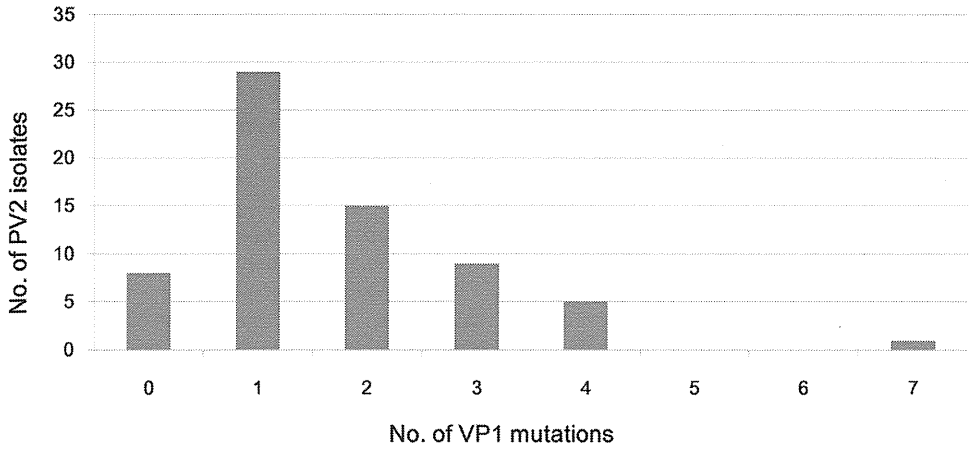

Figure 2. VP1 mutation numbers of environmental PV2 in Shandong from May 2012 to June 2013.
doi:10.1371/journal.pone.0083975.g002

2, VDPV2 strain E12–221 still maintains the temperature sensitive phenotype. The three times of repeat showed the similar results.

Phylogenetic analysis

According to the estimated evolution time of 160 or 176 days of VDPV2 strain E12–221, the VP1 coding regions of all other PV2 strains isolated from sewage collected from May to December 2012 (n = 28) were sequenced. Also, the VP1 coding regions of the environmental PV2 strains isolated from sewage collected from January to June 2013 (n = 38) were sequenced. The results showed they were all Sabin strains. The VP1 substitution numbers of these strains only ranged from 0 to 4 and no other VDPV strain was identified. The phylogenetic tree based on the VP1 sequences of these environmental strains (n = 67, including strain E12–221) revealed that no close relationship was observed between strain E12–221 and any other strains isolated six months before and after (Figure 1). Only four environmental strains shared identical substitutions with this VDPV2 strain. Strains E12–179, E12–180 and E12–188 isolated in October 2012 shared one substitution (A to G at position 2557 of the genome) with the strain E12–221. Strain E12–220 isolated in December 2012 shared one substitution (A to G at position 2986) with the strain E12–221.

Table 3. Hot sites of amino acid substitutions in the VP1 of 67 PV2 isolates in comparison to Sabin strain.

VP1 amino acid position	Substitution	Number of isolates
26	S to G	4
36	A to T	5
40	E to D	4
43	A to V	10
	A to T	1
56	V to E	5
143	I to T	13
	I to N	9
	I to V	2
	I to S	1

doi:10.1371/journal.pone.0083975.t003

The mutation numbers in VP1 nucleotide and amino acid sequences of all environmental PV2 strains in this study was illustrated in Figure 2. PV2 strains with 1 nucleotide or amino acid mutation were the most frequently isolated. A total of 29 amino acid substitutions were found in the 67 environmental strains in VP1 sequence, most of which located near the amino terminal of VP1. Six hot sites for amino acid substitution were identified (Table 3). Unsurprisingly, the Ile143Thr mutation was one of these hot sites.

Reported vaccination rate and AFP surveillance data

The vaccination rate of OPV dose 1, 2, 3, and 4 in Jinan city in 2012 is reported to be 99.75%, 99.67%, 99.75% and 99.70%, respectively. The incidence of AFP cases in Jinan city is reported to be 5.05, 4.80 and 5.92 per 100 000 in 2010, 2011 and 2012, respectively.

Rapid assessment of vaccination rate

A total of 1800 children in Jinan city were investigated, and 98.89% had completed the OPV immunization.

Discussion

Since the last WPV associated paralytic poliomyelitis patient in 1991, Shandong Province had maintained polio free for 22 years. However, VPDV had been detected in Shandong AFP surveillance system in 2007 (P1, 9 nt; P1, 13 nt), 2009 (P2, 11 nt) and 2011 (P1, 10 nt), respectively. They were all ambiguous VPDV (aVDPV) which were clinical isolates from persons with no known immunodeficiency [4].

As a supplemental method to AFP surveillance for global poliomyelitis eradication, environmental surveillance is of great importance in investigating the circulation of WPV or VDPV [23–26]. Enterovirus environmental surveillance had been conducted in Shandong Provincial Poliovirus Laboratory since February 2008. Till June 2013, a total of 351 PVs has been isolated but no WPV was detected. Currently, OPV is still the major vaccine used for routine vaccination in Mainland China, therefore, resulting in the frequent detection of Sabin strains from sewage. By far strain E12–221 was the only VDPV strain detected in the environmental surveillance. In alignment with other environmental PVs collected six months before and after the collection date of the strain, no close relationship was found among themselves, suggesting the VDPV strain did not form a continuous transmission chain in local

population. Also no closely related virus was obtained in local population via acute flaccid paralysis surveillance. So, the evidence available so far is still insufficient to conclude whether the VDPV was derived from importation from other regions or from local persons with primary immunodeficiency.

