acid motifs Ser379-Ser-Asn (SSN) and Lys512-Thr-Gly (KTG) are responsible for β-lactam resistance [8–12]. A strain possessing a single amino acid substitution of Asn526Lys or Arg517His is termed genetically (g) β-lactamase-nonproducing AMP-intermediate-resistant (gLow-BLNAR) *H. influenzae*. A strain possessing amino acid substitutions of Met377Ile, Ser385Thr, and/or Leu 389Phe in addition to Asn526Lys or Arg517His is termed gBLNAR. A strain showing both β-lactamase production and PBP3 substitutions is termed β-lactamase-producing amoxicillin-clavulanic-acid-resistant (gBLPACR-I or gBLPACR-II) *H. influenzae*.

Genetically identified gBLNAR strains are difficult to distinguish from susceptible strains based on antibiotic susceptibility because some gBLNAR strains show MICs indicating susceptibility to β-lactam antibiotics. Nonetheless, these strains are potentially resistant to β-lactam antibiotics. To avoid missing these gBLNAR strains, we constructed a conventional polymerase chain reaction (PCR) procedure to identify resistance genes and confirm species and b serotype in *H. influenzae* simultaneously [10]. This method was a little laborious and time consuming, taking about 3 h to obtain a result because it required gel electrophoresis.

For easier and more rapid detection, we recently constructed a new real-time PCR method using cycling probes to detect amino acid substitutions in the *ftsI* gene that affect β-lactam resistance in *H. influenzae* in addition to the 16S ribosomal RNA (rRNA) gene specific to *H. influenzae*, the *capB* gene encoding the serotype b capsule, and the bla_{TEM} gene encoding TEM-1 β-lactamase.

Here we compare results obtained by real-time PCR with those determined by the conventional PCR. We also evaluated the correlation between combinations of amino acid substitutions in the *ftsI* gene detected by real-time PCR and minimum inhibitory concentrations (MICs) of β-lactam antibiotics.

Materials and methods

Strains and clinical samples

The 206 clinical *H. influenzae* strains used in this study were isolated from pediatric patients with meningitis in 106 Japanese medical institutions between January 2007 and December 2008. Pediatricians requesting full rapid genetic analysis of these isolates provided a copy of the informed consent form completed by each patient's family. Bacteria adherent to sterile swabs were cultured using routine methods on chocolate II agar plates (Nippon Becton–Dickinson, Tokyo, Japan) at 37°C in a 5% carbon dioxide (CO_2) atmosphere.

DNA preparation

A single colony of *H. influenzae* grown on a chocolate II agar plate was suspended in a 0.5-ml microcentrifuge tube containing 30 μl of lysis solution. Composition of the lysis solution was reported previously [13]. The tubes were incubated at 60°C for 10 min and then at 90°C in a thermal cycler (Gene Amp PCR System 9600-R, Perkin-Elmer, Norwalk, CT, USA) for 5 min to lyse the cells, and 2μl of bacterial lysate were used for each PCR.

Conventional PCR

Conventional PCR was performed for *H. influenzae* strains using six sets of primers described previously [10]. The six targets were the *p6* gene encoding P6 membrane protein, identifying the species [14]; the bla_{TEM} gene encoding TEM-1 β-lactamase [15]; the bla_{ROB} gene encoding ROB-1 β-lactamase [16]; the amino acid substitution Asn526Lys in PBP3 encoded by the *ftsI* gene [17]; the amino acid substitution Ser385Thr in PBP3 [8]; and the Hib-specific *capB* locus [18]. Conditions for PCR were 35 cycles at 94°C for 15 s; at 53°C for 15 s; and at 72°C for 15 s.

Real-time PCR

The five sets of primers and probes used in this study are listed in Table 1. Although we sought to construct a real-time PCR procedure targeting the amino acid substitution of Arg517His, we did not succeed because of nonspecific reactions. Amplification and simultaneous cleavage of RNase H were carried out in a total volume of 25 μl. The reaction mixture included 1× CycleavePCR buffer, with final concentrations of 3 mM for Mg^{2+}, 0.3 mM for each deoxynucleoside triphosphate, 0.2 μM of each PCR primer (forward and reverse), 0.2 μM of each probe that labeled with carboxyfluorescein (FAM) or carboxy-X-rhodamine (ROX), 100 U of *Tli* RNase HII, and 1.25 U of Ex *Taq* HS (TaKaRa Bio, Shiga, Japan). Two microliters of sample DNA were added to the reaction mixture as a DNA template. DNA amplification and fluorescence detection were performed using a TP800 thermal cycler Dice real-time PCR system (TaKaRa Bio). Amplification was initiated by incubation at 95°C for 10 s, followed by 40 PCR cycles at 95°C for 5 s, at 55°C for 15 s, and then 72°C for 20 s. Resistance class was determined based on the combination of genes detected, as shown in Table 2.

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

Table 1 Primers and probes for real-time polymerase chain reaction (PCR)

Target gene	Primers and probes	Sequence	Amplicon size (bp)
16S rRNA	16S rRNA-forward primer	5′-TTGACATCCTAAGAAGAGCTC-3′	167
	16S rRNA-reverse primer	5′-TCTCCTTTGAGTTCCCGACCG-3′	
	16S rRNA-probe[b]	5′-(FAM[c])-CCAAC***A***TTTCACA-(Eclipse[d])-3′	
capB	*capB*-forward primer	5′-GCGAGGCTATACACAAGATAC-3′	245
	capB-reverse primer	5′-CTCGGTGATTAATAATTGGT-3′	
	capB-probe[b]	5′-(ROX[c])-GCGAAC***A***GAT-(Eclipse[d])-3′	
bla$_{TEM-1}$	*bla*$_{TEM-1}$-forward primer	5′-GCGATCTGTCTATTTCGTTC-3′	112
	bla$_{TEM-1}$-reverse primer	5′-GGTCTCGCGGTATCATTG-3′	
	bla$_{TEM-1}$-probe[b]	5′-(ROX[c])-GGGAGG***G***CT-(Eclipse[d])-3′	
Asn526Lys[a]	Asn526Lys-forward primer	5′-TAAACGCGCAATGGTGGAAG-3′	142
	Asn526Lys-reverse primer	5′-CACTAAAGCATAACGAGGGTC-3′	
	Asn526Lys-probe[b]	5′-(FAM[c])-AA***G***AAATATGTGG-(Eclipse[d])-3′	
Ser385Thr[a]	Ser385Thr-forward primer	5′-AAATTGTGGACGTTGCACCT-3′	204
	Ser385Thr-reverse primer	5′-AGCGTTTACGGTTTGCGTTC-3′	
	Ser385Thr-probe[b]	5′-(FAM[c])-TGGTGTG***A***CT-(Eclipse[d])-3′	

[a] Gene encoding the amino acid substitution Ser385Thr or Asn526Lys in the *ftsI* gene

[b] Fluorescent dye- and quencher-labeled DNA-RNA chimeric probe. The boldface italic letter in the sequence of these probes indicates the nucleotide replaced by RNA

[c] Fluorescent molecules: carboxyfluorescein (FAM) and carboxy-X-rhodamine (ROX)

[d] Quenching molecules

Table 2 Correlation between resistance class and gene mutations

Resistance classes[a]	Gene[b]				
	16S rRNA	*capB*	*bla*$_{TEM-1}$	Asn526Lys[c]	Ser385Thr[c]
gBLNAS	+[d]	+ or −	−[e]	−	−
gLow-BLNAR	+	+ or −	−	+	−
gBLNAR	+	+ or −	−	+ or −	+
gBLPAR	+	+ or −	+	−	−
gBLPACR-I	+	+ or −	+	+	−
gBLPACR-II	+	+ or −	+	+ or −	+

[a] See text for definitions

[b] Detected by real-time polymerase chain reaction

[c] Alteration in the *ftsI* gene encoding the amino acid substitution Ser385Thr or Asn526Lys

[d] Positive

[e] Negative

sense primer 5′-GTTGCACATATCTCCGATGAG-3′ and reverse primer 5′-CAGCTGCTTCAGCATCTTGC-3′, as described previously [8]. Amplified DNA fragments were purified with a QIAquick PCR purification kit (Qiagen, Tokyo, Japan) and used as templates. Sequencing reactions were carried out with 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).

