Prevalence of *H.influenzae* biotypes and their clinical significance in a University Hospital

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ABSTRACT

Objective: *Hemophilus influenzae* is an important pathogen that is responsible for invasive and non-invasive infections in both children and adults. This study aims to assess the relationship of biotypes to the sites of infection, serotypes, antimicrobial susceptibility, β-lactamase production and age.

Methods: A total of 200 isolates of *H.influenzae* were obtained from clinical specimens over a period of 12 months from January 2001 through to January 2002 from King Abdul-Aziz University Hospital, Riyadh, Kingdom of Saudi Arabia.

Results: Most of the strains were non-typable and were isolated from patients with non-invasive infections. The typable isolates from invasive infections mostly serotype b were isolated from blood, cerebrospinal fluid and hip joint aspirate. Biotype II accounted for 37% of the isolates followed by biotypes III and I (29.5% and 23%). The remaining 10.5% were made up of biotypes IV, V, VI and VII. A significantly high resistance to cotrimoxazole (33.5%) and ampicillin (19%) was observed. Two point five percent of the isolates were resistant to chloramphenicol. All the isolates resistant to ampicillin were β-lactamase producers and susceptible to cefuroxime, ceftriaxone, ciprofloxacin and rifampicin.

Conclusion: This study revealed that biotypes II and III are the predominant biotypes of *H.influenzae* found in non-invasive infections. There is an apparent relationship between biotype and site of infection which could be useful as an epidemiological marker.

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Hemophilus influenzae is an important pathogen causing infections mainly in pediatric population worldwide. These include both invasive and non-invasive infections. The strains responsible for non-invasive infections such as otitis media, conjunctivitis and sinusitis commonly colonize the upper respiratory tract of healthy people.[1] The invasive infections mostly caused by serotype b include meningitis, septicemia, epiglottitis, cellulitis, arthritis and osteomyelitis.[2,3] Due to immunization, the mortality rate of *H.influenzae* invasive infections has been reduced considerably.[4] ampicillin and chloramphenicol used to be drugs of choice for the treatment of life threatening infections; but have now been superseded by the third generation cephalosporins such as ceftriaxone and cefotaxime. In 1976, Kilian[5] in his extensive taxonomic study on *Hemophilus* showed that strains of *H.influenzae* could be divided into biotypes on the basis of 3 biochemical

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Table 1 - Tests scheme for biotyping *Hemophilus influenzae* isolates.

Indole	Urease	Ornithine decarboxylase	Biotype
+	+	+	I
+	+	-	II
-	+	-	III
-	+	+	IV
+	-	+	V
-	-	+	VI
+	-	-	VII
-	-	-	VIII

tests: ability to produce (a) Urease, (b) Indole, and (c) Ornithine decarboxylase. Initially, biotypes 1-V were designated but later on other workers using the same 3 tests recognized biotypes VI, VII and VIII.[6-8] The ability to biotype *H.influenzae* irrespective of serotypes, provide a useful epidemiological marker, such as for monitoring the acquisition and loss of carriage from the upper respiratory tract.[7] A number of investigators[6,9] have studied the relationship of biotypes to serotypes, source of isolation and antimicrobial susceptibility and found correlation between these parameters.

This study was undertaken to evaluate any relationship of biotype to isolation sites, serotypes, different age groups, antimicrobial susceptibility and β-lactamase production in both children and adult population from a referral Ear, Nose and Throat & Ophthalmology Hospital, King Abdul-Aziz University Hospital (KAUH) in Riyadh, Kingdom of Saudi Arabia.

Methods. Strains: 200 isolates of *H.influenzae* causing either invasive or non-invasive infections in pediatric and adult population were obtained from different sites. Only a single isolate per patient was included. Primary isolation of the isolates was carried out on Chocolate Agar (Oxoid Limited, Basingstoke, United Kingdom). The isolates were then subcultured on Chocolate agar and their identity confirmed by X and V factor requirement (Oxoid Limited, Basingstoke, United Kingdom) and biochemically by Analytical Profile Index Neisseria and Hemophilus (API NH) (bioMérieux Sa, France). *Hemophilus influenzae* ATCC 49247 was used as control organism.

