ORIGINAL ARTICLE

Polymorphism of *ftsI* gene in *Haemophilus influenzae* and emergence of cefotaxime resistance in two Tunisian hospitals

S. Ferjani[1], I. Sassi[2], M. Saidani[1,2], E. Mhiri[3], A. Ghariani[3], I. Boutiba Ben Boubaker[1,2], L. Slim[3] and S. Amine[1,2]

1) *University of Tunis El Manar, Faculty of Medicine of Tunis, LR99ES09 Laboratory of Research 'Resistance to Antimicrobial Agents,* 2) *Charles Nicolle Hospital, Laboratory of Microbiology, Tunis and* 3) *Abderrahmen Mami Hospital, Laboratory of Microbiology, Ariana, Tunisia*

Abstract

The decreased affinity to β-lactams *in Haemophilus influenzae* is usually caused by specific alterations in penicillin-binding protein 3 due to varieties of substitutions in *ftsI* gene. This study aimed to characterize the polymorphism of *ftsI* gene in 19 *H. influenzae* strains, isolated between 2014 and 2016 (different resistance phenotypes to β-lactams (n = 9) and susceptible strains (n = 10) used for comparative purposes). All strains were characterized for capsular type by PCR and agglutination tests and for β-lactam resistance by amplification and sequencing of *ftsI*. Biotyping and clonality were performed by API-NH and pulsed-field gel electrophoresis, respectively.

Four strains were β-lactamase-negative ampicillin-resistant and five were β-lactamase-positive clavulanic-acid-resistant. One strain from each group was resistant to cefotaxime. Our isolates belonged mainly to biotype IV and I and were non-typeable and genetically unrelated. According to mutation profiles of their *ftsI*, strains were classified as group I (n = 3), group II (n = 4), group−III−like (n = 1) and group III (n = 1). All group II strains were further classified as subgroup IIb, except for one strain, which harboured a new mutation (N422I). Ampicillin MICs of β-lactamase-negative ampicillin-resistant strains were 6 to 12 times the MICs of susceptible strains. Only bla_{TEM-1} was detected in β-lactamase-positive clavulanic-acid-resistant strains, and was responsible for high MICs for ampicillin (>256 mg/L), whatever the *ftsI* mutational resistance group.

The emergence of cefotaxime-resistant isolates in our country is a matter of concern and requires strict surveillance and rationalization of antibiotic use to preserve these molecules.

Keywords: β-lactamases genes, Cefotaxime resistance, *ftsI* gene, *Haemophilus influenzae*, Penicillin-binding protein 3
Original Submission: 23 April 2019; **Revised Submission:** 19 March 2020; **Accepted:** 27 April 2020
Article published online: 5 May 2020

Corresponding author. F. Sana, Faculty of Medicine of Tunis, LR99ES09 Laboratory of Research 'Resistance to Antimicrobial Agents', 1007, Tunis. Tunisia.
E-mail: ferjsana@yahoo.fr

Introduction

Haemophilus influenzae is a commensal bacterium of the human upper respiratory tract, oropharynx and nasopharynx. It is one of the most frequent pathogens responsible for broncho-pulmonary, ear, nose and throat infections [1]. Also, it represents the principal aetiology of invasive infections such as purulent meningitis, bacteraemia and epiglottitis mainly in infants [1]. β-Lactams, mainly third-generation cephalosporins, are active against *H. influenzae*. However, the emergence and spread of resistant strains worldwide can severely affect their efficacy [2]. Resistance to β-lactams in *H. influenzae* is predominantly mediated by TEM-1 or ROB-1 β-lactamase production and is associated with resistance to aminopenicillins, of which the activity spectrum is limited to penicillins. Strains producing β-lactamases are termed β-lactamase-positive ampicillin-resistant. The second mechanism of resistance is non-enzymatic, due to decreased affinity of β-lactams for the altered transpeptidase domain of penicillin-binding protein 3 (PBP3), involved in septal peptidoglycan synthesis and encoded by the *ftsI* gene [1]. Strains expressing this mechanism are termed β-lactamase-negative ampicillin-resistant (BLNAR).

