RESISTANCE TO SEVERAL ANTIBIOTICS IN HAEMOPHILUS INFLUENZAE STRAINS

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ABSTRACT

Introduction: *H. influenzae is a bacterium that causes respiratory diseases principally in children. In this report, we determined the antibiotic susceptibilities of 200 H. influenzae strains isolated from children in Tunisia between 2010 and 2011.*

Materials and methods: *All strains were investigated by PCR to identify the resistance genes (bla_{TEM-1}, bla_{ROB-1}, and ftsI). Antibiotic susceptibility was controlled by disc diffusion. The antibiotics tested were ampicillin, amoxicillin-clavulanate, cefotaxime, kanamycin, gentamycin, rifampicin, pristinamycin, and nalidixic acid.*

Results: *Two hundred strains of H.influenzae were analyzed: 91 (were resistant to ampicillin (66 were beta-lactamase positive), 43 to kanamycin (39 were β-lactamase positive), 6 to gentamycin (all were β-lactamase positive), 23 to rifampicin (20 were β-lactamase positive) and 11 to pristinamycin (9 were β-lactamase positive). Strains demonstrating resistances to many antibiotics, according to their mechanism of resistance have been detected in this work: 24 strains were ampicillin- kanamycin resistant, 2 were ampicillin - gentamycin resistant, 5 were ampicillin - rifampicin resistant, 3 were ampicillin - pristinamycin resistant, 3 were kanamycin - pristinamycin resistant and 8 strains were kanamycin - rifampicin resistant. Cefotaxime and nalidixic acid were the most intense agents against our strains.*

Conclusion: *This work demonstrates an unexpected rise of resistances to several antibiotics in H. influenzae strains which show different mechanisms of resistance in children. Thus, the evaluation of new antibiotics for the treatment of multiply resistant Haemophilus influenzae diseases seems necessary.*

Keywords: *H. influenzae, antibiotic, resistance, PCR.*

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Introduction

H. influenzae is a human-specific pathogen that causes respiratory diseases in children. This bacterium is distinguished by colony morphology, gram staining, the growth on chocolate agar supplemented with polyvitex, and the requirement of X and V factors. H.influenzae has traditionally been characterized based on differences in the capsular polysaccharide (serotypes a to f).

H.influenzae can cause invasive and non-invasive diseases in children. Treatment of such diseases can be seriously influenced by antibiotic resistance. In H.influenzae, resistance to antibiotics, has, for various years, turn into a major issue(1).

H. influenzae can obtain ampicillin resistance through two mechanisms. One is the production of beta-lactamase, TEM-1, and ROB-1 types, which hydrolyze ampicillin enzymatically. Another is a conformational change in the penicillin-binding proteins (PBP3), enzymes in charge of peptidoglycan combination, which cause a diminished affinity to ampicillin(2). Strains that carry mutations in the ftsI gene affecting PBP3 were called beta-lactamase-negative ampicillin resistant H.influenzae strains. Wrong use of antibiotics may cause the spread of antibiotic resistant H.influenzae strains. This may have suggestions for the treatment of H. influenzae diseases in children. This report determines the antibiotic susceptibilities of 200 H. influenzae strains (β-lactamase positive ampicillin resistant strains, β-lactamase negative ampicillin resistant strains, and β-lactamase positive amoxicillin - clavulanic acid resistant strains) distinguished by PCR.

Materials and methods

Bacterial strains

Two hundred strains of H. influenzae were collected from children. All were isolated in the children's healing center of Tunis between August 2010 and December 2011. 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.

Culture strategies

Chocolate agar supplemented with polyvitex (bioMérieux) is used for the growth of Haemophilus influenzae, when incubated at 35-37°C in a 5% CO_2 air for 24 hours. H. influenzae is small, pleomorphic, gram-negative bacilli, or coccobacilli, and requires X (Hemin) and V (NAD) factor for growth.

