Low β -Lactamase-Negative Ampicillin-Resistant *Haemophilus influenzae* Strains Are Best Detected by Testing Amoxicillin Susceptibility by the Broth Microdilution Method[∇]

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Ampicillin resistance in *Haemophilus influenzae* due to alterations in penicillin-binding proteins (β -lactamase negative ampicillin resistant [BLNAR]) is acquiring increasing clinical and epidemiological importance. BLNAR strains with low ampicillin MICs (0.5 to 4 $\mu\text{g/ml}$) represent the majority of this population in Europe and the United States, but separating them from susceptible isolates is challenging. To investigate the best method to identify low-BLNAR strains, we studied the antibiotic susceptibilities of 94 clinical isolates of *H. influenzae* by microdilution, Etest, and disk diffusion: 25 had no resistance mechanisms (gBLNAS), 34 had mutations in the *ftsI* gene only (gBLNAR), 20 were β -lactamase producers only (gBLPAR), and 15 showed β -lactamase production and mutations in the *ftsI* gene (gBLPACR). By current CLSI breakpoints, most gBLNAR isolates were ampicillin susceptible by microdilution (76.5%) or by Etest (88.2%). Most gBLNAR strains (79.4%) were nonsusceptible to amoxicillin (the most widely used community antibiotic in the United States and Europe) when tested by microdilution. By Etest, 15% of β -lactamase-positive isolates were nonresistant to ampicillin or amoxicillin. The poorest agreement between Etest and microdilution results was for the gBLPAR isolates (25% for ampicillin, 15% for amoxicillin, and 10% for cefaclor). Low-strength disks of ampicillin and amoxicillin-clavulanic acid poorly identified low-BLNAR isolates and are not recommended as a screening method. We suggest new amoxicillin breakpoints for BLNAR isolates as follows: susceptible, MIC $\leq 0.5 \mu\text{g/ml}$ (no resistance mechanisms; pharmacokinetic/pharmacodynamic [PK/PD] data favorable); intermediate, MICs = 1 to 2 $\mu\text{g/ml}$ (resistance mechanisms present but PK/PD data favorable), and resistant, MICs $\geq 4 \mu\text{g/ml}$ (resistance mechanisms present and PK/PD data unfavorable).

Haemophilus influenzae is an important pathogen associated with pneumonia and other acute and chronic respiratory infections in children and adults. Although β -lactamase production is the most common mechanism of resistance to β -lactam antibiotics, alterations in penicillin-binding proteins (PBPs) are acquiring an important role in ampicillin resistance, and the β -lactamase-negative ampicillin-resistant (BLNAR) phenotype is found worldwide (12, 17, 20, 29). In addition, strains that produce β -lactamase and carry mutations in the *ftsI* gene affecting PBP3 (BLPACR strains) have been described (8, 14, 18).

BLNAR strains with low ampicillin MICs currently represent the majority of the BLNAR population in Europe and the United States (8, 12, 20). Previous studies have identified BLNAR isolates with mutations in the *ftsI* gene encoding PBP3 that have ampicillin MICs of $\leq 1 \mu\text{g/ml}$ (7, 14, 15, 33). Therefore, currently recommended ampicillin breakpoints (6) may fail to detect a significant number of the low-BLNAR population, leading to significant clinical and epidemiological consequences.

Detecting BLNAR strains by PCR and direct sequencing of the *ftsI* gene is difficult and impractical. High-BLNAR isolates (with MICs of 8 to 16 $\mu\text{g/ml}$) are effectively identified by regular susceptibility-testing methods, but such isolates are

very rare in clinical laboratories. In contrast, low-BLNAR strains are commonly isolated, and their incidence may be increasing in many parts of the world (12, 29). Low-strength disks of ampicillin (2 μg) and amoxicillin-clavulanic acid (2 and 1 μg) have been proposed as alternative screening methods to discriminate between BLNAR and β -lactamase-negative ampicillin-susceptible (BLNAS) strains (23, 34), but in these proposals, the mutation status of the *ftsI* gene was not determined. In addition, an Epsilon test (Etest) (AB-Biodisk, Solna, Sweden) alone may not define the actual MICs of BLNAR and BLPACR strains for β -lactam antibiotics (2).

Recently, we observed that β -lactamase-negative *H. influenzae* isolates with ampicillin MICs of $\geq 2 \mu\text{g}$ and PBP3 amino acid substitutions increased in Spain from 13.6% in 1997 to 28.9% in 2007 (14a). We also realized that laboratory recognition of these isolates by standard susceptibility methods was difficult, as the methods lacked sensitivity and specificity. In addition, we have reported that cefixime and cefpodoxime resistances in *H. influenzae* have recently increased in Spain (14).

