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Haemophilus influenzae Strains in Tunisian Children

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ABSTRACT

Background: *Haemophilus influenzae, a major pathogen causing respiratory tract infections and meningitis, is becoming increasingly resistant to narrow spectrum penicillin. Investigating the reasons for this resistance is challenging.* ***Methods:*** *The sequences of the ftsI gene, encoding the transpeptidase domain of penicillin binding protein (PBP3), were determined for 44 strains of Haemophilus influenzae with reduced susceptibility to β-lactam antibiotics. Strains, isolated from children, were analyzed for genetic relationship by pulsed-field gel electrophoresis (PFGE).* ***Results:*** *Sequence analysis of the ftsI gene revealed different mutations. We used this polymorphism to classify the different strains into three groups: (I (n=3), II (n=36, including H. influenzae ATCC 49247), this group was divided into four subgroups and III (n=5)). In each group various substitutions were observed.* ***Conclusion:*** *The increasing number of Haemophilus influenzae strains with reduced susceptibility to β-lactam due to mutations in the ftsI gene, is becoming a serious health issue in Tunisia. The resistance to β-lactam was observed in both strains that produce β-lactamase and those that do not. The level of resistance is remarkably high in our country.*

Keywords: *H. influenzae*, ftsI, PBP3, Antibiotic resistance

INTRODUCTION

Haemophilus influenzae is an opportunistic pathogen found naturally in human upper respiratory tract. *Haemophilus influenzae* is the main cause of bronchopulmonary and ear, nose, and throat infections in children. these infections are caused by non-encapsulated (non typable) and encapsulated (typable) strains [1]. The main challenge in treating these infections is antibiotic resistance.

Two mechanisms are responsible for resistance to aminopenicillins, the main and the common mechanism is due to enzymatic hydrolysis of the antibiotic by β-lactamase (TEM-1 and ROB-1 type) [2]. The second mechanism of resistance is due to mutations in PBPs affecting their affinity to penicillin.

The incidence of strains producing β-lactamase is particularly high in many countries [3,4]. Resistance by mechanisms other than the production of β-lactamase is based on decreased affinity of the PBPs involved in septal peptidoglycan synthesis [5]. The first observations of β-lactamase negative ampicillin-resistant (BLNAR) strains were reported in the early 1980s and concerned *H. influenzae* type b [6,7] and non-encapsulated *H. influenzae* strains [8,9]. In contrast to the incidence of strains producing β-lactamase, the incidence of BLNAR strains remains low in many countries [10,11]. Although rare by comparison to strains producing β-lactamase, the prevalence of BLNAR strains has increased rapidly during the last decade [12].

In *H. influenzae*, resistance to ampicillin without the production of β-lactamase was shown to be chromosomally mediated and was correlated with alterations in PBP 3 (3A and 3B) [13]. In 2001, Ubukata, et al. demonstrated that mutations in the *ftsI* gene are important for the resistance to β-lactams in BLNAR *H. influenzae* strains [14]. In BLNAR strains, the amino acid sequences, deduced from the *ftsI* gene encoding the transpeptidase domain of PBP 3 (3A and 3B), show several common amino acid substitutions. On the basis of amino acid substitutions deduced from the *ftsI* gene, the BLNAR strains were classified into three Groups I, II and III according to Ubukata, et al. [14], while Dabernat, et al. [15] divided Group II into four parts, subgroup IIa, IIb, IIc and IId.

We studied a series of *H. influenzae* strains with BLNAR characteristics and β-lactamase-producing strains with reduced susceptibility to β-lactams. Investigating the reasons for this resistance is challenging. The sequence of the *ftsI* gene encoding for the transpeptidase domain was determined and compared to those of the Rd strain (ampicillin-susceptible). We report in this work the diversity of amino acid substitutions arising from alterations of the *ftsI* gene observed among Tunisian isolates. This study will provide descriptive data for the antibiotic susceptibility of *H. influenzae* in Tunisia.

