

Biofilm Formation & β - lactamase Production of *Haemophilus influenzae* Isolated from Lower Respiratory Tract Infections

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Nazar Shiyaa Al-Azawi*, Sabeha Al-Jubori, Abdul-Razzak SH.Hasan*****

*M.Sc., College of Medical Technologies

**Ph.D., College of Medical Technologies

***Ph.D., Al-Anbar College of Medicine

Abstract:

Objectives: To explore the biofilm formation and β -lactamase production of *H. influenzae* isolated from lower respiratory tract infections.

Materials and methods: For the period from 1st Jun/2004 to 30th April/2005, 142 sputum samples were collected from patients with lower respiratory tract infections. These patients were attending TB and Chest disease center or Baquba general hospital. The specimens were cultured on chocolate agar plates with multivitex (Multi-vitamins, BDH-UK) under 10% CO₂. Isolation and identification of *H. influenzae* were based on standard bacteriological and biochemical criteria plus serotyping with specific antisera (Bio-RAD Laboratories-Japan). An *in vitro* biofilm formation was assayed by microtiter plate technique. The β - lactamase production was determined by direct capillary tube method.

Results: Forty isolates of *H. influenzae* were recovered throughout this study. The isolation rate from males was higher than females (72.5% vs 27.5%). The highest isolation rate was recovered from patients with 40 years and older 17(42.5%) compared to other age groups. 17.5% isolates were *H. influenzae* type b (Hib), and the remaining 82.5% isolates were nontype b. twenty percent of the isolates were found to be β -lactamase producers. Furthermore, 37.5% of isolates were found to have the ability for biofilm formation and the majority (66.7%) was β -lactamase producers.

Conclusion: Characterization of biofilms may be important in understanding the pathogenesis of *H. influenzae* infection in human.

Introduction:

H. influenzae is recognized as an important cause of community-acquired pneumonia, acute exacerbation of chronic bronchitis, acute sinusitis and acute otitis media (Doern et al., 1999). Several studies have reported that relatively high rates of β -lactamase positive *H. influenzae* strains particularly among *H. influenzae* type b invasive isolates (Campos et al., 2004; Yagci et al., 2004).

Doern and Brown (2004) reported that 28.3% of *H. influenzae* isolated from respiratory tract infection were β –lactamase producers. In another study on *H. influenzae* isolated from children less than 3 years old, 44.5% were β –lactamase producers (Daberant, et al., 2003).

Experimentally induced otitis media by *H. influenzae* provide evidence that mucosal biofilm forms, suggesting that biofilm formation may be an important factor in the pathogenesis of chronic otitis

media with effusion (Ehrlich et al., 2002; Bouchet et al., 2003; Sword et al., 2004). In an *in vitro* assay on clinical isolates of non-typeable *H. influenzae* from middle ear fluid of children and from sputum of adults with chronic obstructive pulmonary disease, showed substantial variability to grow as biofilms (Murphy and Kirkham, 2002).

Materials and methods:

This study was conducted from 1st Jun/2004 to 30th April/2005. One hundred and forty early morning sputum samples were collected from patients with lower respiratory tract infections. These patients were attending TB and chest disease center or Baquba general hospital. Sputum samples were collected in wide-mouth sterile containers with a tightly fitting screw caps. Before culturing, the specimens were mixed thoroughly using sterile glass beads, then a loopful was taken and streaked on chocolate agar plate supplemented with multivitex ((Multi-vitamins, BDH-UK). The plates were placed in a candle jar (5-10% CO₂) and incubated overnight at 37°C.

Isolation and identification of *H. influenzae* were based on colonial appearance and standard bacteriological and biochemical criteria. These include indole production, hydrolysis of urea agar, positive oxidase and catalase tests and production of hemolysis on blood agar plates (Baron et al., 1994; Collee et al., 1996; Brooks, et al., 2001). Serotyping with specific antisera (Bio-RAD Laboratories-Japan) was carried out by slide agglutination method.

Biofilms formation using microtiter plate assay was carried out according to the method described by Sandoe *et al.* (2003). β - lactamase production was determined by direct capillary tube method (Koneman *et al.* 1992).

Results:

Out of 142 sputum samples, a total of 40 isolates of *H. influenzae* were recovered. Twenty nine isolates were recovered from male patients with mean age (46 $\pm$ 18) years and 11 from female patients with mean age (41.1 $\pm$ 13.3) years.

