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Genetic Characteristics and Clonal Dissemination of β-Lactamase-Negative Ampicillin-Resistant *Haemophilus influenzae* Strains Isolated from the Upper Respiratory Tract of Patients in Japan▽

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We evaluated the recent prevalence of antimicrobial-resistant *Haemophilus influenzae* isolated from the upper respiratory tracts (URT) of patients in Japan. Mutations in the *ftsI* gene, which encodes penicillin binding protein 3 (PBP3), and the clonal dissemination of the resistant strains were also investigated. A total of 264 *H. influenzae* isolates were collected from patients with URT infections. According to the criteria of the Clinical and Laboratory Standards Institute for the susceptibility of *H. influenzae* to ampicillin (AMP), the isolates were distributed as follows: 161 (61.0%) susceptible strains (MIC ≤ 1 μg/ml), 37 (14.0%) intermediately resistant strains (MIC = 2 μg/ml), and 66 (25.0%) resistant strains (MIC ≥ 4 μg/ml). According to PCR-based genotyping, 172 (65.1%) of the isolates had mutations in the *ftsI* gene and were negative for the β-lactamase (*bla*) gene. These 172 isolates were thus defined as genetically β-lactamase-negative ampicillin-resistant (gBLNAR) strains. The *ftsI* mutant group included 98 (37.1%) strains with group I/II mutations in the variable mutated region (group I/II gBLNAR) and 74 (28.0%) strains with group III mutations in the highly mutated region (group III gBLNAR). Eighty-seven (33.0%) of the isolates were genetically β-lactamase-negative ampicillin-susceptible (gBLNAS) strains. The group III gBLNAR strains showed resistance to β-lactams. Only five strains (1.9%) were positive for a *bla* gene encoding TEM-type β-lactamase. The three clusters consisting of 16 strains found among the 61 BLNAR strains (MIC ≥ 4 μg/ml and without the *bla* gene) showed identical or closely related DNA restriction fragment patterns. Those isolates were frequently identified among strains with a MIC to AMP of 16 μg/ml. The current study demonstrates the apparent dissemination and spread of a resistant clone of *H. influenzae* among medical centers in Japan. The gBLNAR strains show a remarkable prevalence among *H. influenzae* isolates, with the prevalence increasing with time. This fact should be taken into account when treating URT infections.

Haemophilus influenzae is a frequently isolated bacterium responsible for various infections of the respiratory tract, including acute otitis media, sinusitis, acute purulent exacerbation of bronchitis, and pneumonia (14, 23, 29, 34). Since the first reports of ampicillin-resistant strains of *H. influenzae* in the United States in 1974, the major mechanism of antimicrobial resistances of *H. influenzae* has been considered to be related to either TEM-1 or ROB-1 types of β-lactamase (4–6, 35). The prevalence of β-lactamase-producing strains in the United States has increased progressively: up to 15.2% in 1983 and 1984, 36.4% in 1994 and 1995, and 31.3% in 1997 and 1998 (4–6). The issue was further complicated in the 1980s by the identification of β-lactamase-negative ampicillin (AMP)-resistant (BLNAR) strains (21). The BLNAR strains generally continued to be isolated at low frequencies in the 1980s (24, 25). However, a surveillance study conducted in Japan in the 1990s showed a marked 19.5% increase in the frequency of BLNAR strains (28). Systematic surveillance studies are essential tools in the effort to define trends in the antimicrobial resistance of bacteria.

The mechanism of resistance in the BLNAR strains involves decreased affinities of penicillin binding proteins (PBPs) for β-lactam antibiotics (26, 30). Among the several PBPs of *H. influenzae*, alterations in PBP3-mediating septal peptidoglycan synthesis during cell division are essential for developing resistance (2, 4). Recent studies characterizing mutations of the *ftsI* gene encoding PBP3 classified the BLNAR strains into three groups based on deduced amino acid substitutions (36). These are the group I strains, with the substitution of Arg-517 for His-517 (Arg-517-His) near the conserved Lys-Thr-Gly (KTG) motif; the group II strains, with the substitution Asn-526-Lys; and the group III strains, with the substitution of three amino acid residues (Met-377, Ser-385, and Leu-389) positioned near the conserved Ser-Ser-Asn (SNN) motif for Ile-377, Thr-385, and Phe-389, respectively, in addition to the substitution Asn-526-Lys (3, 9, 10, 17, 36). Group II BLNAR strains were further divided into four subgroups: subgroup IIa, with the substitution Asn-526-Lys without the substitution for Ala-502; subgroup IIb, with the substitution Val-502-Ala; subgroup IIc, with the substitution Thr-502-Ala; and subgroup IId, with the substitution Val-449-Ile. Of the various missense mutations of the *ftsI* gene, resistance to β-lactam antibiotics

