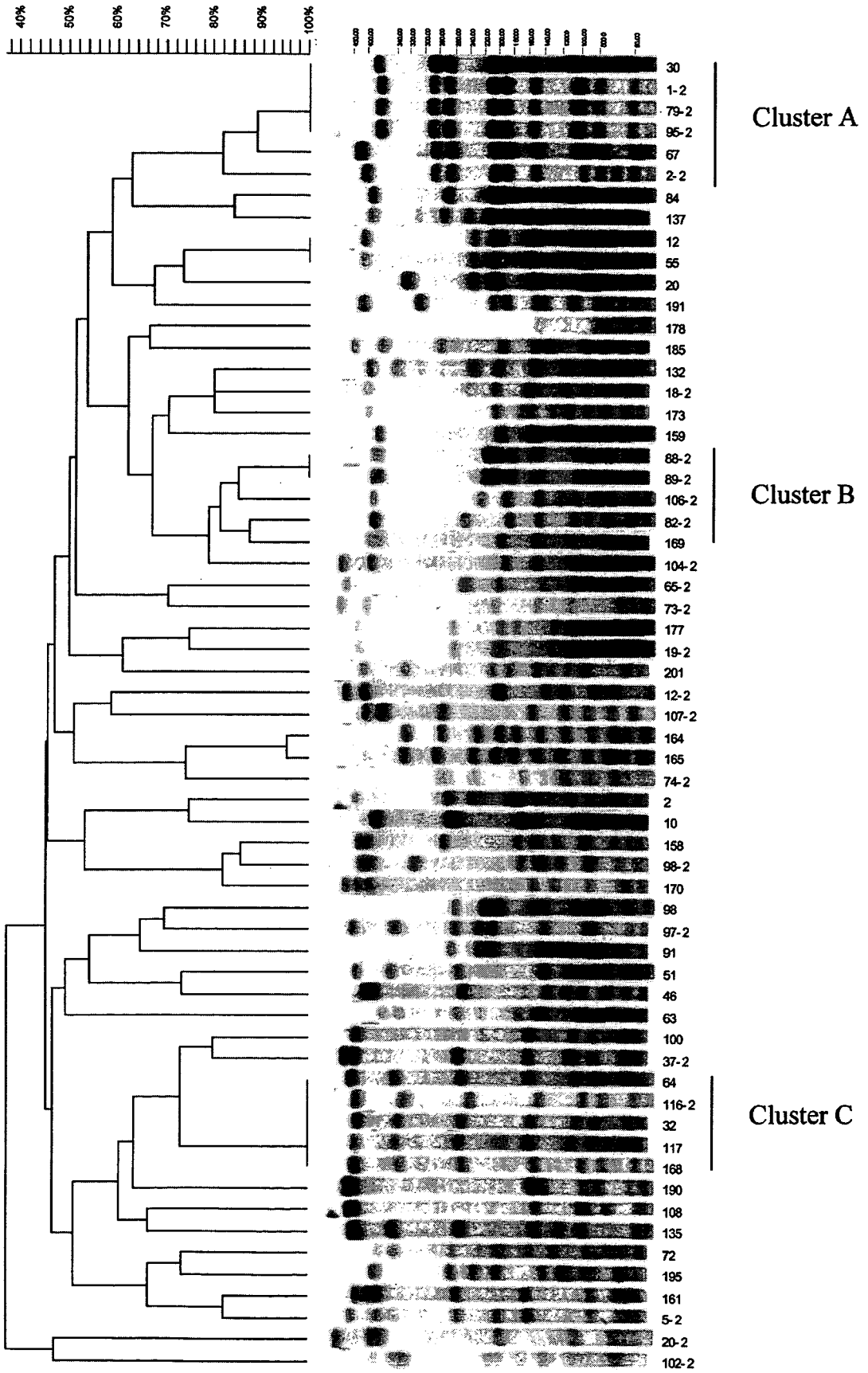

FIG. 2. Genetic identity and/or high relatedness among BLNAR isolates. PFGE dendrogram (unweighted pair group method with arithmetic means) of 61 BLNAR strains (MIC $\geq$ 4 μg/ml). Dice coefficients are shown above the dendrogram. Isolates with $\geq$80% relatedness on the dendrogram are considered highly genetically related.

Group III gBLNAR strains have been proven to be absent among isolates from European countries. Since 68.4% of group I/II BLNAR strains had AMP MICs of ≤1 μg/ml in Japan, strains with group I/II substitutions may exist in Europe or the United States but not be recognized because they are phenotypically AMP susceptible. The inappropriate use of oral antibiotics for the treatment of community-acquired bronchopulmonary and URT infections appears to be responsible for the selection for BLNAR strains. The use of antibiotics might be related to the dissemination of gBLNAR strains in Japan. Some group I/II gBLNAR strains show higher MICs to AMP.

We did not evaluate the substitution Arg-517 in the *ftsI* gene in this study. The substitution in this locus causes an increase of the MIC, but the mutations are varied. Ubukata et al. suggested the necessity of evaluating the sequences of this locus as part of a further investigation of the correlation between genetic mutations and decreasing susceptibilities to antimicrobial agents (36).

We found that some group III gBLNAR strains exhibit a relatively high MIC to AMP (MIC = 32 μg/ml). Kaczmarek et al. suggested that BLNAR strains with mutations of the AcrAB repressor gene *arcR* can occur clinically and that such dual-target mutants can have higher MICs to AMP (MIC range, 8 to 16 μg/ml) (16). Further precise investigations of those BLNAR strains should be considered in future studies.

Future studies should also examine the dissemination of BLNAR strains. A previous study in the United States found that two BLNAR isolates collected from a single institution were clonal (13). A second study of 29 BLNAR isolates collected in France showed 20 unique SmaI PFGE patterns and suggested limited clonality of the BLNAR strains (8). Karlowsky et al. reported the clonal dissemination of BLNAR strains in hospital settings (17). Recent reports have suggested less clonal dissemination of the BLNAR strains (7). However, these previous reports evaluated BLNAR according to broad MIC ranges. In the current study, the PFGE profiles showed a clonal dissemination among strains with increased resistance to AMP (MIC = 16 μg/ml). There was no significant difference in the distributions of BLNAR strains according to age (data not shown), while penicillin-resistant *Streptococcus pneumoniae* cases are predominant among young children. Although penicillin-resistant *S. pneumoniae* shows clonal dissemination worldwide, most of the BLNAR strains in Japan are classified into nonencapsulated, nontypeable strains. In contrast to the encapsulated strains, the nonencapsulated BLNAR strains are genetically diverse and occasionally appear independently in countries, depending on the antibiotic use patterns. Selective pressures, such as frequent prescription of antibiotics, especially consumption of oral cephems, may be the impetus for the clonal dissemination of BLNAR strains. The dissemination patterns of nontypeable *H. influenzae* infections caused by BLNAR strains might be different from those of *S. pneumoniae*.

In conclusion, there is an alarming increase in Japan in the occurrence of BLNAR strains with mutations of the *ftsI* gene. The resistant *H. influenzae* pathogen will disseminate in different ways than penicillin-resistant *S. pneumoniae*. Consequently, we need to continue careful surveillance for BLNAR strains of *H. influenzae* in patient populations and continue our efforts to understand why these antibiotic-resistant strains are becoming more prevalent. PCR-based genotyping and study of molecular characteristics bring us useful information to continue our surveillance of this resistant pathogen.

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Can the Etest Correctly Determine the MICs of β-Lactam and Cephalosporin Antibiotics for Beta-Lactamase-Negative Ampicillin-Resistant *Haemophilus influenzae*?[▽]

A recent study reporting surprisingly high beta-lactam MICs against beta-lactamase-negative ampicillin-resistant (BLNAR) strains when tested by the Etest prompted us to compare results of the Etest with those of the standard broth microdilution concerning BLNAR strains (5). In that study, the MIC_{90}s of BLNAR and beta-lactamase-positive amoxicillin-clavulanate-resistant (BLPACR) strains in response to ampicillin, amoxicillin-clavulanate, cefuroxime, cefaclor, cefixime, and meropenem were 256 and 256, 256 and 256, 8 and 256, 256 and 256, 32 and 256, and 32 and 256 μg/ml, respectively. In previous studies, BLNAR isolates almost always showed low ranges of MICs. For instance, Dabernat et al. reported MIC ranges of the *ftsI* mutant gene strains as 0.5 to 8, 0.5 to 8, 4 to 16, and 0.03 to 0.25 μg/ml for amoxicillin-clavulanate, cefuroxime, cefaclor, and cefixime, respectively (2). Similarly in a recent study, mean MICs of meropenem were <0.3 μg/ml, although BLNAR strains showed increasing MIC trends (8). For the antibiotic susceptibility method, the author performed only the Etest; however, both of the previous studies with lower MICs used the broth microdilution method (2, 6). Concerning the reliability of the Etest, the authors referred to two studies; however, the numbers of *Haemophilus influenzae* isolates with relatively low MICs was small, and the mutation status of the *ftsI* gene was not evaluated in either study (4, 7).