Antibiotic susceptibility

Susceptibility testing was performed using an agar dilution method [19]. Antibiotics used in this study were AMP, cefditoren (CDN), and tebipenem (TBM; Meiji Seika Kaisha, Tokyo, Japan); cefotaxime (CTX; Aventis Pharma, Tokyo, Japan); ceftriaxone (CRO; Chugai Pharmaceutical, Tokyo, Japan), and meropenem (MEM; Dainippon Sumitomo Pharma, Osaka, Japan). *H. influenzae* ATCC 49247 and ATCC 49766 were used as quality control strains.

Results

Sensitivity and specificity of real-time PCR compared with those of conventional PCR

Sensitivities and specificities of the real-time PCR assay for 206 *H. influenzae* strains were compared with results of conventional PCR (Table 3). Relative sensitivities of real-time PCR were 90.5% for gLow-BLNAR, 94.3% for gBLNAR, and 100% for the remaining four resistance classes. Similarly, specificities of real-time PCR were 97.7% for β-lactamase-nonproducing ampicillin-susceptible (gBLNAS), 96.3% for gLow-BLNAR, and 100% for the remaining four resistance classes, including gBLNAR. Of all *H. influenzae* strains, 201 (97.6%) were identified as Hib by real-time PCR. Sensitivities and specificities of real-time PCR for Hib were 100%.

Sensitivity and specificity of real-time PCR compared with those of sequencing

As shown in Table 4, sensitivities and specificities of the real-time PCR assay for all subjects were determined comparing these results with *ftsI* gene sequencing. Sequencing results for only amino acid substitutions Asn526Lys and Ser385Thr are shown in the table. Relative sensitivities of real-time PCR were 97.4% for Asn526Lys and 95.8% for Ser385Thr. Specificities of real-time PCR were 100% for both of the amino acid substitutions.

In four strains in which Asn526Lys was not detected by real-time PCR, sequencing results showed that they possessed this amino acid substitution in the *ftsI* gene. The reason real-time PCR failed to detect this amino acid substitution was the presence of a 1-bp discrepancy in the probed region from the Asn526Lys-probe sequence. Accordingly, fluorescence was not produced by the Asn526Lys-probe in these four strains. Similarly, six strains in which Ser385Thr was not detected by real-time PCR possessed this amino acid substitution in the *ftsI* gene. A 1-bp discrepancy in the probed region from the Ser385Thr-probe sequence was present in these six strains.

Correlation between amino acid substitutions detected by real-time PCR and MICs

Table 5 shows the correlation of amino acid substitutions detected by real-time PCR with MICs. MIC_{90}s of AMP for strains showing Asn526Lys, Ser385Thr, and both Asn526Lys and Ser385Thr were two, four, and eight times higher than those for strains not showing both these amino acid substitutions. Similarly, MIC_{90}s of CTX, CRO, and CDN for strains showing Asn526Lys, Ser385Thr, and both Asn526Lys and Ser385Thr, respectively, were two, 32, and 32 times; two, 32, and 32 times; and two, 16, and 16 times higher than those for strains not showing either amino acid substitution. Combinations of amino acid substitutions detected by real-time PCR corresponded to MICs of AMP

Table 3 Sensitivities and specificities of real-time polymerase chain reaction (PCR) compared with those of conventional PCR

Resistance classes[a]	Real-time PCR results	No. (%) of strains showing results similar to conventional		Total no. of strains
		Positive	Negative	
gBLNAS	Positive	30 (100.0)[b]	4 (2.3)	34
	Negative	0 (0.0)	172 (97.7)[c]	172
	Total	30	176	206
gLow-BLNAR	Positive	38 (90.5)[b]	6 (3.7)	44
	Negative	4 (9.5)	158 (96.3)[c]	162
	Total	42	164	206
gBLNAR	Positive	100 (94.3)[b]	0 (0.0)	100
	Negative	6 (5.7)	100 (100.0)[c]	106
	Total	106	100	206
gBLPAR	Positive	7 (100.0)[b]	0 (0.0)	7
	Negative	0 (0.0)	199 (100.0)[c]	199
	Total	7	199	206
gBLPACR I	Positive	7 (100.0)[b]	0 (0.0)	7
	Negative	0 (0.0)	199 (100.0)[c]	199
	Total	7	199	206
gBLPACR II	Positive	14 (100.0)[b]	0 (0.0)	14
	Negative	0 (0.0)	192 (100.0)[c]	192
	Total	14	192	206

[a] See text for definitions

[b] Sensitivity

[c] Specificity

Table 4 Correlation between results of real-time polymerase chain reaction (PCR) and those of sequencing

Gene	Real-time PCR results	No. (%) of strains also showing sequencing results		Total no. of strains
		Positive	Negative	
Asn526Lys[a]	Positive	151 (97.4)[b]	0 (0.0)	151
	Negative	4 (2.6)[d]	51 (100.0)[c]	55
	Total	155	51	206
Ser385Thr[a]	Positive	114 (95.8)[b]	0 (0.0)	114
	Negative	6 (5.0)[e]	86 (100.0)[c]	92
	Total	120	86	206

[a] Gene encoding the amino acid substitution Ser385Thr or Asn526Lys in the *ftsI* gene

[b] Sensitivity

[c] Specificity

[d] These strains showed a sequence of AAGAA(G)TATGTGG in the Asn526Lys-probe region. The letter in parenthesis represents a 1-bp discrepancy with the Asn526Lys-probe sequence

[e] These strains showed a sequence of TGGTGT(A)ACT in the Ser385Thr-probe region. The letter in parenthesis represents a 1-bp discrepancy with the Ser385Thr-probe sequence

and the cephalosporins. On the other hand, the combinations of amino acid substitutions did not correspond to MICs of MEM and TBM.

Discussion

In 2001, Ubukata et al. [8] first reported that BLNAR strains had altered PBP3 caused by the amino acid substitutions. 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 affected MICs of β-lactam antibiotics. Eighty to ninety percent of gBLNAR strains also were reported to possess the amino acid substitutions Ser385Thr and Asn526Lys, and the remaining strains, possessed Ser385Thr and Arg517His [19–21].

Considering the associations described above, we sought to construct a real-time PCR procedure targeting each of the three amino acid substitutions Ser385Thr, Asn526Lys, and Arg517His. We succeeded in constructing new cycling probes and primers targeting each of Ser385Thr and Asn526Lys but encountered difficulties with Arg517His because of nonspecific reactions. Although this real-time PCR could not reliably detect Arg517His, it showed high sensitivities (90.5–100%) and specificities (96.3–100%) for all resistance classes according to the much slower conventional PCR. The real-time PCR shortened time required from 3 h by conventional PCR to 1.5 h. Furthermore, relative sensitivities (97.4% and 95.8%) and specificities (100%) of the real-time PCR for Asn526Lys and Ser385Thr, respectively, comparing with sequencing results were high. The real-time PCR, however, failed to detect amino acid substitutions in several strains due to the presence of only 1-bp discrepancy in the probed region. It might be difficult to solve this problem because of the limitation on this method using cycling probes.

Individual amino acid substitutions in PBP3 described above affect MICs of the various β-lactam antibiotics in different ways [8, 20, 21]. MIC_{90}s of AMP for gLow-BLNAR strains with Asn526Lys, gBLNAR strains with Ser385Thr, and gBLNAR strains with both Asn526Lys and Ser385Thr were two, four, and eight times higher than those for gBLNAS strains, respectively. MIC_{90}s of cephalosporins including CTX for these strains were two, 16–32, and 16–32 times higher than for gBLNAS strains, respectively. Specifically, the amino acid substitution Ser385Thr affects MIC_{90}s of AMP and cephalosporins more than does Asn526Lys and more greatly affects these when present together with Asn526Lys. In the clinical field, CTX or CRO are often used together with MEM or carbapenems empirically to treat pediatric patients with meningitis. When causative pathogen of meningitis turned out to be Hib-BLNAR, pediatricians increase the dose of these antibiotics up to maximum within the permitted range. Recently, bactericidal effect of these antibiotics against BLNAR are extremely decreased, and thus protract meningitis caused by Hib-BLNAR have increased. Identifying Ser385Thr in gBLNAR strains therefore is highly clinically important. Thus, combinations of amino acid substitutions in the *ftsI* gene detected by real-time PCR correlated clearly with MICs of AMP and cephalosporins, permitting detailed prediction of MICs of β-lactam antibiotics.