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TABLE 1. Oligonucleotide primers used in this study

Primer	Gene	Sequence	Product size (bp)
PBP3-S	*ftsI*	Forward 5′-GATACTACGTCCTTTAAATTAAG-3′ Reverse 5′-GCAGTAAATGCCACATACTTA-3′	551
PBP3-BLN	*ftsI*	Forward 5′-TTCAAGTAACCGTGGTGTGAC-3′ Reverse 5′-GCAGTAAATGCCACATATTTC-3′	465
BLP	*bla*	Forward 5′-TAAGAGAATTATGCAGTGCTGCC-3′ Reverse 5′-TCCATAGTTGCCTGACTCCCC-3′	458
P6	*p6*	Forward 5′-ACGATGCTGCAGGCAATGGT-3′ Reverse 5′-TCCATAGTTGCCTGACTCCCC-3′	198
CPSB	*cps* b	Forward 5′-ACGATGCGCAGGCAATGGT-3′ Reverse 5′-CATCAGTATTACCTTCTACTAAT-3′	224

largely depends on the substitutions Arg-517-His, Asp-526-Lys, Ser-385-Thr, and Leu-589-Phe. Intermediate AMP resistance is commonly found in groups I and II; however, isolates in group III are associated with a higher level of AMP resistance (3, 10, 36).

In 2003, the Japanese Society of Infectious Diseases in Otolaryngology conducted its fourth nationwide surveillance to define the causative pathogens of infectious diseases of the upper respiratory tract (URT) and their contemporary resistance status in Japan. In this report, we present the first part of the surveillance data regarding *H. influenzae*, including the genetic characteristics and the clonal pattern of BLNAR strains.

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MATERIALS AND METHODS

Populations. Between January and May 2003, the Japanese Society of Infectious Diseases in Otolaryngology conducted the fourth nationwide surveillance of the bacterial pathogens responsible for otolaryngological infections. Informed consent was obtained from patients or their parents or guardians according to the guidelines of the institutional review board on clinical samples. Duplicate isolates from the same patients were excluded from the study to prevent repeated isolates.

Identification of *H. influenzae*. Specimens were collected aseptically with small cotton swabs and placed in an anaerobic transport jar (Eiken Chemical Co., Tokyo, Japan). All strains were identified at the Mitsubishi Kagaku Bio-Clinical Laboratories (Tokyo, Japan). *H. influenzae* strains were identified and confirmed by colony morphology, Gram staining, growth in chocolate agar but not in blood agar, the catalase test, and the X and V factor requirement. Production of β-lactamase was examined using a nitrocefinase disc (Nippon Becton Dickinson Company Ltd., Tokyo, Japan). Until the study began, the isolates were stocked in litmus milk broth (Difco Laboratories, Detroit, MI) containing 10% glycerol at −80°C. Serotyping of *H. influenzae* was determined by a slide agglutination procedure using type b antiserum (Denka Seiken, Tokyo, Japan). The method used to identify *H. influenzae* in this study could not accurately distinguish these strains from *Haemophilus haemolyticus*. Thus, some of the *H. influenzae* strains may in fact be *H. haemolyticus* (27).

Antimicrobial susceptibility. Antimicrobial susceptibilities were determined by measuring the isolates' MICs at Mitsubishi Kagaku Bio-Clinical Laboratories using the broth microdilution method according to the procedure set forth by the Clinical and Laboratory Standards Institute (CLSI) (2a). The 10 antibiotics tested in this study were AMP, clavulanate-amoxicillin, cefpodoxime, cefditoren, cefuroxime, clarithromycin, azithromycin, telithromycin, levofloxacin, and meropenem. The breakpoints recommended by the CLSI were used to define susceptibility patterns.

