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Antimicrobial Activities of Piperacillin-Tazobactam against *Haemophilus influenzae* Isolates, Including β-Lactamase-Negative Ampicillin-Resistant and β-Lactamase-Positive Amoxicillin-Clavulanate-Resistant Isolates, and Mutations in Their Quinolone Resistance-Determining Regions[▽]

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β-Lactamase-negative ampicillin-resistant (BLNAR) isolates of *Haemophilus influenzae* have been emerging in some countries, including Japan. The Clinical and Laboratory Standards Institute has only a susceptible MIC breakpoint (≤1 μg/ml) for piperacillin-tazobactam and a disclaimer comment that BLNAR *H. influenzae* should be considered resistant, which was adapted without presentation of data. In addition, fluoroquinolone-resistant *H. influenzae* isolates have recently been occasionally reported worldwide. To address these problems, we examined susceptibilities to β-lactams, including piperacillin-tazobactam, and ciprofloxacin by microdilution and disk diffusion (only for piperacillin-tazobactam) methods, against a total of 400 recent *H. influenzae* clinical isolates, including 100 β-lactamase-negative ampicillin-susceptible, β-lactamase-positive ampicillin-resistant, BLNAR, and β-lactamase-positive amoxicillin-clavulanate-resistant (BLPACR) isolates each. BLNAR and BLPACR isolates were tested by PCR using primers that amplify specific regions of the *ftsI* gene. We also detected mutations in quinolone resistance-determining regions (QRDRs) by direct sequencing of the PCR products of DNA fragments. Among β-lactams, piperacillin-tazobactam exhibited potent activity against all isolates of *H. influenzae*, with all MICs at ≤0.5 μg/ml (susceptible). A disk diffusion breakpoint for piperacillin-tazobactam of ≥21 mm is proposed. We confirmed that all BLNAR and BLPACR isolates had amino acid substitutions in the *ftsI* gene and that the major pattern was group III-like (87.5%). One ciprofloxacin-resistant isolate (MIC, 16 μg/ml) and 31 ciprofloxacin-susceptible isolates (MICs, 0.06 to 0.5 μg/ml) had amino acid changes in their QRDRs. Piperacillin-tazobactam was the most potent β-lactam tested against all classes of *H. influenzae* isolates. It is possible that fluoroquinolone-resistant *H. influenzae* will emerge since several clinical isolates carried mutations in their QRDRs.

Haemophilus influenzae is one of the most common causes of community-acquired respiratory tract infections, such as pneumonia, and serious invasive diseases, including bacteremia and meningitis. The first β-lactamase-positive ampicillin-resistant (BLPAR) *H. influenzae* isolate was reported in the 1970s in the United States (20, 30), and the first β-lactamase-negative ampicillin-resistant (BLNAR) isolate of *H. influenzae* was reported in 1980 (23); it caused endocarditis and meningitis in a 79-year-old female patient in 1977, also in the United States. The mechanism of resistance of the BLNAR isolates involves mutations of the *ftsI* gene encoding penicillin binding protein 3 (PBP3), resulting in decreased affinity of PBP3 (2, 31). Although the frequencies of BLNAR isolates of *H. influenzae* are still low in the United States (8, 19) and have remained relatively constant in longitudinal European surveillance (18), BLNAR isolates of *H. influenzae* have been emerging in some countries (14), particularly in Japan (13, 15, 31), Spain (10), and recently Korea (21). In 1997, β-lactamase-positive amoxicillin (amoxicilline)-clavulanate-resistant (BLPACR) isolates of *H. influenzae* were also reported from the United States (8) and exhibited two characteristics of both BLPAR and BLNAR.

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Thus, on the basis of production of β-lactamase and susceptibilities to ampicillin and amoxicillin-clavulanate, clinical isolates of *H. influenzae* can be divided into four types, β-lactamase negative ampicillin susceptible (BLNAS), BLPAR, BLNAR, and BLPACR.

Data on in vitro susceptibilities to β-lactams, including piperacillin-tazobactam, are not sufficient for *H. influenzae* isolates, particularly in the case of BLNAR and BLPACR types. The Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards) established *H. influenzae* susceptibility breakpoints between 1990 and 1995, especially for penicillins, to delineate β-lactamase-positive isolates. Few BLNAR strains were available for analysis at that time, but with their increase in prevalence, modification of breakpoints and surrogate testing guidelines should be considered. Although CLSI has disk and/or MIC susceptibility testing breakpoints for multiple agents, for piperacillin-tazobactam it has only a MIC breakpoint (≤1 μg/ml) and a disclaimer comment that BLNAR *H. influenzae* should be considered resistant regardless of MIC results, despite the finding that piperacillin alone is very active against BLNAR *H. influenzae* (24).

Furthermore, fluoroquinolone-resistant *H. influenzae* isolates have recently been occasionally reported worldwide (1, 11, 25, 27), though they have rarely been reported in Japan (16, 17). More recently, however, Yokota et al. (32) reported that fluoroquinolone-resistant *H. influenzae* isolates have been emerging in the elderly in Japan, with some of the strains exhibiting levofloxacin MICs of 16 μg/ml or higher.

