TABLE 3. (CONTINUED)

Serotype	*CC*	n	*Genotype*	*ST (n: PMEN clone)*
7F	191	2	S	191(2: Netherlands^{7F}-39)
6C	490	8	2x	**2923(8)**
	5832	3	2x+2b	**5832(3)**
	5241	2	2x+2b	**5241(2)**
	2924	2	2x	**2924(2)**
	3787	1	2x	3787(1)
10A	113	1	2x	**5236(1)**
11A	99	2	S	99(2)
12F	1527	2	2b	**4846(1)**
			2x+2b	**4846(1)**
15A	63	8	R	63(4: Sweden^{15A}-25), 2105(1), **8354(1)**
			1a+2x	63(2: Sweden^{15A}-25)
15B	199	3	1a+2x	199(1: Netherlands^{15B}-37)
			2x	199(2: Netherlands^{15B}-37)
15C	199	5	1a+2x	199(1: Netherlands^{15B}-37)
			2x	199(4: Netherlands^{15B}-37)
16F	3117	1	R	**8351(1)**
22F	433	6	2x	433(6)
	2572	1	S	**5496(1)**
23A	156	6	2x+2b	338(3: Colombia^{23F}-26), **5242(1)**, **6685(1)**,**8340(1)**
24	2572	6	S	2572(1), **5496(5)**
35B	558	1	R	558(1)
	1816	1	2x	2755(1)
38	393	3	S	393(3)
20	4745	1	S	4745(1)
21	1381	1	2x	1233(1)
33	717	3	S	717(3)
34	Group 363	1	S	**3116(1)**
37	447	1	S	**7970(1)**
NT	2572	1	S	**5496(1)**

Bold type indicates STs registered from Japan.
CC, clonal complex; ST, sequence type; PMEN, Pneumococcal Molecular Epidemiology Network.

important priority.[28] Increases of β-lactam and ML resistances in this pathogen pose ongoing difficulties in selecting therapeutic agents.[9]

Aiming for the prevention of pneumococcal infections in infants, PCV7 was licensed in 2000 and recommended for all children aged 2 to 23 months in the United States.[1] PCV7 was licensed in Europe in 2001, but 6 nations, including the UK, have only included it in their national immunization programs since 2006.[20] Currently, PCV7 has been introduced in more than 100 countries as a voluntary vaccination or routine practice for young children.[18] Rapid implementation of PCV7 in young children has resulted in a dramatic reduction in the incidence of IPD and non-IPD in many countries.[15,18,24]

However, increases of pneumococcal infections due to NVT after the introduction of PCV7, especially PRSP of serotypes 6A and 19A,[12] have occurred in some countries, even as the prevalence of VT serotypes was decreasing.[15,24]

PCV13, including serotypes 6A and 19A, replaced PCV7 in vaccination schedules in the United States in 2010.[5] Presently, PCV7 is gradually being replaced with PCV13 worldwide.[8,10] Additionally, an increase serotypes of 15A and 35B has been reported in the United States.[13]

As previously described, PCV7 was approved in Japan in 2009; presently, PCV7 immunization of children under 5 years old has been promoted nationwide by the Ministry of Health, Labour and Welfare (Provisional Special Fund for the Urgent Promotion of Vaccination) since November 2010. The immunization rate was estimated to have reached 50% to 60% in 2011. PCV7 will be formally added to the immunization schedule for Japanese infants in 2013.

In the present study, we aimed to investigate the impact of PCV7 on the serotype of the causative *S. pneumoniae* isolates from children with IPD. Unfortunately, IPD reduction could not be studied in terms of incidence because Japanese data for incidence of IPD per 100,000 persons is not available.

We found that IPD caused by VT strains decreased significantly for serotypes 14 and 19F after promotion of PCV7 vaccination in 2011. Interestingly, the relative decrease in every VT serotype resembled to the kinetics of the serotype-specific immune responses described by Rennels *et al.*[23] This is reflected by a significant decrease in the onset of IPD in children under 2 years old.

In contrast, NVT serotypes 15A and 22F have increased as causative pathogens. The obvious change from VT to NVT serotypes appears to be a consequence of PCV7 vaccination.

By MLST analysis of VT serotype strains, the already well-known PMEN clone and CCs predominated among gPRSP, such as Spain^{6B}-2 in 6B, CC490 and CC2224 in 6B, ST343 evolving from Sweden ST554 in serotype 14, Taiwan^{19F}-14 in 19F, and Taiwan^{23F}-15 in 23F. The occurrence of many new ST numbers suggest that housekeeping gene(s) evolved by mutation or genetic recombination.

Focusing on gPRSP and gPISP among the NVT serotypes, diversities of STs occurred easily as a result of mutations in housekeeping genes and *pbp* genes originating in other countries. The PMEN clone Sweden^{15A}-25 of ST63 was found among serotype 15A with gPRSP in Japan. This had an MIC of 0.12 μg/ml for PEN in 1992, but evolved to PISP showing an MIC of 0.5 μg/ml for PEN in 2008 in France. This worsened to MIC of 2.0 μg/ml for PEN in Japan. A new ST8354 evolved from ST63 by mutations in the *gdh* gene encoding glucose-6-phosphate dehydrogenase.

Recently, capsular switching occurring between different ST strains has been reported by Brueggemann *et al.*[3] As an example, ST2923 including serotypes 6A, 6B, and 6C in this study suggests that capsular switching occurred readily by recombination of the capsular locus region that was sandwiched between the *pbp1a* and *pbp2x* genes, although the original strain was recorded from Bulgaria as serotype 6A and CC490 (ST490).

In conclusion, serotype, genotype, and MLST analyses indicate that spread of microorganisms, especially potential respiratory pathogens occasionally carried as normal flora, is commonplace in the era of globalization. Peumococcal strains first identified abroad were then influenced by antibiotic selection, vaccination status, and population density in subsequent countries, with the emergence of mutations of housekeeping genes and the *pbp* gene, as well as capsular switching. Prevention and control of pneumococcal infections in young children and adults will require the development of a new vaccine including all pneumococcal serotypes. Further surveillance studies on clinical and molecular epidemiology of IPD caused by *S. pneumoniae* is needed to determine the impact of future conjugate vaccines on serotype and clone distribution.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Kimiko Ubukata, PhD
Laboratory of Molecular Epidemiology for Infectious Agents
Kitasato Institute for Life Sciences Kitasato University
5-9-1 Shirokane
Minato-ku
Tokyo 108-8641
Japan

E-mail: ubukatak@lisci.kitasato-u.ac.jp

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ORIGINAL ARTICLE

Antibiotic susceptibility in relation to genotype of *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Mycoplasma pneumoniae* responsible for community-acquired pneumonia in children

Miyuki Morozumi · Naoko Chiba · Takafumi Okada · Hiroshi Sakata · Keita Matsubara · Satoshi Iwata · Kimiko Ubukata

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Abstract *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Mycoplasma pneumoniae* are the main pathogens causing community-acquired pneumonia (CAP). We identified *S. pneumoniae* ($n = 241$), *H. influenzae* ($n = 123$), and *M. pneumoniae* ($n = 54$) as causative pathogens from clinical findings and blood tests from pediatric CAP patients ($n = 903$) between April 2008 and April 2009. Identification of genes mediating antimicrobial resistance by real-time PCR was performed for all isolates of these three pathogens, as was antibiotic susceptibility testing using an agar dilution method or broth microdilution method. The genotypic (g) resistance rate was 47.7 % for penicillin-resistant *S. pneumoniae* (gPRSP) possessing abnormal *pbp1a*, *pbp2x*, and *pbp2b* genes, 62.6 % for β-lactamase-nonproducing, ampicillin-resistant (gBLNAR) *H. influenzae* possessing the amino acid substitutions Ser385Thr and Asn526Lys, and 44.4 % for macrolide-resistant *M. pneumoniae* (gMRMP) possessing a mutation of A2063G, A2064G, or C2617A. Serotype 6B (20.3 %) predominated in *S. pneumoniae*, followed by 19F (15.4 %), 14 (14.5 %), 23F (12.0 %), 19A (6.2 %), and 6C (5.4 %). Coverage for the isolates by heptavalent pneumococcal conjugate vaccine (PCV7) and PCV13, respectively, was calculated as 68.5 and 80.9 %. A small number of *H. influenzae* were identified as type b (6.5 %), type e (0.8 %), or type f (0.8 %); all others were nontypeable. Proper use of antibiotics based on information about resistance in CAP pathogens is required to control rapid increases in resistance. Epidemiological surveillance of pediatric patients also is needed to assess the effectiveness of PCV7 and Hib vaccines after their introduction in Japan.

