J Antimicrob Chemother
doi:10.1093/jac/dkq533

Journal of Antimicrobial Chemotherapy

Polymorphism in *ftsI* gene and β-lactam susceptibility in Portuguese *Haemophilus influenzae* strains: clonal dissemination of β-lactamase-positive isolates with decreased susceptibility to amoxicillin/clavulanic acid

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Received 1 September 2010; returned 19 September 2010; revised 20 December 2010; accepted 21 December 2010

Objectives: The aim of this study was to characterize ampicillin resistance mechanisms in clinical isolates of *Haemophilus influenzae* from Portugal. Association between specific patterns of amino acid substitutions in penicillin-binding protein 3 (PBP3) (with or without β-lactamase production) and β-lactam susceptibility as well as genetic relatedness among isolates were investigated.

Methods: Two-hundred and forty non-consecutive *H. influenzae* isolates chosen according to their different ampicillin MICs [101 β-lactamase-non-producing ampicillin-resistant (BLNAR) isolates, 80 β-lactamase-producing ampicillin-resistant (BLPAR) isolates and 59 β-lactamase-non-producing ampicillin-susceptible (BLNAS) isolates] were analysed. The β-lactamase-encoding bla_{TEM-1} gene was detected by PCR. The *ftsI* gene encoding PBP3 was sequenced. Genetic relatedness among isolates was examined by PFGE.

Results: Of the 240 *H. influenzae* isolates, 141 had mutations in the transpeptidase domain of the *ftsI* gene, including most BLNAR strains (94/101, 93.1%) and a high percentage of BLPAR strains (47/80, 58.8%). As previously reported, the latter have been described as β-lactamase-positive amoxicillin/clavulanic acid resistant (BLPACR). The most common amino acid substitutions were identified near the KTG motif: N526K (136/141, 96.5%), V547I (124/141, 87.9%) and N569S (121/141, 85.8%). The 141 strains were divided into 31 *ftsI* mutation patterns and included six groups (I, IIa, IIb, IIc, IId and III-like). BLNAR strains were genetically diverse but close genetic relationships were demonstrated among BLPACR strains.

Conclusions: This study shows that the non-enzymatic mechanism of resistance to β-lactams is widespread among *H. influenzae* isolates in Portugal. Clonal dissemination of BLPACR strains showing high resistance to ampicillin and reduced susceptibility to amoxicillin/clavulanic acid was documented.

Keywords: penicillin-binding proteins, ampicillin resistance, genetic relatedness, β-lactamases

Introduction

Haemophilus influenzae remains a key aetiological agent of upper and lower respiratory tract infections in both adults and children. Aminopenicillins have been extensively used in therapy for *H. influenzae* infection, but, since the beginning of the 1970s, ampicillin-resistant strains have emerged and spread. Two major mechanisms are involved in ampicillin resistance: the enzymatic mechanism, which consists of β-lactam hydrolysis due to the production of β-lactamase, either TEM-1 type or, rarely, ROB-1 type; and the non-enzymatic mechanism, i.e. decreased affinity of β-lactams for altered penicillin-binding proteins (PBPs). Strains exhibiting this latter mechanism of resistance are referred to as β-lactamase-non-producing ampicillin-resistant (BLNAR) strains and have been increasingly described worldwide.[1–4] Moreover, *H. influenzae* strains possessing both β-lactamase and altered PBPs have been reported. Since the presence of both mechanisms in the same strain may result in a phenotype characterized by resistance or decreased susceptibility to amoxicillin/clavulanic acid, such strains are defined as β-lactamase-positive amoxicillin/clavulanic acid-resistant (BLPACR) strains[3–5] and seem to be increasing after their first description in the USA.[6]

Multiple mutations have been described in the *ftsI* gene among strains classified as BLNAR based on their phenotype. The amino acid substitutions near the conserved motifs SSN (S379SN) and KTG (K512TG) within the transpeptidase domain of PBP3 appear to be mainly responsible for resistance. According to the presence/absence of specific substitutions, BLNAR strains have been classified into three major mutational groups (I to III).[7] Strains with decreased ampicillin susceptibility (MICs $\geq$ 1 mg/L) were commonly found in groups I and II, while isolates belonging to group III were normally associated with high resistance levels to ampicillin, as well as cephalosporins.[3,7–9] In 2002, this classification scheme was partially modified when, according to the criteria developed by Dabernat *et al.*,[10] group II was further divided into four subgroups: subgroups IIa to IId. To our knowledge, group III strains have not been found in Europe until now. More recently, García-Cobos *et al.*[5] described a new group called 'III-like' with mutations at the KTG and STVK motifs, additional to the ones described in group III in Japan[7] with mutations only at the SSN motif. The resistance of *H. influenzae* to β-lactam antibiotics has important clinical implications since empirical antimicrobial therapy is normally established in patients with respiratory tract infections. The aim of this study was to characterize ampicillin resistance mechanisms in clinical strains of *H. influenzae* isolated in Portugal. In particular, associations between specific patterns of amino acid substitutions in PBP3 (with or without β-lactamase production) and susceptibility to the different β-lactam antibiotics were determined. Finally, genetic relatedness among strains showing the BLNAR and BLPACR genotypes (for convenience designated gBLNAR and gBLPACR) was investigated.