Accordingly, our main objective was to determine the most appropriate methods to detect low-BLNAR and BLPACR strains. We studied the antibiotic susceptibilities of clinical *H. influenzae* isolates with well-characterized mechanisms of ampicillin resistance in order to (i) evaluate the abilities of ampicillin, amoxicillin, and amoxicillin-clavulanic acid to distinguish the low-BLNAR and BLPACR populations; (ii) compare broth microdilution and Etest methods for the detection of *H. influenzae* with

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TABLE 1. Amino acid substitutions present in *H. influenzae* isolates with mutations in the *ftsI* gene

Amino acid substitution(s) in <i>ftsI</i> gene	β -Lactamase production ^a	Group ^b	Genotype ^c	No. of isolates
Asn526Lys	—	IIa	gBLNAR	2
Asn350Asp, Gly490Glu, Asn526Lys, and Ala530Ser	—	IIa	gBLNAR	2
Asp350Asn, Met377Ile, Ala502Val, and Asn526Lys	—	IIb	gBLNAR	9
	+	IIb	gBLPACR	5
Ala502Thr and Asn526Lys	—	IIc	gBLNAR	5
	+	IIc	gBLPACR	2
Asp350Asn, Ala502Thr, and Asn526Lys	—	IIc	gBLNAR	8
	+	IIc	gBLPACR	8
Asp350Asn, Ser357Asn, Met377Ile, Ser385Thr, Arg517His, and Thr532Ser	—	III-like	gBLNAR	6
Asp350Asn, Ser357Asn, Met377Ile, Ser385Thr, Leu389Phe, Arg517His, and Thr532Ser	—	III-like	gBLNAR	2
Total				49

^a +, present; —, absent.^b The strains with *ftsI* gene mutations were classified into groups according to the criteria reported by Dabernat et al. (7).^c gBLNAR, *n* = 34; gBLPACR, *n* = 15.

different mechanisms of resistance to β -lactam antibiotics; (iii) determine the usefulness of low-strength disks of ampicillin and amoxicillin-clavulanic acid for screening low-BLNAR strains; (iv) determine the most suitable method to detect isolates with increased resistance to cefixime; and, finally, (v) propose interpretative criteria for the more accurate distinction of low-BLNAR and BLPACR isolates.

MATERIALS AND METHODS

Test isolates. Ninety-four clinical *H. influenzae* isolates and three reference strains from the American Type Culture Collection (ATCC) were included in this study. All isolates were nonencapsulated and were isolated from community-acquired respiratory tract infections (88.3%) or invasive infections (11.7%); 47.9% were from children, 45.7% from adults, and 6.4% from patients of unknown age.

The study collection was selected so that the most important and common mechanisms of ampicillin resistance were represented. Four genotypes of ampicillin susceptibility were included: 25 strains had no detectable resistance mechanism (gBLNAS), 34 had mutations in the *ftsI* gene (gBLNAR), 20 produced β -lactamase but had no *ftsI* mutations (gBLPAR), and 15 produced β -lactamase and also had mutations in the *ftsI* gene (gBLPACR). Genotype determination carried out by PCR amplification and DNA sequencing of *ftsI* and *blaTEM* genes was the gold standard method against which all other methods were tested.

Clinical isolates with alterations in the *ftsI* gene (gBLNAR and gBLPACR genotypes) were selected on the basis of their amino acid substitutions and represented the most common mutation patterns described in BLNAR isolates (14) (Table 1). β -Lactamase-positive isolates (all of the TEM 1 type) were chosen so that the most common promoter regions (26, 31) were represented: 14 isolates had the Prpt promoter (6 gBLPAR and 8 gBLPACR), 12 isolates contained the Pdel promoter (7 gBLPAR and 5 gBLPACR), and 9 isolates had the Pa/Pb promoter (7 gBLPAR and 2 gBLPACR).

The clinical isolates belonging to the gBLNAS, gBLNAR, and gBLPACR genotypes have been described in detail elsewhere (14); the 20 gBLPAR strains were clinical isolates chosen from the collections submitted to our Spanish *Haemophilus* Reference Laboratory.

Strain ATCC 49766 was included as a fully susceptible control and strain ATCC 49247 as an *H. influenzae* BLNAR strain, as recommended by the CLSI (6). Strain ATCC 51907 is a strain whose whole genome has been sequenced (11). These three ATCC strains were studied in parallel and were included for quality control and comparative purposes.

Susceptibility testing. The reference broth microdilution method was performed following CLSI guidelines (4, 5), except that the plates were incubated with 5% CO₂ before the results were read because many strains did not grow well without this modification. The antibiotic susceptibilities of all *H. influenzae* strains were determined by three methods: broth microdilution, Etest, and disk diffusion.

Mueller-Hinton broth (Oxoid Ltd., Basingstoke, United Kingdom) was supplemented with HTM supplement (Oxoid) and yeast extract (5%; Difco, Detroit,

MI). Microtiter plates (Sensititre Enzyme 13 and STRHAE1; Trek Diagnostics Inc. Westlake, OH) were inoculated to produce a final density of approximately 5×10^5 CFU/ml, which was regularly controlled by colony counting on chocolate agar after overnight incubation. The inoculated plates were incubated at 35°C for 20 to 24 h in 5% CO₂ before interpretation of the results. The MIC was defined as the lowest concentration of antibiotic that inhibited growth. Ampicillin, amoxicillin, amoxicillin-clavulanic acid (2:1 ratio), cefaclor, cefuroxime, cefotaxime, and cefixime were evaluated.

The disk diffusion agar method was performed by following the CLSI guidelines (4, 5) using HTM base (Oxoid) supplemented with HTM supplement (Oxoid) and yeast extract (5%; Difco, Detroit, MI). Plates were inoculated with each bacterial suspension adjusted to a McFarland standard of 0.5 and incubated for 20 to 24 h at 35°C in 5% CO₂. Standard disks of ampicillin (10 μ g), amoxicillin-clavulanic acid (30 μ g), cefaclor (30 μ g), cefuroxime acid (30 μ g), cefotaxime (30 μ g), and cefixime (5 μ g) were purchased from Oxoid. Also, low-strength disks of ampicillin (2 μ g) and amoxicillin-clavulanic acid (3 μ g) (Oxoid) were tested.