We therefore examined the susceptibilities to β-lactams, including piperacillin-tazobactam, and ciprofloxacin of a total of 400 recent *H. influenzae* clinical isolates gathered from throughout Japan, including 100 each of BLNAS, BLPAR, BLNAR, and BLPACR isolates. We confirmed mutations of *ftsI* in BLNAR and BLPACR *H. influenzae* isolates. We also detected mutations in quinolone resistance-determining regions (QRDRs) in isolates with ciprofloxacin MICs of 0.06 μg/ml or higher (susceptibility breakpoint of ≤1 μg/ml) and an additional 50 isolates with ciprofloxacin MICs of 0.03 μg/ml or lower.

(Some of these findings were presented at the Annual Meeting of the American Thoracic Society 2008, Toronto, Canada, May 2008, abstract no. 858.)

MATERIALS AND METHODS

Bacterial isolates. In the initial study, a total of 400 recent *H. influenzae* clinical isolates from 138 hospitals and clinics throughout Japan stored at Mitsubishi Chemical Medience Corporation, Tokyo, Japan, were used. Consecutive isolates were studied without repetition of patients. Table 1 summarizes numbers of affiliations, duration of isolation, and the age range of patients for each group of *H. influenzae* isolates. In the additional study, a total of 50 *H. influenzae* clinical isolates, which were different from the above isolates and were associated with low MICs of ciprofloxacin (≤0.03 μg/ml), were used. *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 X and V factor requirements. Production of β-lactamase was examined using a nitrocefinase disk (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan). Prior to the study, the isolates had been kept in litmus milk broth (Difco Laboratories, Detroit, MI) containing 10% glycerol at −80°C.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed by both the microdilution method and the disk diffusion method. MICs were determined by the reference broth microdilution methods of the CLSI using *Haemophilus* test medium (3) for ampicillin, ampicillin-sulbactam, amoxicillin-clavulanate, piperacillin, piperacillin-tazobactam, ceftriaxone, imipenem, meropenem, and a non-β-lactam control, ciprofloxacin. Disk diffusion testing was simultaneously performed using the CLSI M2-A9 (4) method for piperacillin-tazobactam. Two strains of *H. influenzae*, ATCC 49247 (a BLNAR strain) and ATCC 49766 (a BLNAS strain), were used for quality control for both methods. Interpretations of the test results were made using the criteria published in M100-S17 (5).

H. influenzae isolates that had elevated ampicillin MICs (intermediate [2 μg/ml] or resistant [≥4 μg/ml]) but were negative for β-lactamase were judged to be BLNAR. Of β-lactamase-producing isolates, those resistant to amoxicillin-clavulanate were judged to be BLPACR.

***ftsI* mutations.** *H. influenzae* isolates with BLNAR and BLPACR phenotypes were tested by PCR using primers that amplify specific regions of the *ftsI* gene. The resistance-associated mutations in PBP3 were determined according to the method of Hasegawa et al. (12).

Mutations in QRDRs. We determined the nucleotide sequences of the QRDRs of the quinolone target genes *gyrA*, *gyrB*, *parC*, and *parE*. Genomic DNA was isolated from bacteria by using InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA). PCR amplification was performed using HotStarTaq DNA polymerase (Qiagen). The primer sets used to obtain DNA fragments of the *gyrA*, *gyrB*, *parC*, and *parE* QRDRs were previously described (7). Direct sequencing of the PCR products was carried out with an ABI Prism 3130 (Applied Biosystems, Foster City, CA). For the additional 50 isolates with ciprofloxacin MICs of 0.03 μg/ml or lower, mutations in QRDRs were also examined as described above.

TABLE 1. Clinical isolates of *H. influenzae* used in the initial study

Phenotype	No. of isolates	No. of affiliations	Duration of isolation	Range of patient ages (yr)
BLNAS	100	36	28 March 2006 to 27 June 2007	0–84
BLPAR	100	53	12 October 2001 to 22 June 2007	0–92
BLNAR	100	34	13 March 2006 to 7 May 2007	0–86
BLPACR	100	58	1 August 2005 to 14 July 2007	0–97

RESULTS

MICs of eight β-lactams and ciprofloxacin against 400 clinical isolates of *H. influenzae*. Tables 2 to 5 show the MIC distributions for eight β-lactams and ciprofloxacin against 100 clinical isolates each of BLNAS, BLPAR, BLNAR, and BLPACR *H. influenzae*.

In the case of BLNAS *H. influenzae* isolates, all β-lactams and ciprofloxacin exhibited very strong activities against all 100 isolates. On the other hand, in the case of BLPAR *H. influenzae* isolates, whereas amoxicillin-clavulanate was active against all isolates, susceptibility to ampicillin-sulbactam was 75%. Piperacillin alone was not active, while piperacillin-tazobactam was highly potent (MIC at which 90% of the isolates tested are inhibited [MIC_{90}], 0.06 μg/ml; MIC range, ≤0.03 to 0.25 μg/ml; 100% susceptible). Although the MIC ranges of ceftriaxone and meropenem were slightly higher than those for BLNAS *H. influenzae* isolates, their susceptibilities were still 100%. One isolate was resistant to imipenem (MIC, ≥8 μg/ml).

