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Serotype Distribution and β-Lactam Resistance in *Haemophilus influenzae* Isolated from Patients with Respiratory Infections in Korea

Songmee Bae*, Jaehoon Lee, Eunah Kim, Jaehwa Lee, Jaeyon Yu, and Yeonho Kang

Division of Bacterial Respiratory Infections, Center for Infectious Diseases, National Institute of Health, Korea Centers for Disease Control and Prevention, Seoul 122-701, Republic of Korea

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Haemophilus influenzae **is a frequent causative bacterial pathogen of respiratory tract infections. Resistance to β-lactam antibiotics has been a significant clinical problem in treatment for *H. influenzae* respiratory infections. This study describes the serotype, antibiotic resistance and distribution of TEM-1 or ROB-1 β-lactamase in *H. influenzae* isolates from local private hospitals from 2002 to 2004. Among the 100 *H. influenzae* respiratory isolates, only 7% were identified as serotypes a, b, e, and f, with the remaining 93% being nontypeable. Resistance to ampicillin, cefaclor, and tetracycline was 57%, 46%, and 16%, respectively. All strains were susceptible to azithromycin and ciprofloxacin, whereas amoxicillin/clavulanate, cefotaxime, and imipenem exhibited reduced susceptibilities of 99%, 99%, and 91%, respectively. All 57 ampicillin-resistant strains (minimum inhibitory concentration, MIC≥4 μg/ml) were β-lactamase-positive and possessed the TEM-1 type β-lactamase. One β-lactamase-positive amoxicillin/clavulanate-resistant isolate that was resistant to ampicillin (MIC>128 μg/ml) had the TEM-1 type β-lactamase and not susceptible to cefaclor and cefotaxime. Analysis of penicillin binding protein 3 revealed six residues (Asp-350, Met-377, Ala-502, Asn-526, Val-547, and Asn-569) that were substituted by Asn, Ile, Val, Lys, Ile, and Ser, respectively.**

Keywords**:** ampicillin-resistance, *H. influenzae*, TEM-1 β-lactamase

Haemophilus influenzae is a frequent cause of respiratory tract infections (RTIs) including community-acquired pneumonia, sinusitis, otitis media and chronic bronchitis in both children and adults (Tristram *et al*., 2007). This pathogen is classified into six capsular types (a-f) and non-encapsulated (nontypeable) strains (Pittman, 1931; Falla *et al*., 1994). It is well-known that the most virulent strain is encapsulated type b, which is the major pathogen of invasive diseases such as epiglottitis, meningitis, and sepsis (Cao *et al*., 2004). However, the introduction of conjugate *H. influenzae* type b (Hib) vaccine generated a novel epidemiological situation. Recent reports have disclosed a remarkable decline in the number of Hib diseases and an emergence of invasive disease caused by other encapsulated, non-type b or nonencapsulated *H. influenzae* (Waggoner-Fountain *et al*., 1995; Adderson *et al*., 2001; Heath *et al*., 2001).

Resistance to β-lactam antibiotics is a significant clinical problem in treatment of *H. influenzae* RTIs. The prevalence of resistance to ampicillin in *H. influenzae* varies from 10-60% depending on the geographical region and is predominantly due to production of TEM-1 or ROB-1 lactamase (Nissinen *et al*., 1995; Doern *et al*., 1997; Abdel-Rahman *et al*., 2000; Kaczmarek *et al*., 2004). In addition, a high incidence of β-lactamase-negative, ampicillin-resistant (BLNAR) *H. influenzae* strains have been reported in Japan, but are extremely uncommon in other countries (Bozdogan *et al*., 2006). More recently, β-lactamase-positive and amoxicillin/clavulanate-resistant (BLPACR) isolates of *H. influenzae* have also been reported (Kaczmarek *et al*., 2004).

Until now, some reports of tertiary-care hospitals have indicated the high prevalence of β-lactamase-positive *H. influenzae* and the recent emergence of BLNAR strains in Korea (Hoban and Felmingham, 2002; Kim *et al*., 2007). This study describes the serotype, antibiotic resistance and distribution of TEM-1 or ROB-1 type β-lactamase in *H. influenzae* isolates from local hospitals in Korea from 2002 to 2004.

