International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 12 (10), pp. 001-012, October, 2018. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Biochemical and molecular characterization of *Haemophilus influenza* isolated from Riyadh, Kingdom of Saudi Arabia

Afaf I. Shehata*, Amal Abdulaziz Al-Hazani, Hesham Al-Aglaan and Hanan O. Al Shammari

Department of Botany and Microbiology, College of Science, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia.

Accepted 17 June, 2018

The objective of this study focused on the prevalence of *Haemophilus influenza* to confirm the colonies of *H. influenza* on the basis of their growth requirements and serotype distribution. This study prepared 80 isolates of *H. influenze* isolated from five different sources (eye, ear, sputum (SP), lower genital tract (TA), and nasopharyngeal (NPA)) with different ages for infants and elderly persons. The phenotypic characteristics, which included the biotype, serotype, antibiogram and β-lactamase production, were applied by using APINH and minimum inhibitory concentration (MIC). Also, the study focused on the identification of selected serotype using PFGE analysis. The discussion of this study differentiates the age groups occurrence in the isolates, alongside with non-typeable strain versus the typeable ones and their percentages in the sample of isolates. This clustering of most strains in one PFGE pattern might be explained with the colonel population structure of the encapsulated *H. influenza.*

Key words: *Haemophilus influenza,* biotype, serotype, antibiogram, β-lactamase.

INTRODUCTION

Haemophilus influenzae is an important opportunistic pathogen that is rapidly evolving toward widespread distribution among the human population. It colonizes the nasopharynx of approximately 75% of healthy children and adults and becomes a leading cause for a variety of infectious disease such as acute otitis media (AOM), sinusitis, pneumonia, sepsis and meningitis. Although the overwhelming majority of the invasive *Haemophilus* species infections are caused by *H. influenzae* Serotype b, other Serotype as well as nontypeable influenzae (NTI) have been associated with invasive diseases. The pathogens of *H. influenzae* are classified into six encapsulated strains called typeable depending on the serologically distinct capsular polysaccharides (serotype a-f) and non-capsule or nontypeable strains. *H. influenzae* are obligate normal flora of the upper respiratory tract of humans. These pathogen/commensally whose carriage populations are likely to be restricted in size can in some hosts cause life threatening invasive diseases.

Different pathogenic potential which occur almost exclusively in the most Vulnerable population sector-children between 6 months and 6 years of age are usually caused by the encapsulated strain. Non-encapsulated strain is mainly a cause of acute otitis media in both children and adults with exacerbations of chronic bronchitis complicating chronic obstructive pulmonary disease in adults (Murphy and Apicella, 1987).

Biochemical characterization of *H. influenzae* have yield valuable epidemiological information's as specific biotypes have shown to associate different infections, to vary with the source of isolation as well as the antigenic properties and antimicrobial resistance patterns (Berhofer and Back, 1979).

Although most of *H. influenzae* strain recovered from systemic infection belongs to serotype b, the other serotypes have also been isolated from infection in all age groups. Moreover none capsulated strain proved to cause serious ones following breakdown of persons-host defense mechanism (Cerquetti et al. 2000). Antibiotic resistance has emerged to ampicillin, chloramphenicol and tetracycline which are antimicrobial agents traditionally used in treatment of *H. influenzae* infection *H. influenzae* producing β- lactamase have increased globally since it was first recognised in 1972 (Jorgensen, 1992).

*Corresponding author. E-mail: afafsh@ksu.edu.sa.

Chlormaphenicol was consequently recommended in the initial therapy of invasive disease caused by this organism (Ister et al., 1984). Unfortunately chloramphenicol resistance has thereafter been reported worldwide after the first chloramphenicol-resistance strains were isolated in 1976 (van Klingeren et al., 1977).

β- lactamase prevalence rate was found to vary considerably among the different capsulated strain type and the non-capsulated. It also differs with the geographical locality (Garica-Rodriguez et al., 1999) and with patient age.

Consequently future spread of antibiotic resistance in *H. influenzae* may be halted or delayed by a more restricted use of antibiotics guided by continuous epidemiological monitoring and predicated therapeutic outcome.

The widespread infections of *H. influenzae* in The Kingdom of Saudi Arabia among children's and adults have resulted in the initiation of studies on this pathogen. In the present study focus is made on the isolation of *H. influenzae* from patients hospitalized in Riyadh region (KSA) and characterizes these isolate by a biochemical and molecular technique.

This study aims to assess the relationship of biotypes for *H. influenzae* to serotyping, antimicrobial susceptibility age and antibiogram typing. Also this study will evaluate the bacterial applicability for identification of genotype of the bacterial isolates. The main objectives of this study are:

i) To confirm the colonies of *H. influenzae* on the basis of their growth requirements for hemin and Nicotinamide dinucleotid (NAD).

ii) Investigate the prevalence and serotype distribution of *H. influenzae* isolated from patients with lower respiratory tract infection (LRTIS). In order to provide reliable data on-the prevalence of *H. influenzae* serotypes.

iii) Determine the prevalence, age-group distribution, serotyping, antibigram typing and susceptibility patterns of invasive *H. influenzae* in KSA children and adults.

iv) Identify the selected serotypes of the isolated bacteria on the basis of genetic diversity through molecular typing.

MATERIALS AND METHODS

Clinical specimens

The 80 *H. influenzae* isolates were collected from five sources that are eye, ear, sputum, lower genital tract, and nasopharyngeal, by the patients at the Clinical Microbiology Laboratory of the Riyadh Military Hospital 2007-2008. *H. influenzae* strains were grown on sterile Brain Heart infusion broth and the subculture was made by the use of chocolate blood agar that was ready for biotyping.

Identification of *H. influenza* isolate

Bacterial culture of *H. influenzae* is performed on nutrient agar, Chocolate agar, plate with added X & V factors at 37°C in an enriched 5% CO_2 incubator (*OXOID Wade Road, Basingastoke, Hampshire, RG24 8PW, England*). Gram-stained and microscopic observation of a specimen of *H. influenzae* will show Gram-negative, coccobacilli, with no specific arrangement. The APINH kit is a miniaturized rapid system for the identification of *Haemophilus* species which enable the performance of 12 identification tests (enzymatic reactions or sugar fermentations, detection of a penicillinase. Antimicrobial susceptibility testing confirmad by detection β-lactamase. The Kirby-Bauer disk diffusion method, or culture susceptibility test, is applied. *H. influenzae* agglutinating sera are intended for use in the qualitative slide agglutination test to identify serologically the type antigen of pathogenic strain of *H. influenzae* (types *a* to *f*) for epidemiological and diagnostic purposes. Biochemical testes (catalase and oxidase) and pulsed-field gel electrophoresis (PFGE) were applied. The preparation of genomic DNA suitable for PFGE begins for cultures by suspending them in Tris-EDTA (TE) buffer (usb Corporation Cleveland. OH USA) 100 mM and adjusted to a turbidity of 0.5 using a Dade Behring MicroScan turbidity meter.DNA in plugs for PFGE was then digested using the ApaI- (Promega Corporation.2800 Woods Hollow Road. Medison, WI53711-5399 U.S.A) restriction enzyme.