In the case of BLNAR *H. influenzae* isolates, ampicillin-sulbactam and amoxicillin-clavulanate were not active enough, nor was ampicillin, while piperacillin (MIC_{90}, 0.25 μg/ml; MIC range, ≤0.03 to 0.5 μg/ml) and piperacillin-tazobactam

TABLE 2. MIC distributions for eight β-lactams and ciprofloxacin against 100 clinical isolates of BLNAS *H. influenzae*

Antimicrobial agent(s)	No. of isolates with MIC (μg/ml):										MIC (μg/ml)			% of isolates susceptible[a]
	≤0.12	0.25	0.5	1	2	4	8	16	32	≥64	Range	50%	90%	
Ampicillin	11	64	17	8	0	0	0	—[b]	—	—	≤0.12–1	0.25	0.5	100
Ampicillin-sulbactam	11	64	17	8	0	0	0	0	0	0	0.12–1	0.25	0.5	100
Amoxicillin-clavulanate	0	29	60	6	5	0	0	0	0	0	0.25–2	0.5	1	100
Piperacillin	100	0	0	0	0	0	0	0	0	0	≤0.03–0.12	≤0.03	0.06	
Piperacillin-tazobactam	100	0	0	0	0	0	0	0	0	0	≤0.03–0.12	≤0.03	0.06	100
Ceftriaxone	100	0	0	0	0	0	0	0	—	—	≤0.008–0.12	≤0.008	0.015	100
Imipenem	9	13	24	52	2	0	0	—	—	—	≤0.12–2	1	1	100
Meropenem	100	0	0	0	0	0	0	—	—	—	≤0.12	≤0.12	≤0.12	100
Ciprofloxacin	99	1	0	0	0		—	—	—	—	≤0.03–0.25	≤0.03	≤0.03	100

[a] CLSI MIC breakpoints (5).
[b] —, untested concentration.

(MIC_{90}, 0.12 μg/ml; MIC range, ≤0.03 to 0.5 μg/ml; 100% susceptible) retained strong activity. The MIC ranges of ceftriaxone, imipenem, and meropenem were higher than those for BLNAS and BLPAR *H. influenzae* isolates, and four isolates and one isolate were resistant to imipenem and meropenem, respectively. The potency ranking of β-lactams for the BLNAR *H. influenzae* isolates was as follows (MIC_{90} in μg/ml): piperacillin-tazobactam (0.12) > piperacillin (0.25) = ceftriaxone (0.25) > meropenem (0.5) > imipenem (4).

In the case of BLPACR *H. influenzae* isolates, ampicillin, piperacillin, ampicillin-sulbactam, and amoxicillin-clavulanate were not active, while piperacillin-tazobactam retained strong activity (MIC_{90}, 0.25 μg/ml; MIC range, ≤0.03 to 0.5 μg/ml; 100% susceptible). Against imipenem, two isolates were resistant. The potency ranking of β-lactams for the BLPACR *H. influenzae* isolates was as follows (MIC_{90} in μg/ml): piperacillin-tazobactam (0.25) = ceftriaxone (0.25) > meropenem (0.5) > imipenem (2).

While ciprofloxacin was consistently very active against *H. influenzae* isolates of BLNAS, BLPAR, and BLNAR types (MIC, ≤0.25 μg/ml), one BLPACR isolate was fully resistant to ciprofloxacin (MIC, 16 μg/ml) and the range of MICs of ciprofloxacin in BLPACR isolates shifted higher than those of the others tested.

Disk diffusion testing. Even though the phenotypes of *H. influenzae* included BLNAR and BLPACR, piperacillin-tazobactam control ranges were ≤0.03 to 0.5 μg/ml and all zones at ≥21 mm (range, 21 to 50 mm) were considered to indicate high degrees of susceptibility.

Mutation patterns in the *ftsI* gene. All 200 BLNAR and BLPACR isolates had amino acid substitutions in their *ftsI* gene, corresponding to three different mutation patterns. We followed the BLNAR classifications proposed by Dabernat et al. (6) and Ubukata et al. (31). Of the 200 BLNAR/BLPACR isolates, 100% had amino acid mutations of the Lys-Thr-Gly (KTG) motif, including Asn526 to Lys (97.5%) and Arg517 to His (2.5%), analogous to the previously described original groups IIa and I, respectively (31). However, none of the above BLNAR/BLPACR strains could be classified as group I, since all strains with amino acid mutations of Arg517 to His also had an additional Ser385-to-Thr mutation in the Ser-Ser-Asn (SSN) motif and thus belonged to the group III-like strains (10). The most common (88.5%) pattern also appeared to be group III-like, which had Ser385 to Thr in the SSN motif in addition to Asn526 to Lys in the KTG motif. Consequently, pure group I strains, with Arg517 to His alone, and group IIa, which had Asn526 to Lys alone, accounted for 0% and 11.5% of the total, respectively.