Keywords Antibiotic susceptibility · Community-acquired pneumonia · *Streptococcus pneumoniae* · *Haemophilus influenzae* · *Mycoplasma pneumoniae*

M. Morozumi · N. Chiba · T. Okada · K. Ubukata (✉)
Laboratory of Molecular Epidemiology for Infectious Agents, Kitasato Institute for Life Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan
e-mail: ubukatak@lisci.kitasato-u.ac.jp

T. Okada · K. Matsubara · S. Iwata
Department of Pediatrics, National Hospital Organization Tokyo Medical Center, Tokyo, Japan

H. Sakata
Department of Pediatrics, Asahikawa Kosei Hospital, Hokkaido, Japan

S. Iwata
Center for Infectious Diseases and Infection Control, Keio University School of Medicine, Tokyo, Japan

Introduction

Among bacterial pathogens in pediatric patients with community-acquired pneumonia (CAP), *Streptococcus pneumoniae* accounts for 30–35 %, *Haemophilus influenzae* for 5–20 %, and *Mycoplasma pneumoniae* for 10–20 % [1–6]. These overall percentages vary according to patient age, presence or absence of underlying disease, and epidemic occurrences involving a specific pathogen. Emergence and increased prevalence of isolates resistant to antimicrobials among these three organisms are of great concern in clinical pediatrics [7–11]. However, determination of *S. pneumoniae* and *H. influenzae* infection based on nasopharyngeal swab samples is very difficult because these organisms frequently colonize the nasopharynx [12–14]. Alternative specimens such as sputum samples are difficult to collect from pediatric patients, especially those

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less than 1 year old. Highly accurate determination of etiological agents in pediatric CAP therefore is an important need that is difficult to satisfy.

We constructed a real-time polymerase chain reaction (PCR) assay for rapid identification of CAP pathogens, complementing conventional culture methods [15, 16]. Agents responsible for CAP were assessed comprehensively based on the real-time PCR results for 6 bacteria and 11 viruses, bacterial cultures, and blood test results, as well as chest radiographic and clinical findings. All isolates in this study were determined from this evidence to be etiological agents of CAP in pediatric patients [5].

In this article, we describe correlation of susceptibility of oral and intravenous agents with genotypic (g) resistance based on identification of resistance genes by real-time PCR in *S. pneumoniae* and *H. influenzae*, and detection of a mutation in the sequence of the 23S rRNA gene in *M. pneumoniae*.

We also describe coverage rates of heptavalent pneumococcal conjugate vaccine (PCV7) and PCV13 based on serotype of *S. pneumoniae* shortly before approval of PCV7 for clinical use in Japan.

Patients and methods

Microorganisms

We identified *S. pneumoniae* ($n = 241$), *H. influenzae* ($n = 123$), and *M. pneumoniae* ($n = 54$) as etiological agents in pediatric inpatients with CAP treated at institutions belonging to the Acute Respiratory Diseases (ARD) study group. These agents were determined by real-time PCR results using nasopharyngeal swab samples, blood test results at admission, and clinical findings combined with chest radiography, as reported previously [5].

The ten institutions and affiliated pediatricians participating in the ARD study group between April 2008 and April 2009 included the National Hospital Organization Tokyo Medical Center (T. Okada, K. Matsubara, and S. Iwata), Asahikawa Kosei Hospital (H. Sakata), Tohoku Rosai Hospital (R. Takayanagi), Chiba University Hospital (N. Ishiwada), Health Insurance Society of General Ota Hospital, Fuji Heavy Industries Ltd. (Y. Sato), Niigata University Medical and Dental Hospital (T. Oishi), Hakujikai Memorial Hospital (T. Tajima), Kobe City Medical Center General Hospital (T. Haruta), Osaka Rosai Hospital (N. Kawamura), and Kawasaki Medical School (K. Ouchi).

Real-time PCR

Identification of resistance genes in *S. pneumoniae* and *H. influenzae* isolates by culture were performed by real-time PCR methods that we previously devised [8, 17].

To confirm that an isolate was *S. pneumoniae*, the *lytA* gene encoding the autolysin enzyme specific to this agent was amplified simultaneously during real-time PCR to amplify the three penicillin-binding protein (PBP) genes: the *pbp1a*, *pbp2x*, and *pbp2b* genes encoding PBP1A, PBP2X, and PBP2B enzymes, respectively. Positive reactions in the real-time PCR occurred only in susceptible strains possessing normal PBP genes because each probe and primer set was constructed to amplify part of a normal PBP gene. Additionally, *mef* (A) and *erm* (B) genes, which confer resistance to macrolide (ML) antibiotics, were identified. Resistance genotype (g) was represented as penicillin (PEN)-susceptible *S. pneumoniae* (gPSSP), PEN-intermediate *S. pneumoniae* (gPISP), and PEN-resistant *S. pneumoniae* (gPRSP).

For *H. influenzae*, a 16S rRNA gene specific to this organism and the *capB* gene encoding the serotype b capsule were identified by real-time PCR. Genes affecting antibiotic resistance were the *ftsI* gene encoding PBP3, where two amino acid substitutions, Asn526Lys and Ser385Thr, can cause β-lactam resistance; and a functioning bla_{TEM} gene, which encodes TEM-1 β-lactamase. On the basis of PCR results, genotypic resistance was classified as β-lactamase-nonproducing ampicillin (AMP)-susceptible (gBLNAS), β-lactamase-producing AMP resistance (gBLPAR), β-lactamase-nonproducing low-level AMP resistance (glow-BLNAR), β-lactamase-nonproducing AMP resistance (gBLNAR), or β-lactamase-producing amoxicillin (AMX)-clavulanic acid resistance (gBLPACR-I or gBLPACR-II).

Sequencing

The full length of the 23S rRNA gene of all *M. pneumoniae* strains was sequenced with an ABI Prism 3130/3130xl genetic analyzer (Applied Biosystems, Carlsbad, CA, USA) by methods described previously [18]. ML-resistant *M. pneumoniae* (MRMP) possessing the A2063G, A2064G, or C2617A mutation was indicated as gMRMP.

Susceptibility testing

Antibiotic susceptibility testing of *S. pneumoniae* and *H. influenzae* was performed by an agar dilution method using Mueller–Hinton II agar (MH; Becton–Dickinson, Franklin Lakes, NJ, USA) as described previously [19, 20]. Susceptibility of *M. pneumoniae* isolates was determined by a microdilution method using pleuropneumonia-like organism (PPLO) broth [18].

The antibiotics tested were AMP, AMX, cefdinir (CDR), cefditoren (CDN), cefotaxime (CTX), tebipenem (TBM), meropenem (MEM), panipenem (PAM), tosufloxacin (TFX), erythromycin (ERY), clarithromycin (CLR),

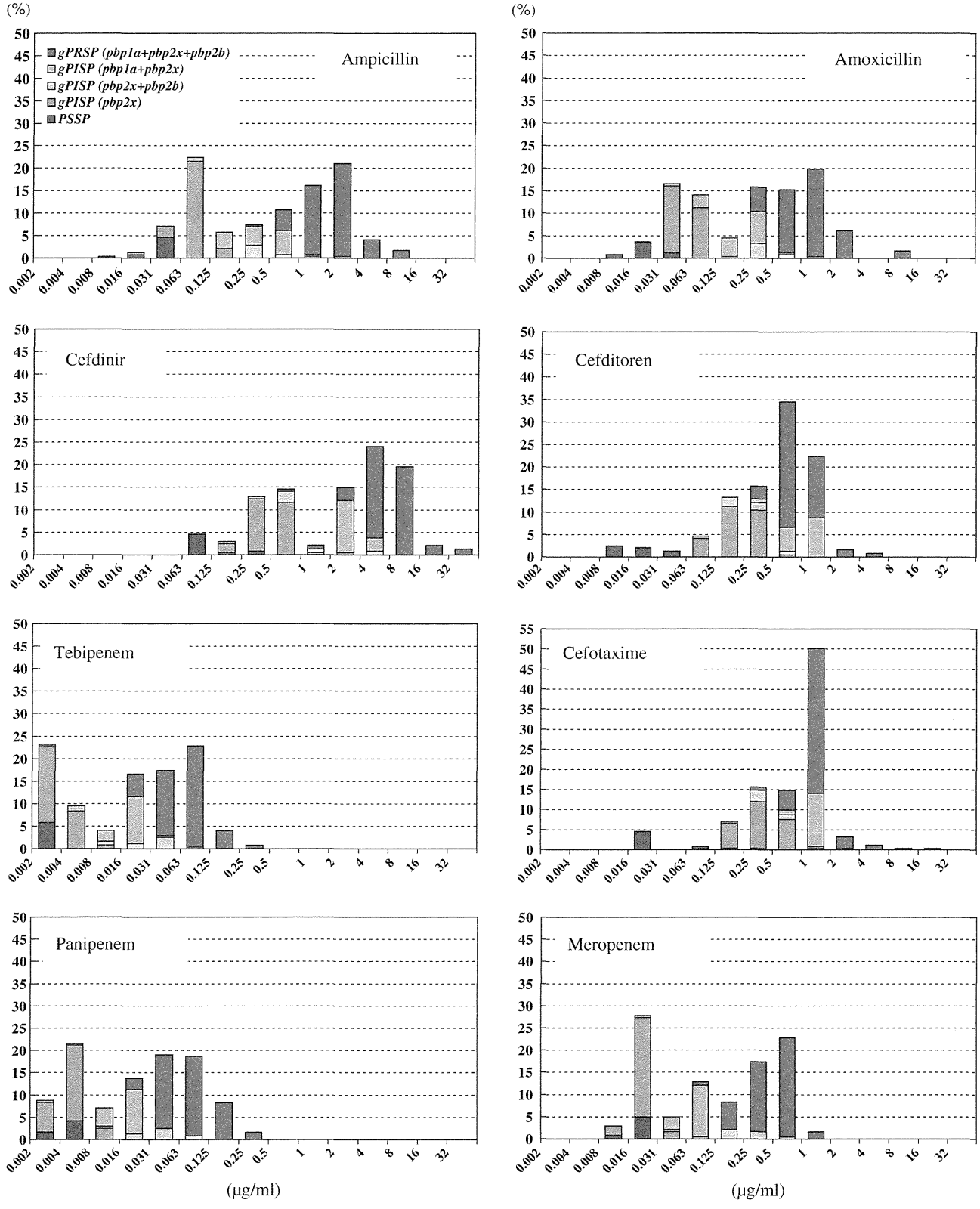

Fig. 1 Susceptibility distributions and genotypes of eight β-lactam agents for *Streptococcus pneumoniae* ($n = 241$)

azithromycin (AZM), telithromycin (TEL), and minocycline (MIN). Standard strains of *S. pneumoniae* ATCC 49619, *H. influenzae* ATCC 49247 and ATCC 49766, and *M. pneumoniae* M129 were used as quality control for susceptibility testing.

