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Antimicrobial Resistance in *Haemophilus influenzae* Respiratory Tract Isolates in Korea: Results of a Nationwide Acute Respiratory Infections Surveillance[▽]

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Antimicrobial susceptibility patterns and β-lactam resistance mechanisms of 544 *Haemophilus influenzae* isolates through the nationwide Acute Respiratory Infections Surveillance (ARIS) network in Korea during 2005 and 2006 were determined. Resistance to ampicillin was 58.5%, followed by resistance to cefuroxime (23.3%), clarithromycin (18.7%), cefaclor (17.0%), amoxicillin-clavulanate (10.4%), and chloramphenicol (8.1%). Levofloxacin and cefotaxime were the most active agents tested in this study. β-Lactamase production (52.4%) was the main mechanism of ampicillin resistance, affecting 96.1% of TEM-1-type β-lactamase. According to their β-lactam resistance mechanisms, all isolates were classified into the following groups: β-lactamase-negative, ampicillin-sensitive (BLNAS) strains (n = 224; 41.5%); β-lactamase-positive, ampicillin-resistant (BLPAR) strains (n = 255; 47.2%); β-lactamase-negative, ampicillin-resistant (BLNAR) strains (n = 33; 6.1%); and β-lactamase-positive, amoxicillin-clavulanate-resistant (BLPACR) strains (n = 28; 5.2%). Among the BLNAR and BLPACR strains, there were various patterns of multiple-amino-acid substitutions in penicillin-binding protein 3. Particularly, among BLNAR, group III isolates, which had three simultaneous substitutions (Met377Ile, Ser385Thr, and Leu389Phe), were identified for the first time in Korea. Three group III strains displayed the highest MIC of cefotaxime (1 to 2 μg/ml). The results indicate the importance of monitoring a changing situation pertaining to the increase and spread of BLNAR and BLPACR strains of *H. influenzae* for appropriate antibiotic therapy for patients with respiratory tract infections in Korea.

Acute respiratory infections (ARIs) frequently account for outpatient visits in primary care practices (2, 9). While many ARIs have a viral etiology (20), physicians often prescribe antibiotics to satisfy a patient or to prevent a worsening of symptoms. The resulting frequent and inappropriate usage of antibiotics has contributed to the acquisition and spread of antibiotic-resistant respiratory bacteria. The high rate of antibiotic resistance of major respiratory pathogens has spurred the increasing use of newer, broad-spectrum antibiotics in the primary care setting (2, 7).

Haemophilus influenzae is one of the major bacterial pathogens of respiratory tract infections (RTIs) in children and adults (18). *H. influenzae* resistance to β-lactam antibiotics is an increasing problem. The resistance to ampicillin in this organism varies from 10% to 60%, depending on the geographical region, and is predominantly mediated by TEM-1 or ROB-1 β-lactamase production (1, 11, 18). Although it is rare in other countries, a high incidence of β-lactamase-negative, ampicillin-resistant (BLNAR) *H. influenzae* strains showing a decreased affinity of penicillin-binding protein 3 (PBP 3) for β-lactam antibiotics due to amino acid substitutions has been reported in Japan (15, 19). More recently, β-lactamase-positive, amoxicillin-clavulanate-resistant (BLPACR) isolates of *H. influenzae* have also been reported (15). These changing trends have brought about the need for local resistance data to optimize the antimicrobial activity against *H. influenzae* in the community.

The Korean Center for Diseases Control (KCDC) started the Acute Respiratory Infections Surveillance (ARIS) as a nationwide, multi-private hospital survey of major respiratory bacteria and their antimicrobial resistance in December 2005. Through the first nationwide survey, we strove to understand the current status of *H. influenzae* resistance from patients with ARIs admitted at private hospitals in Korea. Here, we describe the serotype and antimicrobial susceptibilities of *H. influenzae* isolates through the nationwide ARIS network from December 2005 to December 2006. This study also assessed the prevalence of TEM-1 or ROB-1 type β-lactamase in ampicillin-resistant *H. influenzae* isolates, PBP 3 amino acid substitutions, and molecular epidemiology of BLNAR and BLPACR strains.