Of 22 low-level BLNAR isolates with an ampicillin MIC of 2 μg/ml, five belonged to group IIa (Asn526 to Lys), while the others were group III-like (16 had Ser385 to Thr and Asn526 to Lys, while one had Ser385 to Thr and Arg517 to His). All 78 fully resistant BLNAR isolates were classified as group III-like; 76 of 78 strains had the amino acid changes Ser385 to Thr and

TABLE 3. MIC distributions for eight β-lactams and ciprofloxacin against 100 clinical isolates of BLPAR *H. influenzae*

Antimicrobial agent(s)	No. of isolates with MIC (μg/ml):										MIC (μg/ml)			% of isolates susceptible[a]
	≤0.12	0.25	0.5	1	2	4	8	16	32	≥64	Range	50%	90%	
Ampicillin	0	0	0	0	0	6	94	—[b]	—	—	4–≥8	≥8	≥8	0
Ampicillin-sulbactam	0	0	9	41	25	17	8	0	0	0	0.5–8	0.25	0.5	75
Amoxicillin-clavulanate	0	9	41	25	20	13	0	0	0	0	0.5–4	0.5	1	100
Piperacillin	0	0	0	1	18	12	14	12	21	22	1–>64	16	>64	
Piperacillin-tazobactam	99	1	0	0	0	0	0	0	0	0	≤0.03–0.25	≤0.03	0.06	100
Ceftriaxone	97	3	0	0	0	0	0	0	—	—	≤0.008–0.25	≤0.008	0.015	100
Imipenem	13	19	18	18	2	2	1	—	—	—	≤0.12–≥8	1	1	99
Meropenem	95	3	0	0	0	0	0	—	—	—	≤0.12	≤0.12	≤0.12	100
Ciprofloxacin	98	2	0	0	0	—	—	—	—	—	≤0.03–0.25	≤0.03	≤0.03	100

[a] CLSI MIC breakpoints (5).
[b] —, untested concentration.

TABLE 4. MIC distributions for eight β-lactams and ciprofloxacin against 100 clinical isolates of BLNAR *H. influenzae*

Antimicrobial agent(s)	No. of isolates with MIC (μg/ml):										MIC (μg/ml)			% of isolates susceptible[a]
	≤0.12	0.25	0.5	1	2	4	8	16	32	≥64	Range	50%	90%	
Ampicillin	0	0	0	0	22	44	34	—[b]	—	—	2–≥8	4	≥8	0
Ampicillin-sulbactam	0	0	0	0	22	46	30	2	0	0	2–16	4	8	22
Amoxicillin-clavulanate	0	0	0	0	7	27	60	5	1	0	2–32	8	8	27
Piperacillin	85	13	2	0	0	0	0	0	0	0	≤0.03–0.5	0.12	0.25	
Piperacillin-tazobactam	90	9	1	0	0	0	0	0	0	0	≤0.03–0.5	0.06	0.12	100
Ceftriaxone	28	64	7	1	0	0	0	0	—	—	0.03–1	0.25	0.25	100
Imipenem	0	3	16	52	15	10	4	—	—	—	0.25–≥8	1	4	96
Meropenem	21	59	19	1	0	0	0	—	—	—	≤0.12–1	0.25	0.5	99
Ciprofloxacin	100	0	0	0	0	—	—	—	—	—	≤0.03–0.06	≤0.03	≤0.03	100

[a] CLSI MIC breakpoints (5).
[b] —, untested concentration.

Asn526 to Lys, and the other two had Ser385 to Thr and Arg517 to His. Of 100 BLPACR isolates, 18 had the amino acid change in Asn526 to Lys alone (BLPACR I), 80 had Ser385 to Thr and Asn526 to Lys, and the remaining two had Ser385 to Thr and Arg517 to His (BLPACR II).

Mutations in QRDRs. We first examined the isolates with a ciprofloxacin MIC of 0.25 μg/ml or higher, but all such isolates had at least one mutation in their QRDRs. We therefore expanded our examination to include isolates with a ciprofloxacin MIC of 0.06 μg/ml or higher, since most clinical isolates of *H. influenzae* usually exhibit a MIC of ≤0.03 μg/ml for ciprofloxacin. Table 6 summarizes the results of analysis of mutations in QRDRs for a total of 37 of the initial 400 *H. influenzae* isolates which had a ciprofloxacin MIC of 0.06 μg/ml or higher, including one BLNAS strain, seven BLPAR strains, four BLNAR strains, and 25 BLPACR strains.

Of these 37 isolates, 32 had amino acid changes in at least one of their QRDRs in *gyrA*, *gyrB*, *parC*, and *parE*. Two BLPAR isolates with a ciprofloxacin MIC of 0.06 μg/ml, two BLPACR isolates with a ciprofloxacin MIC of 0.12 μg/ml, and a BLPACR with a ciprofloxacin MIC of 0.06 μg/ml did not have mutations in QRDRs with the method that we used.