The A481G change in 5-noncoding region, along with an amino acid substitution (I143T) at nucleotide position 2909 (U to C) in VP1 have been well known to be responsible for the neuovirulent reversion and the loss of temperature sensitivity phenotype in type 2 VDPVs. In strain E12–221, only at position 481, nucleotide A has mutated to G but the I143T mutation at position 2909 (U to C) in VP1 was not observed even though a total of 7 amino acid mutations located in the VP3, VP1, 3C and 3D region were identified (Table 1). The lack of the I143G neurovirulent reversion mutation may help to explain why the E12–221 still maintains the temperature sensitive phenotype. Instead, for the 67 environmental strains, I143T in VP1 appears to be one of the hot spots of mutation (Table 3). It would be interesting to know, whether, among these strains with I143T, some of them also have the G481A mutation. Altogether, these results suggest that all these environmental type 2 vaccine strains have the tendency to evolve by gaining mutations. The acquirement of the two important ones at position 481 and 2909 is just a matter of time if their circulation in population cannot be interrupted by the high OPV coverage.

Shandong is a province with a high OPV coverage. According to the results of PV neutralization antibody examination in healthy population in 2011, the positive rate of PV1, 2, and 3 was 96.6%, 96.8% and 90.5%. The positive rate for children <2 years of age was 98.0%, 99.0% and 94.0%, respectively (data not shown). However, under such high OPV immunization circumstances, the VDPVs were still occasionally detected from AFP and environmental surveillance system in recent years, suggesting that the evolution of VDPV is not necessarily related to the poor level of local OPV immunization. The phylogenetic analysis revealed no close evolutionary relationship of other environmental PV2 to the VDPV in this study. Taken the relative less numbers of mutations of these VDPVs in Shandong (≤13 nt) into account, the high OPV coverage is suggested to play an important role in interrupting the further transmission of VDPV in local population. Hence, it is concluded that high OPV coverage cannot completely prevent the emergence of VDPV, but can block the occurrence of cVDPV.

Ever since the last WPV associated paralytic poliomyelitis cases was observed in Mainland China in 1994, four incidents of WPV importation has been reported. The most recent importation in Xinjiang had caused an outbreak that claimed 21 poliomyelitis cases. The poor local AFP surveillance network and OPV immunization may be the impulse to the spread of WPV. Environmental surveillance has been demonstrated to play an important role in early-warning of outbreak [27]. Previously, we reported the isolation of a type 3/type 2 recombinant poliovirus with chimeric capsid VP1 protein from sewage in Shandong in 2009 [28]. No such virus was identified from local AFP surveillance system at that time. In this study, the VDPV was isolated from sewage while no related AFP cases were reported. Hence, AFP surveillance combined with continuous environmental surveillance should be of great importance in improving the sensitivity of poliovirus detection.

Conclusions

The present study describes the characterization a type 2 VDPV isolated from sewage in China. The surveillance of VDPVs will become increasingly important since OPV becomes the only remaining source of poliovirus infection in polio free regions. The results presented here confirmed the importance of environmental surveillance in GPEI.

Author Contributions

Conceived and designed the experiments: ZT YZ HY AX WX. Performed the experiments: ZT YZ XL PX SZ SW DY NC. Analyzed the data: ZT YZ YL HY LS. Contributed reagents/materials/analysis tools: ZT SZ HW. Wrote the paper: ZT YZ.

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ORIGINAL ARTICLE

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

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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

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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

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 %).

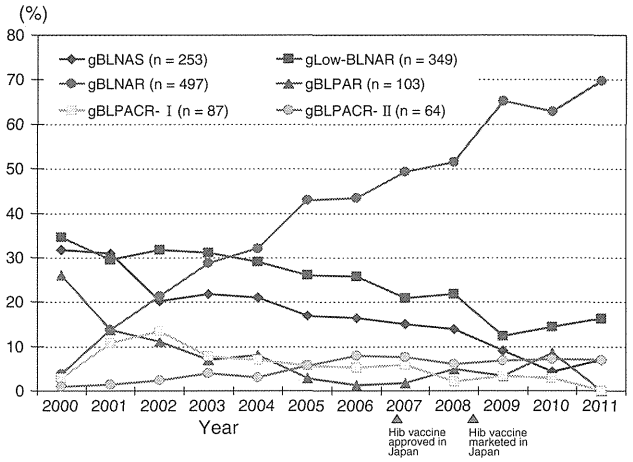

Fig. 1 Year-to-year changes in genotypically classified resistance types among strains isolated between 2000 and 2011. *g* genotype; *Hib* type b *Haemophilus influenzae*; *gBLNAS* β-lactamase-nonproducing, ampicillin (AMP) susceptible *H. influenzae*; *gBLNAR* β-lactamase-nonproducing, AMP-resistant *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]

