ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2009, p. 267–270 Vol. 53, No. 1
0066-4804/09/$08.00+0 doi:10.1128/AAC.00402-08

Genotypic versus Phenotypic Characterization, with Respect to β-Lactam Susceptibility, of *Haemophilus influenzae* Isolates Exhibiting Decreased Susceptibility to β-Lactam Resistance Markers▽

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Received 25 March 2008/Returned for modification 24 June 2008/Accepted 17 October 2008

Among 165 Spanish *Haemophilus influenzae* isolates with mutations in the *ftsI* gene (*ftsI*$^+$) (2005 to 2007), 73% were β-lactamase negative and 26.7% were positive. The proportion of β-lactamase-negative isolates to β-lactamase-positive isolates was 2:1 to 4:1 in general, versus 1:3 in pediatric hospitals. Among 44 β-lactamase-positive strains, 8 strains produced ROB-1 (5 from the pediatric hospital). β-Lactamase-positive *ftsI*$^+$ strains were phylogenetically closer than were β-lactamase-negative strains.

Since previous studies showed that ampicillin-susceptible β-lactamase-negative *Haemophilus influenzae* strains showing an ampicillin MIC of 1 μg/ml should be interpreted with caution because they may carry *ftsI* gene mutations (5), the presence of these mutations in β-lactamase-positive strains susceptible to amoxicillin-clavulanic acid exhibiting MICs of 2/1 to 4/2 μg/ml can be suspected. The aim of this study was to genotypically and phenotypically characterize (with respect to β-lactam susceptibility) *H. influenzae* isolates with ampicillin MIC of ≥1 μg/ml for β-lactamase-negative or amoxicillin-clavulanic acid MIC of ≥2/1 μg/ml for β-lactamase-positive strains. Spanish hospitals were contacted with a request for isolates with these susceptibility characteristics that were collected from March 2005 to March 2007. Six hospitals sent isolates that were retested in triplicate, and those showing the required or 1-dilution-lower modal MICs were included. Of the 252 strains received, 199 were recovered and exhibited the susceptibility requirements. Susceptibility to β-lactams was determined by microdilution (1). The susceptibility breakpoints considered were ≤1 μg/ml for ampicillin, ≤4/2 μg/ml for amoxicillin-clavulanic acid, ≤8 μg/ml for cefaclor, ≤4 μg/ml for cefuroxime, ≤1 μg/ml for cefdinir, and ≤2 μg/ml for cefotaxime (2). Nonsusceptibility was considered when MICs were above the susceptibility breakpoints. β-Lactamase production was determined by the chromogenic cephalosporin test (7).

For amplification and sequencing of the *ftsI* gene, DNA was obtained using the QIAamp DNA kit (Qiagen, Hilden, Germany). PCR amplification of the *ftsI*, *acrR*, bla_{TEM}, and bla_{ROB} genes was performed using referenced primers (6, 8, 9). Strains with mutations in the *ftsI* gene were genotypically defined as BLNAR (β-lactamase negative, ampicillin resistant) or BLPACR (β-lactamase positive, amoxicillin-clavulanic acid resistant) that, when possible, were classified into the groups and subgroups proposed by Dabernat et al. (3) and Ubukata et al. (9). The ClustalW2 program (http://www.ebi.ac.uk) was used to construct phylogenetic trees of a 1,030-bp sequence from the *ftsI* gene.

Of the 199 strains exhibiting the susceptibility requirements, amplification failed in three isolates excluded for further analysis (all were β-lactamase negative; two strains had an ampicillin MIC of 2 μg/ml and one of 1 μg/ml).

Of the 196 strains tested, 31 (15.8%) did not present mutation in the *ftsI* gene, 10 were β-lactamase negative, and 21 were β-lactamase positive by the chromogenic cephalosporin test. Of the 165 strains showing *ftsI* mutations, 121 (73%) were β-lactamase negative (BLNAR), and 44 (26.7%) were β-lactamase positive (BLPACR). The proportion of BLNAR to BLPACR strains was approximately 2:1 to 4:1 in all general hospitals, but the proportion was reversed (approximately 1:3) in the pediatric hospital (H. S. Joan de Deu) (Table 1).

Table 2 shows the MIC_{50}, MIC_{90}, and nonsusceptibility rates to the different antibiotics tested. Susceptibility problems with cefaclor, cefuroxime, and cefdinir are present even in strains without mutations in the *ftsI* gene, with nonsusceptibility rates of 20% to cefuroxime, 33% to 40% to cefdinir, and 40% to 76% to cefaclor. It is remarkable that among isolates without *ftsI* gene mutations, three β-lactamase-negative strains were nonsusceptible to ampicillin, and one β-lactamase-positive strain was nonsusceptible to amoxicillin-clavulanic acid.