The highest isolation rate of *H. influenzae* was recovered from patients with 40 years and older 17(42.5%) compared to the age groups 20-39 and < 20 years (37.5% & 20%) respectively.

Eight out 40 (20%) of *H. influenzae* isolates were found to be β -lactamase producers. Five (62.5%) of them were recovered from males and 3 (37.5%) from females. The difference between the two groups was statistically insignificant (P= 0.66)

The serotyping results revealed that 7 (17.5%) isolates were *H. influenzae* type b (Hib), and the remaining 33 (82.5%) isolates were nontype b. Furthermore, 6 out of 7 (85.7%) of β - lactamase producing isolates were Hib, whereas only 2 out of 33(6.1%) of β - lactamase producers were nontype, table (1).

Among 16 *H. influenzae* isolates, 6(37.5%) were found to have the ability for biofilm formation, and of these 4(66.7%) were type b, table (2).

The results also revealed that the majority of *H. influenzae* isolates that have the ability to form biofilms were β -lactamase positive, table (3).

Table (1): The relationship between β -lactamase production & serotypes.

Serotype	β -lactamase production		Total
	positive	Negative	
<i>H. influenzae</i> type b	6(85.7%)	1(14.3%)	7(100%)
Non type b <i>H. influenzae</i>	2(6.1%)	31(93.9%)	33(100%)

Table (2): The relationship between biofilm formation & serotypes.

Serotype	Biofilm formation		Total
	Positive	Negative	
<i>H. influenzae</i> type b	4(66.7%)	1(10%)	5(31.2%)
Non type b <i>H. influenzae</i>	2(33.3%)	9(90%)	11(68.7%)
Total	6(100%)	10(100%)	16(100%)

Table (3): The relation between biofilm formation & β -lactamase production.

β – lactamase production	Biofilms formation		Total
	Positive	Negative	
Yes	4(66.7%)	1(10%)	5(31.2%)
No	2(33.3%)	9(90%)	11(68.7%)
Total	6(100%)	10(100%)	16(100%)

Discussion:

The high isolation rate of *H. influenzae* from patients at the age 40 years and older compared to other age groups are consistent with previous studies affirming that chronic respiratory disease due to *H. influenzae* were more frequent in elderly patients (Berghmans et al., 2003).

The high rate of non-type b isolates obtained in this study is consistent with other reports (Campos et al., 2004). Although nontypeable *H. influenza* strains lack a polysaccharide capsule, they possess a number of adhesive factors that promote their colonization and invasiveness especially in young children and patients with underlying medical conditions (Geme, 1997; Dawid et al., 2001; O'Neill et al., 2003). Additionally, this evidence suggests a possible high rate of interfamilial transmission of nontypeable *H. influenzae* among children and their parents (Watanabe et al., 2004). It is generally accepted that the carrier rate in the upper respiratory tract for *H. influenzae* type b is 2 – 4 %, while the carrier rate for nontypeable *H. influenzae* is 50-80% (Brooks et al., 2001). Additionally, adults with chronic respiratory diseases also have higher carriage rate (Gracia & Frenadillo, 2002). Furthermore, in lower respiratory tract infection, the mixed virus-bacteria coinfections were mostly associated with nontypeable *H. influenzae*. Probably the selective

pressure exerted by implementation of *H. Influenzae* type b vaccination may on the other side flourish the nontypeable strains to take on greater relative importance as a cause of invasive disease (LaClaire et al., 2003).

Regarding the rate of β -lactamase production by *H. influenzae* obtained in this study is not unusual, since it has been documented that the rate of β -lactamase production by *H. influenzae* isolates varied greatly according to geographic areas (McVernon et al., 2004; Doren & Brown; 2004; Felmingham et al 2004).

Unfortunately, studies regarding biofilm formation by *H. influenzae* were limited in the literature. However, the present results were consistent with those reported by Sward et al. (2004) who reported that 30% of *H. influenzae* isolates were biofilm former. It has been reported that clinical isolates of NTHi recovered from otitis media and from chronic obstructive pulmonary disease showed substantial variability to grow as biofilm *in vitro*, suggesting that biofilm formation may be an important factor in the pathogenesis of *H. influenzae* infection (Ehrlich et al., 2002; Murphy & Kirkham, 2002; Bouchet et al., 2003).

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