https://doi.org/10.1016/j.nmni.2020.100690

Resistance by the latter mechanism can affect penicillins, penicillin and penicillinase inhibitor associations, cephalosporins and carbapenems, depending on the number and the type of mutations in the *ftsI* gene [1]. Strains that accumulate the two mechanisms are termed β-lactamase-positive clavulanic-acid-resistant (BLPCAR) [1]. In BLNAR strains, amino acid substitutions are usually surrounding the conserved motifs Lys512-Thr-Gly (KTG) and Ser379-Ser-Asn (SSN) of the PBP3 transpeptidase domain. More than 40 substitutions have been described in the literature and it has been found that a single BLNAR isolate could accumulate from one to 11 substitutions [1]. According to specific substitutions, BLNAR isolates have been classified into four major mutational groups (I, II, III and III-like). In group I, His-517 was substituted by Arg and in group II Lys-526 was substituted by Asn. Ampicillin MICs of these groups varied between 0.5 and 8 mg/L and they are considered low BLNAR. Group III and group III-like were defined by the second substitution S385T in addition to the first one N526K or R517H, respectively. They present full resistance to ampicillin (MIC range 1−32 mg/L) and cephalosporins (MIC range 0.12−2 mg/L) and considered high BLNAR [3,4]. Furthermore, isolates from group III and group−III−like with the additional substitution L389F showed generally higher MICs to extended-spectrum cephalosporins and meropenem. Hence, two new groups—III+ and group−III−like+—have been proposed by Skaare et al. for these strains [2]. In *H. influenzae* enzymatic resistance has historically predominated, with a prevalence >20% in many European countries, Australia and Canada. Recent studies showed that the prevalence of β-lactamase-positive ampicillin-resistant strains is stabilizing or decreasing. By contrast, a significant increase of BLNAR phenotype was observed in these same countries [1]. The situation in Japan is different and usually marked by high prevalence of BLNAR strains. In addition, high BLNAR strains have been rarely reported outside Asian countries, particularly Japan and Korea [5].

In Tunisia, multicentric studies on antimicrobial resistance of *H. influenzae* showed that β-lactamase production was the most common mechanism of resistance to β-lactams during all years of surveillance (1999−2017). The prevalence of β-lactamase-positive ampicillin-resistant strains varied from 17.3% in 1999 to 36.6% in 2017. BLNAR isolates emerged in 2006 at low frequencies with significant increase from 2.9% in 2007 to 8.2% in 2017 (www.infectiologie.org.tn). In Tunisia, BLNAR strains are routinely detected by phenotypic methods and few data are available on the genetic classification of their *ftsI* gene. Accordingly, we aimed to characterize the polymorphism of *ftsI* gene in a Tunisian collection of 19 *H. influenzae* strains, isolated between 2014 and 2016, and to assess the clonality among them.

Materials and methods

Strain collection

Haemophilus influenzae isolates included in the study were distributed as follows:

- Seven strains recovered from the microbial Laboratory of Charles Nicolle Hospital, including three low BLNAR isolates (β-lactamase-negative, ampicillin MIC >1 mg/L) and four BLPCAR isolates (ampicillin/clavulanic acid MIC >2 mg/L).
- Two cefotaxime-resistant *H. influenzae* strains (Hi16 and Hi19) isolated at the microbiology laboratories of the Charles Nicolle (Tunis city) and Abderrahman Mami (Ariana city) hospitals, respectively.
- Ten control isolates, fully susceptible to β-lactams: β-lactamase-negative ampicillin-susceptible strains, randomly selected, used for comparative purposes.

Strain identification

Isolates were identified through Gram-staining that usually showed a pleomorphic Gram-negative bacilli, their requirements for β-NAD^+ (V factor) and haemin (X factor) for growth and by API NH (bioMérieux, Marcy-l'Étoile, France). Chocolate agar plates (bioMérieux) were routinely used for subcultures of *H. influenzae*. Biotypes were determined using indole, urease and ornithine decarboxylase reactions revealed by API NH [6].

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by disc diffusion method, according to the European Committee on Antimicrobial Susceptibility Testing recommendations (CA-SFM/EUCAST) [7]. The antibiotics tested were penicillin G (1 µg), ampicillin (2 µg), amoxicillin/clavulanic acid (2 µg/1 µg), cefotaxime (5 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), tetracycline (30 µg), chloramphenicol (30 µg), rifampicin (5 µg) and trimethoprim-sulfamethoxazole (1.25−23.75 µg). β-Lactamase production was determined by the chromogenic cephalosporin test (cefinase test) with nitrocefin as the substrate (bioMérieux). MICs of ampicillin, amoxicillin/clavulanic acid and cefotaxime were determined by E-test strips (bioMérieux) and were interpreted according to the EUCAST breakpoints.

Amplification and sequencing of ftsI gene

Mutations in *ftsI* gene encoding PBP3 were identified by PCR and sequencing as previously described [8].