In the current study, our main concern was whether remarkably high MIC patterns of BLNAR strains in response to beta-lactam antibiotics (beta-lactams) and meropenem arise from the test method or their actual status. To confirm the high MIC values, we compared results of the Etest with those of broth microdilution using *H. influenzae* isolates with known mechanisms of beta-lactam resistance.

We examined MICs of 153 *H. influenzae* strains by using the Etest and the broth microdilution method simultaneously (Table 1). MICs of all strains were determined by broth microdilution using the standard method (1) and by the Etest according to the manufacturer's instructions. Two persons independently took readings of MICs, and the agreement was almost $\pm\log_2 1$. *H. influenzae* ATCC 49247 was included in each batch as a control. MICs of the Etest were rounded up to the next $\log_2$ concentration, and agreement was defined as the results of broth microdilution and those of Etest in the range of $\pm\log_2 1$ of each other. The mutation status of the *ftsI* gene in all strains was checked by using primers described previously (3, 8). MICs of BLNAS and beta-lactamase-positive ampicillin-resistant (BLPAR) strains showed consistent results by both tests, and the agreement was 92.59 to 100% for beta-lactams and 90 to 92.59% for meropenem, respectively. However, BLNAR and BLPACR strains showed diverse results, and the agreement for beta-lactam and meropenem decreased to a range of 48.27% to 100% and 42.10% to 44.82%, respectively

TABLE 1. Comparison of Etest and broth microdilution MICs determined for BLNAS and BLNAR *H. influenzae* isolates[a]

Drugs	Genotype	No. of isolates	MIC or MIC range (μg/ml)		No. of E-test MICs within the indicated concn ($\log_2$) of HTM broth dilution MIC										% of agreement within 1 $\log_2$ concn
			Microbroth	E-test	−2	−1	Same	+1	+2	+3	+4	+5	+6	>+6	
Ampicillin	BLNAS	20	0.25–1	0.25–0.5		11	8	1							100
	BLNAR	87	1–32	1–256	1	4	31	7	1	3	7	4	1	28	48.27
	BLPAR	27	32–256	4–256	2		25								92.59
	BLPACR	19	256	256			19								100
Amoxcillin/clavulanate	BLNAS	20	0.5–1	0.5–1		2	8	10							100
	BLNAR	87	1–32	1–256	4	7	20	16	2	11	13	6	2	6	49.42
	BLPAR	27	0.5–2	0.5–2	2		25								92.59
	BLPACR	19	2–256	2–256			10	1	2	5				1	57.89
Ceftriaxone	BLNAS	20	<0.06	0.008–0.19			13	7							100
	BLNAR	87	<0.06–1	0.012–32		3	44	5	1	2				32	59.77
	BLPAR	27	<0.06	0.008–0.12			26	1							100
	BLPACR	19	<0.06–1	0.25–32			5	8	1			1		4	68.42
Meropenem	BLNAS	20	<0.06	0.064–32			7	11						2	90
	BLNAR	87	<0.06–1	0.125–32	3	11	16	12	1	2	3		8	31	44.82
	BLPAR	27	<0.06–0.25	0.064–32		3	14	8	1			1			92.59
	BLPACR	19	<0.06–4	0.125–32			5	3		1	1		4	5	42.10
Levofloxacin	BLNAS	20	<0.06	0.008–0.12		1	19	1							100
	BLNAR	87	<0.06–0.12	0.016–0.094		4	80	2	1						98.85
	BLPAR	27	<0.06	<0.06			27								100
	BLPACR	19	<0.06	<0.06			19								100

[a] MICs of BLNAS and BLNAR *H. influenzae* isolates were determined by using *Haemophilus* test medium (HTM) agar with HTM broth microdilution. BLNAS, beta-lactamase-negative, ampicillin susceptible.

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(Table 1). Furthermore, 9.2% of the intermediate BLNAR strains determined by the broth microdilution method were highly resistant to ampicillin (256 μg/ml) by the Etest method. Similarly, all strains were susceptible to ceftriaxone by broth microdilution, but 42.5 to 68.42% of BLNAR and BLPACR strains showed high MICs to ceftriaxone (32 μg/ml). The fluoroquinolone levofloxacin showed high agreement (98.85% to 100%) between microbroth and Etest results (Table 1).

From these results, we considered that determining MICs to beta-lactams by the Etest is not a reliable method for strains with the *ftsI* mutant gene but is acceptable for BLNAS and BLPAR strains. High MICs to beta-lactams and meropenem may be due to the presence of small colonies within the inhibition ellipses of the Etest and the variable expression of *ftsI* during bacterial growth between agar and broth media. Matic et al. reported low MICs for BLPACR strains to amoxicillin-clavulanate and excluded the possibilities of extended-spectrum beta-lactamase associated with amoxicillin-clavulanate resistance in BLPACR strains (6). We considered that remarkably high MICs of BLNAR strains might arise from an antibiotic susceptibility method such as the Etest rather than an actual elevation of MICs.

In conclusion, we considered that Etest results alone might not represent the actual MIC status of BLNAR and BLPACR strains for beta-lactams and meropenem. Considering the essential roles of beta-lactams in the treatment of invasive diseases, utilizing only the Etest may misguide a community about the susceptibility of *H. influenzae* and would enhance the development of BLNAR strains by selection of an inappropriate antibiotic. Although the Etest is not a national or international reference method, it is a convenient commercial product for generating MICs and can be applicable in regions with a low prevalence of BLNAR strains. Finally, we suggest that MICs of *H. influenzae* isolates should be measured by the broth microdilution method in conjunction with the PCR technique.

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Determination of pneumococcal serotypes/genotypes in nasopharyngeal secretions of otitis media children by multiplex PCR

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Abstract The appropriate clinical applications of pneumococcal polysaccharide vaccines against recent increases in antimicrobial resistant *Streptococcus pneumoniae (S. pneumoniae)* urgently require accurate analytical methodologies for determining and characterizing the serotypes. The results of current immunological determinations of serotypes with anti-capsular polysaccharide-specific sera are difficult to interpret in terms of quellung changes of the pneumococci. In this study, we applied the multiplex PCR technique for the rapid identification of pneumococci and simultaneous rapid determinations of their serotypes and genotypes that directly correlated with antimicrobial susceptibilities from nasopharyngeal secretions (NPS). Serogroups 6, 19F and 23F were the predominant capsular types of *S. pnuemoniae* in the NPS samples. Strains of serotypes 19F and 23F frequently had mutations in *pbp1a, pbp2x* and *pbp2b* and expressed *ermB* and *mefA*; they also were mostly resistant to both penicillin G (PCG) and clarithromycin (CAM). Two NPS samples contained the strain of serotype 19F together with the strain of serotype 23F, although only the strain of serotype 19F was identified by a conventional bacterial culture. Pneumococci were identified in six NPS samples and their serotypes determined by the multiplex PCR, while a conventional bacterial culture failed to identify the pathogens. Our findings suggest that PCR-based serotyping and genotyping can provide an accurate and rapid distribution of pneumococcal serotypes and antimicrobial resistance. The relatively minor populations in the nasopharynx may be determined using molecular techniques.

Keywords Acute otitis media · Genotype · Nasopharyngeal secretion · Serotype · *Streptococcus pneumoniae*

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Introduction

Streptococcus pneumoniae is a leading causative pathogen responsible for acute otitis media (AOM) that frequently colonizes the nasopharynx [3, 6]. This pathogen has long been susceptible to penicillin, and AOM caused by pneumococci are easily improved by oral antimicrobial therapy. However, recent dramatic increases of antimicrobial resistance in *S. pneumoniae* are making the treatments of AOM with oral antibiotics more difficult [5, 8, 9]. There are urgent demands to prevent pneumococcal AOM through vaccinations. Naopharyngeal colonization with causative pathogens is one of the more important risk factors for developing AOM. Consequently, reducing the frequency of nasopharyngeal carriage of *S. pneumoniae* is an important step towards preventing the development of AOM. However, there is less evidence for preventing AOM by vaccines [19]. A newly developed 10-valent vaccine conjugated with *H. influenzae* protein D shows more efficacy for reducing nasopharyngeal carriage [13]. Nevertheless, recent reports have shown a decrease in the carriage of vaccine serotypes and a parallel increase in non-vaccine serotypes following vaccination [1, 14, 20].