PCR-based genotyping. The oligonucleotide primers used in this study are listed in Table 1. Primers for the *ftsI* gene were used to amplify both the variable mutated locus (Asn-526 or Arg-517; primer set PBP3-S) and a highly mutated locus (Ser-385; primer set PBP3-BLN) (9, 11, 30). To determine the strain having a β-lactamase gene, the *bla* locus was amplified by specific primers (9). To confirm that the isolated pathogen was *H. influenzae*, the P6 gene was identified (9). Outer membrane protein P6 is a member of the class of outer membrane proteins known as peptidoglycan-associated lipoproteins, which are highly conserved among *H. influenzae* isolates. To identify the type b *H. influenzae* strain, the *cps* b locus, which encodes type b capsular polysaccharides, was examined by PCR (9). A single colony of *H. influenzae* on chocolate agar plates was lysed in 30 μl of lysis solution (1 M Tris, pH 8.9, 4.5% [vol/vol] NP-40, 4.5% [vol/vol] Tween 20, and 10 mg/ml proteinase K) for 10 min at 60°C and for 5 min at 94°C in a programmable thermal cycler (Gene Amp PCR System 9700; Perkin-Elmer, Norwalk, CT). The reaction mixture (total volume, 50-μl) consisted of 2 μl of bacterial lysate, 0.8 μl of a 10 mM deoxynucleoside triphosphate mixture, 0.1 μl of *Taq* DNA polymerase, 2.5 μl of 10× PCR buffer, 0.5 μl of 25 mM $MgCl_2$, 5.0 μl Q-solution (QIAGEN, Valencia, CA), and 0.125 μl (100 μM) each of primer and distilled water. The mixture was subjected to 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 then further extension at 72°C for 10 min. The amplified DNA fragments were analyzed using 3% agarose gel electrophoresis. On the basis of the PCR-based genotyping, the *H. influenzae* strains were classified into four genotypes: genetically β-lactamase-negative AMP-susceptible (gBLNAS) strains, without amino acid substitutions in the *ftsI* gene and β-lactamase (*bla*) gene; genetically BLNAR (gBLNAR) strains, with an amino acid substitution in the *ftsI* gene; genetically β-lactamase-positive ampicillin-resistant (gBLPAR) strains, with the *bla* gene but without an amino acid substitution in the *ftsI* gene; and genetically β-lactamase-positive amoxicillin-clavulanate-resistant (gBLPACR), with the *bla* gene and an amino acid substitution in the *ftsI* gene. The gBLNAR strains were further divided into two subgroups: group I/II gBLNAR strains, with an amino acid substitution in the variable mutated locus of *ftsI* (at Asn-526 or Arg-517), and group III gBLNAR strains, with an amino acid substitution in the highly mutated locus of *ftsI* (at Ser-385). The strains positive for the *bla* gene were also further divided into three groups based on amino acid substitutions in the *ftsI* gene: strains with group I/II amino acid substitutions in the *ftsI* gene (group I/II gBLPACR), strains with group III amino acid substitutions in the *ftsI* gene (group III gBLPACR), and strains without amino acid substitutions in the *ftsI* gene (BLPAR) (Fig. 1). In this study, we have designated PCR-based genotypes gBLNAS, gBLNAR, gBLPACR, and gBLPAR to distinguish them from phenotypes, which are written without the introductory "g."

DNA restriction fragment polymorphism analyzed by PFGE. The restriction fragment polymorphisms of SmaI-digested chromosomal DNA from *H. influenzae* isolates were evaluated by pulsed-field gel electrophoresis (PFGE) (12). PFGE patterns were analyzed by using Fingerprinting software (Bio-Rad Laboratories, Hercules, CA).