β-lactamases gene detection

Haemophilus influenzae strains with BLPCAR phenotype were screened for bla_{TEM} and bla_{ROB} genes using multiplex PCR as previously described [9].

Capsular typing and genetic relationship

Capsular type was identified by slide agglutination using specific anti-sera (Difco, BD, Le Pont de Claix, France) and was confirmed by PCR [10]. The *H. influenzae* ATCC 10211 (strain with capsular type b was used as a positive control.

The genetic relationship between isolates was analysed by pulsed-field gel electrophoresis (PFGE). The PFGE Pulse Net protocol of *Escherichia coli* was adapted to *H. influenzae* strains using *Sma*I restriction enzyme (New England BioLabs, Ipswich, MA, USA; https://www.cdc.gov/pulsenet/pathogens/pfge.html). DNA profiles were examined with FP-Quest software (BioRad, Marnes la Coquette, France) and using the Dice coefficient and UPGMA (unweighted pair group method with arithmetic mean). Clusters were defined as DNA patterns sharing ≥70% similarity, which corresponds to the possibly related criteria of Tenover et al. [11].

Results

Clinical data

Demographic and clinical data of patients are summarized in Table 1. Most *H. influenzae* strains were isolated from sputum (n = 15). They were mainly recovered from pneumology (n = 5), otorhinolaryngology (n = 3) and paediatrics (n = 3) wards. Fifteen (78.9%) infections were classified as community-acquired (Table 1).

For the two *H. influenzae* strains resistant to cefotaxime (Hi16 and Hi19) and given the importance of this novel resistance, detailed clinical histories of patients are given below.

Clinical observation no. 1

Hi16 was recovered from a 61-year-old man hospitalized in the gastroenterology ward for decompensated cirrhosis post-viral hepatitis C. Two weeks previously, he was treated with cefotaxime (4 g/day for 15 days) for bacteraemia caused by *E. coli*. Four days after having completed his course of antimicrobial therapy, his clinical state deteriorated and he developed stage II encephalopathy, with dyspnoea and recurrence of fever. An infectious investigation was conducted including chest X-ray, which revealed diffuse alveolar images. Sputum and urine cultures were positive for *H. influenzae* resistant to cefotaxime and *Enterococcus faecium* resistant to glycopeptides, respectively. The patient was treated with ofloxacin (800 mg/day) for 10 days, with clinical and biological improvement.

Clinical observation no. 2

Hi19 was isolated from a 72-year-old man hospitalized in thoracic surgery for acute coronary syndrome. He was a former smoker and was previously hospitalized for acute exacerbation of his chronic obstructive pulmonary disease in 2006 and in 2015. The patient did not receive antibiotics in the previous 6 months. For the current episode, an infectious investigation, including sputum and urine cultures was carried out. The two specimens were positive for *H. influenzae* resistant to cefotaxime and *E. coli*, respectively. The patient was treated with a high dose of cefotaxime (6 g/day) for 10 days and with ciprofloxacin (1500 mg/day) on discharge.

Strain characterization

The *H. influenzae* strains were classified into four biotypes. Biotypes IV and I were identified in nine and eight strains, respectively. Susceptible isolates belonged mainly to biotype I (60%), whereas 55.5% of resistant isolates belonged to biotype IV. Biotypes II and VIII were identified in two strains each (Table 1).

All strains were non-typeable by slide agglutination as well as by PCR amplification. PFGE analysis of *H. influenzae* isolates showed 19 unrelated pulsotypes (Fig. 1). They were susceptible to nalidixic acid, chloramphenicol and rifampicin, Eight and three strains were resistant to tetracycline and cotrimoxazole, respectively. All four β-lactamase-producing isolates harboured bla_{TEM-1} gene (Table 2).

ftsI genotypes and correlation with β-lactam MICs

Sequence analysis of the transpeptidase region of PBP3 showed a total of 19 substitutions in 18 positions. The most frequent mutations were (D350N), (S357I), (R517H) and (N526K). In the Hi15 BLPCAR isolate, a new mutation was observed at position 422 (N422I). For this strain, MICs of ampicillin, amoxicillin/clavulanic acid and cefotaxime were >256, 2 and 0.032 mg/L, respectively.

Among strains with a β-lactamase-negative ampicillin-susceptible phenotype (control group), four did not harbour mutations in the *ftsI* gene and six displayed different point mutations (Table 2). Ampicillin MICs varied from 0.19 to 0.75 mg/L (MIC_{50} 0.25 mg/L).