Antimicrobial susceptibility testing

The antimicrobial resistance of all H. influenzae isolates was determined by disc diffusion with an inoculum of 0,5 Mc Farland on chocolate agar supplemented with polyvitex (bioMerieux)[3]. Impregnated paper disks, containing 2μg of amoxicillin (AMX), 20+10μg of amoxicillin + clavulanic acid (AMC), 30μg of cefotaxime (CTX), 30 UI of kanamycin (K), 15μg of gentamicin (GM), 30μg of rifampicin (RA), 30μg of nalidixic acid (NAL) and 15μg of pristinamycin (PT) were connected to the surface of the chocolate agar supplemented with polyvitex (bioMerieux). Plates were incubated for 24 to 48 h at 37°C in 5% CO_2.

Beta-lactamase production

β-Lactamase production was determined by the chromogenic cephalosporin test with nitrocephin (Oxoid) as a substrate.

PCR

PCR primers HI-I and HI-II (bexA gene) have been used to recognize capsulated H. influenzae isolates from non capsulated strains[4]. The bexA gene is in charge of transporting capsular material.

PCR amplification of the type b gene encoding the serotype b capsule is done for bexA (+) strain and bexA (-) strain[4]. Primers sets TEM and ROB were used to recognize the presence of blaTEM and blaROB in the strains giving a positive and negative cefinase test[5]. PCR amplification of the ftsI gene encoding the transpeptidase domain of PBP 3 was done with primers J1 and J2[2].

Amplification with these primers was performed in a volume of 10μl containing 50μM primers (each), 10mM dNTP, 25mM MgCl2, 2μl of 10X Tampon, 0,5U of Taq DNA polymerase and 0,4μl of DNA. PCR cycling was determined as described by Dabernat et al.[6].

Results

The bexA gene was amplified to recognize capsulated H.influenzae isolates from non capsulated ones. Most of the isolates were non capsulated (75%), they gave a negative result with bexA primers. 43 were of type b, 1 of type a, and 6 of types d, e and f. No strain was distinguished as b-. Two hundred strains of H. influenzae were analyzed: 91 (were resistant to ampicillin (66 were beta-lactamase positive), 3 to amoxicillin-clavulanate (low-level), 43 to kanamycin, 6 to gentamycin, 23 to rifampicin and 11 to pristinamycin (low-level) (Figure 1).

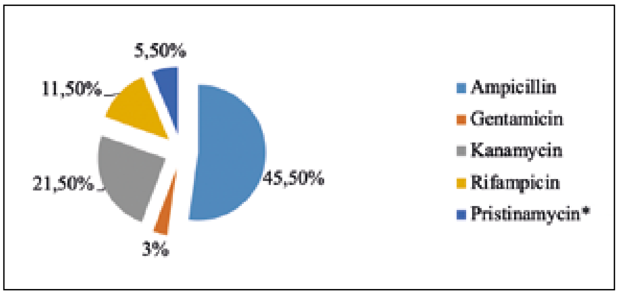

Figure 1: Antibiotic resistance in H.influenzae strains. *Pristinamycin*: low-level resistance.*

The H. influenzae strains were subdivided into 3 groups according to their mechanisms of resistance: strains which had transformations in the ftsI gene called β-lactamase negative, ampicillin-resistant, BLNAR, these strains were β-lactamase negative and negative for the ftsI gene); Strains producing β-lactamase TEM-1 type (bla_{TEM-1} (+)) and had no ftsI changes called β-lactamase positive, ampicillin-resistant, BLPAR and strains producing β-lactamase (bla_{TEM-1} (+)) and had transformations in the ftsI gene called β-lactamase positive, amoxicillin-clavulanate-resistant, BLPACR.

Table 1 demonstrates the prevalence of each antibiotic among the 200 strains according to their mechanisms of resistance.

Antibiotic	Genotype	% of resistance
Ampicillin	BLPAR BLNAR BLPACR	23,5 12,5 9,5
Kanamycin	BLPAR BLNAR BLPACR	18 2 1,5
Gentamycin	BLPAR BLNAR BLPACR	3 0 0
Rifampicin	BLPAR BLNAR BLPACR	10 1,5 0
Pristinamycin (low level resistance)	BLPAR BLNAR BLPACR	4,5 1 0

Table 1: Prevalence of each antibiotic among the 200 strains according to their mechanisms of resistance.