RESULTS

Characteristics of *H. influenzae* isolates. A total of 264 *H. influenzae* isolates were collected from 264 patients during the period of surveillance. The patients ranged in age from 0 to 83 years old, with 143 males and 121 females (Table 2). Among the isolates, 66 (25.0%) were from middle ear fluid, 77 (29.1%) were from the nasopharynges of patients with acute otitis media, 58 (22.0%) were from the crypts of the palatine tonsils of patients with pharyngotonsillitis, and 63 (23.9%) were from the nasal discharges or sinus aspirates of patients with acute rhi-

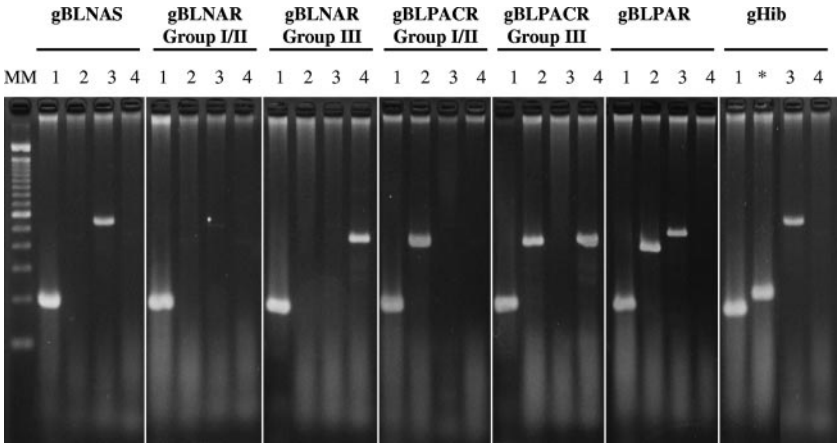

FIG. 1. PCR-based genotypes of *H. influenzae.* Lane 1, P6 gene encoding outer membrane protein P6; lane 2, *bla*; lane 3, *pbp3-S*; lane 4, *pbp3-BLN*; lane *, *cps* b encoding type b capsular polysaccharides. MM, molecular weight marker.

nosinusitis. The isolates from middle ear fluid, sinus aspirates, and tonsillar crypts represented true infections, while the remaining isolates from the nasopharynx and nasal discharge might have represented colonization of the upper airway. There were 37 (14.0%) isolates collected in university hospitals, 84 (31.8%) collected in general hospitals, and 143 (54.2%) collected in private clinics.

Out of the 264 *H. influenzae* isolates, 259 (98.1%) were *bla* gene negative. Only five (1.9%) strains had *bla* genes encoding TEM-type β-lactamase. According to the criteria for the susceptibility of *H. influenzae* to AMP set forth by the CLSI, the 259 strains negative for the *bla* gene were divided into three groups: 161 (62.2%) susceptible strains (MIC ≤ 1 μg/ml), 37 (14.3%) intermediately resistant strains (MIC = 2 μg/ml), and 61 (23.5%) resistant strains (MIC ≥ 4 μg/ml). Serotype b *H. influenzae* was identified in only two isolates. The phenotypic and genotypic tests completely agreed as to species level identification, β-lactamase production, and capsular type. There were no significant differences in the distributions of resistant strains based on gender, age, disease, and type of clinic.

PCR-based genotypes and susceptibility to AMP. The prevalence of each PCR-based genotype among the 264 *H. influenzae* isolates was as follows: 87 (33.0%) were gBLNAS strains, 98 (37.0%) were group I/II gBLNAR strains, 74 (28.0%) were group III gBLNAR strains, 1 (0.4%) was a group I/II gBLPACR strain, 2 (0.8%) were group III gBLPACR strains,

