

Full Length Research Paper

***Haemophilus influenzae* strains in children: Increasing resistance to Beta-lactam antibiotics**

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The aim of this study was to determine the bacteriological characteristics of *Haemophilus influenzae* (Hi) strains isolated from children, namely serotyping, biotyping and their antibiotic susceptibilities specifying the mechanisms and the β -lactams resistance genes. This study made use of 138 Hi strains isolated from 2009 to 2010 at the Microbiological Laboratory of the Children's Hospital in Tunisia. Antimicrobial susceptibility for all Hi isolates was determined as recommended by the CA-SFM (Comité de l'antibiogramme de la Société Française de Microbiologie). Beta-lactam resistance genes (*bla*_{TEM}, *bla*_{ROB} and *ftsI*) were detected by PCR (Polymerase Chain Reaction) as well as their capsular genes (*bexA*). Ampicillin resistance was 44.92% (62/138) of Hi isolates. PCR showed that all of the strains were identified as non-capsulated. These isolates were subdivided into 3 groups according to the ampicillin resistance molecular mechanisms as follows: the group of β -lactamase positive ampicillin resistant isolates (BLPAR) (22.4%), the group of the β -lactamase negative ampicillin resistant strains (BLNAR) (18.1%) and finally the group of the β -lactamase positive amoxicillin-clavulanate resistant strains (BLPACR) (4.3%). All isolates showed high amoxicillin, cefuroxime and imipenem MICs (Minimum Inhibitory Concentration). Among these, the less active one was imipenem. However, cefotaxime, cefixime and cefpodoxime were the most active agents tested against our strains. Finally, we confirmed the dissemination of BLNAR and BLPACR Hi strains with incremental increases in beta-lactam resistance. These strains were previously rare in Tunisia.

Key words: Beta-lactams resistance, *Haemophilus influenzae*, polymerase chain reaction (PCR).

ABBREVIATIONS: **AMC:** amoxicillin-clavulanate; **AMP:** ampicillin; **β -lactam:** Beta-lactam; **BLNAR:** β -lactamase negative ampicillin resistant strains; **BLPACR:** β -lactamase positive amoxicillin-clavulanate resistant strains; **BLPAR:** β -lactamase positive ampicillin resistant strains; **CTX:** cefotaxime; **GM:** gentamicin; **H.i:** *Haemophilus influenzae*; **K:** kanamycin; **MIC:** minimum inhibitory concentration; **NAL:** nalidixic acid; **NTHi:** non-typable *Haemophilus influenzae*; **PBP3:** penicillin binding protein 3; **PCR:** polymerase chain reaction; **RA:** rifampin.

INTRODUCTION

Haemophilus influenzae (Hi) is a human-restricted pathogen which forms part of the normal nasopharyngeal flora. Non-capsulated *H. influenzae*, also known as nontypeable *H. influenzae* (NTHi) are found as both respiratory tract commensals and respiratory and invasive pathogens. This organism can be non-capsulated [also known as nontypeable (NTHi)] or classified into 6 serotypes (a to f) on the basis of capsular polysaccharide characteristics. *H. influenzae* type b (Hib) is the most virulent serotype. Before routine vaccination was implemented, Hib was responsible for invasive *H. influenzae* infections, mainly in children. Routine

immunization with Hib vaccine, however, has reduced the incidence of invasive Hib disease worldwide. In Tunisia, Hib conjugate vaccine was introduced into the routine childhood immunization programme in 2002, then stopped for reasons of costs. It was re-introduced in 2011.

The resistance of this microbe to beta-lactam

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antibiotics can have important clinical implications. This bacterium can acquire ampicillin resistance through two different mechanisms. The first mechanism is frequently related to β -lactamase production (Fareel et al., 2005). The second mechanism is related to the decreased affinity of penicillin binding protein 3 (PBP3) (Ubukata et al., 2001). Laboratory detection of these mechanisms of resistance, especially in strains showing both of them, may be difficult using routine methods.

