表1 2005-06年シーズン本邦6県におけるA/H1N1とA/H3N2のAm耐性株頻度とAm耐性A型インフルエンザ罹患患者の年齢と性別

県*	分離数	Am耐性株/分離数				Am耐性株患者・年齢	Am耐性株患者・性
	/検体数 †	H1N1	(%)	H3N2	(%)	平均（分布）, y	男性 %
宮城	22/22	0/0	(0.0)	15/22	(68.2)	21.3 (1–74)	54.5
山形	18/18	0/0	(0.0)	18/18	(100.0)	3.5 (0.4–8)	38.9
新潟	152/426	0/51	(0.0)	101/101	(100.0)	5.4 (0.2–15)	52.6
群馬	17/28	0/3	(0.0)	14/14	(100.0)	21.2 (1–58)	58.8
福岡	20/20	0/1	(0.0)	7/19	(36.8)	8.6 (1–35)	45.0
長崎	186/236	0/6	(0.0)	76/180	(42.2)	40.5 (5–106)	53.8
計	415/750	0/61	(0.0)	231/354	(65.3)	22.7 (0.2–106)	52.5

Am; アマンタジン耐性

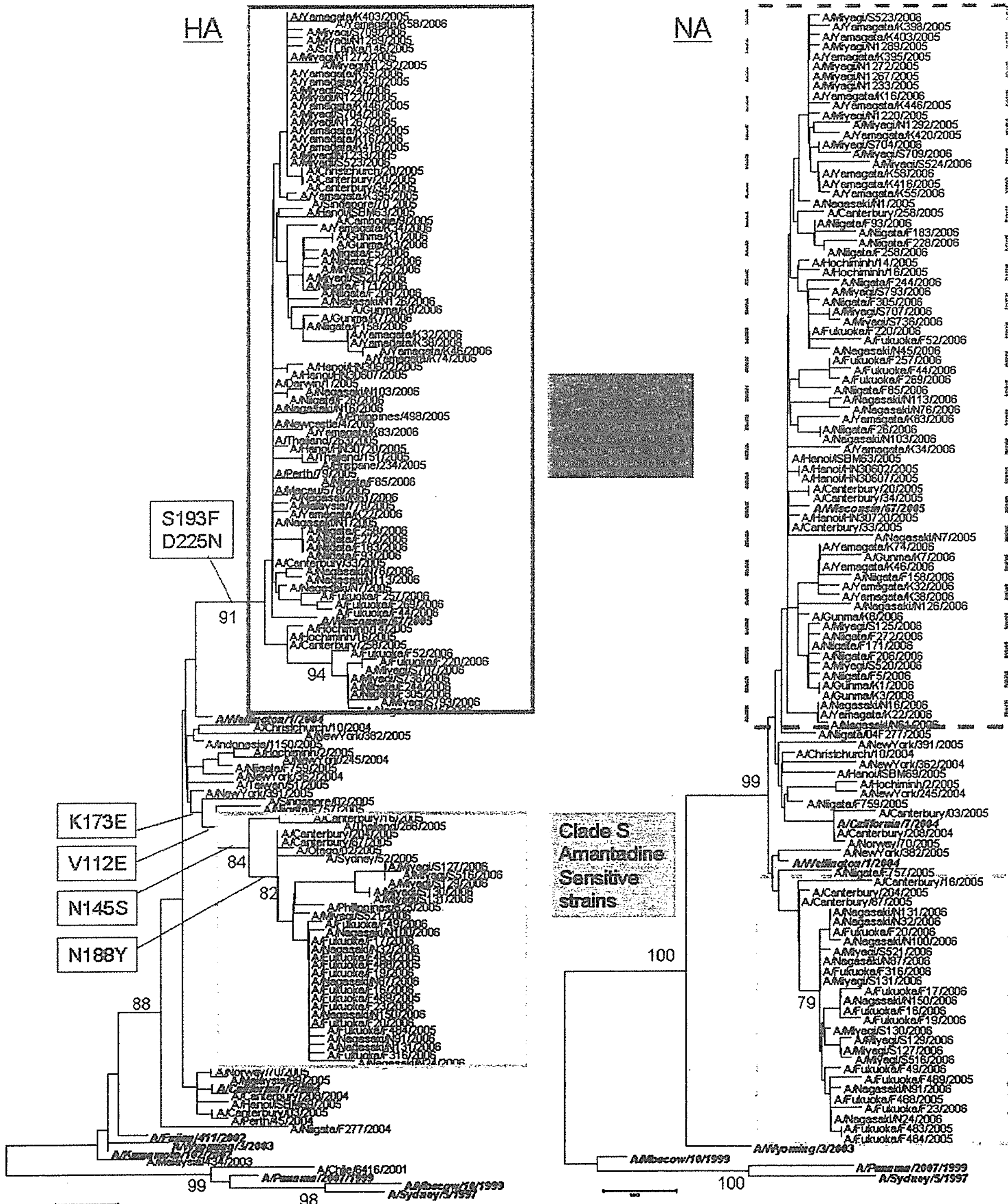
† 新潟、群馬、長崎の検体は咽頭ぬぐい液からウイルス分離、宮城、山形、福岡の検体は分離株から当教室で継代分離

