Phylogenetic tree of *emm* genes

Fig. 4 shows the phylogeny of the 12 types of M protein in *S. dysgalactiae* subsp. *equisimilis* (n=25) isolated from the patients with invasive infections. A neighbour-joining tree was constructed for the M protein pattern using amino acid sequences corresponding to the complete M protein together with several M-like proteins in other previously analysed streptococcal species: seven strains of GAS, four strains of *S. dysgalactiae* subsp. *equisimilis* and one each of *S. dysgalactiae* subsp. *dysgalactiae*, *S. equi*, *S. iniae* and *S. equi* subsp. *zooepidemicus*.

Although M proteins have a hypervariable region at the N-terminal end, *S. dysgalactiae* subsp. *equisimilis* and GAS harbour extremely homologous M proteins compared with those of other *Streptococcus* species. The phylogenetic tree suggested that the M protein of *S. dysgalactiae* subsp. *equisimilis* was an orthologue of that of GAS.

We recently determined the complete genomic sequence of *S. dysgalactiae* subsp. *equisimilis* GGS_124 (*stG480.0*) isolated from patients with STSS (GenBank accession no. AP010935). The genome size was 2.1 Mbp, and sequence coverage with GAS genomes (Ferretti *et al.*, 2001) was 61–63 % identity. Interestingly, many genes encoding virulence factors in GAS were identified in *S. dysgalactiae* subsp. *equisimilis*. The occurrence of serious infections caused by *S. dysgalactiae* subsp. *equisimilis* in elderly persons with underlying diseases is likely to involve both compromised host defences and GAS-like virulence factors. However, it is unknown how this micro-organism invades deep tissues and vessels. Further investigation is needed to clarify this issue.

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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 *pbp* 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]		Univariate analysis	
	Non-survivors ($n=43$)	Survivors ($n=147$)	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]		
$\geqslant15$ 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

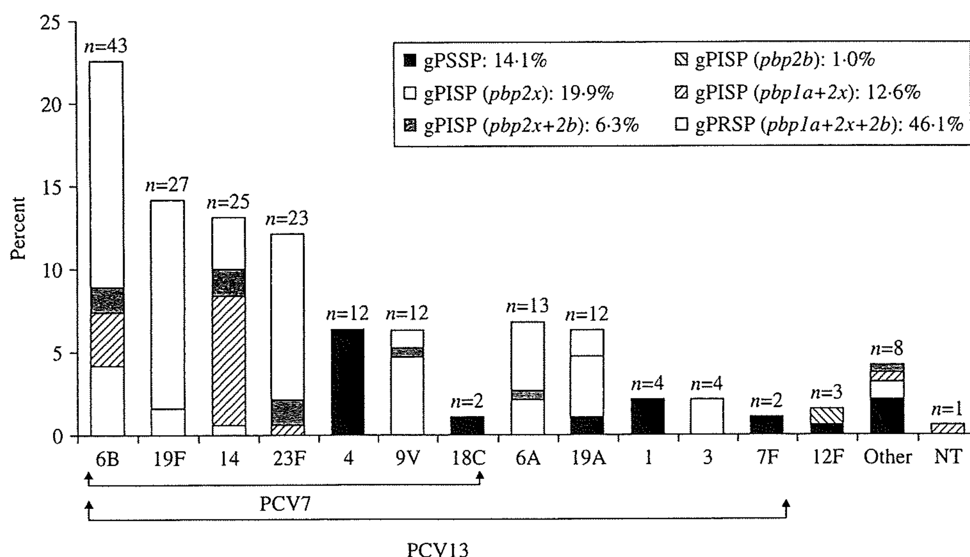

Fig. 1. Serotype distribution and resistance genes identified by PCR in *S. pneumoniae* isolated from children. 'Other' category includes serotypes 15B, 23A, 8, 24, 34, 35, and 38.