One application of the new real-time PCR involved direct detection in a sample of cerebrospinal fluid. DNA preparation for the cerebrospinal fluid obtained from a 7-month-old male patient with meningitis was performed

Table 5 Correlation of combinations of amino acid substitutions in the *ftsI* gene with minimum inhibitory concentrations (MICs)

Resistance classes[a]	No. of strains	Genes[b]		Amino-acid substitution in the *ftsI* gene		MICs (μg/mL)											
						Ampicillin		Cefotaxime		Ceftriaxone		Cefditoren		Meropenem		Tebipenem	
		16S rRNA	bla_{TEM-1}	Asn526Lys[c]	Ser385Thr[c]	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
gBLNAS	34	+[d]	−[e]	−	−	0.25	0.5	0.008	0.031	0.004	0.008	0.008	0.016	0.031	0.063	0.063	0.125
gLow-BLNAR	44	+	−	+	−	1	1	0.031	0.063	0.016	0.016	0.031	0.031	0.125	0.25	0.25	0.5
gBLNAR	10	+	−	−	+	2	2	0.5	1	0.125	0.25	0.125	0.25	0.063	0.063	0.125	0.125
gBLNAR	90	+	−	+	+	2	4	0.5	1	0.25	0.25	0.25	0.25	0.125	0.25	0.25	0.5
gBLPAR	7	+	+	−	−	8	32	0.016	0.016	0.004	0.004	0.016	0.016	0.031	0.063	0.063	0.125
gBLPACR I	7	+	+	+	−	32	64	0.063	0.063	0.016	0.016	0.031	0.031	0.063	0.125	0.25	0.25
gBLPACR-II	4	+	+	−	+	16	32	0.125	0.25	0.031	0.125	0.031	0.063	0.031	0.031	0.063	0.063
gBLPACR-II	10	+	+	+	+	64	64	0.5	0.5	0.25	0.25	0.125	0.25	0.063	0.125	0.25	0.5

[a] See text for definitions

[b] Detected by real-time PCR

[c] Gene encoding amino acid substitution, Ser385Thr or Asn526Lys, in the *ftsI* gene

[d] Positive

[e] Negative

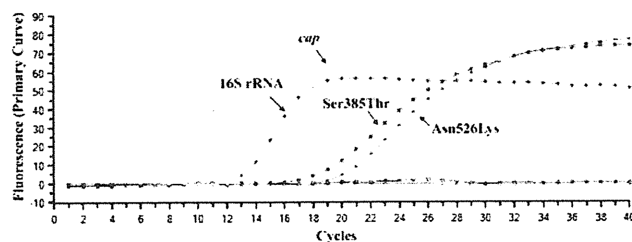

Fig. 1 Result of a real-time polymerase chain reaction (PCR) for a sample of cerebrospinal fluid obtained from a patient with meningitis. Four DNA amplifications representing the 16S rRNA gene, *cap* gene, Asn526Lys, and Ser385Thr are shown

as described previously [22]. Figure 1 shows the results of real-time PCR. Four DNA amplifications—the 16S rRNA gene, *cap* gene, Asn526Lys, and Ser385Thr—were observed. We determined with high accuracy that the etiologic agent in this patient was gBLNAR. Our real-time PCR thus proved highly valuable clinically.

In preventing severe infections caused by Hib and nontypable *H. influenzae* (NTHi), rapid detection of the causative pathogen at the onset is highly important in Japan, as is routine vaccination against Hib. Moreover, determination of resistance class using this rapid real-time PCR greatly facilitates evidence-based antibiotic use.

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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.

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.

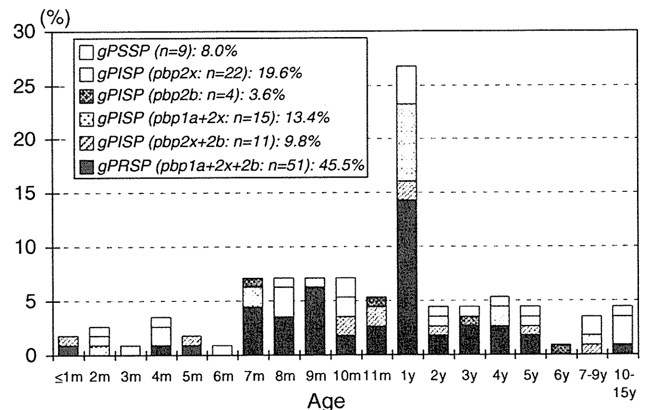

Fig. 1 Age distributions among children ($n = 112$) with pneumococcal meningitis and genotypic resistance classes among pneumococcal isolates. *m* month, *y* years

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*).

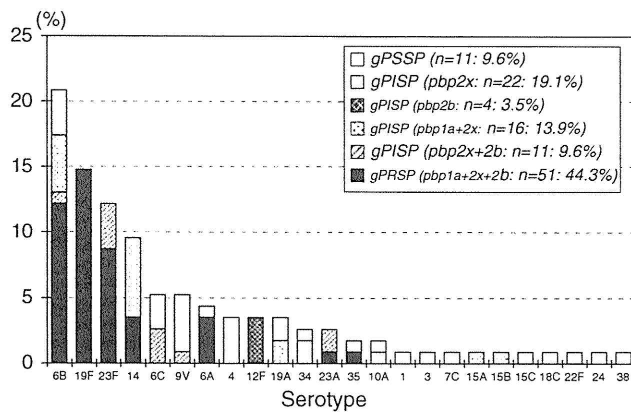

Fig. 2 Serotype distributions ($n = 115$) and genotypic resistance classes among pneumococcal strains

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

Table 1 Association of serotypes with multilocus sequence typing profiles and genetic *pbp* patterns among *S. pneumoniae* strains

Serotype	ST	CC	Genotype	No. of strains	Allele gene							First reported	
					aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*	Year	Country (city)
1	**5239**	306	gPSSP	1	12	8	13	5	16	**336**	20		
3	**5234**	180	*2x*	1	7	15	2	10	6	1	**383**		
4	246	246	gPSSP	4	16	13	4	5	6	10	18	1997	UK
6A	3115	3115	gPRSP	2	7	32	6	1	6	14	14	1989	Korea
	2756	3787	gPRSP	1	8	8	19	16	77	1	68	2004	China
	5243	3787	gPRSP	1	8	8	4	16	77	1	26		
	3787	3787	*2x*	1	8	8	19	16	6	1	68	UN	Singapore
6B	90	156	gPRSP	7	5	6	1	2	6	3	4	1986	Spain
	3387	156	gPRSP	1	5	6	1	2	6	3	26	2002	Korea
	2983	156	*2x*, *1a+2x*	3	5	6	1	2	6	1	271	2003	Japan (Okayama)
	902	490	gPRSP	1	2	13	2	1	6	121	121	2000	Singapore
	2923	490	*2x*, *1a+2x*	2	2	13	2	5	6	121	29	2003	Japan (Kurume)
	5233	490	*2x+2b*	1	2	13	2	5	6	121	14		
	5232	Singleton	gPRSP	1	2	29	4	1	6	121	121		
	5238	Singleton	gPRSP	1	2	5	2	**149**	6	121	121		
	5244	Singleton	gPRSP	1	2	168	2	5	6	121	**385**		
	5245	Singleton	*2x*	1	2	13	19	5	6	124	29		
	2224	2224	gPRSP, *1a+2x*	2	7	12	7	1	116	14	29	1996	UK
	5235	2224	*1a+2x*	1	7	12	7	1	116	20	29		
	5230	180	gPRSP	1	7	15	2	10	6	1	**382**		
6C	2924	2924	*2x*	1	1	5	2	6	6	1	14	2003	Japan (Hyogo)
	2924	2924	*2x*	1	1	5	2	6	6	1	14	2003	Japan (Hyogo)
	5247	156	*2x*	1	1	29	8	6	6	6	14		
	2923	490	*2x*	1	2	13	2	5	6	121	29	2003	Japan (Kurume)
	5241	Singleton	*2x+2b*	3	7	9	8	6	1	6	**384**		
7C	2758	2758	gPSSP	1	10	5	1	1	9	220	8	2005	China
9V	280	280	*2x*, *2x+2b*	3	15	17	4	16	6	1	17	1998	Vietnam
	5231	280	*2x*	3	15	17	4	**148**	6	1	17		
10A	1263	280	*2x*	1	15	13	4	16	6	1	17	1998	USA
	5236	Singleton	gPSSP	1	7	12	1	1	10	1	11		
12F	4846	1527	*2b*	4	12	32	111	1	13	48	6	UN	Japan (Osaka)
14	343	554	gPRSP	2	8	8	4	15	39	12	14	1998	Norway
	236	320	gPRSP	1	15	16	19	15	6	20	26	1993	Taiwan
	5240	230	gPRSP, *1a+2x*	4	5	19	2	17	6	22	14		
	13	15	*1a+2x*	1	1	5	4	5	5	27	8	1997	Australia,USA
	2922	15	*1a+2x*	3	1	5	4	5	5	20	8	2003	Japan (Hyogo)
15A	63	63	*1a+2x*	1	2	5	36	12	17	21	14	1992	Sweden
15B	199	199	*1a+2x*	1	8	13	14	4	17	4	14	1987	Netherlands
15C	199	199	*2x*	1	8	13	14	4	17	4	14	1987	Netherlands
18C	3594	3594	gPSSP	1	10	13	34	16	6	1	31	2007	South Korea
19F	236	320	gPRSP	16	15	16	19	15	6	20	26	1993	Taiwan
	115	115	gPRSP	1	15	16	19	15	30	20	39	1994	Taiwan
19A	3111	3111	*2x*,*1a+2x*	2	61	60	67	16	10	104	14	1989	USA
	2331	2331	*2x*	1	10	16	150	1	17	1	29	1999	Czech
	5237	2331	*1a+2x*	1	10	16	150	1	30	1	29		
22F	433	433	*2x*	1	1	1	4	1	18	58	17	1997	Poland
23F	242	242	gPRSP	7	15	29	4	21	30	1	14	1996	Taiwan
	1437	1437	gPRSP	1	1	32	6	6	6	1	14	2000	Japan
	63	63	gPRSP	1	2	5	36	12	17	21	14	1992	Sweden
	338	156	gPRSP, *2x+2b*	3	7	13	8	6	1	6	8	1995	Colombia
	5246	156	*2x+2b*	1	7	13	8	6	1	138	8		
	5242	156	*2x+2b*	1	7	13	8	6	1	**337**	8		
23A	338	156	gPRSP, *2x+2b*	3	7	13	8	6	1	6	8	1995	Colombia
24	4982	4982	gPSSP	1	7	120	8	8	25	28	14	2001	USA
34	3116	Singleton	*2x*, gPSSP	3	10	8	6	1	9	1	279	2004	Japan (Okayama)
35	558	558	gPRSP	1	18	12	4	44	14	77	97	1998	South Korea
	2755	2755	*2x*	1	10	12	2	1	152	28	14	2004	China
38	393	393	gPSSP	1	10	43	41	18	13	49	6	1998	USA