RESULTS

Descriptive epidemiology

A total of 80 invasive isolate of *H. influenzae* were identified , 36 of 80 isolates were nontypeable strain, 25 was a type f strain, 6 a type e strain, 7 a type a, 2 a type b strain, 2 a type c strain, and 2 a type d strain. Patients with invasive *H. influenzae* disease ranged in age from 1 day to older than 50 years old. 53 (65%) occurred in children less than 2 years of age; 12 (22%) occurred in age ranging from 3-19 years; the remaining 15 occurred in adults ranging from 20 years to older than 50 years old, (Figure 1). There are 7 serotypes in the 80 isolates. 7(8.75%) were serotype "a", 2 (2.5%) serotypes "b", 25 (31.25%) serotype "f", 2 (2.5%) serotype "d", 2 (2.5%) serotype "c", 36 (45%) NT, and 6 (7.5%) serotype "e", (Table 1). Moreover, all isolate were biotypeable as types I through V, and all encapsulated strain were serotype specific. No encapsulated biotype or non-capsulated biotypes VI, VII, and VIII organism were isolated. Of the serotypeable strain (96.25%) belonged to biotype II,III, and IV. 4 of 7 type A were typed as biotype II, 1 as biotype I and 2 biotype III. 2 of 6 type e were typed as III, 2 as IV ,1 as biotype II ,and 1 as biotype V. 18 of 26 type f were typed as biotype II and 7 as biotype III. 18 of 36 NT were typed as II, 15 as biotype III and 3 as type IV. 1of 2 biotype D was biotype II and 1 as biotype V. Serotype b organism belonged to biotype III and the 2 serotype c isolate were biotype II, Table 1.

Biotype *and H. influenza*

H. influenza may be subdivided into eight biotype (I through VII) each on the basis of three biochemical reactions that are also important in the differentiation between some of the *Haemophilus* SP; indole production, urease, and ornithine decarboxylase activities. Biotype of *H. influenzae* shows a relationship to the source of

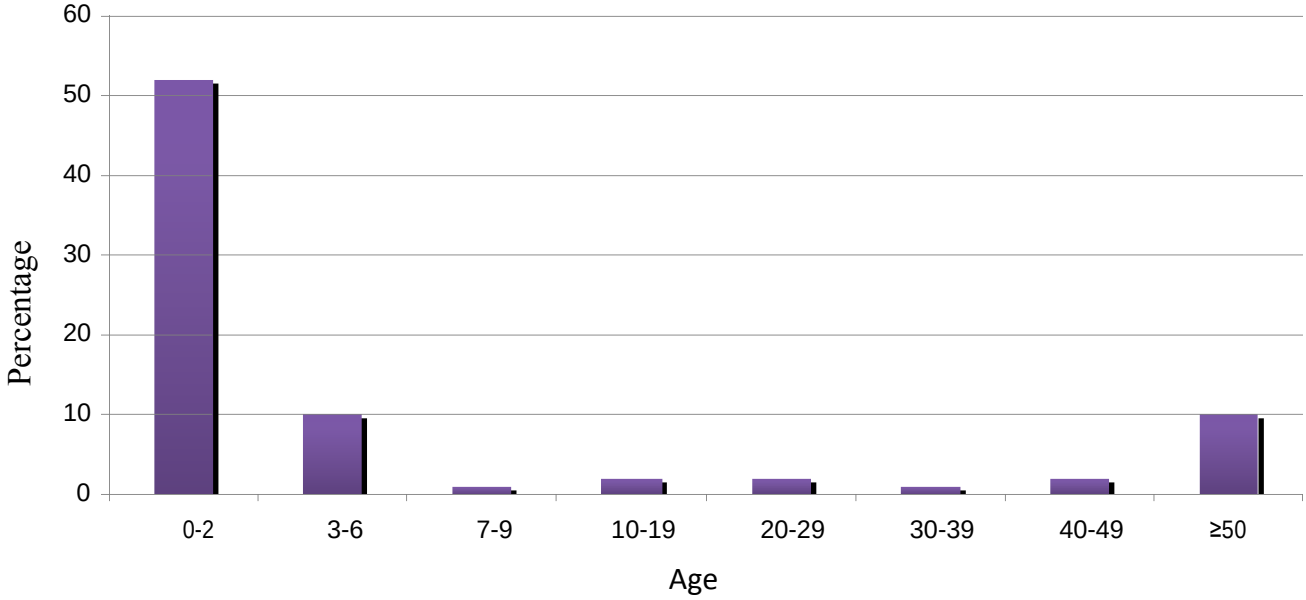

Figure 1: Age distribution of 80 with *H. influenzae* isolate.

Table 1. Relationship between biotypes and serotypes of *H .influenzae* isolate from different side of body from children and elderly.

Serotype	No. of isolate	No. of strain with following biotype				
		I	II	III	IV	V
A	7	1	4	2	–	–
B	2	–	–	2	–	–
C	2	–	2	–	–	–
D	2	–	1	–	–	1
E	6	–	1	2	2	1
F	25	–	18	7	–	–
NT	36	–	18	15	3	–
Total	80	1	46	26	5	2

isolation. Moreover, the biotypes of 80 *H. influenza* strains were determined. The results are presented in Table 2.

The most prevalent biotype was biotype II (57.5%) taken from the eye with a high isolate of 20 (43%) and the lowest one was taken from the ear with a low isolate of 4 (8%), followed by biotype III (32.5%) taken from the eye with a high isolate of 12 (46%) and the lowest one taken from the ear with a low isolate of 1 (4%). This was then followed by biotypes IV (6.25%), V (2.5%) and I (1.25%), respectively.

Serotyping and *H. influenza*

Although serologic typing of *H. influenzae* may be used to establish for isolate *H. influenze* as being any one of the six serotypes "a" to "f", it is usually only used to identify type "b" strain. All *H. influenzae* from cases of invasive infection should be serotyped to rule in/out type "b" infections. Testing can be performed using a slide agglutination test. Table 3 shows the distribution of serotypes *H. influenze* strains and different clinical specimens. From Table 3, most of the two common serotypes in all clinical specimens were NT (43% occurrence), and f which occur at 43 and 31%, respectively. The least common serotypes were a (9% occurrence), and e (7.5% occurrence) that were taken from 4 different clinical specimens that are SP, NPA, TA and EYE. Moreover, serotypes c and d occur only 2.5% in all specimens. Most of serotypes were isolated from EYE (43.7%), NPA (23.7%), SP (13.75%), and TA (11.25%) while only 11.25% serotypes were isolated from the ear. invasive infection should be serotyped to rule in/out type "b" infections.

Table 2. Distribution of biotype by source of isolate in patients.

Source	Biotype					
	I	II	III	IV	V	Total
SP	–	5	4	1	1	11
NPA	–	8	9	1	1	19
TA	–	9	–	–	–	9
EYE	1	20	12	2	–	35
EAR	–	4	1	1	–	6
Total	1	46	26	5	2	80

Table 3. Distribution of serotype *H. influenze* strains in different clinical specimens.