Among the 21 β-lactamase-positive isolates without mutations in the *ftsI* gene, 19 (90.5%) isolates produced TEM-1, concomitantly with ROB-1 in two strains. The remaining two

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▽ Published ahead of print on 27 October 2008.

TABLE 1. Number of isolates exhibiting mutations in the *ftsI* gene, by institution

Center	No. of isolates with the *ftsI* gene sequenced	No. (%) of isolates with mutations in the *ftsI* gene		
		Total	β-Lactamase negative[a]	β-Lactamase positive[a]
H. General Univ. Gregorio Marañon	115	103 (89.6)	82 (79.6)	21 (20.4)
H. S. Joan de Deu	31	21 (67.7)	8 (26.6)	13 (73.4)
H. Clinic	26	22 (84.6)	18 (81.8)	4 (18.2)
H. Univ. Marques de Valdecilla	20	19 (95.0)	13 (68.4)	6 (31.6)
Other	4	0.0 (0)		
Total	196	165 (84.2)	121 (73.3)	44 (26.7)

[a] Percentage values in parentheses represent the proportion of the number of the indicated isolates to the total number of isolates with mutations in the *ftsI* gene.

strains were positive by the chromogenic cephalosporin test, but TEM and ROB β-lactamases were not detected. Among the 44 β-lactamase-positive isolates with mutations in the *ftsI* gene (BLPACR isolates), 37 (84.1%) strains produced TEM-1, and 8 (18.2%) produced ROB-1, with two strains (4.5%) producing both β-lactamases. The remaining strain was positive by the chromogenic cephalosporin test, but TEM and ROB β-lactamases were not detected. Of the eight BLPACR strains producing ROB-1 β-lactamase, five were from the pediatric hospital (H. S. Joan de Deu), representing 38.5% (5 out of 13) of the BLPACR strains from that center.

Nonsusceptibility rates in strains showing mutations in the *ftsI* gene were similar regardless of β-lactamase production, except in the case of amoxicillin-clavulanic acid, where nonsusceptibility rates (MIC ≥ 8/4 μg/ml; resistance, in this case, since no intermediate category is CLSI defined) increased from 9.9% in β-lactamase-negative strains with mutations in the *ftsI* gene (*ftsI*$^+$) to 25% in *ftsI*$^+$ β-lactamase-positive strains (Table 2). More than 50% of these *ftsI*$^+$ β-lactamase-positive strains (MIC range, 8/4 to 32/16 μg/ml) were from the pediatric hospital H. S. Joan de Deu. This hospital also showed the highest prevalence of β-lactamase production among *ftsI*$^+$ strains (13/31 [41.9%], versus 31/165 [18.8%] in general hospitals) (Table 1) and the highest prevalence of amoxicillin-clavulanic acid resistance (MIC ≥ 8/4 μg/ml) among *ftsI*$^+$ β-lactamase-positive strains (6/13 [46.2%], versus 5/31 [16.1%] in general hospitals). This may be related to different patterns of antibiotic consumption in children and adults. It has been suggested to be due to a relationship between amoxicillin-clavulanic acid consumption and the evolution of BLNAR strains (4), which can be extended to BLPACR strains and children, with higher amoxicillin-clavulanic acid consumption.

Cefotaxime exhibited MIC_{50}/MIC_{90}s of ≤0.06/≤0.5 μg/ml and cefditoren values of ≤0.06/≤0.06 μg/ml regardless the presence of *ftsI* mutations and/or β-lactamase production. Only one BLNAR strain was nonsusceptible to cefotaxime (cefotaxime MIC = 4 μg/ml, cefditoren MIC = 0.06 μg/ml).

Eleven strains presented changes that predicted early termination of the *acrR* reading frame. Of them, 10 strains had *ftsI* mutations (one of them is a β-lactamase producer). These strains, presumably hyperproducers of an AcrAB efflux pump, did not present higher ampicillin MICs, suggesting, as in previous studies (4), unrelatedness to high-level ampicillin resistance.

