



Ampicillin Resistance in *Haemophilus influenzae* Isolated from Acute Respiratory Infections in Pediatrics

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Abstract: *Haemophilus influenzae* is a bacterium that can cause severe infections, occurring mostly in infants and children younger than five years of age. Antibiotic treatment may cause the emergence of resistant *H. influenzae* strains, particularly ampicillin-resistant strains. Antimicrobial resistance is a public health threat worldwide, particularly in the developing world. *H. influenzae* strains have been isolated from broncho-alveolar lavages (BALs), nasopharyngeal swabs, and otitis media from children in two paediatric centers at Dakar, Senegal. Antibiotic susceptibility testing was carried out using strips E Test[®] method that provides the ability to precisely determine the minimum inhibitory concentration (MIC). A total of 16 *H. influenzae* strains have been isolated and identified, including 16.7% of ampicillin-resistant patterns (all β -lactamase-negative), 9.4% of the isolates were resistant to cefaclor (MIC₉₀ = 16 μ g/ml) while 100% were susceptible to cefixime to (MIC₉₀ = 0.38 μ g/ml). Interestingly, fluoroquinolones were fully active with very low MIC₉₀. Macrolide were still active against *H. influenzae* isolates although with higher MIC azithromycin MIC₉₀ = 3 μ g/ml, clarithromycin MIC₉₀ = 12 μ g/ml. Ampicillin-resistance has become increasingly reported in *H. influenzae*, suggesting a continuous laboratory based surveillance for antimicrobial resistance pattern for a better management of acute respiratory infections, particularly in low incomes settings.

Keywords: Respiratory Tract Infections, *Haemophilus influenzae*, Ampicillin Resistance

1. Introduction

Haemophilus influenzae is a major community-acquired pathogen causing significant morbidity and mortality worldwide [1]. It is one of the most important bacterial pathogens of pediatric infection [2] and can cause a severe infection, occurring mostly in infants and children younger than five years of age. Pneumonia and meningitis are the

most frequent manifestations. An estimated 3 million cases of meningitis and severe pneumonia and approximately 386,000 deaths occur every year worldwide in children below the age of five years due to type b *H. influenzae* (Hib) [3]. Antibiotic treatment may cause the emergence of resistant *H. influenzae* strains, particularly ampicillin-resistant strains [4]. Ampicillin (AMP) resistance in this organism is due to two well-known mechanisms. One is

resistance mediated by the production of TEM-1 and ROB-1, β -lactams, and the other is decreasing affinity of AMP for penicillin-binding proteins (PBPs) involved in peptidoglycan synthesis. Strains with resistance due to the latter mechanism are termed β -lactamase-negative AMP-resistant (BLNAR) *H. influenzae* [5]. Antibiotics resistance is an issue of great significance for public health at the global level. However, it is of particular concern in limited resources settings.

The objective of this study was to assess the susceptibility of Ampicillin (AMP) resistance profile of *H. influenzae* strains isolated from ARI's cases in children younger than 5 years of age in Dakar.

2. Methods

2.1. Sample Collection

H. influenzae strains were isolated between January 2015 and January 2016, from the Paediatric Department of Abass Ndao University Teaching Hospital and Paediatric Department of Roi Baudouin Hospital in Dakar (Senegal). The strains were isolated from broncho-alveolar lavages (BAL), nasopharyngeal swabs and middle ear secretion in children younger than 5 years of age.

2.2. Isolation and Culture of *H. influenzae*

The samples were cultivated on chocolate agar medium addition by an antibiotic a bacitracin for isolation *H. influenzae*. *H. influenzae* are required X (hemine) and V (NAD) growth factors, present in chocolate agar. Therefore, isolation from clinical specimens on solid medium requires the use of chocolate agar or other X and V factor supplemented media.

H. influenzae strains were identified if the bacterial load was at least 10^5 CFU/ml.

2.3. Identification of *H. influenzae*

H. influenzae was identified by its macroscopic aspects in culture (such as the growth of tiny, moist, and smooth gray colonies) as well as biochemical characteristics: absence of hemolysis, positive catalase and oxidase tests, growth in simultaneous presence of X and V factors, satellite growth

around streaks of *Staphylococcus aureus*, and other biochemical features using API NH[®] galleria (BioMerieux, La Balme-les-Grottes, France).

2.4. Antibiotic Susceptibility Testing

The antibiotic susceptibility was carried out using strips E-Test[®] (AB biodisk, Solna, Sweden) and minimum inhibitory concentration (MIC) was determined according European Committee of susceptibility testing guideline (EUCAST). Bacterial suspensions were diluted to obtain a final concentration of 10^5 CFU/ml (an optical density of 0.5 on the McFarland scale), and inoculated on *Haemophilus* test medium according to EUCAST guidelines 2015. Ampicillin, amoxicillin/clavulanic acid, cefuroxime, cefixime, azithromycin, clarithromycin, levofloxacin, ofloxacin and sulfamethoxazole/trimethoprim were tested. The quality control for antimicrobial susceptibility testing was performed using the ATCC 49247 strain of *H. influenzae*.