MATERIALS AND METHODS

Strains

Two hundred strains were isolated from children at the children's hospital of Tunis between 2010 and 2011. The strains were identified with the required X and V factors for growth. PCR amplification of *p6* gene, encoding the *P6* outer membrane protein (OMP6) specific for *H. influenzae* was utilized to confirm the identification of *H. influenzae*. All strains were positive for the *p6* gene. The production of β-lactamase was detected by a chromogenic cephalosporin test (cefinase; BioMérieux). Serotyping was done by slide agglutination technique with a to f antisera (Difco). Since serotyping using slide agglutination is not enough specific, PCR was used as a confirmation test. The biotype was determined as described by Kilian [16].

The strains were stored at -80°C in 10% glycerol brain heart infusion broth. *H. influenzae* ATCC 49247 (Ampicillin-resistant, β-lactamase negative), *H. influenzae* C425 (blaTEM-1 positive), *H. influenzae* C322 (blaROB-1 positive) and *H. influenzae* ATCC 10211 (*H. influenzae* type b) were used as controls. These strains were afforded by Pr H. Dabernat (Toulouse, France).

Antibiotics susceptibility

Antimicrobial susceptibility was determined for all *H. influenzae* strains. The medium for determination of the minimum inhibitory concentration (MIC) was chocolate agar containing polyvitex (Oxoid) [17]. The β-lactams used in this study were ampicillin, amoxicillin/clavulanic acid, cefuroxime, cefotaxime, cefixime, cefpodoxime and imipenem. MICs were determined by gradient agar diffusion (Epsilometer test; E-Test) method with an inoculum of 0.5 McFarland (2×10^8 CFU/ml). After 24 h of incubation at 37°C under 5% CO_2, the MIC was characterized as the minimal concentration of antibiotic that inhibited bacterial development.

DNA extraction and PCR

After 24 h of development on chocolate agar, 2 to 3 colonies of *H. influenzae* were delayed in 500 µl of sterile distilled water and then boiled for 5 minutes at 100°C. The suspension was centrifuged for 20 min at 14500 rpm. The supernatant containing bacterial DNA was stored at -20°C for additional use.

PCR amplification of the *ftsI* gene encoding the transpeptidase domain of PBP 3 was performed using two sets of primers: primers J1 and J2 (Table 1) to amplify the sequence between nucleotides 1048 to 1598 [18], and primers F1 and F2 (Table 1) to amplify the sequence between nucleotides 936 to 1640 (based on the *ftsI* sequence of *H. influenzae* Rd; accession no. L42023). When we use the J1-J2 primer set, a lack of response is detected for some BLNAR strains. PCR products obtained with primers F1 and F2 were used for sequencing in this work.

Table 1 List of primers used in PCR reactions (*blaTEM, blaROB* and *ftsI*)

Genes	Primers	Nucleotide sequences	Reference
blaTEM	TEM-1	5′ TGG GTG CAC GAG TGG GTT AC 3′	Dabernat, et al. [15]
	TEM-2	5′ TTA TCC GCC TCC ATC CAG TC 3′	
blaROB	ROB-1	5′ ATC AGC CAC ACA AGC CAC CT 3′	Dabernat, et al. [15]
	ROB-2	5′ GTT TGC GAT TTG GTA TGC GA 3′	
ftsI	J1	5′ GAT ACT ACG TCC TTT AAA TTA AG 3′	Ubukata, et al. [14]
	J2	5′ GCA GTA AAT GCC ACA TAC TTA 3′	
Sequencing	F1	5′ GTT AAT GCG TAA CCG TGC AAT TAC C 3′	Dabernat, et al. [15]
	F2	5′ ACC ACT AAT GCA TAA CGA GGA TC 3′	

Primers

TEM (Table 1; nucleotides 321 to 846), and ROB (Table 1; nucleotides 419 to 1110), were utilized to recognize the presence of *blaTEM* and *blaROB* genes in the isolates giving a positive cefinase test according to Tenover, et al. [19].

Amplifications with primers J1, J2, TEM, and ROB were accomplished in 10 µl involving 50 µM of each primer, 5 mM dNTP, 50 mM $MgCl_2$, 1 µl of 10x reaction buffer, Taq DNA polymerase (0.5 U) (Promega), and 1 µl of DNA. Amplification with F1 and F2 for sequencing was realized in 50 µl.