MATERIALS AND METHODS

Bacterial isolates. A total of 540 *H. influenzae* isolates were obtained from 540 patients with RTIs admitted to 20 primary hospitals located in eight different regions as the part of the ARIS from December 2005 to December 2006 in Korea. Participating physicians took nasal aspirates from the recruited patients who had not taken antibiotics preceding the outpatient visit. All samples were sent to the coordinating diagnostic laboratory at the NeoDin Medical Institute, Seoul, South Korea, for microbiological analyses. Upon receipt at the laboratory, specimens were immediately inoculated onto both a blood agar plate (BAP) around a central streak of *Staphylococcus aureus* and a chocolate agar plate. Following incubation, a colony were selected and identified by conventional

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methods including β-NAD^+ (V factor) and hemin (X factor) requirements and API NH kit (bioMérieux, La Balme-les-Grottes, France). The production of β-lactamase was detected using a nitrocefin disk test (BD Biosciences, Franklin Lakes, NJ). All isolates were then sent to a KCDC reference laboratory for further analysis of serotype and antimicrobial resistance.

Serotyping. Serotyping was determined by a slide agglutination method with type a- to f-specific polyclonal antisera (BD Biosciences), and the serotypes were further confirmed using a previously described PCR method (5).

Antimicrobial susceptibility testing. The MIC of ampicillin, amoxacillin-clavulanate, cefaclor, cefuroxime, cefotaxime, chloramphenicol, clarithromycin, and levofloxacin was determined by the broth microdilution method in freshly prepared *Haemophilus* test medium (HTM) according to the 2008 guidelines of the Clinical and Laboratory Standards Institute (3). *H. influenzae* ATCC 49247 and *H. influenzae* ATCC 49766 were used as control strains for MIC testing.

PCR and sequencing. Chromosomal DNA was extracted with an Exgene GeneAll Cell SV (Geneall Biotechnology, Seoul, Korea) according to the manufacturer's protocol. The presence of TEM-1-type and ROB-1-type β-lactamase genes was detected using previously described primer sets (17). The DNA fragment encoding the transpeptidase region of PBP 3 was PCR amplified from the chromosomal DNA of BLNAR or BLPACR isolates as reported previously (11). Amplified products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced with the ABI PRISM dye terminator cycle sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) according to the protocol of the manufacturer with the ABI 377 automated sequencer (Applied Biosystems). The web-based ClustalW (www.ebi.ac.uk/clustalw) and ExPASY Translate tool (www.expasy.ch/tools/dna.html) programs were used for sequence analysis. Amino acid sequences of PBPs were compared with the reference sequence obtained from the *H. influenzae* Rd strain.

PFGE. Bacteria grown on chocolate agar were recovered and suspended in a buffer containing 10 mM Tris and 1 mM EDTA (suspension buffer, pH 8.0), and mixed with an equivalent volume of 2% low-melting-point agarose. After solidification, the plugs were incubated in 100 mM EDTA buffer containing 50 μg/ml lysozyme at 37°C for 1 h and then treated with 250 mM EDTA buffer containing 10 mg/ml proteinase K and 1% *N*-laurolysarcosine overnight at 50°C. After the plugs were washed with extensive volumes using the aforementioned suspension buffer, the total DNA was digested with SmaI (Promega, Madison, WI). The resulting DNA fragments were separated on 1% agarose gel in 0.5× TBE (0.045 M Tris-borate and 1 mM EDTA) buffer with a CHEF III electrophoresis system (Bio-Rad Laboratories, Hercules, CA). The initial pulse time of 1 s was increased linearly to 25 s at 6 V/cm and 14°C. The gels were visualized with ethidium bromide and analyzed by the Fingerprinting II informatix software (Bio-Rad). Pulsed-field gel electrophoresis (PFGE) patterns with coefficients of similarity >85% were considered to define a particular clone.

Statistical analysis. Statistical analysis was performed with a χ^2 test using SAS software version 9.2 (SAS Institute, Cary, NC) as appropriate.

RESULTS

Clinical characteristics of patients. Among 540 patients with RTIs, 318 (58.9%) were men and 221 (40.9%) were women, except for one patient with no data (Table 1). The mean age of the patients was 4.8 ± 8.1 years (range, 0 to 73 years of age), and most of the patients were children <5 years of age (84.3%). Most of strains (at a rate of 76.8%) were recovered from patients suffering from ARIs, such as sinusitis, common cold, and pharyngitis.