In order to be able to carry out a comprehensive evaluation of vaccine efficacies, it is first necessary to understand the prevalence of vaccine serotypes as well as the antimicrobial resistances of pneumococci associated

with AOM. The determination of serotypes by immunological methods requires the isolation of pneumococci, is time consuming and expensive, and the results are difficult to interpret. In an earlier publication, we reported the successful applications of the multiplex PCR approach for determining pneumococcal serotypes [2]. We also showed that there was a correlation between the gene mutation and the minimal inhibitory concentration (MIC) to beta-lactam antibiotics. However, in that study, the gene mutation was investigated using isolated pneumococci [6]. To date, there has been no study which simultaneously evaluates the pneumococcal serotypes and gene types present in nasopharyngeal secretions (NPS). We hypothesized that the PCR technique could be used for identifying serotyping, and genotyping pneumococci present in NPS, thereby also enabling us to determine whether more than one serotype can co-exist there.

The aims of this study were (1) to simultaneously identify and determine serotypes of *S. pneumoniae* in the NPS of acute otitis media (AOM) patients, (2) to determine the genotypes of penicillin-binding proteins (PBPs) and macrolide-resistant traits in NPS of AOM patients and, in addition, (3) to examine whether one or more serotypes of *S. pneumoniae* may co-exist as nasopharyngeal flora in patients.

Materials and methods

Nasopharyngeal secretions

A total of 60 NPS were collected from pediatric patients (12–60 months old) with AOM at the clinics of Otolaryngology – Head and Neck Surgery, Wakayama Medical University Hospital. The NPS samples were collected by suction using a fine, flexible plastic catheter (no 5, French) and syringe. Informed consent was obtained from the parents or guardians of the patients prior to the collection of samples, in accordance with the guidelines of the institutional review board of Wakayama Medical University.

Bacterial culture

A portion of each of the 60 NPS samples was cultured on 5% sheep blood agar plates and chocolate agar plate (Nippon Becton Dickinson, Tokyo, Japan) for 48 h at 37°C in a humidified atmosphere suppled with 5% CO_2. *S. pneumoniae* was identified on the basis of alpha-hemolysis and colony morphology, Gram-stained smear, optochin disk sensitivity and bile solubility. Determinations of the MICs to penicillin G (PCG) and clarithromycin (CAM) were performed using CLSI methods [12], and the serotypes were determined using a standard laboratory method. Briefly, a bacterial suspension was mixed with group-specific or type-specific antisera (The Statens Serum Institute, Copenhagen, Denmark) [15]. The quellung and agglutination were assessed by phase-contrast microscopy.

Preparation of genomic DNA

Total genomic DNA was purified from both NPS and *S. pneumoniae* isolates. Prior to purification of the genomic DNA from the NPS, the samples were diluted three times with sterilized saline and then centrifuged to remove inhibitory substances. The NPS pellets and a single colony of pneumococci were then digested with a lysis solution [1 *M* Tris, pH 8.9, 4.5 (v/v) nonident P-40, 4.5 (v/v) Tween 20, 10 mg/ml Proteinase K) for 1 h at 60°C. Following centrifugation, the supernatant was mixed with 3 *M* sodium acetate and the total genomic DNA was precipitated by ethanol.

PCR-based genotyping

Seven sets of oligonucleotide primers were used to amplify *pbp* genes (*pbp1a, pbp2x, pbp2b*), macrolide-resistant genes (*mefA* and *ermB*), the pneumococcal common autolysin gene (*lytA*) and the pneumolysin gene (*ply*) (Table 1) [16–18]. Each primer mixture contained multiplex PCR5, multiplex PCR6, multiplex PCR7 and PCR8 primers for *lytA* and *pbp1a, pbp2x* and *pbp2b, mefE* and *ermB*, and *ply*, respectively. The PCR reaction mixtures consisted of 1 μl of bacterial lysate or purified DNA from NPs, 8 μl 25 m*M* of dNTP mixture, 2.5 U of Tth DNA polymerase (Takara Biomedicals, Kyoto, Japan), 10 μl 10×PCR buffer, pH 8.3 and 60 ng of primer mixtures (multiplex PCR5, multiplex PCR6, multiplex PCR7 and PCR8) in 100 μl of solution. The reaction mixture was then subjected to 30 cycles (20 s at 94°C, 20 s at 55°C and 15 s at 72°C) of amplification in the programmable thermal cycler (Gene Amp PCR System 9700; Perkin-Elmer, Norwalk, Conn.). Amplified DNA fragments were analyzed using 3% agarose gel electrophoresis (Fig. 1).

PCR-based serotyping

The oligonucleotide primers were used to amplify capsular serotypes 1, 3, 4, 14, 19A, 19F, 23F and serogroups 6, 18,19 and 23 for multiplex PCR-based serotyping (Table 1) [4, 10]. The PCR reaction mixtures consisted of 1 μl of bacterial lysate or purified DNA, 0.5 μl 10 m*M* of dNTP mixture, 0.5 μl *Taq* DNA polymerase, 2.5 μl 10×PCR buffer, 4 μl 25 m*M* $MgCl_2$, 5.0 μl Q-solution (Qiagen, Hilden, Germany), 1.25 μl 1 *M* KCl and 0.25 μl of each primer sets (multiplex PCR1, multiplex PCR2, multiplex PCR3, multiplex PCR4) in 25 μl of solution. For serotyping, the reaction mixture was subjected to denaturation at 94°C for 10 min, followed by 32 cycles of amplification

Table 1 Primers used in this study

Primers	Target gene	Sequences	PCR products (bp)
Multiplex PCR 1	Serogroup 6	5′-TATAGATCCGATACGACGTAAC-3′	200
		5′-ATACCAATTACACCAAAGTCTG-3′	
	Serogroup 19	5′-CTAATGAGCCTAAACGTCTCT-3′	222
		5′-TTGACTGCACCAAGTACACT-3′	
	Serogroup 18	5′-GCATCTGTACAGTGTGCTAATTGGATTGAAG-3′	478
		5′-CTTTAACATCTGACTTTTTCTGTTCCCAAC-3′	
	Serogroup 23	5′-GATGCAAGAAATGTCGGTA-3′	126
		5′-TCTGCCTCATTGTTCTCC-3′	
Multiplex PCR 2	Serotype 1	5′-GTCGTTATGAGAAGGTGGA-3′	108
		5′-TGACCAAATAGAACCTGATG-3′	
	Serotype 19F	5′-GTTCAACGACTAGGACGC-3′	130
		5′-TAGGCACCAATGTTTCACT-3′	
Multiplex PCR 3	Serotype 3	5′-ATGTGGATTCGCAGAGTG-3′	152
		5′-GATTACGCTCAGGGTCAA-3′	
	Serotype 14	5′-AACCGACAAAAACAACTAAG-3′	220
		5′-AACCGACAAAAACAACTAAG-3′	
	Serotype 23F	5′-TGGTAGTGACAGCAACGA-3′	177
		5′-CAAAGGCTAATTCAGCATC-3′	
Multiplex PCR 4	Serotype 4	5′-CTGTTACTTGTTCTGGACTCTCGTTAATTGG-3′	430
		5′-GCCCACTCCTGTTAAAATCCTACCCGCATTG-3′	
	Serotype 19A	5′-GTTAGTCCTGTTTTAGATTTATTTGGTGATGT-3′	478
		5′-GAGCAGTCAATAAGATGAGACGATAGTTAG-3′	
Multiplex PCR 5	*pbp1a*	5′-AAACAAGGTCGGACTCAACC-3	430
		5′-ATATACATTGGTTTATAGTAAGTT-3′	
	lytA	5′-TGAAGCGGATTATCACTGGC-3	273
		5′-GCTAAACTCCCTG TATCAAGCG-3′	
Multiplex PCR 6	*pbp2x*	5′-CCAGGTTCCACTATGAAAGTG-3	292
		5′-ATCCCAACGTTACTTGAGTGT-3′	
	pbp2b	5′-CCTATATGGTCCAAACAGCCT-3′	147
		5′-GGTCAATTCCTGTCGCAGTA-3′	
Multiplex PCR 7	*mefA*	5′-CTGTATGGAGCTACCTGTCTGG-3	402
		5′-CCCAGCTTAGGTATACGTAC-3	
	ermB	5′-CGTACCTTGGATATTCACCG-3	224
		5′-GTAAACAGTTGACGATATTCTCG-3	
PCR8	*ply*	5′-ATTTCTGTAACAGCTACCAACGA-3′	348
		5′-GAATTCCCTGTCTTTTCAAAGTC-3′	

