

Prevalence of type b *Haemophilus influenzae* and antibiotic resistance in 52 clinical isolates in north Lebanon

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معدل انتشار المستدمية النزلية من النمط (ب) ومقاومة المضادات الحيوية في 52 عزلة سريرية بشمال لبنان

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الخلاصة: تهدف هذه الدراسة إلى تحديد النمط المحفطي ومعدل انتشار سلالات المستدمية النزلية من النمط (ب) بشمال لبنان في كل من المرض الباضع وغير الباضع، وتحديد نمط الحساسية وآلية مقاومة بيتا لاكتام (الذراي المتنتجة لبيتا لاكتاماز والسلالات السالبة لبيتا لاكتاماز والمقاومة للأميسيلين). واستُفردت 52 سلالة من المستدمية النزلية من 312 عينة سريرية، وتم تحديد نمط المقاومة لبيتا لاكتام باستخدام طرق التفشي للأقراص واختبار الحساسية للمضادات الحيوية، ثم الطرق الجزيئية مثل تفاعل البوليميراز السلسلي للجينات bla_{ROB} و bla_{TEM} . وكانت تسع من الذراي الاثنيتين والخمسين (نسبة 17.4%) مقاومة للأميسيلين، جميعها تنتج بيتا لاكتاماز أو عُدّت ذراي منخفضة سالبة لبيتا لاكتام ومقاومة للأميسيلين. أما الذراي الحساسة فكان 15.3% منها غير حساسة تماماً للبيتا لاكتاماز وأظهر التنميط المصلي بواسطة التراص على الشريحة الزجاجية أن 30.7% من الذراي هي من النمط (ب).

ABSTRACT The aim of this study was to determine the capsular typing and type b prevalence of clinical *Haemophilus influenzae* strains in north Lebanon in both invasive and non-invasive disease and to determine the susceptibility pattern and the mechanism of resistance to β -lactams [β -lactamase-producing strains and β -lactamase-negative ampicillin resistant (BLNAR) strains]. Fifty-two strains of clinical *H. influenzae* were isolated from 312 clinical specimens; the resistance pattern to β -lactams of these strains was determined by using the disc diffusion and E-test methods followed by molecular methods such as PCR of bla_{TEM} et bla_{ROB} genes. Nine (17.4%) of the 52 strains were resistant to ampicillin; all of them produced type TEM-1 β -lactamase. In the susceptible strains 15.3% were not fully susceptible to β -lactams or considered low BLNAR strains. Slide agglutination serotyping showed that 30.7% of the strains were type b.

Prévalence d'*Haemophilus influenzae* de type B et résistance aux antibiotiques chez 52 isolats cliniques dans le nord du Liban

RÉSUMÉ La présente étude visait à déterminer le typage capsulaire et la prévalence du type B chez des souches cliniques d'*Haemophilus influenzae* dans le nord du Liban, à la fois pour les pathologies invasives et non invasives, et à identifier le profil de sensibilité et le mécanisme de résistance aux β -lactamines (souches productrices de β -lactamases et souches résistantes à l'ampicilline sans production de β -lactamases [souches BLNAR]). Cinquante-deux souches cliniques d'*H. influenzae* ont été isolées à partir de 312 spécimens cliniques. Nous avons identifié le mécanisme de résistance aux β -lactamines de ces souches en utilisant les méthodes de diffusion par disque et du E-test, puis les méthodes moléculaires, comme la PCR pour les gènes bla_{TEM} et bla_{ROB} . Sur les 52 souches, neuf (17,4 %) étaient résistantes à l'ampicilline ; toutes étaient productrices de β -lactamases de type TEM-1. Parmi les souches sensibles, 15,3 % n'étaient pas complètement sensibles aux β -lactamines ou étaient considérées comme des souches BLNAR résistantes à bas niveau. Le sérotypage réalisé par agglutination sur lame a montré que 30,7 % des souches étaient de type B.

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Introduction

In spite of the widespread use of the anti-*Haemophilus influenzae* b vaccine in the industrialized world and the decreased incidence of invasive diseases [1], *Haemophilus influenzae* remains a key species in bronchopulmonary and ear, nose, and throat (ENT) infections in both adults and children. These diseases are most often caused by non-encapsulated strains or non-typeable *Haemophilus influenzae* (NTHi) [2,3]. Invasive disease due to *H. influenzae* may produce various clinical syndromes including meningitis [4].