According to the mutation profile of their *ftsI* gene, strains with BLNAR phenotype (n = 4) were classified as group I (n = 3) and group III+ (n = 1) and those with BLPCAR phenotype (n = 5) were classified as group II (n = 4) and group−III−like (n = 1) (Table 2). Three strains (Hi4, Hi17 and Hi18) from group II were further assigned to subgroup II-b, and were also TEM-1 β-lactamase producers. The remaining strain (Hi15) that had a new mutation (N422I) could not be assigned to any of the previously described group II subgroups. Ampicillin MICs varied between 2 and 4 mg/L in group I and was >256 mg/L in group II.

Resistance to cefotaxime in strains Hi16 and Hi19 was associated with S385T and L389F mutations, respectively. Strain

TABLE 1. Clinical characteristics and biotype of *Haemophilus influenzae* strains (n = 19)

	Reference strains	Date of isolation	Patient age/Gender	Specimens	Wards	Infection origin	Biotype
β-lactam-susceptible strains (Control group)	Hi 2	18/07/2014	79 years/F	Bronchial secretion	Intensive care unit	CA	I
	Hi 3	11/03/2015	–/F	Sputum	External consultation	CA	IV
	Hi 5	12/05/2015	–/M	Sputum	Pneumology	CA	IV
	Hi 6	01/06/2015	81 years/M	Bronchial secretion	Intensive care unit	HA	IV
	Hi 7	10/07/2015	–/F	Sputum	Pneumology	CA	I
	Hi 8	28/07/2015	–/M	Bronchial secretion	Pneumology	CA	I
	Hi 9	12/08/2015	75 years/M	Bronchial secretion	Surgery	HA	IV
	Hi 11	12/09/2015	14 years/M	Sputum	Paediatrics	CA	I
	Hi 12	30/10/2015	–/F	Sputum	Paediatrics	CA	I
	Hi 13	11/11/2015	14 years/F	Pus	Otorhinolaryngology	CA	I
β-lactam-resistant strains	Hi 1	17/07/2014	55 years/F	Sputum	Otorhinolaryngology	CA	I
	Hi 4	10/04/2015	–/F	Sputum	Pneumology	CA	II
	Hi 10	23/09/2015	9 years/F	Sputum	Paediatrics	CA	VIII
	Hi 14	30/10/2015	–/F	Sputum	Pneumology	CA	I
	Hi 15	30/12/2015	1 year/M	Pus	ForensicMedicine	CA	IV
	Hi 16	20/01/2016	62 years/M	Sputum	Gastroenterology	HA	IV
	Hi 17	05/03/2016	–/M	Pus	Otorhinolaryngology	CA	IV
	Hi 18	11/03/2016	–/F	Sputum	Paediatrics	CA	IV
	Hi 19[a]	30/08/2016	72 years/M	Sputum	Thoracic surgery	HA	IV

CA, community-acquired infections were defined as infections in which the onset of patient symptoms occurred before admission or within 48 h of admission to the hospital; HA, hospital-acquired infections were defined as infections in which the onset of symptoms occurred more than 48 h after admission.
[a]Isolate from Abdurrahman Mami hospital.

