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Molecular Characterization and resistance of *H. influenzae* isolated from Nasopharynx of Students in North Lebanon

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Abstract

Introduction: *Haemophilus influenzae* is an important cause of respiratory infections, including acute otitis media, sinusitis, and chronic bronchitis, which are preceded by asymptomatic *H. influenzae* colonization of the human pharynx. The aim of this study is to investigate the rate of *H.influenzae* nasopharyngeal colonization among students ages 2 to 3 years.

Material and methods: A total of 21 isolates of clinical *H. influenzae* were isolated from 87 nasopharyngeal specimens of children between April and June 2011. The isolates were identified by using molecular techniques (PCR), biotypes were determined by using the following tests: ornithin decarboxylase, urease and tryptophanase, and capsular typing was performed by SAST by using polyclonal and specific b antisera (Difco-BD®-USA).

The prevalence of β -lactams resistance, β-lactamase production, the level of macrolide resistance was recorded for each strain by using disc diffusion and E-test strip methods and chromogenic cephalosporin test (cefinase). β -lactams resistance genes (bla_{TEM} and bla_{ROB}) were determined using PCR.

Results: 42.8 % of the *H. influenzae* isolates were type b, and biotypes I, II and III were the majority, whereas biotypes IV, VI and VIII was not found. The majority of capsule type b was belonged to biotype II. Antibiotics susceptibility showed that 19% of the isolates were resistant to ampicillin and produced type TEM-1 β-lactamase.

Conclusion: This study shows the carriage rate of *H. influenzae* in North Lebanon children. The incidence of resistance rate of 19% to ampicillin signals an important warning to the future prophylaxis use of beta-lactam in treatment of *H. influenzae* infections in Lebanon.

Key words: *H.influenzae*, Antibiotic resistance, biotyping.

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Introduction

Haemophilus species constitute approximately 10% of the total bacterial flora in the human upper respiratory tract. *Haemophilus influenzae (H. influenzae)* is an opportunistic pathogen in humans that asymptomatically colonizes the pharyngeal mucosa (1).

In spite of the widespread use of the anti-*Haemophilus* b vaccine in the industrialized countries, and the decreased incidence of invasive *Haemophilus* diseases (2), *H. influenzae* remains a key organism causes infections in lungs, ear, nose, and throat in both adults and children. Most *Haemophilus* infections are caused by non-capsulated strains (3, 4). Following the introduction of *H. influenzae* type-b vaccination, more than 95% of *H. influenzae* disease was caused by type-b, but as the incidence of type-b decreases the relative importance of the other capsular type (a and c–f) and non-capsulated strains have increased (5). Treatment of *Haemophilus* infections can be severely affected by antibiotic resistance (6). *H. influenzae* was highly susceptible to all β-lactam antibiotics few decades ago.

The species of *H.influenzae* have been subdivided into 8 biotypes (biovars) on the basis of their biochemical reactions to urease, ornithin decarboxylase and indole production. In the late 1970s the use of amoxicillin has been decreased following the emergence of strains producing β-lactamases (7). In the 1980s, the 3rd generation cephalosporin provided an alternative that has always efficacy in treating local infections, particularly ear infections.

The percentage of β-lactamases producing *H.influenzae* strains ranged from 25% to 30% among respiratory isolates and nearly 40% of isolates were associated with ear infections. TEM-1 penicillinase which inactivates amoxicillin has been controlled by using clavulanic acid. But resistance developed by modifying the PBP (β-lactamases Negative Ampicillin Resistant strains; BLNAR) is also of concern as it has been found in 20% of non-capsulated strains isolated in Europe (8).

The aim of our study is to determine the colonization rate of *H. influenzae* isolates from 87 nasopharyngeal specimens collected from asymptomatic and healthy children at AL Jil Al WAED School, North Lebanon and to detect antibiotics resistance pattern among the isolates.

Material and methods

Period and place of the study

This study was carried out between April 2011 and June 2011 at AZM Research Center of Biotechnology-Tripoli, North Lebanon.

Specimen collection and Culture

A total of 87 nasopharyngeal samples were collected from asymptomatic children belonging to medium to high incomes families at Al Jil Al WAED School – Tripoli, and aged between 2 and 3 years. All examined children were vaccinated with anti-type b *H. influenzae*. The samples were collected by using swabs media (VWR®-France) and transmitted directly within 15 minutes from the school to our laboratory center according to the recommendation of Remic Groupe (RG) at "Société Francaise de Microbiologie" (SFM) (9). Each sample was treated according to the bacteriological culture standard protocols proposed by the RG- SFM. All samples were cultured on chocolate agar which has supplemented with polyvitex & bacitracin (Biorad®-France).

Phenotypic identification , biotyping and Capsular typing

After 24 hours of incubation at 37 °C, a Gram-stain was performed for suspected colonies and the identification of each isolate was completed by using Rapid I NH (Remel®-USA). The biotypes of *H. influenzae* isolates were determined according to biochemical data obtained from Rapid I NH. Capsular serotyping of *H. influenzae* isolates was performed by SAST by using polyclonal and specific b antisera (Difco-BD®-USA).