Serotyping

Serotype of *S. pneumoniae* strains was determined by the capsular swelling reaction using antiserum purchased from the Statens Serum Institute (Copenhagen, Denmark). Except for capsule type b, serotypes in *H. influenzae* were determined using antiserum (Denka Seiken, Tokyo, Japan).

Results

Streptococcus pneumoniae

Figure 1 shows minimum inhibitory concentration (MIC) distributions of eight β-lactam antibiotics for *S. pneumoniae* ($n = 241$). Genotype was based on real-time PCR results for the *pbp1a*, *pbp2x*, and *pbp2b* genes as already described. The resistance rate of gPRSP (*pbp1a* + *2x* + *2b*) was highest at 47.7 %, followed by 26.1 % for gPISP (*pbp2x*), 14.9 % for gPISP (*pbp1a* + *2x*), and 4.9 % for gPISP (*pbp2x* + *2b*). Occurrence of gPSSP having normal PBP genes was only 6.2 %.

Table 1 shows MIC_{90}s of antimicrobial agents in every resistant genotype together with comparison of the MIC_{90}s with that of gPSSP. MIC_{90}s of AMP, AMX, and TBM for gPISP (*pbp2x*) strains were influenced slightly by the *pbp2x* alterations, resulting in MIC_{90}s about 2 times greater than that for gPSSP. MIC_{90}s of the cephalosporin agents CDN, CDR, and CTX showed greater increases from the *pbp2x* alterations (4–32 times). No carbapenem agents (PAM, MEM, and TBM) were affected by this abnormal gene.

In contrast, MIC_{90}s of AMP, AMX, TBM, PAM, and MEM were affected slightly by *pbp2b* alterations. MIC_{90}s of all agents for gPRSP (*pbp1a* + *2x* + *2b*) generally were 32–128 fold that for gPSSP.

Table 1 MIC_{90} of β-lactam agents affected by *pbp* gene alterations in *Streptococcus pneumoniae*

	gPSSP ($n = 15$)	gPISP (*pbp2x*) ($n = 63$)	gPISP (*pbpla* + *2x*) ($n = 36$)	gPISP (*pbp2x* + *2b*) ($n = 12$)	gPRSP (*pbpla* + *2x* + *2b*) ($n = 115$)
Ampicillin	0.031	0.063 (×2[a])	0.5 (×16)	0.5 (×16)	4 (×128)
Amoxicillin	0.031	0.063 (×2)	0.25 (×8)	0.5 (×16)	2 (×64)
Cefdinir	0.125	0.5 (×4)	4 (×32)	4 (×32)	8 (×64)
Cefditoren	0.031	0.25 (×8)	1 (×32)	0.5 (×16)	1 (×32)
Cefotaxime	0.016	0.5 (×32)	1 (×64)	0.5 (×32)	2 (×128)
Tebipenem	0.002	0.004 (×2)	0.016 (×8)	0.031 (×16)	0.125 (×64)
Panipenem	0.004	0.004 (×1)	0.016 (×4)	0.063 (×16)	0.125 (×32)
Meropenem	0.016	0.016 (×1)	0.063 (×4)	0.25 (×16)	0.5 (×32)

gPSSP penicillin (PEN)-susceptible *S. pneumoniae*, *gPISP* PEN-intermediate *S. pneumoniae*, *gPRSP* PEN-resistant *S. pneumoniae*

[a] The × symbol throughout indicates multiple numbers compared to the minimum inhibitory concentration (MIC) of gPSSP

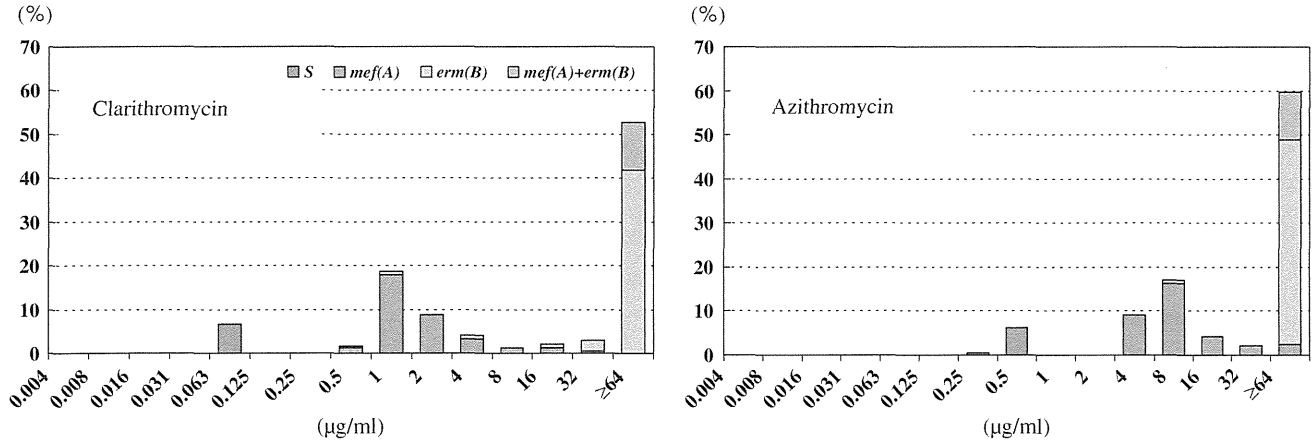

Fig. 2 Susceptibility distributions of clarithromycin (CLR) and azithromycin (AZM) for *Streptococcus pneumoniae* ($n = 241$)

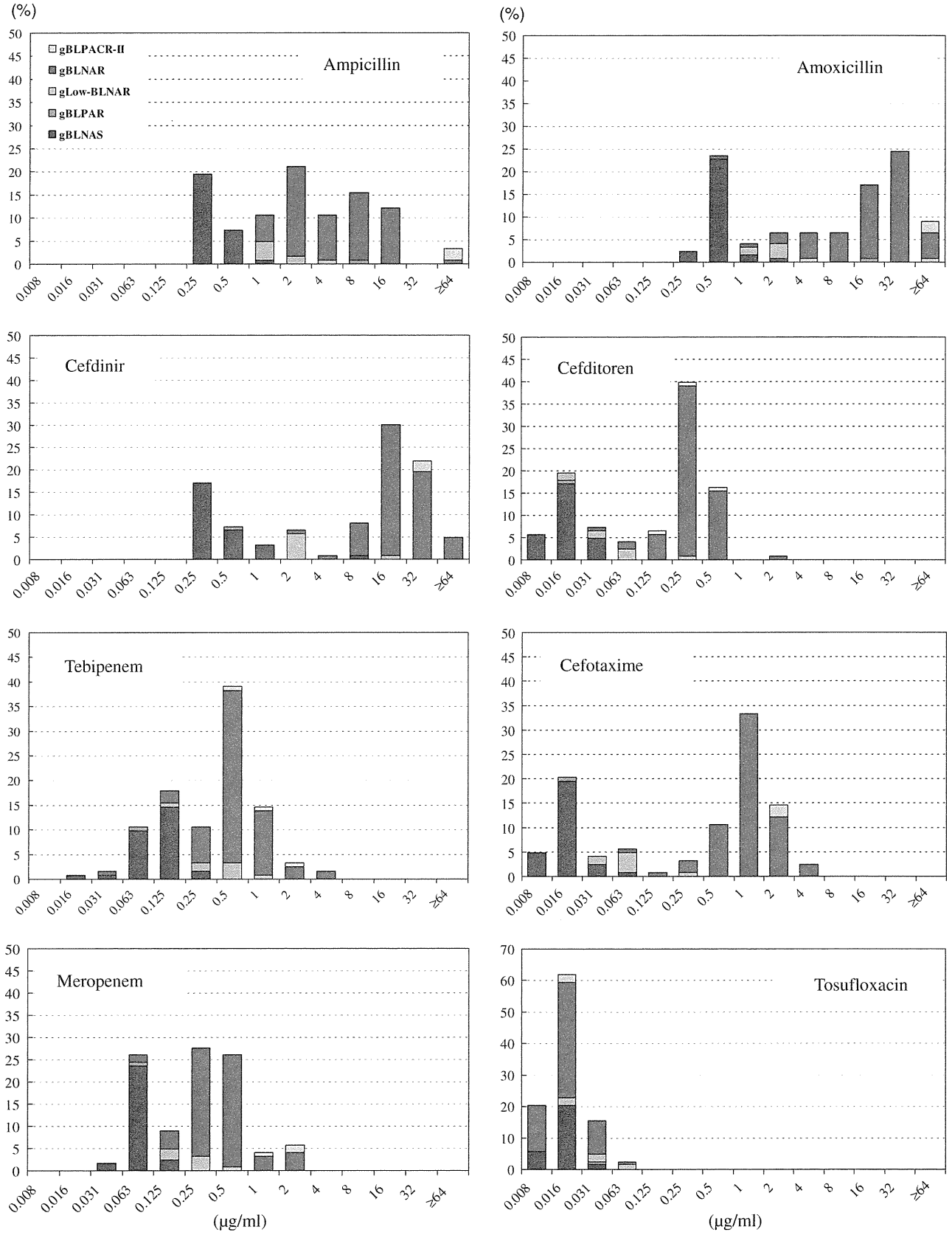

Fig. 3 Susceptibility distributions and genotypes of seven β-lactam agents and tosufloxacin (TFX) for *Haemophilus influenzae* (n = 123)