Serotype	Side of isolate					
	SP	NPA	TA	EYE	EAR	Total
A	2	1	3	1	0	7
B	1	0	0	1	0	2
C	0	0	1	1	0	2
D	0	1	1	0	0	2
E	2	2	1	1	0	6
F	3	3	2	15	2	25
NT	3	12	1	16	4	36
Total	11	19	9	35	6	80

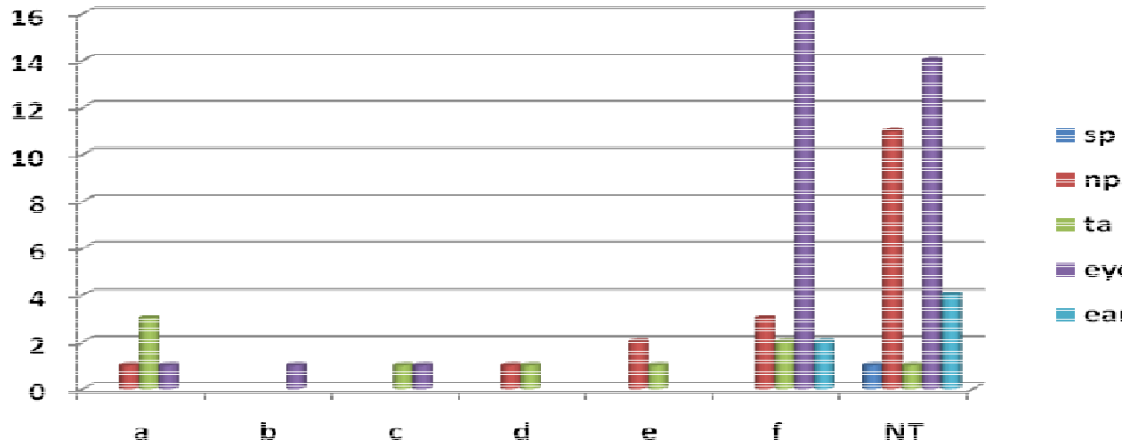

Figure 2. Distribution of serotype *H. influenze* strain in different clinical specimens.

Testing can be performed using a slide agglutination test. Table 3 shows the distribution of serotypes *H. influenze* strains and different clinical specimens. From Table 3, the most two common serotypes in all clinical specimens were NT (43% occurrence), and f which occur at 43 and 31%, respectively. The least common serotypes were a (9% occurrence), and e (7.5% occurrence) that were taken from 4 different clinical specimens that are SP, NPA, TA and EYE. Moreover, serotypes c and d occur only at 2.5% in all specimens. Most of serotypes were isolated from EYE (43.7%), NPA (23.7%), SP (13.75%), and TA (11.25%) while only 11.25% sero-types were isolated from the ear (Figure 2). Figure 3 shows that children are infected by *H. influenze* more than adult. The most serotypes infected children are NT (38.7%), "f" (28.6%) while the least infected serotypes are "b" (only 1.25%) comparing to serotypes "a" (6.25%) and "c" (2.5%). On adult, the most infected serotype was NT (25%) while "b" (1.25%) was the least infected one. Moreover, there are no serotypes "c" and "d" among adult

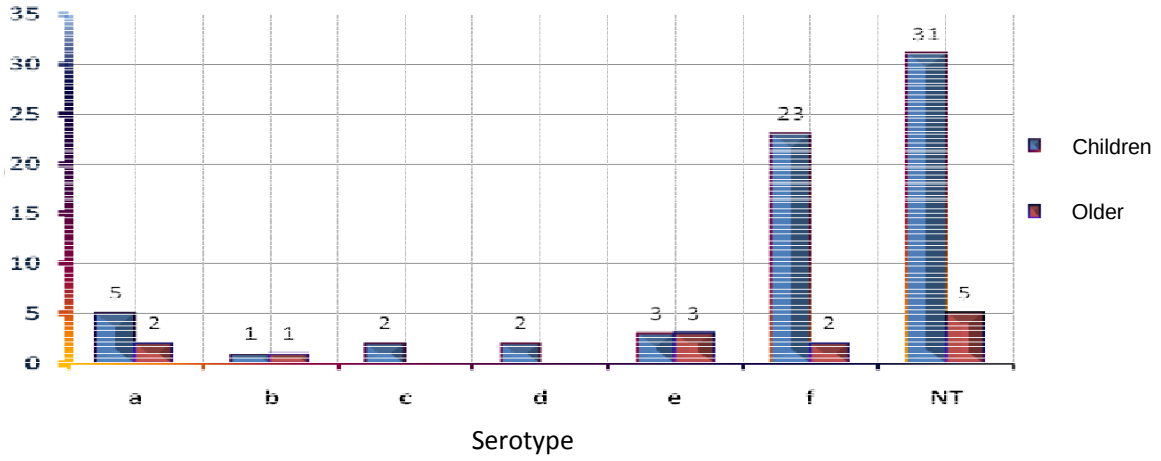

Figure 3. The comparison of serotypes of *H. influenza* between children and elderly.

Table 4. The comparison of serotypes of *H. influenza* between children and elderly.

Group	Serotype							
	a	b	c	d	e	f	NT	Total
Children	5	1	2	2	3	23	31	79
Elderly	2	1	0	0	3	2	5	13
All isolate	7	2	2	2	6	25	36	80

and only 2.25% among children.

The most serotypes infected children are NT (38.7%), f (28.6%) while the least infected serotypes are b (only 1.25%) comparing to serotypes a (6.25%) and c (2.5%).

On adult, the most infected serotype was NT (25%). While b (1.25%) was the least infected one. Moreover, there are no serotypes c and d among adult and only 2.25% among children. Looking at the different sources of isolating *H. influenze* among children. Table 4 shows that the *H. influenze* is mostly isolated from the EYE (49.2%).

Figure 4 shows that children are infected by *H. influenze* more than adult. The most serotypes infected children are NT (38.7%), "f" (28.6%) while the least infected serotypes are "b" (only 1.25%) comparing to serotypes "a" (6.25%) and "c" (2.5%). On adult, the most infected serotype was NT (25%) while "b" (1.25%) was the least infected one. Moreover, there are no serotypes "c" and "d" among adult and only 2.25% among children (Table 5).

Antibiotic susceptibility

Table 6 shows the relation between antibiotic and the isolated specimens. 20 of the 80 *invasive H. influenzae* produced a β-lactamase ampicillin resistant, of the 10 β-lactamase ampicillin resistant were typeables and 10 of the β-lactamase ampicillin resistant were nontypeable. The most effective antibiotics on *H. influenze* are AUG and AZI. All the isolated serotypes are sensitive to AUG expect F. For AZI, two serotypes are resistant to it which are F and NT. On the other hand for the antibiotic TE; there are 8 (10%) isolated bacteria that are resistant to it. Antibiotic C and CRM have a strong resistant from the bacteria with 13.75% of the total number of isolated bacteria from the 80 patients (Figure 5).

The following result were obtained from the isolate, the high sensitive was to Aug 95% with MIC50 0.5 µg/ml and MIC90 2 µg/ml, and AZI 93.7% with MIC50 and MIC90 were 2 µg/ml. However, out of 80 isolate of *H. influenzae* strains 30% resistant to Amp with MIC50 0.5 µg/ml AND mic90 >8 µg/ml, Te resistant came next where 18.5% with MIC50 1 µg/ml AND MIC90 4 µg/ml. Table 7. The following result were obtained from the isolate in children, the high sensitive was to Aug and Azi 97% with MIC50 1 µg/ml and MIC90 2 µg/ml (Figure 6).

