two additional rSeVs to protect against hPIV-1, hPIV-2, hPIV-3, and RSV. Vaccine 2009;27(12):1848–57.
[40] Desprès P, Combredet C, Frenkiel MP, Lorin C, Brahic M, Tangy F. Live measles vaccine expressing the secreted form of the West Nile virus envelope glycoprotein protects against West Nile virus encephalitis. J Infect Dis 2005;191(2):207–14.
[41] Wild TF, Buckland R. Functional aspects of envelope-associated measles virus proteins. Curr Top Microbiol Immunol 1995;191:51–64.
[42] Haga T, Murayama N, Shimizu Y, Saito A, Sakamoto T, Morita T, et al. Analysis of antibody response by temperature-sensitive measles vaccine strain in the cotton rat model. Comp Immunol Microbiol Infect Dis 2009;32(5):395–406.
[43] Liniger M, Zuniga A, Tamin A, Azzouz-Morin TN, Knuchel M, Marty RR, et al. Induction of neutralising antibodies and cellular immune responses against SARS coronavirus by recombinant measles viruses. Vaccine 2008;26(17):2164–74.
[44] del Valle JR, Devaux P, Hodge G, Wegner NJ, McChesney MB, Cattaneo R. A vectored measles virus induces hepatitis B surface antigen antibodies while protecting macaques against measles virus challenge. J Virol 2007;81(19):10597–605.
[45] Liniger M, Zuniga A, Morin TN, Combardiere B, Marty R, Wiegand M, et al. Recombinant measles viruses expressing single or multiple antigens of human immunodeficiency virus (HIV-1) induce cellular and humoral immune responses. Vaccine 2009;27(25–26):3299–305.
[46] Boukhvalova MS, Prince GA, Blanco FCG. The cotton rat model of respiratory viral infections pathogenesis and immunity. Biologicals 2009;37:152–9.

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Serotype and antibiotic resistance of isolates from patients with invasive pneumococcal disease in Japan

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SUMMARY

Invasive pneumococcal disease (IPD) is of concern in Japan, where the heptavalent pneumococcal conjugate vaccine (PCV7) is unavailable. We determined serotypes, genotypes indicating β-lactam resistance, and antibiotic susceptibilities of 496 isolates from normally sterile sites in patients (193 children, 303 adults) from 186 institutions between August 2006 and July 2007. Disease presentations included sepsis (46·2%), pneumonia (31·5%), and meningitis (17·5%). Mortality was 1·4% in children and 22·1% in adults, many of whom had underlying diseases. In children, serotype 6B (22·5%) was followed by 19F (14·1%), and 14 (13·1%); potential coverages of PCV7 and PCV13 were 75·4% and 93·7%, respectively. In adults, serotype 12F (14·3%) was followed by 3 (11·3%), and 6B (10·3%); 23-valent polysaccharide vaccine (PPV23) coverage was 85·4%. Most serotype 12F strains were gPISP, with *pbp2b* gene alteration; carbapenem had an excellent MIC_{90}. PCV7 is recommended for children and PPV23 for adults to increase prevention against IPD.

Key words: Antibiotic resistance, molecular epidemiology, *Streptococcus pneumoniae* (pneumococcus), surveillance, vaccines.

INTRODUCTION

Streptococcus pneumoniae is a leading cause of invasive infections such as lobar pneumonia, septicaemia, and meningitis, which are major contributors to morbidity and mortality in children and adults. Since the discovery of pneumococcal strains resistant to penicillin G (PEN) [1], these strains have spread rapidly worldwide [2, 3] and have been the subject of several epidemiological surveillance studies of capsule serotype distribution and antibiotic susceptibility in many countries [4–8].

In Japan, the prevalence of PEN-resistant *S. pneumoniae* (PRSP) and PEN-intermediate *S. pneumoniae* (PISP) in clinical isolates has increased rapidly since the late 1990s, especially in younger children [9, 10]. Characteristically, PRSP strains show simultaneous

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resistance to cephalosporin antibiotics used in ambulatory practice [9]. In PRSP and PISP, genotypic abnormalities in three penicillin-binding protein (PBP) genes, *pbp1a*, *pbp2x*, and *pbp2b*, which encode the PBP1A, PBP2X, and PBP2B enzymes, respectively, have been identified by polymerase chain reaction (PCR) using primers to detect mutations in these genes [9, 11]. The prevalence of PRSP possessing the three abnormal *pbp* genes currently exceeds 50% in Japan [12].

Given this background, therapeutic choices for patients with invasive pneumococcal disease (IPD) in Japan have been gradually eroded. A carbapenem antibiotic such as panipenem (PAM), which has been used only in Japan, Korea, and China, was administered in preference to intravenous third-generation cephalosporins such as cefotaxime (CTX) and ceftriaxone (CRO). Additionally, rapid increases in numbers of adults and elderly persons with various underlying diseases, is thought to increase the threat of IPD.

A heptavalent pneumococcal conjugate vaccine (PCV7) for children has been introduced in many countries [13], beginning with the USA [14]. This vaccine has been reported to contribute to a decrease in IPD when causative strains are covered [15–18]. In contrast, IPD caused by non-PCV7 serotypes of *S. pneumoniae*, such as 19A, continues to increase [19–21]. As a result, a second-generation pneumococcal conjugate vaccine such as PCV13 is now being developed to cover a wider range of serotypes.

We therefore focused on understanding the serotype distribution and antibiotic susceptibility of isolates from IPD in children and adults throughout Japan, where clinical trials of PCV7 for children have been concluded and approval is expected. Here we describe the serotype distribution and antibiotic susceptibility of the isolates according to their *php* genotype by PCR. We also extrapolate from the data the expected PCV7 and PCV13 coverage rates for children and those of PPV23 and PCV13 for adults.

MATERIALS AND METHODS

We examined 496 *S. pneumoniae* isolates from patients with IPD [22]. Isolates were cultured from clinical samples processed in the laboratories of 186 medical institutions from August 2006 to July 2007 throughout Japan and then sent to our laboratory with an anonymous application form written by the reporting doctor. All isolates were from normally sterile samples such as cerebrospinal fluid (CSF), blood, or pleural or joint fluid.

Haematological tests in IPD patients

To statistically determine risk factors in adults, we requested an anonymous report including patient's age, disease presentation, underlying disease, white blood cell count (WBC), C-reactive protein (CRP), and platelet count (PLT); and outcome, including presence or absence of neurological sequelae.

Serotype and antimicrobial susceptibility

Serotypes of all *S. pneumoniae* isolates were determined by the capsule swelling reaction using antiserum purchased from the Statens Serum Institute (Denmark) [23]. Minimal inhibitory concentrations (MICs) of penicillin (PEN), ampicillin (AMP), cefotaxime (CTX), meropenem (MEM) and vancomycin (VAN) were determined by agar dilution methods using Muller–Hinton II agar (MH; Becton Dickinson, USA) supplemented with 5% defibrinated sheep blood [24]. *S. pneumoniae* ATCC49619 was used as a quality control strain.

Genotypic identification of resistance by PCR

To confirm that isolates were *S. pneumoniae*, the *lytA* gene encoding the autolysin enzyme specific to *S. pneumoniae* [25] was amplified simultaneously with the three PBP genes. Each primer set used for detection of the three PBP genes was designed to amplify a part of the normal *pbp1a*, *pbp2x*, and *pbp2b* genes detected only in susceptible strains [9]. Portions of each gene corresponding to the primers were positioned in blocks of highly divergent sequences within or near conserved amino-acid motifs. Each reaction tube for PCR contained two primer sets, for detecting *lytA* and *pbp1a* in tube A; *pbp2x* and *pbp2b* in tube B; and *mef*(A) and *erm*(B) in tube C. These tubes contained 30 μl reaction mixture as previously described [9, 22, 26].

One colony was chosen from sheep blood agar and suspended in 30 μl lysis solution [11]. The tube then was placed in a thermal cycler (Gene Amp PCR System 9600R; PerkinElmer Cetus, USA) and heat-treated for 10 min at 60 °C and for 5 min at 94 °C to obtain template DNA. Next, 2 μl template DNA was added to each of the three tubes marked A, B, and C containing 30 μl reaction mixture. PCR cycling

Table 1. *Outcome based on presence or absence of underlying disease**

	Children			Adults		
	Underlying disease			Underlying disease		
Outcome	(+)	(−)	Subtotal	(+)	(−)	Subtotal
Fatality	2	0	2 (1·4)	37	6	43 (22·1)
Sequelae (+)	1	3	4 (2·9)	13	4	17 (8·7)
Sequelae (−)	17	115	132 (95·7)	85	50	135 (69·2)
Total	20	118	138 (100·0)	135	60	195 (100·0)

* Patients with unknown status concerning underlying disease and outcome were excluded from analysis.

conditions consisted of 30 cycles at 94 °C for 15 s, 53 °C for 15 s, and 72 °C for 15 s and amplified using a Takara PCR Thermal Cycler (Model TP600; Takara Bio, Japan). Amplified DNA fragments were analysed by electrophoresis on a 3% agarose gel. In the presence of all three DNA fragments corresponding to *pbp1a*, *pbp2x*, and *pbp2b*, the PBP genes were regarded as having essentially the same sequences as the sensitive R6 strain (PEN-susceptible *S. pneumoniae*, PSSP). We regarded the absence of DNA fragments as indicative of sequences other than those in PSSP. Genotypic determination is indicated by adding 'g' to designations as follows: gPSSP, gPISP (*pbp2x*), gPISP (*pbp2b*), gPISP (*pbp1a+2x*), gPISP (*pbp2x+2b*), and gPRSP (*pbp1a+2x+2b*).

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed using a modification of a method described previously [12]. For digestion, DNA plugs were incubated in 1 ml restriction enzyme buffer with 100 U of *Apa*I at 37 °C for 16 h. Electrophoresis was performed with a CHEF Mapper (Bio-Rad Laboratories, USA) at 5·7 V/cm at 14 °C for 18 h.

RESULTS

IPD

IPD was classified into five groups as follows: septicaemia and bacteraemia (including two cases of bacterial endocarditis); pneumonia, where *S. pneumoniae* was isolated from blood cultures; meningitis diagnosed by clinical findings, where *S. pneumoniae* was isolated from CSF or blood; suppurative arthritis or osteomyelitis; and others. In 193 children aged ⩽17 years, septicaemia was predominant with 114 (59·1%) cases, followed by pneumonia with 44 (22·8%) cases, and meningitis with 30 (15·5%) cases; other diseases were rare. Almost 92% of IPD cases in children were aged ⩽4 years. In the 303 adults, septicaemia and pneumonia predominated with 115 (38·0%) cases and 112 (37·0%) cases, respectively, followed by meningitis with 57(18·8%) cases. The median age of adults with septicaemia and meningitis was 66 years, but was somewhat higher in patients with pneumonia (73 years).

