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Diversity of Ampicillin Resistance Genes and Antimicrobial Susceptibility Patterns in *Haemophilus influenzae* Strains Isolated in Korea[▽]

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By Etest determination of the susceptibilities of 229 *Haemophilus influenzae* strains isolated in Korea to 10 antibiotics, the isolates were found to be antibiotic nonsusceptible in the following order: ampicillin (58.1%), trimethoprim-sulfamethoxazole (52%), cefaclor (41.1%), clarithromycin (25.8%), chloramphenicol (14.0%), amoxicillin-clavulanic acid (13.5%), meropenem (11.7%), cefixime (10.9%), cefuroxime (9.2%), and levofloxacin (1.3%). The prevalences of each resistance class were 23.6% for β-lactamase-negative ampicillin-susceptible (BLNAS) strains; 37.6% for strains with the TEM-1 type β-lactamase gene; 1.3% for strains with the ROB-1 type β-lactamase gene; 29.3% for the β-lactamase-negative ampicillin-resistant (BLNAR) strains with a mutation in the *ftsI* gene, which encodes PBP 3; and 8.3% for β-lactamase-positive amoxicillin-clavulanate-resistant (BLPACR) strains, which showed both resistance mechanisms (i.e., a β-lactamase gene and a mutation in the *ftsI* gene). The MIC_{50}s of all β-lactams, including cephem and meropenem agents, for the BLNAR strains were two to three times higher than those for the BLNAS strains. This study confirms that the prevalence of BLNAR and BLPACR strains is relatively high and for the first time confirms the presence of *H. influenzae* strains carrying bla_{ROB-1} in Korea. Even though mutations in another gene(s) might be involved in β-lactam resistance, these results suggest that mutations in the *ftsI* gene are important for the development of resistance to β-lactams in *H. influenzae* strains in Korea.

Despite the extensive use of antimicrobial therapies and the availability of the *Haemophilus influenzae* type b vaccine, *H. influenzae* remains a major pathogen in bronchopulmonary infections as well as ear, nose, and throat infections. Since the broad use of the *H. influenzae* type b vaccine in developed countries, noninvasive, nonencapsulated *H. influenzae* strains have been the main source of such respiratory tract infections (24, 26). *H. influenzae* can acquire ampicillin resistance through two different mechanisms. One is the production of β-lactamases, which are referred to as TEM-1 (33) and ROB-1 (16) and which hydrolyze ampicillin enzymatically. Another is a conformational change in the penicillin-binding proteins (PBPs), which are enzymes responsible for peptidoglycan synthesis, which results in a reduced affinity to ampicillin (17, 19, 20). Strains with resistance due to the latter mechanism were isolated in New Zealand for the first time from 1978 to 1980 (20) and were termed β-lactamase-negative ampicillin-resistant (BLNAR) *H. influenzae* strains. Recently, strains showing both resistance mechanisms were found in clinical isolates, and such *H. influenzae* strains have been termed β-lactamase positive ampicillin-clavulanic acid resistant (BLPACR).

From the genetic analysis of the *ftsI* gene, which encodes PBP 3 in BLNAR strains, it can be determined that the amino acid substitution mutations surrounding the highly conserved KTG (Lys512-Thr-Gly) and SSN (Ser379-Ser-Asn) motifs would be relevant to β-lactam resistance (4, 9, 10, 31). The substitutions in the *ftsI* gene have been classified into the following three groups: group I, His is substituted for Arg-517 (Arg517His) near the KTG motif; group II, Lys is substituted for Asn-526 (Asn526Lys) near the KTG motif; and group III, three residues (Met-377, Ser-385, and Leu-389) near the SSN motif are replaced by Ile, Thr, and/or Phe (Met377Ile, Ser385Thr, and/or Leu389Phe, respectively), in addition to the replacement of Asn526Lys. Isolates with intermediate ampicillin resistance are commonly found in groups I and II, and isolates in group III are associated with a higher level of ampicillin resistance (9, 10, 31). In addition, according to the genetic characterization of BLNAR isolates in France, group II BLNAR strains were divided into four subgroups according to the criteria reported by Dabernat et al. (4). Therefore, BLNAR strains are classified into six groups: I, IIa, IIb, IIc, IId, and III. Subgroup IIa includes the strains that have a substitution at amino acid 526 without a substitution for Ala-502. Subgroup IIb is defined by a Val-502 substitution for Ala-502. Subgroup IIc is defined by a Thr-502 substitution for Ala-502. Subgroup IId is defined by a Val-449 substitution

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for Ile-449. Of the various missense mutations occurring in the *ftsI* gene, resistance to β-lactams, at least to cephalosporins, depends largely on the *ftsI* mutations Arg517His, Asp526Lys, Ser385Thr, and Leu389Phe (23).