For the Etest method, the same antibiotics mentioned above were evaluated. Briefly, colonies grown overnight on chocolate agar were suspended in saline solution to a turbidity equivalent to a McFarland standard of 0.5, and 150-mm-diameter agar plates were inoculated by confluent swabbing of the surface with the adjusted suspensions. Etest strips were placed in an equidistant radial fashion on the surfaces of the plates, and the plates were incubated at 35°C in 5% CO₂ for 20 to 24 h. The MIC end point was read where the growth inhibition ellipse intersected the MIC on the Etest gradient strip.

β -Lactamase production was determined by the chromogenic cephalosporin test with nitrocephin as a substrate (28).

To date, amoxicillin susceptibility breakpoints have not been specifically established by the CLSI; instead, the results of the ampicillin susceptibility tests are to be used to predict the activity of amoxicillin (6). In accordance with this and with other authors (12), we considered isolates with an amoxicillin MIC of ≤ 1 μ g/ml to be susceptible, isolates with an amoxicillin MIC of 2 μ g/ml to be intermediate, and isolates with an amoxicillin MIC of ≥ 4 μ g/ml to be resistant.

Amplification and sequence analysis. Sequencing and analysis of the *ftsI* and *blaTEM* gene sequences were done as described previously (14; García-Cobos and Campos, submitted).

Analysis of results. MICs obtained by the Etest method were rounded up to the next log₂ concentration. Agreement between broth microdilution and Etest results was achieved when the results of both methods were in the range of ± 1 log₂ unit of each other. Pearson correlation coefficients (*r* values) were generated to compare the Etest and reference microdilution methods. Sensitivity and specificity values were calculated as published previously (27).

Very major errors were defined as results that indicated a lack of resistance mechanism (gBLNAS) by disk diffusion, Etest, or microdilution when they were gBLNAR or gBLPACR by the gold standard method. Major errors were classified as results that indicated the presence of mutations in the *ftsI* gene (gBLNAR or gBLPACR) by disk diffusion, Etest, or microdilution when they had no resistance mechanisms (gBLNAS) by the gold standard method.

For the best separation of the gBLNAR and gBLPACR populations, the following criteria were taken into account: sensitivity and specificity values of

TABLE 2. Susceptibilities to ampicillin, amoxicillin, and cefaclor of *H. influenzae* isolates according to resistance class and susceptibility-testing method

Genotype ^a	Method	MIC (μg/ml)			Susceptibility ^b (% of isolates)		
		50%	90%	Range	S	I	R
Ampicillin							
gBLNAS	Microdilution	≤0.25	0.5	≤0.25–0.5	100	0	0
	E-test	0.25	0.5	0.12–0.5	100	0	0
gBLPAR	Microdilution	≥32	≥32	8–≥32	0	0	100
	E-test	4	16	1–32	5	10	85
gBLNAR	Microdilution	1	2	0.5–4	76.5	20.6	2.9
	E-test	1	2	0.5–2	88.2	11.8	0
gBLPACR	Microdilution	≥32	≥32	≥32	0	0	100
	E-test	32	64	8–64	0	0	100
Overall	Microdilution	1	≥32	≤0.25–≥32	54.3	7.40	38.3
	E-test	1	32	0.12–64	59.6	6.40	34.0
Amoxicillin							
gBLNAS	Microdilution	0.5	0.5	0.5–1	100	0	0
	E-test	0.5	0.5	0.25–0.5	100	0	0
gBLPAR	Microdilution	≥64	≥64	8–≥64	0	0	100
	E-test	4	16	1–32	5	10	85
gBLNAR	Microdilution	2	4	1–4	20.6	44.1	35.3
	E-test	1	2	0.5–2	67.6	32.4	0
gBLPACR	Microdilution	≥64	≥64	32–≥64	0	0	100
	E-test	32	64	4–128	0	0	100
Overall	Microdilution	2	≥64	0.5–≥64	34.0	16.0	50.0
	E-test	1	32	0.25–128	52.1	13.9	34.0
Cefaclor							
gBLNAS	Microdilution	2	4	1–4	100	0	0
	E-test	1	2	0.5–8	100	0	0
gBLPAR	Microdilution	8	16	1–32	85	10	5
	E-test	1	2	1–2	100	0	0
gBLNAR	Microdilution	8	8	4–16	94.1	5.9	0
	E-test	4	16	1–32	76.5	17.6	5.9
gBLPACR	Microdilution	8	16	4–16	80	20	0
	E-test	4	32	2–32	73.3	6.7	20
Overall	Microdilution	4	8	1–32	91.5	7.4	1.1
	E-test	2	16	0.5–32	87.2	7.4	5.3

^a gBLNAS, $n = 25$; gBLPAR, $n = 20$; gBLNAR, $n = 34$; gBLPACR, $n = 15$.

^b S, susceptible; I, intermediate; R, resistant. The S/I/R classification for ampicillin and cefaclor is based on the interpretative criteria of the CLSI, and that for amoxicillin is based on the interpretative criteria previously described (12).

$\geq 90\%$, categorical agreement at $\geq 95\%$, and a tolerance of very major and major errors at $\leq 5\%$ (32).