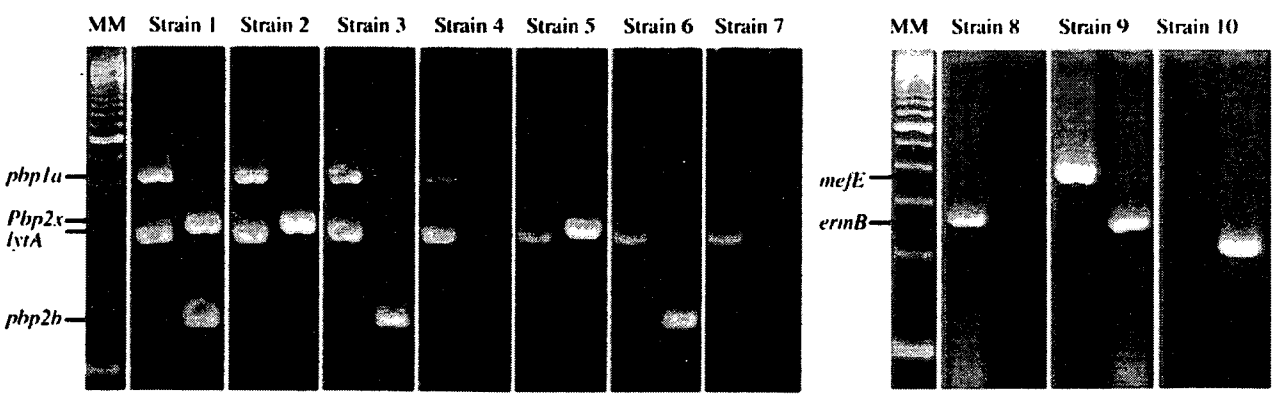

Fig. 1 PCR-based genotypes of seven clinical isolates of *Streptococcus pneumoniae*. *MW* Molecular-weight marker (100 bp). *Strain:1* No mutation in *pbp* genes, *2* mutation in *pbp2b*, *3* mutation in *pbp2x*, *4* mutations in *pbp2b* and *pbp2x*, *5* mutations in *pbp1a* and *pbp2b*, *6* mutations in *pbp1a* and *pbp2x*, *7* mutations in *pbp1a*, *pbp2x* and *pbp2b*, *8 ermB*-positive, *9 ermB*- and *mefE*-positive, *10 mefE*-positive

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 a further extension at 72°C for 5 min for serotyping. Strains of serotype 4 (ATCC BAA-334) and 19F (ATCC 49619) obtained from the American Type Culture Collection (ATCC, Manassas, Va.) were used for quality control in every reaction (Fig. 2).

Results

Identification of *S. pneumoniae* in NPS

S. pneumoniae were identified in 30 (50.0%) and 36 (60.0%) of the 60 NPS samples tested by conventional culture and PCR analysis, respectively. Not only were culturable *S. pnuemoniae* identifiable by conventional bacterial culture methodology and PCR analysis, but even samples containing pneumococci that were unidentifiable by culture techniques showed amplifications of both *lytA* and *ply* with PCR analysis.

Based on the CLSI criteria, the pneumococcal isolates consisted of 12 penicillin-sensitive *S. pneumoniae* (PSSP; 40.0%), 11 penicillin-intermediate *S. pneumoniae* (PISP) (36.7%) and seven penicillin-resistant *S. pneumoniae* PRSP; 23.3%). They were also classified into six (20.0%), one (3.33%) and 12 (76.67%) strains that showed sensitivity, intermediate resistance and resistance to CAM, respectively. Based on the serology, the isolates were classified into serotypes 3 (1; 3.3%), 14 (3; 10.0%), 19F (5; 16.7%), 23F (6; 20.0%), 6B (10; 33.3%), 6A (1; 3.3%), 15 (1; 3.3%), 9V (1; 3.3%) and non-11 (2; 6.7%) (Table 2). All serotype 23F strains and three (60.0%) 19F strains were resistant to both PCG and CAM.

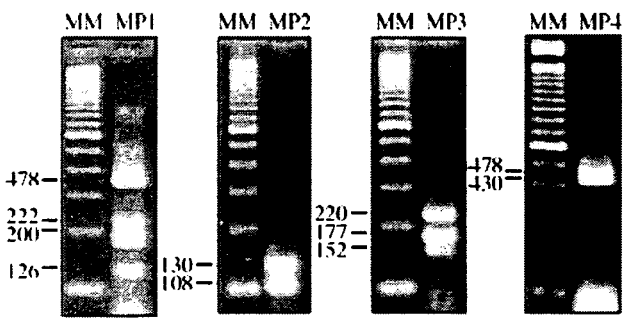

Fig. 2 A multiplex of PCR-based serotypes. *MM* Molecular-weight marker. *MP1* Serogroup 6, serogroup 19, serogroup 18 and serogroup 23, *MP2* serotype 1 and serotype 19F, *MP3* serotype 3, serotype 14 and serotype 23F, *MP4* serotype 4 and serotype 19A

PCR-based genotypes

Among 36 NPS samples, 19 (52.8%) samples contained strains with mutations in the three PBP genes (Table 2), eight (22.2%) contained either strains with mutations in *pbp1a* and *pbp2x* (four samples; 11.1%) or in *pbp2x* and *pbp2b* (four samples; 11.1%), eight (22.2%) contained either strains with a mutation in *pbp1a* (one sample; 2.8%), in *pbp2b* (one sample; 2.8%) and in *pbp2x* (six samples; 16.7%) and only one (2.8%) contained the strain without mutations in the three PBP genes.

Susceptibilities to PCG of strains with mutations in three PBP genes, in two types of PBP genes, in one type of PBP gene and without mutations in the PBP genes were 0.12–2, ≤0.06–1, ≤0.06 and ≤0.06 μg/ml, respectively. Twenty-six (72.2%) samples contained strains with macrolide-resistant genes: 15 (44.4%) samples with strains possessing *mefE*, 13 (19.4%) strains possessing *ermB* and three (8.3%) strains possessing both macrolide-resistant genes, respectively. Ten (27.8%) samples contained strains having neither type of macrolide-resistant gene. Susceptibilities to CAM of strains with the *mefE* gene, *ermB* gene and both were 1–4, >64 and >64 μg/ml, respectively.

PCR-based serotypes

The multiplex PCR was able to determine the serotypes/serogroups of 36 NPS samples; in contrast, conventional methodology determined the serotype/serogroups in only 30 samples (Table 2); these were serotypes 3 (1; 2.8%), 14 (3; 8.3%), 19F (7; 19.4%), 23F (10; 27.8%) and 6 (11; 30.6%) as well as seven 'others' (19.4%). With the exception of the typeable pneumococci, all of the serotypes identified by multiplex PCR showed a similar quellung reaction with antisera. Four untypeable samples by multiplex PCR were classified into serogroups 15 (one), 9V (one) and non-11 (two) types by serological determination. In this study we compared the ratio of accurate determination of serotypes by both procedures. The correct determination of the serotypes when both procedures showed same results was determining. If one procedure failed to identify serotypes determined by the other procedure, the cases were defined as false determinations. The multiplex PCR determined the pneumococcal serotypes in 35 (97.2%) of the 36 NPS samples, while a conventional serological method determined the serotypes in 29 (80.5%) of the 36 NPS samples ($p<0.05$). Two NPS samples were culture-negative but contained pneumococcal genomes. One sample (no. 8) showed serogroup 23 by multiplex PCR, but showed serotype 6B by serological determination. Two different serotypes were determined concurrently in two NPS by multiplex PCR. The positive and negative predictive value of PCR based on the serotyping of NPS

Table 2 Results of the multiplex PCR and conventional culture analyses in terms of detecting, genotyping and serotyping strains of *S. pneumoniae* in pneumococcal-positive nasopharyngeal secretions