In this paper, we presented bacteriological characteristics of *H. influenzae* strains isolated from Tunisian children between January 2009 and August 2010 at the Children's Hospital of Tunis, namely: serotyping, biotyping and their antibiotic susceptibilities. We specified also the molecular mechanisms of β -lactams resistance by amplification of *bla*_{TEM-1} gene and *bla*_{ROB-1} gene encoding types TEM-1 and ROB-1 beta-lactamases respectively, as well as the amplification of the *ftsI* gene, encoding the transpeptidase domain of PBP3 (penicillin-binding protein 3).

MATERIALS AND METHODS

Strains

This work included 138 *H. influenzae* strains isolated between January 2009 and August 2010 at the Children's Hospital Béchir Hamza of Tunis (CHBH). Strains were isolated from different clinical samples. *H. influenzae* ATCC 49247 (Ampicillin-resistant, β -lactamase negative), *H. influenzae* C425 (*bla*_{TEM-1} positive), *H. influenzae* C322 (*bla*_{ROB-1} positive) and *H. influenzae* ATCC 10211 (Strain with capsular type b) were used as controls. All these strains were kindly provided by Professor H. Dabernat (Toulouse, France). However, *H. influenzae* strains were stored at -80°C for subsequent testing.

Bacterial identification

H. influenzae strains were identified using colony morphology, Gram staining and the X and V factors (Oxoid) requirement. Serotyping was determined by slide agglutination method using polyvalent and specific a to f antisera (Difco). Biotyping was performed using Api 10 S (bioMérieux).

Antimicrobial susceptibility

Disk diffusion method

Antimicrobial susceptibility of all *H. influenzae* strains were determined by disk diffusion method on chocolate agar containing polyvitex (Oxoid). We used an inoculum of 0.5 McFarland diluted to one tenth following CA-SFM guidelines (CA-SFM.2012). Plates were incubated for 24 h at 37°C under 5% CO₂. Antibiotics tested were ampicillin (AMP) (concentration: 2 μ g), amoxicillin-

clavulanate (AMC) (20+10 μ g), cefotaxime (CTX) (30 μ g), kanamycin (K) (30 UI), gentamicin (GM) (15 μ g), rifampin (RA) (30 μ g) and nalidixic acid (NAL) (30 μ g). β -lactamase production was examined using a nitrocefin-impregnated disk (Oxoid). We determined reduced susceptibility to ampicillin using a 2 μ g ampicillin disc. All beta-lactamase negative strains with a diameter less than 20 mm around ampicillin disc were classified as beta-lactamase negative ampicillin resistant (BLNAR).

E-Test method

In this study, the minimum inhibitory concentration (MIC) of amoxicillin, amoxicillin-clavulanate, cefuroxime, cefpodoxime, cefotaxime and imipenem was determined by E-test (AB-BIODISK) method (CA-SFM.2012). Medium used was chocolate agar containing polyvitex (Oxoid). After an overnight incubation at 37°C under 5% CO₂, the MIC was defined as the lowest concentration of antibiotic that inhibited bacterial growth.

DNA extraction

After 24 h of growth on chocolate agar containing polyvitex, 2 to 3 colonies of *H. influenzae* were suspended in 600 μ l of sterile distilled water and then boiled at 100°C for 5 min. The suspension was centrifuged for 20 min at 12500 rpm. The supernatant containing bacterial DNA was stored at -20°C for further use.

PCR-based genotyping

PCR amplification (primers, reaction mixture and PCR cycling) was carried out as described previously. PCR amplification of *p6* gene, which encodes the P6 outer membrane protein specific for *H. influenzae* was used to confirm phenotypic identification of *H. influenzae* (Nelson et al., 1988).