However, out of 67 isolate of *H. influenzae* strain in children 22% were resistant to Amp with MIC50 0.5µg/ml and MIC90 >8µg/ml. The resistant came next where 13% with MIC50 1µg/ml and MIC90 8 µg/ml, Table 8. It found that 33% of the TA isolate and 26.3% of the NPA were Ampicillin resistant. For the EYE was 23%, Ear 17%, and SP 10% resistant to Ampicillin. Isolate tested against CRM showed resistance in 44% in TA, 1 8% SP, 10.5% NPA, and EYE was 5.7%. For C, it has been found 22%

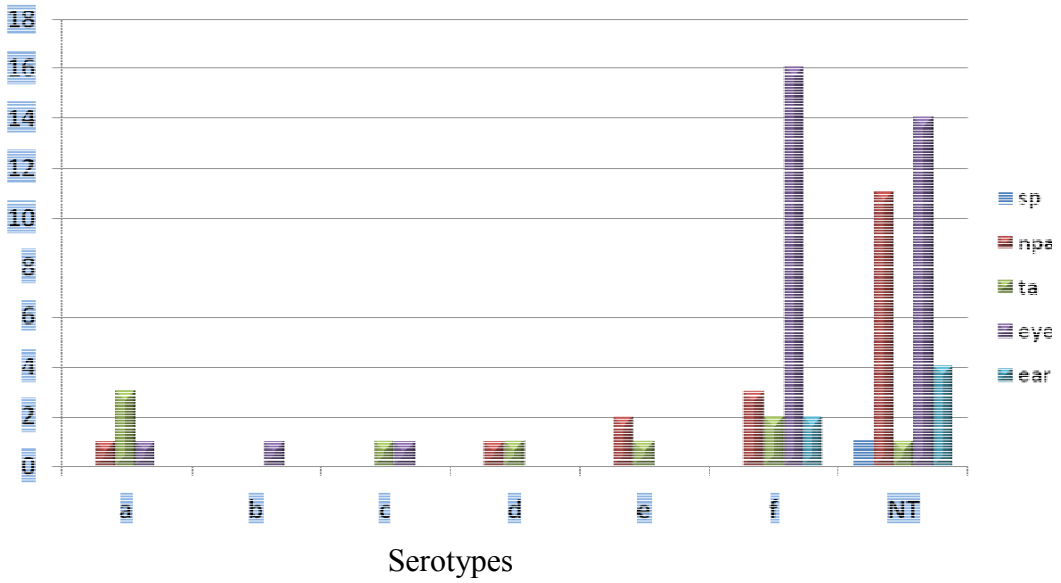

Figure 4. Comparison of serotypes of *H. influenza* in children

Table 5. The comparison between serotypes and sources of isolate of *H. influenzae* in children.

Side of isolate	Serotype						
	a	**b**	**c**	**d**	**e**	**F**	**NT**
SP	0	0	0	0	0	0	1
NPA	1	0	0	1	2	3	11
TA	3	0	1	1	1	2	1
Eye	1	1	1	0	0	16	14
Ear	0	0	0	0	0	2	4

Table 6. MIC 50%, MIC 90% range interpretation of 80 *H.influenzae* invasive isolate.

Antibiotic	MIC mg/l		% of isolate	
	MIC50%	**MIC90%**	**SEN**	**RES**
AMP	0.5	>8	75	25
CRM	1	8	82.50	17.5
TE	1	4	81.25	18.5
AUG	0.5	2	95	5
C	1	8	88.70	11.10
AZI	2	2	93.70	6.25

isolate from TA, 17% of isolate from EAR, NPA 15%, and EYE 14%. Isolate from SP were no resistance in C. The lowest resistant 3% isolate from EYE by using AUG and AZI. Isolate from EAR and NPA were showed no resistant by using AUG and AZI, (Figure 7). Moreover, it was found that isolate from SP is of higher resistant at 18% by using CRM, AZI, the lowest resistant was 0% by using AUG, C. Isolate from NPA were found to be higher in resistant at 26.3% in AMP, the lowest resistant 0% in AUG and AZI. Source Isolate from TA, the lowest resistant 0% by using AZI and higher resistant 44% in CRM. Source isolate from the EYE is of lowest resistant at 3% in AZI and higher at 23% in AMP. Finally, source of isolate from Ear, high one were 17% in AMP. C, and TE. The lowest resistant was 0% by using CRM, AUG, and AZI, Figure 8.

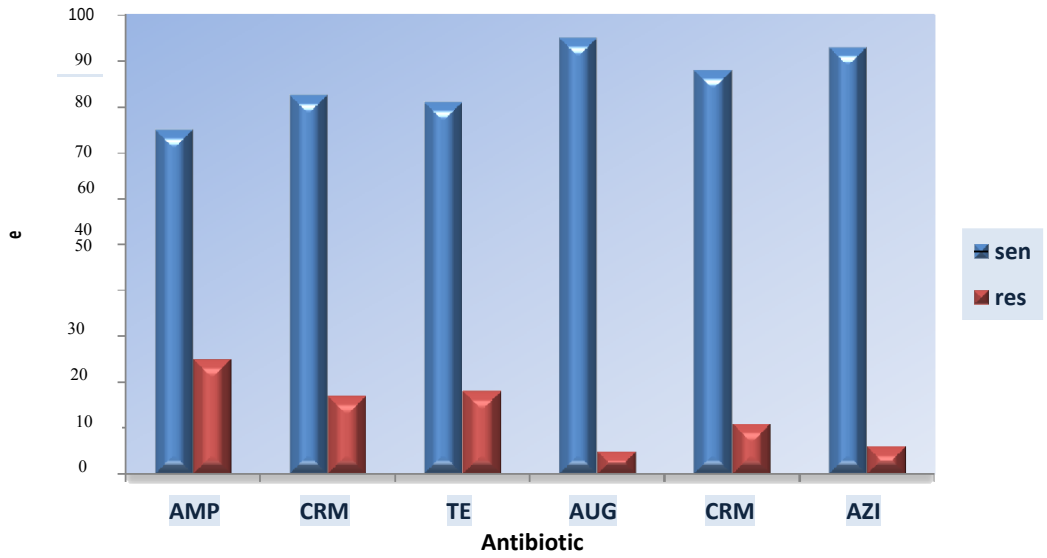

Figure 5. MIC 50%, MIC 90% range interpretation of 80 *H. influenzae* invasive isolate

Table 7. MIC 50%, MIC 90% range interpretation in children.

Antibiotic	MIC mg/l		% of isolate	
	MIC50%	MIC90%	SEN	RES
AMP	0.5	>8	77	22
CRM	1	8	88	12
TE	1	8	86	13
AUG	1	2	97	3
C	1	8	89	10
AZI	1	2	97	3

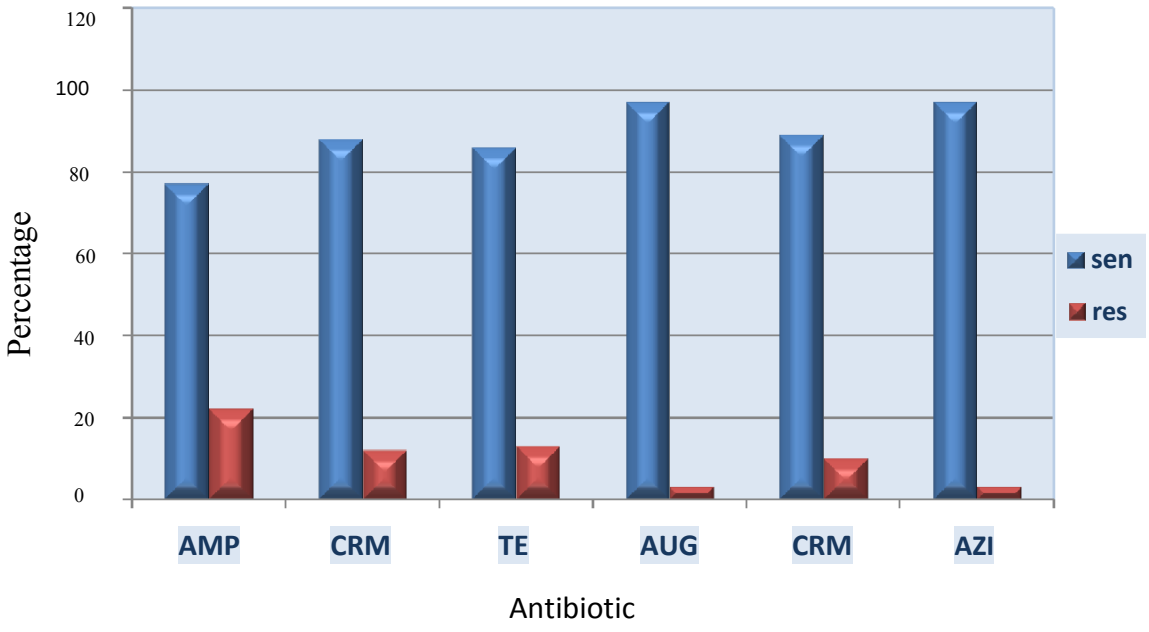

Figure 6. MIC50%, MIC90% range interpretation in children.