In the United States and Europe, the proportion of BLNAR isolates remains very low (5, 10, 15), while the proportion of BLNAR strains in Japan has rapidly increased over the last decade (9, 21, 23). The high prevalence of these resistant organisms is attributed to the frequent use of oral and intravenous cephem antibiotic agents in Japan (28, 29). These BLNAR strains show higher levels of resistance to expanded- and broad-spectrum cephems than non-BLNAR strains, which can cause serious clinical problems.

This report describes the prevalence and antibiotic susceptibilities of β-lactamase-negative ampicillin-susceptible (BLNAS) and β-lactamase-positive ampicillin-resistant (BLPAR) strains; BLNAR strains in groups I, II, and III; and BLPACR strains identified by PCR, as well as the substitutions in the *ftsI* gene in BLNAR and BLPACR strains.

MATERIALS AND METHODS

Strains. A total of 229 *H. influenzae* strains, which were collected from outpatients and hospitalized patients from two tertiary-care hospitals in Seoul, Korea, from 2000 to 2005, were examined in this study. Of the 229 *H. influenzae* strains, 196 strains (85.6%) caused clinical infections, which were treated with various antibiotics; the rest of the strains ($n = 33$ [14.4%]) did not cause clinical infections. Testing for the presence of the β-lactamase enzyme was performed with cefinase disks impregnated with nitrocefin (Becton-Dickinson Microbiology Systems, Cockeysville, MD), as instructed by the manufacturer. The strains *H. influenzae* ATCC 49766 (ampicillin susceptible) and *H. influenzae* ATCC 49247 (BLNAR) were used as controls. The isolates were analyzed by PCR as described below, and the tested strains were stored at −80°C for subsequent testing after PCR.

PCR. The PCR cycling conditions were 35 cycles of the following: 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s. PCR was carried out for the *H. influenzae* isolates by using the following five sets of primers, which have been reported previously (9, 10): P6 primers were used to amplify the p6 gene, which encodes the P6 membrane protein specific for *H. influenzae* (22). TEM-1 primers were used to amplify a part of the $bla_{\text{TEM-1}}$ gene, and ROB-1 primers were used to amplify a part of the $bla_{\text{ROB-1}}$ gene (27). PBP3-S primers were used to amplify a portion of the *ftsI* gene present in BLNAS strains. The binding positions of the PBP3-S primers corresponded to the nonsubstituted sequences for the Asn-526 amino acid in the *ftsI* genes; when Lys was substituted for Asn-526 (Asn-526Lys), DNA amplification with the PBP3-S primers did not occur. The tested strains that showed no amplification signal were identified as members of group II (9, 10). PBP3-BLN primers were used to identify group III strains, which contain Asn526Lys and Ser385Thr amino acid substitutions in the *ftsI* gene (9, 10). Serotype b primers were used to amplify a portion of the gene encoding the serotype b capsule (32). There were no primers that could be used to detect all isolates suspected of being group I BLNAR strains with the Arg517His substitution, according to the results of disk testing of their susceptibilities. Therefore, this substitution was detected by subjecting these strains to direct sequencing (31). On the basis of the PCR results, all *H. influenzae* strains tested could be placed in one of four classes: BLNAS strains, which lack all resistance genes; BLPAR strains, which have the $bla_{\text{TEM-1}}$ or the $bla_{\text{ROB-1}}$ gene; BLNAR strains, which have amino acid substitutions in the *ftsI* gene; and BLPACR strains, which have the $bla_{\text{TEM-1}}$ or the $bla_{\text{ROB-1}}$ gene and amino acid substitutions in the *ftsI* gene.

Antimicrobial susceptibility tests. The antimicrobial susceptibilities were determined by Etest, which is a reliable and appropriate method for the determination of MICs (13, 25). A broth suspension equivalent to a 0.5 McFarland standard was inoculated evenly onto Haemophilus test medium agar (Oxoid, Basingstoke, United Kingdom). After the medium was dry, Etest strips (AB Biodisk, Solna, Sweden) for 10 antibiotics were placed on the medium, which had been incubated at 35°C under 5% CO_2 for 20 h. *H. influenzae* ATCC 49766 and *H. influenzae* ATCC 49247 were included in each batch as controls. The MICs were read at the point of the complete inhibition of growth. The following antibiotics were examined: ampicillin, amoxicillin-clavulanic acid, cefuroxime, cefaclor, cefixime, meropenem, clarithromycin, levofloxacin, trimethoprim-sulfamethoxazole, and chloramphenicol.