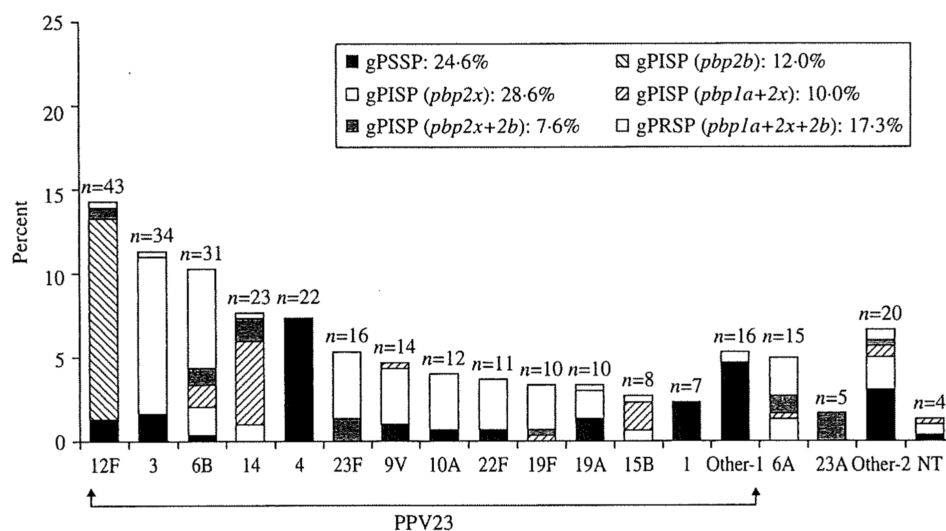

Fig. 2. Serotype distribution and resistance genes identified by PCR in *S. pneumoniae* isolated from adults. 'Other-1' category includes serotypes 9N, 11A, 33, 18C, 20, 2, 7F, 8. 'Other-2' category includes serotypes 35, 7C, 15A, 38, 15C, 31, 16, and 36.

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

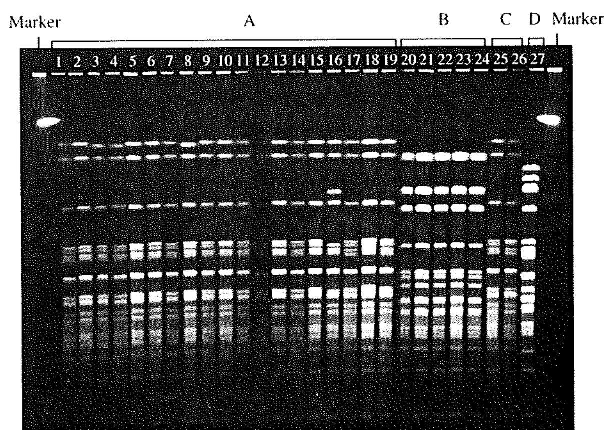

Fig. 3. PFGE patterns of *ApaI* digests of chromosomal DNA from serotype 12F isolates. A, gPISP (*pbp2b*) (lanes 1–19); B, gPSSP (lanes 20–24); C, gPISP (*pbp2x+2b*) (lanes 25, 26); D, gPRSP (*pbp1a+2x+2b*) (lane 27).

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 $P<0·0001$).

PFGE pattern of strains serotyped 12F

Figure 3 shows PFGE patterns of *ApaI* 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 multi-drug-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.

ACKNOWLEDGEMENTS

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DECLARATION OF INTEREST

None.

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特集　世界標準にはるかに及ばないわが国の予防接種体制

肺炎球菌ワクチン

生　方　公　子

別　刷

日本医師会雑誌

第138巻・第4号

平成21（2009）年7月

肺炎球菌ワクチン

生方公子

キーワード　肺炎球菌　7価コンジュゲートワクチン　23価ポリサッカライドワクチン　薬剤耐性

はじめに

肺炎球菌は，小児，あるいは成人が市中において罹患する呼吸器系感染症の起炎菌として最も検出頻度の高い重要な菌である．抗菌薬が発達した今日においても，本菌によって肺炎や敗血症，あるいは化膿性髄膜炎等を発症すると，しばしば急速な臨床経過をたどり，重篤な後遺症や致命的な結果となる場合もある．また，ペニシリン系薬やセフェム系薬に耐性を示す菌，すなわちペニシリン耐性肺炎球菌（penicillin-resistant *Streptococcus pneumoniae*；PRSP）が欧米と同様に増加している点も見逃せない[1,2]．

ここでは，肺炎球菌ワクチンとして主として成人が対象となっている23価ポリサッカライドワクチン（23-valent pneumococcal polysaccharide vaccine；PPV23），小児用として導入が期待されている7価コンジュゲートワクチン（7-valent pneumococcal conjugate vaccine；PCV7）［Prev(e)nar®］と13価コンジュゲートワクチン（PCV13）のカバー率について，筆者らが最近1年間に全国各地から収集した侵襲性感染症由来の肺炎球菌を基に述べる．