Table 8. Antibiotic sensitive & resistant of *H. influenzae* isolate by MIC test.

Source of isolate	No.	AMP (%)		CRM (%)		TE (%)		C (%)		AUG (%)		AZI (%)	
		SEN	RES	SEN	RES	SEN	RES	SEN	RES	SEN	RES	SEN	RES
SP	11	90	10	82	18	90	10	100	-	100	-	82	18
N PA	19	37.60	26.40	89.40	10.50	79	21	84	15	100	-	100	-
TA	9	67	33	56	44	89	11	78	22	89	11	100	-
EAR	6	83	17	100	-	83	17	83	17	100	-	100	-

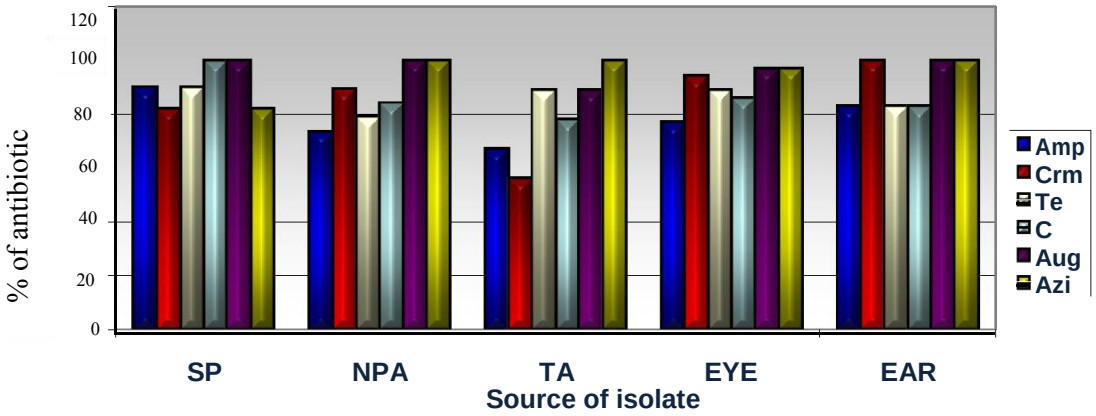

Figure 7. Antibiotic sensitive of *H. influenzae* isolate by MIC test.

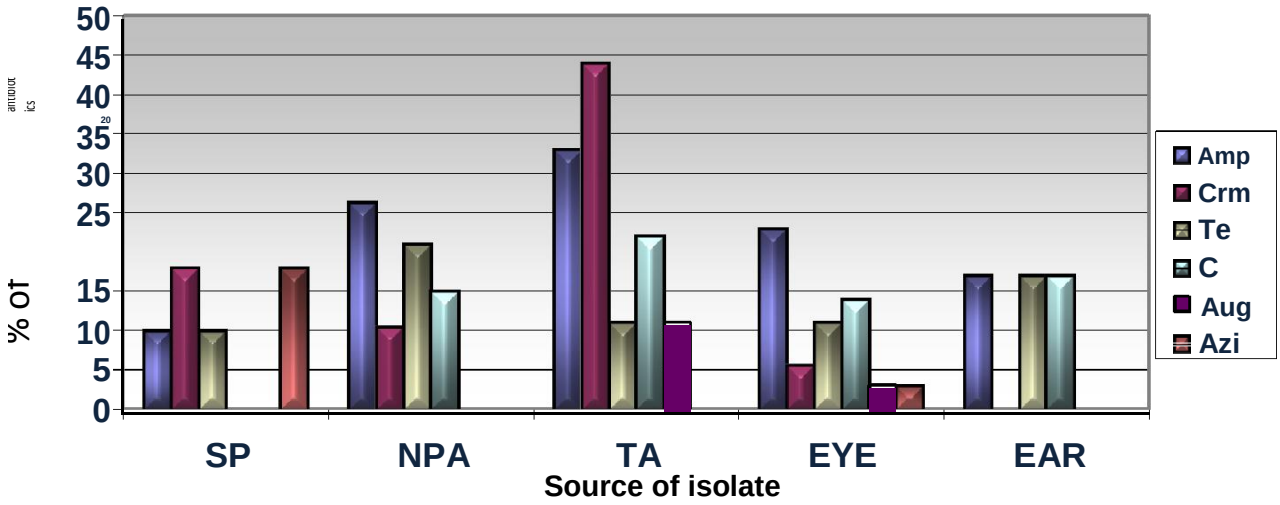

Figure 8. Antibiotic resistant of *H. influenzae* isolate by MIC test.

The susceptibility test was observed on *H. influenza* by AMP and AUG was more than C and TE using MIC and Kirby Bauer methods (Table 9 and Figure 9).

Pulse-field gel electrophoresis (PFGE)

PFGE was performed and compared with the fingerprints of genomic DNA for the typeable *H. influenzae* (serotype a to f). Many distinct restriction patterns were identified among the 6 strains available for PFGE, reflecting the expected genetic diversity among *H. influenzae* strains (Figure 10).

DISCUSSION

This study has been conducted to get a pilot profiling

Table 9. Comparative antibiotic susceptibility result using Kirby Bauer disc diffusion method and MIC.

Susceptibility method	% Resistance			
	AMP	AUG	C	TE
MIC	24	2.5	15	15
Kirby Bauer	27	5	15	17

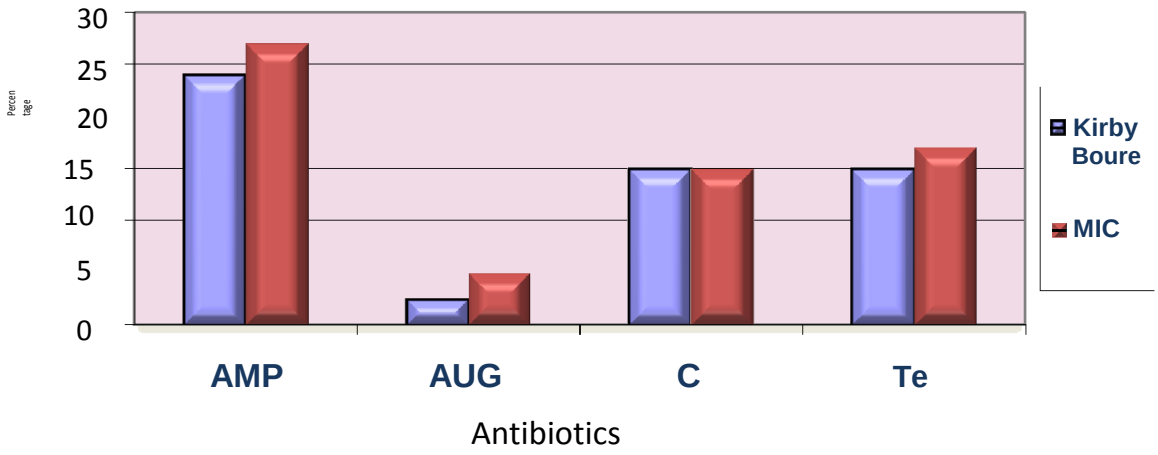

Figure 9. Comparative antibiotic susceptibility result using Kirby Bauer disc diffusion method.

biotype, serotype and antibiotic susceptibility. *H. influenzae* strain prevalent in Riyadh area through studying clinical specimens received in routine bacteriology laboratory of Riyadh Military hospital for the period from 2007-2008. The 80 *H. influenzae* isolates are collected from 5 sources that are eye, ear, sputum, lower genital tract, and nasopharyngeal. *H. influenzae* isolates ere almost equally prevalent in both sex males and females (Vidya and Devarajan, 2001.).