The frequency of decreases in MICs of ciprofloxacin associated with QRDR mutations was highest for the BLPACR isolates. The BLPACR strain most resistant to ciprofloxacin, with a MIC of 16 μg/ml, had five amino acid changes in its QRDRs, including all those of *gyrA*, *gyrB*, *parC*, and *parE*. Ser84 to Leu and Asp88 to Asn in the *gyrA* product were most often found. Mutations in *gyrB* were rare. BLPACR isolates with ciprofloxacin MICs of 0.06 or 0.12 μg/ml frequently had a Ser84-to-Cys mutation in the *parC* product and an Ala369-to-Thr mutation in the *parE* product.

In the additional 50 isolates with ciprofloxacin MICs of 0.03 μg/ml or lower, mutations of QRDRs were found in 13 (26.9%) isolates. These mutations included five patterns: (i) Glu142 to Lys in the *gyrA* product (four isolates), (ii) Asn138 to Ser in the *parC* product (three isolates), (iii) Ser133 to Ala in the *parC* product (two isolates), (iv) Asn138 to Ser and Ser133 to Ala in the *parC* product (three isolates), and (v) Gly143 to Glu in the *parC* product (one isolate). Some of these mutations were found in the isolates for the initial analysis summarized in Table 6.

DISCUSSION

Among β-lactams, piperacillin-tazobactam clearly exhibited very strong activities against all classes of *H. influenzae* isolates (MIC ranges, MIC_{50}s, and MIC_{90}s [μg/ml] were as follows: BLNAS isolates, ≤0.03 to 0.12, ≤0.03, and 0.06; BLPAR isolates, ≤0.03 to 0.25, ≤0.03, and 0.06; BLNAR isolates, ≤0.03 to 0.5, 0.06, and 0.12; and BLPACR isolates, ≤0.03 to 0.5, 0.12, and 0.25, respectively). A total of 400 isolates, including four different phenotypes, were all susceptible according to the current CLSI breakpoint (5). The warnings in CLSI documents that BLNAR *H. influenzae* strains should be considered resistant to some β-lactam compounds are found in M2-A2/M100-

TABLE 5. MIC distributions for β-lactams and ciprofloxacin against 100 clinical isolates of BLPACR *H. influenzae*

Antimicrobial agent(s)	No. of isolates with MIC (μg/ml):										MIC (μg/ml)			% of isolates susceptible[a]
	≤0.12	0.25	0.5	1	2	4	8	16	32	≥64	Range	50%	90%	
Ampicillin	0	0	0	0	0	0	100	—[b]	—	—	≥8	≥8	≥8	0
Ampicillin-sulbactam	0	0	0	0	0	0	89	11	0	0	8–16	8	16	0
Amoxicillin-clavulanate	0	0	0	0	0	0	96	4	0	0	8–16	8	8	0
Piperacillin	0	0	0	0	0	1	17	15	25	42	4–≥64	32	≥64	
Piperacillin-tazobactam	74	24	2	0	0	0	0	0	0	0	≤0.03–0.5	0.12	0.25	100
Ceftriaxone	56	37	7	0	0	0	0	0	—	—	≤0.008–0.5	0.12	0.25	100
Imipenem	1	3	7	37	45	5	2	—	—	—	≤0.12–≥8	2	2	98
Meropenem	26	40	34	0	0	0	0	—	—	—	≤0.12–0.5	0.25	0.5	100
Ciprofloxacin	91	6	2	0	1	—	—	—	—	—	≤0.03–≥2	≤0.03	0.12	99

[a] CLSI MIC breakpoints (5).
[b] —, untested concentration.

TABLE 6. Phenotypes, MICs of ciprofloxacin, and QRDR mutations in *H. influenzae* isolates in the initial study

Phenotype	MIC of ciprofloxacin (μg/ml)	No. of isolates with mutation(s)	Mutation(s) in QRDR:			
			gyrA	*gyrB*	*parC*	*parE*
BLNAS	0.25	1	Asp88Gly	Thr472Ile	Asp83Asn	
BLPAR	0.25	1	Ser84Leu		Ser84Arg/Asn138Ser	Asp364Gly
		1	Asp88Asn		Ser84Cys	Ala369Thr
	0.12	1		Ala400Val		
		1	Asp88Asn		Ser84Cys	Ala369Thr
	0.06	1	Asp88Thr/Glu142Lys			
		2[a]				
BLNAR	0.06	1	Ser84Leu		Ser133Ala/Asn138Ser	
		1	Ser84Leu			
		1	Ser84Leu		Asn138Ser	Ser474Asn
		1	Asp88Thr		Glu88Lys	
BLPACR	16	1	Ser84Leu/Asp88Gly		Glu88Lys	Val392Ile/Asp420Asn
	0.5	2	Ser84Leu		Ser84Ile/Asn138Ser	
	0.25	6	Asp88Asn		Ser84Cys	Asp364Thr
	0.12	9	Asp88Asn		Ser84Cys	Ala369Thr
		1	Asp88Asn			Ala369Thr
		2[a]				
	0.06	1	Asp88Asn		Ser84Cys	Ala369Thr
		1	Asp88Asn			Ala369Thr
		1	Asp88Thr			
		1[a]				

[a] Number of isolates having no mutations in the indicated regions.