Table 2 - Distribution and relationship between biotypes and isolation sites.

	Biotypes								
Site	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**Total isolates (%)**
Eye	10	39	34	1	2	-	-	-	**86 (43)**
Ear	11	18	13	1	4	2	1	-	**50 (25)**
Respiratory tract	13	16	10	2	5	2	-	-	**48 (24)**
Blood	10	-	-	-	-	-	-	-	**10 (5)**
Genital tract	-	1	2	-	1	-	-	-	**4 (2)**
Cerebrospinal fluid	1	-	-	-	-	-	-	-	**1 (0.5)**
Hip aspirate	1	-	-	-	-	-	-	-	**1 (0.5)**
Total biotypes (%)	**46 (23)**	**74 (37)**	**59 (29.5)**	**4 (2)**	**12 (6)**	**4 (2)**	**1 (0.5)**	**-**	

The 3 biochemical tests scheme proposed by Kilian[5] was used for the biotyping. After the biochemical identification, the result of the 3 biochemical tests: Urea, Indole, and Ornithine decarboxylation were retrieved from the API NH for biotyping **Table 1**. Known ATCC strains of *H.influenzae* biotypes I-III were used as control organisms. analytical Profile Index 10 Screen (API 10S) (bioMérieux, Sa, France) was also used to supplement the results of the 3 tests obtained from the API NH to avert any discrepancy in the biotyping with API NH.[10] Enterobacter aerogenes was used as control for Ornithine decarboxylation, *Escherichia coli* for indole production and *proteus mirabilis* for urease production.The isolates were screened for the presence of capsular antigens a, b, c, d, e and f with *H.influenzae* polyvalent a-f antiserum (Bacto-Difco laboratories, Detroit, United States of America) by slide agglutination. The isolates showing

positive agglutination in the polyvalent antisera were then serotyped using the individual typing sera. A known positive culture of *H.influenzae* type b and a negative culture of untypable *H.influenzae* were used as controls. Each isolate of *H.influenzae* was tested for β-lactamase production by chromogenic cephalosporin (Nitrocefin)[11] with Cefinase disk (BBL, Becton Dickinson Microbiology System, Cockeysville) using β-lactamase negative ampicillin sensitive *H.influenzae* and β-lactamase positive ampicillin resistant *H.influenzae* as control organisms.

Microbial susceptibility testing. Susceptibility testing was performed on all isolates by the disk diffusion method on Mueller Hinton Chocolate Agar as defined in the guide line of the National Committee for Clinical Laboratory Standards (NCCLS)[12] using *H.influenzae* ATCC 4976 as control. Antimicrobial agents tested were ampicillin, amoxicillin/clavulanate, cotrimoxazole, cefaclor, cefuroxime, cefotaxime, ceftriaxone, gentamicin, chloramphenicol, polymyxin B, neomycin and tetracycline. All antimicrobial disks were obtained from Mast Diagnostics (Bootle, Merseyside, United Kingdom). After incubating at 35ºC over night, the zone sizes of the test organisms were compared with the zone size of *H.influenzae* control to determine resistance or sensitive (Stokes method).[13] Minimum inhibitory concentration was performed for ceftriaxone, ciprofloxacin and rifampicin by the E-test method (AB Biodisk, Solna, Sweden) using Mueller Hinton Chocolate Agar (BBL, Becton Dickinson Microbiology system, Cockeysville). All the isolates were stored at -70ºC in 10% Glycerol broth for further procedures.