Outcomes and underlying diseases

Table 1 shows outcomes and underlying diseases in 138 children (71·5% of those studied), and 195 adults (64·4%), according to reports returned by collaborating institutions. In children, 20 (14·5%) had underlying diseases, mostly congenital abnormalities. Adverse outcomes for children included death in two (1·4%) cases and neurological sequelae in four (2·9%) cases.

In adults, 135 (69·2%) had underlying diseases, the most common being cancer surgery (38), diabetes (30), cardiovascular disease (18), hepatic disease (16), kidney disease (9), immunological deficiency (3), and splenectomy (2). Deaths were numerous [43 (22·1%)], but 37 of those patients had underlying diseases, and the cause of death was not considered in detail. The median hospital stay in adults who did not survive was 2 days. Seventeen patients, including 13 with underlying disease, had severe neurological sequelae. When outcomes in cases with underlying diseases and those without underlying diseases were compared separately for children and adults, the mortality and sequelae rates were statistically higher in both children and adults having underlying

Table 2. *Clinical laboratory findings associated with fatal outcome in adults with invasive pneumococcal disease*

	Median or % (25/75 percentiles) and [no./total]			
	Non-survivors ($n=43$)	Survivors ($n=147$)	Univariate analysis OR (95% CI)	P value
WBC (10^9 cells/l)	5·1 (2·3–8·8) [37/43]	13·2 (8·2–19·1) [136/147]		
$<5{\cdot}0\times10^9$ cells/l	48·6% [18/37]	11·0% [15/136]	7·64 (3·30–17·68)	$P<0{\cdot}0001$
C-reactive protein (mg/dl)	24·8 (16·3–31·7) [36/43]	20·6 (8·9–33·6) [131/147]		
$\geqslant$15 mg/dl	77·8% [28/36]	65·6% [86/131]	1·83 (0·77– 4·35)	$P=0{\cdot}1661$
PLT (10^9 cells/l)	119 (69–171) [36/43]	197 (130–262) [134/147]		
$<130\times10^9$/l	55·6% [20/36]	23·1% [31/134]	4·15 (1·92–8·97)	$P=0{\cdot}0002$

OR, Odds ratio; CI, confidence interval; WBC, white blood cell count; PLT, platelet count.

Table 3. *MIC_{90} and resistance genes identified by PCR in* S. pneumoniae

		MIC_{90} (μg/ml)					
Resistance class	n	PEN	AMP	CTX	MEM	PAM	VAN
gPSSP	101	0·031	0·031	0·125	0·016	0·004	0·5
gPISP (*pbp2b*)	38	0·125	0·031	0·063	0·031	0·008	0·5
gPISP (*pbp2x*)	124	0·063	0·125	0·5	0·016	0·008	0·5
gPISP (*pbp1a+2x*)	54	0·5	0·5	1	0·125	0·031	0·5
gPISP (*pbp2x+2b*)	35	0·5	0·5	2	0·125	0·031	0·5
gPRSP (*pbp1a+2x+2b*)	140	2	4	2	0·5	0·125	0·5

Each *pbp* gene alteration detected by PCR appears within parentheses.
MICs were determined for the following antibiotics: PEN, penicillin; AMP, ampicillin; CTX, cefotaxime; MEM, meropenem; PAM, panipenem; VAN, vancomycin.
Strains tested MICs: 492 isolates grown on sheep blood agar plate from stock at −80 °C.

diseases (Fisher's test: children, $P=0{\cdot}0395$; adults, $P=0{\cdot}0043$).

Haematological findings and outcomes in adults

We compared WBC, CRP, and PLT at time of admission between the non-surviving and surviving adults. Analysis was carried out using a non-parametric Kruskal–Wallis test and the results are shown in Table 2. The median WBC in non-survivors and survivors was $5{\cdot}1\times10^9$ and $13{\cdot}2\times10^9$ cells/l, respectively; the odds ratio between patients with WBC below and above $5{\cdot}0\times10^9$ cells/l was calculated as 7·64. A clear difference in the PLT was also noted between the two groups; and the odds ratio for mortality between patients with PLT below and above 130×10^9 cells/l was 4·15. No significant difference in CRP was evident between non-survivors and survivors. In addition, no significant difference in resistance type of gPSSP, gPISP, and gPRSP or in serotype (PPV23) was found between the non-survivors and survivors ($P=0{\cdot}1200$, $P=0{\cdot}9891$, respectively).

PBP gene alterations and β-lactam susceptibility

Table 3 shows results of MIC_{90} of PEN, AMP, CTX, MEM, and VAN. Genotype was based on PCR results for the *pbp1a*, *pbp2x*, and *pbp2b* genes. PEN susceptibility declined according to addition of altered *pbp* genes, from a MIC_{90} of 0·063 μg/ml for gPISP (*pbp2x*) to 2 μg/ml for gPRSP (*pbp1a+2x+2b*). In particular, susceptibility to CTX was affected by alterations of *pbp2x*, a pattern markedly different from that of susceptibility to PEN. In contrast, although susceptibility to MEM was affected by the gene alterations, the effect was much less. The MIC_{90} of VAN for all *S. pneumoniae* strains was 0·5 μg/ml.

Relationship between serotype and resistance genotype for β-lactams

The serotypes of *S. pneumoniae* isolates from children, classified as either PCV7 or non-PCV7 types, in decreasing order of prevalence are shown in Figure 1 and the percentage rate of resistance genotypes for β-lactams is also given for each serotype. Serotype 6B

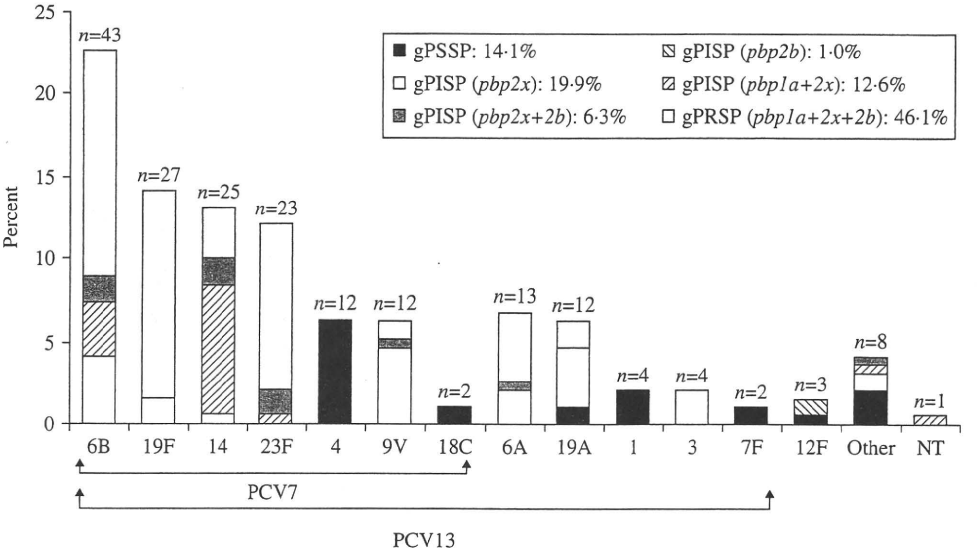

Fig. 1. Serotype distribution and resistance genes identified by PCR in *S. pneumoniae* isolated from children. 'Other' category includes serotypes 15B, 23A, 8, 24, 34, 35, and 38.

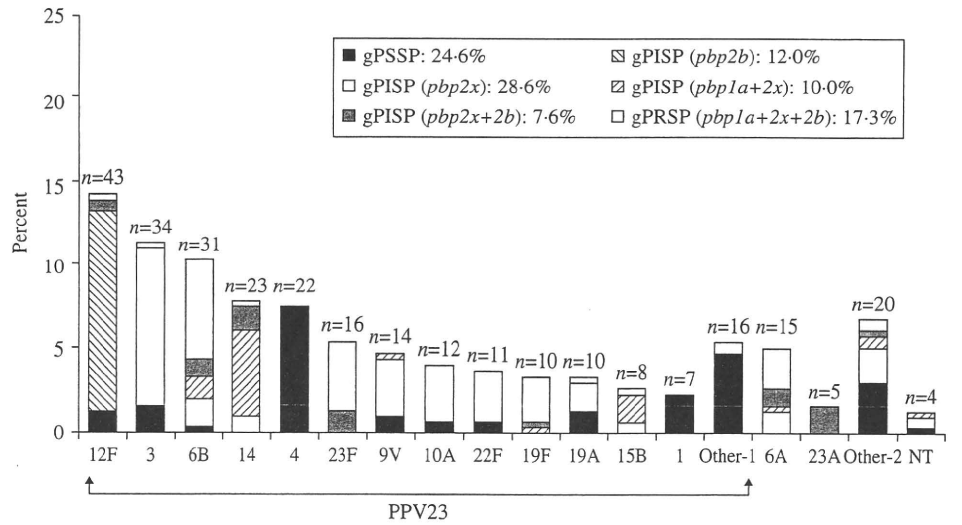

Fig. 2. Serotype distribution and resistance genes identified by PCR in *S. pneumoniae* isolated from adults. 'Other-1' category includes serotypes 9N, 11A, 33, 18C, 20, 2, 7F, 8. 'Other-2' category includes serotypes 35, 7C, 15A, 38, 15C, 31, 16, and 36.

predominated in the PCV7 types, followed in order by 19F, 14, and 23F. Coverage by PCV7, to which types 9V, 4, and 18C were added, was calculated as 75·4%. PCV7 covered types 6B, 19F, 14 and 23F, all of which showed high rates of gPRSP. In addition, coverage by PCV13 was calculated as 93·7%. The resistance rate of gPRSP (*pbp1a+2x+2b*) was highest, at 46·1%, followed by gPISP (*pbp2x*) at 19·9%, gPISP (*pbp1a+2x*) at 12·6%, gPISP (*pbp2x+2b*) at 6·3%, and gPISP (*pbp2b*) at 1·0%. The rate of gPSSP was only 14·1%.

