2		2			56
Azithromycin				2		14	6	8	14	3	5		2		1	59
Levofloxacin								3	50	59	1			1		
Vancomycin						1	93	20								

↓ Breakpoints for each antibiotic.

difference in profile needed to distinguish between PFGE types.

## RESULTS

### Antimicrobial susceptibility test

The MICs of the nine antibiotics tested for the 114 *S. pneumoniae* isolates are shown in Table 1. Using NCCLS breakpoints all isolates were found to be susceptible to ceftriaxone, imipenem and vancomycin. The majority were resistant to erythromycin, clarithromycin and azithromycin; only two isolates were not susceptible to levofloxacin. Forty-eight isolates (42.1%) were susceptible to penicillin G, 40 (35.1%) showed intermediate resistance and 26 (22.8%) were fully resistant to  $\geq 2.0 \mu\text{g/ml}$  of this antibiotic.

### Serotyping

All isolates but one, were grouped into 21 different serotypes (Table 2). The serotypes of penicillin-susceptible isolates varied widely but with serotypes 3 (22.9%), 6B (12.5%) and 19F (12.5%) predominating among 18 other serotypes. In contrast, the 26 fully penicillin-resistant isolates fell into only six serotypes, the most frequent being 19F (50.0%), and 23F (23.1%); intermediate susceptible isolates

were represented by 11 serotypes with 23F (20.0%), 14 (17.5%), 6B (15.0%) and 19F (15.0%) being the most common.

### Molecular characterization of serotypes 19F and 23F

PFGE typing revealed 10 different DNA profiles (A–J) among the 25 strains of serotype 19F (Fig. 1). One pattern, type A, accounted for 14 strains and this was indistinguishable from the profile of the Taiwan multidrug-resistant serotype 19F clone. Three strains of serotype 19F were classified as pattern B and the other eight strains gave unique patterns. Twelve of the pattern A strains were not susceptible to penicillin and all 14 harboured the *mef(A)* gene. Overall 21 (84%) of the serotype 19F strains were *mef(A)* and/or *erm(B)* positive. Eighteen of the serotype 19F strains had alterations in *pbp1a*,  $-2x$ , and  $-2b$ ; four in *pbp2x* and  $-2b$ ; two in *pbp2x*; and one had no alteration. These strains originated from different geographical regions of Japan (Table 3).

The 15 strains of serotype 23F were characterized by nine different DNA profiles (b–j) with pattern b predominating and represented by six strains (Fig. 2). Two strains were typed as pattern g and the remainder were unique. None of the DNA profiles of serotype 23F matched that of the Spanish multidrug-resistant 23F clone but pattern b was similar to the profile of the Taiwan multidrug-resistant serotype

Table 2. Serotypes of susceptible, intermediate, and fully penicillin-resistant pneumococci

Serotype	Susceptible (n=48)	Intermediate (n=40)	Resistant (n=26)
3	11	2	0
6A	3	1	3
6B	6	6	2
7F	1	0	0
9A	1	0	0
9V	4	0	1
11A	2	2	1
14	2	7	0
15A	1	0	0
15C	2	0	0
16F	0	3	0
18A	1	0	0
18B	1	0	0
18C	1	0	0
19A	3	2	0
19F	6	6	13
22F	1	0	0
23A	0	1	0
23F	1	8	6
29	0	1	0
34	1	0	0
Non-typable	0	1	0

23F clone although there were at least four band differences between them (Fig. 2). Strains of pattern b were not susceptible to penicillin and all positive by PCR for either the *mef(A)* or *erm(B)* gene as were strains of the other DNA types. These strains were also widespread in the country (Table 3). Thirteen of the serotype 23F strains were altered in *pbp1a*,  $-2x$ , and  $-2b$ ; and one each in *pbp2x* and  $-2b$ , and *pbp2x* respectively.

Two strains each of serotype 19F (patterns A2 and B) and 23F (patterns e and i) had both *mef(A)* and *erm(B)* genes, and these four strains were all penicillin non-susceptible (Table 3).