Results. The predominant biotypes isolated from eye and ear specimens were biotypes II and III. Distribution of biotypes isolated from various sites is shown in **Table 2**. In all age groups, the predominant biotype was biotype II followed by biotypes III and I **Table 3**. No biotype VIII was found in this study. Of the 200 isolates serotyped, 181 (90.5%) were non-typable. All the isolates from blood, CSF and hip were serotype b and biotype I. One isolate from sputum was serotype d, 2 isolates from ear were serotype b and d, and 2 isolates from eye were serotype b and one isolate was serotype e. All the serotype b isolates were biotype 1.

Out of 200 isolates 38 (19%) were resistant to ampicillin due to β-lactamase production. No ampicillin resistant β-lactamase negative isolates were detected and 67 (33.5%) isolates showed resistance to cotrimoxazole. Resistance to chloramphenicol was 2.5%, cefaclor 4% and tetracycline 8.5% **Table 4**. All the isolates were sensitive to ceftriaxone, cefotaxime, cefuroxime, amoxicillin/clavulanate, ciprofloxacin, gentamicin, polymyxin B and rifampicin. Minimum Inhibitory Concentration (MIC) of ceftriaxone ranged from 0.008-0.023 μg/ml, ciprofloxacin 0.002-0.032 μg/ml and rifampicin 0.038-1.0 μg/ml. Most of the resistant isolates were mostly biotype II and III. Twelve isolates were resistant to ampicillin alone, 2 resistant to ampicillin and tetracycline, 10 resistant to ampicillin and cotrimoxazole, 11 resistant to ampicillin, tetracycline and cotrimoxazole, and 3 resistant to ampicillin, tetracycline, cotrimoxazole and chloramphenicol. The relationship between β-lactamase production and biotypes is shown in **Table 4**. Most β-lactamase producers were biotypes II and III.

Table 3 - *Hemophilus influenzae* biotypes in relation to age group.

Age group (years)	Biotypes I	II	III	IV	V	VI	VII	VIII
0-5	26	43	42	2	-	2	1	-
6-15	9	12	6	1	2	1	-	-
16-25	1	5	3	1	1	-	-	-
>25	10	14	8	-	2	1	-	-

Discussion. The most predominant biotype among the isolates was biotype II accounting for 37% followed by biotype III and I (29.5% and 23%). Other biotypes were observed in comparatively small percentage making up 10.5% of the total isolates. These results are in agreement with other Observations.[6,14-16] In this study, strains of biotypes IV, V, VI and VII accounted for only a small percentage of isolates which seems to be less common as reported by Herper and her associates.[14] No biotype VIII isolates were observed in this study. In invasive infections, all the isolates from blood and CSF were biotype I which support several previous reports[6,9,15,17] which showed that biotype I is predominant in blood and CSF isolates followed by biotype II both of which account for more than 90%. In non-invasive infections, the predominant biotypes were biotypes II and III. This study revealed an apparent relationship between biotypes and sites of infection in both invasive and non-invasive infections. The result of this study, therefore, confirmed previous reports indicating a correlation between biotypes and sites of infection.[5,9,18,19] All the isolates from blood and CSF were serotype b. Other studies[20,21] have shown that non-capsulated non-biotype I strains of *H.influenzae* could cause invasive infections in human. In this study, since most of the isolates were non-serotypable from non-invasive infections, we cannot therefore, comment on whether there is any relationship between serotype and biotype. It was also observed that few non-serotype b such as d, e, f could be involved in eye, ear and respiratory infections.

In this study, it was observed that in all age groups biotype II was the overall predominant biotype

Table 4 - Relationship between biotypes, antibiotic resistance and β-lactamase production.