Sample no.	*S. pneumoniae* isolates								Nasopharyngeal secretions							
	Culture	Serotypes	PCR-based genotypes						PCR		PCR-based serotypes	PCR-based genotypes				
			PBP gene mutaion			MLR genes		MIC	*ply*	*lytA*		PBP gene mutaion			MLR gene	
			pbp1a	*pbp2b*	*pbp2x*	*mefE*	*ermB*	PCG	CAM				*pbp1a*	*pbp2b*	*pbp2x*	*mefE*	*ermB*
1	+	3	−	+	−	−	−	≤0.06	≤0.06	+	+	3	−	+	−	−	−
2	+	6A	+	+	+	−	−	0.5	1	+	+	G6	+	+	+	−	−
3	+	6B	−	−	+	−	−	≤0.06	0.5	+	+	G6	−	−	+	−	−
4	+	6B	−	+	+	−	−	1	0.25	+	+	G6	−	−	+	−	−
5	+	6B	−	−	+	−	−	≤0.06	1	+	+	G6	−	−	+	−	−
6	+	6B	+	−	+	+	−	≤0.06	1	+	+	G6	+	+	+	−	−
7	+	6B	+	+	+	−	−	0.12	0.25	+	+	G6	+	+	+	−	−
8	+	6B	+	+	+	−	−	0.5	0.25	+	+	G23	+	+	+	−	−
9	+	6B	+	+	+	+	+	2	>64	+	+	G6	+	+	+	+	−
10	+	6B	+	−	+	+	−	0.12	2	+	+	G6	+	+	+	+	−
11	+	6B	+	+	+	+	−	≤0.06	2	+	+	G6	+	+	+	+	−
12	+	6B	+	−	+	−	+	≤0.06	>64	+	+	G6	+	+	+	+	+
13	+	9V	+	−	+	−	+	≤0.06	>64	+	+	others	+	−	+	−	+
14	+	14	+	−	+	+	+	0.12	>64	+	+	14	−	−	+	+	−
15	+	14	+	+	+	−	−	0.5	1	+	+	14	+	−	+	−	−
16	+	14	+	+	+	+	−	0.25	4	+	+	14	−	+	+	+	−
17	+	G15	−	−	−	−	+	≤0.06	>64	+	+	others	−	−	−	−	+
18	+	19F	+	−	+	+	−	≤0.06	>64	+	+	19F	+	−	−	+	−
19	+	19F	+	−	+	−	−	≤0.06	≤0.06	+	+	19F	+	+	+	−	−
20	+	19F	+	+	+	+	−	2	2	+	+	19F	+	+	+	+	−
21	+	19F	+	+	+	+	−	2	2	+	+	19F, 23F	+	+	+	+	−
22	+	19F	+	+	+	+	−	1	2	+	+	19F, 23F	+	+	+	+	−
23	+	23F	+	+	+	+	−	1	>64	+	+	23F	+	+	+	+	−
24	+	23F	+	+	+	+	−	2	>64	+	+	23F	+	+	+	+	−
25	+	23F	+	+	+	+	+	2	>64	+	+	23F	+	+	+	+	+
26	+	23F	+	+	+	−	+	2	>64	+	+	23F	+	+	+	+	−
27	+	23F	+	+	+	−	+	1	>64	+	+	23F	+	+	+	+	+
28	+	23F	+	+	+	+	−	2	4	+	+	23F	+	+	+	+	−
29	+	Non-11	+	−	+	−	+	≤0.06	>64	+	+	others	+	−	+	−	+
30	+	Non-11	−	−	+	−	−	≤0.06	≤0.06	+	+	others	−	−	+	+	−
31	−	NA	NA	NA	NA	NA	NA	NA	NA	+	+	G6	+	−	+	+	−
32	−	NA	NA	NA	NA	NA	NA	NA	NA	+	+	19F	−	+	+	−	+
33	−	NA	NA	NA	NA	NA	NA	NA	NA	+	+	19F	−	+	+	−	+
34	−	NA	NA	NA	NA	NA	NA	NA	NA	+	+	23F	−	+	+	−	+
35	−	NA	NA	NA	NA	NA	NA	NA	NA	+	+	others	+	+	+	+	−
36	−	NA	NA	NA	NA	NA	NA	NA	NA	+	+	others	−	−	+	−	+

+, Pneumococcus-positive or mutation of *pbp* gene; −, pneumococcus-negative or no mutation of *pbp* gene; NA, not applicable; Non11, other than the 11 valents conjugated pneumococcal vaccine serotypes; others, other than capsular serotype 1, 3, 4, 14, 19A, 19F, 23F and serogroup 6, 18, 19 and 23; PCG, penicillin G; CAM, clarithromycin

compared with the serological method was 96.3% (26/27 samples) and 100%, respectively.

The multiplex PCR was able to determine the serotypes/serogroups of four NPS samples in which pneumococci were not identified by a conventional bacterial culture and in which it had not been possible to determine pneumococcal serotypes/serogroups.

Discussion

In this era of antimicrobial resistant *S. pneumoniae* and vaccine development against this pathogen, it is important to be able to evaluate the characteristics of the pneumococci colonizing the nasopharynx [3, 5, 6]. However, the current serological determinations of capsular serotypes are difficult to interpret in terms of the quellung reactions of pneumococcal cells with anti-polysaccharide sera [2]. Consequently, attempts have made to determine the accurate pneumococcal characteristics by molecular biological procedures. Earlier studies in our laboratory determined that the application of PCR-based serotyping and genotyping to pneumococcal isolates facilitates the identification of pneumococcal isolates [2] and that the multiplex PCR serotyping approach is able to determine particular types of capsular polysaccharides accurately. PCR-based genotyping also revealed that the frequencies of mutations in *pbp* and the expressions of *mefA* and *ermB* are closely related with susceptibilities to β-lactams and macrolides [6]. However, most of the studies applied PCR to pneumococcal isolates – and not directly to clinical samples [4, 10]. In the present study, we applied the multiplex PCR technique directly to NPS specimens and were able to identify and determine the characteristics of 11 pneumococcal serotypes and serogroups simultaneously. Those identified consisted of most of the serotypes used for current pneumococcal vaccines. We also applied PCR-based genotyping to the *pbp* genes and macrolide resistance genes.

Serogroups 6, 19F or 23F were the predominant capsular types in this study. Approximately 52.8% of the NPS samples contained pneumococci having mutations in the three *pbp* genes and could be classified as PRSP according to the CLSI criteria. Approximately 72.2% of the NPS samples contained pneumococci expressing either type of macrolide-resistant trait and were resistant to CAM. Most of these strains with mutations in the three *pbp* genes belonged to serogroups 6, 19F or 23F. The multiple PCR analysis was able to determine the pneumococci and the pneumococcal serotypes and serogroups isolated from the NPS samples more accurately than conventional culture and immunological methodology. In six samples, pneumococcal genomes were identified by the multiplex PCR, while a conventional culture method failed to identify pneumococci. In two cases, strains of serotypes 19F and 23F were simultaneously identified in the NPS, although only one strain of serotype 19F was identified by a conventional culture method. Recent reports on vaccine efficacies against nasopharyngeal colonization with pneumococci have shown that the pneumococci in the nasopharynx can change from vaccine serotypes to non-vaccine serotypes following vaccination [1, 14, 20]. The underlying factors determining these changes are still unclear, although one hypothesis is a replacement of pneumococcal strains in the nasopharynx. Nasopharygeal pneumococcal flora consist of several different strains, with minor pneumococcal populations present in the nasopharynx. Current conventional serological methodology requires the isolation of the pneumococcal strains. Huebner *et al.* suggested that if the less common serotype represents only 5% of the total pneumococcal population, 59 colonies from each specimen would need to be serotyped to have a 95% probability of picking the second pneumococcal type [7]. In addition, non-PCR methods can be prone to mis-interpretation. In the present study, a strain was determined to be serogroup 23 by the multiplex PCR, while based on the serology, the strain was serotype 6B. It is somewhat difficult to interpret the quellung changes and the serological determination sometimes failed to determine the accurate prevalence of pneumococci in the nasopharynx.

In this study, we have analyzed a limited number of serotypes by the multiplex PCR techniques because of the unavailability of the sequences of other pneumococcal serotypes. However, multiplex PCR-based serotyping can provide an accurate and rapid distribution of pneumococcal serotypes, including minor populations, among the *S. pneumoniae* populations of the nasopharynx. The multiplex PCR method does not need expertise to interpret the results and can be used to run many samples at one time. Moreover, multiplex PCR-based serotyping and genotyping can replace conventional microbiological methods that are used to identify and determine S. pneumoniae, pneumococcal capsular serotype, penicillin susceptibility and macrolide resistance traits. A follow-up study involving the quantitative evaluation by real-time PCR is necessary, and continuous monitoring of pneumococcal serotypes is essential since it has been shown that the incidence of types responsible for AOM can change over time [11]. We have shown that the direct application of PCR to the NPS enables a feasible analysis of minority strains in a pneumococcal population and confirmed this by studies on the carriage of multiple pneumococcal capsular types.