Two strains showing high MICs of 8 μg/ml or more for AMP, AMX, and CTX had two amino acid (aa) substitutions in a conserved motif of the *pbp2x* gene. This Ser-Thr-Met-Lys (STMK) motif was altered to become Ser-Ala-Phe-Lys (SAFK), although the first of these amino acid substitutions, from Thr to Ala, usually can be identified in most gPRSP [21]. Another two strains showing MICs of 8 μg/ml or more for AMP and AMX and 2 μg/ml for CTX had amino acid substitutions in the *pbp2b* gene.

Figure 2 shows MIC distributions of CLR and AZM for *S. pneumoniae*. ML-resistant strains were distinguished according to presence of *mef*(A), *erm*(B), or both genes, at respective frequencies of 34.4, 48.1, and 10.4 %. Susceptible strains possessing no ML resistance genes were less common (7.1 %). MIC_{90}s of CLR for strains with *mef*(A) or *erm*(B) were 4 μg/ml and ≥64 μg/ml, respectively; those of AZM were 16 μg/ml and ≥64 μg/ml.

Haemophilus influenzae

MIC distributions of eight antibiotics for *H. influenzae* are shown in Fig. 3. Resistance classes of 123 *H. influenzae* strains were distinguished according to real-time PCR results. Prevalence of each class among them was 27.6 % for gBLNAS, 0.8 % for gBLPAR, 6.5 % for glow-BLNAR, 62.6 % for gBLNAR, and 2.4 % for gBLPACR-II, respectively, showing predominance of gBLNAR among all isolates.

Table 2 shows MIC_{90}s and ranges of seven agents in every resistant genotype together with comparison with the gBLNAS MIC_{90}s. MIC_{90}s of seven β-lactam antibiotics for gBLNAR were affected in the following order: 64 μg/ml or grater (×64) for AMX and CDR, 2 μg/ml (×64) for CTX, 16 μg/ml (×32) for AMP, 0.5 μg/ml (×16) for CDN, and 1 μg/ml (×8) for TBM and MEM. Substitutions of Arg517His or Asn526Lys, plus Ser385Thr in PBP3, especially influenced MICs of penicillin and cephalosporin antibiotics [22, 23]. Influence of the substitutions on TBM and MEM MICs was less than for cephalosporins.

Table 2 MIC_{90} of β-lactam agents affected by *ftsI* gene mutations in *Haemophilus influenzae*

	gBLNAS ($n = 34$)	glow-BLNAR ($n = 8$)	gBLNAR ($n = 77$)
Ampicillin	0.5	2 (×4[a])	16 (×32)
Amoxicillin	1	4 (×4)	≥64 (×64)
Cefdinir	1	2 (×2)	≥64 (×64)
Cefditoren	0.031	0.063 (×2)	0.5 (×16)
Cefotaxime	0.031	0.063 (×2)	2 (×64)
Tebipenem	0.125	0.5 (×4)	1 (×8)
Meropenem	0.125	0.25 (×2)	1 (×8)

gBLNAS β-lactamase-nonproducing ampicillin (AMP)-susceptible, *glow-BLNAR* β-lactamase-nonproducing low-level AMP resistance, *gBLNAR* β-lactamase-nonproducing AMP resistance

[a] The × symbol throughout indicates multiple numbers compared to the MIC of gBLNAS

The only excellent MIC for gBLNAR was TFX, ranging from 0.008 to 0.063 μg/ml, with an MIC_{90} of 0.031 μg/ml.

Among all isolates, only 8.1 % could be serotyped: type b, 6.5 %, type e, 0.8 %, and type f, 0.8 %.The remainder were nontypeable (NT).

Mycoplasma pneumoniae

MIC distributions of six antibiotics for 54 *M. pneumoniae* isolates are shown in Fig. 4. ML-resistant strains possessing a mutation in domain V of the 23S rRNA were identified most frequently: A2063G (40.7 %), A2064G (1.9 %), or C2617A (1.9 %). Although MIC_{90}s of four ML for isolates without such mutations were excellent (0.0156 μg/ml or less), MRMP if possessing either an A2063G or an A2064G mutation showed high resistance to ERY, CLR, and AZM (MIC, 16 μg/ml or greater). A C2617 mutation was associated with a much smaller decrease in susceptibility to ML.

Although the MICs of MIN and TFX for *M. pneumoniae* were not excellent, these agents were equivalent in ML-susceptible and ML-resistant strains with MIC_{90}s of 1 and 0.5 μg/ml, respectively. No isolate showed resistance to both agents.

Serotype of *Streptococcus pneumoniae*

Figure 5 shows serotype distribution in relationship to genotypic resistance in 241 *S. pneumoniae* isolates. Serotype 6B (20.3 %) predominated in the isolates, followed, in order, by 19F (15.4 %), 14 (14.5 %), 23F (12.0 %), 19A (6.2 %), and 6C (5.4 %). Strains with the first four serotypes were almost all gPISP and gPRSP. The top four serotypes included no gPSSP.

Coverage of PCV7 and PCV13 for all isolates were calculated as 68.5 and 80.9 %, respectively; among gPRSP, coverage of both vaccines were high, calculated as 89.7 and 96.6 %, respectively. Notably, gPRSP strains were detected among non-vaccine types 19A, 6A, 35, and 15A.

Discussion

The three species *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Mycoplasma pneumoniae* are the main causative bacteria in patients with CAP [1–6]. Worldwide, the increase of resistant strains among these microorganisms poses problems in treatment for patients with CAP [7, 9, 11].

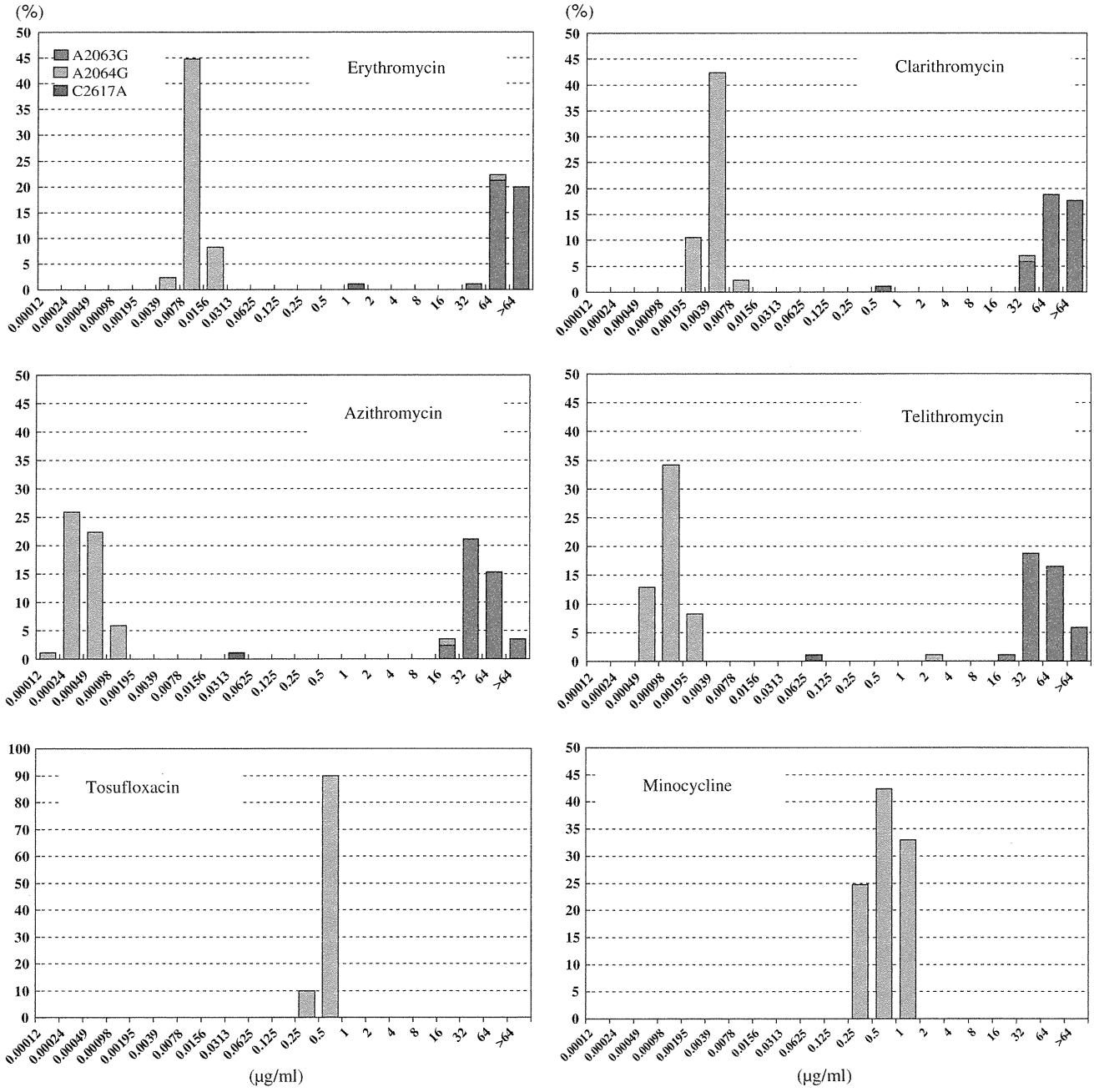

Fig. 4 Susceptibility distributions of macrolides, minocyclin (MIN), and TFX for *Mycoplasma pneumoniae* ($n = 54$)