TABLE 2. Sources of clinical isolates of *H. influenzae*

Characteristic	Total no. (%) of isolates	No. (%) with susceptibility to AMP			*P* value[a]
		Susceptible	Intermediate	Resistant	
Total	264	161 (61.0)	37 (14.0)	66 (25.0)	
Gender					
Male	143 (54.2)	91 (63.6)	16 (11.2)	36 (25.2)	NS
Female	121 (45.8)	70 (57.9)	21 (17.4)	30 (24.8)	
Age (yr)					
0–2	108 (40.9)	56 (51.9)	19 (17.6)	33 (30.6)	NS
3–5	38 (14.4)	27 (71.1)	5 (13.2)	6 (15.8)	NS
6–12	19 (7.2)	12 (63.2)	1 (5.3)	6 (31.6)	NS
13–20	15 (5.7)	10 (66.7)	1 (6.7)	4 (26.7)	NS
21–50	72 (27.3)	48 (66.7)	9 (12.5)	15 (20.8)	NS
≥51	12 (4.5)	8 (66.7)	2 (16.7)	2 (16.7)	NS
Specimens type					
Middle ear fluid	66 (25.0)	34 (51.5)	14 (21.2)	18 (27.3)	
Nasopharyngeal secretion	77 (29.2)	46 (59.7)	10 (13.0)	21 (27.3)	NS
Nasal discharge/sinus aspirate	63 (23.9)	38 (60.3)	9 (14.3)	16 (25.4)	NS
Tonsil swab	58 (22.0)	43 (74.1)	4 (6.9)	7 (19.0)	NS
Type of clinic					
University medical center	37 (14.0)	23 (62.2)	7 (18.9)	11 (18.9)	NS
General hospital	84 (31.8)	49 (58.3)	14 (16.7)	21 (25.0)	NS
Private clinic	143 (54.2)	89 (62.2)	16 (11.2)	38 (26.6)	NS

[a] NS, nonsignificant.

TABLE 3. Correlation between PCR-based genotyping and susceptibility to AMP

PCR-based genotype	No. of isolates	No. of isolates with MIC to AMP (μg/ml) of:										MIC (μg/ml)			Susceptibility (%)[a]		
		≤0.12	0.25	0.5	1	2	4	8	16	32	≥64	MIC_{50}	MIC_{90}	Range	S	I	R
gBLNAS	87	3	35	46	3							0.5	0.5	0.12–1	100	0	0
Group I/II gBLNAR	98	5	23	21	18	23	6	2				0.5	2	0.12–8	68.4	23.5	8.1
Group III gBLNAR	74				7	14	18	24	10	1		4	16	1–32	9.5	18.9	71.6
Group I/II gBLPACR	1										1	128	128	128	0	0	100
Group III gBLPACR	2										2	>128	>128	>128	0	0	100
gBLPAR	2										2	64	>128	64–>128	0	0	100
Total	264	8	58	67	28	37	24	26	10	1	5	0.5	8	0.12–>128			

[a] S, susceptible; I, intermediate; R, resistant.

and 2 (0.8%) were gBLPAR strains (Table 3). Forty-nine strains (18.6%) had group I/II mutations apparently affecting PBP3, although their AMP MICs were at or below the CLSI susceptible breakpoint. The MIC_{50}s of AMP for the group I/II gBLNAR strains were similar to those for the gBLNAS strains, while the MIC_{90}s of AMP for the group I/II gBLNAR strains were higher than those for the gBLNAS strains. The MIC_{50}s and MIC_{90}s of AMP for the group III gBLNAR strains were 8 times and 32 times higher than those for the gBLNAS isolates, respectively.

PCR-based genotypes and antimicrobial susceptibilities to other antibiotics. The susceptibilities of gBLNAS and gBLNAR isolates of *H. influenzae* to other antibiotics are listed in Table 4. Most of the gBLNAS isolates were susceptible to β-lactams and exhibited relatively reduced susceptibilities (MIC_{50} = 8 μg/ml) to clarithromycin. On the other hand, the MIC_{50}s of clavulanate-amoxicillin and cephalosporin for the group III gBLNAR isolates were 4 to 64 times higher than those for gBLNAS isolates. Most of the group III gBLNAR isolates were susceptible to azithromycin, telithromycin, and meropenem. Among cephalosporins, cefditoren was the most active agent tested, with a MIC_{50} and MIC_{90} of 0.12 and 0.5 μg/ml for the group III gBLNAR isolates, respectively. The group I/II gBLNAR isolates usually showed susceptibilities similar to those of the gBLNAS isolates. Levofloxacin was the most potent antimicrobial agent against *H. influenzae* isolates.