Materials and Methods

Bacterial isolates

A total of 100 *H. influenzae* isolates were collected from two diagnostic laboratories located in Seoul, Korea from 2002-2004. These laboratories were requested to provide all *H. influenzae* isolated from patients with respiratory tract infections at local hospitals. Most strains were isolated from clinical specimens such as sputum, ear discharge and nasopharynx. Identification of *H. influenzae* was confirmed by conventional methods including Gram staining, growth on chocolate agar but not blood agar, catalase test, β-NAD^+ (V factor)/hemin (X factor) requirements and API NH kit. The strains were routinely grown on chocolate agar at 37°C in an atmosphere of 5% CO_2 and kept frozen at -70°C until further use.

Serotyping

Serotyping was determined by a slide agglutination method with type a-f-specific polyclonal antisera (BD Biosciences, USA). The serotypes were further confirmed by polymerase chain reaction (PCR) using six pairs of primers as previously described (Falla *et al*., 1994).

* For correspondence. E-mail: songmee@hanmail.net; Tel: +82-2-380-2134; Fax: +82-2-385-8043

Table 1. Source of primers used to amplify conserved transpeptidase region of each *pbp*

Target gene	Primer name	Primer sequence (5'→3')	Product size (bp)
pbp1a	PBP1A-F PBP1A-R	5'-gacaggcagaagcaatcctgtg 5'-catcaaacccgacataagttgtg	1,128
pbp1b	PBP1B-F PBP1B-R	5'-ctttacaagcagatttacgccggtcagcgacctttaggtgtg 5'-gagagagggagatgagtttcggagcgggtggattgggattatcc	1,155
pbp2	PBP2-F PBP2-R	5'-ggactaaaaggtgctgtagttgtgc 5'-gcggtaaaccaagcgtgatcatg	925
pbp3	PBP3-F PBP3-R	5'-gttgcactatctccgatgag 5'-cagctgcttcagcatcttgc	945

β-Lactamase assay

The production of β-lactamase was confirmed by a nitrocefin disk test (BD Biosciences) according to the manufacturer's instructions.

Susceptibility testing

Antimicrobial susceptibilities to ampicillin, amoxacillin/clavulanate, azithromycin, cefaclor, cefotaxime, ciprofloxacin, imipenem, and tetracycline were determined by the disk diffusion method using Haemophilus Test Medium (HTM). The minimum inhibitory concentration (MIC) of ampicillin was determined by the broth microdilution method in freshly prepared HTM medium according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2008). *H. influenzae* ATCC 49247 and *H. influenzae* ATCC 49766 were used as the reference strains for MIC testing and disk diffusion procedures.

PCR detection of TEM-1 and ROB-1 genes

Chromosomal DNA from each isolate was extracted with a QIAmp Tissue kit (QIAGEN, USA) according to the manufacturer's protocol. The presence of the TEM-1-type and ROB-1-type β-lactamase genes was detected using previously described primer sets (Tenover *et al*., 1994). PCR products were resolved by electrophoresis on a 1% agarose gel for 1 h at 100 V. The gels were stained with ethidium bromide and photographed under ultraviolet (UV) light.

PCR and sequencing of Penicillin Binding Proteins (PBPs)

The primers used to amplify the transpeptidase region of *pbp*-1a, -1b, -2, and -3 from BLPACR isolates are listed in Table 1. Amplified products were purified with the QIAquick PCR purification kit (QIAGEN) and sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit according to the protocol of the manufacturer with the ABI 377 automated sequencer (Applied Biosystems, USA). The web-based programs CLUSTAL W (www.ebi.ac.uk/clustalw) and the ExPASY Translate tool (www.expasy.ch/tools/dna.html) were used for sequence analysis. Amino acid sequences of PBPs were compared with the reference sequence obtained from *H. influenzae* Rd strain.

Results

A total of 100 *H. influenzae* were isolated from sputum (n=58), ear discharge (n=12), nasopharynx (n=6), sinus (n=2), blood (n=1), and cerebrospinal fluid (CSF; n=1) of patients with RTIs.