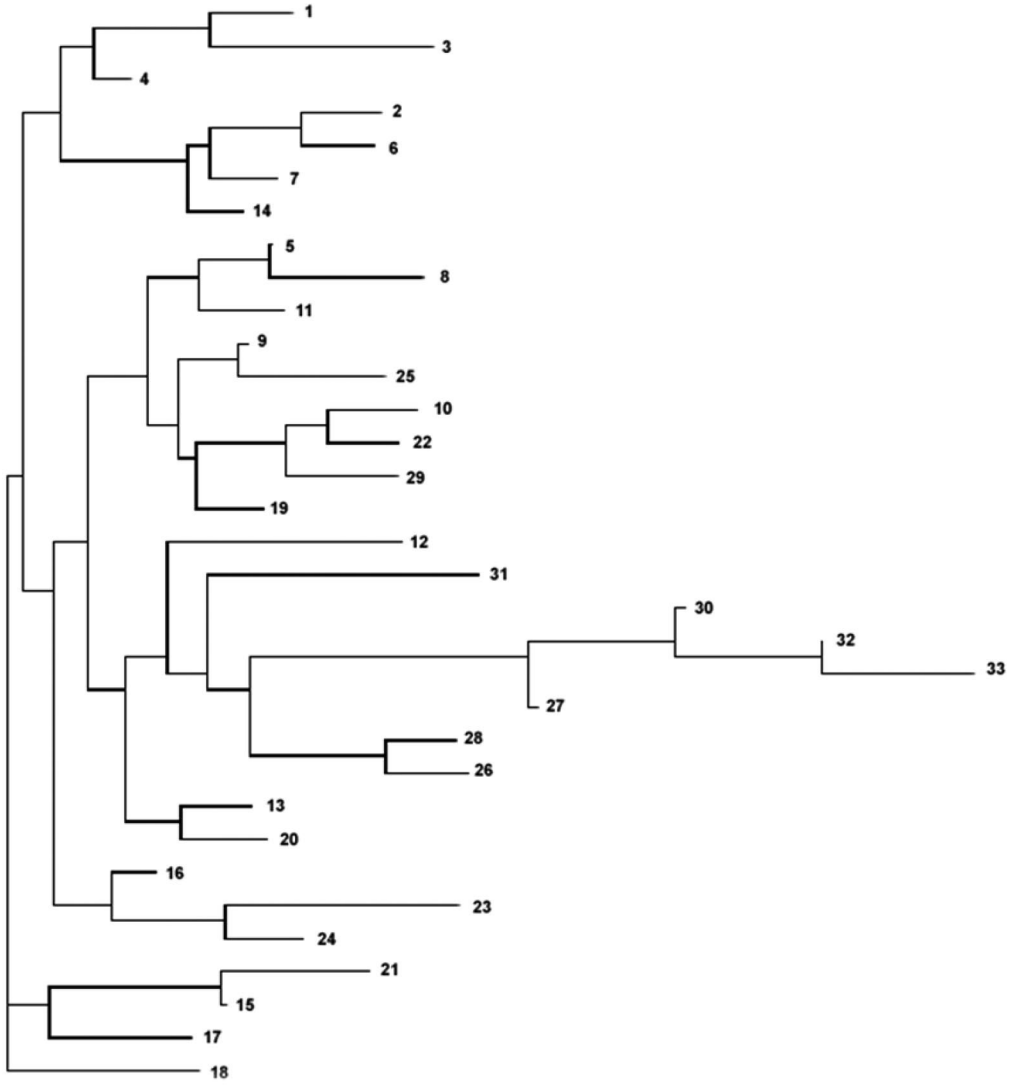
Figure 1 shows phylogenetic trees of a 1,030-bp sequence of the *ftsI* gene. The most frequent group was IIc in BLNAR (62/121 strains [51.2%]) and IIb in BLPACR (25/44 strains [56.8%]). Among BLPACR strains, the number of patterns of amino acid substitutions in the *ftsI* gene was 11, with 47% of isolates belonging to one single pattern (350N, 377I, 502V, 526K, 545I [phylogenetic no. 9]) that represented only 3% (phylogenetic no. 27) of BLNAR strains. Among BLNAR strains, the amino acid substitution profile 350N, 502T, 526K, 545I was the most prevalent pattern (27%) that was also represented in BLPACR strains (18%). BLPACR strains showed a closer phylogenetic relationship than did BLNAR strains, among which 33 patterns were found, only two of them (different than the prevalent pattern in BLPACR strains) showing >10% prevalence.

The study of the prevalence of resistance mechanisms (β-lactamase and *ftsI* gene mutations) to β-lactams in *H. influenzae* and its genetic relatedness may help to establish adequate therapeutic and preventive measures to counter their selection/diffusion.

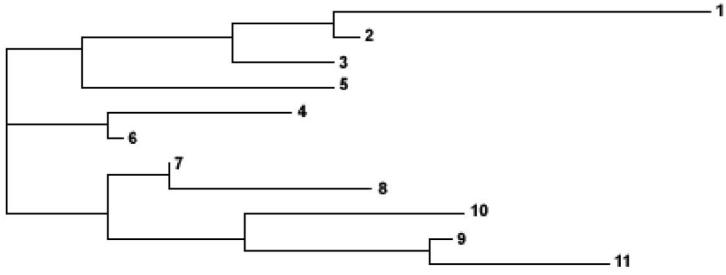
TABLE 2. Antimicrobial susceptibility of strains with (*ftsI*$^+$) and without mutations in the *ftsI* gene distributed by β-lactamase production