Strains demonstrating resistances to several antibiotics have been isolated for in this work: 24 strains (12%) were ampicillin-kanamycin resistant, 2 (1%) were ampicillin - gentamycin resistant, 5 (2.5%) were ampicillin - rifampicin resistant, 3 (1.5%) were ampicillin - pristinamycin resistant, 5 (2.5%) were kanamycin - pristinamycin resistant and 8 strains (4%) were kanamycin - rifampicin resistant. 2 strains (1%) were ampicillin-kanamycin-gentamycin resistant and 2 strains (1%) were ampicillin-kanamycin-rifampicin resistant. Table 2 demonstrates the multiple resistances to several antibiotics in strains according to their mechanisms of resistance.

Cefotaxime and nalidixic acid were the most powerful agents against our H. influenzae strains.

Resistance	Genotype	Number of strains
Ampicillin-Kanamycin	BLPAR BLNAR BLPACR	17 4 3
Ampicillin-gentamicin	BLPAR BLNAR BLPACR	2 0 0
Ampicillin-rifampicin	BLPAR BLNAR BLPACR	2 3 0
Ampicillin-pristinamycin	BLPAR BLNAR BLPACR	1 2 0
Kanamycin-pristinamycin	BLPAR BLNAR BLPACR	5 0 0
Kanamycin-rifampicin	BLPAR BLNAR BLPACR	7 1 0
Ampicillin-kanamycin-gentamycin	BLPAR BLNAR BLPACR	2 0 0
Ampicillin- kanamycin- rifamicin	BLPAR BLNAR BLPACR	1 1 0

Table 2: Multiple resistances to several antibiotics in strains according to their mechanisms of resistance.

Discussion

In this report, 150 strains (75%) were non capsulated and no strain was recognized as b-. In Venezuela, among H. influenzae isolates, 62.9% were non-capsulated and 31.4% were capsulated[7]. In England, 184 isolates underwent serotyping with 79% identified as non-capsulated Haemophilus influenzae and 3% were serotype b (Hib)[8].

Ampicillin resistance in H. influenzae strains is a difficult issue in our country. It's expanding persistently in our hospital: 42,7% in 2009[9] and 45,5% in this investigation. It varies from one country to another[1, 10, 11]. H. influenzae can acquire ampicillin resistance through two different mechanisms. The significant one is betalactamase production. In this report, 66 (33%) were β-lactamase positive. The distribution of this enzyme isn't the same out of my country[12]. Although most ampicillin-resistant H. influenzae strains could be appeared to produce beta-lactamase, 25 strains (12,5% of the isolates) were beta-lactamase negative in our study. Such strains are more frequent in Japan[13].

Today, H. influenzae strains in Tunisian's children are characterized by one drug resistance as well as by many antibiotic resistances. We are the first to examine the various resistances to antibiotics in multiply resistant H.influenzae strains as indicated by the mechanisms of resistance in children in Tunisia. Strains demonstrating different resistances to several antibiotics have been detected in this work: Ampicillin-kanamycin (12%), kanamycin-rifampicin (4%), ampicillin-rifampicin (2,5%), kanamycin-pristinamycin (2,5%), ampicillin-pristinamycin (1,5%), ampicillin-gentamycin (1%), ampicillin-kanamycin-gentamycin (1%), and ampicillin-kanamycin-rifampicin (1%). The rise of multiply resistant H. influenzae strains is a difficult issue in this nation. The uncontrolled and the widespread use of antimicrobials in Tunisia over the years assumed a part in the rise of these multiply resistant strains. The most frequent mechanism of resistance in these strains is beta-lactamase production followed by the alteration of PBP3. No strain appears to have multiple resistances to antibiotics by the relationship of the two mechanisms of resistance, beta-lactamase production, and PBP modification in this paper.

This study shows that in our country, cefotaxime and nalidixic acid should be used in light of their best activity against H. influenzae producing or not beta-lactamase. This study agrees well with those of past investigations in this hospital[9].

Conclusion

This work demonstrates an unexpected rise of resistances to several antibiotics in H. influenzae strains which show different mechanisms of resistance in children. Thus, the evaluation of new antibiotics for the treatment of multiply resistant Haemophilus influenzae diseases seems necessary.

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