表2　2005-06年シーズンAm耐性および感受性罹患患者の患者背景、初診時臨床症状と投薬内容（長崎のデータのみ解析）

	Am感受性 (N=104) *		Am耐性 (N=75) *		P-value †
患者背景					
年齢 - yr					
平均 ± 標準偏差	37.9 ± 17.5		44.7 ± 20.0		0.017
分布	8.3 – 83.8		5.8 – 83.0		
男性 - no. (%)	62	(59.6)	33	(44.0)	0.034
来院までの日数, days					
平均 ± 標準偏差	1.5 ± 1.1		1.5 ± 1.4		ns
インフルエンザワクチン接種- no. (%) ‡	15	(14.4)	20	(26.7)	0.056
海外渡航歴- no. (%)	0	(0.0)	1	(1.3)	ns
初診時の臨床症状- no./total no. (%)					
発熱 ℃ (average ± standard deviation)	38.4 ± 1.0		38.1 ± 0.9		0.041
頭痛	71	(68.3)	53	(70.7)	ns
咳	88	(84.6)	63	(84.0)	ns
痰	59	(56.7)	45	(60.0)	ns
鼻汁	83	(79.8)	60	(80.0)	ns
下痢	5	(4.8)	3	(4.0)	ns
腹痛	6	(5.8)	6	(8.0)	ns
吐き気	12	(11.5)	8	(10.7)	ns
筋・関節痛	74	(71.2)	50	(66.7)	ns
倦怠感	87	(83.7)	59	(78.7)	ns
治療 - no./total no. (%)					
オセルタミビル	96	(92.3)	63	(84.0)	0.083
ザナミビル	7	(6.7)	11	(14.7)	ns
アマンタジン	1	(1.0)	1	(1.3)	ns
計	104	(100.0)	75	(100.0)	

Note: ns, not significant
* 長崎の症例のみ解析
† 統計解析は2×2表は χ^2 testで、平均値はstudent's t testを用いた。
‡ 2005-06年シーズンのワクチン接種歴

図1　2005-06年シーズンに採取されたA/H3N2株のHA及びNA遺伝子の系統樹解析
Neighbor-joining法
HA長=853bp-HA1subunit、NA長=1388bp



Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧

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Ⅳ．研究成果の刊行物・別刷

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Evaluation of Serotypes of *Streptococcus pneumoniae* Isolated from Otitis Media Patients by Multiplex Polymerase Chain Reaction

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Key Words
Streptococcus pneumoniae · Polymerase chain reaction · Serotype · Serogroup · Acute otitis media

Abstract
The increasing difficulty in the management of pneumococcal acute otitis media in parallel with increases in antimicrobial-resistant strains has led to much interest in pneumococcal capsular types for the adoption of effective prevention by vaccines. This study shows that multiplex polymerase chain reaction is a valuable and expeditious method for the capsular typing of pneumococci. The multiplex polymerase chain reaction method accurately detects the majority of serotypes and serogroups frequently isolated from pediatric patients with acute otitis media, allowing the characterization of the colonization patterns for further implications of pneumococcal vaccines.

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Introduction

Acute otitis media (AOM) is a common infectious disease among children, with *Streptococcus pneumoniae* as the most frequent causative pathogen responsible for this infectious disease [1]. Treatments of pneumococcal AOM become more difficult due to a recent dramatic increase in antimicrobial resistance [2–5]. Thus, prevention of pneumococcal AOM through effective vaccination is gaining further importance. The ability of pneumococci to cause disease is closely related to capsular polysaccharides that are major virulent factors and provide resistance to phagocytosis [6]. Current pneumococcal vaccines are based on the capsular polysaccharides. Although clinical application of 23-valent polysaccharide vaccines successfully reduce the prevalence of severe invasive pneumococcal infections, the effectiveness against AOM is still controversial [7]. A 7-valent protein conjugate vaccine licensed in the United States and Europe has much efficacy against AOM, but sometimes causes switching of causative serotypes [8–10]. It is important to evaluate serotypes covering the current widespread vaccines [11, 12]. However, the current determination of serotypes by immunological methods is time consuming, expensive, and provides difficulties in interpreting the results. Recent studies focus on the pneumococcal typing by molecular biological method.

In this study, we evaluated 4 serogroups and 7 serotypes responsible for AOM among *S. pneumoniae* iso-

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Table 1. Primers for multiplex PCR serotyping