Distinct age predilection was observed where Patients with invasive *H. influenzae* disease ranged in age from 1 day to adult older than 50 years. 53 (66.25%) occurred in children less than 2 years of age; 12 (15%) occurred in age ranging from 3-19 years; the remaining 15 occurred in adults ranging from 20 years to older than 50 years old (Figure 1). The least prevalence was found in middle age groups varying from 2-6% of *H. influenzae*. in many other studies 86, 88, 90, and 95% invasive *H. influenzae* infection have been found to occur under 5 years of age and 9% in adults (Anderson, 1983; Wang et al., 2008). This emphasizes the fact that *H. influenza* type b (Hib) disease should be recognized as potentially contagious in young children and elderly. Based on the result of three tests-indole production, urease activity, and ornithine decarboxylase activity *H. influenzae* strain have been identified and characterized into 8 biotype I-VIII. Biochemical characterization of *Haemophilus* sp. has lead to important epidemiologic information and certain biotypes have been found paralleled with different origins of isolate (Barry et al., 1991). Almost of the total isolate belonged to biotype II 57.5% followed but two folds less frequently 33.75% equally by each biotype I and III. Biotype IV and V constituted only 6.25 and 2.5% of stain respectively. Rarely were biotypes VII and VI met with. The most prevalent biotype was biotype II (57.5%) that were taken as the highest isolate from eye 20 (43%) and lowest one was taken from the ear 4(8%) , followed by biotype III (32.5%) that were taken as the highest isolate from eye 12 (46%) and lowest one taken was from ear (4%) (Table 2). Biotype distribution varied considerably in different infection. Biotype II was principally isolated from sputum (sp), eye, next from ear, nasopharyngeal (NPA) and genital tract (TA). Of interest was the fact that in all specimens this biotype was equally representing almost 50% of all and any infection. For instance biotype I and serotype b are commonly associated with meningitis in children, and biotypes II and III are commonly associated with upper respiratory tract infections (Gratten, 1983; Olsen et al., 2005). Serotyping of *H. influenzae* isolate differentiated them into 55% as typeable (THi) and 45% as nontypeable *H. influenzae* (NTHi) using slide agglutination test (Table1).Co agglutination test of nontypeable strains of *H. influenza* showed no false-positive reactions. The use of co agglutination reagents for serological identification of clinical isolates of *H. influenza* proved more advantageous than conventional slide agglutination in several respects. The reactions were rapid, sensitive, and easy to visualize and interpret (Farmer, and Tilton. 1980;

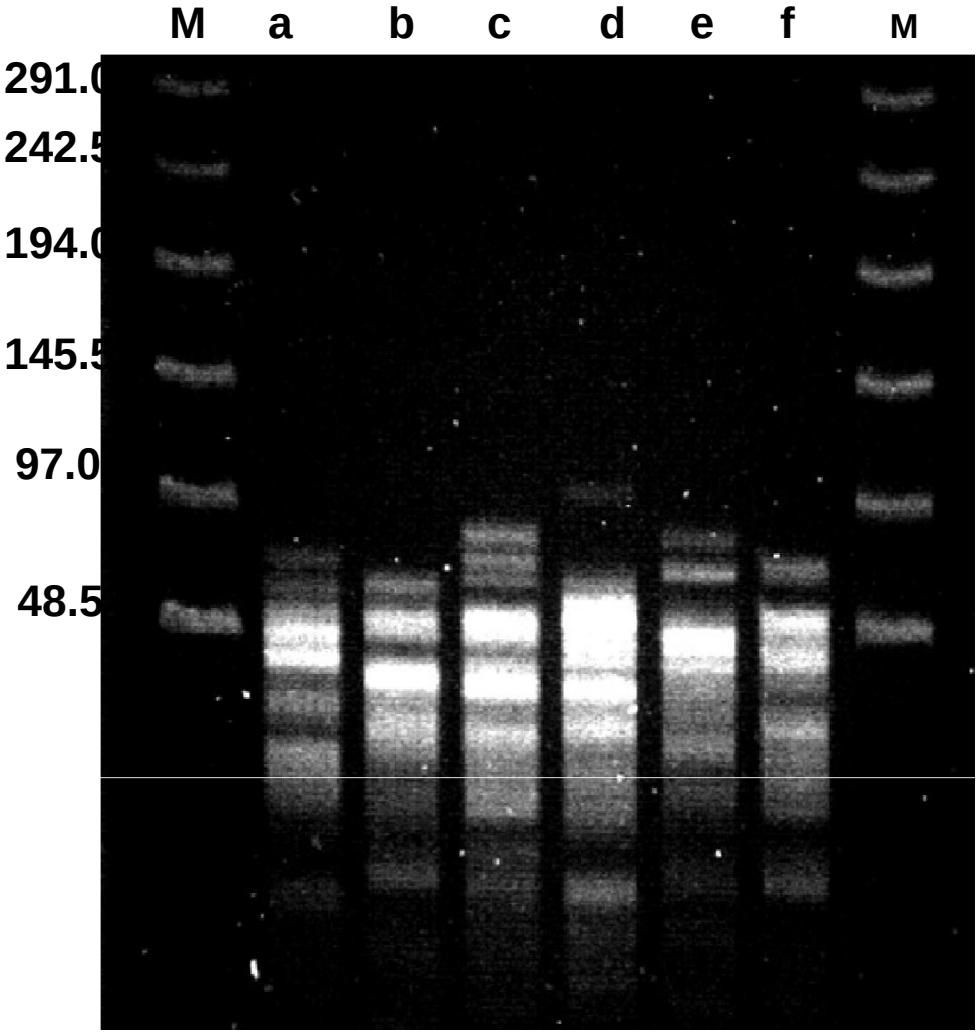

Figure 10. Pulsed-field gel electrophoresis (PFGE) patterns of ApaI-digested chromosomal DNAs of *Haemophilus influenzae* isolates. Lan M, Lan serotype a, Lan serotype b, Lan serotype c, Lan serotype d, Lan serotype e, Lan serotype f, Lan M.

Silverman et al., 1977). *H. influenza* asymptomatically colonizes the nasopharynx of healthy individuals, and causes systemic disease and mucous membrane infections. Eight biotypes and six serotypes are used as epidemiological markers for studying the pattern of colonization of *H. influenzae* and to identify the strains of bacterium commonly known to be pathogenic (Alrawi et al., 2002; Amita et al., 2006). In acute infection, most cases of primary invasive diseases, secondary chronic pulmonary infection with serious complication as well as meningitis with predisposing factor as sinusitis or otitis media were found to be due to nontypeable *H. influenzae* (Bilton et al., 1995; Meats et al., 2003.).