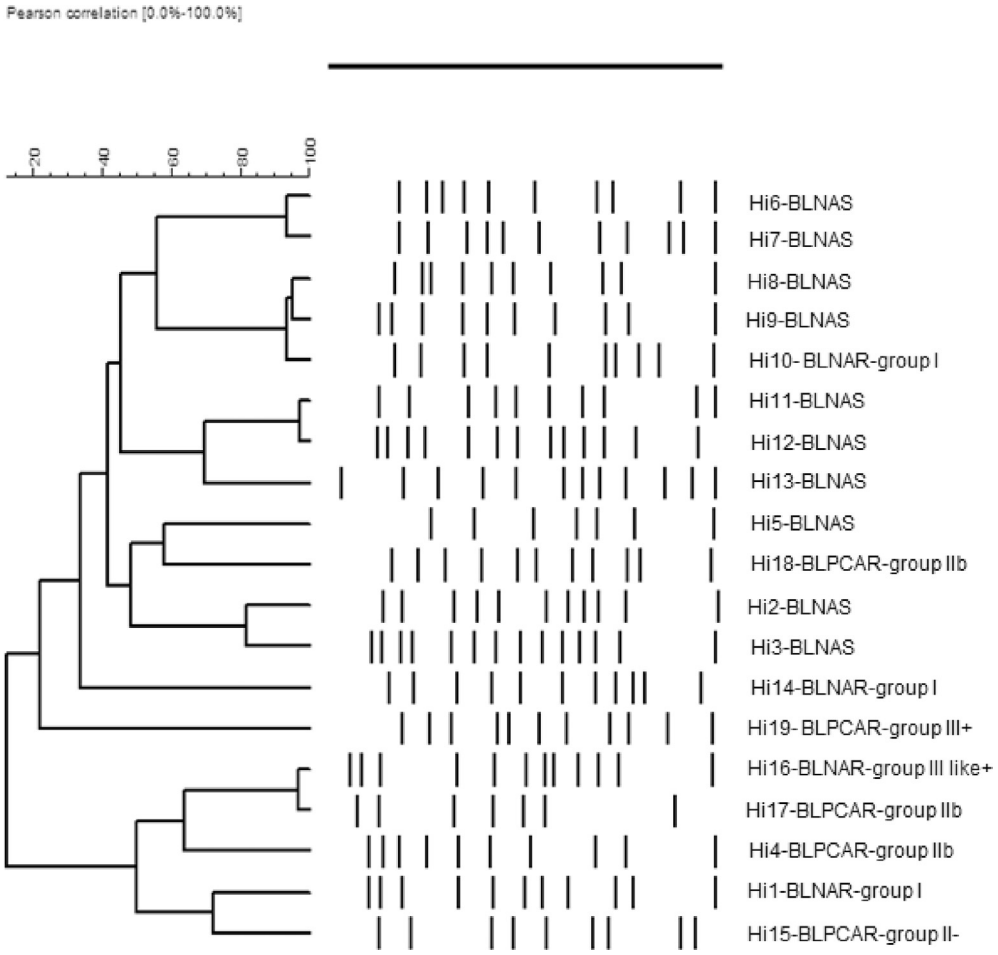

FIG. 1. Dendrogram of pulsed-field gel electrophoresis DNA patterns of *Haemophilus influenzae* strains obtained after the UPGMA analysis of the Dice's coefficient. BLNAS, β-lactamase-negative ampicillin-susceptible; BLNAR, β-lactamase-negative ampicillin-resistant; BLPCAR, β-lactamase-positive clavulanic-acid-resistant).

TABLE 2. **Antimicrobial resistance profiles and deduced amino acid substitution patterns in the transpeptidase region of penicillin-binding protein 3 (PBP3) of *Haemophilus influenzae* strains**

Reference Strains	Resistance profile	Cefinase/*bla* genes	MICs			ftsI groupa		Mutations in ftsI gene																
			AMP	AMC	CTX		D350	S357	M377	S385	L389	P392	N422	G490	A502	V511	R517	N526	S532	F535	V547	I549	Y557	N569
Hi 3	—	—	0.25	0.25	0.012	NA																		
Hi 7	—	—	0.25	0.38	0.016																			
Hi 9	TET	—	0.38	0.25	0.012																			
Hi 12	TET	—	0.19	0.125	0.023																			
Hi 13	—	—	0.25	0.125	0.016							P												
Hi 11	TET	—	0.125	0.125	0.016															I				
Hi 8	SXT	—	0.25	0.25	0.012															I				
Hi 6	—	—	0.75	0.25	0.047																I			
Hi 5	TET	—	0.5	0.38	0.08				s												I			
Hi 2	—	—	0.25	0.25	0.016																	F		
Hi 14	AMX-TET	—	2	0.125	0.064	Group I											H							
Hi 1	AMX	—	4	0.125	0.023											A	H							
Hi 10	AMX-TET	—	4	0.38	0.023											A	H							
Hi 17[a]	AMX-AMC-SXT	+/bla_{TEM-1}	>256	2	0.064	Group IIb	N		I						V			K		I				
Hi 18 [a]	AMX-AMC	+/bla_{TEM-1}	>256	4	0.047	Group Iib	N		I					E	V			K						
Hi 4 [a]	AMX-AMC-TET-SXT	+/bla_{TEM-1}	>256	2	0.047	Group Iib	N		I					E	V			K			I			
Hi 15[b]	AMX-AMC	+/bla_{TEM-1}	>256	2	0.032	Group II	N		I				I*		V			K						
Hi 19	AMX-AMC-CTX	+/bla_{TEM-1}	>256	>256	I	Group−III−like+	N	N	I	T	F						H		T		I		H	S
Hi 16	AMX-AMC-CTX-TET	—	24	4	0.5	Group III+	N	N	I	T	F				T			K						

AMC, ampicillin/clavulanic acid; AMP, ampicillin, CTX, cefotaxime.
[a] Classification according to Ubukata et al. [30] (Group I, II and III), Dabernat et al. [3] (Subgroup II: IIa, IIb, IIc and IId), García-Cobos et al. [4] (Group−III−like), Skaare et al. [23] (Group III+ and Group−III−like+): −, negative; NA, non-assigned resistance group, *, new mutation.