Materials and methods

Clinical isolates

A total of 240 non-consecutive clinical strains of *H. influenzae* isolated in Portugal between 2001 and 2008 were included in this study. Hospital laboratories sent *H. influenzae* isolates to the reference laboratory on a voluntary basis and irrespective of their antibiotic susceptibility pattern. Out of 5536 *H. influenzae* isolates collected during the study period, 2160 were screened for antibiotic susceptibility. Among the latter, 270 isolates were found to be non-β-lactamase producers with ampicillin MICs $\geq$1 mg/L (BLNAR), 523 isolates were β-lactamase producers [β-lactamase-producing ampicillin-resistant (BLPAR)] and the remaining 1367 isolates were ampicillin susceptible with ampicillin MICs $\leq$0.5 mg/L [β-lactamase-non-producing ampicillin-susceptible (BLNAS)]. The 240 *H. influenzae* isolates used in the present study were randomly chosen from the three groups of BLNAR, BLPAR and BLNAS isolates as follows: 101 BLNAR (101/270, 37.4%), 80 BLPAR (80/523, 15.3%) and 59 BLNAS. The age of patients was known in 235 cases; 151 isolates were from children ($\leq$18 years old) and 84 were from adult patients. Most of the isolates (166/240, 69.2%) were from patients with respiratory tract infections, 43 (17.9%) isolates were responsible for conjunctivitis, 11 (4.6%) for otitis, 9 (3.7%) for invasive infections and the remaining 11 (4.6%) for other kinds of infections. Bacteria were grown on chocolate agar plates supplemented with Isovitalex (bioMérieux, Portugal) and incubated for 18–24 h at 35 $\pm$ 1°C in 5% CO_2.

Antimicrobial susceptibility testing

β-Lactamase production was determined by the chromogenic cephalosporin assay with nitrocefin as substrate (Oxoid Limited, UK). For each isolate, MICs of ampicillin, amoxicillin/clavulanic acid (2:1 ratio), cefaclor, cefuroxime, cefotaxime, cefepime and meropenem were determined by the broth microdilution method (Siemens Healthcare Diagnostics Inc., UK) in *Haemophilus* test medium according to the CLSI guidelines and interpreted according to the established CLSI breakpoints for *H. influenzae*.[11] Quality control strains used for MIC testing were ATCC 49247 (BLNAR), ATCC 10211 (BLNAS) and NCTC 11315 (BLPAR).

PCR amplification and gene sequencing

The presence of the β-lactamase-encoding bla_{TEM-1} gene was investigated in all the β-lactamase-producing isolates by PCR amplification of a segment of 1091 bp using primers and conditions previously described.[12]

Alterations in PBP3 were investigated in all 240 *H. influenzae* isolates included in this study by sequencing the region of the *ftsI* gene encoding the transpeptidase domain of PBP3. The *ftsI* gene was first amplified by PCR using primers and conditions previously described.[13] The resulting PCR product was then purified with a PCR purification kit (Exosap, USB Corporation, Cleveland, OH, USA) and the sequence of the *ftsI* gene portion encoding the transpeptidase domain (between 1198680 and 1199553, which corresponds to nucleotides 840–1713 in the *ftsI* gene and amino acids 280–571 in PBP3) was determined by direct sequencing with the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Porto, Portugal) with an ABI 3130 XL sequencer (Applied Biosystems), using primers and PCR conditions previously described.[13] Both strands were sequenced. The *ftsI* sequences were analysed by comparison with those from the *H. influenzae* RD KW20 (accession number NC_000907).

Nucleotide sequence accession numbers

The sequences of the 31 different mutation patterns of the *ftsI* gene reported in this paper (Table 2) have been submitted to the EMBL Nucleotide Sequence Database. The accession numbers of the *ftsI* gene regions encompassing the transpeptidase domain of PBP3 are FR748003 to FR748033.