We used PCR amplification of *bexA* gene, responsible for transporting capsular material, and the type b gene encoding the serotype b capsule to confirm strains serotypes (Falla et al., 1994), after which we determined beta-lactams mechanisms of resistance by amplification of *bla*_{TEM-1} gene and *bla*_{ROB-1} gene encoding types TEM-1 and ROB-1 beta-lactamases respectively (Dabernat et al., 2002). Also the *ftsI* gene, encoding the transpeptidase domain of PBP3 (penicillin-binding protein 3), was amplified in all *H. influenzae* strains (Ubukata et al., 2001; Dabernat et al., 2002). The strains which had altered penicillin-binding proteins are *fts I* (-), characterized by the absence of the *fts I* gene. However, PCR products were visualized on a 2% agarose gel electrophoresis stained with Redsafe (Promega).

RESULTS

During January 2009 and August 2010, 138 *H. influenzae*

strains were isolated. Among them, 62 were ampicillin resistant (44.92%). The majority of the strains were non invasive. Only one strain was invasive and isolated from blood culture. On the basis of ornithine decarboxylase, urease and indole activities, the strains used for this study were classified into eight biotypes. Biotype III was the most frequent followed by biotype II.

Phenotypic identification using X and V factors of all isolates had been confirmed by molecular methods. So, all our isolates had positive results for the *p6* gene, which encodes the P6 outer membrane protein specific for *H. influenzae*.

All isolates were identified as non-typeable by slide agglutination. This result was confirmed using the *bexA* PCR amplification. So, all isolates were non capsulated because they gave a negative result in these PCRs. No strain was identified as b negative. In other words, b negative strains were positive for the b capsular gene but negative for the *bexA* gene (Falla et al., 1994).

Among the *H. influenzae* isolates, 26% (37/138) were beta-lactamase positive. Our strains were susceptible to cefotaxime, gentamicin and nalidixic acid (Figure 1). According to their phenotypic and genotypic patterns, the *H. influenzae* strains were subdivided into three groups. Thirty-one strains (22.4%) produced β -lactamase TEM-1 type [*bla*_{TEM-1} (+)] and were characterized by the presence of the *fts I* gene [*fts I* (+)] (genetically defined β -lactamase positive, ampicillin-resistant, gBLPAR). gBLPAR strains are only resistant to ampicillin but susceptible to amoxicillin-clavulanate. No strain was positive for the *bla*_{ROB-1} gene. Twenty-five strains (18.1%) were characterized by the absence of the *fts I* gene [*fts I* (-)] (The *fts I* PCR system detects resistant mutations of *fts I*). The negative result of PCR means that this strain shares low-level-resistant mutation(s) in PBP, but not defection of this gene, and were beta-lactamase negative (genetically defined β -lactamase negative, ampicillin-resistant, gBLNAR). Six strains (4.3%) produced β -lactamase [*bla*_{TEM-1} (+)] and were also characterized by the absence of the *fts I* gene [*fts I* (-)] (genetically defined β -lactamase positive, amoxicillin-clavulanate-resistant, gBLPACR). gBLPACR strains are resistant to both ampicillin and amoxicillin-clavulanate.

Amoxicillin MIC ranges were different between gBLPAR, gBLNAR and gBLPACR strains. The gBLNAR strains had relatively low MICs of amoxicillin (1.5 - 2 mg/l) compared with gBLPAR (4 - >256 mg/l) and gBLPACR (>256 mg/l) which had high resistance levels following CA-SFM guidelines (CA-SFM.2012). The clavulanate has restored the activity of amoxicillin for the gBLPAR strains (0.5-4 mg/l), but in gBLNAR strains, MICs of amoxicillin-clavulanate was as higher as the MICs of amoxicillin. The decreased susceptibility to amoxicillin was accompanied by a decreased susceptibility at different levels to other β -lactam antibiotics (Table 1). This study describes relatively high MICs of cefuroxime in gBLNAR strains (0.5 - 6 mg/l) and β -lactamase positive including isolates with

both resistance mechanisms (2 - 4 mg/l). Imipenem resistance in *H. influenzae* is studied for the first time in this paper at the Children's Hospital of Tunis. Although imipenem MICs were high (>32 mg/l) in all strains (gBLNAR, gBLPAR and gBLPACR), cefotaxime, cefixime and cefpodoxime were the most active agents tested with low MICs in all *H. influenzae* groups.