S17 tables (amoxicillin-clavulanate, ampicillin-sulbactam, cefaclor, cefetamet, cefonicid, cefprozil, cefuroxime, and loracarbef); in M7-A7/M100-S17 tables, piperacillin-tazobactam and cefamandole were additionally listed; many of those statements have been present since the early 1990s. The current findings are presented to stimulate reevaluation of the *H. influenzae* breakpoints in the era of escalation of BLNAR and BLPACR problems worldwide. This problem appears to be greatest in Asia and Western Europe but may be underestimated in other geographical areas. The high potency of piperacillin-tazobactam has been demonstrated against these strains, but resistance disclaimers are found in the CLSI tables and in U.S. FDA package inserts. Piperacillin-tazobactam, previously considered inactive against BLNAR *H. influenzae* due to limited data, has now been found to have great potency against these strains and should be assigned accurate susceptibility breakpoints.

In the present study, we confirmed that all BLNAR and BLPACR isolates had amino acid substitutions in their *ftsI* gene products and that the major pattern was group III-like (87.5%), followed by the isolates belonging to group IIa.

Moreover, we found that many isolates susceptible to ciprofloxacin had mutations in their QRDRs, particularly in the case of BLPACR isolates, and that one ciprofloxacin-resistant isolate (MIC, 16 μg/ml) had five mutations in its QRDRs. Mutations Ser84Ile and Ser84Arg in ParC and Ser84Leu and Asp88Asn in GyrA are previously known mutations associated with quinolone resistance in *H. influenzae* (11, 22, 26, 33). Asp420Asn in ParE corresponds to resistance-associated mutation sites in *Staphylococcus aureus* ParE (Asp432) and in *Streptococcus pneumoniae* ParE (Asp435) (28, 29). The Asn138Ser mutation in ParC was also found in susceptible *H. influenzae* strains and was suggested not to contribute to fluoroquinolone resistance (32), though it is possible that this is the first mutation in a pattern of stepwise mutation. It is thus still unclear whether all mutations in QRDRs found in the present study contribute to the decreased susceptibility to ciprofloxacin. However, since the MICs of ciprofloxacin for most clinical isolates are usually ≤0.03 μg/ml, those for the isolates associated with QRDR mutations appear to be elevated. Since the isolates with higher ciprofloxacin MICs tended to have several mutations in their QRDRs, it is possible that numerous *H. influenzae* isolates have silent mutations as first mutations and could acquire full resistance to quinolones by stepwise QRDR mutation, as found in *S. pneumoniae* (9). In an additional 50 clinical isolates of *H. influenzae* with ciprofloxacin MICs of 0.03 μg/ml or lower, 26.0% had mutations in their QRDRs, which had not been reported as the causes of ciprofloxacin resistance in *H. influenzae*, and some of them were found in the initial 400 clinical isolates as summarized in Table 6. These mutations would not contribute to ciprofloxacin resistance in *H. influenzae*, if additional significant mutations do not occur in their QRDRs.

In conclusion, piperacillin-tazobactam, a penicillin–β-lactamase inhibitor combination, was the most potent β-lactam tested, with all MICs being ≤0.5 μg/ml against all groups of *H. influenzae* isolates, including BLNAS, BLPAR, BLNAR, and BLPACR isolates. The susceptible disk diffusion breakpoints for piperacillin-tazobactam are proposed to be ≥21 mm. It is possible that quinolone-resistant *H. influenzae* strains may emerge, since several clinical isolates with elevated MICs of ciprofloxacin were found to have specific mutations in their QRDRs, while some isolates with low ciprofloxacin MICs carried mutations in their QRDRs, which do not contribute to ciprofloxacin resistance in *H. influenzae*. Checking for β-lactamase alone is performed for clinical isolates of *H. influenzae*, and antimicrobial susceptibilities are not accurately determined in several laboratories. This may result in lack of detection of BLNAR, BLPACR, and silent quinolone-resistant isolates. Susceptibility testing should be performed in order not to

underestimate the frequency of such emerging resistant strains.