Sequencing. For confirmation of a mutation in the *ftsI* gene, we excluded only 54 strains, which showed positive PCR results with PBP3-S primers and ampicillin-susceptible patterns and which were designated BLNAS. Therefore, we included 175 *H. influenzae* strains without reference to β-lactamase production in the *ftsI* gene sequencing part of the study. The 1.0-kb DNA fragment encoding the PBP 3 transpeptidase domain was amplified from the chromosomal DNA of *H. influenzae* by PCR (31). For confirmation of the presence of $bla_{\text{ROB-1}}$, we sequenced three *H. influenzae* strains carrying $bla_{\text{ROB-1}}$ by PCR. Direct sequencing was performed with a BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) on an ABI Prism 3100 genetic analyzer (Applied Biosystems).

Statistical analysis. One-way analysis of variance and Tukey's multiple-comparison methods were used to compare the MICs for the BLNAS, BLPAR, BLNAR, and BLPACR strains. All the analyses were carried out with SPSS (version 11.5; SPSS Inc., Chicago, IL). A P value of <0.05 was considered significant.

RESULTS

Antimicrobial susceptibility patterns among *H. influenzae* strains by year. Table 1 shows the MIC ranges, the MIC_{50}s, and the MIC_{90}s of six β-lactam agents and four non-β-lactam agents for the 229 *H. influenzae* strains by year of isolation. The percentages of isolates nonsusceptible to the 10 antibiotics, in order, were as follows: ampicillin, 58.1%; trimethoprim-sulfamethoxazole, 52%; cefaclor, 41.1%; clarithromycin, 25.8%; chloramphenicol, 14.0%; amoxicillin-clavulanic acid, 13.5%; meropenem, 11.7%; cefixime, 10.9%; cefuroxime, 9.2%; and levofloxacin, 1.3%. Although the percentage of ampicillin-resistant isolates appears to have increased each year, this increase was not statistically significant. However, the rates of resistance to cefixime and chloramphenicol were significantly higher in 2004 and 2005 ($P < 0.002$ and $P < 0.008$, respectively). Levofloxacin was the most potent agent against *H. influenzae*.

The incidence of serotype b among *H. influenzae* strains. The incidence of serotype b was 3.1% (7/229 strains). The rates of isolation of these strains were one strain in 2000, two strains in 2001, two strains in 2002, and two strains in 2003. All had the resistance genes. Five of the seven isolates (71.4%) were BLPAR strains carrying $bla_{\text{TEM-1}}$, and two isolates (28.6%) were group II BLNAR strains. With the exception of one BLNAR strain, all showed high levels of resistance to ampicillin (MIC ranges, 12 to 256 μg/ml). Table 2 shows the clinical information for the patients from whom the serotype b isolates were recovered.

Antimicrobial susceptibility patterns among *H. influenzae* strains according to whether the strains caused clinical infections that had been treated. Table 3 shows the MIC ranges, MIC_{50}s, and the MIC_{90}s of 10 antibiotics for the 196 *H. influenzae* strains causing clinical infections and the 33 *H. influenzae* strains classified as normal flora according to the clinical information and antibiotic treatment history. The antimicrobial susceptibility patterns between the two groups were not statistically significantly different.

Susceptibilities among *H. influenzae* strains according to resistance gene. The prevalence of each class among the 229 *H. influenzae* strains was as follows: 23.6% ($n = 54$) for BLNAS strains, 37.6% ($n = 89$) for BLPAR strains carrying $bla_{\text{TEM-1}}$ or $bla_{\text{ROB-1}}$, 29.3% ($n = 67$) for BLNAR strains, and 8.3% ($n = 19$) for BLPACR strains. In particular, group III BLNAR

TABLE 1. Distributions of MICs of 10 antibiotics for 229 *H. influenzae* strains isolated in Seoul, Korea, by year