Primers	Serogroup/ serotype	Sequences	Nucleotide number	PCR products, bp
Multiplex PCR 1	serogroup 6	5'-TATAGATCCGATACGACGTAAC-3'	9407–9428	200
		5'-ATACCAATTACACCAAAGTCTG-3'	9580–9592	
	serogroup 19	5'-CTAATGAGCCTAAACGTCTCT-3'	5777–5798	222
		5'-TTGACTGCACCAAGTACACT-3'	5979–5999	
	serogroup 18	5'-GCATCTGTACAGTGTGCTAATTGGATTGAAG-3'	15411–15442	478
		5'-CTTTAACATCTGACTTTTTCTGTTCCCAAC-3'	15735–15765	
	serogroup 23	5'-GATGCAAGAAATGTCGGTA-3'	10379–10398	126
		5'-TCTGCCTCATTGTTCTCC-3'	10486–10505	
Multiplex PCR 2	serotype 1	5'-GTCGTTATGAGAAGGTGGA-3'	9091–9110	108
		5'-TGACCAAATAGAACCTGATG-3'	9174–9194	
	serotype 19F	5'-GTTCAACGACTAGGACGC-3'	8351–8369	130
		5'-TAGGCACCAATGTTTCACT-3'	8462–8481	
Multiplex PCR 3	serotype 3	5'-ATGTGGATTCGCAGAGTG-3'	7511–7529	152
		5'-GATTACGCTCAGGGTCAA-3'	7646–7664	
	serotype 14	5'-AACCGACAAAAACAACTAAG-3'	7488–7509	220
		5'-TGTATGGTGATATGGACTATT-3'	7688–7708	
	serotype 23F	5'-TGGTAGTGACAGCAACGA-3'	9660–9678	177
		5'-CAAAGGCTAATTCAGCATC-3'	9818–9837	
Multiplex PCR 4	serotype 4	5'-CTGTTACTTGTTCTGGACTCTCGTTAATTGG-3'	9558–9589	430
		5'-GCCCACTCCTGTTAAAATCCTACCCGCATTG-3'	9957–9988	
	serotype 19A	5'-GTTAGTCCTGTTTTAGATTTATTTGGTGATGT-3'	12118–12150	478
		5'-GAGCAGTCAATAAGATGAGACGATAGTTAG-3'	12566–12596	

lated from patients with AOM by both multiplex polymerase chain reaction (PCR) and serological determination.

Material and Methods

S. pneumoniae Strains

A total of 64 *S. pneumoniae* isolates from the nasopharynx or middle ear fluids of pediatric patients with AOM were used in this study. *S. pneumoniae* was identified by α-hemolysis and colony morphology on 5% sheep blood agar plates (Nippon Becton Dickinson Company Ltd., Tokyo, Japan), Gram's stained smear, optochin disk sensitivity, and bile solubility. Bacteria were routinely cultured on the 5% sheep blood agar plates in humidified atmosphere supplemented with 5% CO_2 and in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich., USA) supplemented with 0.5% of yeast extract (Difco Laboratories).

A Multiplex PCR-Based Serotyping Determination

The oligonucleotide primers to amplify capsular serotypes 1, 3, 4, 14, 19A, 19F, 23F and serogroups 6, 18, 19, and 23 used for the multiplex PCR in this study are listed in table 1 [13, 14]. A single colony of *S. pneumoniae* on a 5% sheep blood agar plate was lysed in 30 μl of lysis solution (1 *M* Tris, pH 8.9, 4.5 v/v nonident P-40, 4.5 v/v Tween 20, and 10 mg/ml proteinase K) for 10 min at 60°C and for 5 min at 94°C in the programmable thermal cycler (Gene Amp PCR System 9700, Parkin Elmer, Norwalk, Conn., USA). The PCR reaction mixtures consisted of 1 μl of bacterial lysate, 0.5 μl of 10 m*M* dNTP mixture, 0.5 μl of *Taq* DNA polymerase, 2.5 μl of 10× PCR buffer, 4 μl of 25 m*M* $MgCl_2$, 5.0 μl Q-solution (Qiagen GmbH, Hilden, Germany), 1.25 μl of 1 *M* KCl, and 0.25 μl of each primer set in 25 μl solution. The reaction mixture was subjected to denaturation at 94°C for 10 min, 32 cycles of amplification consisting of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 1 min, and further extension at 72°C for 5 min. Strains of serotypes 4 (ATCC BAA-334) and 19F (ATCC 49619) were obtained from the American Type Culture Collection (ATCC Manassas, Va., USA) as positive control in every reaction (fig. 1).

Immunological Serotype Determination

Immunological serotypes were determined by the standard capsular reaction test. Briefly, a pure bacterial culture suspension was mixed with group-specific and/or type-specific antisera (The Statens Serum Institute, Copenhagen, Denmark) [15]. The swelling of capsules and agglutination were assessed under the phase-contrast microscope.

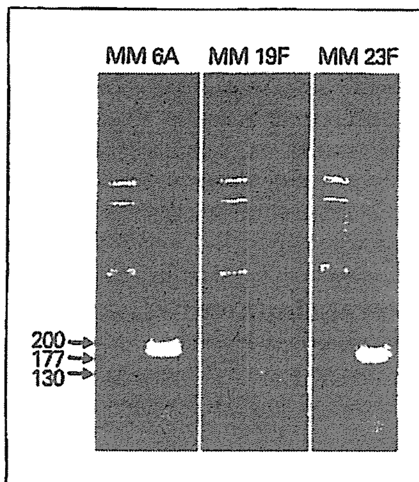

Fig. 1. Multiplex PCR-based serotypes 6A, 19F, and 23F. MM = Molecular marker.