H. influenzae type b (Hib) is the only type for which there is a vaccine and for which control measures are considered necessary. Before the widespread use of Hib conjugate vaccines, Hib was a leading cause of bacterial meningitis in the world among children under five years of age and a major cause of other life-threatening invasive bacterial disease in this age group [5]. The impact of vaccination on Hib epidemiology was observed early. In Lebanon the Hib vaccination has been mandatory since 2004; this vaccine provided free by the Lebanese Ministry of Public Health [6].

We do not have any data concerning the major types involved in non-invasive and invasive disease in our community. This lack of data is due to there being no serotyping in Lebanese clinical laboratories.

Treatment of such invasive or non-invasive infections can be severely affected by antibiotic resistance [7]; for example, in the 1970s the use of amoxicillin was thwarted by the emergence of β -lactamase-producing strains [7], then in the 1980s, the third-generation cephalosporin provided an alternative that has always been effective in treating local infections, particularly some ear infections.

In France, the percentage of β -lactamase-producing strains ranged from 25% to 30% of respiratory isolates and nearly 40% of isolates of otorrhoea. The activity of the enzyme produced almost exclusively TEM-1 penicillinase, which inactivates amoxicillin, but it can

be restored in the presence of clavulanic acid [8].

Resistance by modifying the penicillin binding protein (PBP) is also of concern as it relates to 20% of non-encapsulated strains isolated in Europe [9].

There are no data concerning the prevalence and the evolution of antibiotic resistance of clinical *H. influenzae* in Lebanon, while the most recent Middle East data concerning the prevalence were from 2007 [10]. The aim of our study is to evaluate the efficiency of the standard phenotypic identification by comparing it with the molecular method, serotyping the clinical isolates to start following the prevalence of serotype b in both invasive and non-invasive disease, evaluate the prevalence of this serotype in different infection sites and detect the mechanisms of antibiotic resistance to β -lactams by molecular methods.

Methods

This study was carried out between October 2010 and June 2011 at the AZM Centre for Biotechnology Research and its Applications, Tripoli, north Lebanon.

Specimen collection

A total of 312 clinical samples (ear discharge, nose discharge, sputum, broncho-alveolar aspiration, cerebrospinal fluid (CSF) and conjunctivitis pus), were collected from patients admitted at Nini Hospital, Tripoli, aged between 7 months and 67 years old. Nini Hospital is a peripheral hospital with 120 beds; all specialties are present except organ transplantation. The samples were collected and transmitted directly (< 15 min) from the hospital to the AZM Centre according to the recommendations of the Groupe Rémic at the Société française de Microbiologie [11].

Bacteriological study

H. influenzae isolation

Each sample was treated according to the bacteriological culture standard

protocols put forward by the Groupe Rémic at the Société française de Microbiologie. In all cases each sample was cultured on chocolate agar + polyvitamin supplement + bacitracin (Bio-rad, France).

Phenotypic identification

After 24 hours of incubation at 37 °C, a Gram stain was performed for the suspect colony and the identification of each isolate was completed using Rapid NH (Remel, USA).

Capsular typing

Serotyping was performed using the standard antimicrobial susceptibility test (SAST) method using polyclonal and specific b antisera (Difco-BD, USA).

Molecular study

DNA extraction

After overnight growth on chocolate agar, a loopful of *H. influenzae* bacteria was suspended in 500 μ L of sterile ultra pure water and then extracted using the QiAmp DNA mini kit (Qiagen, Germany); the protocol suggested by the manufacture was followed.

Molecular identification

All isolates identified as *H. influenzae* by phenotypic methods were identified again by detecting their specific genes, such as the *p6* [12] and *16S rRNA* [13] genes by PCR; the primers used are listed in Table 1.

PCR amplification was performed with 25 μ L reaction mixtures that contained 10 pmol of each relevant oligonucleotide primer, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 3.0 mM MgCl₂, 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 MyCycler (Bio-Rad, Germany) with the following thermal profile: 7 minutes at 95 °C, 35 cycles at 95 °C for 30 seconds, 58 °C for 30 seconds, 72 °C for 45

seconds and final extension at 72 °C for 5 minutes. Ten microlitres of the 25 µL of the amplification products were visualized by 1.2% agarose gel electrophoresis and ethidium bromide staining.

Detection of resistance genes

PCR amplification of the sequence encoding the TEM and ROB β-lactamases (*bla_{TEM-1}* and *bla_{ROB}* genes) was performed for all strains [14].