PCR cycling was realized in a PCR Thermal cycler (Applied Biosystems). Twenty amplifications of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and primer extension at 75°C for 30 s were performed. Then, an extension at 72°C for 10 min. PCR products were observable by 2% agarose gel electrophoresis and ethidium bromide (BET) staining.

DNA sequencing and sequence analysis

DNA fragments acquired by PCR with F1 and F2 primers (Table 1) were employed for sequencing. Sequences were detected in a DNA sequencer (ABI Prism 3700; Applied Biosystems).

An unweighted pair group method with arithmetic mean (UPGMA) phylogram was created using ClustalW2 (www.ebi.ac.uk) and TreeDyn (www.phylogeny.fr). *H. influenzae* Rd KW20 was used as reference (GenBank accession no. L42023). Deduced PBP3 amino acid sequences were compared with *H. influenzae* Rd KW20 strain.

Pulsed-field gel electrophoresis (PFGE)

From a culture grown for 24 h on chocolate agar, we prepared genomic DNA. Cells were suspended in phosphate-buffered saline (PBS; 10^9 CFU/ml); 100 µl of suspension was combined with 100 µl of 1% low-melting-point agarose (bioRad) in order to form plugs. These plugs were incubated in a buffer prepared with 1 ml of 0.5 M EDTA (pH 9), 1% sodium dodecyl sulfate (SDS); 25 µl of proteinase K (5 mM) (Promega) for 24 h at 50°C. This lysis was blocked with 40 µl of phenyl methane sulfonyl fluoride (PMSF) (0.25 M) at 25°C for 1 h. The plugs were rinsed 3 times at 25°C for 45 min with Tris EDTA buffer (10 mM Tris, 1 mM EDTA) (bioRad).

Then, the plugs were digested at 25°C for 24 h with SmaI (25 U; Invitrogène) added to 300 µl of the enzyme buffer. The slices were positioned on the wall comb, and diluted 1% agarose (Bio-Rad) was poured into the gel mold. They were run with an angle of 120° at 6 V/cm and with an initial switch time of 5.3 s to a final switch time of 34.9 s at 14°C in Tris-borate-EDTA buffer (TBE; 0.5×) for 24 h by using the CHEF DR III variable angle system (Bio-Rad). The DNA ladder (48.5 kb) (Bio-Rad) was utilized as a marker.

BioNumerics software was used to analyze the gel. The resemblance of the PFGE patterns was predictable using the Dice coefficient.

RESULTS

In the current study we analyzed two hundred different strains. These strains are isolated between 2010 and 2011 from children at the children's hospital of Tunis. Strains were isolated from pulmonary (161) (80.5%), superficial (3) (1.5%) and other (12) (6%) samples (Osteomyelitis, intra-abdominal cyst, newborn, hydrostatic cyst and abscesses). Additional strains were isolated from blood culture (7) (3.5%), puncture liquid (4) (2%) and CSF (13) (6.5%). Serotyping was determined for the 200 strains by slide agglutination technique with polyvalent and specific a to f antisera (Difco), results were confirmed by PCR. The results showed that the majority of strains studied were non-typable (NT) (150) (75%), 43 strains (21.5%) of type (b) (Hib), 1 (0.5%) of type (a) and 6 (3%) of type d, e and f (d (n=2); e (n=2); f (n=2)).

Antibiotic susceptibility

Among the 200 strains studied 91 were resistant to ampicillin (45.5%). Total 66 strains produced β-lactamase (33%). Twenty-five strains (12.5%) were resistant without β-lactamase production and were called β-lactamase negative ampicillin resistant (BLNAR).