Table 2. Multiplex PCR-based serotypes of *S. pneumoniae*

Multiplex PCR	Strains	Percentage	Immunological serotypes	Strains	Percentage
1	1	1.6	1	1	1.6
3	2	3.1	3	2	3.1
4	0	0.0	4	0	0.0
14	3	4.7	14	3	4.7
19A[c]	1	1.6	19A	1	1.6
19F[c]	22	34.4	19F	22	34.4
23F	6	9.4	23F	6	9.4
G23[c]	4	6.3	G23	4	6.3
G6[c]	10	15.6	6A	5	7.8
			6B	5	7.8
No group	15	23.4	9V	1	1.6
			G15	1	1.6
			only D[a]	3	4.7
			non 11[b]	10	15.6
Total	64	100		64	100

[a] Only positive for pool serum D.
[b] Serotypes that are not included in 11-valent vaccine.
[c] Serogroups 6, 19, and 23, respectively.

Results

A total of 49 (76.6%) pneumococcal isolates could be typed by the multiplex PCR-based serotyping and fully agreed with the results obtained by conventional immunological methods (table 2). Serogroup 19 was most frequently identified at 36.0% among patients with AOM, followed by serogroup 23 (15.7%), and serogroup 6 (15.6%). Twenty-two (95.7%) out of 23 strains in serogroup 19 were determined as serotype 19F. Six (60.0%) isolates of serogroup 23 belonged to serotype 23F. However, the multiplex PCR-based serotyping could not further classify serotype 6, because sequence data were not available. Serogroup 6 isolates were further classified into either serotype 6A (7.8%) or 6B (7.8%) by usual immunological determination. Fifteen (23.4%) isolates could not be classified by multiplex PCR-based serotyping. By the immunological method, the strains contained serotype 9V and serogroup 15. The remaining 10 isolates (15.6%) were not classified into certain serotypes, including 11-valent conjugate vaccine.

Discussion

S. pneumoniae produces about 90 immunologically distinct capsules as a major virulence factor [16]. Despite the large variety of capsular types, limited numbers of serotypes are most often associated with diseases. Serotypes 3, 6, 9V, 14, 19F, and 23F are mostly responsible for AOM [1, 17–19]. Among these serotypes, serotypes 6, 19, and 23 are also related with antimicrobial resistant strains [15, 20]. In this study, we applied a multiplex PCR method to determine serotypes 1, 3, 4, 14, 19A, 19F, and 23F, and serogroups 6, 19, 18, and 23 of *S. pneumoniae*. The 49 isolates could be classified into serotype or serogroup by a multiple PCR method. No strain gave discrepant results to the current immunological determination. Depending on both a multiplex PCR and the immunological assay, serotype 19F was the most frequently prevalent type among strains isolated from AOM patients.

Only 23-valent capsular polysaccharide-based pneumococcal vaccine is implemented in Japan, although the 7-valent vaccine was approved in both the United States and Europe [8, 21]. An investigational 11-valent second-generation vaccine will soon be available in the United States. To optimize the development of future conjugate vaccines and to evaluate their efficacy, it is necessary to understand the serotype- or serogroup-specific epidemiology of pneumococci associated with AOM. Continuous monitoring of *S. pneumoniae* serotypes is essential since it has been shown that the incidence of types responsible for AOM can change over time [22].

There are many advantages in using a multiplex PCR to determine serotypes of pneumococci. PCR can easily be implied among microbiology laboratories, apply to many samples at once, and quickly produce reliable results without specialized expertise required in immunological procedures. PCR also allows a feasible analysis of minority strains in a pneumococcal population, confirmed by studies on the carriage of multiple pneumococcal capsular types.

In conclusion, the possibility to use a multiplex PCR method as a qualitative assay to evaluate the true composition of possibly diverse populations of pneumococci increases its usefulness as a new capsular typing technique.

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High Prevalence of *Streptococcus pneumoniae* with Mutations in *pbp1a, pbp2x,* and *pbp2b* Genes of Penicillin-Binding Proteins in the Nasopharynx in Children in Japan

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Key Words
Penicillin resistant *Streptococcus pneumoniae* · Acute otitis media · Penicillin-binding protein · Polymerase chain reaction

Abstract
Objective: To evaluate the resistances of *Streptococcus pneumoniae* to β-lactams developed by stepwise alterations in high-molecular-weight penicillin-binding proteins (PBPs) with a reduced binding affinity of β-lactams. Among the numerous mutations in *pbp* genes that alter the affinity for β-lactams, the decreased affinity of PBP1A, 2X and 2B is especially important in the development of resistances to β-lactams. ***Study Design:*** Retrospective review. ***Methods:*** In this study, we investigated the mutations in *pbp1a, pbp2x,* and *pbp2b* genes evaluated by polymerase chain reaction (PCR) in 866 pneumococcal isolates collected from the nasopharynx of Japanese children with acute otitis media. ***Results:*** 210 strains (24.3%) exhibited no mutations in the three *pbp* genes. 333 strains (38.5%) had mutations in the three *pbp* genes, 78 (9.0%) in two *pbp* genes, whereas 245 (28.3%) displayed mutations in only one *pbp* gene. Among the 656 strains with mutations in *pbp* genes, 620 (94.5%) strains had mutations in *pbp2x*. The annual prevalence of antimicrobial-resistant *S. pneumoniae* showed a gradual increase in strains with mutations in the three *pbp* genes and a parallel decrease in strains without mutations. ***Conclusions:*** PCR-based genotyping can characterize the antimicrobial resistances in pneumococci along with minimal inhibitory concentrations (MICs). Physicians should pay attention to the recent increase in antimicrobial-resistant *S. pneumoniae* when treating pediatric acute otitis media.