The serotypes of *S. pneumoniae* isolates from adults that were covered by PPV23 are shown in Figure 2, in decreasing order of prevalence. These results differed markedly from those for children. The most prevalent type, 12F, accounted for 14·3% of the total; interestingly, almost all had gPISP (*pbp2b*). Serotype 3 (11·3%), with a high incidence of gPISP (*pbp2x*), was second only to 12F. Other common serotypes were, type 6B (10·3%), with a high frequency of gPRSP (*pbp1a+2x+2b*), while type 14 (7·6%) showed a high frequency of gPISP (*pbp1a+2x*). PPV23 and PCV13

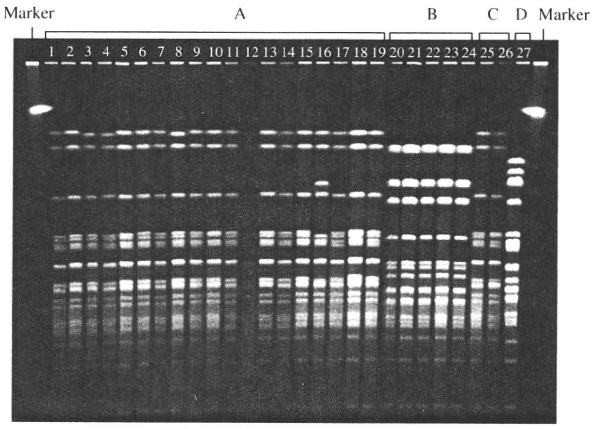

Fig. 3. PFGE patterns of *Apa*I digests of chromosomal DNA from serotype 12F isolates. A, gPISP (*pbp2b*) (lanes 1–19); B, gPSSP (lanes 20–24); C, gPISP (*pbp2x*+*2b*) (lanes 25, 26); D, gPRSP (*pbp1a*+*2x*+*2b*) (lane 27).

provided coverage in 85·4% and 61·5%, respectively. Non-survivors and patients with sequelae had developed IPD involving strains of various serotypes. The predominant resistance genotype in adults was gPISP (*pbp2x*) at 28·6%, followed by gPSSP at 24·6%, gPRSP (*pbp1a*+*2x*+*2b*) at 17·3%, gPISP (*pbp2b*) at 12·0%, gPISP (*pbp1a*+*2x*) at 10·0%, and gPISP (*pbp2x*+*2b*) at 7·6%. The serotype and the resistance genotype of strains differed significantly between children and adults (both $P<0{\cdot}0001$).

PFGE pattern of strains serotyped 12F

Figure 3 shows PFGE patterns of *Apa*I DNA digests of serotype 12F strains. The 27 strains pictured, namely five gPSSP, two gPISP (*pbp2x*+*2b*), one gPRSP, and 19 gPISP (*pbp2b*), were selected randomly from 38 strains which were isolated from patients throughout Japan. DNA restriction patterns of strains with the same resistance genotype were homogeneous, suggesting that *S. pneumoniae* strains possessing the same *pbp* alterations had spread widely. There has been a rapid increase in the prevalence of serotype 12 in Japan and this serotype is present in 18% of cases with a poor prognosis in adults. This increase is therefore considered to be of clinical significance.

DISCUSSION

S. pneumoniae is a major causative agent of diseases such as pneumonia, meningitis, and acute otitis media (AOM), as well as various other serious invasive infections. In the USA, the PCV7 vaccine was developed for children and approved in 2000, and has been incorporated into the paediatric vaccination schedule [14]. Immunization programmes using PCV7 have spread widely, and are presently conducted in almost 70 countries worldwide [27]. The incidence of IPD involving vaccine-type *S. pneumoniae* has been reported to have decreased significantly [15, 17, 18], and a related decrease in IPD in adults has been noted [16]. However, the incidence of IPD caused by non-vaccine-type *S. pneumoniae* has increased; particularly type 19A [19–21]. In order to provide increased coverage, a new vaccine, PCV13, is being developed, which will include types 19A, 6A, and 3 [28].

Much clinical attention has been drawn to a rapid increase in PRSP in *S. pneumoniae* isolates. These strains have been causative agents of paediatric AOM [29] and meningitis [22] in Japan since 1990 and this increase is strongly related to a shift from prescribing oral penicillins for outpatients to using oral cephalosporins. The increase may also be related to use of macrolides, considering that most PRSP are multidrug-resistant *S. pneumoniae* (MDRSP) also resistant to macrolides [30]. In addition, Japan's high population density tends to accelerate increases in resistant organisms.

We previously compared *pbp* gene alterations in *S. pneumoniae* strains that had been isolated in the same time period from the USA and Japan [10]. In the USA, where use of penicillins predominated, increases were evident in resistant strains with the *pbp2b* gene alteration whereas in Japan, where cephalosporins predominated, many strains characteristically had the *pbp2x* gene alteration. As shown in this study, the latter pattern still persists in Japan.

According to USA guidelines [31], the use of third-generation cephalosporins – CTX, CRO, or either of these in combination with VAN – is recommended for meningitis caused by PRSP. In Japan, however, carbapenems such as PAM and MEM are recommended as first-choice antibiotics in this situation. A major reason for this practice is that 60% of Japanese paediatric meningitis cases are caused by *Haemophilus influenzae* type b (Hib), of which about 36·2% show resistance to AMP and CTX, reflecting β-lactamase non-producing and AMP-resistant *H. influenzae* as the causative pathogens [32]. Therefore, in Japan, the preferred paediatric treatment increasingly involves concomitant use of a carbapenem, with its superior bactericidal effect against *S. pneumoniae*, plus CTX or CRO, with superior activity

against *H. influenzae*; treatment now is basically the same for adults.

As for vaccines against *S. pneumoniae*, PPV23 has been introduced in Japan, where it is used mainly on a voluntary basis for elderly people as well as adults and children with underlying diseases. The PCV7 vaccine is currently under review by the Japanese Ministry of Health, Labour and Welfare, and approval is expected soon. Nevertheless, one needs to know the extent to which PCV7 covers IPD. According to our epidemiological surveillance in the current study, PCV7 covers 75·4% of strains isolated from children with IPD. However, the incidence of types 6A and 19A, which are non-vaccine types, is significant, so the introduction of PCV13 will be beneficial.

In Japan, a recent rapid increase in IPD in adults may reflect the rapid ageing of society and an increase of lifestyle-related diseases in the adult population. The current situation whereby PPV23 vaccination is voluntary, limits its effectiveness against this increase. Development of disease caused by *S. pneumoniae* in adults with underlying disease often triggers disseminated intravascular coagulation (DIC), leading to death or serious sequelae for which the prognosis is extremely poor. Also of concern is the poor prognosis for adults who develop IPD caused by *S. pneumoniae* with intermediate PEN resistance. In addition, serotype 12F was very rare in 2000, but in the current study accounted for 12·0% of IPD cases and strains show essentially the same PFGE pattern as gPISP (*pbp2b*). The reason why this type of *S. pneumoniae* has increased so rapidly in adults is unknown, and requires further investigation. Finally, but importantly, the impact of the forthcoming introduction of PCV7 will need to be assessed by continued epidemiological surveillance of IPD throughout Japan.

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DECLARATION OF INTEREST

None.

REFERENCES

1. **Hansman D, Bullen MM.** A resistant pneumococcus. *Lancet* 1967; **2**: 264–265.
2. **Klugman KP.** Pneumococcal resistance to antibiotics. *Clinical Microbiology Reviews* 1990; **3**: 171–196.
3. **Appelbaum PC.** Antimicrobial resistance in *Streptococcus pneumoniae*: an overview. *Clinical Infectious Diseases* 1992; **15**: 77–83.
4. **Phongsamart W, *et al.*** Serotype distribution and antimicrobial susceptibility of *S. pneumoniae* causing invasive disease in Thai children younger than 5 years old, 2000–2005. *Vaccine* 2007; **25**: 1275–1280.
5. **Heffernan HM, *et al.*** Invasive pneumococcal disease in New Zealand 1998–2005: capsular serotypes and antimicrobial resistance. *Epidemiology and Infection* 2008; **136**: 352–359.
6. **Bruce MG, *et al.*** International circumpolar surveillance system for invasive pneumococcal disease, 1999–2005. *Emerging Infectious Diseases* 2008; **14**: 25–33.
7. **Pebody RG, *et al.*** Pneumococcal disease surveillance in Europe. *Eurosurveillance* 2006; **11**: 171–178.
8. **Centers for Disease Control and Prevention.** Progress in introduction of pneumococcal conjugate vaccine – worldwide, 2000–2008. *Morbidity Mortality Weekly Report* 2008; **57**: 1148–1151.
9. **Ubukata K, *et al.*** Identification of penicillin and other beta-lactam resistance in *Streptococcus pneumoniae* by polymerase chain reaction. *Journal of Infection and Chemotherapy* 1997; **3**: 190–197.
10. **Ubukata K.** Problems associated with high prevalence of multidrug-resistant bacteria in patients with community-acquired infections. *Journal of Infection and Chemotherapy* 2003; **9**: 285–291.
11. **Ubukata K, *et al.*** Combinational detection of autolysin and penicillin-binding protein 2B genes of *Streptococcus pneumoniae* by PCR. *Journal of Clinical Microbiology* 1996; **34**: 592–596.
12. **Chiba N, *et al.*** 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. *Journal of Antimicrobial Chemotherapy* 2005; **56**: 756–760.
13. **Reinert RR.** Pneumococcal conjugate vaccines – a European perspective. *International Journal of Medical Microbiology* 2004; **294**: 77–94.
14. **Advisory Committee on Immunization Practices.** Preventing pneumococcal disease among infants and young children. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recommendations and Reports* 2000; **49**: 1–35.
15. **Centers for Disease Control and Prevention.** Direct and indirect effects of routine vaccination of children with 7-valent pneumococcal conjugate vaccine on incidence of invasive pneumococcal disease – United States, 1998–2003. *Morbidity Mortality Weekly Report* 2005; **54**: 893–897.
16. **Lexau CA, *et al.*** Changing epidemiology of invasive pneumococcal disease among older adults in the era of

pediatric pneumococcal conjugate vaccine. *Journal of the American Medical Association* 2005; **294**: 2043–2051.