PFGE analysis

Genetic relatedness among 74 *H. influenzae* isolates representative of the different *ftsI* mutational groups was examined by PFGE after digestion of DNAs with SmaI restriction enzyme, following procedures previously described.[14] The isolates included in PFGE analysis were distributed as follows: 43 were selected among 94 (45.7%) gBLNAR strains (27 from mutational subgroup IIb, 9 from IIc, 5 from IId and 2 from III-like) and 31 among 47 (66%) gBLPACR strains (26 from mutational subgroup IIb and 5 from IIc). These strains were not epidemiologically related since they were isolated from different hospitals in geographically distinct regions. PFGE patterns were analysed using Dice coefficients of similarity with a tolerance level of 1.1% and the dendrogram was generated by the unweighted pair group method with arithmetic means (UPGMA) using BioNumerics software (Applied Maths, Kortrijk, Belgium). Isolates with a Dice band-based similarity coefficient value of $\geq$80.0% were considered to belong to the same cluster, which indicates a two- to three-fragment difference in gels with an average of 12 bands.[15]

Results

Antimicrobial susceptibility

Table 1 shows the MIC_{50}, MIC_{90}, MIC range and susceptibility categories of β-lactam antibiotics for the 240 *H. influenzae* isolates classified into the four different genotypes (gBLNAS, gBLPAR,

Table 1. Susceptibilities to β-lactam antibiotics of 240 *H. influenzae* isolates grouped in genotypes based on the presence/absence of *ftsI* mutations

		MIC (mg/L)			Susceptibility class (%)		
Antibiotic	Genotype[a]	MIC_{50}	MIC_{90}	MIC range	S	I	R
Ampicillin	gBLNAS	0.25	1	0.12–2	100	0	0
	gBLPAR (TEM-1)	64	256	4–512	0	0	100
	gBLPACR	128	512	8 to >512	0	0	100
	gBLNAR	2	2	1–8	42.6	52.1	5.3
Amoxicillin/clavulanic acid	gBLNAS	0.5	1	≤0.25–4	100	0	0
	gBLPAR (TEM-1)	2	2	0.5–4	100	0	0
	gBLPACR	2	4	1–4	100	0	0
	gBLNAR	2	4	≤0.25–4	100	0	0
Cefepime	gBLNAS	≤0.12	0.25	≤0.12–0.5	100	0	0
	gBLPAR (TEM-1)	≤0.12	≤0.12	≤0.12–0.5	100	0	0
	gBLPACR	≤0.12	0.25	≤0.12–1	100	0	0
	gBLNAR	0.25	0.5	≤0.12–1	100	0	0
Cefotaxime	gBLNAS	≤0.03	0.06	≤0.03–0.12	100	0	0
	gBLPAR (TEM-1)	≤0.03	≤0.03	≤0.03–0.06	100	0	0
	gBLPACR	≤0.03	0.06	≤0.03–0.25	100	0	0
	gBLNAR	0.06	0.12	≤0.03–1	100	0	0
Cefuroxime	gBLNAS	0.5	4	≤0.25 to >8	92.4	4.6	3
	gBLPAR (TEM-1)	0.5	2	≤0.25 to >8	97	0	3
	gBLPACR	2	4	1 to >8	97.9	0	2.1
	gBLNAR	4	>8	≤0.25 to >8	67	18.1	14.9
Cefaclor	gBLNAS	2	16	≤1 to >16	87.8	6.1	6.1
	gBLPAR (TEM-1)	8	>16	≤1 to >16	72.7	12.1	15.2
	gBLPACR	8	>16	8 to >16	57.4	29.8	12.8
	gBLNAR	16	>16	≤1 to >16	45.8	28.7	25.5
Meropenem	gBLNAS	≤0.06	0.12	≤0.06–0.25	100	0	0
	gBLPAR (TEM-1)	≤0.06	0.12	≤0.06–0.25	100	0	0
	gBLPACR	0.12	0.25	≤0.06-0.5	100	0	0
	gBLNAR	0.25	0.5	≤0.06–2	92.6	0	7.4[b]

S, susceptible; I, intermediate; R, resistant.
[a]Classification based on mutations characterized in *ftsI* gene: gBLNAS ($n=66$), strains without mutations; gBLPAR (TEM-1) ($n=33$), β-lactamase-producing strains (TEM-1) lacking mutations; gBLPACR ($n=47$), β-lactamase-producing strains with mutations, classified into groups I and II; gBLNAR ($n=94$), strains with ampicillin MIC ≥1 mg/L and with mutations, classified into groups I, II and III-like.
[b]Percentage of meropenem non-susceptible strains.