On the basis of the PCR genotyping results, the *H. influenzae* strains were subdivided into 3 groups. We define the groups according to production of β-lactamase TEM-1 and detection of the *ftsI* gene. The used PCR primers

are able to discriminate between strains with or without mutations in the *ftsI* gene. PCR primers will bind only on mutated *ftsI* gene, subsequently amplification will be abolished in strains with wt *ftsI* gene and only in strains with resistant mutation PCR product will be detected. About 47 strains (23.5%) produced β-lactamase TEM-1 and were characterized by the detection of the *ftsI* gene (genetically defined β-lactamase positive, ampicillin-resistant, gBLPAR). gBLPAR strains are only resistant to ampicillin but susceptible to amoxicillin-clavulanate. No isolate was positive for *blaROB-1*. In 25 strains (12.5%) no PCR product was detected. This means that these strains share low-level-resistant mutation (s) in PBP, but not defective for this gene, and were β-lactamase negative (genetically defined β-lactamase negative, ampicillin resistant, gBLNAR). Total 19 strains (9.5%) produced β- lactamase and were detected by the lack of detection of the *ftsI* gene (genetically defined β-lactamase positive, amoxicillin-clavulanate-resistant, gBLPACR). gBLPACR strains are resistant to ampicillin and amoxicillin-clavulanate.

Table 2 MICs of β-lactams (mg/L) according to resistance mechanism in *H. influenzae* strains

Variables	Ampicillin			Amoxicillin-clavulanate			Cefuroxime			Cefotaxime			Cefixime		
MIC	50	90	-	50	90	-	50	90		50	90	-	50	90	-
BLPAR (N=47)	>256	>256	96- >256	4	4	0.5-4	3	3	0.5-3	0.047	0.047	0.008-0.047	0.19	0.19	<0.016-0.19
BLNAR (N=25)	4	4	1-4	4	4	1-4	6	6	1.5-6	0.125	0.125	0.023-0.125	0.25	0.25	0.023-0.25
BLPACR (N=19)	>256	>256	>256	6	6	1-6	6	6	0.75-6	0.19	0.19	0.023-0.19	0.25	0.25	<0.016-0.25

All isolates were tested by E-test technique. The criteria used are those of the Antibiogram Committee of the French Microbiology Society (CA-SFM) [20]. The MICs of the seven antibiotics tested on all the strains and according to the resistance phenotype are reported in Table 2. The production of β-lactamase conferred a high level of resistance to ampicillin (96-> 256 mg/L). The absence of β-lactamase production conferred a lower level of ampicillin resistance (MIC between 1 and 4 mg/L). For these strains, clavulanic acid does not restore the activity of ampicillin. The other β-lactams showed modified activity on the β-lactamase producing strains; MICs are increased for strains of lesser sensitivity. Maximum values are 6 mg/L for cefuroxime, 0.38 mg/L for cefpodoxime, 0.25 mg/L for cefixime and >32 mg/L for imipenem.

Mutation patterns in the *ftsI* gene

PCR amplification of the 551bp fragment (nucleotides 1048 to 1598) using J1and J2 primers, failed to give any detectable PCR product in 44 isolates (including *H. influenzae* ATCC 49247). PCR product was detected in all strains when primers set (F1 and F2) were used.

F1 and F2 primers were used to perform sequencing of the *ftsI* gene (bp 960 to 1618) in the 44 strains that failed to give any amplification with the J1 and J2 primer set. *H. influenzae* ATCC 49247 were subjected to sequencing and used as control.

The amino acid sequences of the critical region (AA 327 to AA 540) of the 44 sequenced strains were compared to the same sequence of the standard Rd strain (Figure 1). Table 3 represents the amino acid substitutions encoded by the *ftsI* genes of the 44 *H. influenzae* strains and *H. influenzae* ATCC 49247.

Table 3 Deduced amino acid substitutions detected from *H. influenzae* strains with resistance to β-lactam antibiotics

Groups	Number of strains	Amino acid substitutions												CMI (mg/L)			Type of Strains
		Asp 350	Ser 357	Ala 368	Met 377	Ser 385	Leu 389	Ala 437	Ile 449	Gly 490	Ala 502	Arg 517	Asn 526	Ampicillin	Cefuroxim	Imipinem	
I	1				Ile							His		1,5- >256	2-3	>32	2BLPACR; NT 1BLNAR; NT