Hi19 was also a TEM-1 producer. Cefotaxime MICs were 0.5 mg/L and 1 mg/L for Hi16strain (group III+) and Hi19 strain (group III like+), respectively.

High resistance level of ampicillin (>256 mg/L) was associated with TEM-1 production whatever the *ftsI* mutational resistance group. ROB enzyme has not been found in our collection.

Discussion

In this study, molecular mechanisms of resistance to β-lactams in clinical *H. influenzae* isolates showing different β-lactam resistance phenotypes were investigated. All strains were non-typeable by slide agglutination as well as by PCR amplification. The predominance of non-typeable strains was also reported in other Tunisian studies, and in Korea, Spain and France [12,13]. However, previous reports demonstrated the limitation of slide agglutination for *H. influenzae* serotyping in comparison with the results provided by PCR [14,15]. This may be related to the individual characteristics of expression of capsule and/or other antigens on the bacterial surface [16,17].

In Tunisia, until 2002, all invasive infections in young children were caused by *H. influenzae* b strains [18], justifying the introduction of the anti-Hib conjugate vaccine. However, given its high cost, it was abandoned at the beginning of 2006. Then, based on extensive evidence demonstrating the economic impact of the anti-Hib conjugate vaccine through direct and indirect cost savings, as well as through contributions to the Tunisian economy in general, this vaccine was reintroduced into the vaccination schedule in 2011 [19].

In the present study, PFGE analysis of the *H. influenzae* isolates showed diverse pulsotypes, which is in agreement with the genetic heterogeneity of non-capsulated *H. influenzae* previously reported [1]. Also, according to resistance phenotype, many studies have shown that BLNAR and BLPCAR strains of *H. influenzae* are genetically diverse with a general absence of related PFGE DNA profiles [1]. Otherwise, clonal spread of BLNAR strains of serotype b was reported by a limited number of studies in Japan [20,21]. In comparison, some local outbreaks caused by BLNAR *H. influenzae* strains have been reported, in Norway, Spain and Canada. of BLNAR *H. influenzae* strains that were caused by closely related clones [4,8,22].

Among the β-lactamase-negative ampicillin-susceptible phenotypes (n = 10), four strains of our collection had the prototype amino acid sequence of the transpeptidase region of PBP3. In the remaining six strains, different point mutations were found. These observations were previously reported [2,8,23]. Many silent mutations have been reported in the DNA region encoding the transpeptidase domain of PBP3 in susceptible strains [23]. The results of García-Cobos et al. showed that β-lactams susceptible *H. parainfluenzae* strains presented various substitutions not previously assigned to any *ftsI*-resistant groups [4].

According to mutational profiles of *ftsI* genes, BLNAR and BLPCAR isolates of our collection were classified as group I, II, IIb, III-like+ and III+. Strain Hi15 belonging to group II, but not assigned to any group II subgroups, showed a new mutation (N422I) in its *ftsI* gene, suggesting a novel subgroup II. The β-lactam resistance in *H. influenzae* due to *ftsI* mutations is increasing worldwide [22]. Low-level resistance isolates, mainly with the N526K substitutions, predominated in most geographical regions, whereas high-level resistance isolates with additional L389F substitution (ampicillin MIC_{50}: 128 mg/L and cephalosporins MIC_{50}: 1 mg/L) are common in Japan and South Korea. Epidemiological data from European countries and Canada showed a gradual increase in resistant isolates from group II with sporadic cases of cefotaxime-resistant strains [1,13,22,24,25].

In Tunisia, resistance to cefotaxime has been recently reported among six *H. influenzae* isolates from Habib Bourguiba hospital. These strains belonged to group IIa, group IIb, group III and group−III−like [26]. The two cefotaxime-resistant strains described in our study belonged to group III+ and group−III−like+. According to clinical data, *H. influenzae*-resistant strains were isolated from two elderly individuals with severe underlying diseases, chronic cirrhosis in one and chronic obstructive pulmonary disease in the other. It has been demonstrated that people suffering from chronic obstructive pulmonary disease are frequently colonized by *H. influenzae* in their respiratory tract [12,27]. In addition, previous antimicrobial treatment by cefotaxime and iterative hospitalizations are contributing factors for selection of such resistant strains. According to Dabernat et al., the inappropriate use of oral antibiotics for the treatment of community-acquired bronchopulmonary and upper respiratory tract infections seems to be responsible for the selection of BLNAR strains [3].