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Introduction

Streptococcus pneumoniae is the most commonly identified bacterial cause of meningitis, acute otitis media (AOM), rhinosinusitis, and community-acquired pneumonia [1–4]. Since a penicillin-resistant strain of *S. pneumoniae* was firstly isolated in South Africa in 1967, the increase in penicillin-resistant *S. pneumoniae* (PRSP) is causing serious clinical problems worldwide [5, 6]. PRSP now represent an important risk factor for intractable acute otitis media [7–9]. As the nasopharynx plays an important role as a reservoir for the potential causative pathogens, it is important to characterize the *S. pneumoniae* isolates identified from the nasopharynx of children with AOM. In addition to the current bioassays for evaluating antimicrobial susceptibilities by the microbroth dilution method, recent studies attempted to evaluate the genetic characteristics of pneumococci [10, 11].

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This advanced procedure brings up fascinating information on this pathogen. Resistance of *S. pneumoniae* to β-lactams becomes established via stepwise alterations in the high-molecular-weight penicillin binding proteins (PBPs) and the reduction of the binding affinity of β-lactams to the PBPs [12–15]. Among those, PBPs 1A, 2X, and 2B possess a transpeptidase activity and contain the conserved amino acid motif SXXK, SXN and KT(S)G in an active serine residue [11]. *S. pneumoniae* acquires exogenous low-affinity genes and causes genetic mutations that alter PBP affinity for β-lactams [16, 17].

In this study, we evaluated polymerase chain reaction (PCR)-based genotyping of *pbp* genes in *S. pneumoniae* isolated from the nasopharynx of children and compared their antimicrobial susceptibilities.

Materials and Methods

S. pneumoniae Strains

A total of 866 nasopharyngeal isolates of *S. pneumoniae* were collected from pediatric patients with acute otitis media (AOM) at the Clinic of Otolaryngology-Head and Neck Surgery, Wakayama Medical University Hospital, and its affiliated hospitals in Japan, between January 1998 and December 2002. Duplicated isolates were excluded from the study by discarding repeat isolates taken from the same patient within a time period of 3 months after the first isolates. Nasopharyngeal cultures were obtained with a small rayon-tipped flexible swab at the first medical examination. *S. pneumoniae* was identified by α-hemolysis and colony morphology on 5% sheep blood agar, gram-stained smear, optochin disk sensitivity and bile solubility. The autolysin gene *(lytA)* was also amplified to confirm *S. pneumoniae* by PCR. Bacteria were routinely cultured on the 5% sheep blood agar plates (Nippon Becton Dickinson, Tokyo, Japan) in a humidified atmosphere supplemented with 5% CO_2. The isolates were stocked at –80°C in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich., USA) supplemented with 0.5% of yeast extract (Difco Laboratories) containing 10% glycerol pending analysis.

Antimicrobial Susceptibility Test

Out of 866 pneumococcal isolates, 710 (82.0%) were evaluated for susceptibility to penicillin G (PCG) (156 strains could not be recovered from the frozen stock). Minimal inhibitory concentrations (MICs) of *S. pneumoniae* to PCG were determined either by the microbroth dilution method according to the NCCLS recommendations or E test (AB Biodisk, Sweden). The definition of the susceptibility of *S. pneumoniae* to PCG was based on the criteria established by the NCCLS [18]. Strains with MICs of PCG ≥2 μg/ml are interpreted as PRSP, strains with MICs from 0.1 to 1 μg/ml as penicillin intermediately-resistant *S. pneumoniae* (PISP) and strains with MIC below 0.06 μg/ml as penicillin-susceptible *S. pneumoniae* (PSSP).

Subsets of isolates (107 isolates for ampicillin and 165 for others) were randomly selected to determine the susceptibility to other β-lactams. MICs to ampicillin (ABPC), cefaclor (CCL), cefditoren (CDTR), cefdinir (CFDN), and cefpodoxime (CPDX) were determined by the microbroth dilution method.

PCR Analysis of pbp *Genes of* S. pneumoniae

The oligonucleotide primers to amplify parts of the *pbp1a*, *pbp2x*, and *pbp2b* used in this study were as follows: for *pbp1a*: 5′-AAACAAGGTCGGACTCAACC-3′, 5′-AGGTGCTACAAATTGAGAGG-3′: product size 430 bp, for *pbp2x*: 5′-CCAGGTTCCACTATGAAAGTG-3′, 5′-CATCCGTCAAACCGAAACGG-3′: product size 292 bp; for *pbp2b*: 5′-CCTATATGGTCCAAACAGCCT-3′, 5′-GGTCAATTCCTGTCGCAGTA-3′, product size 147 bp. To confirm the isolated pathogen as *S. pneumoniae*, primers for *lytA*: 5′-TGAAGCGGATTATCACTGGC-3′, 5′-GCTAAACTCCCTG TATCAAGCG-3′, 273 bp were used. Primer mixture A contained primers for amplifying *lytA* and *pbp1a*, and primer mixtures B contained primers for amplifying *pbp2x* and *pbp2b* [19].