Genetic distribution of BLNAR strains by PFGE. PFGE was used to evaluate the clonal disseminations of the BLNAR strains by detecting the restriction enzyme polymorphism of chromosomal DNA. PFGE analysis of the 61 BLNAR isolates showed that 16 (26.3%) isolates shared either identical or highly similar (≥80%) banding patterns (Fig. 2). Both of these clusters had MICs to AMP of 4 μg/ml and were *bla* negative. One cluster (cluster A) was made up of a group I/II gBLNAR strain and its related clones, which mostly showed MICs to AMP of 16 μg/ml. Another cluster (cluster B; 100% identical) was a group III gBLNAR strain and its related clones, which mostly showed MICs to AMP of 4 to 8 μg/ml. The last cluster (cluster C) had five isolates, and all shared ≥80% pattern similarity. In contrast to strains with MICs to AMP of 16 μg/ml, most of strains with MICs to AMP of 4 to 8 μg/ml showed various PFGE patterns (similarity ≤ 70%). All of the BLNAR strains in the three clusters were isolated in different clinics and different places in Japan.

DISCUSSION

The BLNAR strains of *H. influenzae* are becoming the latest problem for medical doctors who have to deal with the antimicrobial resistance of *H. influenzae* (7, 16). In the current surveillance, we focused on BLNAR strains isolated from the URT and investigated the clonal dissemination of the resistant strains in Japan. The BLNAR strains were identified in 25.0% of *H. influenzae* samples isolated from the URT, with *H. influenzae* strains having the *bla* gene being identified in only five (1.9%) isolates. Interestingly, in the United States and Europe, the BLNAR strains were uncommon and represented less than 1 to 2% of *H. influenzae* strains until the early 1990s, with their prevalence gradually increasing from 2.5% in 1994 to 10.1% 1995 (4–6, 16). In Japan, the BLNAR strains were not identified before 1984 and then had a prevalence of only 2.1% in 1988 and 5.0% in 1991 (32). The strains with MICs for AMP of 1.0 μg/ml then increased from 23.1% to 37.8% from 1996 to 1999 (32). Furthermore, a prospective prevalence study in Japan in 1999 showed that 55.1% of the *H. influenzae* strains were BLNAS, 3% were BLPAR, 26.4% were intermediate-resistant strains, and 13.2% were BLNAR (35). The increase in the percentage of BLNAR strains has led to serious problems in the treatment of infectious disease in Japan (26, 31–33). Unfortunately, there is still limited information about the precise prevalence and dissemination of antimicrobial-resistant pathogens.

The detection of BLNAR strains with decreased susceptibilities to β-lactams is controversial. The CLSI definition of BLNAR strains consists of those strains with no detectable β-lactamase and with MICs for AMP of 4 μg/ml. Strains with MICs for AMP of 2 μg/ml are defined as having intermediate, or indeterminate, resistance because such strains can show themselves to be susceptible, intermediate, or resistant to AMP if retested another day or by another method. There is no universal consensus concerning the breakpoint of BLNAR, with several different values having been proposed (1, 15). Kim et al. reported antimicrobial susceptibilities by Etest and showed reduced susceptibilities to AMP (256 μg/ml) for BLNAR strains (18). However, the Etest sometimes fails to exhibit the actual MIC, especially for BLNAR strains, while the test is useful to determine the antimicrobial susceptibilities of BLNAS strains (1a). An effort to acquire a genetic understanding of the intricacies of the resistance mechanism led to the development of reliable tests for detecting BLNAS strains.

TABLE 4. Antimicrobial susceptibilities of *H. influenzae* isolates to other antibiotics according to PCR-based genotypes