gBLPACR and gBLNAR), according to the presence/absence of *ftsI* gene mutations. Comparing gBLNAS and gBLNAR isolates, MIC_{50} values of the latter were four to eight times higher for all antibiotics tested, with the exception of cefepime and cefotaxime, where there was only a slight increase. However, only 57.4% of the gBLNAR isolates were non-susceptible to ampicillin, since 42.6% of isolates with this genotype presented an ampicillin MIC of 1 mg/L, a value categorized as susceptible according to CLSI breakpoints.[11] Analysing ampicillin MICs, gBLPACR isolates showed higher MIC values ($MIC_{50}=128$ mg/L and $MIC_{90}=512$ mg/L) than both gBLPAR ($MIC_{50}=64$ mg/L and $MIC_{90}=256$ mg/L) and gBLNAR (MIC_{50} and $MIC_{90}=2$ mg/L). In relation to amoxicillin/clavulanic acid, gBLPAR (MIC_{50} and $MIC_{90}=2$ mg/L) as well as gBLPACR and gBLNAR (MIC_{50} and $MIC_{90}=2$ and 4 mg/L, respectively) isolates showed decreased susceptibility to this antibiotic for the three classes, when compared with gBLNAS.

No strains were found to be resistant to cefotaxime or cefepime in any of the genotype classes; however, a few *H. influenzae* isolates showed decreased susceptibility to these antibiotics when compared with the susceptible population: two gBLNAR isolates with a cefotaxime MIC of 1 mg/L and six isolates (five gBLNAR and one gBLPACR) with a cefepime MIC of 1 mg/L. Cefuroxime and cefaclor showed the highest non-susceptibility rates (I+R), against gBLNAR isolates: 33% and 54.2%, respectively. However, cefaclor also exhibited reduced activity against gBLPAR and gBLPACR, with non-susceptibility rates of 27.3% and 42.6%, respectively. The MIC_{50} and MIC_{90} values of meropenem were both four times higher for gBLNAR isolates compared with gBLNAS. Seven gBLNAR isolates (7/94, 7.4%) were found to be non-susceptible to meropenem; of these, six had an MIC of 1 mg/L and one had an MIC of 2 mg/L. All the 80 BLPAR isolates harboured the bla_{TEM-1} gene, as detected by PCR amplification; although there is a description of

rare *H. influenzae* strains harbouring both enzymes,[16] we did not look for the presence of ROB type β-lactamase.

Mutation patterns in ftsI gene

Of the 240 *H. influenzae* isolates analysed, 141 presented mutations in the transpeptidase domain of the *ftsI* gene that have previously been shown to be associated with characterization of groups and subgroups already described (Table 2). Of note, 27 of the 59 BLNAS isolates presented point mutations in the *ftsI* gene, but these have not been previously associated with any resistance mechanism to β-lactams. The 141 isolates included 94 gBLNAR out of 101 BLNAR strains (93.1%) and 47 gBLPACR out of 80 BLPAR strains (58.8%). Therefore, seven isolates that exhibited a BLNAR phenotype did not present mutations in the *ftsI* gene and were classified as gBLNAS according to their genotype. In these seven isolates, the possible presence of other mechanisms affecting ampicillin susceptibility, such as overexpression of the AcrAB efflux pump or alterations in outer membrane protein 2 (OMP2), cannot be ruled out. The 47 BLPAR isolates found to possess both the bla_{TEM1} gene and mutations in the *ftsI* gene were re-designated as gBLPACR, according to previous studies.[3,6,17] Overall, the most frequent amino acid substitutions were identified near the KTG motif, as follows: N526K (136/141, 96.5%), V547I (124/141, 87.9%) and N569S (121/141, 85.8%). The 141 strains were then divided into 31 different *ftsI* mutation patterns, which included the six groups previously defined[5,10] (I, IIa to IId and III-like) (Table 2). The substitution N526K was shared by all the group II strains, which were divided into different subgroups according to the presence/absence of additional substitutions. In particular, 8 strains were classified as subgroup IIa (8/136, 5.9%), 96 isolates were classified as subgroup IIb (96/136, 70.6%), 23 belonged to subgroup IIc (23/136, 16.9%) and 9 to subgroup IId (9/136, 6.6%). Three strains (3/141, 2.1%) with the R517H amino acid substitution were classified as group I, whereas two strains (2/141, 1.4%) presented a pattern suggestive of the previously described group III-like,[5] characterized by the following amino acid substitutions: M377I and S385T in the SSN motif; R517H and T532S in the KTG motif; D350N in the STVK motif and S357N.