I．発症年齢と肺炎球菌の耐性化

図1には，肺炎球菌性侵襲性感染症に罹患した症例の年齢分布と，耐性遺伝子解析に基づく収集菌株（$n=496$）のペニシリン系薬耐性化状況を示す．小児では敗血症が最も多く，次いで肺炎や化膿性髄膜炎であったが，そのほかに感染性心内膜炎や蜂窩織炎例も少数認められている．また，発症例は1歳までが63％を占め，大半が4歳までに集中している．それに対し，成人では肺炎が最も多く，次いで敗血症や化膿性髄膜炎，そのほかに膿胸，化膿性関節炎，骨髄炎といったさまざまな疾患が認められている．30歳代でも発症例がみられ，年齢の上昇と共に症例数は次第に増加し，70歳代にピークが認められる．近年注目されるのは，後述するように成人例が増加してきていることである．

図1中の棒グラフには耐性化状況も併せて示してあるが，小児と成人ではPRSPの割合が明らかに異なっている．すなわち，小児ではPRSPが45％，ペニシリン軽度耐性菌（PISP）が40％を占め，ペニシリン感性菌（PSSP）はわずか14％しか分離されていない．しかし成人由来株ではPRSPは18％と少なく，PISPが58％，そしてPSSPは24％の割合となっている．

また，肺炎球菌全体の80％はマクロライド系薬に耐性であり，ニューキノロン系薬にも耐性菌が出現しはじめている．

Pneumococcal Conjugate Vaccine
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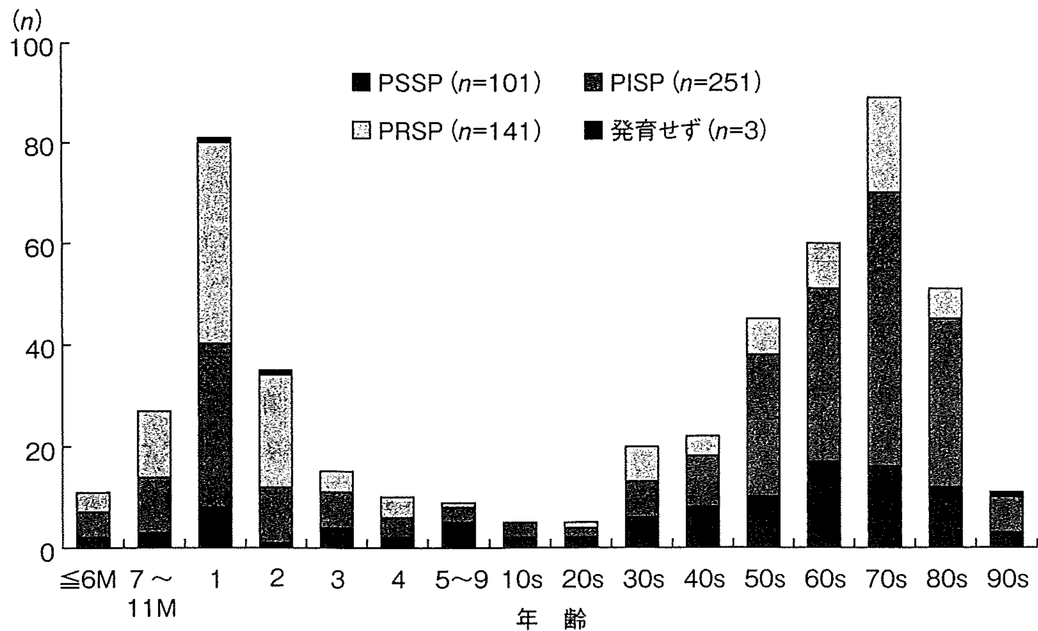


図1　肺炎球菌性侵襲性感染症例の年齢分布と分離株のペニシリン系薬耐性化状況（収集期間：2006年8月～2007年7月，n = 496株）

［生方公子：我が国における侵襲性感染症例の患者背景の解析と分離されたβ溶血性レンサ球菌と肺炎球菌の分子疫学解析．平成19年度厚生労働科学研究費補助金新興・再興感染症研究事業「新規に発生しているレンサ球菌による劇症型感染症の臨床的・細菌学的解析と診断・治療法に関する研究」（主任研究者：砂川慶介）総括研究報告書，平成20年3月；8-22より引用］