Capsular types of the 100 *H. influenzae* isolates are summarized in Table 2. Ninety-three isolates were nontypeable and seven isolates were typeable. Of these seven, two each were type a, b, and f, and one was type e. One isolates each from CSF and blood were identified as type b, and were predominantly involved in serious invasive infections.

Of the 100 *H. influenzae* isolates tested, 57 displayed resistance to ampicillin (Table 3). All 57 isolates produced β-lactamase. β-Lactamase production was associated with the resistance to tetracycline and cefaclor when compared with β-lactamase-negative isolates. Excluding ampicillin, *H. influenzae* demonstrated the highest level of resistance to cefaclor (46%) and tetracycline (16%). The majority of *H. influenzae* isolates were highly susceptible to amoxicillin/clavulanate (99%), cefotaxime (99%), and imipenem (91%), and all isolates were susceptible to azithromycin and ciprofloxacin.

The presently-examined *H. influenzae* isolates displayed ampicillin MICs ranging from 0.125 to >128 μg/ml (Table 4).

Table 2. Distribution of capsule serotypes in the 100 *H. influenzae* isolates obtained from local private hospitals in Korea

Source	No. of strains	No. of strains with the following serotype						
		a	b	c	d	e	f	Non-type
Sputum	58	2					2	54
Ear discharge	12							12
Nasopharynx	6							6
Sinus	2							2
Blood	1		1					
CSF[a]	1		1					
Others[b]	20					1		19

[a] CSF, cerebrospinal fluid
[b] Others include eye pus, throat, wound, and conjunctival swab

Table 3. Antibiotic susceptibilities of β-lactamase-positive and β-lactamase-negative *H. influenzae* isolates by disk diffusion test

Antimicrobial agents	Overall (n=100)		β-Lactamase positive (n=57)				β-Lactamase negative (n=43)			
	No. of strains		No. (%) of strains				No. (%) of strains			
	S[a]	R	S		R		S		R	
Ampicillin	43	57	0	(0.0)	57	(100.0)	43	(100.0)	0	(0.0)
Amoxicillin/Clavulanate	99	1	6	(98.2)	1	(1.8)	43	(100.0)	0	(0.0)
Azithromycin	100	NA[b]	57	(100.0)	NA		43	(100.0)	NA	
Cefaclor	54	46	25	(43.9)	32	(56.1)	29	(67.4)	14	(32.6)
Cefotaxime	99	NA	56	(98.2)	NA		43	(100.0)	NA	
Ciprofloxacin	100	NA	57	(100.0)	NA		43	(100.0)	NA	
Imipenem	91	NA	51	(89.5)	NA		40	(93.0)	NA	
Tetracycline	84	16	41	(71.9)	16	(28.1)	43	(100.0)	0	(0.0)

[a] S, susceptible; R, resistant
[b] NA: no NCCLS interpretative breakpoints available

Table 4. Distribution of TEM-1 and ROB-1 β-lactamase genes according to the ampicillin MICs of the examined *H. influenzae* isolates

Ampicillin MIC (μg/ml)	No. of strains inhibited by MIC (μg/ml) of :	No. of strains with gene	
		TEM-1	ROB-1
0.125	1	0	0
0.25	15	0	0
0.5	13	0	0
1	14	0	0
2	0	-	-
4	2	2	0
8	0	-	-
16	3	3	0
32	6	6	0
64	9	9	0
128	5	5	0
> 128	32	32	0

Of the 57 β-lactamase-positive isolates, all possessed the TEM-1 type β-lactamase gene, but no strains had the ROB-1 type β-lactamase gene. The TEM-1 type β-lactamase was the most prevalent *H. influenzae* ampicillin-resistant genotype.

A single BLPACR strain was identified using phenotypic screening. This isolate was a nontypeable strain isolated from the sputum of a patient with a RTI. The BLPACR isolate was resistant to ampicillin (MIC>128 μg/ml) and had a TEM-1 type β-lactamase (Table 5). This isolate was not susceptible to cefaclor and cefotaxime, but was susceptible to tetracycline and ciprofloxacin.