2.4.1. Beta-lactamase Tests

β -lactamase production of *H. influenzae* isolates was determined using a nitrocefin-based test (Cefinase, Becton Dickinson Microbiology Systems, and Cockeysville, Md).

2.4.2. Analysis of Results

The geometric mean values of MIC₅₀ and MIC₉₀ obtained from the antibiotic susceptibility testing were calculated and analyzed using the Whonet 5.6 software (WHO Collaborating Centre for Surveillance of Antimicrobial Resistance, Boston, Massachusetts).

3. Results

A total of 16 strains of *H. influenzae* were isolated and identified from 150 samples collected from children under 5 years of age. These strains of *H. influenzae* were tested for antibiotics susceptibility.

3.1. Antibiotic Susceptibility Testing

Table 1 summarizes the results of the susceptibility testing of *H. influenzae* to commonly used antibiotics in ARIs treatment.

Table 1. Antibiotics Susceptibility of *Haemophilus Influenzae*.

Antibiotics	Critical Values	%R	%I	%S	MIC ₅₀	MIC ₉₀	Range μ g/ml
Ampicillin	S \leq 1 R \geq 4	16.7	16.7	66.7	1	4	0.064 - 16
Amoxicillin/clavulanic acid	S \leq 4 R \geq 8	0	0	100	.75	1.5-	0.125 - 2
Cefuroxime	S \leq 4 R \geq 16	0	0	100	1	4	0.125 - 4
Cefaclor	S \leq 8 R \geq 32	9.4	3.1	87.5	3	16	0.125 - 48
Cefixime	S \leq 1	0	0	100	.064	.38	0.016 - 0, 38
Ciprofloxacin	S \leq 1	0	0	100	.016	.047	0.003 - 1-
Levofloxacin	S \leq 2	0	0	100	.012	.023	0.002 - 0.75
Sulfamethoxazole/trimethoprim	S \leq .5 R \geq 4	100	0	0	32	32	0.75 - 32
Azithromycin	S \leq 4	0	0	100	1	3	0.032 - 4
Clarithromycin	S \leq 8 R \geq 32	0	18.8	81.2	6	12	0.032 - 16

3.2. Susceptibility to β -lactam Antibiotics

All strains *H. influenzae* isolates were susceptible to amoxicillin/clavulanic acid ($MIC_{90} = 1.5 \mu\text{g/ml}$), and cephalosporins (cefixim $MIC_{90} = 0.38 \mu\text{g/ml}$; cefuroxim $MIC_{90} = 4 \mu\text{g/ml}$). However, resistance patterns to cefaclor (9.4%), and BLNAR (16.7%) were detected.

The Cefinase test used for detection of *H. influenzae* β -lactamase producing strains was negative.

3.3. Susceptibility to Fluoroquinolones

Fluoroquinolones were fully active (100%) with very low MIC_{90} for ciprofloxacin: $MIC_{90} = 0.047 \mu\text{g/ml}$ and levofloxacin: $MIC_{90} = 0.023 \mu\text{g/ml}$.

3.4. Susceptibility to Macrolides

The results showed that macrolides like azithromycin ($MIC_{90} = 3 \mu\text{g/ml}$) and clarithromycin ($MIC_{90} = 12 \mu\text{g/ml}$) had good activity for *H. influenzae* strains with respectively 100% and 81.2%.

3.5. Susceptibility to Sulfamethoxazole/Trimethoprim

All strains were resistant to sulfamethoxazole / trimethoprim (100%).

4. Discussion

H. influenzae is an important pathogen able to cause a wide spectrum of diseases in children [6]. Infections due to *H. influenzae* are usually treated with β -lactam antibiotics including aminopenicillins or cephalosporins [7]. These last years the emergence of resistant strains is questioning the classic antibiotic treatment. This resistance mainly concerns β -lactams in particular aminopenicillins [8].

In this study, 16.7% and 9.4% of isolates showed resistance to ampicillin and cefaclor, respectively. While 16.7% of *H. influenzae* strains were intermediate susceptible to ampicillin. All strains were susceptible to cefixime, cefepime, cefuroxime as well as to the association amoxicillin/clavulanic acid. In the genus *Haemophilus*, the most common mechanism of ampicillin resistance is the production of the TEM-1 β -lactamase [9]. However, recently, reduced susceptibility to ampicillin without β -lactamase production (BLNAR phenotype) has become widespread in *H. influenzae* [7]. High prevalence rate (46%) of ampicillin-resistant *H. influenzae* strains had been reported in 2000 in Turkey [10].