Associations between specific substitutions in PBP3 and antimicrobial susceptibility

Among gBLNAR strains, higher ampicillin MICs have been previously associated with group III-like isolates due to the unique patterns of amino acid substitutions exhibited by these strains.[5] However, in this study only one of the two strains belonging to the III-like group showed a particularly high ampicillin MIC (8 mg/L), while for the other this value was 1 mg/L. An association between reduced susceptibility/resistance to ampicillin and the presence of V547I and N569S substitutions, which were present in most strains belonging to the different subgroups of group II, could be hypothesized in comparison with group I isolates lacking the above substitutions (MIC_{90}=128 mg/L and 1 mg/L for group II and I isolates, respectively). Concerning susceptibility to cefaclor, our gBLNAR isolates with both V547I and N569S substitutions presented MICs between 8 and >16 mg/L, but the remaining group I strains (without either of these mutations but showing the R517H substitution) presented similar MICs to this antibiotic (MIC range 8–16 mg/L), suggesting that cefaclor susceptibility may be affected by V547I and N569S substitutions as well as by R517H. In relation to V511A substitution, we only have two strains carrying it in our study (one gBLNAR and one gBLPACR) (Table 2). In agreement with previous data,[18] there may be an association between V511A substitution and both resistance to ampicillin and reduced susceptibility to cefepime since the corresponding MICs were particularly high for both strains carrying this substitution (ampicillin MIC=8 mg/L and 512 mg/L for the gBLNAR and the gBLPACR isolate, respectively; cefepime MIC=1 mg/L for both strains). Moreover, both strains carrying the V511A substitution also showed reduced susceptibility (MIC=4 mg/L) to amoxicillin/clavulanic acid, suggesting a possible role of the above substitution in susceptibility to this antibiotic, although the number of strains carrying the V511A substitution was too low to confirm this.

Several studies[3–5] suggest there is an association between substitutions in the SSN motif (M377I, S385T) and decreased susceptibility to cephalosporins; however, analysis of our data indicated that only one of the eight isolates with decreased susceptibility to cephalosporins (MICs≥1 mg/L) showed the presence of one (specifically M377I) of the two substitutions. In general, the S385T substitution was rare among strains isolated in Portugal. Both M377I and S385T substitutions were exhibited by only two isolates belonging to group III-like, but neither isolate showed decreased susceptibility to cephalosporins. These findings suggest that substitutions other than M377I and S385T might also affect susceptibility to cephalosporins and that the pattern of PBP3 substitutions involved in susceptibility to this class of antibiotics might vary in different geographic settings. Concerning meropenem, most non-susceptible strains (MIC=1 mg/L or MIC=2 mg/L) shared a pattern of amino acid substitutions that included D350N, A502V/T, N526K, V547I and N569S, with the exception of the one group III-like isolate that exhibited only D350N and V547I. A similar pattern of substitutions has been previously found associated with imipenem resistance.[13] Although Kim *et al.*[19] reported that altered PBP3 does not significantly affect resistance to this antibiotic, previous data from some authors of the present study[13] demonstrated that heterogeneous resistance to imipenem depends largely on PBP3 amino acid substitutions, though other mechanisms may also be involved in the heterogeneous expression of such resistance.

PFGE analysis

The 74 *H. influenzae* isolates were distributed into 30 different PFGE patterns; 17 patterns contained individual isolates, but 12 patterns included two to five genetically related isolates (>80% identity) (Figure S1 and Table S1, both available as Supplementary data at *JAC* Online). In particular, one pattern (pattern 10; Figure 1) constituted a major cluster with 18 gBLPACR isolates that had ampicillin MICs ranging from 32 mg/L to 256 mg/L, all belonging to the *ftsI* mutation subgroup IIb and sharing the following substitution pattern: D350N, M377I, A502V, N526K, V547I and N569S. These clonal strains had been isolated in different hospitals between 2005 and 2008 and did not have any epidemiological relationship. The remaining 13 gBLPACR strains were distributed into nine different PFGE patterns, of which three were found in more than one strain

Table 2. Amino acid substitutions identified in the transpeptidase domain of the *ftsI* gene