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Rapid identification of nontypeable and serotype b *Haemophilus influenzae* from nasopharyngeal secretions by the multiplex PCR

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Summary

Objective: *Heamophilus influenzae* (*H. influenzae*) is an important pathogen responsible for both invasive and non-invasive infectious diseases. While encapsulated type b strain recognized as a major cause of severe invasive diseases, nontypeable strains are the major causes of non-invasive infectious diseases. Detection of this pathogen from nasopharyngeal secretions (NPS) is important.
Methods: We developed a multiplex polymerase chain reaction (PCR) for rapid identification of nontypeable and serotype b *H. influenzae* from nasopharyngeal secretions.
Results: A total 25 nasopharyngeal secretions were evaluated in this study. The multiplex PCR provided rapid and unequivocal results for determining either nontypeable or encapsulated typeable especially type b strains including a determination of β-lactamase productions.
Conclusion: The multiplex PCR based serotyping provided more reliable results than slide agglutination test (SAT) and is a valuable and expeditious method for identification of *H. influenzae* with determining capsular serotypes.
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1. Introduction

Haemophilus influenzae colonizes in the human nasopharynx and becomes a leading cause for a variety of infectious disease such as acute otitis

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media (AOM), sinusitis, pneumonia, sepsis and meningitis [1—4]. The pathogens are classified into six encapsulated strains called typeable depending on the serologically distinct capsular polysaccharides (serotypes a—f) and non-capsulated/nontypeable strain. While encapsulated type b strain recognized as a major cause of severe invasive diseases such as meningitis and bacteremia, nontypeable strains are the major causes of non-invasive infectious disease such as otitis media [5,6].

Serotypes of *H. influenzae* have usually been determined by the slide agglutination test (SAT) with six specific antisera against each type of capsular polysaccharides. The test is easy and simple to be applied. However, the results are sometimes unreliable because of atypical agglutinations [7,8]. In the current pre-vaccination era, more exact procedures are required to determine either typeable strains, especially type b strain, or nontypeable strains for reducing risks of severe invasive infections caused by typeable strains among children and for further development of vaccine against nontypeable strains [9,10]. To determine typeable strains more clearly, recent studies focused on detections of *bexA* gene encoding common capsular polysaccharide sequences of *H. influenzae* [7,11—14].

In this study, we applied multiplex polymerase chain reaction (PCR) to determine nontypeable or encapsulated typeable strains and production of β-lactamase.

2. Material and methods

2.1. Strains

Seven encapsulated typeable *H. influenzae* strains (ATCC9327, ATCC9334, ATCC9007, ATCC9332, ATCC8142, ATCC9833, one clinical isolate) and one nontypeable *H. influenzae* (ATCC49247) were used as positive controls in this study. *Staphylococcus aureus* (*S. aureus*) (ATCC25922) and *Streptococcus pneumoniae* (*S. pneumoniae*) (ATCC49619 and BAA-3) were also used as negative controls (Table 1).

2.2. Samples

A total 25 nasopharyngeal secretions (NPS) were collected from children (12—48 months old) with acute otitis media at the outpatient clinic of Otolaryngology-Head and Neck Surgery of Wakayama Medical University Hospital. Nasopharyngeal secretions were collected by the suction with a fine, flexible plastic catheter (no. 5, French) and syringe. Informed consents were obtained from patient's parents or guardians prior to the collection of samples according to the institutional review board.

2.3. Identification of *H. influenzae*

The 100 μl of NPS were cultured on chocolate agar plate and incubated at 37 °C for 24—48 h. *H. influenzae* strains were identified by colony morphology on the chocolate agar plates, no growth in blood agar plate, Gram's staining, catalase test and requirement of X and V factors. The growth of *H. influenzae* were semi-quantitatively determined as 1+, 2+ and 3+ that represent the growth in first, second and third quadrant of chocolate agar plate, respectively. Productions of β-lactamase were examined by nitrocefinase disc (Nippon Becton Dickinson Company Ltd., Tokyo, Japan). Serotypes of *H. influenzae* were determined by the slide agglutination test with six specific antisera against each type of capsular polysaccharides (Denka Seiken Co., Ltd., Tokyo).

Table 1 *H. influenzae* and other strains in this study

Strains	Serological agglutination test	β-lactamase	PCR			
			P6	bexA	typeb	TEM-1
H. influenzae ATCC 9327	Type a	−	+	+	−	−
H. influenzae ATCC 9334	Type b	−	+	+	+	−
H. influenzae ATCC 9007	Type c	−	+	+	−	−
H. influenzae ATCC 9332	Type d	−	+	+	−	−
H. influenzae ATCC 8142	Type e	−	+	+	−	−
H. influenzae ATCC 9833	Type f	−	+	+	−	−
H. influenzae clinical isolates	Type b	+	+	+	+	+
H. influenzae ATCC 49247	Nontypeable	−	+	−	−	−
S. aureus ATCC 25922	N/A	N/A	−	−	−	N/A
S. pneumoniae ATCC 49619	N/A	N/A	−	−	−	N/A
S. pneumoniae ATCC BAA-3	N/A	N/A	−	−	−	N/A

N/A, not assessed.

2.4. Purification of genomic DNA

The genomic DNA was purified from NPS. Briefly, the NPS samples were diluted 1:3 with sterilized saline and centrifuged at 12,000 rpm for 30 min at 4 °C. The pellets were collected and digested with 200 μl of lysis solution (1 M Tris pH 8.9, 4.5 v/v, nonident P-40, 4.5 v/v, Tween 20, 10 mg/ml Proteinase K) for 1 h at 60 °C. After centrifugation at 12,000 rpm for 20 min at 4 °C, the supernatants were mixed with 100 μl of 3 M sodium acetate buffer and then with 1 ml cold ethanol. Total genomic DNA was purified as precipitations. The total genomic DNA was also purified from *H. influenzae* isolates. Lysis of *H. influenzae* total DNA also used for PCR. In brief, a single colony of *H. influenzae* isolates on a chocolate agar plate was lysed in 30 μl of lysis solution for 10 min at 60 °C and for 5 min at 94 °C in the programmable thermal cycler (Gene Amp PCR System 9700, Perkin-Elmer, Norwalk, CT, USA).

2.5. Multiplex PCR

Primers specific for the *bexA* gene encoding capsular polysaccharides (H1 and H2), *p6* gene encoding a common outer membrane protein P6 (P6-S and P6-R), β-lactamase gene (TEM-S and TEM-R) and *cpsb* gene encoding type b capsular polysaccharide (typeB-S and typeB-R) were used for the multiplex PCR to identify nontypeable, type b and β-lactamase producing *H. influenzae* strains, respectively (Table 2) [7,8,14].

Each 25-μl reaction mixture contained 0.5 μl of each primers (final concentrations were 1 μM of H1 and H2, 1 μM of TEM-S and TEM-R, 1 μM of typeB-S and typeB-R, 3 μM of P6-S and P6-R), 3 μl template DNA, 12.5 μl Qiagen master mixture (Qiagen Gmbh, Germany), 2 μl of 25 mM $MgCl_2$ and 4.5 μl distilled water. The reaction mixture was subjected to amplification in the programmable thermal cycler consisting in denaturation at 94 °C for 10 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s and further extension at 72 °C for 10 min. Amplified DNA fragments were analyzed by 2% agarose gel electrophoresis.

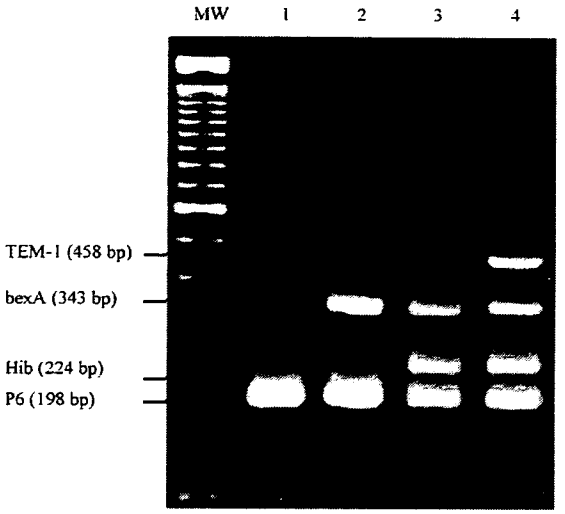

Fig. 1 Multiplex PCR of *H. influenzae*. MW, molecular weight (100 bp); Lane 1, nontypeable *H. influenzae* (ATCC49247); Lane 2, non-type b encapsulated *H. influenzae* (ATCC9327); Lane 3, type b *H. influenzae* (ATCC9334) and Lane 4, β-lactamase producing type b *H. influenzae* (clinical isolate). DNA fragments correspond as follows P6 gene (198 bp), Hib (224 bp) and *bexA* (343 bp), TEM-1 (458 bp).

3. Results

3.1. Specificity and sensitivity of multiplex PCR

The multiplex PCR were specific to *H. influenzae* strains and could identify typeable, type b and nontypeable strains including productions of β-lactamase (Fig. 1, Table 3). The negative controls of *S. aureus* and *S. pneumoniae* did not showed any amplification for the four genes (Table 3).