	1		Asn									His				
	1										Thr	His				
II a	1				Ile								Lys	1-3	2-4	>32	3BLNAR; NT
	3												Lys				1BLPACR; NT
	ATCC 49247												Lys	2	2	-	BLNAR
II b	8										Val		Lys	1 - > 256	2-6	16 - >32	1BLPACR; b 3BLNAR; b 9BLPACR; NT 5BLNAR; NT
	2	Asn									Val		Lys				
	2									Glu	Val		Lys				
	1				Ile						Val		Lys				
	2	Asn			Ile						Val		Lys				
	2	Asn	Asn		Ile						Val		Lys				
	1	Asn			Ile					Glu	Val		Lys				
II c	1								Val		Thr		Lys	1,5 - > 256	2-3	>32	3BLPACR; NT
	4			Thr							Thr		Lys				5BLNAR; NT
	3	Asn									Thr		Lys				1BLNAR; b
	1				Ile						Thr		Lys				
II d	5								Val				Lys	1,5 - > 256	1.5 – 2	>32	1BLNAR; b 1BLPACR; b 1BLPACR; NT
III	5	Asn			Ile	Thr	Phe						Lys	1,5 - > 256	6-12	>32	3BLNAR; NT 1BLNAR; b 1BLPACR; NT

In this fraction of the *ftsI* gene, a variety of mutations were identified. The mutation patterns were categorized into 3 groups owing to diverse amino acid substitutions.

In Group I (n=3), His517 was substituted for Arg517. In Group II (n=36, including *H. influenzae* ATCC 49247), Lys526 was substituted for Asn526. In Group III (n=5), 3 amino acid substitutions were detected: Met377Ile, Ser385Thr and Leu389Phe in addition to the Asn526Lys substitution.

In each group a variety of other substitutions were detected (Table 3). We separated Group II into 4 subgroups. Subgroup IIa (n=4); *H. influenzae* ATCC 49247 is included in subgroup IIa. Subgroup IIb (n=18) is detected by the substitution of Val-502 for Ala-502. Extra substitutions are detected in subgroup IIb. These are the common substitutions in Group II. Subgroup IIc (n=9) is detected by the substitution of Thr-502 for Ala-502; supplementary substitutions were observed. Subgroup IId (n=5) is determined by the replacement of Val-449 for Ile-449. TEM type β-lactamase producing isolates were noted in all groups (I, IIa, IIb, IIc, IId, III).

Pulsed-Field Gel Electrophoresis (PFGE)

DNA from the 44 isolates and *H. influenzae* ATCC 49247 were investigated by PFGE. The profiles accomplished after

enzyme digestion displayed 10 to 12 fragments from 20 to 400 kb. The PFGE results demonstrate the heterogeneity of our isolates (Figure 2).

Figure 1 Alignment of PBP 3 amino acid sequences deduced from the sequences of the *ftsI* genes present in *H. influenzae* Rd and in *H. influenzae* strains with resistance to β-lactam antibiotics. Dots: identical amino acids. Conserved amino acid motifs: STVK, SSN, and KTG

DISCUSSION

H. influenzae is a specific bacterium that causes infections in children. The β-lactams resistance can include significant clinical implications. Two mechanisms are implicated in the β-lactams resistance of *H. influenzae*: The first one is the β-lactamase production and the second one is the decreased affinity of penicillin binding protein 3 (PBP 3A and PBP 3B) [14]. In this study, we defined the molecular mechanisms of the β-lactams resistance of 200 *H. influenzae* strains isolated from Tunisian children.

Following the introduction of the Hib vaccine (*Haemophilus influenzae* type b) in the vaccine schedule in Tunisia, Hib decreased significantly from 69%, according to the Smaoui and Kechrid [21], to 21.5% in the current study. This shows the success of the Hib vaccine in our country. This effectiveness is also shown in the literature [22].

Ampicillin resistance is a problem in Tunisia. In 2006, 28.3% of *H. influenzae* isolates were ampicillin resistant [21]. This rate is increasing continuously in our hospitals: 42.7% in 2009 [23], 44.92% in 2015 [24] and 45.5% as shown in the present study. In this work, *H. influenzae* isolates were categorized into 3 groups, according to their ampicillin resistance mechanisms: genetically defined β-lactamase positive, ampicillin-resistant (gBLPAR) strains (47; 23.5%), β-lactamase negative, ampicillin-resistant (gBLNAR) strains (25; 12.5%) and β-lactamase positive, amoxicillin-clavulanate-resistant (gBLPACR) strains (19; 9.5%).