	No. of isolates[b]		Amino acid substitution																					
							near SSN motif								near KTG motif									
Group[a]	Bla−	Bla+	Ala318	Asp350	Ser357	Ala368	Met377	Ser385	Ala437	Ile449	Leu456	Gly490	Ala502	Val511	Arg517	Asn526	Val529	Ala530	Thr532	Tyr538	Val547	Asp551	Ala554	Asn569
I	1														His									
	2														His								Val	
IIa	2	1		Asn								Glu				Lys		Ser						
	1			Asn			Ile									Lys						Gly		
	2															Lys						Gly		
	1															Lys		Ser			Ile			
	1															Lys								
IIb	1												Val			Lys								
		1		Asn			Ile					Glu	Val			Lys								
	2			Asn			Ile						Val			Lys					Ile	Asn		Ser
	5	9		Asn			Ile					Glu	Val			Lys					Ile			Ser
	2			Asn					Ser				Val			Lys					Ile			Ser
	20	22		Asn			Ile						Val			Lys					Ile			Ser
	1		Thr	Asn			Ile						Val			Lys					Ile			Ser
	1										Pro	Glu	Val			Lys					Ile			Ser
	9	1										Glu	Val			Lys					Ile			Ser
	1			Asn	Asn								Val	Ala		Lys					Ile			Ser
		1		Asn									Val	Ala		Lys					Ile			Ser
	1			Asn			Ile						Val			Lys	Met				Ile			Ser
	5	4		Asn									Val			Lys					Ile			Ser
	1	2					Ile						Val			Lys					Ile			Ser
	6												Val			Lys					Ile			Ser
IIc	3												Thr			Lys								
		1		Asn			Ile						Thr			Lys					Ile			Ser
	2			Asn									Thr			Lys								
	6	4		Asn									Thr			Lys					Ile			Ser
	6	1											Thr			Lys					Ile			Ser
IId	2			Asn					Ser	Val						Lys					Ile			Ser
	1									Val			Thr			Lys					Ile			Ser
	7									Val						Lys					Ile			Ser
III-like	2			Asn	Asn		Ile	Thr							His				Ser		Ile			
Miscellaneous[c]	27																							

[a]The strains with mutations in the *ftsI* gene were classified into six groups: I and II (a, b, c and d) according to Dabernat *et al.*;[10] and III-like according to García-Cobos *et al.*[5]
[b]Bla+, β-lactamase-producing strains; Bla−: β-lactamase-non-producing strains.
[c]Twenty-seven isolates with MICs ≤0.5 mg/L, with point mutations in the *ftsI* gene, not related to β-lactam resistance.

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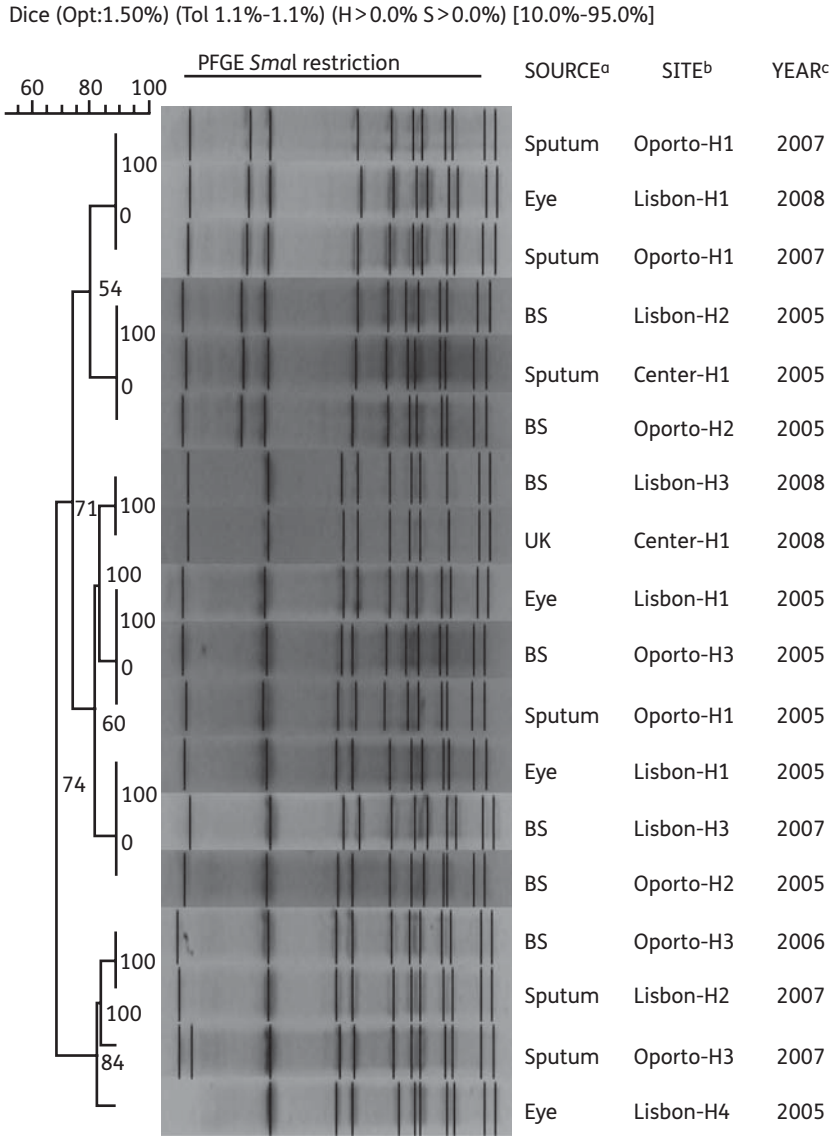