To identify amino acid substitutions in penicillin binding proteins (PBPs) involved in the mechanism of amoxicillin/clavulanate resistance, the nucleotide sequences of *pbp*-1a, -1b, -2, and -3 of BLPACR isolates were determined and, subsequently, the deduced amino acid sequences were compared with those of ampicillin-susceptible Rd strain. Table 6 shows that the multiple mutations detected in the *pbp*-1a, -1b, -2, and -3 genes caused alterations in the deduced amino acid sequences of the corresponding PBPs. From the genetic analysis of *pbp*-3 gene, six residues (Asp-350, Met-377, Ala-502, Asn-526, Val-547, and Asn-569) were substituted by Asn, Ile, Val, Lys, Ile, and Ser, respectively. This strain was classified into the IIb subgroup proposed by the Dabernat *et al*. (2002).

PBP1A had four mutations, His396-Arg, Ile513-Met, Asn626-Ser, and Met657-Phe, and PBP1B had four mutations, Lys380-Glu, Gly411-Val, Thr569-Ser, and Ile738-Val. Five mutations (Leu321-Pro, Ile437-Met, Leu508-Ile, Asn513-Ser, and Ala518-Thr) were evident in PBP2. Various mutation combinations were observed simultaneously in other PBP1A, PBP1B, and PBP2, compared to the Rd sequence, but none presented an obvious correlation with the high level of amoxicillin/clavulanate resistance.

Discussion

Rapidly increasing antibiotic resistance in major respiratory pathogens has presented challenges in empirical antibiotic treatment of patients with RTIs (Alpuche *et al*., 2007). Pediatricians and clinicians face a growing problem of *H. influenzae* β-lactam antibiotic resistance, which is the most common bacterial cause of RTIs. International and national surveillance studies on *H. influenzae* have chronicled large regional differences and changes in antibiotic resistance of this pathogen (Karlowsky *et al*., 2002; Tristram *et al*., 2007). An understanding of resistance in this pathogen may contribute to the establishment of policies for the appropriate choice of prescribing antibiotics for patients with RTIs at the local level (Hoban and Felmingham, 2002; Alpuche *et al*., 2007).

Although antibiotic resistance of *H. influenzae* has been

Table 5. Characteristics of BLPACR

Strain	Isolation site	Serotype	β-Lactamase production	β-Lactamase gene		Antibiotic resistance[a]						MIC
				TEM-1	ROB-1	AMP	AMC	CEC	CTX	CIP	TET	AMP
Hif_03_6	sputum	nontypable	+	+	-	R	R	R	NS	S	S	>128

[a] AMP, ampicillin; CTX, cefotaxime; TET, tetracycline; CIP, ciprofloxacin; AMC, amoxicillin-clavulanate; CEC, cefaclor; S, susceptible; NS, not-susceptible; R, resistant

Table 6. Deduced amino acid substitutions in the transpeptidase region of PBP1A, 1B, 2, and 3 in BLPACR isolates compared to the sequence of strain Rd

Strain	Amino acid substitutions																			
	PBP1A				PBP1B				PBP2					PBP3						
	AA396	AA513	AA626	AA657	AA380	AA411	AA569	AA738	AA321	AA437	AA508	AA513	AA518	AA350	AA377	AA502	AA526	AA547	AA569
Rd	His	Ile	Asn	Met	Lys	Gly	Thr	Ile	Leu	Ile	Leu	Asn	Ala	Asp	Met	Ala	Asn	Val	Asn
Hif_03_6	Arg	Met	Ser	Phe	Glu	Val	Ser	Val	Pro	Met	Ile	Ser	Thr	Asn	Ile	Val	Lys	Ile	Ser

studied in tertiary-care hospitals in Korea, there is little available data to understand the epidemiology of *H. influenzae* isolates from patients admitted to private hospitals in the community (Chong *et al*., 1992; Kwak *et al*., 2000; Kim *et al*., 2007). As a consequence, physicians at private hospitals may not be as inclined to pursue the cause of infections. Therefore, we focused on the serotype and ampicillin resistance of *H. influenzae* isolates from patients with RTIs at private hospitals in Korea.