Serotype distribution. Prevalence of encapsulated *H. influenzae* was only 7.4% (40 of 540): 16 isolates were type a, two were type b, two were type c, eight were type d, six were type e, and six were type f. The remaining isolates (92.6%) were nontypeable *H. influenzae* (data not shown).

Antimicrobial susceptibility of *H. influenzae*. The in vitro activities, MIC_{50}, and MIC_{90} for the eight antimicrobial agents tested against the 540 *H. influenzae* isolates are summarized in Table 2. Resistance to ampicillin was 58.5%, followed by cefuroxime (23.3%), clarithromycin (18.7%), cefaclor (17.0%), amoxicillin-clavulanate (10.4%), and chloramphenicol (8.1%). Levofloxacin (MICs of 0.031 μg/ml) and cefotaxime (MICs of 0.125 μg/ml) were the most active agents tested, and all isolates were fully susceptible to these agents.

TABLE 1. Clinical characteristics of the patients

Patient characteristic	No. (%) of patients (n = 540)
Gender	
Male	318 (58.9)
Female	221 (40.9)
Unknown	1 (0.2)
Age (yr)	
Mean ± SD	4.8 ± 8.1
Range	
0–2	278 (51.5)
3–5	177 (32.8)
6–10	57 (10.6)
11–20	10 (1.8)
21–50	12 (2.2)
51–73	5 (0.9)
Type of RTI[a]	
Sinusitis	153 (28.3)
Common cold	140 (25.9)
Pharyngitis	122 (22.6)
Otitis media	82 (15.2)
Pneumonia	75 (13.9)
Croup	9 (1.7)

[a] RTI, respiratory tract infection. Preliminary diagnosis was made on the basis of the history and physical examinations and included 41 patients with more than one RTI.

Ampicillin-resistant mechanisms of *H. influenzae*. β-Lactamase production was the major mechanism of ampicillin resistance, with an overall rate of 52.4%. All *H. influenzae* isolates were classified into the following groups according to their ampicillin resistance mechanism (Table 2): β-lactamase-negative, ampicillin-sensitive (BLNAS) strains (n = 224; 41.5%); β-lactamase-positive, ampicillin-resistant (BLPAR) strains (n = 255; 47.2%); BLNAR strains (n = 33; 6.1%); and BLPACR strains (n = 28; 5.2%). In the 540 *H. influenzae* isolates, ampicillin MICs ranged from <0.5 to >512 μg/ml. Concerning the isolates found to be β-lactamase positive (MICs of >2 μg/ml), 268 (94.7%) were positive for the *TEM-1* gene only, 9 (3.2%) for the *ROB-1* gene only, 4 (1.4%) for both genes, and 2 (0.7%) for neither gene (Table 3). The ampicillin MICs for the strains expressing *ROB-1*-encoded β-lactamase were 16 to 256 μg/ml. In this study, the production of *TEM-1*-encoded β-lactamase was the most prevalent mechanism of ampicillin resistance in *H. influenzae*.

In the BLNAR strains, the MIC_{50}s of ampicillin, amoxicillin-clavulanate, and cefuroxime were 2- to 4-fold higher than those for the BLNAS strains. In the BLPACR strains, the MIC_{50}s of ampicillin, amoxicillin-clavulanate, and cefuroxime were about 4-fold higher than those for the BLNAS strains. Compared with the MIC range between BLNAR and BLPACR strains, there were no differences in the MICs of amoxicillin-clavulanate, cefaclor, cefuroxime, and cefotaxime. However, the BLNAR strains had relatively lower MICs of ampicillin (2 to 8 μg/ml) than the BLPACR (4 to >512 μg/ml) strains.