The data were managed and the statistics were calculated by using the Computer programs Whonet (WHO/CSR/DRS/99.1; World Health Organization) and GraphPad Prism (GraphPad Software, Inc.).

RESULTS

Antibiotic susceptibility. Susceptibility data obtained with the reference isolates ATCC 49247 and ATCC 49766, either by the microdilution or by the disk diffusion method, were always within the recommended reference values (6). The MICs for amoxicillin, ampicillin, amoxicillin-clavulanic acid, and cefaclor are organized according to their resistance classes in Table 2. In general, higher MIC₅₀ and MIC₉₀ values were obtained by the microdilution method than by the Etest method. Compared to susceptible isolates (gBLNAS), the gBLNAR population had a fourfold increase in ampicillin MIC₅₀ and MIC₉₀ values by both microdilution and Etest. In the case of amoxicillin, the MIC₅₀ and MIC₉₀ values were four- and eightfold higher by microdilution and two- and fourfold higher by Etest (Table 2).

Susceptible *H. influenzae* (gBLNAS) isolates were well iden-

tified by microdilution with both ampicillin and amoxicillin testing; this was also the case for the β -lactamase-producing resistant population (gBLPAR and gBLPACR) (Table 2).

However, important differences in testing methods were observed for the gBLNAR isolates, as 76.5% of them were susceptible to ampicillin by microdilution and 88.2% were found to be susceptible by Etest (Table 2). Moreover, 79.4% of the gBLNAR isolates were identified as nonsusceptible to amoxicillin by microdilution but only 32.4% by Etest (Table 2).

Differences were also observed for the β -lactamase-positive isolates: 100% were correctly identified as resistant by the microdilution method, but 85% were categorized as resistant, 10% as intermediate, and 5% as susceptible by Etest with either ampicillin or amoxicillin (Table 2). This finding may have clinical consequences, as $\sim 15\%$ of β -lactamase-positive *H. influenzae* isolates could be misclassified as nonresistant by the Etest. Accordingly, a β -lactamase test should be used along with the Etest.

The whole collection, independent of resistance class, was categorized as susceptible to amoxicillin-clavulanic acid according to the current CLSI breakpoints (data not shown).

TABLE 3. Comparison of *H. influenzae* susceptibility results obtained by the Etest method and the broth microdilution reference method

Antibiotic	Genotype ^a	MIC range (μg/ml)		No. of Etest MICs within indicated concn (log ₂) of HTM broth microdilution MICs							% Agreement within 1 log ₂ concn	Pearson correlation coefficient (r)
		Microbroth	Etest	>-2	-2	-1	Same	+1	+2	>+2		
Ampicillin	gBLNAS	≤0.25-0.5	0.12-0.5			2	21	2			100	0.63
	gBLPAR	8-≥32	1-64	9	6	4	1				25	
	gBLNAR	0.5-4	0.5-2		1	13	18	2			97	
	gBLPACR	≥32	8-64		2	4	9				86.7	
	Overall			9	9	23	49	2			80.9	
Amoxicillin	gBLNAS	0.5-1	0.25-0.5			11	14				100	0.63
	gBLPAR	8-≥64	1-32	14	3	3					15	
	gBLNAR	1-4	0.5-2	1	8	18	7				73.5	
	gBLPACR	32-≥64	4-128	1	1	9	3	1			86.7	
	Overall			16	12	41	10	1			80.9	
Amoxicillin-clavulanate	gBLNAS	0.5-1	0.25-1		1	13	9	2			96	0.92
	gBLPAR	0.25-2	0.25-1			8	11	1			100	
	gBLNAR	0.5-4	0.25-4	1	8	18	7				73.5	
	gBLPACR	1-4	0.5-2		5	8	1	1			66.7	
	Overall			1	14	47	28	4			84	
Cefaclor	gBLNAS	1-4	0.5-8		7	12	4	1	1		68	0.87
	gBLPAR	1-32	1-2	7	11	1	1				10	
	gBLNAR	4-16	1-32	1	3	14	8	5	3		79.4	
	gBLPACR	4-16	2-32			7	4	1	2	1	80	
	Overall			8	21	34	17	7	6	1	61.7	

^a gBLNAS, *n* = 25; gBLPAR, *n* = 20; gBLNAR, *n* = 34; gBLPACR, *n* = 15; overall, *n* = 94.

With cefaclor, 94.1% and 76.5% of the gBLNAR population were susceptible by microdilution and Etest, respectively, indicating that the nonsusceptible population was 5.9% by microdilution and 23.5% by Etest (Table 2).

The majority of isolates of all resistance genotypes were classified as susceptible to cefuroxime, cefotaxime, and cefixime according to the CLSI breakpoints (6), and no significant differences were observed between the microdilution and Etest methods.

Comparison of broth microdilution and Etest. A comparison of the Etest with microdilution showed an overall essential agreement of 80.9% for both ampicillin and amoxicillin (Table 3). For the resistant gBLNAR class, essential agreement was 97% for ampicillin and 73.5% for amoxicillin. The poorest essential agreement was for the gBLPAR strains (25% for ampicillin and 15% for amoxicillin) (Table 3).

For amoxicillin-clavulanic acid, an overall essential agreement of 84% was obtained (Table 3). However, while essential agreement for gBLNAS and gBLPAR strains was 96% and 100%, respectively, it was lower for gBLNAR (73.5%) and gBLPACR (66.7%) strains (Table 3).