Antibiotic	Value (μg/ml) for strains without mutations						Value(μg/ml) for *ftsI*$^+$ strains					
	β-Lactamase negative (*n* = 10)			β-Lactamase positive (*n* = 21)			β-Lactamase negative (*n* = 121)			β-Lactamase positive (*n* = 44)		
	MIC_{50}	MIC_{90}	%NS	MIC_{50}	MIC_{90}	%NS	MIC_{50}	MIC_{90}	%NS	MIC_{50}	MIC_{90}	%NS
AMP	1	2	30.0	≥128	≥128	100	2	4	98.3	≥128	≥128	95.5
AMC	0.5	4	0.0	2	4	4.8	2	4	9.9	4	8	25.0
CEC	8	32	40.0	32	≥128	76.2	32	≥128	70.2	32	64	86.4
CXM	1	8	20.0	2	8	19	4	16	48.8	4	16	45.4
CDR	1	4	40.0	0.5	4	33.3	2	8	57.0	2	4	52.3
CTX	0.03	0.25	0.0	0.03	0.06	0.0	0.06	0.5	0.8	0.03	0.25	0.0
CDN	0.03	0.06	NA	0.03	0.06	NA	0.06	0.06	NA	0.03	0.06	NA

[a] Nonsusceptibility (NS) defined as MICs above susceptibility breakpoints. Susceptibility breakpoints (μg/ml) ≤1 μg/ml for ampicillin, ≤4/2 μg/ml for amoxicillin-clavulanic acid, ≤8 μg/ml for cefaclor, ≤4 μg/ml for cefuroxime, ≤1 μg/ml for cefdinir, and ≤2 μg/ml for cefotaxime (2). NA, no CLSI breakpoints available; AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CEC, cefaclor; CXM, cefuroxime; CDR, cefdinir; CTX, cefotaxime; CDN, cefditoren.



1; 350N (1/1%), **2**; 449V (1/1%), **3**; 517H (1/1%), **4**; 526K (4/3%), **5**; 502T-526K (7/6%), **6**; 449V- 545I (1/1%), **7**;449V- 526K (2/2%), **8**; 357A- 502T- 526K (1/1%), **9**; 502T-526K-545I (19/16%), **10**; 502T- 511L- 526K (1/1%), **11**; 350N-502T- 526K (4/3%), **12**; 377I-502V-526K (1/1%), **13**; 502V-526K-545I (1/1%), **14**; 449- 526K-545I (8/7%), **15**; 350N-490E-526K (1/1%), **16**; 350N-526K-545I (1/1%), **17**; 490E-526K-545I (1/1%), **18**; 526K-530S-545I (2/2%), **19**; 350N-502T-526K-545I (33/27%), **20**; 350N-502V-526K-545I (2/2%), **21**; 350N-490E-526K-530S (2/2%), **22**; 502T-511L-526K-545I (1/1%), **23**; 350N-437S-511L-526K (1/1%), **24**; 350N-437S-526K-545I (1/1%), **25**; 437S-502T-526K-545I (1/1%), **26**; 350N-377I-449V-526K (1/1%), **27**; 350N-377I-502V-526K-545I (4/3%), **28**; 350N-502T-511L-526K-545I (2/2%), **29**; 350N-357N-377I-385T-545I (1/1%), **30**; 350N-357N-377I-385T-517H-545I 4/3%, **31**; 350N-377I-490E-502V-526K-545I (3/2%), **32**; 350N-357N-377I-385T-517H- 530S-545I (7/6%), **33**; 350N-357N-377I-385T-388F-517H-530S-545I (1/1%)



1; 388V (1/2%), **2**; 545I (1/2%), **3**; 350N-545I (1/2%), **4**; 502T-526K (2/4%), **5**; 490E-526K-545I (1/2%), **6**; 502T-526K-545I (4/9%), **7**; 350N-502T-526K-545I (8/18%), **8**; 350N-357N-502T-526K-545I (1/2%), **9**; 350N-377I-502V-526K-545I (21/47%), **10**; 350N-437S-502V-526K-545I (2/4%), **11**; 350N-377I-490E-502V-526K-545I (2/4%)

FIG. 1. Phylogenetic trees of a 1,030-bp sequence from the *ftsI* gene of *ftsI*$^+$ β-lactamase-negative isolates (top tree) and of *ftsI*$^+$ β-lactamase-positive isolates (bottom tree). Phylogenetic numbers, amino acid substitutions, numbers and percentages of strains are shown below each phylogenetic tree.

L.M.-M. is supported by Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Spanish Network for the Research in Infectious Diseases (REIPI RD06/0008). This study was supported by an unrestricted grant from Tedec-Meiji Farma S.A., Madrid, Spain.

We thank M. Casal and J. L. Gómez-Garcés for participating in the study and M. Gimeno for her collaboration.

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