Amino acid substitutions of PBP 3 in BLNAR and BLPACR strains. The nucleotide sequences of the *ftsI* gene were determined for 33 BLNAR and 28 BLPACR strains identified using

phenotypic screening, and their deduced amino acid sequences were compared with those of the ampicillin-susceptible Rd strain. The amino acid substitutions in PBP3 deduced for BLNAR strains are listed in Table 4. Among the 33 BLNAR isolates, changes of Asp350Asn, Met377Ile, Ala502Val/Thr, and Asn526Lys were most commonly found and 13 different mutation patterns were identified. According to proposed classification strategies (4, 19), most BLNAR isolates (26 isolates; 78.8% of the total BLNAR isolates) belonged into group II, defined by the Asn526Lys substitution (Table 4). Moreover, 26 isolates of group II could be subdivided into four subgroups: IIa ($n = 3$), IIb ($n = 10$), IIc ($n = 10$), and IId ($n = 3$). The Arg517His substitution characteristic of group I was observed in three BLNAR isolates (9.1%). Particularly, BLNAR isolates of group III, which had four simultaneous substitutions (Met377Ile, Ser385Thr, Leu389Phe, and Asn526Lys) are presently reported for the first time in Korea.

In the BLPACR isolates, the most prevalent substitutions were Asp350Asn, Met377Ile, Ala502Val/Thr, and Asn526Lys, all of which were present in the BLNAR strains (Table 5). A total of 92.9% belonged in group II, whereas only 7.1% belonged in group I. No strain classified as group III was evident in the BLPACR strains. Compared with BLNAR strains, BLPACR isolates had similar patterns in PBP 3 amino acid substitution.

PFGE patterns of BLNAR and BLPACR strains. The genetic relatedness of BLNAR and BLPACR strains of *H. influenzae* was investigated by cluster analysis using PFGE. Isolates with greater than 85% similarity were grouped into the same PFGE cluster. There were no epidemiological links between the 33 BLNAR and 28 BLPACR isolates, which were isolated from different patients from several geographically dispersed clinics throughout Korea. As shown in the dendrogram (Fig. 1), there were 27 different PFGE patterns among the 33 BLNAR isolates and 24 different patterns among the 28 BLPACR isolates. Most of the PFGE patterns contained only one isolate. However, eight PFGE clusters, designated I to VIII in order of descending occurrence in Fig. 1, consisted of two to three genetically related isolates. Generally, epidemiologically unrelated BLNAR and BLPACR strains of *H. influenzae* displayed diverse genetic heterogeneity.

Among the 33 BLNAR strains, there were found four PFGE clusters (cluster I to IV). Cluster I consisted of three isolates (H136, H146, and H157), and cluster II had two isolates (H224 and H252). These five isolates had the identical IIc amino acid substitution pattern (Ser311Pro, Ala502Thr, and Asn526Lys). Cluster IV also included three BLNAR isolates (H305, H308, and H331), which had the same IIb mutation pattern (Asp350Asn, Met377Ile, Gly490Glu, Ala502Val, and Asn526Lys). Two of the isolates (H305 and H331) came from the same hospital but were isolated at different times. In addition, two BLNAR strains of cluster III (H162 and H256) presented with the same PFGE cluster, even though they had different *ftsI* mutation patterns.

Among the 28 BLPACR strains, four PFGE clusters were evident (V to VIII). Cluster VII included two isolates (H222 and H223) with the same IIb mutation pattern (Asp350Asn, Met377Ile, Ala502Val, and Asn526Lys), which were isolated from different hospitals in the same region. The BLPACR isolates of clusters V, VI, and VIII showed the same PFGE pattern but different *ftsI* mutation patterns.

TABLE 2. In vitro susceptibilities of the 540 *H. influenzae* isolates to eight antimicrobial agents[a]