Total essential agreement for cefaclor was 61.7%, but it was much lower for gBLPAR strains (10%), as MICs were much lower by Etest than by microdilution (Table 3). The overall essential agreement for cefuroxime, cefotaxime, and cefixime was >90%, and it was >80% for any resistance class (data not shown), suggesting that Etest is a good method for testing these cephalosporins.

Population distribution according to resistance class. Figure 1 shows *H. influenzae* susceptibilities to ampicillin, amoxicillin, and amoxicillin-clavulanic acid according to the resistance class and testing method. The ampicillin MIC range for gBLPAR strains was 8 to ≥32 μg/ml by microdilution and

1 to 64 μg/ml by Etest (Fig. 1); for amoxicillin, a MIC range of 8 to ≥64 μg/ml was observed by microdilution and 1 to 32 μg/ml by Etest (Fig. 1).

By microdilution, gBLNAR isolates had an ampicillin MIC range of 0.5 to 4 μg/ml (peaking at 1 μg/ml), an amoxicillin MIC range of 1 to 4 μg/ml (peaking at 2 and 4 μg/ml), and an amoxicillin-clavulanic acid MIC range of 0.5 to 4 μg/ml (peaking at 2 μg/ml) (Fig. 1). By Etest, the MIC ranges were 0.5 to 2 μg/ml for ampicillin, 0.5 to 2 μg/ml for amoxicillin, and 0.25 to 4 μg/ml for amoxicillin-clavulanic acid (Fig. 1).

By microdilution, gBLPACR isolates had an amoxicillin-clavulanic acid MIC range between 1 and 4 μg/ml and gBLPAR isolates had an amoxicillin-clavulanic acid MIC range of 0.25 to 2 μg/ml (Fig. 1). By Etest, the amoxicillin-clavulanic acid MIC ranges were as follows: gBLPACR, 0.5 to 2 μg/ml; gBLPAR, 0.25 to 1 μg/ml; gBLNAR, 0.25 to 4 (peaking at 1 μg/ml); and gBLNAS, 0.25 to 0.5 μg/ml (Fig. 1).

Detection of increased resistance to cefixime. The gBLNAR strains presenting increased resistance to cefixime, corresponding to the III-like group with mutations at the SSN motif (14), showed a cefixime MIC of ≥0.5 μg/ml by either the microdilution or the Etest method and a cefixime disk diameter of ≤29 mm (Fig. 2). The Etest also distinguished this population with reduced susceptibility to cefixime (data not shown).

Usefulness of the low-strength disks. Table 4 shows the disk inhibition zones of gBLNAR and gBLNAS isolates. The populations were not well separated, as an important overlapping zone was observed with all four disks studied (Table 4). With the 10-μg ampicillin disk, the inhibition zones of 31 gBLNAR isolates (91.2%) overlapped with those of 18 gBLNAS isolates (72%); with the 2-μg ampicillin disk, the inhibition zones of 16 gBLNAR isolates (47.1%) overlapped

FIG. 1. Distribution of ampicillin, amoxicillin, and amoxicillin-clavulanic acid MICs for the *H. influenzae* study collection according to their resistance classes. gBLNAS, $n = 25$; gBLPAR, $n = 20$; gBLNAR, $n = 34$; gBLPACR, $n = 15$.

with those of 21 gBLNAS isolates (84%); with the 20/10 μg amoxicillin-clavulanic acid disks, the inhibitions zones of 32 gBLNAR isolates (94.1%) overlapped with those of 23 gBLNAS isolates (92%); and with the 2/1 μg amoxicillin-clavulanic acid disks, the inhibition zones of 24 gBLNAR isolates (70.6%) overlapped with those of 18 gBLNAS isolates (72%) (Table 4). By current CLSI criteria (6), all gBLNAR isolates would have been identified as ampicillin susceptible for the ampicillin 10- μg and amoxicillin-clavulanic acid 20/10 μg disks.

Breakpoint analysis. Several proposals for sensitivity criteria based on determination of the MIC (by microdilution or Etest) or of the inhibition zones by the disk diffusion method are listed in Table 5. The most consistent way to identify low-gBLNAR isolates (categorical agreement $\geq 95\%$) was by testing amoxicillin and amoxicillin-clavulanic acid by microdilution (cutoff, MIC $\geq 1 \mu\text{g/ml}$). gBLPACR isolates were best identified by testing amoxicillin-clavulanic acid by microdilution (cutoff, MIC $\geq 2 \mu\text{g/ml}$) (Table 5).

Other criteria that might be acceptable include detecting gBLNAR by testing ampicillin susceptibility by the microdilution method (cutoff, MIC $\geq 1 \mu\text{g/ml}$; categorical agreement,

89.8%) or amoxicillin susceptibility by Etest (cutoff, MIC $\geq 1 \mu\text{g/ml}$; categorical agreement, 84.7%) (Table 5).

The analysis of several tentative breakpoints based on disk diffusion was less reliable, as all of them had very major errors (10 to 23% of samples tested) (Table 5). Although a disk diameter of $\leq 23 \text{ mm}$ for the amoxicillin disk (2 μg) and $\leq 20 \text{ mm}$ for the amoxicillin-clavulanic acid disk (2/1 μg) may be indicative of gBLNAR isolates (88% specificity in both cases), the sensitivity values were low (76.5% and 58.8%, respectively) (Table 5).