New sequence types (STs) and alleles are shown in bold face and underlined. Country, first country of isolation for the same ST clone referred from the MLST database

CC clonal complex, *year*, first isolation year of the same ST clone referred from the MLST database

Table 2 Associations of isolation areas with serotypes and sequence types

Serotype	MLST type (n)	Area					
		Hokkaido Tohoku	Kanto	Chubu	Kinki	Chugoku Shikoku	Kyushu
4	246(4)		246(1)	246(2)			246(1)
6A	3115(2)		3115(1)			3115(1)	
	2923(2)				**2923(2)**		
	90(7)	90(3)	90(1)		90(2)		90(1)
6B	2983(3)		2983(3)				
	2224(2)				2224(1)		2224(1)
	2924(1)				**2924(1)**		
	2923(1)	**2923(1)**					
6C	2924(1)				**2924(1)**		
	<u>5241(3)</u>		5241(1)		5241(1)		5241(1)
9V	280(3)	280(1)		280(2)			
	<u>5231(3)</u>		5231(1)	5231(1)		5231(1)	
12F	4846(4)		4846(1)		4846(2)		4846(1)
	343(2)			343(2)			
14	**<u>5240(4)</u>**		5240(1)				5240(3)
	2922(3)		2922(1)		2922(1)		2922(1)
	236(1)		**236(1)**				
15A	63(1)		**63(1)**				
15B	199(1)						**199(1)**
15C	199(1)		**199(1)**				
19A	3111(2)		3111(2)				
19F	236(16)	**236(2)**	**236(4)**		**236(6)**	**236(1)**	**236(3)**
23A	338(3)		**338(2)**	**338(1)**			
	338(3)		**338(1)**		**338(2)**		
23F	63(1)				**63(1)**		
	242(7)	242(1)	242(2)	242(1)	242(1)	242(1)	242(1)
34	3116(4)	3116(2)		3116(1)	3116(1)		

Sequence types (STs) with two or more strains were selected. Novel STs are shown in bold face and underlined

The STs corresponding to the multiple serotypes are indicated in red bold face

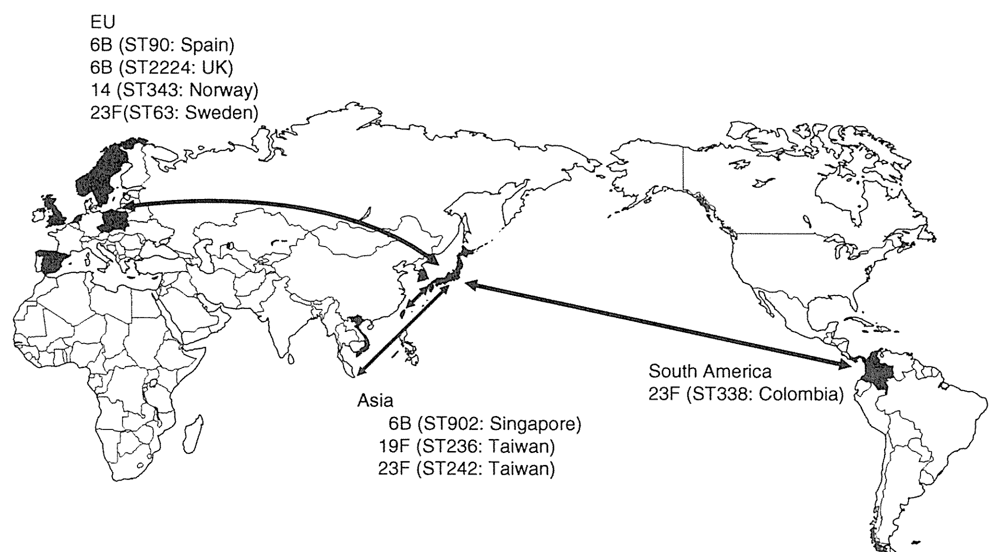

Fig. 3 Possible spread of prevalent serotypes, such as 6B, 19F, 23F, and 14, and corresponding sequence types between foreign countries and Japan

in serotype 19F, and ST63/ST242/ST338 in serotype 23F. As shown in the figure, pneumococcal strains now present in Japan and also other areas may have spread between Japan and European, Asian, or South American countries. In the future, clonal expansion is likely to result from human population drift.

Our manuscript represents the first report regarding associations of serotypes with MLST data and genotypic resistance classes based on PBP alterations in pneumococcal strains from children with meningitis in Japan. Pneumococcal MLST results have already been described in isolates from Japanese adults with community-acquired pneumonia [16]. Invasive pneumococcal disease is an important concern in Japan, and differences in serotype distributions between children and adults should be noted [21].

Continuous, accurate molecular epidemiologic surveillance regarding pneumococcal strains continues to be important in terms of global issues including vaccination and new antibiotic development.

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Application of the Real-Time PCR Method for Genotypic Identification of β-Lactam Resistance in Isolates from Invasive Pneumococcal Diseases

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We sought to identify genotypic resistance classes by real-time PCR in 300 *Streptococcus pneumoniae* isolates from invasive pneumococcal diseases. Primers and molecular beacon probes were designed for the *lytA* gene, 3 *pbp* genes, and the *mef*A/*erm*B genes. Targeted sequences of *pbp1a*, *pbp2x*, and *pbp2b* genes in susceptible strain R6 corresponded to those of penicillin G-nonsusceptible strains, including sites within or adjacent to conserved amino acid motifs. If amplification did not occur, the corresponding penicillin-binding protein (PBP) was considered to possess amino acid substitution(s) affecting minimal inhibitory concentrations (MICs) of β-lactam antibiotics. Real-time PCR required 90 min or less. Strains were assigned to six genotypic classes: Genotypic penicillin-susceptible *S. pneumoniae* (gPSSP) with 3 normal genes (22.3%); genotypic penicillin-intermediate *S. pneumoniae* (gPISP) (*pbp2x*) with an abnormal *pbp2x* gene (25.3%); gPISP (*pbp2b*) with an abnormal *pbp2b* gene (7.3%); gPISP (*pbp1a*+*2x*) with abnormal *pbp1a*+*2x* genes (11.3%); gPISP (*pbp2x*+*2b*) with abnormal *pbp2x*+*2b* genes (4.7%); or genotypic penicillin-resistant *S. pneumoniae* (gPRSP) with 3 abnormal PBP genes (29.0%). Sensitivity and specificity of real-time PCR compared with those of conventional PCR were high, 73.7–100% and 97.7–100%, respectively. As for relationships between genotype and β-lactam MICs, 90% of MICs for every resistance class were distributed within three serial dilutions for almost all antibiotics. MICs of each β-lactam antibiotic were estimated with high probability from genotypic patterns. In conclusion, determination of genotypic classes of *S. pneumoniae* using rapid real-time PCR is useful in selecting effective therapeutic agents for patients with pneumococcal infection.