Antimicrobial agent	All isolates ($n = 540$)				BLNAS ($n = 224$)				BLPAR ($n = 255$)				BLNAR ($n = 33$)				BLPACR ($n = 28$)			
	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC range (μg/ml)	% S	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC range (μg/ml)	% S	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC range (μg/ml)	% S	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC range (μg/ml)	% S	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC range (μg/ml)	% S
Ampicillin	16	256	≤0.5–>512	41.5	1	1	≤0.5–1	100.0	128	256	2–>512	0.0	2	8	2–8	0.0	256	>512	4–>512	0.0
Amoxicillin-clavulanate	2	8	≤0.25–32	89.6	2	8	≤0.25–16	96.4	2	4	0.25–4	100.0	8	8	1–16	39.4	8	16	8–32	0.0
Clarithromycin	8	16	≤1–64	81.3	8	16	≤2–64	84.4	8	16	2–32	83.5	8	32	4–64	51.5	8	16	1–32	71.4
Chloramphenicol	0.5	1	0.25–16	91.9	0.5	0.5	0.25–4	100.0	0.5	8	0.25–16	85.1	0.5	1	0.5–4	93.9	0.5	8	0.25–8	85.7
Cefaclor	8	32	1–128	83.0	8	16	1–128	85.3	8	32	4–128	82.0	8	32	8–64	84.8	8	64	4–128	71.4
Cefuroxime	2	8	0.25–32	76.7	2	8	0.25–32	82.1	2	8	0.5–16	82.0	8	16	1–32	33.3	8	32	1–32	35.7
Cefotaxime	0.063	0.125	≤0.031–2	100.0	0.063	0.25	≤0.031–2	100.0	0.063	0.125	≤0.031–0.25	100.0	0.063	1	0.031–2	100.0	0.063	1	0.063–1	100.0
Levofloxacin	0.016	0.031	≤0.008–0.5	100.0	0.016	0.031	≤0.008–0.031	100.0	0.016	0.031	≤0.008–0.5	100.0	0.016	0.016	0.008–0.031	100.0	0.016	0.031	0.016–0.063	100.0

[a] BLNAS, β-lactamase-negative, ampicillin-susceptible *H. influenzae*; BLPAR, β-lactamase-positive, ampicillin-resistant *H. influenzae*; BLNAR, β-lactamase-negative, ampicillin-resistant *H. influenzae*; BLPACR, β-lactamase-positive, amoxicillin-clavulanate-resistant *H. influenzae*; % S, percentage with susceptibility.

TABLE 3. MIC range of ampicillin and resistance mechanisms in 540 *H. influenzae* isolates

Characteristic	Ampicillin MIC (μg/ml)											
	<0.5	1	2	4	8	16	32	64	128	256	512	>512
Total no. of strains (n = 540)	106	118	28	9	6	15	24	68	86	48	21	11
No. of β-lactamase-positive strains (n = 283)	0	0	3	4	3	15	24	68	86	48	21	11
TEM-1 gene only (n = 268)	0	0	3	4	3	14	21	61	84	46	21	11
ROB-1 gene only (n = 9)	0	0	0	0	0	1	2	5	0	1	0	0
Both *TEM-1* and *ROB-1* genes (n = 4)	0	0	0	0	0	0	1	1	1	1	0	0
No *TEM-1* or *ROB-1* gene (n = 2)	0	0	0	0	0	0	0	1	1	0	0	0
No. of BLNAR strains (n = 33)	0	0	25	5	3	0	0	0	0	0	0	0

DISCUSSION

The high prevalence of antimicrobial resistance in major respiratory pathogens has led to serious concern in the selection of an appropriate antimicrobial agent for the empirical treatment of RTIs worldwide. With the high rate of β-lactamase in *H. influenzae*, amoxicillin-clavulanate and oral cephalosporins have been widely used for oral antibiotic treatments for outpatients with RTIs at private sector hospitals in Korea. Although β-lactamase production is regarded as the major mechanism of ampicillin resistance in *H. influenzae*, there can be enormous regional differences and changes in β-lactamase prevalence, ranging from 3% in Germany up to 65% in Korea (10, 18).

In this study, β-lactamase production (52.4%) was the main mechanism of ampicillin resistance in *H. influenzae*. In β-lactamase-positive strains, the prevalences of TEM-1 and ROB-1 were 94.7% and 3.2%, which is similar to the report in a global survey from the PROTEKT study (18). Also, two isolates, with ampicillin MICs of 64 to 128 μg/ml, were positive for nitrocefin hydrolysis and negative for both *TEM-1* and *ROB-1* genes by PCR. This may suggest the existence of an undiscovered β-lactamase in *H. influenzae*. In addition, the BLNAR strains with an ampicillin MIC of ≥2 μg/ml were detected in 6.1% of all strains, which were collected from geographically diverse areas of Korea. This rate was lower than that (29.3%) described in a recent report based on one tertiary care hospital located in Seoul, South Korea (13), although the different methodologies and definitions of a BLNAR strain used previously and presented may at least partially explain this dichotomy.