PCR amplification was performed with 25 µl reaction mixtures that contained the same content and concentration of the *p6* and *16S* gene amplification; the PCR cycling was processed using the same thermal cyclers with the following thermal profile: 7 minutes at 95 °C, 35 cycles at 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 45 seconds and final extension at 72 °C for 5 minutes. Ten microlitres of the amplification products were visualized by 1.2% agarose gel electrophoresis and ethidium bromide staining; the primers used are listed in Table 2.

Antimicrobial susceptibility

Antimicrobial susceptibility testing for all *H. influenzae* isolates was performed by the diffusion method on *Haemophilus* test medium (HTM) agar plate according to the protocols of the Comité de l'Antibiogramme de la Société française de Microbiologie 2010 [15]. We used the commercial discs (Bio-Rad France) to test the following: ampicillin (10 µg), amoxicillin (amoxi) (20 µg)/clavulanic acid (clav) (10 µg), cefalotin (30 µg), cefotaxime (30 µg), cefuroxime (30 µg), gentamicin (10 UI), kanamycin (30 µg), tetracycline (30 µg), rifampicin (30 µg), nalidixic acid (30 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), pefloxacin (5 µg), trimethoprim (1.25 µg), sulfamethoxazole (23.75 µg), chloramphenicol (30 µg) and erythromycin (15 µg).

All samples were assessed for β-lactamase production by using the Cefinase chromogenic test (BD, USA).

We determined the minimum inhibitory concentrations of amoxicillin, amoxiclav and erythromycin for each isolate by using E-test strips (Biomérieux, France)

Table 1 Primers used in this study for amplification

Gene primer name	Primer (5' to 3') nucleotide
<i>p6</i>	Hi P6 F 5'-CCAGCTGCTAAAGTA TTAGTAGAA G-3' [10] Hi P6 R 5'-TTCACCGTAAGATACTGTGCC-3'
<i>16S rRNA</i>	16S F 5'-CTCAGATTGAACGCTGGCGGC-3' F [11] Nor 5'-TGACATCCTAAGAAGAGC-3'

Table 2 Primers used in this study

Gene primer name	Primer (5' to 3') nucleotide
<i>bla_{TEM-1}</i> [12]	TEM F 5'-TGGGTGCACGAGTGGGTAC-3' TEM R 5'-TTATCCGCCTCCATCCAGTC-3'
<i>bla_{ROB}</i> [12]	Rob F 5'-ATCAGCCACACAAGCCACCT-3' Rob R 5'-GTTTGCATTGGTATGCGA-3'

Table 3 Serotype of *Haemophilus influenzae* isolated from clinical samples by slide agglutination serotyping

Specimen	NTHi (%)	Hib (%)	% of Hib/infection site	Number of type b encapsulated strains (%)
ND	8	8	32.0	9
OD	4	3	25.0	5
S	9	4	28.5	1
CP	0	1	100.0	0
Total	21 (40.5)	16 (30.7)		15 (28.8)

Hib = *H. influenzae* type b; *NTHi* = non-typable *H. influenzae*; *ND* = nose discharge; *OD* = otitis discharge; *S* = sputum; *CP* = conjunctivitis pus.

and we followed the protocol suggested by the manufacture.

Results

Our results showed 100% agreement between phenotypic and molecular identification (data not shown).

As seen in Table 3, 16 (30.7%) of the 52 *H. influenzae* had positive agglutination reaction with type b-specific antiserum, while 15 (28.8%) were typed as non-b encapsulated type. Twenty-one (40.5%) of the isolates were reported to be NT (non-typeable).

Table 4 shows the chronological distribution of serotypes based on different

Table 4 Distribution of isolate according to pathological sites

Pathological site	<i>Haemophilus influenzae</i> isolates (No.)	%
Nose discharge (n = 71)	25	35.2
Sputum (n = 78)	14	17.9
Otitis discharge (n = 54)	12	22.2
Broncho-alveolar aspiration (n = 65)	0	0
Cerebrospinal fluid (n = 32)	0	0
Conjunctivitis pus (n = 12)	1	8.4
Total (n = 312)	52	16.7

Table 5 Percentage of antibiotic resistant strains to all antibiotics tested in this study