**DISCUSSION**

Penicillin-resistant *S. pneumoniae* are already distributed worldwide and resistance appears to be expanding to include multiple antimicrobial agents [1, 4]. In Asia, the proportion of *S. pneumoniae* reported to be non-susceptible to penicillin over the last decade ranges from 68.8% in Thailand [12], to 3.8% in India, 9.0% in Malaysia, 9.8% in China, 21.0% in Indonesia, 23.1% in Singapore, 38.7% in Taiwan, 41.2% in Sri Lanka, 60.8% in Vietnam, 79.7% in

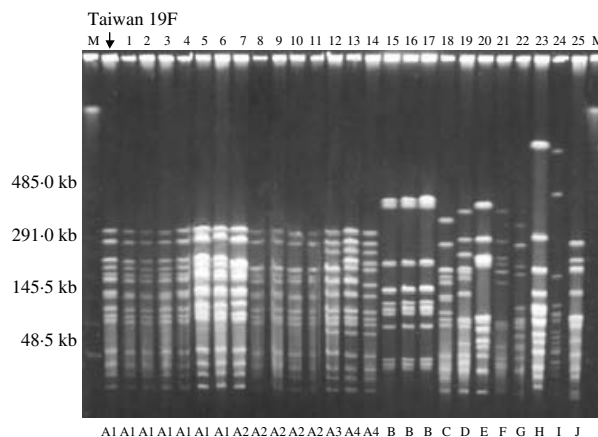


Fig. 1. Pulsed-field gel electrophoresis (PFGE) patterns of *SmaI*-digested DNA from 25 strains of serotype 19F *S. pneumoniae* and a representative of the Taiwan multi-resistant serotype 19F clone. M, Molecular size marker.

Korea and 65.3% in Japan in 1996 to 1997 [13]. Indeed, a survey of a single prefecture in Japan from 2001 to 2003 reported a frequency of 60.9% [6]. In this study, 57.9% of the *S. pneumoniae* isolates from patients with CAP were penicillin non-susceptible and some of these were also resistant to various other antibiotics. Thus, it appears that the frequency of multidrug-resistant *S. pneumoniae* in Japan is among the highest in Asia. A previous study demonstrated that the aetiology of CAP in Japan did not differ substantially from Western countries and that *S. pneumoniae* was the most common pathogen in this disease [3]. Some earlier surveys indicate that the clinical outcome between penicillin-susceptible and non-susceptible isolates does not differ under current resistance levels [5, 14]. However, invasive pneumococcal isolates with very high-level penicillin resistance (MIC  $\geq 8.0 \mu\text{g/ml}$ ) have been reported to be prevalent in the United States [15]. Caution should be exercised concerning such strains, although we did not detect them in Japan.

The 23-valent pneumococcal polysaccharide vaccine (PS23) has been promoted worldwide, although it fails to protect children <2 years old [16, 17]. It is well known that children aged <5 years and adults of  $\geq 65$  years are the most susceptible to invasive pneumococcal infection and have a high mortality rate [16, 18]. Vaccination, therefore, seems to be the only available method for preventing pneumococcal disease [16, 19]. In this study, all the isolates were classified into 21 serotypes, 82.5% of which are accounted for in PS23. Furthermore, 56 (59.6%) of the isolates were penicillin non-susceptible isolates.

Table 3. Correlation of PFGE patterns, penicillin resistance, macrolide resistance genes and mutation in penicillin binding protein genes in strains of *S. pneumoniae* serotypes 19F and 23F