To date, the BLNAR strains of *H. influenzae* have remained rare globally but their prevalence has increased in some countries, including Japan, Spain, and France (6, 18). Particularly, in Japan, recent surveillance data indicate that BLNAR isolates have exponentially increased from 5.8% in 2000 to 34.5% in 2004, perhaps as a result of increased exposure to oral cephalosporins (8). This has implications for the treatment of *H. influenzae* infections. Presently, BLNAR strains are more significantly resistant to amoxicillin-clavulanate, clarithromy-

TABLE 4. Deduced amino acid substitutions in PBP 3 of 33 BLNAR strains compared with the Rd strain in this study

Group[a]	No. of strains	Amino acid substitution											Geometric mean MIC and range (μg/ml)					
													Ampicillin		Cefuroxime		Cefotaxime	
		Ser311	Asp350	Ser357	Met377	Ser385	Leu389	Ile449	Gly490	Ala502	Arg517	Asn526	MIC	Range	MIC	Range	MIC	Range
I	3		Asn	Asp	Ile	Thr					His		2	2	13.3	8–16	0.25	0.25
IIa	1								Glu			Lys	4	2–8	9.3	4–16	0.063	0.063
	1		Asn		Ile							Lys						
	1		Asn		Ile				Glu			Lys						
IIb	2									Val		Lys	2.2	2–8	6	2–8	0.069	0.063–0.125
	3		Asn		Ile					Val		Lys						
	5		Asn		Ile				Glu	Val		Lys						
IIc	7	Pro								Thr		Lys	2.8	2–8	11.4	2–16	0.194	0.063–1
	1							Val		Thr		Lys						
	1		Asn	Asp	Ile	Thr				Thr		Lys						
	1		Asn	Asp		Thr	Phe			Thr		Lys						
IId	3							Val				Lys	2.7	2–4	8	4–16	0.708	0.063–2
III	3		Asn	Asp	Ile	Thr	Phe					Lys	5.3	4–8	13.3	4–32	1.7	1–2
Other[b]	1												2		2		0.063	

[a] A total of 33 BLNAR strains were classified into six groups (I, IIa, IIb, IIc, IId, and III) based on previously proposed criteria (4, 19).
[b] One isolate had no *ftsI* gene mutation.

TABLE 5. Deduced amino acid substitutions in PBP 3 of 28 BLPACR strains compared with the Rd strain in this study

Group[a]	No. of strains	Amino acid substitution											Geometric mean MIC and range (μg/ml)					
													Ampicillin		Cefuroxime		Cefotaxime	
		Asp350	Ser357	Ala368	Met377	Ser385	Ala437	Ile449	Gly490	Ala502	Arg517	Asn526	MIC	Range	MIC	Range	MIC	Range
I	2	Asn	Asp		Ile	Thr					His		192	128–256	32	32	0.188	0.125–0.25
IIa	1	Asn			Ile							Lys	288	64–512	2	2	0.532	0.063–1
	1	Asn			Ile	Thr						Lys						
IIb	14	Asn			Ile					Val		Lys	230	4–512	11.4	1–32	0.188	0.063–1
	2									Val		Lys						
	1	Asn			Ile				Glu	Val		Lys						
IIc	2			Thr						Thr		Lys	208	64–512	6.5	2–16	0.079	0.063–0.125
	1	Asn	Asp				Ser			Thr		Lys						
	1	Asn								Thr		Lys						
IId	2							Val				Lys	512	512	4	4	0.094	0.063–0.125
Other[b]	1	Asn					Ser						512		8		0.125	

[a] A total of 28 BLPACR strains were classified into the six groups based on the previously proposed criteria (4, 19).
[b] One isolate hasn't been included in the subgroups mentioned above in this study.

cin, chloramphenicol, and cefuroxime than BLNAS strains ($P < 0.0001$, $P < 0.0001$, $P = 0.0002$, and $P < 0.0001$, respectively), similar to previous reports (6, 18). Although the clinical efficacy of amoxicillin-clavulanate and oral cephalosporins against BLNAR may be still unclear, the use of first-line oral antibiotics against *H. influenzae* infections should continue to be made cautiously, but is recommended in situations where the emergence of BLNAR strains has been documented.