In many countries, Hib conjugate vaccine and PCV7 have been incorporated into the pediatric vaccination schedule, with the aim of infection control. Presently, immunization with both Hib and PCV7 has been introduced in more than 100 countries worldwide.

Incidence of invasive pneumococcal disease and pneumonia causing vaccine-type *S. pneumoniae* has been reported to have decreased significantly after introduction of the vaccine [24, 25]. In Japan, PCV7 was approved in October 2009 by the government and marketed in February 2010. Although the pneumococcal isolates in this study were obtained just before marketing of PCV7, coverage for PCV 7 and PCV13 compared to our previous results in 2005 [21] has decreased somewhat, from 70.9 to 68.5 % for PCV7 and from 84.9 to 80.9 % for PCV13. Notably, in this study, gPRSP already were identified in non-vaccine types, specifically 19A, 6A, 35, and 15A.

Although reliable statistics are not yet available, the attainable vaccination rate for Japanese infants was considered to be about 60 % according to the 2011 official support provided by "Provisional Special Fund for the Urgent Promotion of Vaccination against Such Diseases as

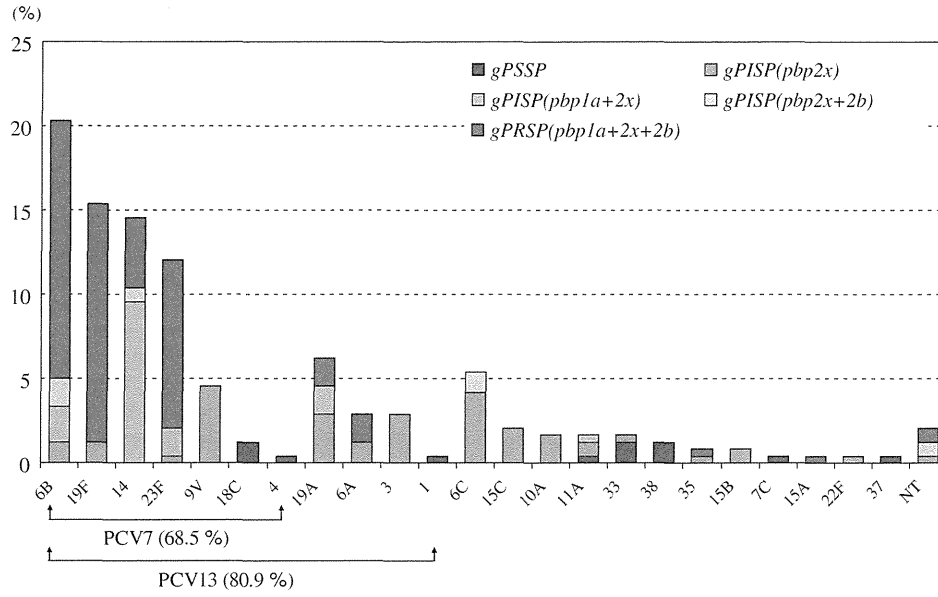

Fig. 5 Serotype distribution and resistance genes identified by polymerase chain reaction (PCR) in *Streptococcus pneumoniae* isolated from children ($n = 241$)

Cervical Cancer." The decrease in invasive pneumococcal diseases (IPD) from 333 cases in 2010 to 113 cases in 2011 (data not shown here) was attributed, in our nationwide surveillance, to the introduction of PCV7. However, the non-vaccine types 35 and 15A of gPRSP in addition to resistant 19A and 6A had increased significantly among IPD isolates in 2011, markedly limiting the efficacy of PCV7 to 46 %. Serotype changes favoring those not covered by PCV7 and PCV13 will become increasingly important clinical problems worldwide.

Nontypeable *H. influenzae* (NTHi) has attracted widespread attention for decreasing susceptibility to many oral and parenteral β-lactam antibiotics. In Japan, gBLNAR among Hib and NTHi isolated from patients with meningitis, pneumonia, and acute otitis media (AOM) has increased rapidly since 2000, to presently exceed 60 %, because the Hib vaccine was not marketed until late 2008 and also because oral cephalosporins were prescribed freely for respiratory diseases in children. Serum concentrations obtained using oral cephalosporins usually are low, exceeding the MIC of causative gBLNAR only on rare occasions.

Meningitis had decreased dramatically in 2011 because of official support as described above [26]. However, the Hib vaccine cannot contribute to the decrease in CAP caused by NTHi. Targeting other antigens in *H. influenzae* such as P6 membrane protein [27, 28] will be required to decrease NTHi diseases associated with resistance to β-lactam agents.

In this study, gMRMP was prevalent (44.4 %) and was associated with high ML resistance; a few patients initially received MIN. Subsequently, an outbreak of *M. pneumoniae* pneumonia occurred among children throughout Japan, beginning in early summer of 2011; 87.1 % of isolates were gMRMP [29]. Clinical findings of prolonged cough and fever characterized patients with gMRMP pneumonia; accordingly, the antibiotic was changed to MIN, doxycycline (DOX), or TFX for almost all patients. The clinical and bacteriological effectiveness of these three agents differed considerably, but only MIN is approved for treating *M. pneumoniae* infections in Japan. MIN or DOX was significantly more effective in achieving defervescence within 24 h and in decreasing numbers of gMRMP DNA copies 3 days after initiation than TFX ($p \leq 0.05$) [29]. Clinical trials of these agents are likely to gain approval for more agents in gMRMP-associated infections.

Finally, the rapid increase of resistant strains in these important causative pathogens contributes to narrowing the range of choice of oral antibiotics among pediatricians. To prevent increase of resistant strains, rapid identification of causative pathogens in CAP is needed, including both bacteria and viruses. Chest radiography and blood tests on admission also make important contributions. In likely bacterial infections, the most appropriate antibiotic must be chosen based on current information concerning antibiotic resistance and with reference to the Japanese Guidelines for the Management of Respiratory Infectious Diseases in Children 2007 [30].

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ORIGINAL ARTICLE

Longitudinal surveillance of *Haemophilus influenzae* isolates from pediatric patients with meningitis throughout Japan, 2000–2011

Kimiko Ubukata · Naoko Chiba · Miyuki Morozumi · Satoshi Iwata · Keisuke Sunakawa · The Working Group of Nationwide Surveillance for Bacterial Meningitis

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Abstract In Japan, β-lactamase-nonproducing, ampicillin-resistant organisms have been evident among *Haemophilus influenzae* type b (Hib) isolates since 2000, when no appropriate vaccine had been approved. We therefore performed molecular analysis of agents causing *H. influenzae* meningitis nationwide over the following 10 years. Some 285 institutions have participated in surveillance since 2000. The capsular type and resistance genes of 1,353 isolates and 23 cerebrospinal fluid samples from pediatric patients with meningitis we had received from 2000 to 2011 were analyzed by polymerase chain reaction. Blood and spinal fluid test results obtained when patients were admitted were examined for correlation with outcomes. Hib was found in 98.9 % of isolates. We received more than 100 Hib isolates per year until vaccination began in December 2008, when these isolates decreased, especially since establishment of a special fund to promote vaccination in November 2010. Decreased incidence among infants 7 months to 2 years old has been particularly notable. However, the rate of ampicillin-resistant organisms has increased to more than 60 % of all isolates since 2009. We received 587 replies to a questionnaire concerning outcomes, indicating 2 % mortality and 17.7 % serious morbidity. Age of 6 months or younger and presence of disseminated intravascular coagulation at admission were related to an unfavorable outcome ($p < 0.05$), but ampicillin resistance was not. Combination therapy with third-generation cephem and carbapenem agents was used initially for 72 % of patients. Routine immunization can prevent Hib meningitis in children.