A single colony of *S. pneumoniae* on a 5% sheep blood agar plate was lysed in 30 μl of lysis solution (1 *M* Tris, pH 8.9, 4.5 v/v nonidet P-40, 4.5 v/v Tween-20, 10 mg/ml proteinase K) for 10 min at 60°C and for 5 min at 94°C in the programmable thermal cycler (Gene Amp PCR System 9800, Perkin Elmer, Norwalk, Conn., USA). The reaction mixtures consisted of 1 μl of bacterial lysate, 8 μl of 25 m*M* of dNTP mixture, 2.5 U of Tth DNA polymerase (Takara Biomedicals, Kyoto, Japan), 10 μl of 10× PCR buffer (pH 8.3) and 60 ng of primer mixtures A or B in 100 μl solution. The reaction mixture was then subjected to 30 cycles of amplification in the programmable thermal cycler: 20 s at 94°C, 20 s at 55°C, and 15 s at 72°C. Amplified DNA fragments were analyzed using 3% agarose gel electrophoresis. Because of the diversities in PBP, *pbp* genes could be amplified in nonmutated genes of PSSP, but failed in PISP and PRSP mutated genes (fig. 1).

Results

Detection of Mutations in pbp *Genes by PCR*

210 strains (24.2%) exhibited no mutations in *pbp* genes. 333 strains (38.5%) had mutations in the three *pbp* genes. 78 strains (9.0%) had mutations in two *pbp* genes, among these 24 (2.8%) in *pbp1a* and *pbp2x*, 16 (1.8%) in *pbp1a* and *pbp2b*, 38 (4.4%) in *pbp2x* and *pbp2b*. 245 strains (28.3%) had mutations in one *pbp* gene, out of these strains 1 (0.1%) had a mutation in *pbp1a*, 225 (26.0%) in *pbp2x*, 19 (2.2%) in *pbp2b*. Of the 656 *pbp*-gene-mutated strains, overall 620 pneumococci (94.5%) had mutations in *pbp2x*.

Relationship between Mutations in pbp *Genes and Susceptibility to PCG*

According to the criteria recommended by NCCLS [18], 281 strains (39.6%) were PSSP, 221 (31.1%) were PISP, and 208 (29.3%) were PRSP. Out of 281 penicillin-susceptible strains, 156 strains (55.5%) had no mutations in *pbp* genes. Among the remaining 125 susceptible

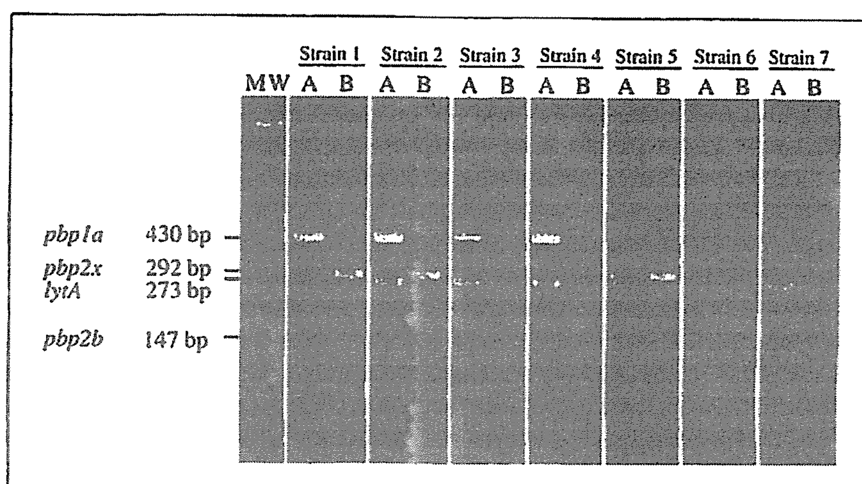

Fig. 1. PCR-based genotypes of seven clinical isolates of *Streptococcus pneumoniae*. MW = Molecular weight (100 bp). A = PCR for *pbp1a* and *lytA*. B = PCR for *pbp2x* and *pbp2b*. Strain 1: no mutation in *pbp* genes; strain 2: mutation in *pbp2b*; strain 3: mutation in *pbp2x*; strain 4: mutations in *pbp2b* and *pbp2x*; strain 5: mutations in *pbp1a* and *pbp2b*; strain 6: mutations in *pbp1a* and *pbp2x*; strain 7: mutations in *pbp1a*, *pbp2x* and *pbp2b*.