In BLNAR strains, PBP 3 amino acid substitutions appear to be correlated with β-lactam resistance by genetic analysis and transformation studies (4, 11, 14, 16). Many substitutions are near sites surrounded by the conserved STVK (Ser327-Thr-Val-Kys), SSN (Ser379-Ser-Asn), and KTG (Lys513-Thr-Gly) motifs in PBP 3. Previous studies of BLNAR strains from Japan, France, and Spain have reported large variations in PBP 3 mutations and categorized them into several distinct groups based on the amino acid substitution patterns (4, 6, 18, 19). In this study, Korean BLNAR isolates had various patterns of multiple PBP 3 amino acid substitutions. We classified them into three groups (I, II, and III) according to the criteria proposed by Ubukata et al. and Dabernat et al. (4, 19). Among the Korean BLNAR isolates, the predominance of group II with the Asn526Lys substitution is in agreement with recent situations from Japan and Spain (6, 18, 19). Interestingly, the amino acid substitutions at positions 377, 385, and 389 around the SSN (Ser379-Ser-Asn) motif occur frequently compared to those described by the previous Korean report (13). In addition, group III with the triple substitutions of Met377Ile, Ser385Thr, and Leu389Phe in the presently detected BLNAR strains are novel in Korea. The Ubukata strains with these triple substitutions are associated with decreased susceptibility to cephalosporins, with an ~250-fold increase of cefotaxime MICs (from 0.008 to 2.0 μg/ml) compared with wild-type susceptible strains (19). The present study also indicated that three strains with group III had an especially high cefotaxime MIC (1 to 2 μg/ml) (Table 4).

Our present study found 28 BLPACR strains (5%) based on ampicillin and amoxicillin-clavulanate MICs and nitrocefin hydrolysis. These 28 isolates of BLPACR showed more resistance to amoxicillin-clavulanate, chloramphenicol, and cefuroxime than BLNAS strains ($P < 0.0001$, $P < 0.0001$, and $P < 0.0001$, respectively). Especially, BLPACR strains had a much higher ampicillin MIC (4 to 512 μg/ml) than BLNAR strains (2 to 8 μg/ml), but no differences in MICs between BLPACR and BLNAR strains were observed in other antibiotics tested. All 28 BLPACR strains had TEM-1 type β-lactamase and multiple mutations within the *ftsI* gene. Although BLPACR strains had amino acid substitutions identical to those described in the BLNAR strains, no isolate displayed the triple substitutions of Met377Ile, Ser385Thr, and Leu389Phe, defined as group III, which confers significant resistance to cefotaxime.

In this study, we found 44 BLNAR and 28 BLPACR strains using phenotypic screening during the ARIS program. These BLNAR and BLPACR strains, which bore no epidemiological links, showed diverse genetic heterogeneity, suggesting that they independently occurred in several regions, similar to other studies (6, 12). In addition, we found the genetically related BLNAR and BLPACR isolates from geographically distinct hospitals in eight PFGE clusters. The molecular evolution of BLNAR strains has been considered to occur through the acquisition of point mutations in the *ftsI* gene by antibiotic pressure. However, the present findings suggest the possibility of clonal spread of BLNAR and BLPACR strains in the future. Today, given the widespread use of oral cephalosporins and amoxicillin-clavulanate against RITs, we cannot completely exclude the possibility of a rapid increase of BLNAR strains for a limited time in our country.

In summary, we confirmed the still-high proportion of TEM-1 β-lactamase-positive ampicillin-resistant nontypeable *H. influenzae* isolates in Korea. Recent emergence of BLNAR and BLPACR strains was detected in *H. influenzae* isolates from the patients at private hospitals of geographically distinct regions. For the first time, we observed BLNAR strains of group III possessing the triple substitutions of Met377Ile,