17. **Poehling KA, *et al.*** Invasive pneumococcal disease among infants before and after introduction of pneumococcal conjugate vaccine. *Journal of the American Medical Association* 2006; **295**: 1668–1674.
18. **Black S, *et al.*** Surveillance for invasive pneumococcal disease during 2000–2005 in a population of children who received 7-valent pneumococcal conjugate vaccine. *Pediatric Infectious Diseases Journal* 2007; **26**: 771–777.
19. **Pelton SI, *et al.*** Emergence of 19A as virulent and multidrug resistant Pneumococcus in Massachusetts following universal immunization of infants with pneumococcal conjugate vaccine. *Pediatric Infectious Diseases Journal* 2007; **26**: 468–472.
20. **Farrell DJ, Klugman KP, Pichichero M.** Increased antimicrobial resistance among nonvaccine serotypes of *Streptococcus pneumoniae* in the pediatric population after the introduction of 7-valent pneumococcal vaccine in the United States. *Pediatric Infectious Diseases Journal* 2007; **26**: 123–128.
21. **Centers for Disease Control and Prevention.** Invasive pneumococcal disease in children 5 years after conjugate vaccine introduction – eight states, 1998–2005. *Morbidity Mortality Weekly Report* 2008; **57**: 144–148.
22. **Ubukata K, *et al.*** Antibiotic susceptibility in relation to penicillin-binding protein genes and serotype distribution of *Streptococcus pneumoniae* strains responsible for meningitis in Japan, 1999 to 2002. *Antimicrobial Agents and Chemotherapy* 2004; **48**: 1488–1494.
23. **Ubukata K, *et al.*** Incidence of penicillin-resistant *Streptococcus pneumoniae* in Japan, 1993–1995. *Journal of Infection and Chemotherapy* 1996; **1**: 177–184.
24. **Clinical and Laboratory Standards Institute.** Performance standards for antimicrobial susceptibility testing: 18th informational supplement. Wayne, PA, USA: CLSI, 2008; CLSI document M100-S18.
25. **Garcia P, *et al.*** Nucleotide sequence and expression of the pneumococcal autolysin gene from its own promoter in *Escherichia coli*. *Gene* 1986; **43**: 265–272.
26. **Nagai K, *et al.*** Evaluation of PCR primers to screen for *Streptococcus pneumoniae* isolates and beta-lactam resistance, and to detect common macrolide resistance determinants. *Journal of Antimicrobial Chemotherapy* 2001; **48**: 915–918.
27. **Center KJ.** Prevenar vaccination: review of the global data, 2006. *Vaccine* 2007; **25**: 3085–3089.
28. **Scott D, *et al.*** Phase 1 trial of 13-valent pneumococcal conjugate vaccine in Japanese adults. *Pediatr Int* 2008; **50**: 295–299.
29. **Yamanaka N, Hotomi M, Billal DS.** Clinical bacteriology and immunology in acute otitis media in children. *Journal of Infection and Chemotherapy* 2008; **14**: 180–187.
30. **Ubukata K, Iwata S, Sunakawa K.** In vitro activities of new ketolide, telithromycin, and eight other macrolide antibiotics against *Streptococcus pneumoniae* having *mef*A and *erm*B genes that mediate macrolide resistance. *Journal of Infection and Chemotherapy* 2003; **9**: 221–226.
31. **Tunkel AR, *et al.*** Practice guidelines for the management of bacterial meningitis. *Clinical Infectious Diseases* 2004; **39**: 1267–1284.
32. **Hasegawa K, *et al.*** High prevalence of type b beta-lactamase-non-producing ampicillin-resistant *Haemophilus influenzae* in meningitis: the situation in Japan where Hib vaccine has not been introduced. *Journal of Antimicrobial Chemotherapy* 2006; **57**: 1077–1082.

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ORIGINAL ARTICLE

Diverse mutations in the *ftsI* gene in ampicillin-resistant *Haemophilus influenzae* isolates from pediatric patients with acute otitis media

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Abstract To clarify molecular changes in β-lactamase-nonproducing, ampicillin-resistant (BLNAR) *Haemophilus influenzae*, which is increasing in pediatric patients with acute otitis media (AOM) in Japan, we identified amino acid (aa) substitutions in penicillin-binding protein 3 for the BLNAR strains. Of 191 *H. influenzae* strains isolated from middle ear fluid of pediatric AOM patients between October 2005 and March 2008, BLNAR strains determined by PCR accounted for 49.2%. Of the BLNAR strains, 91.5% possessed 4 aa substitutions: Met377Ile, Ser385Thr, Leu389Phe, and either Asn526Lys or Arg517His. Additionally, the emergence of BLNAR strains possessing a new aa substitution of Val329Ala in the conserved aa motif of Ser327-Thr-Val-Lys, or Val511Ala adjacent to the conserved aa motif of Lys512-Thr-Gly, was noted. Transformation of the *ftsI* gene into the Rd reference strain (ATCC 51907) demonstrated that these two aa substitutions reduced susceptibility to amoxicillin more than to cephalosporins. Pulsed-field gel electrophoretic profiles of BLNAR strains were highly diverse. These results suggested that inadequate antibiotic use may increase BLNAR strains by selecting mutations in the *ftsI* gene and that such use may have favored the new aa substitutions.

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Introduction

Haemophilus influenzae is an important pathogen causing respiratory tract infection, pneumonia, acute otitis media (AOM), and meningitis. Two well-known mechanisms are implicated in the resistance of *H. influenzae* to ampicillin (AMP). One is the enzymatic hydrolysis of β-lactam agents resulting from the production of TEM-1 and ROB β-lactamases [1–4]; the other is decreased affinity of penicillin-binding protein (PBP) 3 for β-lactam antibiotics reflecting amino acid substitutions in the enzyme [3, 5]. Strains with alterations in PBP3 are termed β-lactamase-nonproducing, AMP-resistant (BLNAR) *H. influenzae*. In addition, strains demonstrating both mechanisms are termed β-lactamase-producing, amoxicillin-clavulanic acid-resistant (BLPACR) *H. influenzae*.

Amino acid substitutions in PBP3 surrounding the conserved Lys512-Thr-Gly (KTG) and Ser379-Ser-Asn (SSN) motifs are responsible for β-lactam resistance [5–9]. Single substitution of Asn526Lys or Arg517His was commonly found in BLNAR isolates with intermediate resistance to AMP (low-BLNAR). Additional amino acid substitutions, Met377Ile, Ser385Thr, and/or Leu389Phe, were characterized by higher than intermediate resistance to AMP (BLNAR).

In the United States, the prevalence of BLNAR strains was reported to be less than 5% in a recent study [10]. Another recent study reported a relatively constant prevalence of BLNAR strains, about 9%, in most of Europe [11]. In Japan and Spain, however, a marked increase in the number of BLNAR isolates has been observed [12–14].

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Currently, an increase in intractable or recurrent AOM caused by BLNAR has become a great concern in pediatrics and otolaryngology in Japan [15, 16]. Despite the high reported prevalence of BLNAR strains from AOM, their evolutionary molecular changes in the *ftsI* gene have not been clarified. In this study, we characterized amino acid substitutions in PBP3 and their correlation with antibiotic susceptibilities in *H. influenzae* isolated from pediatric patients with AOM.

Materials and methods

Strains

In Japanese medical institutions, 191 clinical *H. influenzae* strains were isolated from middle ear fluid collected from pediatric patients with AOM by puncture or incision of the tympanic membrane between October 2005 and March 2008. The middle ear fluid samples were sent to our laboratory, where we immediately carried out bacterial culture and species identification based on the requirement of β-NAD (V factor) and hemin (X factor). Additionally, PCR was performed on all isolates, as described below, to identify species, β-lactamase genes, and *ftsI* mutations.

Polymerase chain reaction (PCR)

We performed PCR for *H. influenzae* using six sets of primers described previously [7]. The six targets were the 16S rRNA gene identifying species [17], the TEM-1 β-lactamase gene [18], the ROB-1 β-lactamase gene [19], an amino acid substitution of Asn526Lys in the *ftsI* gene [20], an amino acid substitution of Ser385Thr in the *ftsI* gene [5], and the Hib-specific *capB* locus [21]. PCR cycling conditions using lysates extracted from colonies of isolates included 35 cycles at 94°C for 15 s; at 53°C for 15 s; and at 72°C for 15 s. On the basis of the PCR results, the resistance class was described by attaching "g" to indicate genetic identification as opposed to other biologic assays, yielding designations such as gBLNAR, gLow-BLNAR, gBLPAR, and gBLNAS.

Serotyping

Serotypes of *H. influenzae* strains, except for type b, were determined by the agglutination test using antiserum purchased from Becton–Dickinson (Franklin Lakes, NJ, USA).

Antibiotic susceptibility

Susceptibility testing was performed by an agar dilution method [22]. Antibiotics used in this study were AMP, amoxicillin (AMX), cefditoren (CDN), and tebipenem (TBM, a new oral carbapenem; Meiji Seika Kaisha, Tokyo, Japan); cefdinir (CDR; Astellas Pharma, Tokyo, Japan); cefotaxime (CTX; Aventis Pharma, Tokyo, Japan); and meropenem (MEM; Dainippon Sumitomo Pharma, Osaka, Japan). *H. influenzae* ATCC 49247 and ATCC 49766 were used as quality control strains.

Sequencing

The 1.0-kb DNA region of the *ftsI* gene corresponding to the transpeptidase domain of PBP3 was amplified from the chromosomal DNA of *H. influenzae* by PCR using a sense primer, 5′-GTTGCACATATCTCCGATGAG-3′, and a reverse primer, 5′-CAGCTGCTTCAGCATCTTGC-3′, as described previously [5]. Amplified DNA fragments were purified using a QIAquick PCR purification kit (Qiagen, Tokyo, Japan) and used as templates. Sequencing reactions were carried out using a BigDye Terminator cycle sequencing kit, version 3.1 (Applied Biosystems, Foster City, CA, USA). DNA sequencing was performed with an ABI Prism 3130/3130xl genetic analyzer (Applied Biosystems).

Transformation

The Rd strain (ATCC 51907) was transformed with an open reading frame corresponding to the *ftsI* gene, which was PCR-amplified. Transformation was carried out using a cuvette with a 0.1-cm electrode gap and a MicroPulser electroporation apparatus (Bio-Rad Laboratories, Hercules, CA, USA), as described previously [5]. Conditions for electroporation were 1.8 kV/cm with time constants of 5.8 to 5.9 ms. Colonies grown on selective agar plates containing CTX at 0.016, 0.063, and 0.25 mg/l were selected at random, and antibiotic susceptibilities for the colonies were determined by the agar dilution method as described above. The *ftsI* gene of the colonies was sequenced to confirm gene transfer.

Pulsed-field gel electrophoresis (PFGE)

PFGE was carried out according to the method described previously, with some modifications [23]. Chromosomal DNAs extracted from each *H. influenzae* strain were digested with *Sma*I. Electrophoresis was performed using CHEF Mapper (Bio-Rad Laboratories). Separation of DNA fragments was achieved at 6 V/cm at 14°C for 20 h and 18 min. Pulse time, which changed in a lineal manner, was 0.47 to 63.08 s.

Results

Resistance classes and susceptibility

The resistance classes of 191 *H. influenzae* isolates were identified by PCR. AMP-resistant strains were extremely

common, representing 60.2% of all isolates; the proportion of each resistance class was 49.2% for gBLNAR, 6.8% for gLow-BLNAR, 3.7% for gBLPACR II, and 0.5% for gBLPAR. Strains without any resistance genes, i.e., gBLNAS, AMP-susceptible strains, represented 39.8%.