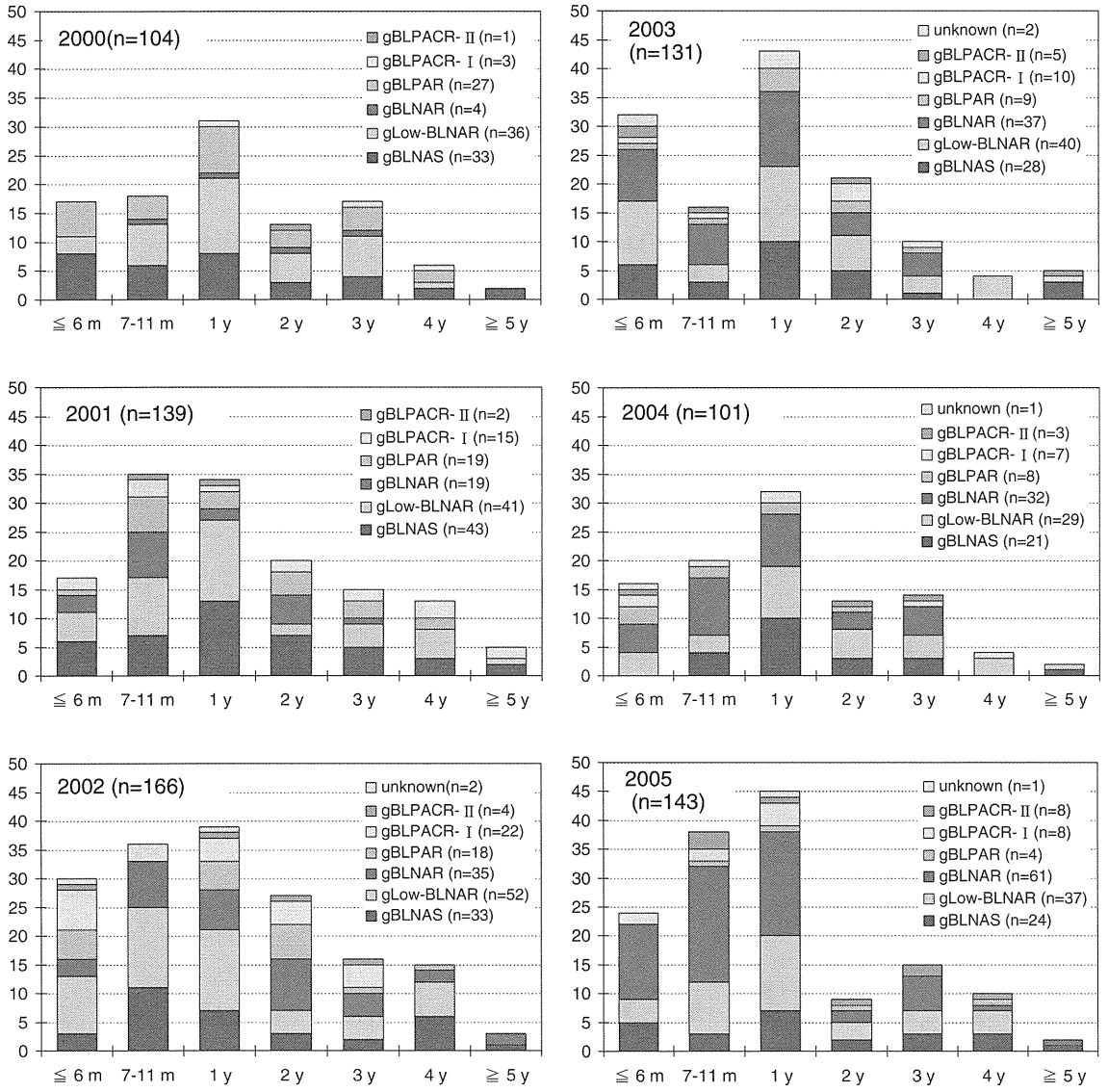

Fig. 2 Year-to-year distribution shows relationships between patient age and genotypically classified β-lactam resistance types among strains isolated between 1999 and 2011