Antibiotic(s)	Yr of isolation (no. of isolates)	MIC (μg/ml)			Susceptibility (% of isolates)[a]			*P* value
		50%	90%	Range	S	I	R	
Ampicillin	2000–2002 (45)	0.75	256	0.016–256	57.8	0.0	42.2	0.342
	2003 (69)	6	256	0.125–256	42.0	5.8	52.2	
	2004 (75)	256	256	0.023–256	36.0	8.0	55.3	
	2005 (40)	256	256	0.094–256	35.0	5.0	60.0	
	Total (229)	24	256	0.016–256	41.9	5.2	52.8	
Amoxicillin-clavulanic acid	2000–2002 (45)	0.75	2	0.016–8	97.8	0.0	2.2	0.16
	2003 (69)	1	8	0.19–256	88.4	0.0	11.6	
	2004 (75)	1	256	0.016–256	81.3	2.7	16.0	
	2005 (40)	1	256	0.19–256	80.0	10.0	10.0	
	Total (229)	1	8	0.016–256	86.5	2.6	10.9	
Cefuroxime	2000–2002 (45)	0.5	2	0.016–4	100.0	0.0	0.0	0.11
	2003 (69)	0.75	8	0.094–256	89.9	1.4	8.7	
	2004 (75)	1	256	0.032–256	86.7	1.3	12.0	
	2005 (40)	1	256	0.094–256	90.0	0.0	10.0	
	Total (229)	0.75	4	0.016–256	90.8	0.9	8.3	
Cefaclor	2000–2002 (45)	2	128	0.05–256	73.3	15.6	11.1	0.066
	2003 (69)	4	256	0.094–256	59.4	10.1	30.4	
	2004 (75)	6	256	0.094–256	60.0	14.7	25.3	
	2005 (40)	12	256	0.5–256	40.0	25.0	35.0	
	Total (229)	4	256	0.047–256	59.0	15.3	25.8	
Cefixime	2000–2002 (45)	0.047	0.19	0.016–32	97.8		2.2	0.002
	2003 (69)	0.047	0.75	0.016–256	92.8		7.2	
	2004 (75)	0.047	256	0.016–256	78.7		21.3	
	2005 (40)	0.032	256	0.016–256	97.4		2.6	
	Total (229)	0.047	24	0.016–256	89.1		10.9	
Meropenem	2000–2002 (45)	0.064	0.19	0.01–32	95.6		4.4	0.174
	2003 (69)	0.094	32	0.023–32	88.4		11.6	
	2004 (75)	0.125	32	0.002–32	84.0		16.0	
	2005 (40)	0.125	32	0.047–32	87.5		12.5	
	Total (229)	0.094	32	0.002–32	88.3		11.7	
Clarithromycin	2000–2002 (45)	12	32	0.016–32	75.6	24.4		0.37
	2003 (69)	12	24	0.016–256	79.7	15.9	4.3	
	2004 (75)	16	24	0.48–256	70.7	24.0	5.3	
	2005 (40)	12	32	1.00–256	74.2	21.8	3.9	
	Total (229)	12	32	0.016–256	74.2	21.8	3.9	
Levofloxacin	2000–2002 (45)	0.016	1	0.004–1.5	100.0		0.0	0.154
	2003 (69)	0.016	0.047	0.008–32	95.7		4.3	
	2004 (75)	0.023	0.032	0.004–2	100.0		0.0	
	2005 (40)	0.016	0.0455	0.002–0.19	100.0		0.0	
	Total (229)	0.016	0.047	0.002–32	98.7		1.3	
Trimethoprim-sulfamethoxazole	2000–2002 (45)	0.75	32	0.032–32	48.9	4.4	46.7	0.802
	2003 (69)	0.38	32	0.016–32	50.7	2.9	46.4	
	2004 (75)	32	32	0.02–32	46.3	0.0	54.7	
	2005 (40)	32	32	0.032–32	48.0	1.7	50.2	
	Total (229)	4	32	0.016–32	48.0	1.7	50.2	
Chloramphenicol	2000–2002 (45)	0.75	16	0.075–48	84.4		15.6	0.008
	2003 (69)	0.5	6	0.19–16	85.5	7.2	7.2	
	2004 (75)	0.5	4	0.064–12	89.3	4.0	6.7	
	2005 (40)	0.5	8	0.016–16	82.5	2.5	15.0	
	Total (229)	0.5	6	0.064–48	86.0	3.9	10.0	

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

strains were not found in Korea, and strains carrying bla_{ROB-1} were found for the first time in Korea. Table 4 shows the MIC ranges, the MIC_{50}s, and the MIC_{90}s of the 10 antibiotics for the 229 *H. influenzae* strains classified into the four groups according to the PCR results and *ftsI* sequences. The MIC_{50}s of the β-lactam and cephalosporin agents for the BLNAR isolates were two to three times greater than those for the BLNAS isolates. The MIC_{50}s of clarithromycin and chloramphenicol for the BLNAR isolates were similar to those for the BLNAS isolates. The MIC_{50}s of trimethoprim-sulfamethoxazole were 256 times higher than those for the BLNAS isolates. The MIC_{50}s of ampicillin, cefaclor, cefixime, and trimethoprim-sulfamethoxazole for the BLNAR, BLPAR, and BLPACR isolates were significantly higher than those for the BLNAS isolates ($P < 0.000$, 0.000, 0.004, and 0.005, respectively).