Ⅱ．侵襲性感染症発症例の背景と予後

肺炎球菌感染症やレンサ球菌感染症は，抗菌薬が潤沢でなかった時代には急速に重篤化する感染症として恐れられたが，多くの抗菌薬が使用できる今日にあって，これらの感染症が再興感染症の1つとして注目されている[3]．疫学研究で集積された成人例では，28％が救命救急を含む時間外受診例であり，病態の急速な進行が窺える．

その背景として，①わが国では急速に高齢社会を迎え，加齢と共に抗体が減弱してきている可能性，②交通網の発達による人々の交流により，日本人が抗体を獲得していない新たな型の菌が持ち込まれ拡散しつつある可能性，加えて，③基礎疾患を有する易感染状態予備軍の増加等，さまざまな要因が複雑に絡んでいると指摘されている．

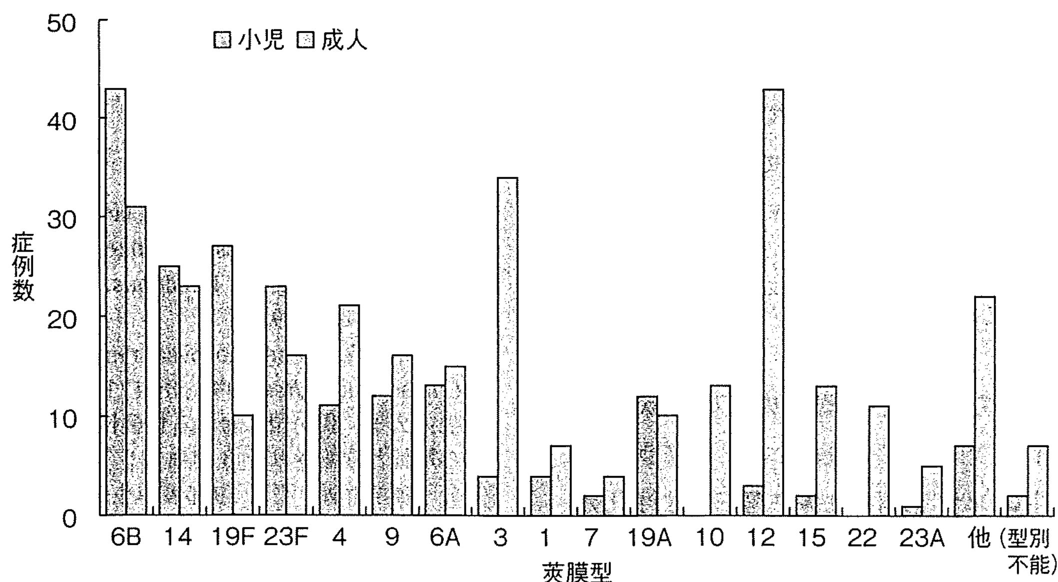
成人発症例に対する調査でも75％が悪性腫瘍の術後，糖尿病，心疾患，肝胆膵疾患，腎疾患，脾臓摘出例であった．ちなみに，成人例に占める死亡率は19.9％，後遺症を残した例は7.8％で，小児の1.4％および2.7％に比して明らかに予後不良である．

Ⅲ．菌の莢膜型とワクチンカバー率

1．小児

肺炎球菌の病原因子としては種々の菌体成分が知られているが，ワクチン抗原として用いられているのは，そのなかの菌体表層に存在する多糖体でできた莢膜で，90種類の型に分類されている[4]．生体内へ侵入した肺炎球菌では，莢膜を有しているためにオプソニゼーションにかかわる補体の活性化が阻害され，菌は多核白血球による貪食作用から逃れて病原性が発揮できる．

ちなみに，小児用に開発されたPCV7は，多糖体に無毒化したジフテリアトキシンCRM_{197}（免疫学的輸送蛋白）を結合させ，防御免疫応答



PCV7 : 4, 6B, 9V, 14, 18C, 19F, 23F
PCV13 : 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F
PPV23 : 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F

図2　侵襲性感染症由来肺炎球菌の莢膜型（$n = 492$）

［生方公子：我が国における侵襲性感染症例の患者背景の解析と分離された β 溶血性レンサ球菌と肺炎球菌の分子疫学解析．平成19年度厚生労働科学研究費補助金新興・再興感染症研究事業「新規に発生しているレンサ球菌による劇症型感染症の臨床的・細菌学的解析と診断・治療法に関する研究」（主任研究者：砂川慶介）総括研究報告書，平成20年3月；8-22より引用］