Of all strains tested, only 6 (3.1%) were serotyped as type b ($n = 5$) or type f ($n = 1$); the remaining strains were nontypable (NT).

Table 1 shows the MIC ranges, MIC_{50}s, and MIC_{90}s of seven β-lactam antibiotics for *H. influenzae* strains classified into four resistance groups, excluding gBLPAR, which consisted of 1 strain. The MIC_{90} of the standard antibiotic AMP for gBLNAR, 8 mg/l, was 16 times higher than the value for gBLNAS, 0.5 mg/l. In contrast, the MIC_{90}s of most cephalosporin antibiotics (CDR, CDN, and CTX) for gBLNAR were markedly increased; the MIC_{90} values of CDR, CDN, and CTX were 64, 8, and 32 times higher than the value for gBLNAS, respectively. The *ftsI* gene mutations affected the MICs of cephalosporin antibiotics more than those of AMP. The MICs of TBM and MEM for AMP-resistant strains were affected slightly by *ftsI* gene mutations. The MIC_{90}s of TBM and MEM for gBLNAR were increased 4 to 8 times relative to those for gBLNAS.

Table 1 MIC distributions and resistance classes of *Haemophilus influenzae* strains

Antimicrobial agent and Resistance class	MIC (mg/l) Range	MIC_{50}	MIC_{90}
Ampicillin			
gBLNAS[a] ($n = 76$)	0.063–1	0.25	0.5
gLow-BLNAR ($n = 13$)	0.5–2	1	1
gBLNAR ($n = 94$)	0.5–32	2	8
gBLPACR-II ($n = 7$)	2–>64	16	>64
Amoxicillin			
gBLNAS	0.125–1	0.5	0.5
gLow-BLNAR	0.5–4	2	4
gBLNAR	0.25–64	8	32
gBLPACR-II	2–>64	64	>64
Cefdinir			
gBLNAS	0.031–1	0.25	0.5
gLow-BLNAR	0.5–4	0.5	2
gBLNAR	2–32	8	32
gBLPACR-II	8–16	16	16
Cefditoren			
gBLNAS	0.002–0.063	0.016	0.031
gLow-BLNAR	0.016–0.063	0.031	0.063
gBLNAR	0.031–1	0.25	0.25
gBLPACR-II	0.125	0.125	0.125
Cefotaxime			
gBLNAS	0.004–0.063	0.016	0.031
gLow-BLNAR	0.016–0.125	0.031	0.125
gBLNAR	0.063–4	0.5	1
gBLPACR-II	0.25–0.5	0.5	0.5
Meropenem			
gBLNAS	0.008–0.125	0.063	0.125
gLow-BLNAR	0.063–0.5	0.125	0.25
gBLNAR	0.031–0.5	0.25	0.5
gBLPACR-II	0.063–0.25	0.063	0.25
Tebipenem			
gBLNAS	0.008–0.25	0.063	0.125
gLow-BLNAR	0.031–0.5	0.25	0.5
gBLNAR	0.031–1	0.25	1
gBLPACR-II	0.25–0.5	0.5	0.5

gBLNAS, gLowBLNAR, low-β-lactamase-nonproducing, ampicillin (AMP)-resistant; gBLPACR-II, β-lactamase-producing, amoxicillin-clavulanic acid-resistant-II

[a] g in the strain name denotes genetic identification

Amino acid substitutions in PBP3

Table 2 shows the deduced amino acid substitutions in PBP3 in gLow-BLNAR ($n = 13$), gBLNAR ($n = 94$), and gBLPACR II ($n = 7$) strains. These strains were classified into groups based on the eight amino acid substitutions reported by Hasegawa et al. [24]. Of these eight amino acid substitutions, three substitutions (Arg517His, Asn526Lys, and Ser385Thr) were considered to importantly affect resistance.

All gLow-BLNAR strains commonly possessed Asn526Lys without Ser385Thr, which had been identified frequently among AMP-resistant *H. influenzae* strains isolated in the late 1990 s. On the other hand, all gBLNAR and gBLPACR II strains possessed Ser385Thr and either Asn526Lys or Arg517His. Furthermore, 91.1% (92/101) of the gBLNAR and gBLPACR II strains possessed Met377Ile and Leu389Phe as well.

The emergence of gBLNAR with a Val329Ala substitution in the conserved amino acid motif of Ser327-Thr-Val-Lys (STVK) and gBLNAR, with a Val511Ala substitution adjacent to the KTG motif, which had not been identified in the early 2000 s in Japan, was noted.

Correlation between Val329Ala and Val511Ala and antibiotic susceptibilities

To investigate the effects of Val329Ala in the STVK motif and Val511Ala adjacent to the KTG motif in the *ftsI* gene upon antibiotic susceptibilities, the AMP-susceptible strain Rd was transformed with PCR-amplified *ftsI* gene fragments from gBLNAR with Val329Ala (KU007) and gBLNAR with Val511Ala (KU026). As controls, PCR-amplified *ftsI* gene fragments from gBLNAR without Val329Ala (KU001), gBLNAR without Val511Ala (KU002), and gLow-BLNAR (KU046) were introduced to Rd as well.

Table 2 Amino acid substitutions identified in the *ftsI* genes from gBLNAR and gBLPACR II *H. influenzae* strains

Resistance class	Subgroup	No. of strains	Amino acid substitution								MIC_{90} (mg/l)						
			STVK[a]	SSN motif[b]			KTG motif[c]										
			Val-329	Met-377	Ser-385	Leu-389	Ala-502	Val-511	Arg-517	Asn-526	AMP	AMX	CDR	CDN	CTX	MEM	TBM
gBLNAS[d]			–	–	–	–	–	–	–	–	0.25	0.5	0.25	0.008	0.008	0.063	0.125
gLow-BLNAR ($n = 13$)		6	–	–	–	–	–	–	–	Lys	1	4	1	0.063	0.063	0.5	0.5
		3	–	–	–	–	Thr	–	–	Lys							
		3	–	–	–	–	Val	–	–	Lys							
		1	–	Ile	–	–	Val	–	–	Lys							
gBLNAR ($n = 94$)	i	1	–	–	Thr	–	–	–	His	–	4	4	32	0.25	1	0.25	0.25
	ii	1	–	Ile	Thr	–	–	–	His	–							
	iii	8	–	Ile	Thr	Phe	–	–	His	–							
	iv	1	–	–	Thr	–	–	–	–	Lys	4	32	32	0.25	1	0.5	1
	v	2	Ala	–	Thr	–	–	–	–	Lys							
	vi	3	–	–	Thr	–	Thr	–	–	Lys							
	vii	75	–	Ile	Thr	Phe	–	–	–	Lys	16	32	32	0.5	1	0.5	1
	viii	1	–	Ile	Thr	Phe	–	Ala	–	Lys							
	ix	2	–	Ile	Thr	Phe	Val	–	–	Lys							
gBLPACR-II ($n = 7$)	ii	1	–	Ile	Thr	–	–	–	His	–	16	64	8	0.125	0.5	0.25	0.25
	iii	1	–	Ile	Thr	Phe	–	–	His	–							
	vii	5	–	Ile	Thr	Phe	–	–	–	Lys	>64	>64	16	0.125	0.5	0.25	0.5

AMP ampicillin, *AMX* amoxicillin, *CDR* cefdinir, *CDN* cefditoren, *CTX* cefotaxime, *MEM* meropenem, *TBM* tebipenem

[a] STVK, Ser327-Thr-Val-Lys

[b] SSN, Ser379-Ser-Asn

[c] KTG, Lys512-Thr-Gly

[d] Control, ATCC49766

Table 3 MICs of *H. influenzae* strains transformed with a *ftsI* DNA fragment and donor strains

Strains	Amino acid substitution							MIC (mg/l)						
	STVK[a]	SSN motif[b]			KTG motif[c]									
	Val-329	Met-377	Ser-385	Leu-389	Ala-502	Val-511	Arg-517	Asn-526	AMP	AMX	CDR	CDN	CTX	MEM	TBM
Recipient Rd	–	–	–	–	–	–	–	–	0.125	0.5	1	0.008	0.016	0.063	0.063
Donor KU 046	–	–	–	–	–	–	–	Lys	1	2	1	0.016	0.031	0.25	0.25
Donor KU 001	–	–	Thr	–	–	–	–	Lys	2	2	16	0.125	0.5	0.5	0.5
Donor KU 007	Ala	–	Thr	–	–	–	–	Lys	1	64	64	0.25	4	0.5	0.25
Donor KU 002	–	Ile	Thr	Phe	–	–	–	Lys	2	4	16	0.25	2	0.25	0.5
Donor KU 026	–	Ile	Thr	Phe	–	Ala	–	Lys	2	64	64	0.25	2	0.5	0.5
Rd^{KU046}	–	–	–	–	–	–	–	Lys	0.5	1	1	0.008	0.031	0.125	0.125
Rd^{KU001}	–	–	Thr	–	–	–	–	Lys	1	2	16	0.063	0.5	0.25	0.25
Rd^{KU007}	Ala	–	Thr	–	–	–	–	Lys	1	32	64	0.25	2	0.25	0.25
Rd^{KU002}	–	Ile	Thr	Phe	–	–	–	Lys	1	2	16	0.125	2	0.25	0.25
Rd^{KU026}	–	Ile	Thr	Phe	–	Ala	–	Lys	1	32	64	0.125	2	0.25	0.5

AMP ampicillin, *AMX* amoxicillin, *CDR* cefdinir, *CDN* cefditoren, *CTX* cefotaxime, *MEM* meropenem, *TBM* tebipenem

[a] STVK, Ser327-Thr-Val-Lys

[b] SSN, Ser379-Ser-Asn

[c] KTG, Lys512-Thr-Gly

Table 3 shows the MICs of seven β-lactam antibiotics for each transformant and donor strains. The MICs of AMX, CDR, CDN, and CTX for transformant Rd^{KU007} were higher than those for transformant Rd^{KU001}. Especially, the AMX MIC of transformant Rd^{KU007} was 16 times higher than that for transformant Rd^{KU001}. On the other hand, the MICs of AMP, MEM, and TBM for transformant Rd^{KU007} were same as those for transformant Rd^{KU001}. The MICs of AMX and CDR for transformant Rd^{KU026} were 16 and 4 times higher than those for transformant Rd^{KU002}. In contrast, the MICs of AMP, CDN, CTX, MEM, and TBM for transformant Rd^{KU026} were equal or almost equal to those for transformant Rd^{KU002}.