Keywords *Haemophilus influenzae* type b (Hib) · Genotypic β-lactamase-nonproducing (gBLNAR) · Polymerase chain reaction · Surveillance · Molecular epidemiology

K. Ubukata (✉) · N. Chiba · M. Morozumi
Laboratory of Molecular Epidemiology for Infectious Agents, Kitasato Institute for Life Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan
e-mail: ubukatak@lisci.kitasato-u.ac.jp

S. Iwata
Center for Infectious Diseases and Infection Control, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 106-8582, Japan

K. Sunakawa
Kitasato University School of Medicine, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

Introduction

Community-acquired bacterial meningitis in children is a serious infection that occasionally is fatal. Pathogens and infection rate differ according to patient age; availability of vaccination against *Haemophilus influenzae* type b (Hib), *Streptococcus pneumoniae*, and others; and location in a developed versus a developing country.

Hib is well known to cause meningitis, epiglottitis, purulent arthritis, pericarditis, pneumonia, and other infections in infants and children over 3 months of age. Based on data from our surveillance [1] and from Ishiwada et al. [2], the incidence of Hib is approximately 10 to 12 per 100,000 children under 5 years of age. However, Hib meningitis already is uncommon in many countries where Hib vaccination has been introduced. Unfortunately, Hib vaccine was not approved by the Japanese Ministry of Health, Labour and Welfare until January 2007, and voluntary vaccination of children only began in late 2008. In November 2010, vaccination of children with Hib and

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heptavalent pneumococcal conjugate vaccine (PCV7) was recommended by the Provisional Special Fund for the Urgent Promotion of Vaccination against Such Diseases as Cervical Cancer. The vaccination rate among infants at risk throughout Japan is estimated to be 40–60 % as of the end of 2011.

Meanwhile, the rate of β-lactamase-nonproducing, ampicillin (AMP)-resistant *H. influenzae* (BLNAR) and Hib isolates from patients with meningitis have increased rapidly in parallel with their exponential increase in patients with respiratory tract infections (RTI) [1, 3, 4]. Although BLNAR strains showing decreased affinity for β-lactam antibiotics were first described in the United States in the 1980s [5, 6], these strains have remained rare in that country [7, 8] and in the European Union (EU) [9–11], except for France.

The resistance mechanism in BLNAR involves mutations in the *ftsI* gene encoding penicillin-binding protein 3 (PBP3), which mediates septal peptidoglycan synthesis in the cell wall. PBP3 is the main target of cephalosporin antibiotics, which differs from that of penicillins and carbapenems. Accordingly, susceptibility to cephalosporin clearly is affected by *ftsI* gene mutations [12]. We have identified amino acid substitutions at three PBP3 positions mainly associated with decreased β-lactam susceptibility: Asn526Lys, Arg517His, and Ser385Thr. Strains with substitutions of either Asn526Lys or Arg517His and also Ser385Thr were classified as genotypic BLNAR (gBLNAR) based on correlations with β-lactam susceptibility. Other strains with only Asn526Lys or Arg517His substitutions were classified as genotypic Low-BLNAR (gLow-BLNAR). In Australia [13], France [14], and Norway [15], the incidence of gLow-BLNAR isolates possessing an Asn526Lys substitution with AAA sequences was significant, but not in Japan, where AAG sequences contributed to this substitution.

In this report, we describe results in *H. influenzae* isolates from meningitis patients collected by the Nationwide Surveillance for Bacterial Meningitis (NSBM) working group and impact on survival outcome and presence or absence of sequelae for the following: yearly changes in genotypic β-lactam resistance, blood and spinal fluid test results, and antibiotics initially used at disease onset.

Materials and methods

Patients and strains

A total of 1,353 *H. influenzae* strains isolated from cerebrospinal fluid (CSF) collected from pediatric patients with bacterial meningitis were sent to Kitasato Institute for Life Sciences from clinical laboratories at 285 Japanese medical institutions between January 2000 and December 2011. CSF samples sent by pediatricians for identification of the causative pathogen also included 23 samples containing *H. influenzae* DNA. These strains and clinical samples were sent to our laboratory accompanied by two documents that protected the anonymity of the patient: one is a record of the informed consent obtained from the guardians of the infants and children, and the other is a survey form that was filled out by the attending physician.

Genotypic determination of β-lactam resistance was performed immediately by polymerase chain reaction (PCR) on all isolates received to determine *ftsI* gene mutations as described in the following section. These PCR results were immediately reported to the referring pediatrician and the laboratory technicians.

Polymerase chain reaction

Conventional PCR [16] was performed on *H. influenzae* isolates using six sets of primers that we had constructed for routine use in our laboratory: P6 primers to amplify the *p6* gene for identification of the *H. influenzae* species; TEM-1 primers to amplify a part of the TEM-1-type β-lactamase gene (bla_{TEM}); ROB-1 primers to amplify a part of the ROB-1-type β-lactamase gene (bla_{ROB}); PBP3-S primers to identify an Asn526Lys amino acid substitution in the *ftsI* gene; PBP3-BLN primers to identify Asn526Lys and Ser385Thr amino acid substitutions in the *ftsI* gene; and serotype b primers to amplify a part of the Hib-specific *capB* locus. PCR cycling conditions were 35 cycles at 94 °C for 15 s; 53 °C for 15 s, and 72 °C for 15 s.

Thereafter, Asn526Lys and Ser385Thr amino acid substitutions were separately identified by the real-time PCR method we constructed in 2007 [17].

Isolates suspected to have an Arg517His substitution based on susceptibility to ampicillin (AMP) and cefotaxime (CTX) were subjected to direct sequencing to detect this substitution, because useful primers could not be designed.

Genotypic resistance patterns were classified as follows: gBLNAS, without any of the three substitutions; gBLPAR, producing β-lactamase TEM-1 or ROB-1; gLow-BLNAR, with substitution of Asn526Lys or Arg517His; gBLNAR, with two or three substitutions, Asn526Lys or Arg517His, as well as Ser385Thr; gBLPACR-I, producing β-lactamase but having a gLow-BLNAR genotype; and gBLPACR-II, also producing β-lactamase but having a gBLNAR genotype.

Statistical analysis

We used Microsoft Excel 2010 for Statistics (SSRI, Tokyo, Japan) and Prism Version 5.0 (GraphPad Software, La Jolla, CA, USA) for data analysis. Categorical variables were compared using chi-squared tests. Continuous variables were compared using Student's *t* test. A *p* value less than 0.05 indicated a significant difference between groups.

Results

Changes in resistance among strains for year to year

A breakdown of Hib and nontypeable *H. influenzae* (NTHi) among 1,353 isolates and 23 spinal fluid samples collected from pediatric inpatients with *H. influenzae* meningitis during the study period is shown in Table 1. Among all isolates, 98.9 % were identified to be serotype b; the remaining 1.1 % represented NTHi. One quarter of spinal fluid samples were shown to contain Hib DNA by real-time PCR. No other serotypes were recognized.

Figure 1 shows year-to-year changes in β-lactam resistance among strains. Resistance was identified molecularly by conventional PCR (from 2000 to 2009) and by real-time PCR (from 2010 to 2011) for the *ftsI* gene encoding PBP3, the bla_{TEM} gene encoding TEM-1 β-lactamase, and the bla_{ROB} gene encoding ROB-1 β-lactamase, respectively [16, 17]. For strains that showed discrepancies between their susceptibility for AMP or CTX and the results of PCR, the *ftsI* gene was analyzed by sequencing.

As shown in Fig. 1, Hib gBLNAR first was identified as a novel resistant strain in 2000. Since then, the resistance rate has increased exponentially over time, exceeding 60 % in 2009 and reaching approximately 70 % in 2011. Over the same interval, gBLNAS and gBLPAR, respectively, decreased from 32 and 26 % in 2000 to 8 and 0 % in 2011.

Distributions of patient age and β-lactam resistance by year

Yearly distribution of patient age and β-lactam resistance according to genotypic identification is shown in Fig. 2. Hib vaccination of children began on a voluntary basis on December 19, 2008. Subsequently, the immunization rate for Hib in Japanese children up to 1 year old is estimated to have been approximately 10 % in 2009, 20 % in 2010, and 50–60 % in 2011, representing an increase every year (data not shown here).

Although longitudinal surveillance demonstrated that the largest number of patients up to 1 year old continued until 2008, these patients decreased beginning in 2009 when Hib vaccination started. In 2011, the total of cases decreased dramatically to 46, about half the usual collected strains. Further, no differences in prevalence of gBLNAR were seen between age groups.

Details of sequelae

Details of sequelae in patients with *H. influenzae* meningitis are listed in Table 2.

This information was obtained from the questionnaires completed by attending physicians. Among the 655 responses, details concerning patients with or without sequelae were recorded in 587 cases. Death was reported in 12 patients (2.0 %), whereas serious sequelae, mainly including brain atrophy or infarction, motor dysfunction, and auditory or visual dysfunction, were noted in 104 (17.7 %).