Susceptibility	AM	AMC	CF	CTX	CXM	K	GM	TE	R	NA	OFX	PFX	CIP	SXT	C
S (No.)	43	52	52	52	52	51	52	49	52	49	50	51	51	32	52
(%)	(82.6)	(100)	(100)	(100)	(100)	(98.1)	(100)	(94.2)	(100)	(94.2)	(96.1)	(98.1)	(98.1)	(61.5)	(100)
R (No.)	9	0	0	0	0	1	0	3	0	3	2	1	1	20	0
(%)	(17.4)					(1.9)		(5.8)		(5.8)	(3.9)	(1.9)	(1.9)	(38.5)	

S = susceptible; R = resistant; N = number of isolates; AM = ampicillin; AMC = amoxi-clav; CF = cefazolin; CTX = cefotaxime; CXM = cefuroxime; K = kanamycin; GM = gentamicin; TE = tetracycline; R = rifampicin; NA = nalidixic acid; OFX = ofloxacin; PFX = pefloxacin; CIP = ciprofloxacin; SXT = sulfamethoxazole and trimethoprim; C = chloramphenicol.

infection sites. We found the most type b and non-b encapsulated isolates in nose discharge; most of NTHi was isolated from sputum.

Table 5 showed the percentage of antibiotic resistant strains to all antibiotics tested in this study, noted that the phenomenon of multiresistance was seen in one (1.9%) of the isolates which were resistant to ampicillin, co-trimoxazole, kanamycin and tetracycline.

Of the 52 isolates tested, nine (17.4%) had an amoxicillin minimum inhibitory concentration > 1 µg/L and were positive when tested with Cefinase; according to their phenotypic and genotypic patterns, these nine strains (100%) were type TEM-1 β-lactamase producing (Table 6). We detected 8 isolates (15.3%) that had an amoxi-clav minimum inhibitory concentration between 0.75 and 1 µg/L.

Regarding susceptibility to macrolides, all of our strains had an intermediate susceptibility to erythromycin according to the critical values given by the Comité de l'Antibiogramme [15].

Discussion

In Lebanon, a sentinel surveillance system network for bacterial meningitis of select sites throughout the country was set up by the Epidemiological Surveillance Unit of the Ministry of Public Health; for 2000–2003; the etiology of approximately 30% of purulent meningitis cases was specified. Among this group, the major causative pathogens were *Streptococcus pneumoniae* 31%, *Neisseria meningitidis* 33% and *H. influenzae* 8% [10]. An unpublished review examined bacterial meningitis cases in patients less than 20 years of age admitted to American University of Beirut hospital from 1980 to 2003. Of 184 cases, 50 (27%) were caused by Gram-negative organisms, and 15 were identified as *H. influenzae* (13 before introduction of vaccine) (G. Dbaiibo, personal communication, 2000).

After the introduction of a conjugate vaccine against *H. influenzae* in 2003—a key public health advance—the incidence of invasive *H. influenzae* isolates in Lebanon has fallen sharply. The Ministry of Public Health reported the prevalence of *H. influenzae* invasive isolates between 2008 and 2011: there was one (0.5%) *H. influenzae* meningitis isolate in 2009 and 2010 and no invasive *H. influenzae* isolates in 2008 and 2011 [6].

There are no national data on the prevalence of *H. influenzae* infections in non-invasive disease. Our study showed that the prevalence was 30.7%.

It is very difficult to compare our results with other studies because the majority followed the prevalence of type b strain isolated from invasive infection such as meningitis while we did not found any invasive case caused by this type in our study.

Antibiotic resistance in *H. influenzae* is more diverse and widespread than is commonly appreciated, and continued surveillance of antimicrobial susceptibility is recommended in order to monitor resistance trends and identify emerging resistance early.

Amoxicillin-resistant strains were 17.4% in our study; they represented 5.6% in Beirut in 2008 [16], based on these two Lebanese results in two different years the prevalence in northern Lebanon is higher compared to that found in Beirut.

Comparing figures for Islamic Republic of Iran, Saudi Arabia and Qatar we found a high prevalence of β-lactam resistant isolates, respectively 43.6% [17], 34% [18] and 35.7% [19]. The difference could be related to dissimilar prescribing habits in the different countries.