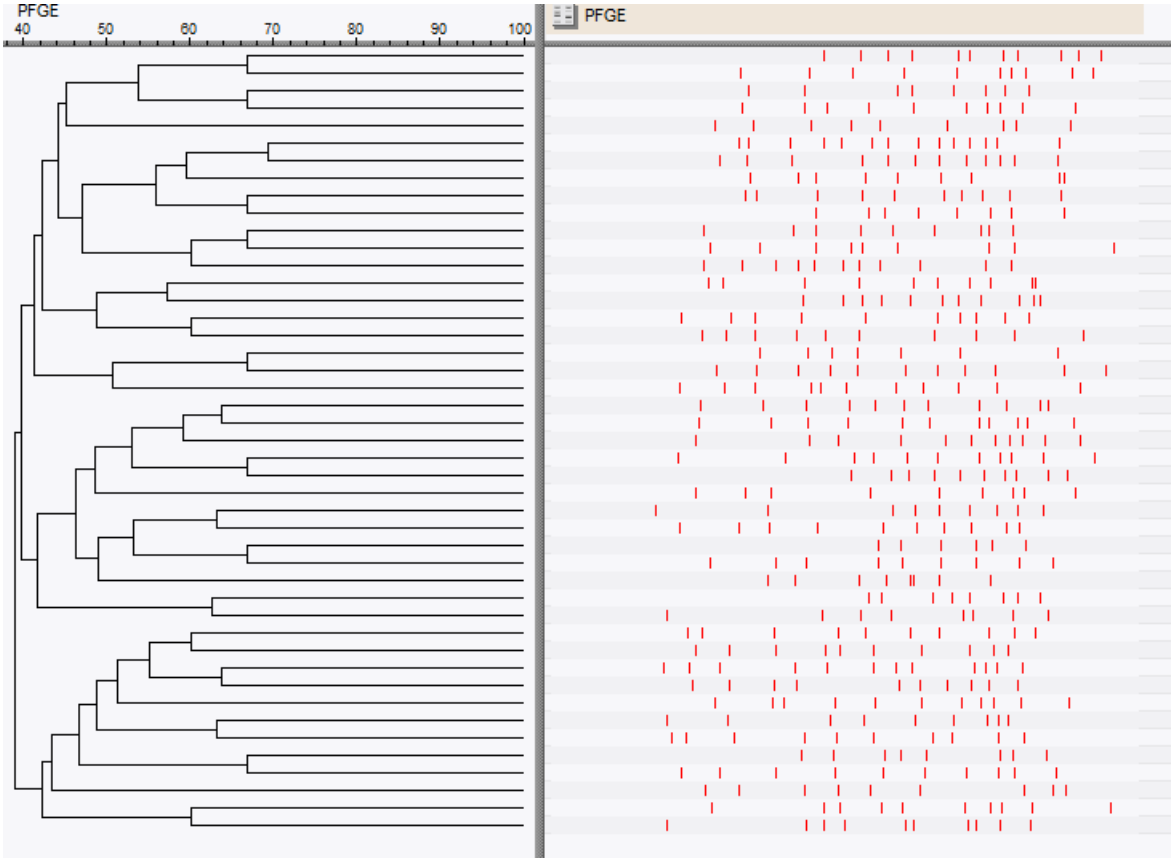

Figure 2 PFGE analysis dendrogram showing the genotypic relationship between *H. influenzae* strains with resistance to β-lactam antibiotics

The percentages of resistance showed in the present study results are considerably higher than those obtained in previous studies carried out in the same laboratory in Tunisia [21,23,24]. Indeed, in an earlier study carried out in our laboratory, the BLNAR strains accounted for only 1.6% of the strains [21]; they had a representation rate of 12.5% in this study. In 2006 only 3.2% of isolated strains have a superposition of the two resistance mechanisms [21] this percentage tripled in only 7 years to reach 9.5% as shown in the current study. This shows that strains of *H. influenzae* with modified PBP are becoming more abundant with a clear increase in the percentage of BLNAR and BLPACR strains, difficult to be detected with the traditional laboratory techniques. All the above-mentioned observations indicate that several antibiotics including ampicillin, amoxicillin and clavulanic acid will be less effective for treating diseases caused by this pathogen.