Table 1. MIC distribution and resistance genes identified by PCR in *S. pneumoniae* strains resistant to PCG in Japan

MIC, μg/ml	≤0.03	0.06	0.125	0.25	0.5	1	2	4	MIC_{50}	MIC_{90}	Range	Total
Without mutation	105	51	8	2	1	1	2	1	≤0.03	0.06	≤0.03–4	171
With mutation in *pbp*												
2x	20	81	74	6	1	4	3	0	0.06	0.125	≤0.03–2	189
2b	8	7	1	0	0	1	0	0	0.06	0.125	≤0.03–1	17
1a+2x	0	0	3	5	1	2	2	2	0.5	4	0.125–4	15
1a+2b	1	0	0	5	3	1	0	0	0.25	0.5	≤0.03–1	10
2x+2b	2	2	9	7	2	2	0	0	0.125	0.5	≤0.03–1	24
1a+2x+2b	2	2	3	2	25	52	174	24	2	2	≤0.03–4	284
Total	138	143	98	27	33	63	181	27				710

strains, 101 (35.9%) had mutations in *pbp2x*, 1 (0.4%) in *pbp1a* and *pbp2b*, 4 (1.4%) in *pbp2x* and *pbp2b*, 15 (5.3%) in *pbp2b*, and 4 (1.4%) in the three *pbp* genes. In the PSSP group, 109 (87.2%) had mutations in *pbp2x*. On the other hand, 198 (95.2%) of PRSP strains had mutations in the three *pbp* genes. The PISP strains had diverse mutations with various combinations. Out of 221 PISP strains, 82 (37.1%) had mutations in the three *pbp* genes. Out of the 82 strains, 77 (93.9%) strains had MICs of 1–0.5 μg/ml, which is very near to resistant strains. Of the remaining 139 PISP strains, 11 strains (5.0%) had mutations in *pbp1a* and *pbp2x*, 9 (4.1%) in *pbp1a* and *pbp2b*, 20 (9.1%) in *pbp2x* and *pbp2b*, 85 (38.5%) in *pbp2x*, 2 (0.9%) in *pbp2b*, whereas 12 (5.4%) strains displayed no *pbp* gene mutations. The MIC_{50} and MIC_{90} of the strains with mutations in the three *pbp* genes to PCG were ≥2 μg/ml, whereas strains without mutation in any *pbp* gene were ≤0.03 and 0.06 μg/ml, respectively. The MIC_{50} and MIC_{90} of the strains with mutations in *pbp2x* were 0.06 and 0.125 μg/ml, respectively. On the other hand, the MIC_{50} and MIC_{90} of strains with mutations in two types of *pbp* genes (*pbp1a* and *pbp2x*, *pbp1a* and *pbp2b* or *pbp2x* and *pbp2b*) ranged between 0.125 and 0.5 μg/ml and 0.5 and 4 μg/ml, respectively (table 1).

Relationship between Mutations in pbp *Genes and Susceptibility to Other β-Lactams*

The relationships between mutations in *pbp1a*, *pbp2x*, and *pbp2b* genes and MICs to other β-lactams are shown in table 2. Strains with mutations in the three *pbp* genes showed high MICs to other β-lactams while strains without mutations in the *pbp* genes showed relatively lower MICs to other β-lactams. Out of the 165 isolates, 6.6, 20.0, 24.2, and 35.8% strains were resistant to CDTR, CFPN, CCL and CFDN, respectively. All strains without mutation in *pbp* genes were susceptible to those cephalosporins.

Table 2. Correlation between susceptibilities to other β-lactams and mutations in *pbp* genes

a ABPC

MIC, μg/ml	≤0.03	0.06	0.125	0.25	0.5	1	2	4	MIC_{50}	MIC_{90}	Range	Total
No mutation	25	7	1	0	0	0	0	0	≤0.03	0.06	≤0.03–0.125	33
With mutation in *pbp*												
2x	6	16	1	0	0	0	1	0	0.06	0.06	≤0.03–2	24
2b	1	0	0	0	0	0	0	0	≤0.03	≤0.03	≤0.03	1
1a+2x	1	0	0	1	0	0	1	1	0.25	4	≤0.03–4	4
2x+2b	2	2	1	0	0	0	0	0	0.06	0.125	≤0.03–0.125	5
1a+2x+2b	0	0		1	6	19	13	1	1	2	0.25–4	40
Total	35	25	3	2	6	19	15	2				107

b CDTR

MIC, μg/ml	≤0.03	0.06	0.125	0.25	0.5	≥1	MIC_{50}	MIC_{90}	Range	Total
No mutation	42	4	4	3	0	0	≤0.03	0.125	≤0.03–0.125	53
With mutation in *pbp*										
2x	3	4	20	7	2	1	0.125	0.25	≤0.03 to ≥1	37
2b	1	0	0	0	0	0	≤0.03	≤0.03	≤0.03	1
1a+2x	0	0	0	1	2	1	0.5	≥1	0.25 to ≥1	4
1a+2b	2	0	0	0	0	0	≤0.03	≤0.03	≤0.03	2
2x+2b	1	0	7	0	1	0	0.125	0.5	≤0.03–0.5	9
1a+2x+2b	2	1	3	5	39	9	0.5	≥1	≤0.03 to ≥1	59
Total	51	9	34	16	44	11				165

c CPDX

MIC, μg/ml	≤0.03	0.06	0.125	0.25	0.5	1	2	4	MIC_{50}	MIC_{90}	Range	Total
No mutation	26	14	2	11	0	0	0	0	0.06	0.25	≤0.03–0.25	53
With mutation in *pbp*												
2x	2	1	4	18	6	3	3	0	0.25	1	≤0.03–2	37
2b	1	0	0	0	0	0	0	0	≤0.03	≤0.03	≤0.03	1
1a+2x	0	0	0	0	1	1	2	0	1	2	0.5–2	4
1a+2b	0	0	2	0	0	0	0	0	0.125	0.125	0.125	2
2x+2b	1	0	0	6	1	0	1	0	0.25	2	≤0.03–2	9
1a+2x+2b	1	0	2	1	3	18	30	4	2	2	≤0.03–4	59
Total	31	15	10	36	11	22	36	4				165