Figure 1. PFGE analysis dendrogram, according to Dice's similarity index (above the dendrogram), of cluster 10, showing the genetic relatedness among 18 gBLPACR *H. influenzae* strains. [a]BS, bronchial secretions; UK, unknown. [b]H1–H4 are different hospitals in the same geographic region. [c]Year of isolation. Characteristics of strains are presented in Table S1.

(patterns 2, 13 and 30). The gBLNAR strains belonging to the *ftsI* mutation subgroups IIb and IIc were widely disseminated throughout the dendrogram, although some small clusters (four or more strains) of closely related isolates were identified (clusters 1, 3, 26, 27 and 29). In particular, cluster 1 included five gBLNAR strains, three from subgroup IIb (sharing the same substitution profile: D350N, M377I, A502V, N526K, V547I and N569S) and two from subgroup IIc (D350N, A502T, N526K, V547I and N569S), with ampicillin MICs between 1 and 2 mg/L, isolated from different hospitals in the years 2007 and 2008, cluster 3 included four gBLNAR isolates from diverse subgroups (IIb, IIc and IId) with ampicillin MICs of 1 mg/L and isolated from different hospitals in 2007 and 2008, and cluster 26 included five strains from subgroup IIb with ampicillin MICs of 2 mg/L and sharing the same mutational profile (G490E, A502V, N526K, V547I and N569S); these five strains were isolated from hospitals all located in the region of Lisbon during the years 2004 and 2006. Cluster 27 comprised four subgroup IIb strains with ampicillin MICs of 1 mg/L (one strain) or 2 mg/L (three strains) that shared the substitution profile D350N, M377I, A502V, N526K, V547I and N569S. Cluster 29, which included four strains from subgroup IIb with ampicillin MICs of 1 mg/L (two strains) or 2 mg/L (two strains), was characterized by the same substitution profile as cluster 27, with the exception of only one strain without the substitution D350N. Clusters 27 and 29 both included strains isolated in the region of Lisbon during 2004–06 and 2002–07, respectively. Finally, the two gBLNAR strains belonging to the III-like *ftsI* mutational group appeared genetically totally unrelated to each other (clusters 8 and 17).

Discussion

This is the first study performed in Portugal concerning non-enzymatic resistance to ampicillin in *H. influenzae*. Our results demonstrate the emergence and dissemination of such a resistance mechanism among *H. influenzae* isolates from Portugal, since a high percentage of β-lactamase-negative strains (94/101, 93.1%) exhibiting reduced susceptibility or resistance to ampicillin were found to carry mutations in the *ftsI* gene. Notably, although only five BLNAR strains with *ftsI* mutations (gBLNAR) were fully resistant to ampicillin (MICs $\geq$ 4 mg/L), thus being categorized as resistant according to CLSI[11] breakpoints, most other gBLNAR strains (89/94, 94.7%) would be classified as susceptible (MIC = 1 mg/L) or with intermediate resistance to ampicillin (MIC = 2 mg/L). This result supports the need to redefine the breakpoints for resistance to ampicillin, as already proposed by others,[20–24] to allow efficient detection and characterization of BLNAR strains. On the other hand, in this study seven isolates considered BLNAR according to the phenotype did not have any mutation of the *ftsI* gene and were categorized as gBLNAS based on the genotype. From our point of view, regulatory bodies should offer some guidance on the laboratory definition of BLNAR strains. Whether BLNAR strains should be defined based on phenotype or genotype has yet to be agreed. In the routine practice of clinical microbiology laboratories, definition based on genotype may be difficult to address, therefore highlighting the importance of clearly defined susceptibility interpretation criteria for detection of BLNAR.