In this study, among the 98 respiratory strains excluding two invasive strains, most (94.9%) were nontypeable and the remaining five were non-type b encapsulated strains (two type a, two type f and one type e); no type b strain was evident. These results seem to cause by the increase of Hib vaccine usage in the private sector of Korea and are similar to the low proportion of type b among respiratory strains reported in Japan and Saudi Arabia (Luong *et al*., 2004). In addition, a recent report from Korea showed that type b strains accounted for 3.1% of all the strains isolated from RTIs at the examined tertiary-care hospitals (Kim *et al*., 2007). In the era of widespread Hib vaccination, the relative importance of infections due to nonencapsulated and non–type b encapsulated *H. influenzae* has increased and many persons remain at risk. Unfortunately, a preventable vaccine against infections caused by nontypeable and non-type b *H. influenza* is not currently possible.

β-Lactamase production is a principle mechanism of ampicillin resistance in *H. influenzae*, with enormous variations in prevalence ranging from 3% in Germany to 65% in South Korea (Hoban and Flemingham, 2002). In this study, β-lactamase production was 57%, and all involved isolates were the TEM-1 type, similar to previous reports from other Korean tertiary-care hospitals.

As the result of the high prevalence of β-lactamase production by *H. influenzae*, amoxicillin/clavulanate has become the first-line oral antibiotic therapy for acute RTIs at private hospitals in Korea. Our study discerned only one BLPACR strain among the 100 *H. influenzae* isolates using phenotypic screening with MICs of ampicillin and amoxicillin/clavulanate and nitrocefin hydrolysis. Although methodological differences hamper comparisons of the incidence of BLPACR strains in other countries, it is apparent that BLPACR strains are very rare.

BLPACR strains are generally ampicillin resistant and are less susceptible to cefaclor, cefuroxime, cefprozil or cefpodoxime than comparative β-lactamase-positive and amoxicillin/clavulanate-susceptible strains (Doern *et al*., 1997). One BLPACR isolate in the present study had a high ampicillin MIC (>128 μg/ml) and was not susceptible to cefaclor and cefotaxime. Although the clear understanding of the clinical significance of this strain is presently lacking, it may influence a declined success in the clinical treatment of respiratory infections.

To find additional substitutions contributing to β-lactam resistance in BLPACR strain, the sequences of the transpeptidase region of PBP 1A, 1B, 2, and 3 were examined. Compared with those of *H. influenzae* RD, the BLPACR strain had a variety of amino substitutions in several PBPs. Of these, amino acid substitutions in the PBP3 were located around the highly conserved penicillin-binding sites, the KTG (Lys512-Thr-Gly) motif and SSN (Ser379-Ser-Asn) motifs, which are essential for function. These substitutions patterns are identical to those of the BLNAR strains reported previously (Tristram *et al*., 2007) and could be classified into subgroup IIb proposed by Dabernat *et al*. (2002). In particular, we identified Ala502-Val and Asn526-Lys substitutions at the KTC motif that conferred increased ampicillin and amoxicillin/clavulanate MICs but had little impact on resistance to the latest oral cephalosporins, and a Met377-Ile substitution at the SSN motif that increased cefotaxime MIC 10-60 fold. Numerous other substitutions except PBP3 were detected in PBPs from Rd strain, but none presented an obvious correlation with the higher level of β-lactam resistance observed (Table 6). The substitutions found presently should be studied if they are responsible for the amoxicillin/clavulanate or cephalosporin resistance phenotypes.

The molecular evolution of *H. influenzae* strains has been considered to occur through the acquisition of point mutations in the *fts*I gene by antibiotic pressure (Tristram *et al*., 2007). However, most clinical laboratories only look for β-lactamase production in *H. influenzae* in our country, and it may be not easy to detect an emergence of a β-lactam antibiotic resistant strain caused by PBP alterations. Physicians and researchers have also noted that PBP alteration is another resistance mechanism of this pathogen against β-lactam antibiotics.

This study revealed the high proportion of the TEM-1 β-lactamase-positive ampicillin-resistant nontypeable *H. influenzae* and one BLPACR strain from patients with RTIs in our community. Therefore, increasingly reliable data concerning antibiotic resistant profiles and mechanisms of *H. influenzae* causing RTIs, and guidance in terms of developing new agents, requires a nationwide, multicenter surveillance study.

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