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

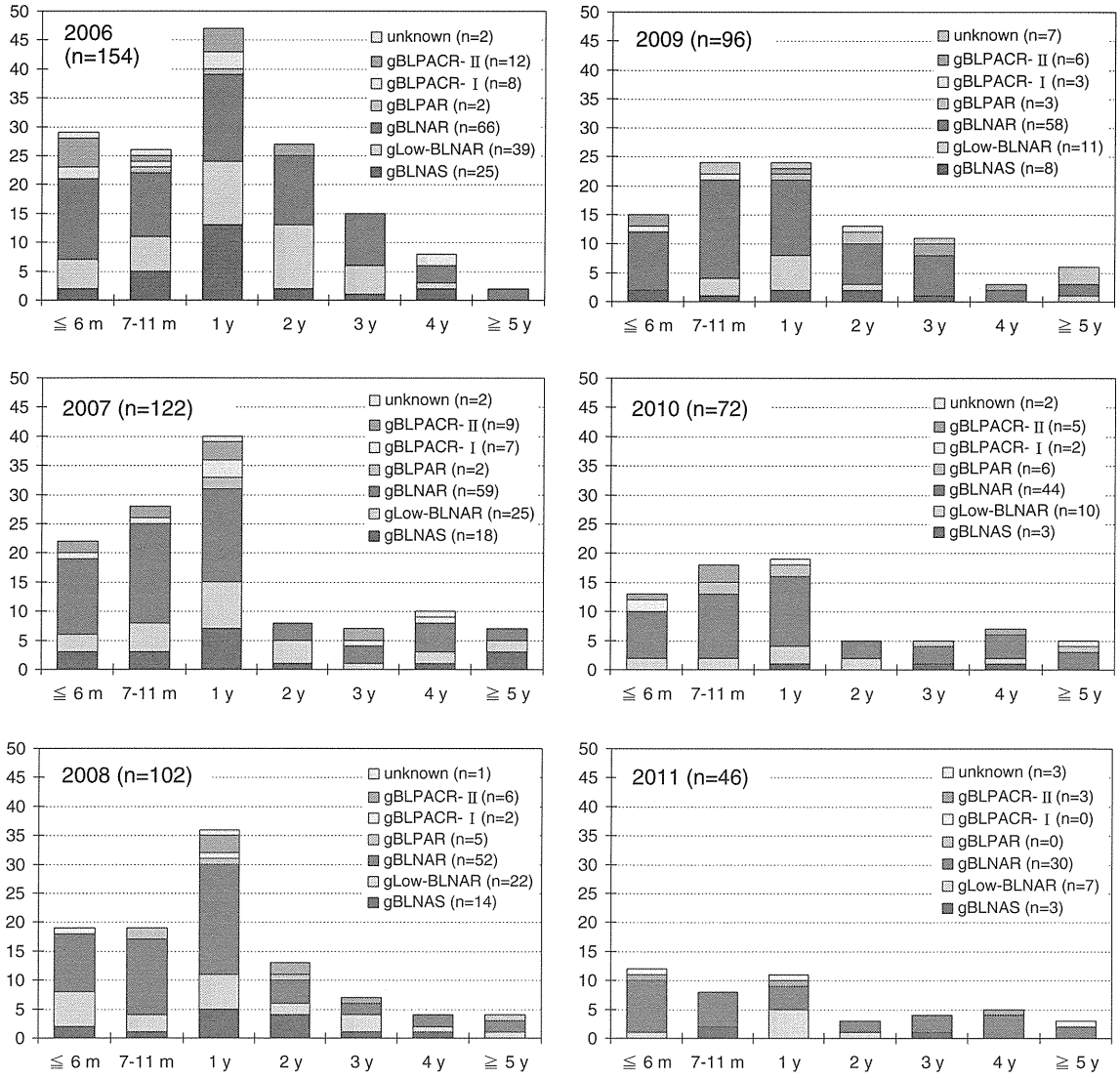

Fig. 2 continued

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

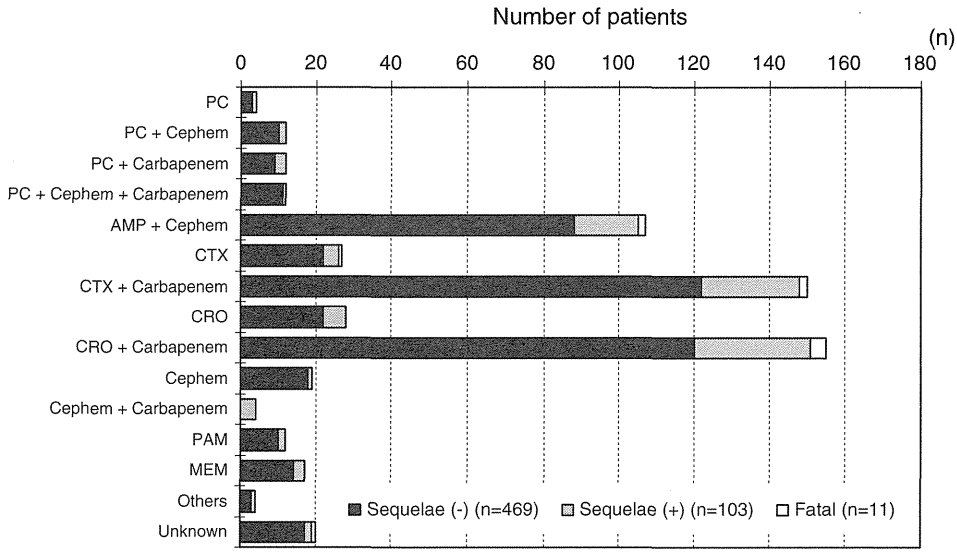

Fig. 3 Correlation between initial antimicrobial therapy given to meningitis patients upon hospitalization and outcomes. *PC* piperacillin (PIPC) and PIPC/tazobactam, *AMP* ampicillin, *CTX* cefotaxime, *CRO* ceftriaxone, *PAM* panipenem, *MEM* meropenem

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

Acknowledgments We deeply thank the pediatricians and laboratory technicians belonging to the 285 medical institutions for active cooperation. We also thank Keiko Hamano-Hasegawa for cooperation in construction of the PCR method and surveillance study. This study was supported in part by a grant under the category "Research on Emerging and Re-emerging Infectious Diseases" (number H21-002 and H22-013) from the Japanese Ministry of Health, Labour and Welfare (to K. Ubukata).

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Rapid Decrease of 7-Valent Conjugate Vaccine Coverage for Invasive Pneumococcal Diseases in Pediatric Patients in Japan

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In Japan, the heptavalent pneumococcal conjugate vaccine (PCV7) has been introduced on a voluntary basis since February 2010, and official financial support for children under 5 years started in November 2010. The impact of PCV7 on invasive pneumococcal diseases (IPD) in children is unknown. There are 340 medical institutions that actively participated in our surveillance project throughout Japan. We collected 252 strains from patients with IPD in 2006 (pre-PCV7), 280 strains in 2010 (under 10% immunization achieved), and 128 strains in 2011 (50% to 60% immunization). Serotypes and penicillin-resistance genotypes (g) were compared between these years. Multilocus sequence typing was also carried out on these strains. Due to the official promotion, IPD significantly decreased in 2011 ($p<0.001$). In particular, meningitis and sepsis caused by vaccine type (VT) strains declined ($p=0.033$, $p<0.001$). In less than 2 years, among nonvaccine types (NVT), 15A and 22F increased in 2011 ($p=0.015$, $p=0.015$). Coverage by PCV7 decreased from 71.8% in 2006 to 51.6% in 2011. Sequence-type diversities accompanied by evolution to gPRSP occurred in both VT and NVT strains. Reduction of IPD caused by VT strains was accomplished, but a rapid increase of NVT raises concern about a future decrease in the efficacy of PCV7.