Introduction

Streptococcus pneumoniae is a leading etiologic agent in children and adults with severe invasive infections that contribute importantly to morbidity and mortality.[20,32] Strains resistant to penicillin G (PEN) have emerged and spread rapidly worldwide.[1,15]

In Japan, clinical isolates of PEN-resistant *S. pneumoniae* (PRSP) and PEN-intermediate *S. pneumoniae* (PISP) have increased rapidly since the late 1990s among school and preschool children as well as patients aged 65 years or older with either respiratory tract infections (RTI) or invasive pneumococcal diseases (IPD).[28,30] The mortality rate reportedly is higher in elderly IPD patients than in pediatric patients.[8]

Characteristically, PRSP and PISP strains show simultaneous resistance to cephalosporin antibiotics used in ambulatory practice.[30] The resistance mechanism for β-lactam antibiotics in PRSP and PISP is a decrease in affinities of three PEN-binding proteins (PBP) involved in peptidoglycan synthesis. These three enzymes, PBP1A, PBP2X, and PBP2B, are encoded by the *pbp1a*, *pbp2x*, and *pbp2b* genes, respectively. Among PEN-nonsusceptible strains (PRSP and PISP), abnormal genetic mosaic patterns of *pbp1a*, *pbp2x*, and/or *pbp2b* were found to differ from those of PEN-susceptible *S. pneumoniae* (PSSP).[9,14] Although a variety of mosaic regions have been detected in each gene, the main contributors to β-lactam resistance are amino acid substitutions identified within or adjacent to conserved amino acid motifs such as Ser-Thr-Met-Lys (STMK), Ser-Ser-Asn (SSN), and Lys-Ser-Gly (KSG).[2,3,13,23,24]

Therefore, we established a conventional PCR method to determine whether or not a pneumococcal isolate is PEN-susceptible according to molecular evidence.[31] This PCR was completed within 2.5 hr from selection of a colony for testing by amplification and gel electrophoresis. The resistance pattern based on the results of conventional PCR

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was defined as genotypic (g) resistance and represented by designations such as gPRSP (*pbp1a+pbp2x+pbp2b*), gPISP(*pbp1 a+pbp2x*), gPISP(*pbp2x*), and gPSSP. Currently, the prevalence of gPRSP possessing three abnormal *pbp* genes exceeds 46% among pediatric patients and 17% among adults in Japan.[8]

Given this situation, therapeutic choices for Japanese IPD patients have gradually eroded, with empirical first-line therapy shifting from penicillins or third-generation cephalosporins to carbapenem antibiotics. At the same time, numbers of adults and elderly persons with various underlying diseases posing high risk of IPD have increased rapidly.

In the present study, we aimed to construct a novel assay using real-time PCR that eliminates the need for gel electrophoresis, allowing completion of all procedures within 90 min. We describe sensitivity and specificity of our real-time PCR compared with conventional PCR and efforts to estimate MICs of therapeutic agents against various strains belonging to different PBP genotypic classes.

Materials and Methods

Strains and serotyping

Clinical isolates of *S. pneumoniae* obtained from IPD patients were collected from 186 clinical laboratories at medical institutions participating in our program of active nationwide surveillance for emerging and re-emerging of infectious diseases. We randomly selected 300 strains as follows: Blood ($n=218$), cerebrospinal fluid ($n=56$), pleural fluid ($n=14$), joint fluid ($n=6$), and others ($n=6$). These strains were sent to our laboratory from August, 2006, to July, 2007, accompanied by application form with a similar format as the Active Bacterial Core Surveillance (ABCs) case report.

The serotypes of all strains were determined in real time by the Quellung reaction using antiserum purchased from the Statens Serum Institute (Copenhagen, Denmark). The serotypes of these strains were mainly 6B ($n=47$), 12F ($n=28$), 14 ($n=27$), 3 ($n=26$), 4 ($n=22$), 9 ($n=20$), 19F ($n=19$), 23F ($n=18$), 6A/6C ($n=16$), 19A ($n=14$), 15 ($n=12$), and others ($n=51$).

Real-time PCR primers and molecular beacon probes

Sequences of six sets of primers and molecular beacon (MB) probes and amplicon sizes (bp) applied for our real-time PCR are shown in Table 1. Target genes and the DNA amplification positions were the *lytA* gene encoding the autolysin enzyme specific to *S. pneumoniae*[12]; the *pbp1a* gene detected in susceptible strains,[18] located in the region including a conserved amino acid motif, STMK, corresponding to that of resistant strains; the *pbp2x* gene detected in susceptible strains,[16] located in the region surrounding the STMK motif corresponding to divergent sequences of resistant strains; the *pbp2b* gene detected in susceptible strain,[10] located in the region adjacent the SSN motif; the *mefA* gene

Table 1. Primers and Molecular Beacon Probes for Real-Time PCR

Target gene	*Sequence (5′ to 3′)*	*Position*	*Amplicon size (bp)*	*Target amino acid substitution*
Autolysin (*lytA*)				
Sense primer	CAGAATTAGGTTTTTTCTCGC	723–743	188	—
Reverse primer	TAAGAGTTCGATATAAAGGCG	890–910		
Probe	FAM-CGCGATCAGGTCTCAGCA TTCCAACCGCCGATCGCG-BHQ1	809–830		
PBP 1A (*pbp1a*)				
Sense primer	AAACCGCGACTGGGGATCAAC	2037–2057	239	S(T)MK ↓ A or S
Reverse primer	GGTTGAGTCCGACCTTGTTT	2275–2256		
Probe	FAM-CGCGATCACTGGGATAGGGG CTACTTTGGCGATCGCG-BHQ1	2174–2196		
PBP 2X (*pbp2x*)				
Sense primer	CCAGGTTCCACTATGAAAGTG	1255–1275	197	S(T)(M)K ↓ ↓ A F
Reverse primer	ATCCCAACGTTACTTGAGTGT	1451–1431		
Probe	FAM-CGCGATCAGATGCCACGATTC GAGATTGGGGATCGCG-BHQ1	1353–1375		
PBP 2B (*pbp2b*)				
Sense primer	CCTATATGGTCCAAACAGCCT	1566–1586	147	SSN(T) ↓ A or S
Reverse primer	GGTCAATTCCTGTCGCAGTA	1712–1693		
Probe	FAM-CGCGATCTCGGCACCAGCAAT CTAGAGTCTGATCGCG-BHQ1	1626–1648		
Macrolide efflux (*mef*A)				
Sense primer	GGGACCTGCCATTGGTGTGC	180–199	402	—
Reverse primer	CCCAGCTTAGGTATACGTAC	581–562		
Probe	FAM-CGCGATCCCCCAGCACTCAAT GCGGTTACACGATCGCG-BHQ1	359–382		
Adenine methylase (*erm*B)				
Sense primer	CGTACCTTGGATATTCACCG	721–740	224	—
Reverse primer	GTAAACAGTTGACGATATTCTCG	944–922		
Probe	FAM-CGCGATCCCGCCATACCACAG ATGTTCCGATCGCG-BHQ1	852–872		

encoding the efflux protein for 14-membered macrolide (ML) antibiotics[25]; and the *erm*B gene encoding adenine methylase for 14- and 16-membered ML antibiotics.[27]

Primers and MB probes corresponding to *pbp1a*, *pbp2x*, and *pbp2b* genes were designed to amplify the DNA only in susceptible strains. All MB probes were labeled with a fluorescent reporter of 6-carboxyfluorescein (FAM) at the 5′ end and also with a black hole quencher 1 (BHQ-1) at the 3′ end. Reporters and quenchers were connected to stem oligonucleotides.