This study focused on the distinctive relationship between serotype prevalence and age. Capsulated strain was found to be solely restricted to the first two decodes of life but in a remarkable humble percentage. On the other hand nontypeable *H. influenzae* strain apart from being extensively encompassing all age group were exclusively prevalent beyond decade of life (Table 4).

Distribution of serotypes *H. influenze* strains and different clinical specimens, the most two common serotypes in all clinical specimens were nontypeable (NT) (43% occurrence), and serotype f that occur 43 and 31%, respectively. The least common serotypes were serotypes a (9% occurrence) and e (7.5% occurrence) that were taken from 4 different clinical specimens (SP, NPA, TA and eye), while the major serotype a (43%) was found in TA and the major serotype e (33%) was found in SP and NPA. Moreover, serotypes c and d occur only at 2.5% in all specimens. Most of serotypes were isolated from eye (43.7%), NPA (23.7%), SP (13.75%), and TA (11.25%) while only 11.25% serotypes were isolated from the ear (Table 3) shows that the *H. influenze* is mostly isolated from the eye (49.2%). Moreover, the most serotypes in children are Serotype f (23.8%) and NT (20.8%). Looking at the isolated sources in

H. influenze is less common in the following sources: TA, ear and SP for children. Furthermore, only nontypeable (NT) was found on all the sources for children (Table 5). The study pointed out that, *H. influenza* serotype "f" was the principal serotype encountered in almost 32.5% of capsulated strain and as the exclusive serotype in eye and genital specimens. High relationship has been found between *H. influenzae* strain serotypes and biotypes. Biotype II was the principal one in *H. influenzae* serotype "f" & "a". biotype III were equally belonging to serotype "a"& "f" (Table 1). In fact a substantial correlation has been found between biotypes and serotypes of *H. influenzae* strain (8). in agreement with the fact that the serotypes constitute separate evolutionary lineaging. Intensively was biotype I belonging to serotype "a", "b", & "f"; biotype II to serotype "c"; and biotype IV to serotype "d" or "e "(22). About 85% of *H. influenzae* strains were β-lactamase negative and 25% β-lactamase positive. The majority of the β-lactamase positive strains were ampicillin highly resistant, chloramphenicol and tetracycline 35 and 45% resistant respectively and cefotaxime 90% sensitive. In contrast 90% of β-lactamase negative *H. influenzae* strains were all among the sensitive strain to the tested antibiotic (Table 6). Other prevalence rate of β-lactamase production approached (25%) or showed distinct difference as in isolate from the Middle East being 65.3% in Kuwait, 79.5% in Oman, 82.9% in Saudi Arabia and united Arab Emirates abd 95.7% in Europe in Turkey (26). In the United States, the proportion of BLNAR isolate remains very low while during the last decade the proportion of β-lactamase negative isolate has rapidly increased in Japan (Hasegawa et al., 2004).

In this study β-lactamase positive has *H .influenzae* strain were 10% nontypeable, for all 80 *H. influenzae* isolates were studied for their *in vitro* susceptibility against 6 antibiotics known to be first choice treatment in *H. influenzae* infection. These were ampicillin, chloramphenicol, azithromycin, tetracycline, cefuroxime, and amoxicillin using 1 method run in MIC (Table 6). Influenzae isolate were found susceptible to amoxicillin clavulanate in wide scale conducted study in 4 European countries in France, Germany, Italy and Spain (Blosser et al., 2002). In the present work almost all *H. influenzae* (89%) were sensitive to chlorophenicol (Tables 7, and 8). In fact wild type strain of *H. influenzae* is known to be susceptible to chloramphenicol and to the macrolide groups of antimicrobials. However, because of the spread of conjugative plasmids, a vast proportion of clinical isolates have become among others, chloramphenicol resistant (Jacobs et al., 1999).

For *H. influenza* isolate, tetracycline and cefuroxime showed a lesser effect than the rest other tested antibiotics where 81.25 and 82.5% of strain were sensitive to respectively. Resistance to amoxicillin was negligible (5%) by susceptibility test (Table 7).

In France resistance of *H. influenzae* to tetracycline and chloramphenicol was fond to be less than 10 and 2% respectively in 1997 (Abdurahman et al., 2000). In Portugal 20% of *H. influenzae* was proved to be resistant to ampicillin (Shibl, 2001). Data from the Alexander Project 1999 for Saudi Arabia indicated that 27.9 and 29% af *Haemophilus* isolate were ampicillin and trimethoprime sulphamethoxazole resistant respectively (Felmingham and Washington, 1999).

Regarding the specimen site antibiotic relationship studied in the present work, it was found that Ciproloxacin and fotaxime were universally potent regardless of their specimen origin (Table 9); although Chlorophenicol meningitis showed 100% potency in SP as expected. However, Tetracycline and Ampicillin almost had the same efficiency on *H. influenzae* isolate from SP (90%). However, prevalence of ampicillin resistant *H. influenzae* β-lactamase positive strain increased conreased considerably (Hasegawa et al., 2003) in both typeable and non typeable strain posing a serious problem in Europe and USA (10) and Middle East (Shible and Gaillot, 1994). However Ampicillin resistant prevalence, showed large different between individual countries, where it was highest in Spain 30.6% and lowest in Germany 1.6%. Moreover, the prevalence of chloramphenicol resistance remains below 1% in USA and European countries, yet resistant has become a major problem in Spain 24.9% and Belgium 10.9% and are often additionally resistant to ampicillin, tetracycline (Machka et al., 1988). This study shows, in β-lactamase positive strain, a high level of resistant to ampicillin was displayed with MIC90 of >8 µg/ml and resistance to chloramphenicol and tetracycline with MIC90 of 4 or >4 µg/ml (Table 7). *H. influenzae* genotype was identified by PFGE.

Briefly, the bacterial effective resistance to antibiotic or its susceptibility were identified and discussed in the sample of isolates. Out of the 80 isolates, there were 53 (65%) occurred in children less than 2 years of age. 12 (22%) occurred in age ranging from 3-19 years. The remaining15; occurred in adults ranging from 20 years to older than 50 years old. During the study period, a total of 80 invasive isolates of *H. influenza* were identified, 36 of 80 isolates were nontypeable strain, 34 isolates were typeable. There were 6 serotypes in the 80 isolates. There percentages found to be, 7 (8.75%) were serotype "a", 2 (2.5%) serotypes "b", 25 (31.25%) serotype "f", 2 (2.5%) serotype "d", 2 (2.5%) serotype "c", 36 (45%) nontypeable, and 6 (7.5%) serotype "e".

Nontypeable (38, 7%) was the most serotypes that infect children , ranging from one day to ten years old, serotype "f" (28.6%) while the least infected serotypes was "b" (only 1.25%) comparing to serotypes "a" (6.25%) and "c" (2.5%). For adult, the most infected serotype *was* nontypeable (25%) while "b" (1.25%) was the least infected one. Looking at the different sources of isolated *H. influenza* among children were mostly isolated from the eyes (49.2%). Moreover, the most serotypes in children were "f" (23.8%) and nontypeable (20.8%).The order of the most prevalent biotype for all isolates were