In the present study, all β-lactamase-positive strains (17.4%) produced TEM-1 β-lactamase. It is the most frequent resistance mechanism reported for *H. influenzae*. The distribution of *bla*_{TEM} and *bla*_{ROB} genes differs significantly from one country to another:

Table 6 Minimum inhibitory concentrations of 52 *Haemophilus influenzae* isolates, determined by E-testing

Minimum inhibitory concentrations										
Amoxicillin			<i>bla</i> _{TEM-1} present (<i>n</i> = 9)		Amoxi-clav			Erythromycin		
Range	No.	%	No.	%	Range	No.	%	Range	No.	%
0.06–0.5	36	51.6	0	0	0.06–0.5	37	71.2	< 0.5	0	0
0.75–1	6	11.5	0	0	0.75–1	15	28.8	0.5–3	25	48
1.5–3	2	3.8	2	22.2	1–256	0	0	3–8	27	52
3–16	2	3.8	2	22.2						
16–32	1	2	1	11.1						
32–64	1	2	1	11.1						
64–256	2	3.8	2	22.2						
>256	1	2	1	11.1						

Amoxi-clav: amoxicillin–clavulanic acid, N = number of strains, n = number of amoxicillin-resistant strains.

in Turkey, *bla*_{TEM} gene was detected in 100% of *H. influenzae* isolates [20], and in Tunisia, the frequency was 62.5% [7].

In our study, there was no fully BLNAR strain, but we found eight strains (15.3%) with amoxi-clav minimum inhibitory concentration values (MIC) > 0.5 µg/mL; three of them were isolated from otitis pus with MIC = 1, three isolated from nasal discharge with 0.75 < MIC < 1 and two isolated from sputum with MIC = 1; all of these eight isolates were β-lactamase–negative producing strains.

These strains are considered not to be fully susceptible to β-lactams and can therefore be classified as low BLNAR strains according to Dabernat et al. who reported in 2007 that the MIC range of the *ftsI* mutant gene strains was 0.5–8 µg/L for amoxi-clav, after examination of amoxi-clav MICs using the E-test and the broth micro-dilution method [21].

As *H. influenzae* has low sensitivity to erythromycin due to an efflux mechanism and methylation of 23S rRNA [22], we tested erythromycin in order to detect acquired resistance to all macrolide antibiotics as recommended by EUCAST (European Committee on Antimicrobial Susceptibility Testing) [23]. Our results showed low susceptibility for all isolates; compared with those in Turkey we found that 7.5% of

the Turkish isolates were resistant to erythromycin [24].

The fluoroquinolones are considered to be the first choice antimicrobial agents in cases of community-acquired pneumonia and otitis media in adults, so the emergence of fluoroquinolone-resistant *H. influenzae* and *S. pneumoniae* is a concern. Quinolone resistance is imparted by mutations in a particular domain referred to as the quinolone-resistance–determining region (QRDR) of one or both of the principal target enzymes, DNA gyrase (an A₂B₂ complex encoded by the *gyrA* and *gyrB* genes) and topoisomerase IV (a C₂E₂ complex encoded by the *parC* and *parE* genes) [25].

Of our strains 5.8% had a quinolone resistance; compared with other studies we found that this resistance is growing; the Centre National de Référence de *Haemophilus influenzae* found that 0.5% of its strains were resistant [26], while there was a high prevalence of resistance present in Taiwan 43.8% [27]. In Japan, a team working on the prevalence of quinolone resistance reported that of 457 *H. influenzae* strains, 12 (2.6%) were fluoroquinolone resistant; note that these strains were derived from patients over 58 years of age [25]. All *H. influenzae* strains in Turkey were susceptible to fluoroquinolones [28]. The same results were found in Beirut [16].

Co-trimoxazole resistance is very high; it was about 39.5% in our isolate. The percentages of co-trimoxazole resistant isolates varied between different countries, ranging from 14% to 50% [27,28]; they accounted for 31.6% in Turkey [24]. For tetracycline, 5.8% of our strains were resistant compared to 16.7% in Beirut [16] and 2.6% in France. Finally we found that 1.9% of our strains were resistant to kanamycin.

In all our strains we found one strain with multidrug resistance; it was resistant to ampicillin, tetracycline, kanamycin, nalidixic acid and co-trimoxazole.

Conclusion

This study looked at the prevalence of *H. influenzae* serotypes in both invasive and non-invasive infections.

The phenotypic identification used by the majority of Lebanese clinical laboratories is effective since it gave the same results as using molecular methods. It is essential to screen the antibiotic resistance of these bacteria to the β-lactams by MIC methods because we found that 15.3% of our strains were not fully susceptible to amoxi-clav; this type of strain can develop at any time inside or outside the human body to be completely transformed into BLNAR strains leading to β-lactam

treatment failure. We look forward to studying the genetic mutation in the *ftsI* gene encoding the penicillin-binding proteins in our isolates with amoxi-clav MIC > 0.5.

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