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REFERENCES

1. **Biedenbach, D. J., and R. N. Jones.** 2003. Five-year analysis of *Haemophilus influenzae* isolates with reduced susceptibility to fluoroquinolones: prevalence results from the SENTRY antimicrobial surveillance program. Diagn. Microbiol. Infect. Dis. **46:**55–61.
2. **Clairoux, N., M. Picard, A. Brochu, N. Rousseau, P. Gourde, D. Beauchamp, T. R. Parr, Jr., M. G. Bergeron, and F. Malouin.** 1992. Molecular basis of non-β-lactamase-mediated resistance to β-lactam antibiotics in strains of *Haemophilus influenzae*. Antimicrob. Agents Chemother. **36:**1504–1513.
3. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, M7-A7. Approved standard, 7th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Clinical and Laboratory Standards Institute.** 2006. Performance standards for antimicrobial disk susceptibility tests, M2-A9. Approved standard, 9th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Clinical and Laboratory Standards Institute.** 2007. Performance standards for antimicrobial susceptibility testing, 15th edition. Approved standard M100-S17. Clinical and Laboratory Standards Institute, Wayne, PA.
6. **Dabernat, H., C. Delmas, M. Seguy, R. Pelisser, G. Faucon, S. Bennamani, and C. Pasquier.** 2002. Diversity of β-lactam resistance-conferring amino acid substitutions in penicillin-binding protein 3 of *Haemophilus influenzae*. Antimicrob. Agents Chemother. **46:**2208–2218.
7. **Davies, T. A., L. M. Kelly, D. B. Hoellman, L. M. Ednie, C. L. Clark, S. Bajaksouzian, M. R. Jacobs, and P. C. Appelbaum.** 2000. Activities and postantibiotic effects of gemifloxacin compared to those of 11 other agents against *Haemophilus influenzae* and *Moraxella catarrhalis*. Antimicrob. Agents Chemother. **44:**633–639.
8. **Doern, G. V., A. B. Brueggemann, G. Pierce, H. P. Holley, Jr., and A. Rauch.** 1997. Antibiotic resistance among clinical isolates of *Haemophilus influenzae* in the United States in 1994 and 1995 and detection of β-lactamase-positive strains resistant to amoxicillin-clavulanate: results of a national multicenter surveillance study. Antimicrob. Agents Chemother. **41:**292–297.
9. **Fukushima, K. Y., Y. Hirakata, K. Sugahara, K. Yanagihara, A. Kondo, S. Kohno, and S. Kamihira.** 2006. Rapid screening of topoisomerase gene mutations by a novel melting curve analysis method for early warning of fluoroquinolone-resistant *Streptococcus pneumoniae* emergence. J. Clin. Microbiol. **44:**4553–4558.
10. **García-Cobos, S., J. Campos, E. Lázaro, F. Román, E. Cercenado, C. García-Rey, M. Pérez-Vázquez, J. Oteo, and F. de Abajo.** 2007. Ampicillin-resistant non-β-lactamase-producing *Haemophilus influenzae* in Spain: recent emergence of clonal isolates with increased resistance to cefotaxime and cefixime. Antimicrob. Agents Chemother. **51:**2564–2573.
11. **Georgiou, M., R. Munoz, F. Roman, R. Canton, R. Gomez-Lus, J. Campos, and A. G. De La Campa.** 1996. Ciprofloxacin-resistant *Haemophilus influenzae* strains possess mutations in analogous positions of GyrA and ParC. Antimicrob. Agents Chemother. **40:**1741–1744.
12. **Hasegawa, K., K. Yamamoto, N. Chiba, R. Kobayashi, K. Nagai, M. R. Jacobs, P. C. Appelbaum, K. Sunakawa, and K. Ubukata.** 2003. Diversity of ampicillin-resistance genes in *Haemophilus influenzae* in Japan and the United States. Microb. Drug Resist. **9:**39–46.
13. **Hasegawa, K., R. Kobayashi, E. Takada, A. Ono, N. Chiba, M. Morozumi, S. Iwata, K. Sunakawa, and K. Ubukata.** 2006. High prevalence of type b β-lactamase-non-producing ampicillin-resistant *Haemophilus influenzae* in meningitis: the situation in Japan where Hib vaccine has not been introduced. J. Antimicrob. Chemother. **57:**1077–1082.
14. **Hoban, D. J., G. V. Doern, A. C. Fluit, M. Roussel-Delvallez, and R. N. Jones.** 2001. Worldwide prevalence of antimicrobial resistance in *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* in the SENTRY Antimicrobial Surveillance Program, 1997–1999. Clin. Infect. Dis. **32**(Suppl. 2)**:**S81–S93.
15. **Hotomi, M., K. Fujihara, D. S. Billal, K. Suzuki, T. Nishimura, S. Baba, and N. Yamanaka.** 2007. Genetic characteristics and clonal dissemination of β-lactamase-negative ampicillin-resistant *Haemophilus influenzae* strains isolated from the upper respiratory tract of patients in Japan. Antimicrob. Agents Chemother. **51:**3969–3976.