Antimicrobial agent	PCR-based genotype	No. of isolates	MIC (μg/ml)			Susceptibility (%)[a]		
			MIC_{50}	MIC_{90}	Range	S	I	R
Clavulanate-amoxicillin	gBLNAS	87	0.5	1	0.25–2	100	0	0
	Group I/II gBLNAR	98	1	4	0.25–16	92.9	NA	7.1
	Group III gBLNAR	74	8	32	2–32	26.7	NA	73.3
	Total	259	1	16	0.25–32			
Cefpodoxime	gBLNAS	87	0.12	0.12	≤0.06–0.12	100	NA	0
	Group I/II gBLNAR	98	0.25	2	≤0.06–8	90.8	NA	9.2
	Group III gBLNAR	74	8	16	≤0.06–64	9.1	NA	91.9
	Total	259	0.25	8	≤0.06–64			
Cefditoren	gBLNAS	87	≤0.06	≤0.06	≤0.06–0.12	NA	NA	NA
	Group I/II gBLNAR	98	≤0.06	0.12	≤0.06–0.5	NA	NA	NA
	Group III gBLNAR	74	0.25	0.5	≤0.06–8	NA	NA	NA
	Total	259	≤0.06	0.5	≤0.06–8			
Cefuroxime	gBLNAS	87	1	2	0.25–16	97.7	1.1	1.2
	Group I/II gBLNAR	98	2	16	0.25–64	66.3	12.3	21.4
	Group III gBLNAR	74	64	128	0.25–>128	1.4	4.1	94.6
	Total	259	2	128	0.12–>128			
Clarithromycin	gBLNAS	87	8	16	2–32	79.3	17.2	3.5
	Group I/II gBLNAR	98	8	16	2–32	75.5	22.5	2.0
	Group III gBLNAR	74	8	16	4–16	60.8	39.2	0
	Total	259	8	16	2–32			
Azithromycin	gBLNAS	87	2	4	0.25–8	98.8	NA	1.2
	Group I/II gBLNAR	98	2	4	0.5–16	95.9	NA	4.1
	Group III gBLNAR	74	2	4	0.5–8	97.3	NA	2.7
	Total	259	2	4	0.25–16			
Telithromycin	gBLNAS	87	2	4	0.5–8	97.7	2.3	0
	Group I/II gBLNAR	98	2	4	0.5–8	93.9	6.1	0
	Group III gBLNAR	74	2	4	1–8	87.8	12.2	0
	Total	259	2	4	0.5–8			
Levofloxacin	gBLNAS	87	≤0.06	≤0.06	≤0.06	100	0	0
	Group I/II gBLNAR	98	≤0.06	≤0.06	≤0.06–0.12	100	0	0
	Group III gBLNAR	74	≤0.06	≤0.06	≤0.06–0.5	100	0	0
	Total	259	≤0.06	≤0.06	≤0.06–0.5			
Meropenem	gBLNAS	87	≤0.06	≤0.06	≤0.06–0.12	100	0	0
	Group I/II gBLNAR	98	0.12	0.25	≤0.06–0.5	100	0	0
	Group III gBLNAR	74	0.25	0.5	≤0.06–2	90.5	0	9.5
	Total	259	0.12	0.5	≤0.06–2			

[a] S, susceptible; I, intermediate; R, resistant; NA, not available.

Thus, in this study, we used a PCR-based method to determine *H. influenzae* genotypes. This method allowed us to evaluate mutations of the *ftsI* gene, which encodes PBP3. This, in turn, led to a better understanding of the genetic characteristics of the pathogen.

The group I/II gBLNAR strain has mutations at the Asn-526 locus in the *ftsI* gene. The group III gBLNAR strain has a Thr-385-Ser substitution in the *ftsI* gene around the SSN motif, in addition to a Lys-526 substitution that mostly affects the increases of MICs (3, 17–19, 22, 31, 36). The Asn-526 locus in the *ftsI* gene may not affect the PBP3 structure at the β-lactam binding site and thus may not result in phenotypic amipicillin resistance, whereas the Thr-385-Ser substitution may have a direct effect. The Asn-526 region may not represent a significant mutation site. For PCR-based genotyping, we used primers for the *ftsI* gene to amplify the variably mutated locus of Asn-526 in BLNAS strains and the highly mutated locus of Ser-385 frequently identified in BLNAR strains (9, 11). We found that the Asn-526 locus can be amplified in BLNAS strains while the highly mutated Ser-385 locus in the BLNAR strains failed to be amplified. On the other hand, the latter locus frequently showed similar mutations among BLNAR strains.

According to the PCR-based genotyping of *H. influenzae*, gBLNAR strains were highly prevalent in Japan (65.1%), and about 86.9% of gBLNAR strains were classified as group III gBLNAR. About 62.2% of intermediately AMP-resistant strains were also classified as group I/II gBLNAR strains, and about 41.6% of AMP-susceptible strains were classified as group I/II gBLNAR strains. The AMP-susceptible isolates with an amino acid substitution in the *ftsI* gene have the potential to develop further resistance to penicillin and cephalosporin.