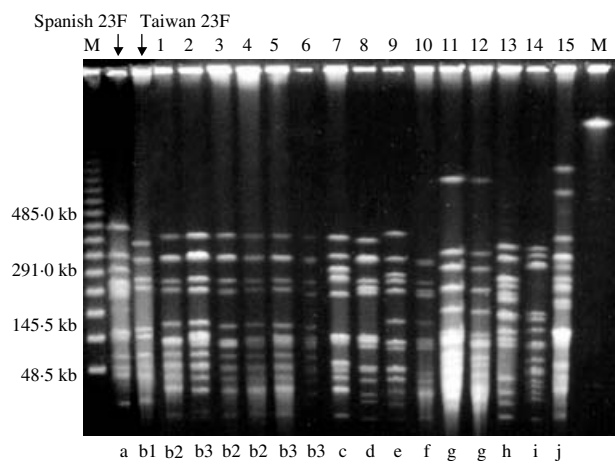
PFGE line	PFGE pattern	Penicillin resistant	autolysin	<i>pbp1a</i>	<i>pbp2x</i>	<i>pbp2b</i>	<i>mef(A)</i>	<i>erm(B)</i>	Region
19F									
1	A1	R	+	+	+	+	+	-	Tohoku
2	A1	S	+	-	+	-	+	-	Kyushu
3	A1	S	+	-	+	-	+	-	Kyushu
4	A1	R	+	+	+	+	+	-	Kinki
5	A1	R	+	+	+	+	+	-	Kanto
6	A1	R	+	+	+	+	+	-	Kinki
7	A2	R	+	+	+	+	+	+	Kyushu
8	A2	I	+	+	+	+	+	-	Chubu
9	A2	R	+	+	+	+	+	-	Kanto
10	A2	I	+	+	+	+	+	-	Kanto
11	A2	R	+	+	+	+	+	-	Kanto
12	A3	R	+	+	+	+	+	-	Chubu
13	A4	R	+	+	+	+	+	-	Kinki
14	A4	I	+	+	+	+	+	-	Kanto
15	B	I	+	+	+	+	+	-	Kanto
16	B	R	+	+	+	+	+	+	Kanto
17	B	S	+	-	+	+	-	+	Kyushu
18	C	I	+	+	+	+	+	-	Kanto
19	D	R	+	+	+	+	+	-	Kinki
20	E	I	+	-	+	+	-	+	Kanto
21	F	R	+	+	+	+	-	+	Kanto
22	G	S	+	-	+	+	-	-	Kanto
23	H	S	+	-	+	+	-	-	Chugoku
24	I	S	+	-	-	-	-	-	Kanto
25	J	R	+	+	+	+	-	-	Kanto
23F									
1	b2	R	+	+	+	+	-	+	Kinki
2	b3	I	+	+	+	+	+	-	Kanto
3	b2	I	+	+	+	+	+	-	Kanto
4	b2	I	+	+	+	+	-	+	Kanto
5	b3	I	+	+	+	+	-	+	Chugoku
6	b3	I	+	+	+	+	+	-	Kanto
7	c	I	+	-	+	+	-	-	Kyushu
8	d	R	+	+	+	+	-	+	Kanto
9	e	R	+	+	+	+	+	+	Kanto
10	f	I	+	+	+	+	-	+	Kanto
11	g	S	+	-	+	-	-	+	Kanto
12	g	R	+	+	+	+	-	+	Kyushu
13	h	R	+	+	+	+	+	-	Kanto
14	i	I	+	+	+	+	+	+	Kinki
15	j	R	+	+	+	+	-	+	Kanto

S, penicillin-susceptible. I, penicillin intermediate resistant. R, penicillin-resistant. Penicillin binding protein genes *pbp1a*, *pbp2x*, and *pbp2a*: +, altered; -, not altered. Macrolide resistant genes *mef(A)* and *erm(B)*: +, positive; -, negative.

Although the efficacy of PS23 against pneumonia without bacteraemia is controversial [20], a combination of influenza and pneumococcal vaccination appears to be beneficial for the reduction of mortality from all causes in individuals aged  $\geq 65$  years [21].

In the group of serotype 23F pneumococci, six out of 15 strains showed the predominant PFGE pattern b which was different from the pattern of the Spanish multidrug-resistant 23F clone but similar to the Taiwan multidrug-resistant serotype 23F clone.





**Fig. 2.** Pulsed-field gel electrophoresis (PFGE) patterns of *Sma*I-digested DNA from 15 strains of serotype 23F *S. pneumoniae* and representatives of the Spanish and Taiwan multiresistant serotype 23F clone. M, Molecular size marker.

Although a previous study indicated that the Spanish multidrug-resistant 23F clone was spreading in Asia [13], it was not detected in this survey. On the other hand, 14 out of 25 strains of serotype 19F showed the predominant pattern A which was closely related to the pattern for the Taiwan multidrug-resistant 19F clone. Since these predominant serotypes were collected from different regions in Japan and most were penicillin non-susceptible with multiple alterations in *pbp* and all isolates had macrolide resistant genes, these results suggest that derivatives of the Taiwan multidrug-resistant 19F and 23F clones have the potential to spread further in Japan. Recently, it was reported by Kasahara *et al.* [6] that these clones had already spread in Japan. However, theirs was a pilot study and they recognized the need for an urgent nationwide surveillance for these strains. Our nationwide study focusing on CAP-associated pneumococci has yielded results consistent with their earlier findings.

Moreover, it has been suggested in a recent report that pneumococcal isolates containing both the *mef*(A) and *erm*(B) genes may have originated from the Taiwan multidrug-resistant serotype 19F clone containing the *mef*(A) gene after introduction of the *erm*(B) gene [22]. However, one serotype 19F strain different from the Taiwan serotype 19F clone and two serotype 23F strains containing both the *mef*(A) and *erm*(B) genes were identified in our study suggesting that other types of antibiotic-resistant pneumococci might appear in Japan.

In conclusion, our results indicate that multidrug-resistant pneumococci are spreading rapidly in Japan and that efforts to prevent spread of pandemic multidrug-resistant serotype 19F and 23F clones should be intensified.

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## DECLARATION OF INTEREST

None.

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