Introduction

STREPTOCOCCUS PNEUMONIAE is a leading etiologic agent in children with severe invasive infections that contribute importantly to morbidity and mortality.[19] Invasive pneumococcal diseases (IPD) caused by penicillin G (PEN)-resistant *S. pneumoniae* (PRSP) have emerged and spread rapidly worldwide.[2,17]

In the United States, heptavalent pneumococcal conjugate vaccine (PCV7) was introduced in February 2000 and added to the immunization schedule for children in October 2000.[1] Subsequent surveillance studies have demonstrated a decrease in the prevalence of pneumococcal infection caused by vaccine type (VT) serotypes and PRSP.[4,22]

Currently, children in over 100 countries have undergone PCV7 immunization, followed by substantial regional decreases of IPD.[18] However, increases in pneumococcal infections caused by nonvaccine types (NVT), such as PRSP with serotypes 19A and 6A,[12] suggest that NVTs are emerging and are replacing VT serotypes in some countries.[15,16,21] Other NVT serotypes such as 15A and 35B have been reported to be increasing in the US[13] and elsewhere.[14]

In Japan, PRSP has increased as a causative pathogen among children with respiratory tract infections, acute otitis media, and IPD since the late 1990s.[6,26,27] PCV7 was approved in October 2009 and has been clinically used in infants on a voluntary basis since February 2010, but the vaccination rate was estimated to be under 10% in that year. From November 2010, PCV7 vaccination was encouraged for the children under 5 years old throughout Japan by an official program, the Provisional Special Fund for the Urgent Promotion of Vaccination. As a result, PCV7 immunization was estimated to have reached 50% to 60% in 2011.

In the present study we aimed to clarify the changes in serotypes and genotypes favoring resistance to β-lactam antibiotics in *S. pneumoniae* isolated from pediatric patients with IPD. A possible relationship between the results of multilocus sequence typing (MLST) and serotype or genotype was also evaluated.

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Materials and Methods

Patients and pneumococcal strains

All subjects were pediatric patients under 18 years old with IPD. Isolates from sterile clinical samples such as blood, cerebrospinal fluid, pleural effusion, and joint fluid were examined. Clinical laboratories serving 340 Japanese medical institutions actively participated in this surveillance project after written permission was granted by the laboratory director or hospital director. Surveillance was performed from May 2006 to April 2007 (designated 2006), May 2010 to April 2011 (designated 2010), and May 2011 to April 2012 (designated 2011). The 3 periods, respectively, correspond to the year preceding the introduction of PCV7 (pre-PCV7); the year of voluntary immunization (vol-PCV7; less than 10% immunization achieved); and the year of official promotion (post-PCV7; 50% to 60% immunization).

Clinical isolates were promptly sent to our laboratory, accompanied by a survey form filled out anonymously by the attending physician. The following information was collected from all patients: patient age at onset, sex, specifics of the disease, prognosis, and blood test results, sequel, and outcome. Our survey form was based on the format of the Active Bacterial Core Surveillance case report (ABCs).

Serotypes and antibiotic-resistant genotypes

Serotypes of all isolates were determined by the capsular quellung reaction, using antiserum purchased from the Statens Serum Institute (Copenhagen, Denmark).

Alterations in 3 PBP genes mediating β-lactam resistance in *S. pneumoniae*—*pbp1a* (PBP1A), *pbp2x* (PBP2X), and *pbp2b* (PBP2B)—were identified by real-time PCR methods that we have reported previously.[7] The *lytA* gene encoding the autolysin enzyme specific to *S. pneumoniae* was analyzed similarly. The genes *mef* (A) and *erm* (B), which confer resistance to macrolide (ML) antibiotics, were also identified.[7]

Genotype (g) based on molecular analysis is represented here as PEN-susceptible *S. pneumoniae* (gPSSP) possessing 3 normal *pbp* genes; PEN-intermediate *S. pneumoniae* (gPISP), further classified as gPISP (*pbp2x*), gPISP (*pbp1a+pbp2x*), or gPISP (*pbp2x+pbp2b*); or PEN-resistant *S. pneumoniae* (gPRSP) possessing all 3 abnormal *pbp* genes. The relationship between susceptibility to parenteral agents among phenotype *S. pneumoniae* and resistance genotype was described previously.[7]

Results of serotype and resistance genotype analysis for each strain were immediately relayed to the referring pediatrician and local laboratory technicians.

Multilocus sequence typing

MLST was performed on a total of 408 strains obtained in 2010 and 2011 according to the previously described methods,[11] with slight modifications. Primers posted on the CDC website (www.cdc.gov/ncidod/biotech/strep/alt-MLST-primers.htm) were used except for the forward primer for the *ddl* gene.[25] MLST and eBURST analyses were performed according to the MLST website (http://spneumoniae.mlst.net).

Statistical analysis

Microsoft Excel 2010 for Statistics (SSRI, Tokyo, Japan) was used for data analyses. Categorical variables were compared using chi-squared tests.

Results

Patient age and capsular serotype

The age distribution of patients with IPD according to the serotype of isolates (VT vs. NVT) and the year when isolated are shown in Table 1.

Specimens were collected throughout Japan in 2006 ($n=252$), 2010 ($n=280$), and 2011 ($n=128$). The years corresponded to pre-PCV7, vol-PCV7 (PCV7 immunization rate below 10%), and post-PCV7 (50% to 60% PCV7 immunization rate).

The total number of cases in 2011 was significantly lower compared with that in 2006 or 2010, especially in patients under 2 years old ($p<0.001$). Overall, coverage by PCV7 in the 3 periods decreased from 71.8% in 2006 to 51.6% in 2011. Inversely to the proportion of VT, the proportion of NVT serotypes increased from 28.2% in 2006 to 48.4% in 2011($p<0.001$).

Year-to-year changes in VT and NVT prevalence by disease

Table 2 compares the VT and NVT serotype prevalence among the isolates from various types of IPD: meningitis, sepsis and bacteremia, pneumonia, etc., during each of the 3 years studied. Pneumonia was confined to cases in which *S. pneumoniae* was isolated from blood culture. VT strains decreased significantly in meningitis and sepsis cases through the three periods ($p=0.033$, $p<0.001$).