Electrophoretic profiles

Figure 1 shows the PFGE profiles of 20 gBLNAR and 7 gBLPACR II strains selected randomly from each amino acid substitution subgroup. The profiles were extremely diverse and could not be classified into groups. Interestingly, the strains that shared an amino acid substitution subgroup and had the same resistance type differed in PFGE profiles. This diversity represents evidence that the resistant strains were clonally different from each other.

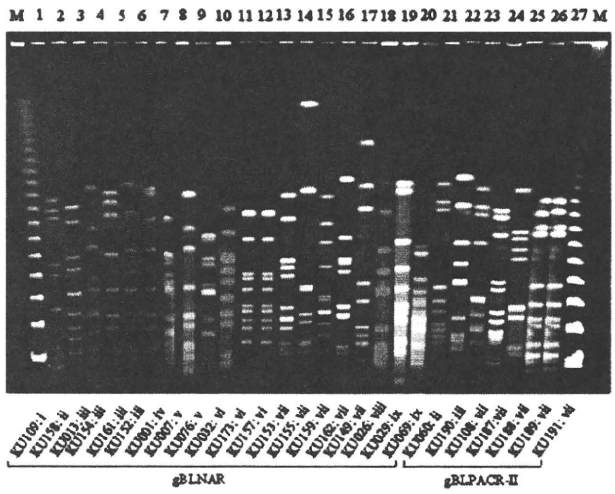

Fig. 1 Pulsed-field gel electrophoresis (PFGE) profiles of chromosomal DNA from gLow-β-lactamase-nonproducing, ampicillin-resistant (*BLNAR*), *gBLNAR*, and g β-lactamase-producing, amoxicillin-clavulanic acid-resistant II (*gBLPACR II*) strains ($n = 27$) digested with *Sma*I restriction enzyme ('g' in the strain name denotes genetic identification). *Lanes M*, λ Ladder molecular size marker; *1*, gBLNAR subgroup i (KU109); *2*, gBLNAR subgroup ii (KU158); *3–6*, gBLNAR subgroup iii (KU013, 154, 161, and 152); *7*, gBLNAR subgroup iv (KU001); *8–9*, gBLNAR subgroup v (KU007 and 076); *10–12*, gBLNAR subgroup vi (KU032, 173, and 157); *13–17*, gBLNAR subgroup vii (KU153, 155, 159, 162, and 149); *18*, gBLNAR subgroup viii (KU026); *19–20*, gBLNAR subgroup ix (KU029 and 069); *21*, gBLPACR-II subgroup ii (KU060); *22*, gBLPACR-II subgroup iii (KU190); *23–27*, gBLPACR-II subgroup vii (KU108, 187, 188, 189, and191)

Discussion

In 2001, Ubukata et al. [5] found three amino acid substitutions near the SSN motif: Met377Ile, Ser 385Thr, and Leu389Phe; and two amino acid substitutions near the KTG motif: Asn526Lys and Arg517His; in PBP3 of BLNAR strains isolated in Japan. They reported that these amino acid substitutions affected the MICs of cephalosporin antibiotics more than those of AMP. Additional new amino acid substitutions of Val329Ala in the STVK motif and Val511Ala adjacent to the KTG motif were identified in 2006 in Japan [14]. However, their effects upon antibiotic susceptibilities have remained to be clarified. In the present study, we demonstrated that Val329Ala affected the MICs of AMX and the cephalosporin antibiotics, CDR, CDN, and CTX, while Val511Ala affected the MICs of AMX and CDR. In particular, we found that either of these amino acid substitutions increased the MIC of AMX by 16 times. AMX is the antibiotic most often used to treat community-acquired respiratory infections in the United States and Europe [25]. Similarly, since around 2005, AMX has commonly been used to treat pediatric outpatients with respiratory infections and AOM in Japan. In the present study, three patients from whom the BLNAR strains with the new amino acid substitutions were isolated had not been treated with AMX for 7 days before the strains were isolated. However, AMX may have been used previously to treat their AOM or respiratory infections, because they were aged 3, 4, and 6 years, i.e., they were older than the pediatric patients commonly seen with AOM, who are usually age 1 and under. We suggest that the change of antibiotic use in Japan, from oral cephalosporin to AMX, may have promoted the emergence of these two amino acid substitutions, Val329Ala and Val511Ala, which influenced the MICs of AMX.

The AMP-resistant strains tested in the present study showed incredible diversity in their PFGE profiles, although some of them had the same amino acid substitution subgroup and the same resistance type. The diversity is equivalent to that in *H. influenzae* as normal flora [26, 27]. From the above finding, we suppose that *H. influenzae* as normal flora in healthy children acquired resistance in some way and turned out to be a causative strain. Horizontal transfer of the mutated *ftsI* gene may be one of the ways of acquiring resistance. Takahata et al. [28, 29] reported horizontal transfer of the mutated *ftsI* gene from clinical isolates of BLNAR to the Rd strain of BLNAS. When we examined this phenomenon using clinically isolated *H. influenzae* as a recipient, horizontal transfer of the mutated *ftsI* gene was not identified (data not shown). The spread of resistant *H. influenzae* by horizontal transfer of the mutated *ftsI* gene is a reasonable possibility, because *H. influenzae* in nature is capable of transformation [30, 31]. However, β-lactam antibiotics at doses that provide inadequate concentrations may more likely favor mutations in the *ftsI* gene and select gBLNAR.

In summary, we have described evolutionary molecular changes in the *ftsI* gene involving the reduced antibiotic susceptibilities of *H. influenzae* isolated from pediatric patients with AOM. These molecular changes seem to be related to a change in antibiotic use. Thus, strict control of antibiotic use, based on evidence such as trends concerning resistant strains and their molecular changes, is important to prevent increases of resistant strains.

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References

1. Karlowsky JA, Verma G, Zhanel GG, Hoban DJ. Presence of ROB-1 ß-lactamase correlates with cefaclor resistance among recent isolates of *Haemophilus influenzae*. J Antimicrob Chemother. 2000;45:871–5.
2. Medeiros AA, Levesque R, Jacoby GA. An animal source for the ROB-1 beta-lactamase of *Haemophilus influenzae* type b. Antimicrob Agents Chemother. 1986;29:212–5.
3. Scriver SR, Walmsley SL, Kau CL, Hoban DJ, Brunton J, McGeer A, et al. Determination of antimicrobial susceptibilities of Canadian isolates of *Haemophilus influenzae* and characterization of their beta-lactamases. Canadian Haemophilus Study Group. Antimicrob Agents Chemother. 1994;38:1678–80.
4. Vega R, Sadoff HL, Patterson MJ. Mechanisms of ampicillin resistance in *Haemophilus influenzae* type B. Antimicrob Agents Chemother. 1976;9:164–8.
5. Ubukata K, Shibasaki Y, Yamamoto K, Chiba N, Hasegawa K, Takeuchi Y, et al. Association of amino acid substitutions in penicillin-binding protein 3 with β-lactam resistance in β-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. Antimicrob Agents Chemother. 2001;45:1693–9.
6. Dabernat H, Delmas C, Seguy M, Pelissier R, Faucon G, Bennamani S, et al. Diversity of ß-lactam resistance-conferring amino acid substitutions in penicillin-binding protein 3 of *Haemophilus influenzae*. Antimicrob Agents Chemother. 2002;46:2208–18.
7. Hasegawa K, Yamamoto K, Chiba N, Kobayashi R, Nagai K, Jacobs MR, et al. Diversity of ampicillin-resistance genes in *Haemophilus influenzae* in Japan and the United States. Microb Drug Resist. 2003;9:39–46.
8. Osaki Y, Sanbongi Y, Ishikawa M, Osaki Y, Kataoka H, Suzuki T, et al. Genetic approach to study the relationship between penicillin-binding protein 3 mutations and *Haemophilus influenzae* ß-lactam resistance by using site-directed mutagenesis and gene recombinants. Antimicrob Agents Chemother. 2005;49:2834–9.
9. Straker K, Wootton M, Simm AM, Bennett PM, MacGowan AP, Walsh TR. Cefuroxime resistance in non-ß-lactamase *Haemophilus influenzae* is linked to mutations in *ftsI*. J Antimicrob Chemother. 2003;51:523–30.
10. Karlowsky JA, Critchley IA, Blosser-Middleton RS, Karginova EA, Jones ME, Thornsberry C, et al. Antimicrobial surveillance of *Haemophilus influenzae* in the United States during 2000–2001 leads to detection of clonal dissemination of a ß-lactamase-negative and ampicillin-resistant strain. J Clin Microbiol. 2002;40:1063–6.

