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In vitro Connection Between Biofilm Formation and Virulence factors of Pathogenic Ear Bacteria

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Abstract
Fifty-two samples of ear bacterial infection discharges were collected from some medical centers in Baghdad. The number and percentage of bacterial isolates were: Acute Otitis Media, 12 (23.1%); Chronic Otitis Media, 16 (30.8%); Acute Otitis Externa, 10 (19.2%); and Chronic Otitis Externa, 14 (26.9%). Nine tested isolates were formed biofilm by Congo red method and micro titer plate method. *S.aureus* produced α-hemolysin, while *Klebsiella pneumoniae* produced ɣ-hemolysin and *P. aeruginosa* produced ß-hemolysin. Production of ESBL was seen with *S. aureus, K. pneumoniae and P. aeruginosa* which showed inhibition zones to both Ceftazidime and Cefotaxime with 30mm, 37 mm; 32mm, 34 mm; and 18mm, 22mm respectively. Protease enzyme was produced by *S. aureus and P. aeruginosa*, and the inhibition zone were (32mm), while *K. pneumoniae* showed no production. Analysis of biofilm formation by scanning electron microscope showed that *Pseudomonas aeruginosa* (isolate no. 4) was able to form a biofilm on both surfaces, glass and polyester, and it is higher on polystyrene than on glass. Salt Aggregation Test results showed that *P.aeruginosa* aggregated with ammonium-sulfate solution of ≤1.0 M concentration, whereas, *Klebsiella pneumoniae and S.aureus* isolates had SAT value ≤1.5 M, and indicate that all tested bacterial isolates were hydrophobic. *P.aeruginosa* have strong capability to adhesion (CHA value 65%), while *Klebsiella pneumoniae* and *Staphylococcus aureus* have the moderate capability when exhibited 50% and 30% respectively. The findings of this search showed that biofilm formation can be seen as an indication of virulence and drug resistance of pathogenic ear bacteria, and biofilm formation has strong correlation with these virulence properties.

INTRODUCTION

Acute Otitis Media is considered the most common bacterial infection in pediatric age group. Treatment needs to be tailored to the specific pathogen because resistance gets increased to these bacteria, accounting for more costs in management. *Pseudomonas aeruginosa and Staphylococcus aureus* account for more than 40% of these infections especially in older children. (Al-Shawwa et al. 2005).

Four basic criteria defining infections caused by biofilm has been proposed by Parsek and Singh, they are: Bacterial cell adherence to or associated with a surface, Observation of clusters of bacterial cells in vivo, Localized pattern of infection, Increased in antibiotics resistance as compared to resistance of genetically similar individual bacteria (Reisner et al 2006). Factors influencing the process of biofilm creation are: Environmental including temperature, nutrient availability, pH, stress agents) and Microbial features (strain, cell surface, metabolic activity and growth phase). Initial attachment of planktonic bacteria is achieved by first changing the cell shape to a more sessile one along with flagellar movement in order to antagonize surface repulsive and hydrodynamic forces. This has been seen in various pathogens including *L. monocytogenes, E.. coli, V. cholerae* and *P. aeruginosa* (Toutain *et al.*, 2007).

Pseudomonas aeruginosa is an important pathogen for hospital infections mostly affecting immunocompromised patients with severe diseases. Infections caused by *P. aeruginosa* are usually difficult to manage because of high resistance to multiple antibiotics due to the intrinsic development of resistance and extrinsic acquisition of genes (Norouzi et al 2010).

Klebsiella pneumoniae subsp. Pneumoniae is known to be a virulent microorganism when it causes disease to human partly due to its ability to form biofilm. It has the ability to form biofilms causing pneumonia when inhaled as well as urinary tract infections, and infections in the biliary tree and wounds (Mirkar et al 2016).

Staphylococcus aureus can cause ear infections when normal nasopharyngeal flora get inhibited and decreased in numbers by frequent use of antibiotics. The biofilm formation ability of *S. aureus* is attributed to the production of Polysaccharide Intercellular Adhesin which is crucial in bacterial adherence to a surface and accumulation in a form of multilayered cell clusters (Gaffar et al, 2002).