In 2007, Uncu *et al.* reported 3.2% of resistance rate among *H. influenzae* isolates [11]. Susceptibility to all β -lactams in *H. influenzae* is generally predicted by susceptibility to ampicillin as defined by the CLSI, MIC breakpoints, which are as follows: 1 $\mu\text{g/ml}$, susceptible; 2 $\mu\text{g/ml}$, intermediate; and 4 $\mu\text{g/ml}$, resistant [12]. For *H. influenzae* β -lactamase production was the primary reason for the high rates of resistance associated with ampicillin. In this study, non- β -lactamase producing strains were detected.

Our findings are in disagreement with data reported from previous study in Dakar between 2005 and 2008, showing β -lactamase production in all ampicillin-resistant *H. influenzae* isolates [13]. However, while some authors use the ampicillin-resistance breakpoint and absence of β -lactams to define BLNAR strains, others include ampicillin-intermediate strains as BLNAR strains. One international surveillance study of almost 3,000 strains from 1999 to 2000 showed an overall prevalence of 16.6% β -lactams-positive strains, ranging from as low as 3% in Germany to as high as 65% in South Korea, and an overall prevalence of only 0.07% for BLNAR [14]. In the SOAR study from 2002 to 2003, 4.5% of *H. influenzae* isolates from Turkey were β -lactamase positive [15]. Another study carried out by Sener *et al.* in 2007, performed in 379 isolates of *H. influenzae* of these, 5.5% produced β -lactamase and 4.7% were resistant to ampicillin ($MIC 0.2 \mu\text{g/ml}$). Among the β -lactamase producers, five isolates were found to be intermediate-resistant to ampicillin; two isolates were tested β -lactamase-negative and ampicillin-resistant (BLNAR). Increasing amino-penicillin resistance, usually occurring as the result of β -lactamase production, but also resistance at other antibiotics further underline the need for effective surveillance [15].

Respiratory tract infections caused by *H. influenzae* should be treated with an expanded-spectrum cephalosporin, with cefepime or chloramphenicol as alternatives [1]. In this present study, in addition to amoxicillin/clavulanic acid complex, all *H. influenzae* strains were susceptible to cefixime, cefepime, and cefuroxime. However, resistance patterns to cefaclor have been detected among isolates. In addition, emergence of cefotaxime and cefepime resistance had been reported in Spain among BLNAR *H. influenzae* strains in 2007 [16].

In this study, fluoroquinolones, including ciprofloxacin and levofloxacin, were fully actives on *H. influenzae* with very low $MIC_{90} = 0.47 \mu\text{g/ml}$ and $0.23 \mu\text{g/ml}$ respectively.

H. influenzae resistance to fluoroquinolones remains an exceptional event and was first described in 2003 [17]. In most published data, the incidence does not exceed 1% of strains. Fluoroquinolones are currently part of first-line treatments for community-acquired lung disease infections [18]. The main of fluoroquinolones resistance is due to mutations occurring in the *gyrA* mechanism and *parC* genes. Several DNA gyrase or topoisomerase IV mutations are required for *H. influenzae* to be resistant to fluoroquinolones.

The results of this study showed that all strains of *H. influenzae* were 100% susceptible to azithromycin with an MIC_{90} of 3 $\mu\text{g/ml}$ and 81.2% for clarithromycin with an MIC_{90} of 12 $\mu\text{g/ml}$. Cardines *et al.* reported in 2010, 10.1% of azithromycin resistance *H. influenzae* strains [19]. Among macrolides, azithromycin had the lowest MICs (0.25 to 2 $\mu\text{g/ml}$) [20]. In France in 2013, the resistance rate to macrolides was 29.9% compared to 50.8% in 2001. In most cases, this is an MLSB type resistance. Resistance by an active efflux, phenotype M affecting only C14 and C15 macrolides, concerns less than 5% of erythromycin-resistant

strains. Macrolide resistance remains most often associated with beta-lactam resistance [21]. A high rate (100%) of *H. influenzae* resistance to sulfamethoxazole/trimethoprim was observed contrasting therefore with result reported by Gueye et AL., in Dakar in 2009 [13]. In the study by Uncu et al. (2007) between 2005 and 2006, the resistance to trimethoprim-sulfamethoxazole, at *H. influenzae* as 25%. Ampicillin and trimethoprim-sulfamethoxazole are excluded from the treatment due to changing or increasing resistance rates [11].

5. Conclusion

The problem of ampicillin resistance became increasingly more frequent in *H. influenzae*. β -lactamase producing strains are more common in children. Emergence of antibiotic resistance is a serious challenge for the management of *H. influenzae* disease, with laboratory based surveillance for antimicrobial resistance. Continued surveillance for resistance and susceptibility testing of *H. influenzae* are crucial to maximize the benefits of antimicrobial therapy and to contain the spread of infection.

Conflict of Interest

There are no conflicts of interest.

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