In our series, the five BLPCAR isolates harboured the bla_{TEM-1} gene. This finding is in agreement with previous Tunisian studies [28,29]. Although the predominance of TEM-1 β-lactamase was largely reported worldwide, ROB-1 is rarely found outside North America [1].

Our study presents two major limitations. First, clinical data were collected retrospectively and detailed information for all patients, such as age, co-morbidities, severity of diseases, antimicrobial treatment history and clinical outcomes could not be obtained. Second, our results gave limited data on the distribution of the *ftsI* group in our country because of the low number of studied strains. Further large studies including other hospital centres will be necessary to assess the real clinical

impact of this emerging resistance and to identify additional risk factors for selection of resistant strains.

In conclusion, this study revealed the diversity of mutation profiles in *ftsI* gene among BLNAR and BLPCAR *H. influenzae* strains. Our results further indicate that these strains were non-capsulated and were genetically unrelated. The emergence of strains resistant to extended spectrum β-lactams is alarming and requires strict epidemiological surveillance. Furthermore, rationalization and strict control of antibiotic use, mainly in the community, is needed to preserve the activity of these molecules.

Conflict of interest

The authors have no conflicts of interest to declare. No funding was received for the study.

Authors' contributions

FS and SM were responsible for the conception or design of the work; SI, ME and SL performed the data collection; and FS performed the data analysis and interpretation. FS drafter the article; BBBI critically revised the article and the final approval for publication was given by FS, SI, SM, ME, GA, SL, SA and BBBI.

Acknowledgements

This work was supported by the Ministry of Higher Education and Scientific Research of Tunisia.

References

[1] Tristram S, Jacobs MR, Appelbaum PC. Antimicrobial resistance in *Haemophilus influenzae*. Clin Microbiol Rev 2007;20:368–89.

[2] Skaare D, Anthonisen IL, Kahlmeter G, Matuschek E, Natås OB, Steinbakk M, et al. Emergence of clonally related multidrug resistant *Haemophilus influenzae* with penicillin-binding protein 3-mediated resistance to extended-spectrum cephalosporins, Norway, 2006 to 2013. Eurosurveillance 2014;19:1–13.

[3] Dabernat H, Delmas C, Seguy M, Pelissier R, Faucon G, Bennamani S, et al. Diversity of β-lactam resistance-conferring amino acid substitutions in penicillin-binding protein 3 of *Haemophilus influenzae*. Antimicrob Agents Chemother 2002;46:2208–18.

[4] García-Cobos S, La E, Roma F, Cercenado E, Abajo F De. 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 2007;51:2564–73.

[5] Yokota S, Ohkoshi Y, Sato K, Fujii N. High prevalence of β-lactam-resistant *Haemophilus influenzae* type b isolates derived from respiratory tract specimens in Japanese patients. Int J Infec Dis 2009;13:584–8.

[6] Kilian M. A taxonomic study of the genus *Haemophilus*, with the proposal of a new species. J Gen Microbiol 1976;93:9–62.

[7] The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 1.0 2014. http://www.eucast.org/clinical_breakpoints.

[8] Skaare D, Allum A, Anthonisen IL, Jenkins A, Lia A, Strand L, et al. Mutant *ftsI* genes in the emergence of penicillin-binding protein-mediated β -lactam resistance in *Haemophilus influenzae* in Norway. Clin Microbiol Infect 2010;16:1117–24.

[9] Tristram SG, Nichols S. A multiplex PCR for β-lactamase genes of *Haemophilus influenzae* and description of a new bla_{TEM} promoter variant. J Antimicrob Chemother 2006;58:183–5.

[10] Falla TJ, Crook DWM, Brophy LN, Maskell D, Kroll JS, Moxon ER. PCR for capsular typing of *Haemophilus influenzae*. J Clin Microbiol 1994;32:2382–6.

[11] Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 1995;33:2233–9.

[12] Puig C, Tirado-vélez JM, Calatayud L, Tubau F, Garmendia J, Ardanuy C. Molecular characterization of fluoroquinolone resistance in nontypeable *Haemophilus influenzae* clinical isolates. Antimicrob Agents Chemother 2015;59:461–6.

[13] Bae S, Lee J, Lee J, Kim E, Lee S, Yu J, et al. Antimicrobial resistance in *Haemophilus influenzae* respiratory tract isolates in Korea: results of a nationwide acute respiratory infections surveillance. Antimicrob Agents Chemother 2010;54:65–71.