TABLE 2. Clinical information for patients from whom serotype b *H. influenzae* strains were recovered

Characteristic	SMC0001	SMC0105	SMC0120	SMC0203	SMC0208	SMC0304	SMC0340
Yr of isolation	2000	2001	2001	2002	2002	2003	2003
Gender[a]	M	M	M	F	F	F	F
Age (yr)	7	59	5	83	38	7	12
Diagnosis	Paransal sinusitis	Community-acquired pneumonia	Paransal sinusitis	Sepsis	Bronchi-ectasis	Chronic sinusitis	Paransal sinusitis
Specimen	Nasopharyngeal	Sputum	Nasopharyngeal	Blood	Sputum	Nasopharyngeal	Nasopharyngeal
Resistance type	BLPAR	BLPAR	BLPAR	BLPAR	BLNAR	BLNAR	BLPAR
Treatment	Clarithromycin	Levofloxacin, ceftriaxone	Amoxicillin-clavulanic acid	Ceftriaxone, metronidazole, gentamicin	None	Cefuroxime	Amoxicillin-clavulanic acid

[a] M, male; F, female.

Sequencing of *ftsI* gene. Table 5 shows the deduced amino acid substitutions in parts of the *ftsI* gene around the KTG motif and the SSN motif in the BLNAR ($n = 67$) and BLPACR ($n = 19$) strains. In this part of the *ftsI* gene, various mutations were identified, and 14 different mutation patterns were detected in 86 clinical strains. The mutation patterns were classified into two groups according to the different amino acid substitutions without reference to β-lactamase production. In groups I ($n = 4$) and II ($n = 81$), His-517 was substituted for Arg-517 and Lys-526 was substituted for Asn-526, respectively. In one isolate, these substitutions (at amino acids 517 and 526) were observed simultaneously in the same strain. None of the clinical isolates from Korea belonged to the group III proposed by Ubukata and colleagues (10, 31). Group II was divided into four subgroups according to the criteria reported by Dabernat et al. (4). Isolates in subgroup IIa ($n = 44$), subgroup IIb ($n = 24$), and subgroup IIc ($n = 13$) but not subgroup IId ($n = 0$) were identified. BLPACR strains, which do carry $bla_{\text{TEM-1}}$ ($n = 19$), were found in all subgroups identified for the BLNAR strains (subgroup I, $n = 3$; subgroup IIa, $n = 10$; subgroup IIb, $n = 3$; subgroup IIc, $n = 2$; and subgroups I and IIb, $n = 1$).

Changes in resistance patterns among *H. influenzae* strains by year. Table 6 shows the annual changes in the incidence of resistant strains between 2000 and 2005. Among those cases, the prevalence of the BLNAS strains decreased markedly from 2000 to 2002: 33.3% ($n = 15$) in 2000 to 2002, 24.6% ($n = 17$) in 2003, 21.3% ($n = 16$) in 2004, and 11.1% ($n = 6$) in 2005. Although the incidence of BLNAR groups I and II was relatively high each year, no strain of BLNAR group III was found.

TABLE 3. Distributions of MICs of 10 antibiotics for *H. influenzae* strains according to whether the strains caused clinical infections that had been treated or were normal flora (no treatment)

Antibiotic(s)	Treatment (no. of isolates)	MIC (μg/ml)			*P* value
		50%	90%	Range	
Ampicillin	No treatment (33)	0.38	256	0.016–256	0.088
	Treatment (196)	24	256	0.023–256	
Amoxicillin-clavulanic acid	No treatment (33)	0.75	256	0.016–256	0.968
	Treatment (196)	1	8	0.016–256	
Cefuroxime	No treatment (33)	0.5	256	0.016–256	0.976
	Treatment (196)	1	8	0.016–256	
Cefaclor	No treatment (33)	2	256	0.094–256	0.452
	Treatment (196)	8	256	0.047–256	
Cefixime	No treatment (33)	0.047	32	0.023–256	0.999
	Treatment (196)	0.047	0.75	0.016–256	
Meropenem	No treatment (33)	0.094	32	0.016–32	0.227
	Treatment (196)	0.094	32	0.002–32	
Clarithromycin	No treatment (33)	16	32	0.016–32	0.284
	Treatment (196)	16	32	0.016–256	
Levofloxacin	No treatment (33)	0.023	1	0.016–6	0.800
	Treatment (196)	0.016	0.32	0.002–32	
Trimethoprim-sulfamethoxazole	No treatment (33)	32	32	0.016–32	0.719
	Treatment (196)	4	32	0.016–32	
Chloramphenicol	No treatment (33)	0.75	4	0.19–8	0.057
	Treatment (196)	0.5	8	0.06–48	