Based on the *H. influenzae* population analyzed in this study, we propose a practical algorithm to detect the low-gBLNAR, gBLPAR, and gBLPACR isolates by the microdilution method (Fig. 3). These simple indications may help clinical laboratories to improve their recognition of the several genotypes of aminopenicillin resistance in *H. influenzae*.

DISCUSSION

Since 1986 (24), several laboratory methods to detect BLNAR *H. influenzae* have been examined. The definition of BLNAR itself is confusing, as the low- and high-BLNAR classifications

FIG. 2. Distribution of cefixime (CFM) MICs and disk diameters for *H. influenzae* isolates according to the mutations in the *ftsI* gene.

that have been proposed show overlapping MIC ranges (30). Several authors have contributed to the improvement of laboratory detection of BLNAR strains by suggesting different culture media and disk strengths and proposing modifications of the CLSI detection criteria (1, 9, 10, 13, 19, 21, 22, 23, 25, 34).

However, most previous studies have analyzed a low number of BLNAR isolates, have not reported the resistance genotypes, and have contained isolates with high MICs that are rarely found in laboratories in the United States or Europe. In addition, ampicillin and amoxicillin susceptibilities in BLNAR

strains have not been compared. The importance of studying *H. influenzae* susceptibility to amoxicillin seems clear, since ampicillin is very little used at the community level in the United States and Europe while amoxicillin, on the other hand, is the most used antibiotic, accounting for about 22% of the total outpatient consumption, mainly for respiratory and other common infections (16).

In this study, low-gBLNAR isolates with well-established PBP3 mechanism of resistance patterns had ampicillin microdilution MICs between 0.5 and 4 μg/ml, with a peak at 1 μg/ml (Fig. 1). Consequently, the majority of these isolates (76.5%) would have been reported as ampicillin susceptible and only 23.5% as nonsusceptible (Table 2) by current CLSI recommendations (6). This same population had a MIC range of 1 to 4 μg/ml for amoxicillin, and the majority of gBLNAR isolates (79.4%) would have been reported as nonsusceptible (Table 2). These data suggest that the best option to detect gBLNAR isolates is by testing amoxicillin by the microdilution method.

Separation of low-gBLNAR and gBLNAS isolates is of epidemiological interest, as an increase in low-gBLNAR strains could indicate adaptive changes in PBP3 in response to increased pressure posed by β-lactamase-resistant antibiotics (amoxicillin-clavulanic acid and new cephalosporins). Also, it is important for clinical reasons, since, although these isolates mainly cause respiratory infections in the United States and Europe, an increasing number of BLNAR isolates cause invasive infections, including meningitis (11.7% in this study).

In this study, detection of low-gBLNAR *H. influenzae* isolates by the disk diffusion method was of limited use because the proportion of overlapping inhibition zones between gBLNAS and gBLNAR populations was unacceptably high. Accordingly, the disk method should not be used alone or as a screening method to detect BLNAR isolates. Other reports (1, 9, 21, 23, 24, 25, 34) have proposed discriminative criteria for the disk diffusion method, particularly for the low-strength disks, but we could not reproduce those results, perhaps due to the different gBLNAR populations analyzed.

Both ampicillin and amoxicillin are currently recommended by the British Society for Antimicrobial Chemotherapy for susceptibility testing of *H. influenzae*, with a breakpoint of ≤1 μg/ml considered susceptible and ≥2 μg/ml considered resistant for both antibiotics (3). For the most commonly recom-

TABLE 4. Distributions of the disk inhibition zone diameters obtained for *H. influenzae* isolates

Disk diam (mm)	No. of isolates ^a							
	AMP (10 μg)		AMP (2 μg)		AMC (20/10 μg)		AMC (2/1 μg)	
	gBLNAS	gBLNAR	gBLNAS	gBLNAR	gBLNAS	gBLNAR	gBLNAS	gBLNAR
12–14								4
15–17				3				6
18–20				15			3	10
21–23			3	8			6	10
24–26			8	7			9	4
27–29		3			2			
30–32	1	16	10	1	2	7	6	
33–35	5	9	3		6	17	1	
36–38	12	6	1		10	6		
39–41	4				5	2		
	3				2			

^a AMP, ampicillin; AMC, amoxicillin-clavulanic acid. The rectangles indicate the overlapping zone, between gBLNAR and gBLNAS isolates.

TABLE 5. Microbiological criteria for the laboratory detection of gBLNAR and gBLPACR *H. influenzae*

Genotype population	Method	Antibiotic (μg) ^c	Proposed criterion ^d	Sensitivity (%)	Specificity (%)	% of tests having errors		Categorical agreement ^e (%)
						Major	Very major	
gBLNAR ^a	Microdilution	AMP	≥1	82.4	100	0	10.2	89.8
		AMX	≥1	100	92	3.4	0	96.6
		AMC	≥1	97	92	3.4	1.7	94.9
	E-test	AMP	≥1	55.9	100	0	25.4	74.6
		AMX	≥1	73.5	100	0	15.3	84.7
		AMC	≥1	58.8	92	3.4	23.7	72.9
	Disk (charge)	AMP (10)	≤32	82.4	76	10.2	10.2	79.7
		AMP (2)	≤23	76.5	88	5.1	13.6	81.4
		AMC (20/10)	≤32	68	76.5	13.6	13.6	72.9
		AMC (2/1)	≤20	58.8	88	5.1	23.7	71.2
gBLPACR ^b	Microdilution	AMC	≥2	93.3	97.8	1.7	1.7	96.7
	E-test	AMC	≥2	33.3	100	0	16.7	83.3