DISCUSSION

H. influenzae is a human specific pathogen that causes respiratory infections in both children and adults. The β -lactams resistance can have important clinical implications. Two primary mechanisms are implicated in the resistance of *H. influenzae* to β -lactams: β -lactamase production (Farrell et al., 2005) and decreased affinity of penicillin binding protein 3 (PBP3) (Ubukata et al., 2001). In this paper, we specified the molecular mechanisms of the β -lactams resistance of *H. influenzae* strains isolated from Tunisian children between January 2009 and August 2010 at the Children's Hospital of Tunis to help the antibiotic therapy in our hospital.

In this work, PCR amplification of *p6* gene, which encodes the P6 outer membrane protein specific for *H. influenzae* was used to confirm phenotypic identification of *H. influenzae*. All strains were positive for the *p6* gene. Although some early studies use P6 PCR to identify *H. influenzae*, recent studies (Abdeldaim et al., 2009) show that it is unable to differentiate *H. influenzae* from *H. haemolyticus*. So, some of our isolates may have been *H. haemolyticus*.

All isolates were identified as non-typeable by slide agglutination method. In fact, all isolates were isolated from respiratory infections. In Korea, the majority of the strains were non capsulated (Bae et al., 2010). The situation is the same in Spain (Puig et al., 2014).

Serotyping using slide agglutination is a phenotypic typing method which is sometimes insufficient because of a lack of sensitivity and specificity. PCR can prevent these errors. In our study, we have a total correlation between these two methods. However, Maria et al. (2006) demonstrate the limitations of slide agglutination for *H. influenzae* serotyping in comparison with the results provided by PCR.

Ampicillin resistance is a significant problem in our country. This resistance concern 28.3% of *H. influenzae* isolates in 2006 (Smaoui and Kechrid, 2006). This rate is increasing continually in our hospital: 42.7% in 2009 (Oueslati et al., 2009) and 44.92% in the present study. Ampicillin resistance varies also from one country to another, whereas ampicillin resistance reaches 58.5% in Korea (Bae et al., 2010).

H. influenzae can acquire ampicillin resistance through two different mechanisms. The major one has been considered to be related to the β -lactamase production. In this report, 37 isolates were β -lactamase positive with an overall rate of 26%. Beta-lactamase production varies

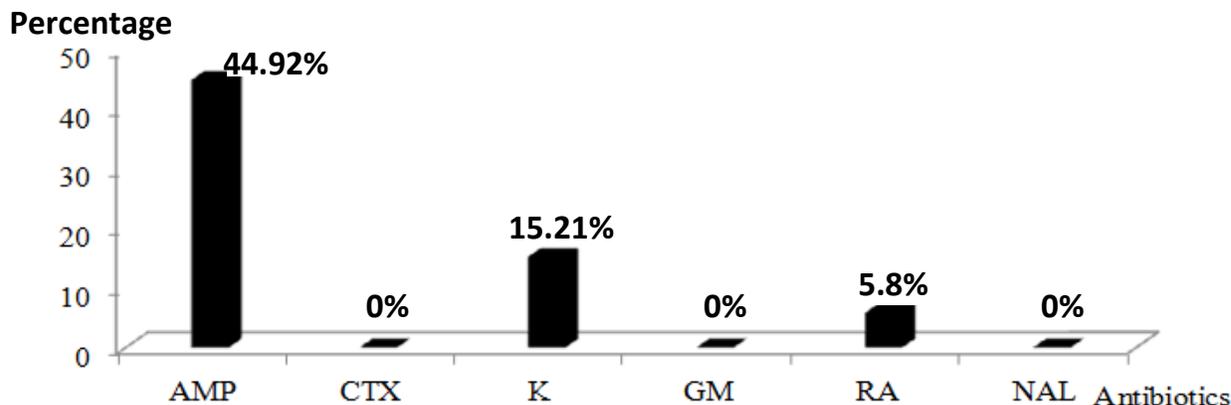


Figure 1. Antimicrobial susceptibility of *H. influenzae* strains.