Year-to-year changes in serotype and resistance genotype

Year-to-year changes in the serotypes and resistance genotypes in children under 5 years old subjected to immunization by the official promotion are shown in Figs. 1 and 2.

TABLE 1. VACCINE-TYPE AND NONVACCINE TYPE FOR 3 YEARS, ACCORDING TO AGE

		Year			
Age	*Serotype*	*2006 (n=252)*	*2010 (n=280)*	*2011 (n=128)*	*p-Value*
≤1 year	VT[a]	113	141	40	<0.001
	NVT[b]	38	45	43	
2–4 years	VT	60	54	20	0.347
	NVT	16	22	10	
≥5 years	VT	8	9	6	0.492
	NVT	17	9	9	
Total	VT	181 (71.8)[c]	204 (72.9)	66 (51.6)	<0.001
	NVT	71 (28.2)	76 (27.1)	62 (48.4)	

[a]VT, serotypes (4, 9V, 18C, 6B, 14, 19F, 23F) included in PCV7.
[b]NVT, serotypes not included in PCV7.
[c]The number of cases is followed by the percentage in parentheses.
VT, vaccine type; NVT, nonvaccine types; PCV7, pneumococcal conjugate vaccine.

TABLE 2. YEAR-TO-YEAR CHANGES VACCINE-TYPE AND NONVACCINE TYPE PREVALENCE BY DISEASE

Diseases	*Serotype*	*2006 (n=252)*	*2010 (n=280)*	*2011 (n=128)*	p-*Value*
Meningitis	VT[a]	46	40	13	0.033
	NVT[b]	21	18	17	
Sepsis and bacteremia	VT	90	98	33	<0.001
	NVT	37	45	40	
Pneumonia	VT	38	51	12	0.715
	NVT	10	9	3	
Other	VT	7	15	8	0.832
	NVT	3	4	2	
Total	VT	181	204	66	<0.001
	NVT	71	76	62	

[a]VT, serotypes (4, 9V, 18C, 6B, 14, 19F, 23F) included in PCV7.
[b]NVT, serotypes not included in PCV7.

Resistance genotypes were classified according to the presence or absence of 3 abnormal PBP genes, *pbp1a*, *pbp2x*, and *pbp2b*, identified by real-time PCR methods.[7] All strains of serotypes 6B, 19F, 14, and 23F were gPRSP, gPISP (*pbp1a+pbp2x*), gPISP (*pbp2x+pbp2b*), or gPISP (*pbp2x*). The number of serotype 14 and 19F decreased in 2011, representing the post-PCV7 period ($p=0.003$, $p=0.044$).

The numbers of NVT 15A and 15C increased significantly in 2011 ($p<0.001$, $p=0.046$). Strains identified as gPRSP, which showed MICs of 0.5 to 4.0 μg/ml for PEN and 1 to 8 μg/ml for cefotaxime, were evident among serotypes 15A, 16F, and 35B.

Changes in the proportion of individual serotypes

Increases and decreases in the proportion of each serotype under 2 years old between 2006 (pre-PCV7) and 2011 (post-PCV7) are shown in Fig. 3. VT serotype 14, which was common among IPD cases, decreased considerably ($p=0.007$). The serotypes of 6B, 18C, and 23F were little changed. Serotype 19A, which is included in a newer vaccine, PCV13, tended to increase ($p=0.056$). Other PCV13 serotypes showed minimal changes. Proportions of NVT serotypes, especially serotypes 15A and 22F, increased (both $p=0.015$).

Multilocus sequence type

MLST was performed on all VT and NVT strains in 2010 and 2011. As presented in Table 3, the sequence type (ST) and clonal complex (CC) were diversified to number 113 for ST and 35 for CC, respectively. Sixty-six of the STs (61.1%) were registered from Japan.

Considering the associations between ST and serotype in gPRSP, 30 strains of serotype 6B were ST90, including CC156, which was submitted as a Pneumococcal Molecular Epidemiology Network (PMEN) clone of Spain^{6B}-2, followed by ST2224 in CC2224, ST902 and ST6413 in CC490, and ST5232 (singleton). Most gPRSP in other VT strains were ST343 in serotype 14, which had evolved from Sweden ST554; ST236 in serotype 19F, representing the Taiwan^{19F}-14 clone; ST1437, which was identified in Japan or ST242 of the Taiwan^{23F}-15 clone in serotype 23F. Notably, gPRSP strains in serotypes 6A and 19A expanded to 11 STs, including ST3111, which had evolved from the original US strain (Alaska; MIC for PEN, 0.03 μg/ml); ST2756, identified in China; and ST282, which developed from the original Vietnam strain. ST81 ($n=2$) and ST156 ($n=1$), recorded as Spain^{23F}-1 and Spain ^{9V}-3 clones, respectively, were very few.

Meanwhile, STs of gPRSP in NVT serotypes were mainly seen ST63 in serotype 15A submitted from Sweden^{15A}-25, originally showing an MIC of 0.12 μg/ml for PEN; ST8351 of serotype 16F, registered in Japan during study; and ST558 in serotype 35B, registered in the US and showing an MIC of 2.0 μg/ml for PEN.