biotype II (57.5%), then biotype III (32.5%), biotype IV (6.25%), biotype V (2.5%) followed by biotype I (1.25%). (Studying the effect of antibiotic showed there were 20 of the 80 invasive *H. influenza* produced a β-lactamase ampicillin resistant , of the 10 β-lactamase ampicillin resistant were typeables and 10 of the β-lactamase ampicillin resistant were nontypeable. The rate of β-lactamase producers was extensively among nontypeable of *H. influenza.* Resistance wise, the most effective antibiotics on *H. influenze* found to be amoxicillin (AUG) and azithromycin (AZI). All of the isolates serotypes found to be sensitive to amoxicillin (AUG) expect F. For: azithromycin (AZI), two serotypes found resistant to it which are type "f" and nontypeable (NT). On the other hand for the antibiotic Tetracycline (Te), there were 8 (10%) isolated bacteria found to be resistant to it. Antibiotic chloramphenicol (C) and cefuroxime (CRM) have a strong resistant from the bacteria with 13.75% of the total number of isolated bacteria from the 80 patients. All isolate exhibited the serotype a, b, c d, e and f capsular serotypes. PFGE with Sma1 restriction enzyme digestion generated not well- resolved profiles, as several very close fragments of 97.0-48.5 kb were obtained (Figure 10). Following the PFGE profiles were easier to compare the isolate with other kb such as Lan 1,3,4,5. The isolate in Lan serotype a, Lan serotype b, Lan serotype c, Lan serotype d, Lan serotype e, Lan serotype f were ranging between 97.0 and 48.5 kb. According to criteria reported by Tenover (1995) and Wang et al. (2008), all isolated were related to *H. influenzae* serotype a to f. According to epidemiologic data, patient with strains that showed indistinguishable information that did not appear to in PFGE patter which cannot be observed in any common risk factor. The result of PFGE in data needed to be assured by marking with more than one restriction enzyme and Multiple PCR analysis.

REFERENCES

Abdurahman EM, Ismael NA, Dixon RA (2000). Antimicrobial resistance and prevalence of β-lactamase in *Haemophilus influenzae* isolate a surveillance study of patients with respiratory infection in Saudi Arabia. Diagn. Microbiol. Infect. Dis., 36: 203-208.

Alrawi AM, Chern KC, Cevallos V, Lietman T, Whitcher JP, Margolis TP, Cunningham ET (2002). Biotypes and serotypes of *Haemophilus influenzae* ocular isolates. Br. J. Ophthalmol., 86: 276-277

Amita J, Pradeep K, Shally A (2006). High ampicillin resistance in different biotypes and serotypes of *Haemophilus influenzae* colonizing the nasopharynx of healthy school-going Indian children. J. Med. Microbiol., 55: 133-137.

Anderson P (1983). Antibody response to *Haemophilus influenzae* type b and diphtheria toxin induced by conjugates of oligosaccarides of the type b capsule with the nontoxic protein. Infect. Immun., 39: 233-238.

Barry AL, Jorgensen JH, Hardy DJ (1991). Reproducibility of disc susceptibility tests with *Haemophilus influenza*. Antimicrob. Chem., 26: 295-301.

Berhofer TR, Back AE (1979). Biotypes of *Haemopilus influenzae* encountered in clinical laboratories. J. Clin. Microbial., 10: 168-174.

Bilton D, Pye A, Johson M (1995). The isolate and characterization of nontypeable *H. influenzae* from the sputum of adult cystic fibrosis patients. Eur. Respir. J., 8: 948-53.

Blosser MRS, Karlowsky JA, Critchely LA (2002). Antimicrobial surveillance of *Haemophilus influenzae* in the United states during 2000-2001 leads to detection of clonal dissemination of β- lactamase negative and Amoicillin resistant strain. J. Clin. Microbiol, 40: 1063-1066.

Cerquetti M, Atti M, Renna G (2000). characterisation of non-type b *Haemophilus influenzae* strain isolated from patients with invasive disease. The HI study group. J. Clin. Microbiol., 38(12): 46-52.

Farmer SG, Tilton RC (1980). Immunoserological and immunochemical detection of bacterial antigens and antibodies: In E. H. Iennette, A. Balows, W . H. Hausler, and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C., pp. 528 -529

Felmingham D, Washington J (1999). Trends in the antimicrobial susceptibility of bacterial respiratory tract pathogens-finding of the Alexander Project 1992-1996. J. Chemother., 11: 5-21.

Garica-Rodriguez JA, Baqiero F, De Lomas G (1999). Antimicrobial susceptibility of 1422 *Haemophilus influenzae* isolates from respiratory tract infection in spain. Result of a 1 year (1996-1997) multicenter surveillance study. Infect. Abstr., 27: 265-267.

Gratten M (1983). *Haemophilus influenzae*, biotype VII. J. Clin. Microbiol., 18: 1015-1016.

Hasegawa K, Chiba N, Kabayashi R, Murayama S (2004). Rapidly increasing prevalence of β-lactamase non producing ampicilin resistant *Haemophilus influenzae* patients with meningitis. Antimicrobial Agents Chemother., 48: 1509-1514

Hasegawa K, Yamamoto K, Chiba N, Kobayashi R, Nagai K, Jacobs MR (2003). Diversity of ampicillin-resistance genes in *Haemophilus influenzae* in Japan and the United States. Microb. Drug Resist. Spring, 9(1): 39-46

Ister GR, Conner JS, Glode MP (1984). Increasing ampicillin resistance rates in *Haemophilus influenzae* meningitis. Am. J. Dis. Child, 138: 366-369.

Jacobs MR, Bajaksouzain S, Zilles A (1999). Susceptibilities of streptopneumonia, and *Haemophilus influenzae* to 10 oral antimicrobial agents based on pharmacodynamic. Antimicrob. Agents Chemother., 43(139): 1091-1908.

Jorgensen JH (1992) Update on mechanisms and prevalence of antimicirobial resistance in *Haemophilus influenzae*. Clin. infect. Dis., 14: 11-23.

Machka K, Barveny I, Daberant H (1988) Distribution and resistance pattern of *Haemophilus influenzae* a European cooperative study. Eur. J. Clin. Microbiol., 7(140): 14-24.

Meats E, Feil EJ, Stringer S, Cody AJ, Goldstein R, Kroll JS, Popovic T, Spratt BG (2003). Characterization of encapsulated and noncapsulated *Haemophilus influenzae* and determination of phylogenetic relationships by multilocus sequence typing. J. Clin. Microbiol., 41: 1623-1636

Murphy TF, Apicella MA (1987). Nontypable *Haemophilus influenzae* review of clinical aspects, surface antigen, and the human immune response to infection. Rev. Infect. Dis., 9: 1-15.

Olsen SJ, Dejsirilert S, Sangsuk L, Chunsutiwat S, Dowell SE (2005). Frequent *Haemophilus influenzae* type b colonization in rural Thailand. Pediatr. Infect. Dis. J., 24: 739-742.

Shibl A (2001). Comparison of the *in vitro* activity of commonly prescrbed antibiotics againt *Haemopilus influenzae* isolate from the Middle East. Clin. Microbiol. Infect., 7(1): 1-394.

Shible AM, Gaillot O (1994). Susceptibility of clinical significal *Haemophilus influenzae* strain to oral antimicrobial agent in Saudi Arabia. Chemotherapy, 40(138): 300-403.

Silverman M, Stratton D, Diallo A, Egler L (1977). Diagnosis of acute bacterial pneumonia in Nigerian children. Arch. Dis. Child. 52: 925-931.

van Klingeren B, Van Embden JDA, Dressens KM (1977). Plasmid mediated chlramphenicol resistamnce in *Haemophilus influenzae*. Antimicrob. Agents Chemother, 11: 383-387.

Vidya R, Devarajan MD (2001). *Haemophilus influenzae* infection. E Med. J., 2: 80.

Wang CC, Kuo HY, Chiang DH, Tsai CC, Lin ML, Chan YJ. (2008). Invasive *Haemophilus influenzae* disease in adults in Taiwan. J. Microbiol. Immunol. Infect., 41(3): 209-214.