Note: AMP: ampicillin, CTX: cefotaxime, K: kanamycin, GM: gentamicin, RA: rifampin, and NAL: nalidixic acid.

from one country to another: 16.7% in Canada, 31.6% in France, 5% in Italy and 27.5% in the United States (Farrell et al., 2005). All of the 37 isolates in our study were positive for the *bla*_{TEM-1} gene. No ROB-1 type β -lactamase is found in our study nor in previous reports in our country (Smaoui and Kechrid, 2006; Oueslati et al., 2009). The distribution of TEM-1 and ROB-1 beta-lactamases varies widely from one country to another: The prevalence of TEM-1 among the β -lactamase positive *H. influenzae* strains is 97.5% in Brazil, 98% in France, 88% in Canada and 85.8% in USA (Farrell et al., 2005). ROB-1 is rarely found outside North America (Farrell et al., 2005). Kim et al. (2007) confirms for the first time in Korea the presence of *H. influenzae* strains carrying *bla*_{ROB-1} genes.

In this work, *H. influenzae* isolates were subdivided into 3 groups, according to their ampicillin resistance mechanisms: genetically defined β -lactamase positive, ampicillin-resistant (gBLPAR) strains, β -lactamase negative, ampicillin-resistant (gBLNAR) strains and β -lactamase positive, amoxicillin-clavulanate-resistant (gBLPACR) strains. The prevalence of gBLPAR strains is 22.4% (31/138). This percentage is lower than that of a previous study reported in our hospital (32.48%) (Oueslati et al., 2009). In Korea, 22.6% of *H. influenzae* strains are for gBLPAR strains (Bae et al., 2013); the percentage is 15.8% in Spain (Garcia et al., 2007). The gBLNAR strains are detected in 18.11% of all strains. This rate was lower (7%) in a previous study in Tunis (Oueslati et al., 2009). The increase in the percentage of gBLNAR strains may cause serious therapeutic problems. The prevalence of gBLNAR strains has rapidly increased in some countries such as Japan (Hotomi et al., 2007), whereas, the incidence of gBLNAR *H. influenzae* is low in Korea (Bae et al., 2010). BLNAR strains are uncommon in Australia, while gBLNAR strains are more common than previously recognized (Witherden et al., 2011).

In this work, the incidence of gBLPACR is higher (4.3%) than in a previous report in Tunisia (3.18%) (Oueslati et al., 2009). The prevalence of the gBLPACR strains is 5.2% in Korea (Bae et al., 2010), 0.3% in Japan (Hotomi et al., 2007) and 10.4% in Spain (Garcia et al., 2007).

This study shows high frequencies of resistance in cefuroxime. Resistance to this antibiotic has been reported previously in our hospital (Oueslati et al., 2009) such as in Korea (Bae et al., 2010). For the first time at the Children's Hospital in Tunisia, all strains were resistant to imipenem. Therefore, imipenem resistance occurred in *H. influenzae* strains with or without beta-lactamase production. Imipenem resistant *H. influenzae* is one of the important issues in this manuscript. Generally, all ampicillin resistant *H. influenzae* strains are not abnormally highly resistant to carbapenems. MIC of another carbapenem such as meropenem should be able to clarify imipenem resistant strains in this study. Further studies are necessary to elucidate the resistance to imipenem among *H. influenzae* strains in this study. *H. influenzae* isolates with imipenem resistance have been observed to be very rare mainly among beta-lactamase negative ampicillin resistant strains in some parts of the world such as the United States (Brown and Traczewski, 2005) and Japan (Sanbongi et al., 2006). In Spain, isolates of ampicillin-resistant *H. influenzae* causing invasive infections remain susceptible to imipenem (García-Cobos et al., 2014). Resistance to imipenem was reported previously in neutropenic patients in a bone marrow transplant center of Tunisia (Touati et al., 2009).