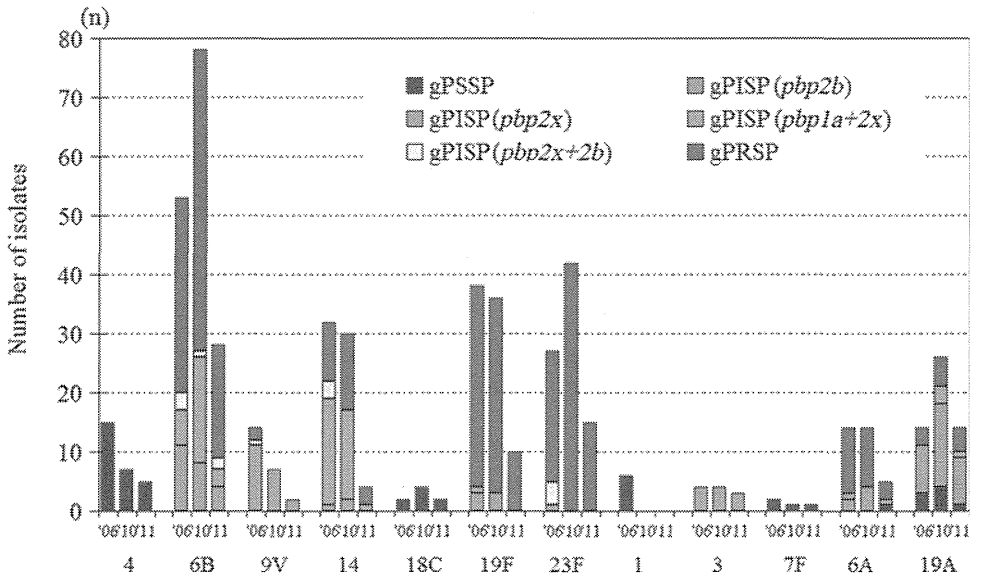

FIG. 1. Changes in the number of heptavalent pneumococcal conjugate vaccine (PCV7) serotypes and those additionally covered by PCV13, and changes in penicillin resistance genotype in the three periods: 2006, 2010, and 2011. These results were limited to isolates from young children under 5 years old. PEN, penicillin G; gPSSP, genotypic PEN-susceptible *Streptococcus pneumonia*; gPISP, genotypic PEN-intermediate *S. pneumonia*; gPRSP, genotypic PEN-resistant *S. pneumonia*.

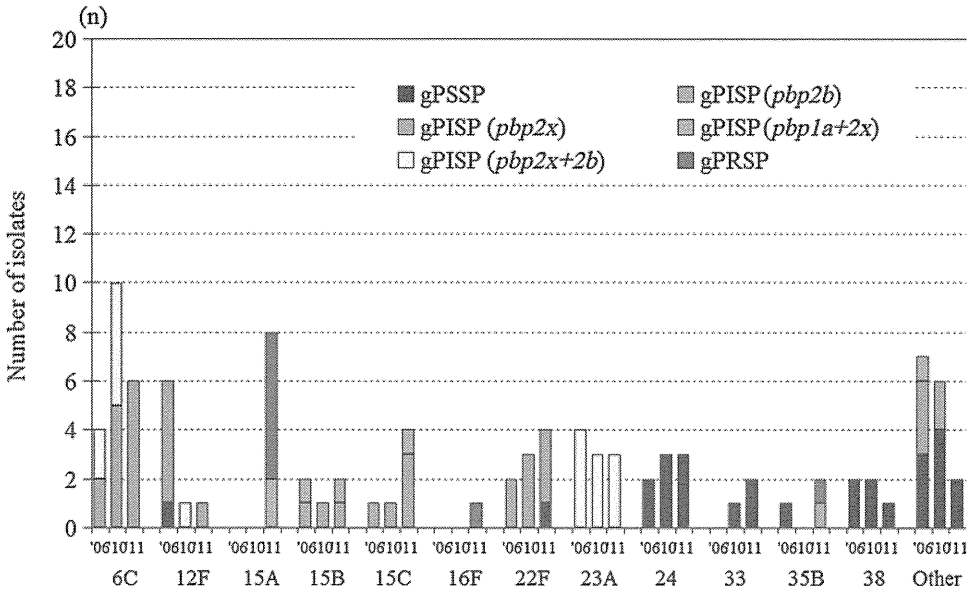

FIG. 2. Changes in the number of nonvaccine serotypes (excluding strains covered by PCV13) and in penicillin-resistant genotypes, in the three periods: 2006, 2010, and 2011.

Variation in the STs and genotypes in serotype 6C and STs in serotype 23A were evident. In addition, gPISP (*pbp2x*) strains with ST199 submitted as the Netherlands[15B]-37 clone had evolved to gPISP (*pbp1a+2x*) by way of *pbp1a* gene alterations in serotypes 15B and 15C.

Notably, 10 cases of capsular switching suggested the following relationships to ST and serotype as follows: between serotypes 15B and 15C in ST199; 14 and 19F in ST236; 6B and 23F in ST242; 23A and 23F in ST338; 6A and 6B in ST2756; 6A, 6B, and 6C in ST2923 and ST3787; 6B and 6C in ST2924; 22F and 24 in ST5496; and 6B and 19F in ST6183.

On the other hand, 93.6% of all strains isolated in 2010 and in 2011 possessed *mef*(A) and/or *erm*(B) genes mediating ML antibiotic resistance.