[14] Bonifácio MEN, Silva P, Medeiros MIC, Neme SN, Macedo C, Marin JM. Comparison of two slide agglutination serotyping methods and PCR-based capsule typing for the characterization of *Haemophilus influenzae* serotypes. Braz J Microbiol 2006;37:39–41.

[15] Monge S, Mollema L, Melker H De, Sanders E, Ende A Van Der, Knol M. Clinical characterization of invasive disease caused by *Haemophilus influenzae* serotype b in a high vaccination coverage setting. J Pediatr Infect Dis Soc 2019;8:261–4.

[16] Gilsdorf JR. Antigenic diversity and gene polymorphisms in *Haemophilus influenzae*. Infect Immun 1998;66:5053–9.

[17] Laclaire LL, Tondella MLC, Beall DS, Noble CA, Raghunathan PL, Rosenstein NE, et al. Identification of *Haemophilus influenzae* serotypes by standard slide agglutination serotyping and PCR-based capsule typing. J Clin Microbiol 2003;41:393–6.

[18] Smaoui H, Kechrid A. Study of *Haemophilus influenzae* strains isolated at the Tunis children' s hospital in the prevaccination era (1999–2002). Med Mal Infect 2006;36:364–8.

[19] Ben Redjeb S, Boutiba Ben Boubaker I. L'antibiorésistance en Tunisie (LART). Haemophilus influenzae; 2011. p. 55–9. Tunis.

[20] Miyahara R, Suzuki M, Morimoto K, Chang B. Nosocomial outbreak of upper respiratory tract infection with β-lactamase-negative ampicillin-resistant nontypeable *Haemophilus influenzae*. Infect Control Hosp Epidemiol 2018;39:652–9.

[21] Kishii K, Chiba N, Morozumi M. Diverse mutations in the *ftsI* gene in ampicillin-resistant *Haemophilus influenzae* isolates from pediatric patients with acute otitis media. J Infect Chemother 2010;16:87–93.

[22] Tsang RSW, Shuel M, Whyte K, Hoang L, Tyrrell G, Horsman G, et al. Antibiotic susceptibility and molecular analysis of invasive *Haemophilus influenzae* in Canada, 2007 to 2014. J Antimicrob Chemother 2017;72:1314–9.

[23] Skaare D, Anthonisen IL, Caugant DA, Jenkins A, Steinbakk M, Strand L, et al. Multilocus sequence typing and *ftsI* sequencing : a powerful tool for surveillance of penicillin-binding protein 3-mediated β-lactam resistance in nontypeable *Haemophilus influenzae*. BMC Microbiol 2014;14:1–16.

[24] Dabernat H, Delmas C. Epidemiology and evolution of antibiotic resistance of *Haemophilus influenzae* in children 5 years of age or less in France , 2001–2008 : a retrospective database analysis. Eur J Clin Microbiol Infect Dis 2012;13:2745–53.

[25] Park C, Kim KH, Shin NY, Byun JH, Kwon EY, Lee JW, et al. Genetic diversity of the *ftsI* gene in β-lactamase-nonproducing ampicillin-resistant and β-lactamase-producing amoxicillin-/clavulanic acid-resistant nasopharyngeal Haemophilus influenzae strains isolated from children in South Korea. Microb Drug Resist 2013;19:224–30.

[26] Mezghani Maalej S, Ktari S, Ben Abdallah R, Mahjoubi F, Hammami A. Emergence of cefotaxime resistant *Haemophilus influenzae* in Tunisia. J Glob Antimicrob Resist 2019;17:130–1.

[27] Fuursted K, Nyvang G, Stegger M, Skytt P. Molecular characterisation of the clonal emergence of high-level ciprofloxacin-monoresistant *Haemophilus influenzae* in the region of southern Denmark. Integr Med Res 2016;5:67–70.

[28] Mzilem S, Ksiaa S, Smaoui H, Kechrid A. *Haemophilus influenzae* strains in children : increasing resistance to β-lactam antibiotics. Nt J Microbiol Immunol Res 2015;3:84–9.

[29] Touati A, Achour W, Ben Hassen A. Phenotypic and molecular characterization of β-lactams resistance and capsular typing of colonizing *Haemophilus influenzae* strains isolated from neutropenic patients in Tunisia. Path Biol 2009;57:353–7.

[30] Ubukata K, Shibasaki Y, Yamamoto K, Chiba N, Hasegawa K, Takeuchi Y, et al. 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 2001;45:1693–9.