TABLE 4. Distributions of MICs of 10 antibiotics for *H. influenzae* strains by resistance gene, as identified by PCR and direct sequencing

Antibiotic(s)	Resistance type (no. of isolates)	MIC (μg/ml)			Susceptibility (% of isolates)[a]			*P* value
		50%	90%	Range	S	I	R	
Ampicillin	BLNAS (54)	0.25	0.38	0.016–256	100.0	0.0	0.0	0.000
	BLPAR (89)	256	256	0.19–256	0.0	2.2	97.8	
	BLNAR (67)	0.75	256	0.016–256	62.7	14.9	22.4	
	BLPACR (19)	256	256	32–256	0.0	0.0	100.0	
Amoxicillin-clavulanic acid	BLNAS (54)	0.5	1	0.016–256	100.0	0.0	0.0	0.054
	BLPAR (89)	1.5	12	0.25–256	80.9	3.4	15.7	
	BLNAR (67)	1	256	0.016–256	86.6	3.0	10.4	
	BLPACR (19)	1.5	256	0.38–256	73.7	5.3	21.0	
Cefuroxime	BLNAS (54)	0.5	1	0.016–256	98.1	0.0	1.9	0.104
	BLPAR (89)	1	256	0.13–256	88.8	0.0	11.2	
	BLNAR (67)	1	8	0.016–256	89.6	3.0	7.5	
	BLPACR (19)	1	256	0.38–256	84.2	0.0	15.8	
Cefaclor	BLNAS (54)	2	6	0.05–256	90.7	5.6	3.7	0.000
	BLPAR (89)	12	256	0.13–256	43.8	13.5	42.7	
	BLNAR (67)	6	256	0.05–256	58.2	22.4	19.4	
	BLPACR (19)	16	256	1–256	42.1	26.3	31.6	
Cefixime	BLNAS (54)	0.023	0.064	0.004–256	98.1		1.9	0.004
	BLPAR (89)	0.047	32	0.016–256	92.0		8.0	
	BLNAR (67)	0.064	32	0.016–256	85.1		14.9	
	BLPACR (19)	0.064	256	0.05–256	73.7		26.3	
Meropenem	BLNAS (54)	0.064	0.125	0.004–32	94.4		5.6	0.135
	BLPAR (89)	0.094	32	0.023–32	83.1		16.9	
	BLNAR (67)	0.125	32	0.01–32	88.1		11.9	
	BLPACR (19)	0.19	0.38	0.09–32	94.7		5.3	
Clarithromycin	BLNAS (54)	16	24	0.016–96	83.3	13.0	3.7	0.092
	BLPAR (89)	16	32	0.038–256	66.3	32.6	1.1	
	BLNAR (67)	12	24	0.016–256	79.1	14.9	6.0	
	BLPACR (19)	16	256	8–32	68.4	21.1	10.5	
Levofloxacin	BLNAS (54)	0.016	0.032	0.004–32	98.1		1.9	0.146
	BLPAR (89)	0.016	0.032	0.008–0.25	100.0		0.0	
	BLNAR (67)	0.023	1	0.004–32	98.5		1.5	
	BLPACR (19)	0.016	0.047	0.016–0.03	94.7		5.3	
Trimethoprim-sulfamethoxazole	BLNAS (54)	0.125	32	0.016–32	63.0	3.7	33.3	0.005
	BLPAR (89)	32	32	0.016–32	42.7	1.1	56.2	
	BLNAR (67)	32	32	0.032–32	47.8	1.5	50.7	
	BLPACR (19)	32	32	0.09–32	31.6	0.0	68.4	
Chloramphenicol	BLNAS (54)	0.5	0.75	0.064–48	96.3	0.0	3.7	0.541
	BLPAR (89)	0.75	12	0.13–48	78.7	5.6	15.7	
	BLNAR (67)	0.5	6	0.016–48	88.1	6.0	6.0	
	BLPACR (19)	0.5	8	0.5–8.0	84.2	0.0	15.8	

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

A BLPACR strain was first identified in 2003. The incidence of BLPAR strains varied every year.