Real-time PCR conditions

The real-time PCR reaction mixture consisted of 15 μl of 2× real-time PCR Master Mix (Toyobo, Tokyo, Japan), each primer at 0.2 μM, and each MB probe at 0.3 μM. The final volume of the mixture was adjusted to 30 μl by addition of DNase- and RNase-free H_2O. After each reaction mixture was pipetted into a 96-well plate, plates were stored at −30°C until use.

One colony grown on a sheep blood agar plate was picked up and suspended in 30 μl of lysis solution.[29] The tube then was placed in a thermal cycler (Gene Amp PCR System 9600R; Perkin-Elmer Cetus, Norwalk, CT) and heat-treated for 5 min at 60°C and for 5 min at 94°C to obtain template DNA. Next, after wells of the frozen real-time PCR reagent were thawed on ice, 2 μl of each template DNA was added to each well. Real-time PCR was performed immediately with a Stratagene Mx3000P (Stratagene, La Jolla, CA). The PCR conditions included an initial DNA denaturation step of 95°C for 30 sec, followed by 40 cycles of 95°C for 15 sec, 50°C for 20 sec, and 75°C for 15 sec. The time required from the lyses reaction to completion of real-time PCR was 90 min.

Conventional PCR

Conventional PCR was performed as a control assay for the real-time PCR in the same strains, using a commercially available kit (Wakunaga Pharmaceuticals, Hiroshima, Japan). The right of commercial production for this kit had been transferred to the company from Ubukata et al.[19,30]

Sequencing of pbp genes with discrepancies between the two PCR methods

Both the *pbp1a* and *pbp2x* genes in *S. pneumoniae* strains for which a discrepancy in the PCR data was recognized between the conventional and real-time methods were sequenced to identify the amino acid substitution. PCR primers used for analysis were a sense primer for *pbp1a*, 5′-TGGGATGGATGTTTACACAAATG-3′; a reverse primer for *pbp1a*, 5′-TGTGCTGGTTGAGGATTCTG-3′; a sense primer for *pbp2x*, 5′-TATGAAAAGGATCGTCTGGG-3′; and a reverse primer for *pbp2x*, 5′-AGAGAGTCTTTCATAGCTGAAGC-3′, as described previously.[2,3]

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). DNA sequencing was performed with an ABI 3130/3130xl genetic analyzer (Applied Biosystems).

Susceptibility testing

MICs of the five β-lactam antibiotics PEN, ampicillin (AMP), cefotaxime (CTX), meropenem (MEM), and panipenem (PAM) were determined by an agar dilution method using Mueller–Hinton II agar (MH, Becton Dickinson, Franklin Lakes, NJ) supplemented with 5% defibrinized sheep blood. Bacterial inoculum size and culture conditions were in accordance with a previously described method.[28]

S. pneumoniae ATCC49619 and R6 reference strains were used as quality controls.

Multilocus sequence typing and eBURST analysis

Multilocus sequence typing (MLST) performed for *S. pneumoniae* strains recognized discrepancy in the data of the two PCR methods. MLST and eBURST analysis was performed according to the MLST site (http://spneumoniae.mlst.net/).

Results

Resistant genotypes determined by real-time PCR

Figure 1 shows four patterns from a computer display connected to the real-time PCR instrument shown just after the PCR reaction was completed. Each tested strain was identified as follows: A, as gPSSP by DNA amplification corresponding to *lytA* (a), *pbp1a* (b), *pbp2x* (c), and *pbp2b* (d) genes; B, as gPISP (*pbp2x*), with only the *pbp2x* gene not amplified; C, as gPISP (*pbp1a*+*pbp2x*), with *pbp1a* and *pbp2x* genes not amplified; and D, as gPRSP (*pbp1a*, *pbp2x*, and *pbp2b*), with all 3 *pbp* genes not amplified. With regard to ML resistance, a strain showing DNA amplification for *mef*A and/or *erm*B genes was identified as ML resistant.

Genotypic classification of β-lactam and macrolide resistance

All strains tested were classified into six genotypic categories by real-time PCR for 3 *pbp* genes: gPSSP with three normal genes (n=67, 22.3%); gPISP (*pbp2x*) with an abnormal *pbp2x* gene (n=76, 25.3%); gPISP (*pbp2b*) with an abnormal *pbp2b* gene (n=22, 7.3%); gPISP (*pbp1a*+*2x*) with abnormal *pbp1a*+*2x* genes (n=34, 11.3%); gPISP (*pbp2x*+*2b*) with abnormal *pbp2x*+*2b* genes (n=14, 4.7%); and gPRSP with three abnormal PBP genes (n=87, 29.0%). Strains examined included 106 from pediatric patients (35.3%) and 194 from adult patients (64.7%). Percentages of the strains representing gPRSP accounted for 50.5% of isolates from children and 17.5% of those from adults.

Although detailed data are not shown, ML resistance in these strains was classified into four genotypic categories based on presence or absence of resistance genes: ML-susceptible strains (n=58, 19.3%); 14-membered ML-resistant strains possessing an *mef*A gene (n=79, 26.3%); 14- and 16-membered ML-resistant strains possessing an *erm*B gene (n=150, 50.0%); or an ML-resistant strain possessing *mef*A and *erm*B genes (n=13, 4.3%). Proportions of strains showing ML resistance were 85.9% of isolates from children and 77.9% of the isolates from adults.

Sensitivity and specificity of real-time PCR

As shown in Table 2, sensitivity and specificity for all strains were compared between conventional and real-time