11. Jansen WTM, Verel A, Beitsma M, Verhoef J, Milatovic D. Longitudinal European surveillance study of antibiotic resistance of *Haemophilus influenzae*. J Antimicrob Chemother. 2006;58: 873–7.
12. García-Cobos S, Campos J, Lázaro E, Román F, Cercenado E, García-Rey C, et al. Ampicillin-resistant non-ß-lactamase-producing *Haemophilus influenzae* in Spain: recent emergence of clonal isolates with increased resistance to cefotaxime and cefixime. Antimicrob Agents Chemother. 2007;51:2564–73.
13. Pérez-Trallero E, García-de-la-Fuente C, García-Rey C, Baquero F, Aguilar L, Dal-Ré R, et al. Geographical and ecological analysis of resistance, co-resistance, and coupled resistance to antimicrobials in respiratory pathogenic bacteria in Spain. Antimicrob Agents Chemother. 2005;49:1965–72.
14. Sanbongi Y, Suzuki T, Osaki Y, Senju N, Ida T, Ubukata K. Molecular evolution of ß-lactam-resistant *Haemophilus influenzae*: 9-year surveillance of penicillin-binding protein 3 mutations in isolates from Japan. Antimicrob Agents Chemother. 2006;50: 2487–92.
15. Hotomi M, Fujihara K, Sakai A, Billal DS, Shimada J, Suzumoto M, et al. Antimicrobial resistance of *Haemophilus influenzae* isolated from the nasopharynx of Japanese children with acute otitis media. Acta Otolaryngol. 2006;126:240–7.
16. Yamanaka N, Hotomi M, Billal DS. Clinical bacteriology and immunology in acute otitis media in children. J Infect Chemother. 2008;14:180–7.
17. Nelson MB, Apicella MA, Murphy TF, Vankeulen H, Spotila LD, Rekosh D. Cloning and sequencing of *Haemophilus influenzae* outer membrane protein P6. Infect Immun. 1988;56:128–34.
18. Sutcliffe JG. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. Proc Natl Acad Sci USA. 1978;75:3737–41.
19. Juteau JM, Levesque RC. Sequence analysis and evolutionary perspectives of ROB-1 beta-lactamase. Antimicrob Agents Chemother. 1990;34:1354–9.
20. Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, et al. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science. 1995;269:496–512.
21. Van Eldere J, Brophy L, Loynds B, Celis P, Hancock I, Carman S, et al. Region II of the *Haemophilus influenzae* type be capsulation locus is involved in serotype-specific polysaccharide synthesis. Mol Microbiol. 1995;15:107–18.
22. Hasegawa K, Chiba N, Kobayashi R, Murayama SY, Iwata S, Sunakawa K, et al. Rapidly increasing prevalence of ß-lactamase-nonproducing, ampicillin-resistant *Haemophilus influenzae* type b in patients with meningitis. Antimicrob Agents Chemother. 2004;48:1509–14.
23. Yano H, Suetake M, Kuga A, Irinoda K, Okamoto R, Kobayashi T, et al. Pulsed-field gel electrophoresis analysis of nasopharyngeal flora in children attending a day care center. J Clin Microbiol. 2000;38:625–9.
24. Hasegawa K, Kobayashi R, Takada E, Ono A, Chiba N, Morozumi M, et al. High prevalence of type b ß-lactamase-nonproducing ampicillin-resistant *Haemophilus influenzae* in meningitis: the situation in Japan where Hib vaccine has not been introduced. J Antimicrob Chemother. 2006;57:1077–82.
25. García-Cobos S, Campos J, Román F, Carrera C, Pérez-Vázquez M, Aracil B, et al. Low β-lactamase-negative ampicillin-resistant *Haemophilus influenzae* strains are best detected by testing amoxicillin susceptibility by the broth microdilution method. Antimicrob Agents Chemother. 2008;52:2407–14.
26. LaCross NC, Marrs CF, Patel M, Sandstedt SA, Gilsdorf JR. High genetic diversity of nontypeable *Haemophilus influenzae* isolates from two children attending a day care center. J Clin Microbiol. 2008;46:3817–21.
27. St. Sauver J, Marrs CF, Foxman B, Somsel P, Madera R, Gilsdorf JR. Risk factors for otitis media and carriage of multiple strains of *Haemophilus influenzae* and *Streptococcus pneumoniae*. Emerg Infect Dis. 2000;6:622–30.
28. Takahata S, Ida T, Senju T, Sanbongi Y, Miyata A, Maebashi K, et al. Horizontal gene transfer of *ftsI*, encoding penicillin-binding protein 3, in *Haemophilus influenzae*. Antimicrob Agents Chemother. 2007;51:1589–95.
29. Takahata S, Kato Y, Sanbongi Y, Maebashi K, Ida T. Comparison of the efficacies of oral β-lactams in selection of *Haemophilus influenzae* transformants with mutated *ftsI* genes. Antimicrob Agents Chemother. 2008;52:1880–3.
30. Goodgal SH, Mitchell MA. Sequence and uptake specificity of cloned sonicated fragments of *Haemophilus influenzae* DNA. J Bacteriol. 1990;172:5924–8.
31. Bakkali M. Genome dynamics of short oligonucleotides: the example of bacterial DNA uptake enhancing sequences. PLoS One. 2007;2:e741.

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ORIGINAL ARTICLE

Molecular epidemiologic characteristics of *Streptococcus pneumoniae* isolates from children with meningitis in Japan from 2007 through 2009

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Abstract We examined associations of serotypes with multilocus sequence typing (MLST) data for 7 housekeeping genes and the genotype concerning penicillin resistance based on penicillin-binding protein (PBP) alterations in *Streptococcus pneumoniae* isolates from children with meningitis. From throughout Japan, we collected 115 pneumococcal isolates from the cerebrospinal fluid of patients 15 years old or younger from January 2007 to December 2009. We then carried out serotyping, MLST, and genotypic classification. Isolates included 24 serotypes and 52 sequence types (STs) according to MLST, of which 18 were novel. The 4 predominant serotypes included a variety of STs: 14 STs in serotype 6B ($n = 24$), 2 STs in 19F ($n = 17$), 6 STs in 23F ($n = 14$), and 5 STs in 14 ($n = 11$). Resistance genotypes included 6 types: 44.3% for gPRSP (*pbp1a* + *2x* + *2b*), 13.9% for gPISP (*pbp1a* + *2x*), 9.6% for gPISP (*pbp2x* + *2b*), 19.1% for gPISP (*pbp2x*), 3.5% for gPISP (*pbp2b*), and 9.6% for gPSSP. Interestingly, the most prevalent serotype of 6B included 7 newly identified STs and a variety of genotypes for resistance. STs in serotypes 23F and 14 were highly diverse, but not in 19F. These results suggest that various genetic elements in *S. pneumoniae* might be intrinsically susceptible to genetic mutations and recombination, with acceleration of emergence reflecting selection pressures such as antibiotic overuse.

Keywords *Streptococcus pneumoniae* · Meningitis · Child · Serotype · Multilocus sequence typing · Genotypic resistance · Penicillin-binding protein

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Introduction

Streptococcus pneumoniae is a major pathogen causing community-acquired infection, including respiratory tract infections, acute otitis media, septicemia, and meningitis. This agent remains a leading cause of morbidity and mortality worldwide, especially among children and the elderly [1, 2].

In particular, penicillin (PEN)-resistant *S. pneumoniae* (PRSP) emerged in the 1980s and spread rapidly to many countries, posing several difficult clinical problems.

In Japan, meningitis caused by this PRSP in children was first reported by Arimasu et al. [3] in 1988. According to an active Nationwide Surveillance for Bacterial Meningitis (NSBM) program organized by Sunakawa and Ubukata et al. [4], which has been operating since 1999, the prevalence of PEN-intermediate resistant *S. pneumoniae* (PISP) and PRSP is frequent, accounting for 43.1 and 39.7% of *S. pneumoniae* isolates from meningitis patients, respectively.

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Resistance to β-lactam antibiotics in PISP and PRSP is mediated mainly by abnormalities in 3 *pbp* genes encoding the PEN-binding proteins (PBP) 1A [5], PBP2X [6], and PBP2B [7], which are enzymes involved in peptideglycan synthesis. PCR-based genotypic identification to demonstrate the presence or absence of abnormalities in the 3 *pbp* genes was designed by Ubukata et al. [8] in order to rapidly and accurately identify these sites of resistance in *S. pneumoniae* isolates. PCR results, designated by expressions such as gPRSP (*pbp1a* + *2x* + *2b*) and gPISP (*pbp2x*), correspond well to the susceptibilities determined by bioassay [9].

Serotyping using antiserum raised against polysaccharide capsule, a virulence factor of *S. pneumoniae*, has received emphasis in worldwide epidemiologic studies concerning this agent [10, 11]. Heptavalent pneumococcal conjugate vaccine (7-PCV) or 13-valent PCV (13-PCV) has been developed based on a great abundance of capsule-type data collected worldwide [12, 13]. However, information about pneumococcal serotypes is less than fully informative concerning individual clones causing invasive illnesses, because a given serotype includes some clones representing horizontal transfer of capsular genes into newly identified lineages [14].

To clarify the clonality of *S. pneumoniae* strains showing the same capsule type that were isolated in different areas and countries in recent years, multilocus sequence typing (MLST) that determines 7 allelic genes was developed [15]. In Japan, Imai et al. [16] first described MLST data relating to pneumococcal isolates from adults with community-acquired pneumonia. However, similar data for isolates from pediatric-age invasive pneumococcal infections have been lacking in Japan.

We therefore applied MLST analytic approaches to *S. pneumoniae* isolates from children with meningitis from 2007 through 2009 to identify linkages between serotypes, genotypic resistance types, and MLST data.

Materials and methods

Strains and serotyping

The active program for NSBM (research representative: Prof. K. Sunakawa, Kitasato University) has been carried out by participating pediatricians since 2000.

Bacterial strains first isolated from cerebrospinal fluid (CSF) were sent to the Laboratory of Molecular Epidemiology for Infectious Agents (Graduate School of Infection Control Sciences, Kitasato University), together with anonymous information concerning patient characteristics provided by attending pediatricians.

Pneumococcal strains ($n = 115$) isolated from the CSF of patients 15 years old or younger at 70 participating hospitals from January 2007 through December 2009 were included in this study. These hospitals were located in 6 areas of Hokkaido-Tohoku, Kanto, Chubu, Kinki, Chugoku-Shikoku, and Kyushu. Strains were immediately grown on sheep blood agar (Nippon Becton–Dickinson, Tokyo, Japan) at 37°C in an atmosphere with 5% CO_2. After single-colony purification and recultivation, isolates were stored in 10% skim milk (Difco Laboratories, Detroit, MI, USA) at −80°C until use. The pneumococcal species from all subjects were confirmed by PCR as described previously, based on amplification of the *lytA* gene encoding the autolysin enzyme specific to *S. pneumoniae* [17].

After speciation, we determined serotypes of *S. pneumoniae* strains with the Quellung reaction, using antiserum purchased from the Statens Serum Institute (Copenhagen, Denmark).

Identification of genotypic classes based on PBP alterations

A single colony of *S. pneumoniae* grown on a sheep blood agar plate was suspended in a microcentrifuge tube (0.5 ml) containing 30 μl of lysis solution [18]. To prepare template DNA solution, the tube was placed in a thermal cycler (Gene Amp PCR System 9600 R; PerkinElmer Cetus, Waltham, MA, USA), heat-treated for 10 min at 60°C, and then for 5 min at 94°C. Each primer set for the detection of the 3 targeted PBP genes was designed to amplify normal *pbp1a*, *pbp2x*, and *pbp2b* that existed among PEN-susceptible strains [4, 17].

Next, we added the 2 μl template DNA to each of 4 tubes containing 30 μl of PCR reaction mixture. Each reaction tube for real-time PCR contained a specific molecular beacon probe and primer set to detect each of the genes *pbp1a*, *pbp2x*, and *pbp2b* (abstr. no. 2074, Chiba et al.; 50th Interscience Conference on Antimicrobial Agents and Chemotherapy, Boston, USA, 2010).

Cycling conditions for real-time PCR consisted of 40 cycles at 95°C for 15 s, 50°C for 20 s, and 75°C for 15 s using Stratagene Mx3000P (Agilent Technologies, La Jolla, CA, USA). Based on the presence of all 3 DNA fragments corresponding to *pbp1a*, *pbp2x*, and *pbp2b*, the genotype was assigned to the PEN-susceptible class. When any of the targeted DNA fragments were absent, we assigned the strain to a PEN-nonsusceptible class, since it had sequence(s) at variance with those in PSSP. Six genotypic classes were indicated by adding "g" to the designation as follows: gPSSP, gPISP (*pbp2x*), gPISP (*pbp2b*), gPISP (*pbp1a* + *2x*), gPISP (*pbp2x* + *2b*), and gPRSP (*pbp1a* + *2x* + *2b*) [19].