DISCUSSION

The in vitro activities and rates of resistance to the 10 antimicrobial agents obtained in the present study showed that ampicillin, trimethoprim-sulfamethoxazole, and cefaclor were problematic in terms of resistance, with overall rates of resistance to these agents of 52.8%, 50.2% and 25.8%, respectively. Except for levofloxacin, the rates of resistance to the other antimicrobial agents were relatively high, with rates ranging from 3.9 to 11.8%. Three isolates (1.3%) had levofloxacin MICs of ≥2 μg/ml. During the 1990s there has been a shift away from the use of amoxicillin and less potent oral cephalosporins, such as cefaclor, loracarbef, and cefprozil, for the treatment of *H. influenzae* infections toward the use of amoxicillin-clavulanate, macrolides, more potent advanced oral cephalosporins, and fluoroquinolones. The quinolones are expected to become increasingly important empirical treatment options if the trends toward increasing rates of resistance among *H. influenzae* pathogens continues, particularly among the oral agents commonly prescribed (2, 12). Data from the SENTRY program, which analyzed *H. influenzae* isolates with

TABLE 5. Amino acid substitutions around KTG motif and SSN motif of *ftsI* genes of BLNAR and BLPACR *H. influenzae* strains[a]

Group	No. of isolates	Amino acid substitution													
		377 Met	385 Ser	389 Leu	386 Arg	398 Glu	437 Ala	448 Ile	454 Ala	465 Thr	490 Gly	495 Ile	502 Ala	517 Arg	526 Asn
I	3												Ala	His	Asn
I	1												Val	His	Asn
IIa[b]	32												Ala		Lys
IIa	4							Val					Ala		Lys
IIa	2				Gly								Ala		Lys
IIa	2								Val				Ala		Lys
IIa	1					Asp	Ser					Met	Ala		Lys
IIa	1						Ser						Ala		Lys
IIa	1									Ala			Ala		Lys
IIa	1										Glu		Ala		Lys
IIb[b]	17												Val		Lys
IIb	7	Ile											Val		Lys
IIc[b]	13												Thr		Lys
I + IIb	1	Ile											Val	His	Lys

[a] A total of 67 BLNAR *H. influenzae* strains and 19 BLPACR *H. influenzae* strains were tested.

[b] Strains that had *ftsI* gene mutations were classified into six groups: groups I, IIa, IIb, IIc, IId, and III. Group II was divided into four subgroups according to the criteria reported by Dabernat et al. (4). Subgroup IIa includes strains that have a substitution at amino acid 526 without a substitution for Ala-502. Subgroup IIb is defined by a Val-502 substitution for Ala-502. Subgroup IIc is defined by a Thr-502 substitution for Ala-502. Subgroup IId is defined by a Val-449 substitution for Ile-449. Subgroup IId was not found in this study.

reduced susceptibilities to fluoroquinolones, determined that the rate of resistance remained very low (0.15%) between 1997 and 2001 in North America, Latin America, and Europe (2). Although the SENTRY study did not evaluate isolates from Asia or the Western Pacific, based on the findings of this study, the prevalence of quinolone-resistant *H. influenzae* strains in Korea was relatively higher from 2000 to 2005. *gyrA* and *parC* mutations, which are located in the *H. influenzae* quinolone resistance-determining region that leads to resistance or reduced susceptibility to ciprofloxacin, have been reported (2, 3, 8). Fluoroquinolones, particularly the newer class of compounds with increased potencies for the treatment of *H. influenzae* infections, are an excellent alternative to other orally administered compounds, such as β-lactams (cephalosporins and penicillins) and macrolides (erythromycin, azithromycin, and clarithromycin). The very low rates of levofloxacin-resistant *H. influenzae* isolates with reduced susceptibilities documented in this study suggest that this drug class will soon be an effective contemporary treatment for *H. influenzae* infections, provided that it is used prudently.

Serotype b strains were not discovered in Korea until after 2003 in this study, and the total incidence was 3.1% (7/229). The introduction of the *H. influenzae* type b vaccine might have contributed to the decreased incidence of *H. influenzae* type b infections after 2003.

The detection of BLNAR strains with decreased susceptibilities to β-lactam antibiotics is controversial. No universal single resistance breakpoint has been chosen, and different values have been proposed (1, 11). Barry et al. (1) stressed the difficulty in standardizing procedures and reaching a universal definition of a BLNAR strain. The clinical relevance of resistance through the modification of PBPs has yet to be demonstrated unambiguously. The role of such resistant strains in therapeutic failure has not been clearly demonstrated, and there are few cases of therapeutic failure when resistant strains are present (18). Understanding of the intricacies of the resistance mechanisms and the use of strains that have been investigated genetically should lead to the development of reliable tests for the detection of such strains.