In the literature, *ftsI* mutations have also been found in BLPAR strains which are designated as gBLPACR.[17] Since their first description in USA[6] until now, only a few gBLPACR strains have been characterized worldwide,[2,16,17,25–28] with the exception of France,[10] with 13.9% of strains of this genotype isolated between 1999 and 2000, and Spain, where 26.7% of gBLPACR strains have recently been reported.[29] In this study, we unexpectedly found a high percentage of gBLPACR strains among our BLPAR strains (47/80, 58.8%). A combination of β-lactamase production and the presence of altered PBP3 resulted in higher ampicillin MICs compared with those obtained for BLPAR and gBLNAR strains. All BLPAR strains we characterized possessed a typical TEM-1 type β-lactamase. We did not investigate TEM gene subtypes and characterize the promoters. Since it is well known[23,30] that differences in the TEM-1 promoter sequences can affect β-lactamase expression, and therefore the level of β-lactam resistance, we cannot rule out the possibility that some differences in the resistance levels observed may be related also to differences in the promoter region.

Using a classification scheme based upon mutations of the *ftsI* gene, we were able to categorize gBLNAR and gBLPACR strains into the previously described groups and subgroups,[7,10] with the majority of strains (96.5%) being classified into group II. Of note, two strains were classified as group III-like, as defined by others.[5] Interestingly, until now there have been no reports of group III strains in Europe.[5,10] The analysis of clonality shows that the 74 *H. influenzae* strains analysed were dispersed into numerous different PFGE patterns, in agreement with the genetic heterogeneity of non-capsulated *H. influenzae*.[31] Contrary to Karlowsky *et al.*[32] and as already described by others,[9,10,33] we did not find close genetic relationships among the gBLNAR strains, although small clusters were observed. In this study, the existence of genotypically diverse strains harbouring identical *ftsI* genes might suggest the occurrence of genetic transfer of this gene in *H. influenzae*. The molecular evolution of gBLNAR strains may be explained by two hypotheses: point mutations in the *ftsI* gene due to antibiotic pressure or homologous recombination from related species,[34] as occurs in other organisms.[35]

As opposed to gBLNAR genetic diversity, we would like to stress the close genetic relationships occurring among the majority of the gBLPACR strains analysed (18/31, 58%, cluster 10). Our findings confirmed previous data obtained by Sevillano *et al.*,[29] which demonstrated closer phylogenetic relationships among gBLPACR than among gBLNAR strains.

This study has some limitations. First, our data are not representative of the whole country. The sample of strains was chosen according to the MICs of ampicillin and not all were consecutive. Second, there is a lack of clinical data on patients, such as specific information about previous treatment with β-lactam antibiotics, and no data on β-lactam antibiotic consumption in Portugal. Thus, we were unable to establish relationships between treatment/consumption and the resistance status of strains. Finally, the possible presence of mechanisms other than PBP3 mutations affecting susceptibility to β-lactam, such as overexpression of the AcrAB efflux pump, was not investigated either.

In conclusion, our results are indicative of wide dissemination of a non-enzymatic mechanism of resistance to β-lactams among *H. influenzae* isolates circulating in our country, which is a matter of concern. Although most gBLNAR strains were found to be genetically diverse, clonal dissemination of gBLPACR strains highly resistant to ampicillin and with reduced susceptibility to amoxicillin/clavulanic acid was documented. Further studies are required to clarify the clinical significance of gBLNAR isolates showing decreased susceptibility towards ampicillin and amoxicillin/clavulanic acid. A better understanding of this issue may help in establishing adequate therapeutic and preventive measures to avoid selection or dissemination of such strains. The inappropriate use of oral antibiotics may be responsible for the selection of this new resistance trait. We would like to emphasize the importance of continuing surveillance studies as essential tools to define trends in the antimicrobial resistance of *H. influenzae*.

Acknowledgements

We thank Vera Manageiro and Deolinda Louro, National Reference Laboratory of Antimicrobial Resistance, National Institute of Health, Lisbon, Portugal, for aid in dendrogram construction and for maintenance of the *H. influenzae* collection, respectively. We are grateful to Tonino Sofia, Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Rome, Italy, for editorial assistance.

Funding

This work was supported by National Institute of Health, Lisbon, Portugal. M. P. B.-L. spent 2 weeks in Istituto Superiore di Sanità, financed by a

research grant from Fundação Calouste Gulbenkian, Portugal (grant no. 21-90222).

Transparency declarations

None to declare.

Supplementary data

Figure S1 and Table S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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