MLST and analysis of clonal complexes (CCs) using the eBURST database

MLST of all strains was performed using methods described by Enright et al. [20]. The same template DNA used

for the genotypic identification of PEN resistance was subjected to MLST.

Internal fragments of 7 housekeeping genes were analyzed: *aroE* (shikimate dehydrogenase), *gdh* (glucose-6-phosphate dehydrogenase), *gki* (glucose kinase), *recP* (transketolase), *spi* (signal peptidase I), *xpt* (xanthine phosphoribosyltransferase), and *ddl* (D-alanine-D-alanine ligase). The corresponding 7 primer sets found on the MLST website and the CDC homepage (http://spneumoniae.mlst.net/misc/info.asp, http://www.cdc.gov/ncidod/biotech/strep/alt-MLST-primers.htm) were used. A *ddl* forward primer (5′-AGGATTCTTGGAAGTTTTGAAAATG-3′) was newly constructed for this study.

PCR was performed using Thermal Cycler Dice 600 (Takara, Kyoto, Japan) with cycling conditions that included an initial DNA denaturation step at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 50°C for 40 s, and 72°C for 1 min.

Amplified DNA fragments were purified using a QIA quick® 96 PCR Purification Kit (Qiagen, Valencia, CA, USA), and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit and an Applied Biosystems 3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Sequences obtained at each of the 7 loci then were compared with those of all known alleles at those loci using the database at the pneumococcal MLST website (http://spneumoniae.mlst.net). A sequence identical to a known sequence was assigned the corresponding allele number, and sequences that were not identical to any known allele sequence were assigned new allele numbers through the MLST website. The allelic profile of each strain as well as its ST designation was determined through the 7 constitutive numbers.

We also used eBURST to analyze the clonal association of strains with strains of identical serotype on the website (http://spneumoniae.mlst.net/eburst/). CCs were established by the eBURST sets, in which 6 of 7 identical allele numbers showed commonality with the 1 different number.

Results

Age distributions among children with pneumococcal meningitis

We analyzed 115 pneumococcal isolates in the CSF of Japanese children ranging in age from newborns to adolescents. CSF sampling dates ranged from January 2007 to December 2009. Boys accounted for 67 isolates (58.3%), while 39 isolates were from girls. The genders of the remaining patients were unspecified.

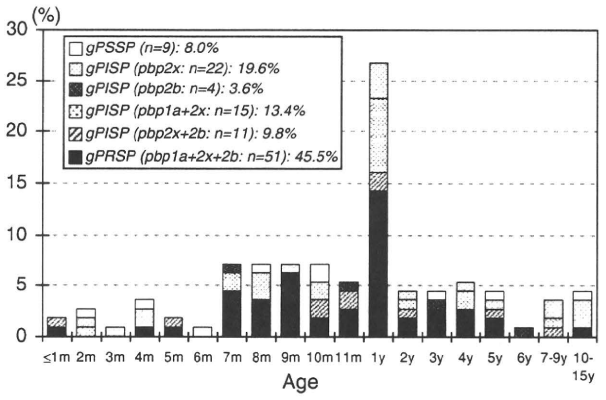

Fig. 1 Age distributions among children ($n = 112$) with pneumococcal meningitis and genotypic resistance classes among pneumococcal isolates. *m* month, *y* years

Figure 1 shows age distributions for the various genotypic resistance types among 112 children with meningitis, except for 3 patients whose ages were unclear. A number of patients were 6 months old or younger (11.6%). Isolation rates were high among infants from 7 to 11 months old (33.9%), and showed a peak (26.8%) among 1-year-old infants, with 72.3% of isolations occurring in infancy (1 year old or younger).

Genotypically resistant strains were predominantly gPRSP (*pbp1a* + *2x* + *2b*) (45.5% of all strains), followed by gPISP (*pbp2x*) (19.6%), gPISP (*pbp1a* + *2x*) (13.4%), gPISP (*pbp2x* + *2b*) (9.8%), and gPISP (*pbp2b*) (3.6%). Isolates of gPSSP with the 3 normal PBP genes accounted for only 8.0%. Importantly, gPRSP (*pbp1a* + *2x* + *2b*) strains were especially prevalent among patients 7 months old or older.

This chronology might reflect a decrease in immunoglobulins transferred from the mother to the newborn.

Serotype distributions and genotypic resistance classes

Figure 2 shows serotype prevalences for all *S. pneumoniae* isolates ($n = 115$) in decreasing order. Types represented numbered 24. Serotype 6B (20.9%, $n = 24$) was most prevalent, followed by 19F (14.8%, $n = 17$), 23F (12.2%, $n = 14$), and 14 (9.6%, $n = 11$). Serotypes 6A ($n = 5$) and 6C ($n = 6$) also were observed in our study. Coverage rates of 7-PCV and 13-PCV by age group were: 70.6 and 82.4% in infants (younger than 1 year old); 72.5 and 80.4% in preschool children (1–5 years old); and 20.0 and 30.0% in school children (older than 5 years old). Except for 6 strains (serotypes 6A, 23A and 35), we found that 88.2% of gPRSP was distributed among the 4 serotypes above, especially 19F, 6B, and 23F. Infrequent serotypes tended to be gPSSP, gPISP (*pbp2x*), or gPISP (*pbp1a* + *2x*).

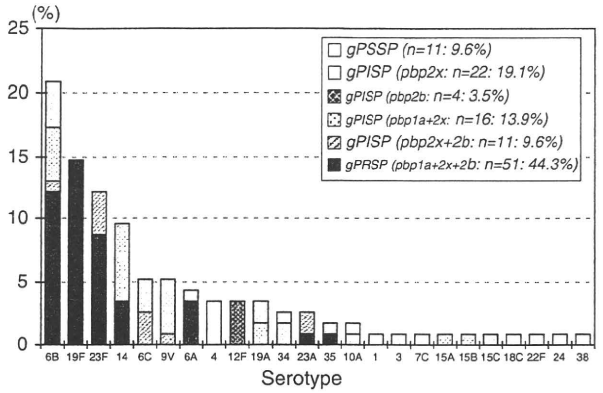

Fig. 2 Serotype distributions ($n = 115$) and genotypic resistance classes among pneumococcal strains

Association of serotype with sequence types (STs) and genotypes

Table 1 shows 52 STs identified by MLST performed on 115 pneumococcal strains, together with information concerning reference strains. We identified 8 novel allele sequences (*recP*148, *recP*149, *xpt*336, *xpt*337, *ddl*382, *ddl*383, *ddl*384, and *ddl*385). Thirty-four of the STs had already been assigned according to the MLST website, and the remaining ones were found to represent 18 new STs identified in our study. Eight ST profiles (STs 5231, 5238, 5239, 5242, 5230, 5234, 5241, and 5244) contained the new allele sequences, and others included novel allelic profiles (STs 5232, 5233, 5235, 5236, 5237, 5240, 5243, 5245, 5246, and 5247) consisting of known allele sequences.

Associations of isolation areas with serotypes and STs are indicated in Table 2. We confirmed that there are no significant distributions of serotypes and STs in Japan.

A correlation was evident between serotypes and STs, except for the 6 STs (STs 63, 199, 236, 338, 2923, and 2924) corresponding to the multiple serotypes in our observations (Tables 1, 2).

Additional eBURST analysis showed the presence of 29 CCs and 7 singletons. Relationships between STs and CCs for the main serotypes were as follows: 14 STs and 9 CCs in serotype 6B, 2 STs and 2 CCs in 19F, 6 STs and 4 CCs in serotype 23F, and 5 STs and 4 CCs in serotype 14 (Table 1).

The serotypes 6B, 6C, and 6A included 10 newly identified STs and a variety of both CCs and genotypic resistance types, suggesting that the 6B, 6C, and 6A serotypes might be susceptible to genetic alterations.

Discussion

Pneumococcal meningitis among young children and the elderly remains an important cause of morbidity and mortality in Japan [21]. Treatment of bacterial meningitis consists primarily of antimicrobial chemotherapy as recommended in the guidelines edited by the Japanese Society of Neurological Therapeutics, the Japanese Society for Neuroinfectious Diseases, and the Japanese Society of Neurology 2007 [22]. Recommended first-line antibiotics include a combination of third-generation cephalosporins and carbapenems for patients older than 4 months.

The aim of this work was an improved understanding of features such as serotypes, resistance genotypes, and STs among the pneumococcal strains most often isolated from children with meningitis. Although 93 serotypes exist among the strains, serotype 6B is most frequent, since strains of this serotype do not bear antigens corresponding to maternal antibodies transferred placentally to newborns.

In this study, we found that the serotypes 6B, 6C, and 6A included 10 newly identified STs and a variety of resistance genotypes and CCs compared with those associated with other serotypes. Our data suggest that strains with serotypes 6B, 6C, and 6A might be intrinsically susceptible to mutations and genetic recombination, considering that infant carriers with the serotypes 6B, 6C, and 6A seemed prevalent in the community. These genetic changes might be accelerated by selection pressures such as overuse of antibiotics and increased human mobility, reflecting progress in transportation.

In our surveillance study, all isolates recovered from cerebrospinal fluid were collected through the cooperation of pediatricians at collaborating medical institutions nationwide. Strains obtained and patient information would appear to be unbiased in terms of the specifics of *S. pneumoniae* and meningitis. We confirmed that serotypes 6B, 19F, 23F, and 14 were prevalent as causes of meningitis in Japanese children.

The Pneumococcal Molecular Epidemiology Network (PMEN) characterized 26 multidrug-resistant clones of *S. pneumoniae*, which were identified worldwide using MLST analysis (http://www.sph.emory.edu/PMEN/) [23]. All STs with allele profiles that were either similar or single- or double-locus variants within the PMEN clones represented the same serotypes, except for ST5241. Associations of serotypes with STs were also evident, except for the 6 STs (STs 63, 199, 236, 338, 2923, and 2924) corresponding to multiple serotypes in our study. In addition, multiple STs were included among the same serotypes (i.e., 6B, 23F, 14, 6A, 6C, and 19A), indicating the diversity of the genetic elements in the *S. pneumoniae* strains.

Comparison with the MLST database showed that most of the clones observed in our study possess allele profiles similar to reference clones obtained worldwide, especially in Asian and European countries. Figure 3 shows the worldwide distribution of ST90/ST2224/ST902 in the commonly seen serotype 6B, ST343 in serotype 14, ST236