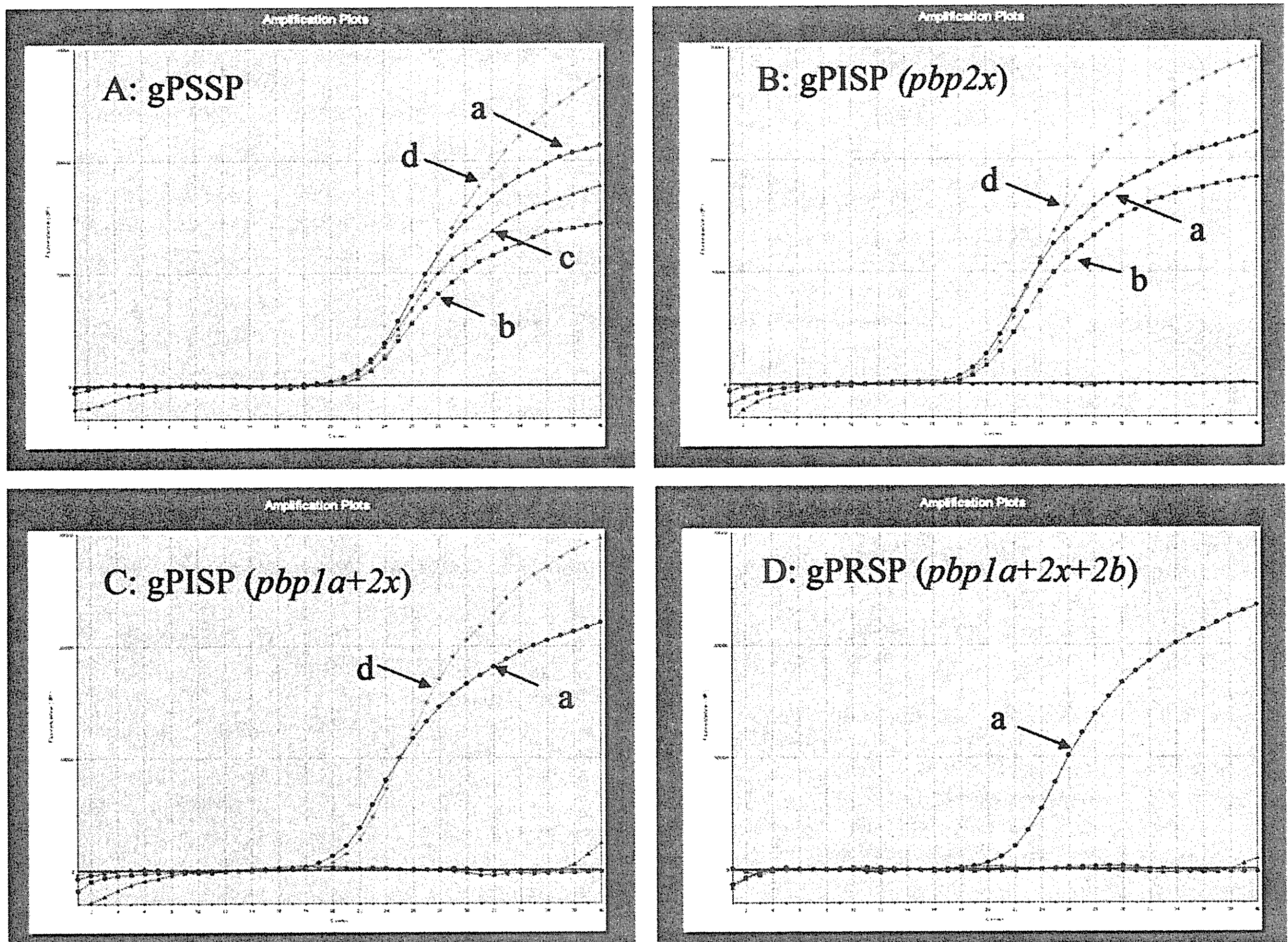

FIG. 1. Four genotypic resistance patterns from a computer display connected to the real-time PCR instrument, seen just after the PCR reaction was completed. (**A**) Genotypic penicillin-susceptible *Streptococcus pneumoniae* (gPSSP) by DNA amplification corresponding to *lytA* (a), *pbp1a* (b), *pbp2x* (c), and *pbp2b* (d) genes. (**B**) Genotypic penicillin-intermediate *S. pneumoniae* (gPISP) (*pbp2x*), with only the *pbp2*x gene not amplified. (**C**) gPISP (*pbp1a*+*pbp2x*), with *pbp1a* and *pbp2x* genes not amplified. (**D**) Genotypic penicillin-resistant *S. pneumoniae* (gPRSP) (*pbp1a*, *pbp2x*, and *pbp2b*), with 3 *pbp* genes not amplified.

PCR. The sensitivity and specificity for the *mefA* gene and the *ermB* gene were calculated to be 100%.

Table 3 shows detailed information for the nine strains (3.0%) showing a discrepancy between real-time PCR and conventional PCR. In these strains, DNA amplification for the *pbp1a* or *pbp2x* gene corresponding to the susceptible strain occurred weakly in conventional PCR but not at all in real-time PCR. According to susceptibility testing for AMP, and CTX, results of real-time PCR proved more accurate than those of conventional PCR. Overall, our new real-time PCR method showed to have excellent sensitivities and specificities compared with those of conventional PCR.

Relationships between PBP gene alterations and MIC of β-lactam agents

Figure 2 shows relationships between MICs of five β-lactam agents and results of real-time PCR for *pbp1a*, *pbp2x*, and *pbp2b* genes in the tested strains. MICs of PEN, MEM, and PAM were affected by *pbp2b* alterations rather than those in *pbp2x*. On the other hand, the MIC of CTX was 4–8 times lower than that of PEN due to *pbp2x* alterations. Notably, 90% of MICs in each genotype resistance class were distributed essentially within three serial dilution concentrations (for instance, gPRSP in PEN, from 0.5 to 2 mg/L) for almost all antibiotics. However, eight gPSSP strains with a CTX MIC ranging from 0.125 to 0.25 mg/L possessed substitutions of Thr550Ala adjacent to a KSG motif in PBP2X that could not be detect with the real-time PCR constructed in this study.

Estimated MIC_{50} values and corresponding ranges for 90% of β-lactam antibiotics among six PBP genotypic categories are listed in Table 4. On the basis of these data, MIC estimation for parenteral β-lactam antibiotics associated with clinical efficacy could be made with high probability.

Discussion

The ultimate global public health goal in the 21st century is to develop and disseminate vaccination to prevent infectious diseases caused by various viruses and bacteria more effectively. For immunity against pneumococcal infections, development of 23-valent pneumococcal polysaccharide

Table 2. Sensitivites and Specificities of Real-Time PCR Compared with those Conventional PCR

Genotype	Real-time PCR	Conventional PCR (%) Positive	Conventional PCR (%) Negative	Total no. of samples
gPSSP	Positive	67 (98.5)[a]	0 (0.0)	67
	Negative	1 (1.5)	232 (100.0)[b]	233
	Total	68	232	300
gPISP (*pbp2x*)	Positive	75 (96.2)	1 (0.5)	76
	Negative	3 (3.8)	221 (99.5)	224
	Total	78	222	300
gPISP (*pbp2b*)	Positive	22 (100.0)	0 (0.0)	22
	Negative	0 (0.0)	278 (100.0)	278
	Total	22	278	300
gPISP (*pbp1a+2x*)	Positive	31 (100.0)	3 (1.1)	34
	Negative	0 (0.0)	266 (98.9)	266
	Total	31	269	300
gPISP (*pbp2x+2b*)	Positive	14 (73.7)	0 (0.0)	14
	Negative	5 (26.3)	281 (100.0)	286
	Total	19	281	300
gPRSP (*pbp1a+2x+2b*)	Positive	82 (100.0)	5 (2.3)	87
	Negative	0 (0.0)	213 (97.7)	213
	Total	82	218	300

[a]Sensitivity.
[b]Specificity.

vaccine (PPV23)[5] began in the early 1980s in the United States, and this vaccine was introduced in Japan in 1988. In Japan, 7-valent pneumococcal conjugate vaccine (PCV7) has just been approved on a voluntary basis to prevent IPD among children with immunologic immaturity.

In countries where PCV7 has been introduced into the vaccine schedule, incidence of pediatric IPD caused by vaccine-type strains has decreased significantly,[4,6,22] while a related decrease of IPD among adults also has been reported.[17] However, prevalence of IPD caused by serotypes 19A and 6A (nonvaccine types) has increased, accompanied by a shift from PEN-susceptible to PEN-resistant strains.[7,11,21] Some investigators also have reported that overall incidence of IPD is little changed.[26]

In Japan, great clinical attention has been paid to the increase of PRSP and PISP among *S. pneumoniae* isolates from IPD,[8] which strongly reflects the difference in use of oral antibiotics between pediatricians and internists. Specifically, in pediatric practice, oral cephalosporins are favored over penicillins for outpatients, although a recent shift back toward amoxicillin and AMP has been noted. On the other hand, in internal medicine, ML and fluoroquinolone agents rather than β-lactam antibiotics are preferred. This might contribute significantly to rates of

Table 3. Details of 9 Strains Showing a Discrepancy in Results in between Real-Time PCR and Conventional PCR

No of strain	Genotype: Conventional PCR	Genotype: Real-time PCR	MIC (mg/L): PEN	AMP	CTX	MEM	PAM	Serotype	ST	CC
Ref R6	gPSSP	gPSSP	0.016	0.016	0.016	0.008	0.004	—	—	—
RS-009	gPISP(*pbp2x*)	gPISP(*pbp1a+2x*)	0.125	0.5	1	0.031	0.008	14	13	15
RS-027	gPISP(*pbp2x*)	gPISP(*pbp1a+2x*)	0.125	0.5	1	0.031	0.008	6B	385	156
RS-083	gPISP(*pbp2x*)	gPISP(*pbp1a+2x*)	0.125	0.5	1	0.031	0.004	6B	2983	156
RS-046	gPISP(*pbp2x+2b*)	gPRSP	0.5	1	2	0.063	0.016	14	343	554
RS-101	gPISP(*pbp2x+2b*)	gPRSP	0.5	2	2	0.063	0.016	14	343	554
RS-193	gPISP(*pbp2x+2b*)	gPRSP	0.5	1	0.5	0.063	0.016	14	343	554
RS-311	gPISP(*pbp2x+2b*)	gPRSP	0.5	1	2	0.125	0.016	14	343	554
RS-065	gPISP(*pbp2x+2b*)	gPRSP	1	4	1	0.25	0.031	6B	6939	None[a]
RS-208	gPSSP	gPISP(*pbp2x*)	0.063	0.125	0.125	0.016	0.004	6A	4542	156

When DNA amplification occurred, the corresponding *pbp* gene showed the same sequences as the susceptible strain; for example, a strain showing amplification of *pbp1a* and *pbp2b* genes was designation gPISP(pbp2x).
[a]ST6939 is not present in any group of clonal complexes.
MIC, minimum inhibitory concentration; PEN, penicillin; AMP, ampicillin; CTX, cefetaxime; MEM, meropenem; PAM, panipenem; ST, sequence type; CC, clonal complex; gPSSP, genotypic penicillin-susceptible *Streptococcus pneumoniae;* gPISP, genotypic penicillin-intermediate *S. pneumoniae;* gPRSP, genotypic penicillin-resistant *S. pneumoniae.*