Molecular study

DNA extraction

A loopful of *H. influenzae* bacteria was taken from overnight growth on chocolate agar, and it was suspended in 500 µl of distilled water and treated using QiAmp DNA mini Kit – Qiagen® Germany, for extraction of DNA as outlined in the protocol of the manufacture.

Molecular identification

All isolates identified as *H. influenzae* by phenotypic methods and by detecting their specific genes of (p6 and 16S RNA) using PCR. The primers used are listed in **Table 1**. PCR amplification was performed with 25µl reaction mixtures that contained of 10 pmol of each relevant oligonucleotide primer, 10mMTris–HCl (pH 8.4), 50 mM KCl, 3.0mM $MgCl_2$, 200 mM (each) deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), and 1.25 U of Taq DNA polymerase (sigma®-Germany). The PCR cycling was processed using the programmable thermal cycler my Cycler (Bio-Rad®-Germany) with the following thermal profile: 7 min at 95 °C, 35 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s and final extension at 72 °C for 5 min. Ten microlitre of 25µl of the amplification products was visualized by 1.2% agarose gel electrophoresis and following ethidium bromide staining.

Table 1. The primers used for detection of *H. influenzae* genes.

Gene Primers name	Primer (5′ to 3′) Nucleotide
P6	Hi P6 F 5′-CCAGCTGCTAAAGTA TTAGTAGAA G-3 (10)
	Hi P6 R 5′-TTCACCGTAAGATACTGTGCC-3′
16S RNA	16S F 5′-CTCAGATTGAACGCTGGCGGC-3′ F (11)
	Nor: 5′-TGACATCCTAAGAAGAGC-3′

Detection of Resistance genes

PCR amplification of the sequence encoding the TEM and ROB β–lactamases (bla_{TEM-1} and bla_{ROB} genes) (11) was performed for all isolates. PCR amplification was performed with 25µl reaction mixtures that contains PCR amplification was performed with the same mixture and concentrations as above; the PCR cycling was processed using the programmable thermal cycler my Cycler (Bio-Rad-Germany) with the following thermal profile: 7 min at 95 °C, 35 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s and final extension at 72 °C for 5 min. Ten microlitre of the amplification products was visualized by 1.2% agarose gel electrophoresis and ethidium bromide staining; Primers used are listed in **Table 2**.

Table 2. Primers used to detect resistant genes (12).

Gene Primers name	Primer (5′ to 3′) Nucleotide
blaTEM-1	TEM F 5′-TGGGTGCACGAGTGGGTTAC-3′
	TEM R 5′-TTATCCGCCTCCATCCAGTC-3′
blaROB	Rob F 5′-ATCAGCCACACAAGCCACCT -3′
	Rob R 5- GTT TGC GAT TTG GTA TGC GA-3′

Antimicrobial susceptibility

Antimicrobial susceptibility testing for all *H. influenzae* isolates was performed by the diffusion disc method on *Haemophilus* Test Medium (HTM) agar plate according to the "Commité Antibiogramme" (CA) of the SFM 2010 (13). The following discs were obtained from (Bio-RAD®-France): ampicillin (10µg), amoxicillin (20µg)-clavulanic-acid (10µg), cefalotin (30µg), cefotaxime (30µg), cefuroxime (30µg), gentamycin (10 UI), tetracycline (30 µg), rifampicin (30µg), ofloxacin (5µg), ciprofloxacin (5µg), pefloxacin (5µg), trimethoprim (1.25µg)-sulfamethoxazole (23.75µg), chloramphenicol (30µg). All *H. influenzae* isolates were assessed for β-lactamase production by using the chromogenic test: cefinase (BD®-USA). The MICs of amoxicillin, amoxi-clav and erythromycin were determined using E-test strips (AB Biodisk-Solna, Sweden) commercialized by Biomérieux – France.

Results

A total of 21/87 (24.1%) *H. influenzae* strains were isolated and confirmed using PCR for detection the genes encoding the p6 protein and 16 S RNA. Table 3 shows the distribution of these isolates into type b capsular and biotypes. The distribution of *H. influenzae* isolates was as follow: 7 (33.3%) were non-typable, 9 (42.8%) were serotype b and 5 isolates (23.8%) were non-type b capsulated isolates. **Table 3** shows the Distribution of *Haemophilus influenzae* biotypes of the isolates was the following: 52.3% biotype II, followed by 28.5% biotype I, 9.5% of biotype III, 4.7% biotype V and 4.7% of biotype VIII. The results of antimicrobial susceptibility test of 13 antibiotics are shown in **Table 4**. Only 4 isolates (19.0%) were resistant to ampicillin and all of them were positive for cefinase test. **Table 5** shows that MICs of the 21 isolates, and of these only 4 (19.0%) had amoxicillin

Table 3. Distribution of type b, biotypes and PCR amplification for the identification of *H. influenzae* isolates.