Antibiotic	Biotypes I	II	III	IV	V	VI	VII	VIII	Total (%)
Cotrimoxazole	7	37	23	-	-	-	-	-	**67 (33.5)**
Amipicillin	7	15	15	-	1	-	-	-	**38 (19)**
Tetracycline	3	4	10	-	-	-	-	-	**17 (8.5)**
Cefaclor	-	3	5	-	-	-	-	-	**8 (4)**
Chloramphenicol	-	2	3	-	-	-	-	-	**5 (2.5)**
β-lactamase production (%) n=38	7 (18.4)	15 (39.5)	15 (39.5)	-	1 (2.6)	-	-	-	

followed by biotypes III and I, this indicates their prevalence in the community. *Hemophilus influenzae* was susceptible to a wide range of antimicrobial agents. But by 1970's, strains resistant to a number of agents had been reported in some countries including KSA.[9,18,22-25] Resistance to ampicillin mediated by β-lactamase production had steadily increased since its description in 1971. In this study, ampicillin resistance was 19% and due to production of β-lactamase. There were no ampicillin resistant β–lactamase negative strains. In previous reports from KSA, ampicillin resistance due to β-lactamase production had shown considerable increase from 4%,[22] 8%,[24] 10.7%,[2] 12%,[18] 17%[23] to 19% in this study. Studies in many countries had shown a high prevalence of β-lactamase production; such as United Kingdom (10.6%),[26] Canada (28.4%)[27] and United States of America (36.4%).[28] This study showed relationship between β-lactamase production and biotype as β-lactamase producing strains were mostly biotypes II and III accounting for 78.9% of the β-lactamase producing isolates. It was also observed that none of the isolates producing β-lactamase were biotype IV, VI and VII, supporting the conclusion reached by Carl Kamme[29] that biotype IV is unable to acquire plasmid carrying transposon A. It could be assumed that, this might also be the case for biotypes VI and VII. This assumption needs to be proved by further studies. Ceftriaxone, ciprofloxacin and rifampicin were selected for MIC by E-test as ceftriaxone is commonly used antibiotic for severe infections caused by *H.influenzae* in this hospital. Also, we wanted to know the MIC of ciprofloxacin for further use in treatment if necessary and rifampicin for prophylaxis. Studies by DeMaria et al[19] showed that all their biotype V strains were β-lactamase producers which is in contrast to this study which showed 1 (8.3%) of biotype V as β-lactamase producer. Some scholars have attempted to equate *H.influenzae* biotype III with *Hemophilus aegyptius* usually isolated from cases of conjunctivitis. *Hemophilus aegyptius* can be identified by its inability to grow on tryptic soy agar with added hemin and NAD, its susceptibility to trooleandomycin and its inability to ferment D-xylose, inability to produce indole and agglutinate human erythrocytes.[30,31] In this study no attempt was made to differentiate *Hemophilus aegyptius* from biotype III eye isolates due to all the above tests either singly or together do not serve to distinguish all *Hemophilus aegyptius* strains from all *H.influenzae* strains.[31,32]

This study showed a considerable increase in the prevalence of cotrimoxazole resistance. The work carried out by Khizzi and Saeed[18] showed that 1.5% of their strains were resistant to cotrimoxazole while in this present study 33.5% of the isolates were resistant and mostly biotype II (55.2%) and biotype III (34.3%). This increase may due to random use of cotrimoxazole in the community. Except for ampicillin and cotrimoxazole resistance there was no substantial difference in the antimicrobial susceptibility pattern of any particular biotype of the isolates. Multiple resistance to 3 or 4 antibiotics was found in 7% of our isolates. Biotypes II and III seem to play important role especially in non-invasive infections. It may be possible that these biotypes II and III from the eye could be involved in the genital infection which may result in ophthalmia neonatorum and other infections in neonates.[33,34] It may also be interested to look for additional virulence factors associated with these biotypes II and III as they are predominant biotypes associated with non-invasive diseases. These 2 areas will be of interest for further investigation.

The efficacy of biotyping as a potential epidemiological marker can be helpful when *H.influenzae* is isolated at the same time from different sites of a patient; and also could be useful for monitoring acquisition and loss of carriage from upper respiratory tract.[7]

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