^a Calculated for gBLNAS and gBLNAR populations (*n* = 25 + 34 = 59).
^b Calculated for gBLNAS, gBLPAR, and gBLPACR populations (*n* = 25 + 20 + 15 = 60).
^c AMP, ampicillin; AMX, amoxicillin; AMC, amoxicillin-clavulanic acid.
^d MIC (μg/ml) or disk diameter (mm).
^e Categorical agreement indicates the percentage of isolates that fulfill each proposal.

mended amoxicillin doses in children, a PK/PD breakpoint of ≤2 μg/ml may indicate susceptibility and ≥4 μg/ml may indicate resistance (30). Our data suggest that all isolates with an amoxicillin MIC of 2 μg/ml are of the gBLNAR genotype and contain amino acid substitutions in PBP3, thus risking an eventual clinical failure, particularly in invasive infections.

Our data support in part the British Society for Antimicrobial Chemotherapy proposed breakpoints, although an important proportion of isolates with an amoxicillin MIC of 1 μg/ml would be gBLNAR. A way to reconcile PK/PD data and the presence of resistance mechanisms would be by establishing for the low-gBLNAR isolates a tentative proposal of an amoxicillin MIC of ≤0.5 μg/ml as susceptible (no resistance mech-

anisms; PK/PD data favorable), 1 to 2 μg/ml as intermediate (resistance mechanisms present but PK/PD data favorable), and ≥4 μg/ml as resistant (resistance mechanisms present and PK/PD data not favorable).

In summary, we have shown that detection of low-gBLNAR *H. influenzae* isolates presenting with amino acid substitutions at the PBP3 protein can be challenging, as they may overlap with the susceptible population (gBLNAS); however, our data indicate that testing amoxicillin by broth microdilution effectively separates gBLNAS and gBLNAR populations. Amoxicillin is the most common antibiotic used to treat community-acquired respiratory infections in the United States and Europe. Based on PK/PD data and the presence of PBP3

FIG. 3. Practical algorithm to detect four genotypes of aminopenicillin susceptibility in *H. influenzae* by the reference microdilution method. bla (+), β-lactamase positive; AMX, amoxicillin; AMC, amoxicillin-clavulanic acid.

mutations, an amoxicillin MIC of ≤ 0.5 $\mu\text{g/ml}$ indicates fully susceptible isolates, a MIC of 1 to 2 $\mu\text{g/ml}$ indicates intermediate isolates with PBP3 mutations for which special clinical precautions should be taken, and a MIC of ≥ 4 $\mu\text{g/ml}$ indicates resistant isolates for which amoxicillin should not be used.