Table 2 Primers for the multiplex PCR

Primer name	Target	Sequence (5′–3′)	Primer length	Position	PCR product (bp)
P6-S	*P6*	ACGATGCTGCAGGCAATGGT	20	141–160	198
P6-R		CATCAGTATTACCTTCTACTAAT	23	316–338	
TEM-S	*TEM-1*	TAAGAGAATTATGCAGTGCTGCC	23	350–372	458
TEM-R		TCCATAGTTGCCTGACTCCCC	21	787–807	
HI-1	*bexA*	CGTTTGTATGATGTTGATCCAGACT	25	3552–3577	343
HI-2		TGTCCATGTCTTTCAAAATGATG	22	3873–3895	
Typeb-S	*cpsb*	AGATACCTTTGGTCGTCTGC	20	5483–5502	224
Typeb-R		CTTACGCTTCTATCTCGGTG	20	5706–5725	

Table 3 Identification of nontypeable and type b *H. influenzae*

Patient ID	Culture	Serological aggligation test	β-lactamase	Multiplex PCR							
				Bacterial lysate				Nasopharyngeal secretion			
				P6	bexA	typeb	TEM-1	P6	bexA	typeb	TEM-1
1	3+	N/T	–	+	–	–	–	+	–	–	–
2	1+	N/T	–	+	–	–	–	+	–	–	–
3	NGHi	N/A	–	N/A	N/A	N/A	–	–	–	–	–
4	3+	N/T	–	+	–	–	–	+	–	–	–
5	3+	N/T	–	+	–	–	–	+	–	–	–
6	NGHi	N/A	–	N/A	N/A	N/A	–	–	–	–	–
7	NGHi	N/A	–	N/A	N/A	N/A	–	–	–	–	–
8	1+	N/T	–	+	–	–	–	+	–	–	–
9	3+	N/T	–	+	–	–	–	+	–	–	–
10	3+	Type b	–	+	+	+	–	+	+	+	–
11	3+	N/T	–	+	–	–	–	+	–	–	–
12	3+	N/T	–	+	–	–	–	+	+	+	–
13	3+	N/T	–	+	–	–	–	+	–	–	–
14	NGHi	N/A	–	N/A	N/A	N/A	–	+	+	+	–
15	2+	Type b	–	+	+	+	–	+	+	+	–
16	NGHi	N/A	–	N/A	N/A	N/A	–	–	–	–	–
41	NGHi	N/A	–	N/A	N/A	N/A	–	–	–	–	–
42	1+	N/T	–	+	–	–	–	+	–	–	–
49	NGHi	N/A	–	N/A	N/A	N/A	–	–	–	–	–
50	1+	Type b	+	+	+	+	+	+	+	+	+
51	3+	N/T	–	+	–	–	–	+	–	–	–
52	3+	N/T	–	+	–	–	–	+	–	–	–
53	NGHi	N/A	–	N/A	N/A	N/A	–	–	–	–	–
58	3+	N/T	–	+	–	–	–	+	–	–	–
59	3+	N/T	–	+	–	–	–	+	–	–	–

NGHi, no growth of *H. influenzae*; +, positive; –, negative; N/A, not applicable; NT, nontypeable; 1+, 2+, 3+, growth in first, second and third quadrant of chocolate agar plate, respectively.

The lowest limit of the multiplex PCR to identify the genomic DNA of *H. influenzae* strain was 2×10^{-3} ng (Fig. 2).

3.2. Identification of *H. influenzae* in NPS

H. influenzae were isolated in 17 (68.0%) samples by conventional culture method. There were 3 (17.6%) serotype b strains and 14 (82.4%) non-encapsulated strains by SAT. Only one strain (4%) produced β-lactamase. All the strains identified in NPS by conventional culture method were confirmed as the similar characteristics by the multiplex PCR.

On the other hand, *H. influenzae* were identified in 18 (72.0%) out of the 25 NPS samples by the multiplex PCR. There were 5 (27.8%) type b strains possessing both *bexA* and *cpsb* genes and 13 (72.2%) non-encapsulated strains without *bexA* gene. The β-lactamase gene was identified in one sample. Two NPS samples (patient nos. 12 and 14) showed amplicons for *bexA* and *cpsb* genes while encapsulated *H. influenzae* strains were not isolated in these samples. One is the sample in which non-encapsulated strain was identified by conventional culture method and another is the sample in which *H. influenzae* were not identified by the conventional culture method (Table 3).

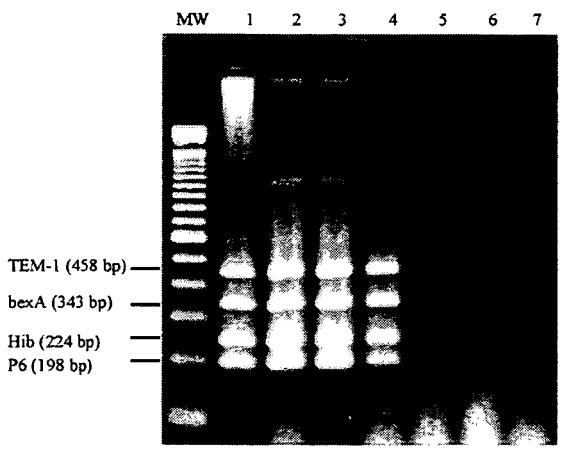

Fig. 2 Sensitivity of multiplex PCR. MW, molecular weight (100 bp); Lane 1, 50 ng; Lane 2, 5 ng; Lane 3, 500 pg; Lane 4, 50 pg; Lane 5, 5 pg; Lane 6, 500 fg and Lane 7, 50 fg.

4. Discussion

Since the type b capsular polysaccharide conjugate vaccine widely used in the United States, the incidence of invasive infectious diseases caused by type b strain in children less than 5 years old have remarkably decreased but increased relative importance of nontypeable strains and other encapsulated typeable strains [15,16]. In contrast to the United States, type b capsular polysaccharide conjugated vaccine has not licensed yet in Japan. The type b *H. influenzae* strain still remains the leading causes of meningitis in Japan [17–19]. It is worthy to identify nontypeable and encapsulated typeable strains. The SAT has been widely applied to determine the types of capsular polysaccharides of *H. influenzae*. However, the test has sometimes shown to be unreliable due to serological cross-reactions and/or lower sensitivity of sera. Laclaire *et al.* reported that two-thirds of serotype b *H. influenzae* isolates reported to CDC were incorrectly classified by SAT in 2003 but actual prevalence of Hib will be overestimated [8]. In addition to these problems, the SAT cannot be applied clinical specimens directly. It requires isolation of *H. influenzae* according to the usual laboratory cultures prior to determine the capsular types and it takes more than 48 h.

In this study, we firstly applied multiplex PCR to identify *H. influenzae* and determine the capsular characteristics of the pathogen isolated in NPS. *H. influenzae* were identified at 68–72% among NPS. About 82.4% by culture and 72.2% by the multiplex PCR of *H. influenzae* isolates were nontypeable strains. Ueyama *et al.* reported that more than 90% of strains in the nasopharynx were nontypeable [20]. About 17.6% by culture and 27.8% by PCR of *H. influenzae* were Hib strains. The surveillance of pediatric respiratory tract infectious diseases during 1980–1991 showed that only 2.6% of isolates were type b strains [21,22]. One sample (patient no. 14) in which we failed to identify *H. inlfuenzae* by conventional bacterial culture had the DNA genome of type b strain. The multiplex PCR based serotyping provided more reliable results than SAT. However, one sample (patient no. 12) in which nontypeable *H. influenzae* was isolated by bacterial culture possessed *bexA* and *cpsb* genes by the multiplex PCR. While the predominant strain in the NPS will be nontypeable, small concomitants of type b strain would be exist in the NPs. By the conventional methods alone, we cannot determine the actual prevalence of Hib that will be the minority of pathogens among the nasopharynx. Hubener *et al.* suggested that if the less common serotype represents only 5% of the total pneumococcal population, 59 colonies from each specimen would need to be serotyped to have a 95% probability of picking the second pneumococcal type [23]. The multiplex PCR allows a feasible analysis of minority strains in the nasopharyngeal *H. influenzae* population. It is necessary to apply real-time quantitative PCR for further quantitative evaluations. However, the method can easily be implied among microbiology laboratories and can assess many samples at once with rapid reliable results.

In conclusion, the possibility to use a multiplex PCR method as a qualitative assay to evaluate the true composition of possibly diverse populations of *H. influenzae* increases the usefulness as a new typeable/nontypeable technique.