16. **Inoue, M., N. Y. Lee, S. W. Hong, K. Lee, and D. Felmingham.** 2004. PROTEKT 1999–2000: a multicentre study of the antibiotic susceptibility of respiratory tract pathogens in Hong Kong, Japan and South Korea. Int. J. Antimicrob. Agents **23:**44–51.
17. **Inoue, M., S. Kohno, M. Kaku, K. Yamaguchi, J. Igari, and K. Yamanaka.** 2005. PROTEKT 1999–2000: a multicentre study of the antimicrobial susceptibility of respiratory tract pathogens in Japan. Int. J. Infect. Dis. **9:**27–36.
18. **Jansen, W. T., A. Verel, M. Beitsma, J. Verhoef, and D. Milatovic.** 2006. Longitudinal European surveillance study of antibiotic resistance of *Haemophilus influenzae*. J. Antimicrob. Chemother. **58:**873–877.
19. **Karlowsky, J. A., I. A. Critchley, R. S. Blosser-Middleton, E. A. Karginova, M. E. Jones, C. Thornsberry, and D. F. Sahm.** 2002. Antimicrobial surveillance of *Haemophilus influenzae* in the United States during 2000-2001 leads to detection of clonal dissemination of a β-lactamase-negative and ampicillin-resistant strain. J. Clin. Microbiol. **40:**1063–1066.
20. **Khan, W., S. Ross, W. Rodriguez, G. Controni, and A. K. Saz.** 1974. *Haemophilus influenzae* type b resistant to ampicillin: a report of two cases. JAMA **229:**298–301.
21. **Kim, I.-S., C.-S. Ki, S. Kim, W. S. Oh, and K. Ran.** 2007. Diversity of ampicillin resistance genes and antimicrobial susceptibility patterns in *Haemophilus influenzae* strains isolated in Korea. Antimicrob. Agents Chemother. **51:**453–460.
22. **Li, X., N. Mariano, J. J. Rahal, C. M. Urban, and K. Drlica.** 2004. Quinolone-resistant *Haemophilus influenzae* in a long-term-care facility: nucleotide sequence characterization of alterations in the genes encoding DNA gyrase and DNA topoisomerase IV. Antimicrob. Agents Chemother. **48:**3570–3572.
23. **Markowitz, S. M.** 1980. Isolation of an ampicillin-resistant, non-β-lactamase-producing strain of *Haemophilus influenzae*. Antimicrob. Agents Chemother. **17:**80–83.
24. **Morikawa, Y., M. Kitazato, J. Mitsuyama, S. Mizunaga, S. Minami, and Y. Watanabe.** 2004. In vitro activities of piperacillin against β-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. Antimicrob. Agents Chemother. **48:**1229–1234.
25. **Nazir, J., C. Urban, N. Mariano, J. Burns, B. Tommasulo, C. Rosenberg, S. Segal-Maurer, and J. J. Rahal.** 2004. Quinolone-resistant *Haemophilus influenzae* in a long-term care facility: clinical and molecular epidemiology. Clin. Infect. Dis. **38:**1564–1569.
26. **Pérez-Vázquez, M., F. Román, B. Aracil, R. Cantón, and J. Campos.** 2004. Laboratory detection of *Haemophilus influenzae* with decreased susceptibility to nalidixic acid, ciprofloxacin, levofloxacin, and moxifloxacin due to *gyrA* and *parC* mutations. J. Clin. Microbiol. **42:**1185–1191.
27. **Pérez-Vázquez, M., F. Román, S. García-Cobos, and J. Campos.** 2007. Fluoroquinolone resistance in *Haemophilus influenzae* is associated with hypermutability. Antimicrob. Agents Chemother. **51:**1566–1569.
28. **Perichon, B., J. Tankovic, and P. Courvalin.** 1997. Characterization of a mutation in the *parE* gene that confers fluoroquinolone resistance in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **41:**1166–1167.
29. **Takahashi, H., T. Kikuchi, S. Shoji, S. Fujimura, A. B. Lutfor, Y. Tokue, T. Nukiwa, and A. Watanabe.** 1998. Characterization of *gyrA*, *gyrB*, *grlA* and *grlB* mutations in fluoroquinolone-resistant clinical isolates of *Staphylococcus aureus*. J. Antimicrob. Chemother. **41:**49–57.
30. **Tomeh, M. O., S. E. Starr, J. E. McGowan, Jr., P. M. Terry, and A. G. Nahmias.** 1974. Ampicillin-resistant *Haemophilus influenzae* type b infection. JAMA **229:**295–297.
31. **Ubukata, K., Y. Shibasaki, K. Yamamota, N. Chiba, K. Hasegawa, Y. Takeuchi, K. Sunakawa, M. Inoue, and M. Konno.** 2001. Association of amino acid substitutions in penicillin binding protein 3 with β-lactam resistance in β-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. Antimicrob. Agents Chemother. **45:**1693–1699.
32. **Yokota, S.-I., Y. Ohkoshi, K. Sato, and N. Fujii.** 2008. Emergence of fluoroquinolone-resistant *Haemophilus influenzae* strains among elderly patients but not among children. J. Clin. Microbiol. **46:**361–365.
33. **Yoshizumi, S., Y. Takahashi, Y. Watanabe, E. Okezaki, Y. Ishii, and K. Tateda.** 2004. In vitro antibacterial activities of new fluoroquinolones against clinical isolates of *Haemophilus influenzae* with ciprofloxacin-resistance-associated alterations in GyrA and ParC. Chemotherapy **50:**265–275.