The activity of various antibiotics tested shows that cefotaxime, cefpodoxime and cefixime have the best activity in terms of MIC. 100% of strains are sensitive to these antibiotics at low MICs. This new generation of antibiotics constitutes a good alternative for ampicillin, amoxicillin, and clavulanic acid. Our results are in a good concordance with results obtained by Oueslati, et al. [23]. Our results showed also that cefuroxime and imipenem are less active regardless of the phenotype of the strains tested. Comparable results have been reported in Europe [25].

In *H. influenzae*, changes in PBPs result in an important level of non-enzymatic mediated β-lactam resistance. The major factors involved in β-lactam resistance are the modifications in two PBPs: PBPs 3A and 3B [14]. Amino acid substitutions in PBP 3 are implicated in β-lactam resistance in BLNAR strains [14].

Depending on the classification system proposed by Ubukata, et al. [14], the different amino acid substitutions observed found in the 44 strains (25 BLNAR and 19 BLPACR) enabled us to subdivide them into 3 groups (I, II and III). We separated group II into 4 subgroups according to Dabernat, et al. [15]. Subgroup IIa (n=4); *H. influenzae* ATCC 49247 is included in subgroup IIa. Subgroup IIb (n=18) is characterized by the substitution of Val-502 for Ala-

502. Extra substitutions are observed in subgroup IIb. These are the commonest substitutions in group II. Subgroup IIc (n=9) is determined by the substitution of Thr-502 for Ala-502; other substitutions were recognized in this work. Subgroup IId (n=5) is determined by the substitution of Val-449 for Ile-449. The substitutions at positions 377, 385 and 389 sited close to the Ser-Ser-Asn (SSN) motif was detected in Tunisian strains.

Beta-lactam resistance in *Haemophilus influenzae* due to low affinity of mutated PBP3 to penicillin is growing throughout the world. For example, nearly all Japanese strains (excluding one isolate) have a substitution of the Asn-350 to an Asp-350 furthermore in greater part of these strains include a substitution of Asn-357 for Ser-357 [14]. The French isolates with mutation in the *ftsI* gene, have diverse amino acid substitutions; No strains related to Group III were found, 63 of the 108 isolates had not any substitution at position 350; Only one strain, in Group IIb, had two substitutions at positions 350 and 357 [15]. In Portugal, among the 240 *H. influenzae* isolates, 141 showed mutations in the *ftsI* gene (in the transpeptidase domain of this gene), with a mastery of the BLNAR isolates (93.1%) and an elevated proportion of BLPAR isolates (58.8%). BLPAR isolates have been characterized as β-lactamase-positive amoxicillin+clavulanic acid resistant (BLPACR); the majority of amino acid substitutions were recognized in close proximity to the KTG motif. The 141 mutated Portuguese strains contained 31 different mutations. Basing on these mutations these strains were classified in 6 groups [26]. In Norway, Skaare, et al. demonstrated that the incidence of PBP3-mediated ampicillin resistance was 9% versus 2.5% before 3 years. Group II PBP3 dominated (96%), with 6% and 20% isolates non-susceptible to cefotaxime and meropenem respectively; Group III was recognized in Northern Europe in 2014 [27].

In this study, strains of *H. influenzae* represent an elevated level of heterogeneity (lack of clonality). Pulsed-field gel electrophoretic profiles of BLNAR isolates were extremely various in Japan [28]. The identical result was achieved in South Korea [29]. The diffusion of strains is clonal in further countries, mostly in Norway [27] and Canada [30].

CONCLUSION

The diversity of amino acid substitutions in strains isolated from our hospital and the occurrence of mutations reported constitute an original situation. Our study showed that Tunisian strains are particularly resistant to cefuroxime and imipenem. The inappropriate use of antibiotics for the treatment of *H. influenzae* infections seems to be responsible for the selection of BLNAR and BLPACR strains which are in perpetual increase in our country.

DECLARATION

Conflict of Interest

The authors and planners have disclosed no potential conflicts of interest, financial or otherwise.

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