d CCL

MIC, μg/ml	0.25	0.5	1	2	4	8	≥16	MIC_{50}	MIC_{90}	Range	Total
No mutation	2	24	19	6	2	0	0	1	2	0.25–4	53
With mutation in *pbp*											
2x	1	3	20	11	1	0	1	1	2	0.25 to ≥16	37
2b	0	1	0	0	0	0	0	0.5	0.5	0.5	1
1a+2x	0	0	0	0	0	2	2	8	≥16	8 to ≥16	4
1a+2b	0	0	0	0	1	1	0	4	8	2–4	2
2x+2b	0	0	5	3	1	0	0	1	4	1–4	9
1a+2x+2b	0	1	2	1	0	1	54	≥16	≥16	0.5 to ≥16	59
Total	3	29	46	21	5	4	57				165

Table 2 (continued)

e CFDN

MIC, μg/ml	≤0.125	0.25	0.5	1	2	4	8	MIC_{50}	MIC_{90}	Range	Total
No mutation	40	11	2	0	0	0	0	≤0.125	0.25	≤0.125–0.5	53
With mutation in *pbp*											
2x	3	23	7	1	1	2	0	0.25	1	≤0.125–4	37
2b	1	0	0	0	0	0	0	≤0.125	≤0.125	≤0.125	1
1a+2x	0	0	1	0	1	1	1	2	8	0.5–8	4
1a+2b	1	1	0	0	0	0	0	≤0.125	0.25	≤0.125–0.25	2
2x+2b	2	3	3	1	0	0	0	0.25	1	≤0.125–1	9
1a+2x+2b	3	0	1	2	6	39	8	4	8	≤0.125–8	59
Total	50	38	14	4	8	42	9				165

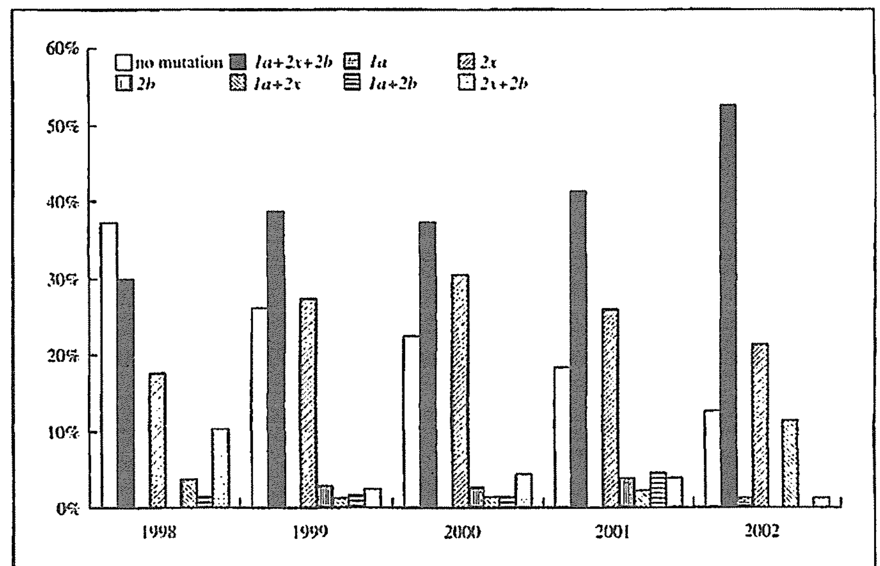

Fig. 2. Year-to-year changes in the prevalence of mutations in *pbp* genes.

While the MIC_{50} and MIC_{90} of strains without mutations in *pbp* genes to ABPC were ≤0.03 and 0.06 μg/ml, those of strains with mutations in the three *pbp* genes were 1 and 2 μg/ml, respectively. The MIC_{50} and MIC_{90} of the strains with mutations in the three genes were 32 times higher than those of the strains without mutations in *pbp* genes. Although the vast majority of strains with mutations in *pbp2x* gene were susceptible to PCG and ABPC, they showed much higher MICs to cephalosporins.

Year-to-Year Change in the Prevalence of Resistant Strains

The annual changes in strains with mutations in the three *pbp* genes and the *pbp2x* gene were assessed from 1998 to 2002. Strains with mutations in the three *pbp* genes gradually increased from 1998 to 1999. The increases then slowed down from 1999 to 2001 and increased again in 2002. On the other hand, the number of strains without mutations in *pbp* genes decreased. The number of strains with mutations in *pbp2x* gradually increased from 1998 to 2000, and then gradually decreased from 2001 to 2002 (fig. 2).

Discussion

The increase in PRSP is causing serious clinical problems worldwide and also in Japan [20, 21]. The countrywide surveillance of *S. pneumoniae* isolated from the upper respiratory tract in Japan between 1998 and 1999