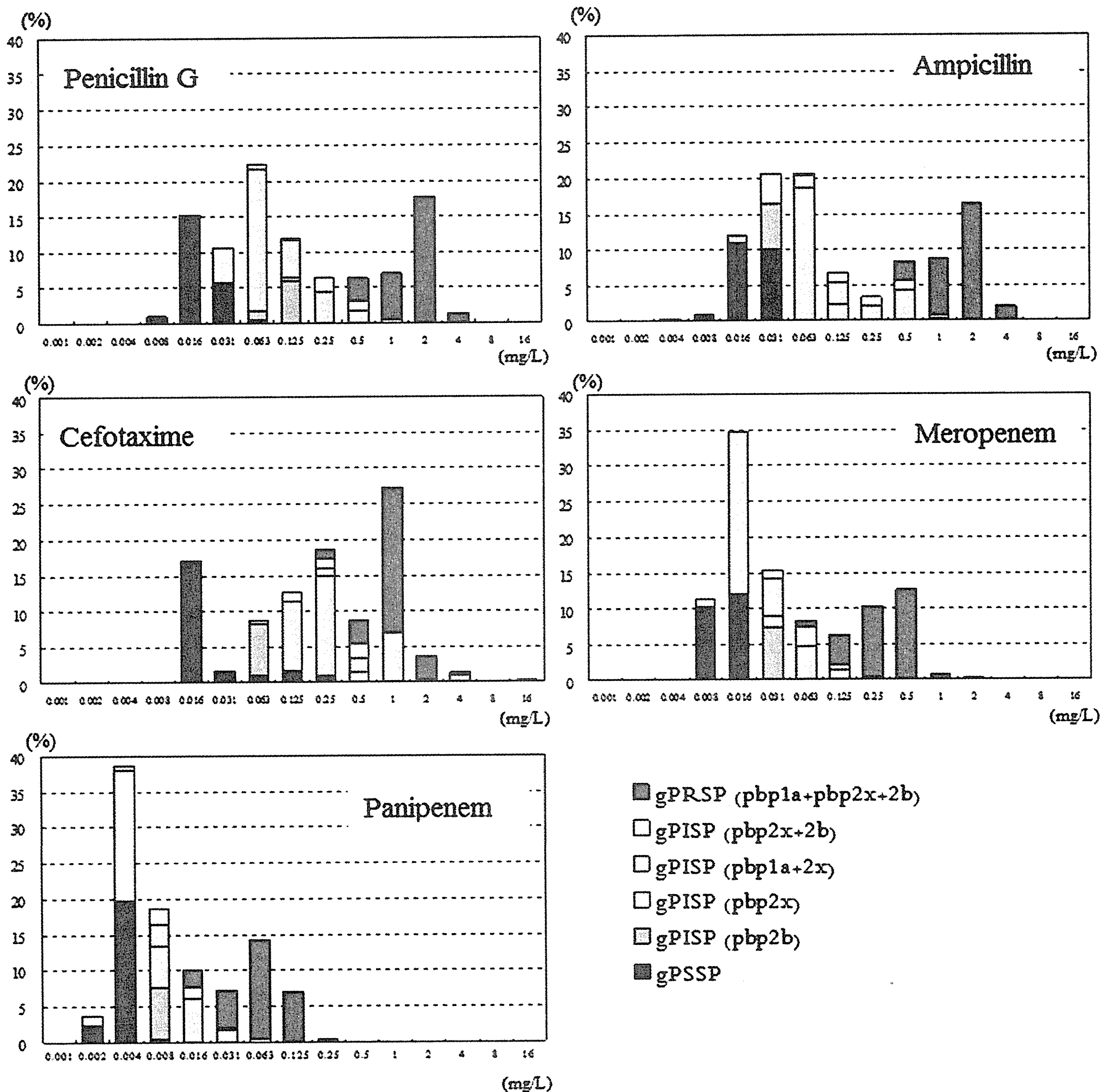

FIG. 2. Correlation between minimal inhibitory concentrations (MICs) of five β-lactam antibiotics and penicillin-binding protein (PBP) gene alterations for 300 *Streptococcus pneumoniae* isolates from invasive infections.

gPRSP isolated from pediatric patients and gPISP isolated from adult patients.

These situations concerning antibiotic resistance, in addition to the present state of pneumococcal vaccination, show that a need for rapid and accurate determination of resistance in clinical isolates is necessary for appropriate selection of chemotherapeutic agents in pneumococcal infections.

We initially identified species and antibiotic resistance using colony samples likely to be *S. pneumoniae* from blood agar plate using a conventional PCR method completed within 2.5 hr using gel electrophoresis.[30] Intrinsically, three primer sets designed on *pbp1a*, *pbp2x*, and *pbp2b* genes detect the most important amino acid substitutions affecting β-lactam susceptibilities, all positioned within or adjacent to conserved amino acid motifs in each PBP—substitutions from STMK to SAMK or SSMK in PBP1A, substitutions from STMK to SAMK or SAFK and from (L)KSG to (V)KSG in PBP2X, and substitution from SSN(T) to SSN(A) or SSN(S) in PBP2B. The genotypic resistance pattern based on the *pbp* gene analysis was divided into six categories: gPSSP, gPISP(*pbp2x*), gPISP(*pbp2b*), gPISP(*pbp1a*+*pbp2x*), gPISP (*pbp2x*+*pbp2b*), and gPRSP (*pbp1a*+*pbp2x*+*pbp2b*).

This was not shown in the results, but each class of resistance genes was not of a single clone. For example, gPRSP was divided into 11 serotypes with various clonal complexes (CCs). The major serotypes and CCs were serotype 6B with

TABLE 4. ESTIMATED MIC_{50s} AND FITTING RANGES OF 90% OF β-LACTAM ANTIBIOTICS FOR 6 PBP GENOTYPE CLASSES

		Estimated MIC (mg/L)				
Genotype	n	*PEN*	*AMP*	*CTX*	*MEM*	*PAM*
gPSSP	67	0.016 (0.016–0.031)	0.016 (0.016–0.031)	0.016 (0.016–0.125)	0.016 (0.008–0.016)	0.004 (0.002–0.004)
gPISP (*pbp2b*)	22	0.125 (0.063–0.125)	0.031 (0.016–0.031)	0.063 (0.063)	0.031 (0.031)	0.008 (0.008)
gPISP (*pbp2x*)	76	0.063 (0.031–0.063)	0.063 (0.031–0.063)	0.25 (0.125–0.25)	0.016 (0.016–0.031)	0.004 (0.002–0.008)
gPISP (*pbp1a*+*2x*)	34	0.25 (0.125–0.5)	0.25 (0.063–0.5)	1 (0.25–2)	0.063 (0.031–0.125)	0.016 (0.008–0.031)
gPISP (*pbp2x*+*2b*)	14	0.25 (0.063–0.5)	0.25 (0.063–0.5)	0.25 (0.125–0.5)	0.063 (0.031–0.125)	0.016 (0.008–0.031)
gPRSP (*pbp1a*+ *pbp2x*+*2b*)	87	2 (0.5–2)	2 (0.5–2)	1 (0.5–2)	0.5 (0.125–0.5)	0.063 (0.031–0.125)

MIC, minimum inhibitory concentration; PEN, penicillin; AMP, ampicillin; CTX, cefetaxime; MEM, meropenem; PAM, panipenem; gPSSP, genotypic penicillin-susceptible *Streptococcus pneumoniae*; gPISP, genotypic penicillin-intermediate *S. pneumoniae*; gPRSP, genotypic penicillin-resistant *S. pneumoniae*.

CC156 and CC490, serotype 19F with CC320, serotype 23F with CC156, CC242 and CC1437, serotype 6A with CC3115, CC3787 and CC81, and serotype 14 with CC320 and CC554.

As stated in the Results section, real-time PCR yielded satisfactory sensitivity and specificity compared with conventional PCR. Accurate estimation of MICs of each β-lactam antibiotic on the basis of genotypic patterns is highly important. Our novel real-time PCR assay also can be completed within 90 min after selection of colony samples, with elimination of gel electrophoresis, saving both time and labor.

Another merit of this assay is possible direct testing of usually sterile specimens (such as cerebrospinal fluid, joint fluid, and pleural fluid) from IPD patients, because primers and MB probes for amplification of the *lytA* gene are included in the real-time PCR. In the future, simultaneous performance of speciation and identification of resistance gene(s) by real-time PCR should optimize cost and benefit in clinical settings.

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