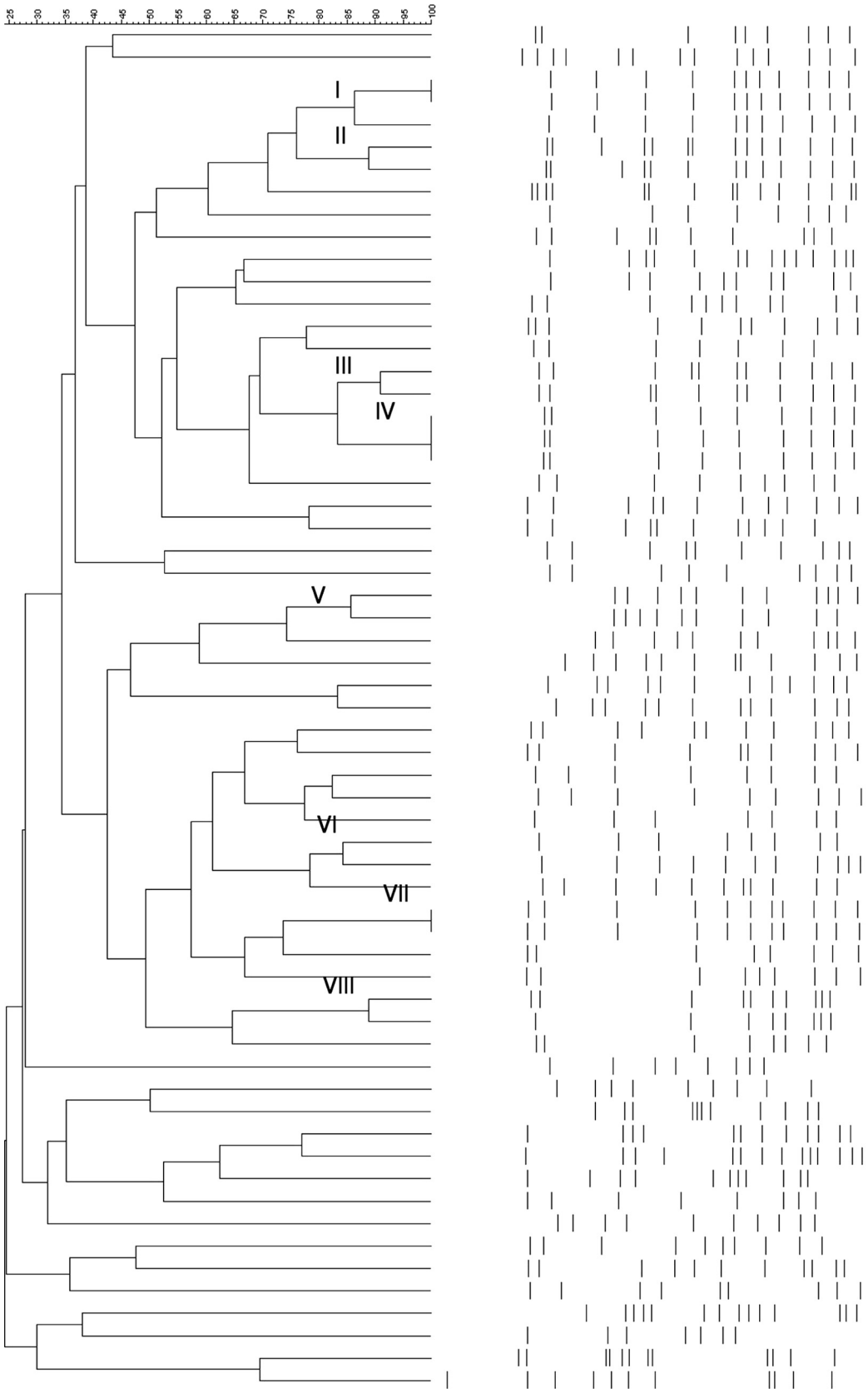

FIG. 1. Dendrogram and PFGE patterns of 33 BLNAR strains and 28 BLPACR strains of *H. influenzae*. The dendrogram was constructed by the unweighted-pair group method of average linkage (UPGMA). The scale measures similarity values. Strains and their subtypes are shown on the right column.

Ser385Thr, and Leu389Phe; in particular, strains with an especially elevated resistance to cefotaxime were detected for the first time in Korea. Although the rate of BLNAR and BLPACR strains is lower, it may also affect the development of the BLNAR and BLPACR *H. influenzae* phenotype by the frequent use of amoxicillin-clavulanate and oral cephalosporins to treat RTIs in our country. Thus, it is important to monitor the increased occurrence and spread of BLNAR and BLPACR strains of *H. influenzae* for appropriate antibiotic therapies for patients with RTIs.

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