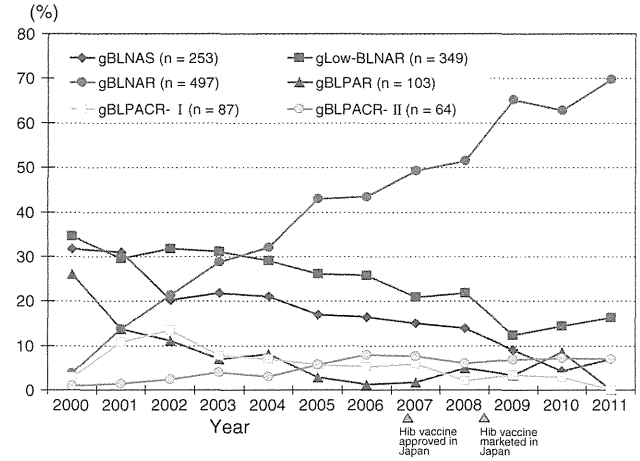

Fig. 1 Year-to-year changes in genotypically classified resistance types among strains isolated between 2000 and 2011. *g* genotype; *Hib* type b *Haemophilus influenzae*; *gBLNAS* β-lactamase-nonproducing, ampicillin (AMP) susceptible *H. influenzae*; *gBLNAR* β-lactamase-nonproducing, AMP-resistant *H. influenzae*; *gLow-BLNAR* β-lactamase-nonproducing, low-level AMP-resistant *H. influenzae*; *gBLPAR* TEM-1 β-lactamase-producing, AMP-resistant *H. influenzae*; *gBLPACR* TEM-1 β-lactamase-producing, amoxicillin/clavulanic acid-resistant *H. influenzae*

Table 1 Strains and samples isolated from pediatric patients throughout Japan with *Haemophilus influenzae* meningitis, by year

Samples	Serotype	Years												
		2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	Total
Strain (n = 1,353)	Hib	104	138	162	129	99	141	150	120	99	89	68	39	1,338
	NTHi		1	2		1	1	2		2		2	4	15
Spinal fluid (n = 23)[a]	Hib						1	1	1	1	2			6
Unknown				2	2	1		1	1		5	2	3	17
Total		104	139	166	131	101	143	154	122	102	96	72	46	1,376

Hib type b *Haemophilus influenzae*, *NTHi* nontypeable *Haemophilus influenzae*

[a] Samples were analyzed by real-time PCR to detect the *capB* gene encoding capsular type b polysaccharide by a method in our laboratory [3]

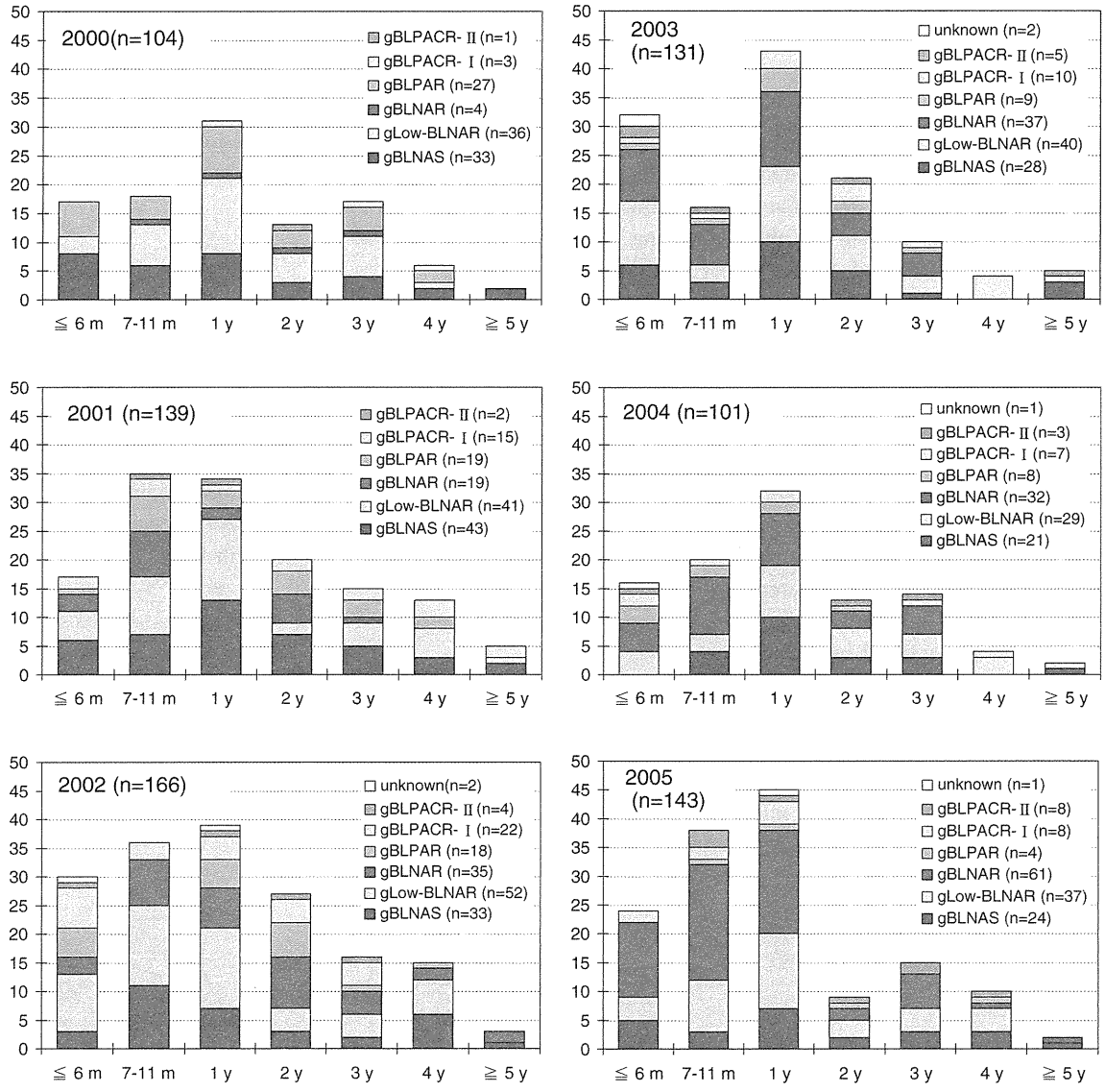

Fig. 2 Year-to-year distribution shows relationships between patient age and genotypically classified β-lactam resistance types among strains isolated between 1999 and 2011

Characteristics of patients with and without sequelae

Table 3 compares characteristics of patients with versus without sequelae after the onset of Hib meningitis. Although sequelae were more frequent in patients 6 months old or younger at the time of onset, significant differences in onset age were observed between the two groups ($p < 0.05$). The presence of disseminated intravascular coagulation (DIC) also significantly affected sequelae.

No significant differences were noted in blood test and spinal fluid test results. Outcome also was not affected by resistance type of the Hib pathogen, namely, whether gBLNAR or not.

Correlation between antimicrobial choice and outcome

Relationships between initial antimicrobial therapy given to meningitis patients on hospital admission and outcomes are shown in Fig. 3. Half the patients ($n = 305$, 52.3 %) received initial therapy with combinations of a third-generation cephem and a carbapenem agent, namely, CTX and meripenem (MEM) or panipenem (PAM), or ceftriaxone (CRO) and MEM or PAM. The next most frequent treatment was AMP and CTX or CRO therapy (18.3 %). Monotherapy with CTX or CRO was given only to 12.7 %.

When the causative agent was identified as *H. influenzae*, the therapeutic regimen was changed to combination