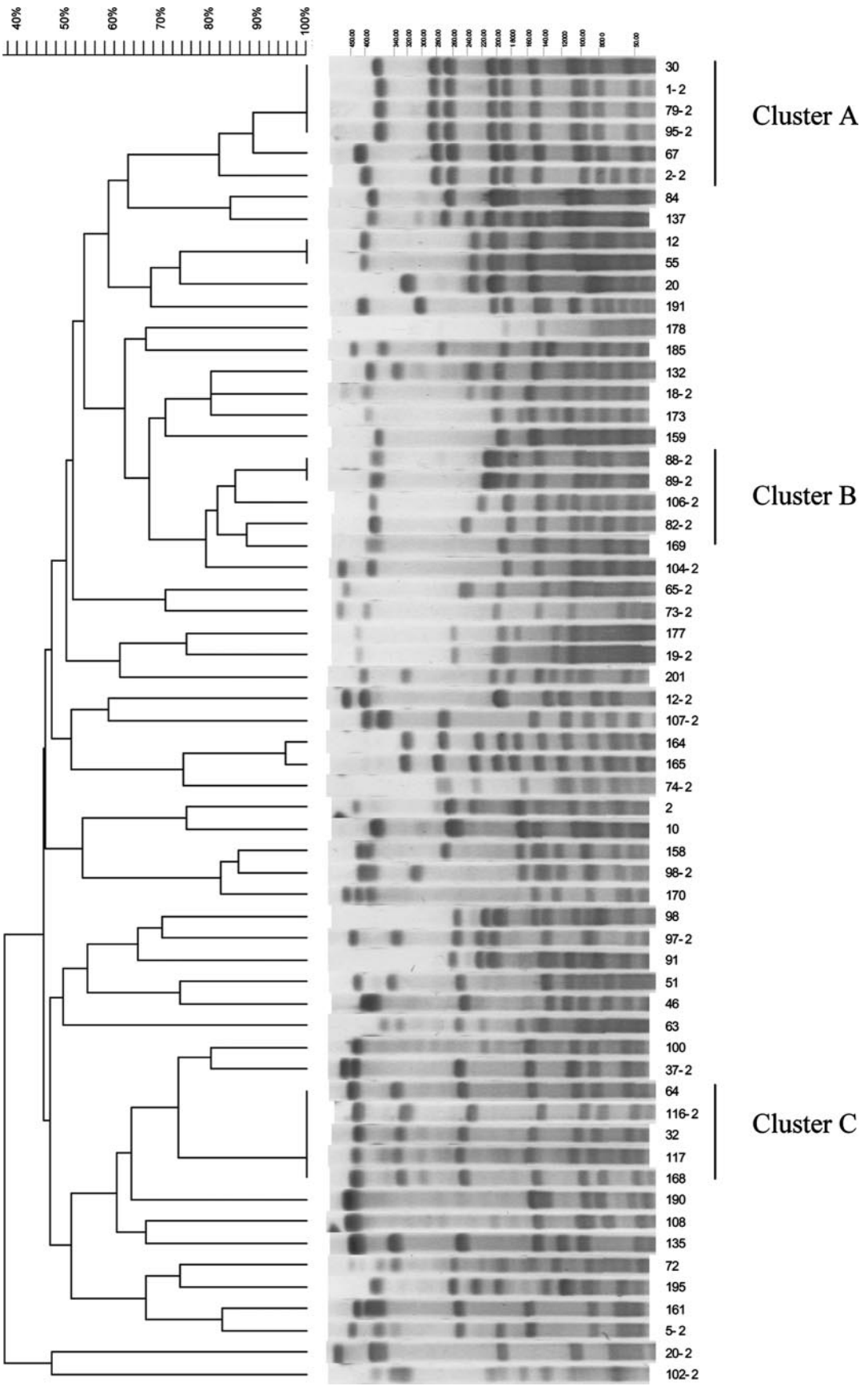

FIG. 2. Genetic identity and/or high relatedness among BLNAR isolates. PFGE dendrogram (unweighted pair group method with arithmetic means) of 61 BLNAR strains (MIC ≥ 4 μg/ml). Dice coefficients are shown above the dendrogram. Isolates with ≥80% relatedness on the dendrogram are considered highly genetically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