Discussion

Prevention of IPD, well known for high morbidity and mortality in immunologically immature infants, is an

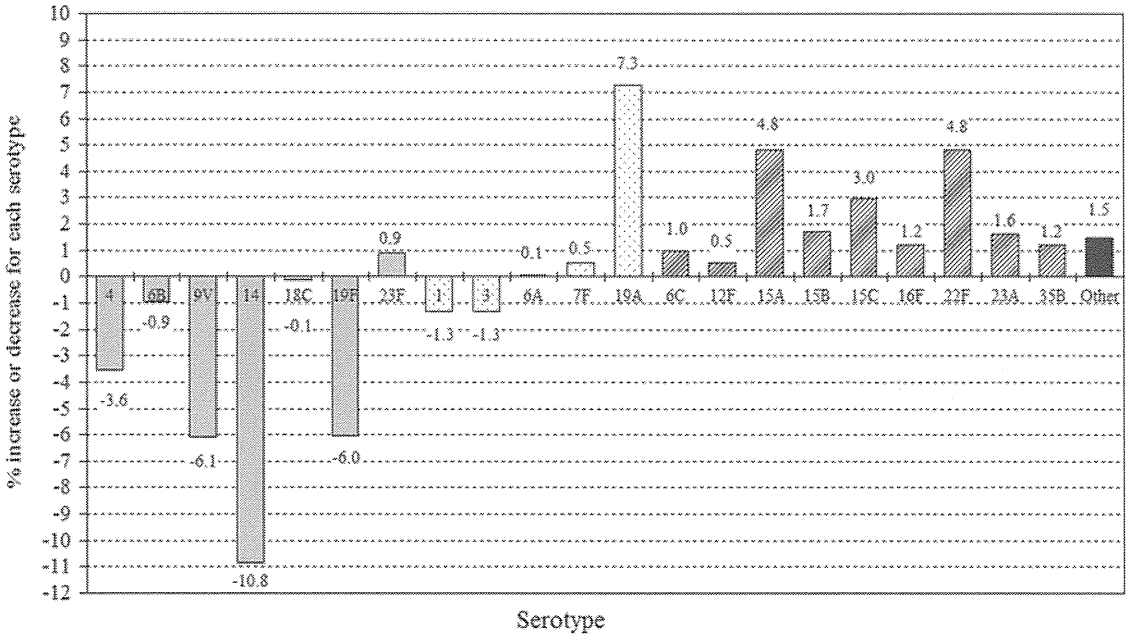

FIG. 3. Proportional increases and decreases in each serotype in 2006 and 2011. This result was limited to isolates from young children under 2 years old. Gray, serotypes covered by PCV7; stippled, serotypes additionally covered by PCV13; diagonal line and black, nonvaccine serotypes not covered by PCV13.

TABLE 3. RELATIONSHIPS BETWEEN MULTILOCUS SEQUENCE TYPES, SEROTYPES, AND PENICILLIN-RESISTANCE GENOTYPES, IN INVASIVE PNEUMOCOCCAL ISOLATES (N=408)

Serotype	*CC*	n	*Genotype*	*ST (n: PMEN clone)*
6B	156	50	R	90(30: Spain^{6B}-2), 95(1), 273(2: Greece^{6B}-22), **5497(1)**, **7870(1)**
			2x+2b	**5830(1)**
			1a+2x	**2983(8)**, **5497(2)**, **7824(1)**
			2x	**2983(3)**
	242	1	R	242(1: Taiwan^{23F}-15)
	490	20	R	902(5), **6413(3)**, **7492(2)**, **8345(1)**, **New(1)**
			2x+2b	902(1)
			2x	**2923(5)**, **5245(1)**, **6430(1)**
	2224	14	R	2224(6), **7835(1)**
			1a+2x	2224(7)
	2924	4	2x+2b	**8348(1)**
			1a+2x	**6183(1)**
			2x	**2924(2)**
	3787	6	R	2756(1), 3787(5)
	7834	2	1a+2x	**7834(2)**
	Group 358	1	R	**7967(1)**
	singleton	8	R	**5232(7)**, **5244(1)**
14	554	12	R	343(9), 3388(1), **7971(1)**, **7974(1)**
	230	9	1a+2x	**5240(7)**, **7966(1)**, **7973(1)**
	15	7	1a+2x	**2922(7)**
	156	3	R	**5493(1)**
			2x	124(1), **7972(1)**
	199	1	R	876(1)
	320	1	R	236(1: Taiwan^{19F}-14)
	singleton	1	R	**New(1)**
19F	320	43	R	236(31: Taiwan^{19F}-14), 926(1), 1421(1), 1428(1), 1464(1), **7873(1)**, **7991(1)**, **8341(1)**, **8342(1)**, **8344(1)**, **8349(1)**
			2x	236(2: Taiwan^{19F}-14)
	156	1	R	**8352(1)**
	242	1		**New(1)**
	2924	1	2x	**6183(1)**
23F	**2924**	31	R	**1437(28)**, **6434(1)**, **7836(1)**, **7872(1)**
	242	25	R	242(21: Taiwan^{23F}-15), 1435(1), 1444(1), **7968(1)**, **8343(1)**
	156	1		338(1: Colombia^{23F}-26)
4	490	11	S	246(10), **New(1)**
	Group 457	1	S	**5872(1)**
9V	156	9	2x	280(6), **5231(3)**
18C	3594	6	S	3594(5), **7829(1)**
6A	3787	11	R	2756(4), **6432(1)**, **6437(1)**, **8350(1)**
			1a+2x	3787(1)
			2x	**3113(1)**, **7969(1)**, 3787(1)
	81	4	R	282(2), 81(2: Spain^{23F}-1)
	3115	1	R	3115(1)
	490	1	2x	**2923(1)**
	singleton	1	R	**7871(1)**
	singleton	1	S	**8347(1)**
19A	3111	25	R	3111(7)
			1a+2x	3111(4)
			2x	3111(13)
			S	**8339(1)**
	2331	13	2x	2331(9)
			S	2331(3), **5842(1)**
	156	1	R	156(1: Spain^{9V}-3)
	320	1	R	320(1)
3	180	7	2x	180(6: Netherlands^{3}-31), **New(1)**

(*continued*)

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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ORIGINAL ARTICLE

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

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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*

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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