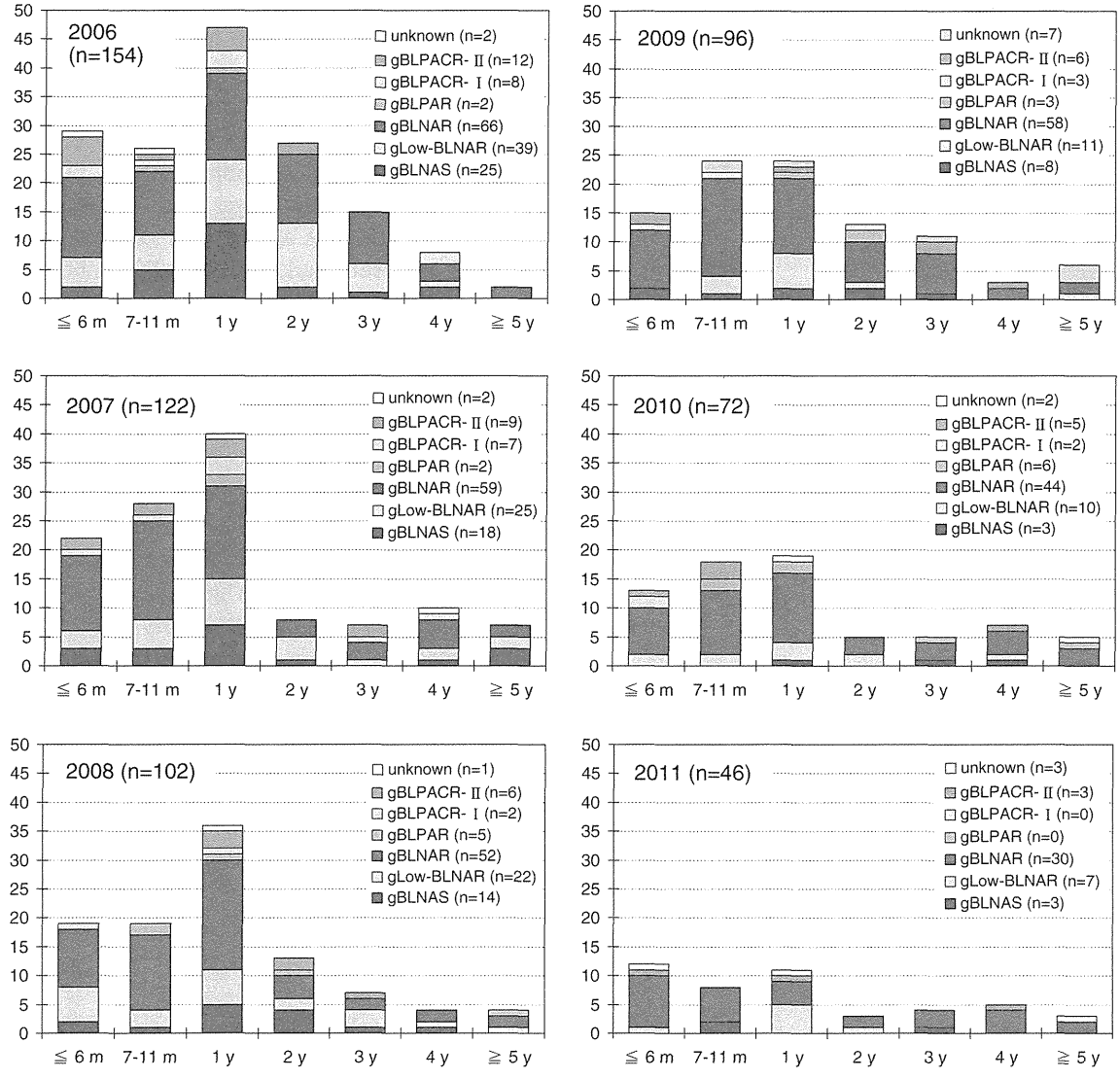

Fig. 2 continued

therapy. A significant relationship between antimicrobial choice and outcome was not observed.

Discussion

Bacterial meningitis is an infectious disease with a high occurrence rate of fatalities and serious sequelae in children. Accordingly, vaccine development has been attempted for many years. The Hib conjugate vaccine was developed to prevent occurrence of bacterial meningitis and severe infections caused by capsular type b *H. influenzae*, the most frequent causative organism. In the United States, Hib vaccine was licensed for use in children in 1987 and entered the standard vaccination schedule in 1990 [18, 19]. As a result, a dramatic decrease in bacterial meningitis from Hib became evident after a few years [19–21]. This vaccination then was introduced in many other countries, where Hib infection now has become a largely eradicated disease.

In Japan, introduction of Hib vaccine was much delayed, for reasons including a low incidence of bacterial meningitis from Hib relative to other countries; high accessibility of Japanese medical institutions; easily used antibiotics; and lack of a feeling of urgency concerning vaccination.

During the decade preceding Japanese adoption of the vaccine, gBLNAR, a new resistant strain, emerged among Hib isolates [1]. These resistant bacteria increased rapidly, causing a major therapeutic problem. Previously, 22–26 % of Hib isolates were β-lactamase-producing strains [1, 3], so the first-choice agent was a third-generation cephem, CTX [22]. To address the problem of gBLNAR, treatment shifted heavily to combination therapy with a third-generation cephem and carbapenem [22], based on the

mechanism of resistance in these strains. The targets of β-lactam antibiotics are the penicillin-binding proteins (PBPs) involved in peptidoglycan synthesis; in BLNAR, the *ftsI* gene encoding PBP3 shows a number of important mutations [12].

Table 2 Sequelae following *Haemophilus influenzae* meningitis

	Number of patients (% of total)
Sequelae (+)	116 (19.8)
Death	12 (2.0)
Brain death	4 (0.7)
Cerebral palsy	1 (0.2)
Hydrocephalus	5 (0.9)
Brain atrophy/brain infarct	22 (3.7)
Epilepsy	5 (0.9)
Motor dysfunction	24 (4.1)
Auditory dysfunction	12 (2.0)
Visual disorder	2 (0.3)
Other[a]	31 (5.3)
Sequelae (−)	471 (80.2)
Total	587

[a] Includes two patients with two sequelae

Therefore, BLNAR susceptibility to cephems, which target mainly PBP3, is 50–100 times decreased compared to that of susceptible organisms. However, because the main target of carbapenems is unrelated to PBP3, susceptibility of BLNAR to carbapenem is not affected [3]. A synergistic effect from a combination of two kinds of agents with their different target sites is expected.

The Hib vaccine was approved in Japan in January 26, 2007, and has been marketed since December 19, 2008. Accurate nationwide vaccination numbers are not available, but the estimated vaccination rate in the infant population (up to 1 year of age), based on numbers of vials delivered, was 10 % in 2009, 20 % in 2010, and 40–60 % in 2011. The high vaccination rate in 2011 is attributable largely to official support provided by the Provisional Special Fund for the Urgent Promotion of Vaccination Against Such Diseases as Cervical Cancer. This initiative includes Hib and PCV7 vaccination of infants.

Incidence of Hib meningitis among children 7 months to 1 year of age has decreased gradually since 2009 in our results, reflecting the effect achieved by Hib vaccination. However, the decrease in incidence among infants under 6 months of age is less evident than that among those between 7 months and 3 years old. Infants vaccinated at such a young age may have difficulty producing antibody titers sufficient to prevent Hib infection at that stage.

Table 3 Characteristics of children with Hib meningitis with and without sequelae

Characteristics	Sequelae (+) (n = 116)	Sequelae (−) (n = 471)	p value
Age			
≤6 months	28 (24.1 %)	77 (16.3 %)	*0.049*
7–11 months	24 (20.7 %)	106 (22.5 %)	0.673
1 year	32 (27.6 %)	118 (25.1 %)	0.575
>2 years	32 (27.6 %)	170 (36.1 %)	0.084
Underlying disease (+/−)	12/95 (11.2 %)	46/389 (10.6 %)	0.847
Initially seen at an other hospital (+/−)	74/27 (73.3 %)	327/96 (77.3 %)	0.389
DIC (+/−)	31/80 (27.9 %)	37/414 (8.2 %)	*<0.001*
Blood values			
WBC (cell/μl)	9,150[a] (5,397–14,375)[b]	11,200 (7,100–16,720)	0.562
PLT (10^4/μl)	18.4 (8.9–31.3)	21.2 (13.8–31.7)	0.656
CRP (mg/dl)	15.4 (7.3–22.8)	14.0 (7.5–20.8)	0.587
Spinal fluid values			
Cells (cell/μl)	6,176[a] (2,718–11,170)[b]	7,872 (3,375–14,720)	0.235
Glucose (mg/dl)	21 (5–42)	31 (10–51)	0.342
Protein (mg/dl)	159 (98–240)	142 (96–212)	0.762
Steroid therapy (+/−)	88/8 (91.7 %)	384/25 (93.9 %)	0.428
gBLNAR + gBLPACR II	41(35.3 %)	166 (35.2 %)	0.983

Fifty percent of all subjects included in the range

DIC disseminated intravascular coagulation, *PLT* platelets, *CRP* C-reactive protein, *gBLNAR* genotypic ampicillin (AMP)-resistant *H. influenzae*, *gBLPACR* genotypic TEM-1 β-lactamase-producing, amoxicillin/clavulanic acid-resistant *H. influenzae*

[a] Median value analyzed by box-and-whisker plots

[b] Value shown in parentheses is 25 percentile and 75 percentile analyzed by box-and-whisker plots

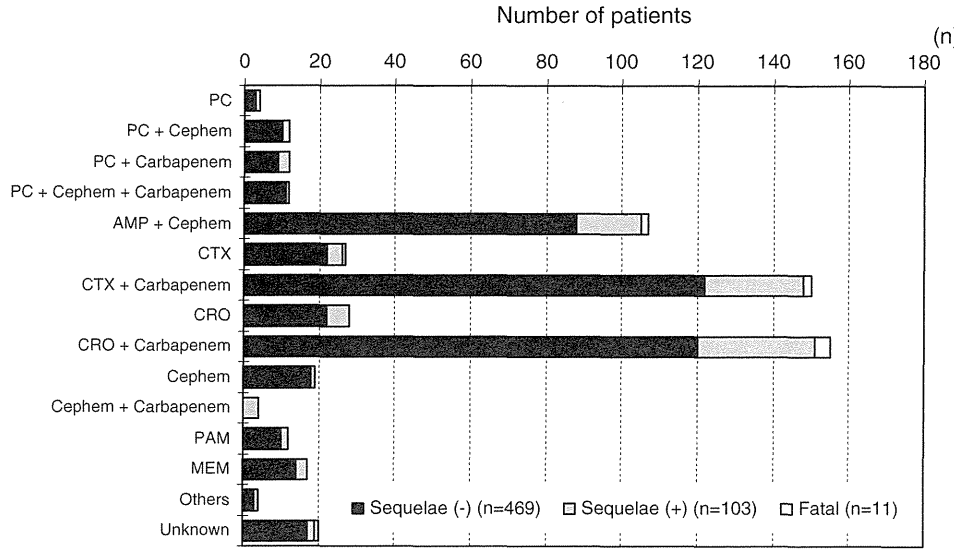

Fig. 3 Correlation between initial antimicrobial therapy given to meningitis patients upon hospitalization and outcomes. *PC* piperacillin (PIPC) and PIPC/tazobactam, *AMP* ampicillin, *CTX* cefotaxime, *CRO* ceftriaxone, *PAM* panipenem, *MEM* meropenem

Continued surveillance is needed to see how the incidence of Hib meningitis may change in the future.

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