No. (%)	biotype I	biotype II	biotype III	biotype V	biotype VIII
21 (100%)	6(28.5)	11(52.3)	2(9.5)	1(4.7)	1(4.7)

Table 4. The antimicrobial susceptibility of 21 *H. influenzae* isolates using disc diffusion test.

	AM	AMC	CF	CTX	CXM	GM	TE	RA	OFX	PFX	CIP	SXT	C
% S	81	100	100	100	100	100	100	100	100	100	100	71.5	95.2
% R	19	0	0	0	0	0	0	0	0	0	0	28.5	4.8

S = Susceptible, R = Resistance

Table 5. Antimicrobial susceptibility of 21 *H. infuenzae* isolates as determined by E-test method.

Amoxicillin E-test range	No. (%)	Amoxi-Clav E-test range	No. (%)	Erythromycin E-test range	No. (%)
≤ 0.5 mg/L	17 (80.8)	≤ 0.5 mg/L	17 (80.8)	< 0.5 mg/L	Null
≤ 1 mg/L	Null	≤ 0.75 mg/L	4 (19.2)	0.5-3 mg/L	6 (28.5)
≤ 3 mg/L	1 (4.8)	≤ 1 mg/L	Null	3-8 mg/L	15 (71.5)
32-256 mg/L	3 (14.4)				

MIC > 1 mg/l and were also intermediate susceptibility to erythromycin and positive for TEM-1 β-lactamase producing. No BLNAR isolates was found.

Discussion

The present study shows that among 87 children aged between 2-3 years in North Lebanon, the incidence rate of *H. influenzae* isolates was 24.1%. The majority of the isolates (66.6%) were capsulated, and 64.2% were type b and 33.3% of the isolates were nontypable.

In general, most studies from western countries have reported high prevalence of *H. influenzae* (48.2% - 95%) in children with otitis media infection or colonization in day-care centers (14). From our geographic area, two recent studies had shown similar results to our study. The prevalence of oropharyngeal colonization in 296 Iranian children with *H. influenzae* was 23.9% (15), whereas in Turkey, the prevalence of nasopharyngeal colonizing with *H.influenzae* type b in children aged between 5 and 6 years was 16.2% (16).

A study in France has demonstrated that the average carrier rate of *H.influenzae* among 1,683 children was 40.9% (17). This study suggests that anti-*Haemophilus* b vaccine is an important factor in reducing colonization with capsulated *H. influenzae* in Lebanese children.

It has been reported that nasopharynx flora is influenced by age and socio-economic settings, since a child's nasopharynx represents a special dynamic environment controlled by interactions between bacterial species, host immune system and immunization with pneumococcal conjugate vaccine (18).

The same study also found that children from lower socio-economic schools were more likely to carry *M. catarrhalis*, *S. aureus* and antibiotic-resistant *S. pneumoniae*, including a high proportion of non-typeable pneumococcal strains. Positive associations between *S. pneumoniae* and *H. Influenza*e, *H. Influenzae* and *M. catarrhalis* and *H. influenzae* and *S. aureus* were detected (18). The present study included children from medium and high incomes families, and all of them were immunization with Hib vaccine.

Prevalence of antimicrobial resistance among human colonizing bacteria is varied widely between different countries, and it has been used to predict response to treatment of invasive infections caused by these organisms. This study revealed that 19.0% of *H. influenzae* strains were ampicillin resistant and all of them were positive produced TEM-1 type ß-lactamase and no BLNAR strain was found. A recent Turkish study examined healthy school children, has reported that 12.9% of nasopharyngeal carriages of *H.influenzae* were ampicillin resistant and all of them were type TEM-1 β-lactamase producing (19), while a French Study reported that 45.8% of nasopharyngeal colonizing *H.influenzae* were ampicillin resistant, and of these , 44.5% were TEM-type beta-lactamase producers and 1.3% were BLNAR strains (17). The susceptibility to erythromycin showed that all of our isolates were intermediate susceptible. A study from Turkey (2006) has reported that 7.5% of *H.influenzae* isolates from children were resistant to erythromycin (20), whereas a study from Iran (2007) showed that resistance rates to azithromycin and clarythromycin was 19.6% and 35.3%, respectively (15).The fluoroquinolones are considered to be candidates for the first choice of antimicrobial agents in cases of community-acquired pneumonia and otitis media in adults, so the emergence of fluoroquinolones-resistant in *H. influenzae* and *S. pneumoniae* can be of clinical and public health concern, particularly in region like Lebanon where levels of antimicrobial resistance among respiratory pathogens are already high (21-22), and emerging of fluoroquinolone resistance among *S. pneumoniae* and *H. influenzae* in children will be highly significant warning. Our study has demonstrated 100% susceptibility of *H. influenzae* isolates to fluoroquinolones, while many countries worldwide reported low- high prevalence of *H. influenzae* fluoroquinolones resistance among children (19-23).

In conclusion, this study reports a preliminary data on the carriage rate of *H. influenzae* isolates among North Lebanon young children . The incidence of a resistance rate among isolates (19%) to ampicillin indicates a warning signal to use of beta-lactams in prophylaxis treatment of *H. influenzae* infections.

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