Cefotaxime, cefixime and cefpodoxime are considered to be an important treatment against *H. influenzae* infections. These antibiotics were also the most potent agents tested against *H. influenzae* isolates in a previous report in Tunisia (Oueslati et al., 2009). The situation is quite different from one country to another. In Thailand,

Table 1. MICs of 7 β -lactams antibiotics for the *H. influenzae* strains classified into 3 groups according to the PCR results.

| Strain | Amoxicillin | | | Amoxicillin-clavulanate | | | Cefuroxime | | | Cefotaxime | | | Cefixime | | | Cefpodoxime | | | Imipenem | | |
|----------------|-------------|--------|-----------|-------------------------|--------|-----------|------------|--------|-----------|------------|--------|-------------|----------|--------|--------------|-------------|--------|------------|----------|--------|-----------|
| | MIC 50 | MIC 90 | MIC range | MIC 50 | MIC 90 | MIC range | MIC 50 | MIC 90 | MIC range | MIC 50 | MIC 90 | MIC range | MIC 50 | MIC 90 | MIC range | MIC 50 | MIC 90 | MIC range | MIC 50 | MIC 90 | MIC range |
| gBLPAR (N*=31) | >256 | >256 | 4- >256 | 2 | 2 | 0.5-4 | 3 | 3 | 0.125-4 | 0.094 | 0.094 | 0.008-0.094 | 0.032 | 0.032 | <0.016-0.032 | 0.25 | 0.25 | 0.032-0.25 | >32 | >32 | 0.5- >32 |
| gBLNAR (N=25) | 2 | 2 | 1.5-2 | 2 | 2 | 1.5-4 | 6 | 6 | 0.5-6 | 0.064 | 0.064 | 0.008-0.064 | 0.047 | 0.047 | 0.016-0.047 | 0.25 | 0.25 | 0.025-0.25 | >32 | >32 | 2- >32 |
| gBLPACR (N=6) | >256 | >256 | >256 | 6 | 6 | 4-8 | 4 | 4 | 2-4 | 0.047 | 0.047 | 0.016-0.047 | 0.032 | 0.032 | 0.016-0.032 | 0.25 | 0.25 | 0.125-0.25 | >32 | >32 | >32 |

N*: number of strains.

all *H. influenzae* isolates were susceptible to cefotaxime, 5% was non-susceptible to cefuroxime (Intakorn et al., 2014). In Spain, Garcia-Cobos et al. (2008) report that the majority of *H. influenzae* isolates are classified as susceptible to cefuroxime, cefotaxime and cefixime, whereas in Korea, resistance to cefixime, cefuroxime among *H. influenzae* strains is reported (Kim et al., 2007).

All of the isolates were susceptible to gentamicin and nalidixic acid. This result was obtained previously in this hospital (Smaoui and Kechrid, 2006). However, resistance to kanamycin occurred in 15.21% of cases respectively. Among the kanamycin resistant strains in this report (21 strains), 13 were β -lactamase positive. In 2003, 26% of the strains were kanamycin resistant with 98% of the strains producing β -lactamase (Dabernat, 2006).

Finally, we confirmed the dissemination of gBLNAR and gBLPACR *H. influenzae* strains with incremental increases in beta-lactam resistance at the Children's Hospital of Tunis. These strains were previously rare in Tunisia. That is why it seems important to monitor the evolution of the mechanisms of resistance among the *H.*

influenzae isolates in our country because the emergence of these strains may have therapeutic implications.

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