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In vitro induction and selection of fluoroquinolone-resistant mutants of *Streptococcus pyogenes* strains with multiple *emm* types

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***Objectives*: To perform a systematic analysis of point mutations in the quinolone resistance determining regions (QRDRs) of the DNA gyrase and topoisomerase genes of *emm* type 6 and other *emm* types of *Streptococcus pyogenes* strains after *in vitro* exposure to stepwise increasing concentrations of levofloxacin.**

***Methods*: Twelve parent strains of *S. pyogenes*, each with a different *emm* type, were chosen for stepwise exposure to increasing levels of levofloxacin followed by selection of resistant mutants. The QRDRs of *gyrA*, *gyrB*, *parC* and *parE* correlating to mutants with increased MICs were analysed for point mutations.**

***Results*: Multiple mutants with significantly increased MICs were generated from each strain. The amino acid substitutions identified were consistent regardless of *emm* type and were similar to the mechanisms of resistance reported in clinical isolates of *S. pyogenes*. The number of induction/selection cycles required for the emergence of key point mutations in *gyrA* and *parC* was variable among strains. For each parent-mutant set, when MIC increased, serine-81 of *gyrA* and serine-79 of *parC* were the primary targets for amino acid substitutions. No point mutations were found in the QRDRs of *gyrB* and *parE* in any of the resistant mutants sequenced.**

***Conclusions*: Despite its intrinsic polymorphism in the QRDR of *parC*, *emm* type 6 is not more likely to develop high-level resistance to fluoroquinolones when compared with other *emm* types. All *emm* types seem equally inducible to high-level fluoroquinolone resistance.**

Keywords: *S. pyogenes*, resistance, laboratory induction and selection, point mutations

Introduction

Since the first report of multiple fluoroquinolone resistance in *Streptococcus pyogenes*,[1] patient isolates with reduced susceptibility to fluoroquinolones have been reported by several investigators throughout the world.[2–6] Published reports have indicated that the prevalence of *S. pyogenes* with reduced susceptibility to ciprofloxacin was 3.5% in 1998–99 in Spain, 5.4% in 1999–2002 in Belgium and 10.9% in 2002–03 in the United States.[4,5,7] Orscheln *et al.*[4] reported that all *S. pyogenes emm* type 6 isolates they investigated had intrinsic reduced susceptibility to fluoroquinolones due to a polymorphism in the quinolone resistance determining region (QRDR) of the *parC* gene, which codes for a change from serine-79 to alanine. Analysis of the *gyrA* and *parC* gene sequences of some of those fluoroquinolone-resistant isolates has demonstrated point mutations along the QRDRs analogous to previously reported mutations in *S. pyogenes*.[1–4,6] The limited sequencing data from the wild-type clinical isolates shows a lack of a systematic correlation between fluoroquinolone resistance level and amino acid substitutions in the QRDRs of *gyrA* and *parC*, leaving uncertainty as to whether resistance to fluoroquinolones in *S. pyogenes* is a continuously evolving process or occurs as random events.

Stepwise acquisition of mutations in the QRDRs of the *parC* and *gyrA* genes of *Streptococcus pneumoniae* has been

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demonstrated *in vitro* after exposure to increasing concentrations of fluoroquinolones.[8] Stepwise acquisition of point mutations in the QRDRs of *S. pyogenes* is implied because wild-type clinical isolates with higher MICs of fluoroquinolones have different amino acid substitutions at the same location as isolates with lower MICs.[1–4,6] Laboratory-generated mutants of *S. pyogenes* strains through serial passages by an exposure and selection process may help investigators correlate the appearance of point mutations with increased MICs of fluoroquinolones.[9–11] Previous reports of laboratory-induced mutants with reduced fluoroquinolone susceptibility did not include *emm* typing data, have incomplete analysis of sequencing information of the QRDRs of *gyrA/parC* genes, or did not evaluate mutations in the QRDRs in a stepwise fashion. A pioneer study of Schmitz *et al.*[11] identified alterations in the *gyrA* and *parC* genes of resistant mutant isolates, but did not report the change in point mutations for each resistant mutant throughout the stepwise exposure to fluoroquinolones.

In this current project, 12 parent strains representing 12 different *emm* types were chosen for stepwise induction and selection by increasing levels of levofloxacin in order to determine whether isolates of various serotypes of *S. pyogenes* may be equally inducible to resistance and whether levels of resistance to fluoroquinolone correlate with particular substitutions of amino acid residues in the QRDRs. The findings of concomitant point mutations with specific amino acid substitutions correlated with increased resistance to fluoroquinolones in *S. pyogenes* of multiple *emm* types are essential for a better understanding of whether the emergence of fluoroquinolone resistance is more likely with certain *emm* types.

Materials and methods

Bacterial strains and growth conditions

Twelve wild-type isolates of *S. pyogenes* of different *emm* types (1, 4-1, 6-1, 9, 11, 12, 28, 73-3, 75-5, 89, 94 and 103) with comparable initial susceptibilities (not more than 4-fold difference in MICs) to levofloxacin were selected for the induction assays. Ten isolates were recovered from swabs collected from patients with tonsillitis and one of each from patients with pharyngitis and rhinosinusitis. These 12 *emm* types are common both in Japan and in the USA. The strains were originally provided by Sugita ENT Clinic (Urayasu, Japan) and Tokyo Metropolitan Institute of Public Health (Tokyo, Japan). *emm* typing of the *S. pyogenes* strains was performed at the Tokyo Metropolitan Institute of Public Health by sequencing according to the recommendation of the Division of Bacterial and Mycotic Diseases, Center for Diseases Control and Prevention, and using the *emm* sequence database (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). *S. pyogenes* was identified using standard methods.[4]

Susceptibility testing

MICs of levofloxacin (Daiichi Pharmaceuticals, Tokyo, Japan) for parent and mutant strains were determined by a reference broth microdilution method in cation-adjusted Muller–Hinton broth (Difco Laboratories, Detroit, MI, USA) supplemented with 5% lysed horse blood, as recommended by the Clinical Laboratory Standards Institute (CLSI, formerly NCCLS).[12] Dilutions of levofloxacin ranged from 0.125 to 128 mg/L. *S. pneumoniae* ATCC 49619 was used for quality control with a QC range of 0.5–2 mg/L. In addition, susceptibility of the parent and mutant strains to other representative fluoroquinolones (ciprofloxacin, gatifloxacin, ofloxacin and norfloxacin) was determined using the Etest (AB Biodisk, Solna, Sweden) following the manufacturer's instructions.

Multicycle induction and selection of resistant mutants

Parent strains were grown in antibiotic-free Todd–Hewitt broth supplemented with 0.5% yeast extract (Difco Laboratories) at 37°C for 18 h, and $\sim 1 \times 10^8$ cfu/mL (0.5 McFarland standard) of each strain was inoculated into 2 mL of Todd–Hewitt broth containing levofloxacin. The levofloxacin concentrations used for mutant induction ranged from 2× to 4× the MIC for the parent strain or sub-parent mutant strain resulting from the prior induction step. After an overnight incubation at 37°C, cells growing in the tubes with the highest levofloxacin exposure concentration were selected for mutants with increased MICs by plating onto levofloxacin embedded agar plates at concentrations of 0, 2, 4, 8 and 16 mg/L, or higher when necessary. Three colonies were randomly selected for MIC determination, and isolates with the highest MIC were subjected to sequence analysis and further induction. This was repeated when a higher exposure concentration was used for the next step of the induction/selection cycle until mutants with significantly high MICs were selected. The stability of the selected resistant mutants was confirmed by sub-culture onto antibiotic-free 5% sheep blood plates (Nippon Becton Dickinson Company Ltd, Tokyo, Japan) for 10 serial passages.

Amplification and DNA sequencing of the QRDRs

Mutational alterations in the QRDRs of all subunits for DNA gyrase and topoisomerase IV of both parent and fluoroquinolone-resistant mutants were investigated by DNA sequence analysis. Table 1 shows the primers used for amplification of fragments of *gyrA/B* and *parC/E* containing the QRDR. Multiple DNA sequencing reactions were performed for each QRDR of individual strains using the Applied Biosystems sequencing kit and ABI Prism 310 Genetic Analyzer (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA).

Results

Fluoroquinolone-resistant mutants

Table 2 shows the MICs of levofloxacin for the parent strains and the mutants. MICs of the other fluoroquinolones tested for all mutants showed increases nearly identical with those observed

Table 1. Primers used for amplification of QRDR fragments of *gyrA*, *gyrB*, *parC* and *parE*

QRDR target	Nucleotide location	Primer sequence
gyrA	3–28	5′-GCAAGATCGAAATTTAATTGACGTC
	595–617	5′-ACTCTCTTGTTGTACAGTCTGG
gyrB	790–811	5′-GCGGCTCTTACTCGGGTCATCA
	1722–1746	5′-TTCTGCGGCATCATCAACTGTCAC
parC	1–24	5′-ATGTCAAACATTCAAAACATGTCC
	500–521	5′-AGCCTGCGGAAATACCAGAAG
parE	970–991	5′-GCTAGACCTATTGTAGAGAGC
	1802–1823	5′-TTATCCTCGATCCACTGACGG