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REFERENCES

- Alou, L., M. J. Giménez, D. Sevillano, L. Aguilar, N. González, O. Echevarría, M. Torrico, P. Coronel, and J. Prieto. 2007. Are β -lactam breakpoints adequate to define non-susceptibility for all *Haemophilus influenzae* resistance phenotypes from a pharmacodynamic point of view? *J. Antimicrob. Chemother.* **59**:652–657.
- Billal, D. S., M. Hotomi, and N. Yamanaka. 2007. Can the E-test correctly determine the MICs of β -lactam and cephalosporin antibiotics for β -lactamase-negative ampicillin-resistant *Haemophilus influenzae*? *Antimicrob. Agents Chemother.* **51**:3463–3464.
- British Society for Antimicrobial Chemotherapy. January 2008. BSAC methods for antimicrobial susceptibility testing, document version 7. British Society for Antimicrobial Chemotherapy, United Kingdom. http://www.bsac.org.uk/_db/_documents/version_7.pdf.
- Clinical and Laboratory Standards Institute. 2002. Performance standards for antimicrobial susceptibility testing, 11th informational supplement M100-S12. CLSI, Wayne, PA.
- Clinical and Laboratory Standards Institute. 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 7th ed. Approved standard M7-A7. CLSI, Wayne, PA.
- Clinical and Laboratory Standards Institute. 2007. Performance standards for antimicrobial susceptibility testing, 17th ed. Approved standard M100-S17. CLSI, Wayne, PA.
- 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.
- 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.
- Doern, G. V., G. S. Daum, and T. A. Tubert. 1987. Ampicillin disk diffusion susceptibility testing of *Haemophilus influenzae*. *J. Clin. Microbiol.* **25**:1675–1678.
- Doern, G. V., J. H. Jorgensen, C. Thornsberry, and H. Snapper. 1990. Disk diffusion susceptibility testing of *Haemophilus influenzae* using haemophilus test medium. *Eur. J. Clin. Microbiol. Infect. Dis.* **9**:329–336.
- Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenney, G. Sutton, W. FitzHugh, C. Fields, J. D. Gocayne, J. Scott, R. Shirley, L. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter. 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**:496–512.
- Fluit, A. C., A. Florijn, J. Verhoef, and D. Milatovic. 2005. Susceptibility of European β -lactamase-positive and -negative *Haemophilus influenzae* isolates from the periods 1997/1998 and 2002/2003. *J. Antimicrob. Chemother.* **56**:133–138.
- Fuchs, P. C., and A. L. Barry. 1994. Interpretative criteria for susceptibilities of *Haemophilus influenzae* to ampicillin, amoxicillin, and amoxicillin-clavulanic acid. *J. Clin. Microbiol.* **32**:2846–2850.
- 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.
- García-Cobos, S., J. Campos, E. Cercenado, F. Román, E. Lázaro, M. Pérez-Vázquez, F. de Abajo, and J. Oteo. Antibiotic resistance in *Haemophilus influenzae* decreased, except for β -lactamase-negative amoxicillin-resistant isolates, in parallel with community antibiotic consumption in Spain from 1997 to 2007. *Antimicrob. Agents Chemother.*, in press.
- García-de Lomas, J., M. Lerma, L. Cebrián, J. L. Juan-Bañón, P. Coronel, M. J. Jiménez, and L. Aguilar. 2007. Influence of *Haemophilus influenzae* β -lactamase production and/or *fisI* gene mutations on in vitro activity of and susceptibility rates to aminopenicillins and second- and third-generation cephalosporins. *Int. J. Antimicrob. Agents* **30**:190–192.
- Goossens, H., M. Ferech, S. Coenen, P. Stephens, and the European Surveillance of Antimicrobial Consumption Project Group. 2007. Comparison of outpatient systemic antibacterial use in 2004 in the United States and 27 European countries. *Clin. Infect. Dis.* **44**:1091–1095.
- Hasegawa, K., R. Kobayashi, E. Takada, A. Ono, N. Chiba, M. Morozumi, S. Iwata, K. Sunakawa, K. Ubukata, and the Working Group of the Nationwide Surveillance for Bacterial Meningitis. 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.
- 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.
- Heelan, J. S., D. Chesney, and G. Guadagno. 1992. Investigation of ampicillin-intermediate strains of *Haemophilus influenzae* by using the disk diffusion procedure and current National Committee for Clinical Laboratory Standards guidelines. *J. Clin. Microbiol.* **30**:1674–1677.
- 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.
- Jones, R. N., J. Hindler, G. V. Doren, C. Thornsberry, and E. H. Gerlach. 1992. The re-evaluation of the NCCLS ampicillin susceptibility tests with haemophilus test medium; new interpretative criteria for the disk diffusion method. *Antimicrob. Agents Chemother.* **35**:600–602.
- Jorgensen, J. H., J. S. Redding, L. A. Maher, and A. W. Howell. 1987. Improved medium for antimicrobial susceptibility testing of *Haemophilus influenzae*. *J. Clin. Microbiol.* **25**:2105–2113.
- Kärpänoja, P., A. Nissinen, P. Huovinen, and H. Sarkkinen. 2004. Disc diffusion susceptibility testing of *Haemophilus influenzae* by NCCLS methodology using low-strength ampicillin and co-amoxiclav discs. *J. Antimicrob. Chemother.* **53**:660–663.
- Mendelman, P. M., D. O. Chaffin, C. Clausen, T. L. Stull, C. Needham, J. D. Williams, and A. L. Smith. 1986. Failure to detect ampicillin-resistant, non- β -lactamase-producing *Haemophilus influenzae* by standard disk susceptibility testing. *Antimicrob. Agents Chemother.* **30**:274–280.
- Mendelman, P. M., E. A. Wiley, T. L. Stull, C. Clausen, D. O. Chaffin, and O. Onay. 1990. Problems with current recommendations for susceptibility testing of *Haemophilus influenzae*. *Antimicrob. Agents Chemother.* **34**:1480–1484.
- Molina, J. M., J. Cordoba, A. Monsoliu, N. Diosdado, and M. Gobernado. 2003. *Haemophilus influenzae* and β -lactam resistance: description of *bla* TEM gene deletion. *Rev. Esp. Quimioter.* **16**:195–203. (In Spanish.)
- Motulsky, H. 1995. Interpreting lab tests: introduction to Bayesian thinking, p. 129 to 136. Intuitive Biostatistics. Oxford University Press, Inc., New York, NY.
- O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shinger. 1972. Novel method for detection of β -lactamase by using a chromogenic cephalosporin substrate. *Antimicrob. Agents Chemother.* **1**:283–288.
- Sanbongi, Y., T. Suzuki, Y. Osaki, N. Senju, T. Ida, and K. Ubukata. 2006. Molecular evolution of β -lactam-resistant *Haemophilus influenzae*: 9-year surveillance of penicillin-binding protein 3 mutations in isolates from Japan. *Antimicrob. Agents Chemother.* **50**:2487–2492.
- Tristram, S., M. R. Jacobs, and P. C. Appelbaum. 2007. Antimicrobial resistance in *Haemophilus influenzae*. *Clin. Microbiol. Rev.* **20**:368–389.
- Tristram, S. G., R. Hawes, and J. Souprounov. 2005. Variation in selected regions of *bla*TEM genes and promoters in *Haemophilus influenzae*. *J. Antimicrob. Chemother.* **56**:481–484.
- Turnidge, J., and D. L. Paterson. 2007. Setting and revising antibacterial susceptibility breakpoints. *Clin. Microbiol. Rev.* **20**:391–408.
- Ubukata, K., Y. Shibasaki, K. Yamamoto, 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.
- Zerva, L., D. J. Biedenbach, and R. N. Jones. 1996. Reevaluation of interpretative criteria for *Haemophilus influenzae* by using meropenem (10-microgram), imipenem (10-microgram), and ampicillin (2- and 10-microgram) disks. *J. Clin. Microbiol.* **34**:1970–1974.