が生じやすくなっている．PCV7は，欧米においては2000年から導入され，侵襲性感染症が劇的に減少したと報告されている[5,6]が，ワクチンに含まれない型の肺炎球菌感染症が増加しているとの報告も散見され[7]，現在さらに多くの型を含むPCV13が米国およびEUにおいて申請中である．

図2は，小児由来株で比較的分離頻度の高い莢膜型から並べ，成人のそれと比較している．6B型が圧倒的に多く，次いで19F，14，23F型と，6A，9，19A，4型も少なからず分離されている．ちなみに4型を除くこれらの型の菌にはPRSPやPISPが多いのも特徴である．PCV7（4，6B，9V，14，18C，19F，23F型抗原を含む）のカバー率は73.8％と計算される．しかし，急性中耳炎で多い3型や化膿性髄膜炎で比較的多い6A型がカバーされていない．PCV13（1，3，5，6A，7F，19A型を追加）ではそれらがカバーされ，カバー率は92.1％と高まる．ちなみに，わが国においては現在PCV7が審査中とのことである．

そのほかに，インフルエンザ菌の表層リポ蛋白Dを担体として莢膜多糖体に結合させ，インフルエンザ菌に対する抗体獲得も同時に狙った11価ワクチンも開発中である．

2．成人

成人由来株の莢膜型は，図2からも明らかなように小児由来株とは著しく異なる．最も多いのは12型で，その大半がPISPである．次いで，やはりPISPの多い3，6B，14型の頻度が高いが，さまざまな型の肺炎球菌によって発症している点が特徴である．

成人に使用可能なワクチンはPPV23であるが，基礎疾患などのリスクファクターを有する2歳以上の小児には，すでに任意で接種が勧められている．PPV23は，1，2，3，4，5，6B，7F，8，9N，9V，10A，11A，12F，14，15B，17F，18C，19A，19F，20，22F，23F，33Fの莢膜抗原を含むが，成人由来の菌に対するカバー率は83.7％である．多糖体のみの精製物

であるため抗原性が低く，抗体価が上がりにくいといわれる．接種後の重症感染症発症の防御率は50～80％といわれ，米国では5年後の再接種が認められているが，日本では残念ながら認められていない．

まとめ

肺炎球菌に限らず，呼吸器感染症の起炎菌として重要なインフルエンザ菌，マイコプラズマ菌，β溶血性レンサ球菌において薬剤耐性化が急速に進行している．

感染症の予防に重点をおく欧米では，細菌に対するワクチン開発にも積極的である．Hib ワクチン，肺炎球菌ワクチンに加え，周産期感染症として重要なB群溶血性レンサ球菌（GBS）に対するワクチンも開発中である．

なお，PCV7 接種後の副反応については，わが国では未認可なので欧米の情報しか得られないが，注射個所の発赤と腫脹，発熱などが記載されているものの，重篤な副反応はみられていない．

肺炎球菌感染症は医療負担が大きいことや，重篤な後遺症を残した場合の社会的リスクを考えると，少子化や保育園児の増加，あるいは高齢者の増加等，急速な社会変化を眼前にして，わが国においても肺炎球菌ワクチンの定期接種化が強く望まれる．

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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].

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.

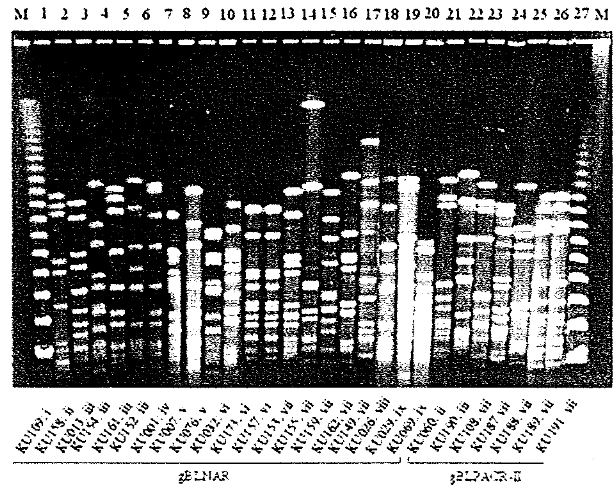

Fig. 1 Pulsed-field gel electrophoresis (PFGE) profiles of chromosomal DNA from gLow-β-lactamase-nonproducing, ampicillin-resistant (*BLNAR*), *gBLNAR*, and g β-lactamase-producing, amoxicillin-clavulanic acid-resistant II (*gBLPACR II*) strains ($n = 27$) digested with *Sma*I restriction enzyme ('g' 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)