A large number of silent mutations occur in the DNA encoding the transpeptidase domain of PBP 3, with the DNA sequences of different strains diverging as much as 7%. The different patterns of amino acid substitution obtained with the strains studied can be attributed to groups I and II proposed by Ubukata et al. (31). The situation is quite different for the Korean strains. The absence of strains belonging to group III has been proven among isolates from France (4, 6), Austria (6), Germany (6), Italy (6), Holland (6), Poland (6), Portugal (6), Spain (6), the United Kingdom (6), and Turkey (6). A triple substitution at positions 377, 385, and 389, located near

TABLE 6. Changes in resistance patterns among *H. influenzae* strains from clinical isolates, by year, as identified by PCR and direct sequencing

Resistance class	No. (%) of strains in the following yr:				
	2000–2002	2003	2004	2005	Total
BLNAS	15 (33.3)	17 (24.6)	16 (21.3)	6 (11.1)	54 (23.6)
BLPAR, $bla_{\text{TEM-1}}$	19 (42.2)	21 (30.4)	26 (34.7)	20 (50)	86 (37.6)
BLPAR, $bla_{\text{ROB-1}}$	0 (0)	0 (0)	2 (2.7)	1 (2.5)	3 (1.3)
BLNAR (groups I and II)	11 (24.4)	23 (33.3)	23 (30.7)	10 (25)	67 (29.3)
BLNAR (group III)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
BLPACR	0 (0)	8 (11.6)	8 (10.7)	3 (7.5)	19 (8.3)
Total	45 (19.7)	69 (30.1)	75 (32.8)	40 (17.5)	229 (100)

the conserved SSN motif, has not been found in Korean or European strains. The Japanese strains classified into group III were resistant to cephem antibiotics: the cefotaxime MICs for these strains were 128 to 256 times higher than those for the susceptible strains. Although none of the Korean strains were found to belong to group III, some strains showed high levels of resistance to cephems. The inappropriate use of oral antibiotics for the treatment of community-acquired bronchopulmonary and ear, nose, and throat infections appears to be responsible for the selection of BLNAR strains. This difference might be related to the dissimilar antibiotic prescription habits in different countries.

Previous studies with group I and group II BLNAR strains of *H. influenzae* from Japan, France, and North America indicate that mutations in *ftsI*, which encodes PBP 3, confer ampicillin MICs of 1 to 4 μg/ml (4, 6, 30, 31). Several BLNAR strains with ampicillin MICs of 4 to 16 μg/ml were recently isolated from North America and were studied (14). The geometric mean ampicillin MICs for several clinical isolates ranged from 8 to 10.56 μg/ml. An analysis of the resistant BLNAR strains revealed frameshift insertions in *acrR* for all the high-level-ampicillin-resistant isolates. *acrR* was intact in all the low-level-ampicillin-resistant and ampicillin-susceptible strains tested. That report showed that the BLNAR strains with high ampicillin MICs have combined resistance mechanisms as a result of changes in the genes for PBP 3 and in the AcrAB efflux pump (14). Kaczmarek et al. (14) suggested that BLNAR strains of *H. influenzae* with mutations in the AcrAB repressor gene *acrR* can occur clinically and that such dual-target mutants can have higher ampicillin MICs (in the 8- to 16-μg/ml range). In this study, 20.9% (14/67) of the BLNAR strains showed higher levels of ampicillin resistance (MICs, ≥8 μg/ml). Now that the resistance mechanisms of the BLNAR strains are being established, all possible measures should be taken to prevent their selection. In addition, the involvement of other resistance mechanisms in current or future strains should be considered.

Different studies carried out with BLNAR strains have shown their genotypic and phenotypic heterogeneities and the absence of the clonal propagation of these strains (6, 7, 10, 19). On the basis of multilocus sequence typing, which was carried out for some BLNAR and BLPACR strains (data not shown), an analysis of the clonality of these strains in this study showed that they had heterogeneous sequence types.

In summary, the emergence of the BLNAR phenotype of *H. influenzae* was demonstrated, with the prevalence ranging from 24.4% to 33.3%. These BLNAR strains are more resistant to cephems than the BLNAS strains. It is important for laboratory technicians to use strategies that not only allow the routine examination of *H. influenzae* strains for the production of β-lactamase but also allow for determination of the presence of the resistance gene by using the PCR techniques described in this report. Even though mutations in another gene(s) might be involved in β-lactam resistance, these results suggest that mutations in the *ftsI* gene are important for the development of resistance to β-lactams in *H. influenzae* strains in Korea. In addition, the patterns of susceptibility to cefixime, cefuroxime, meropenem, and levofloxacin, to which ampicillin-resistant *H. influenzae* strains are traditionally believed to be susceptible, show the presence of resistant strains. Therefore, continued monitoring of the susceptibility trends will be needed to guide the appropriate antimicrobial chemotherapy.

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