The aim of this search was to determine any possible correlation between biofilm forming capability of ear pathogenic bacteria and their virulence properties, and have been demonstrated to play significant role in adhesion and biofilm formation.

METHODS

Samples Collection

Samples were collected from discharges of different types of ear infections, obtained from: (The Teaching Institute of Laboratories, Baghdad Teaching Hospital, Hospital of Surgical Specialties, Medical City and Al-Imamain Al-Kadhimain Medical City) in Baghdad. The samples were collected in the period between December 2016 to May 2017.

Isolation of ear pathogenic Bacteria

Staphylococcus aureus isolates were cultured using Mannitol salt agar which is selective to gram positive bacteria, then incubated for 24 hours at 37 °C, while *P. aeruginosa* and *K. pneumoniae* isolates were cultured using MacConkey agar which is selective to gram negative bacteria, and then cultured on Mueller-Hinton agar and Simmons citrate agar respectively for 24 hours at 37 °C.

Identification of Bacterial Samples

Primary diagnostic of isolates is based on morphological characteristics of the colonies on MacConkey agar and other selected media (MacFaddin, 2000). Also bacteria are observed by stained the isolates with Gram stain (*Benson, 2001).* Biochemical tests performed according to (Forbes *et al.* 2007).

Confirmation of Bacterial Isolates using the VITEK 2 System

The VITEK 2 system is a computerized analytic system dedicated for the detection, susceptibility testing of clinically significant bacteria.

Biofilm Production Assays

1-Congo Red Test

Bacterial isolates were incubated aerobically on Congo red medium for 24-48 hours at 37 ℃. Positive results were indicated by black colonies with a dry crystalline consistency The weak slim producers usually remain pink, while an occasional darkening at the centers of the colonies with the absence of a dry crystalline colonial morphology indicates an indeterminate result (Niveditha et al., 2012).

2-Microtiter Plate Method

The assay of biofilm formation was obtained from O'Toole (2011), and absorbance was measured at 630 nm by ELISA reader.

Hemolysin Production test:

Tested isolates of bacteria were used to detect the hemolysin production onto blood agar medium and incubated overnight at

35º C. Hemolysin production was detected by the presence of a zone of lysis of the erythrocytes around the colony (Johnson and Boese-Marrazzo, 1980).

ESBL Detection test:

Extended-Spectrum Bacterial Lactamase (ESBL) production screening was done according to the criteria recommended by NCCLS (2000). Briefly, two disks of antibiotic, ceftazidime (30 mg) and cefotaxime (30 mg) on Mueller-Hinton agar plate, were used by Kirby-Bauer disk diffusion method, and incubated overnight at 37 ℃. An inhibition zone of ≤ 22 mm for ceftazidime and ≤ 27 mm for cefotaxime indicated an ESBL producing strain.

Protease Production test:

About 100 µl of the suspension of isolated bacteria was added to the plates of milk agar. Inhibition zones were observed and measured in the medium (Mansour et al., 2014).

Antibiotic Sensitivity Test

Discs of antibiotics were put on Mueller-Hinton agar medium. Then, after incubation for 24 hours, the results were assessed and inhibition zone around antibiotic discs were checked (Nair et al., 2013).

Measurement of Bacterial Cell Surface Hydrophobicity

Bacterial hydrophobicity was assayed by Salt Agglutination Test (SAT) (Nalina and Rahim, 2006). Agglutination in salt concentrations of less than 1.0M indicated surface hydrophobicity. Conversely, surface hydrophilicity was indicated by the agglutination of bacteria in high salt concentrations, from 2.0 to 3.0M.

Bacterial Adhesion to Hydrocarbons

Microbial adhesion to hydrocarbon was assessed with xylene according to (Rosenberg and Gutnick, 1980). The degree of adhesion was calculated as [1-(A1/A0)] 100 [%].

Detection of Biofilm by Scanning Electron Microscopy (SEM)

Biofilm formation on polyester and glass strips was monitored visually by scanning electron microscopy model (TESCAN-VEGA/USA) operated at 30 KV, (Hyde et.al., 1997).

Statistical Analysis:

The Statistical Analysis System- SAS (2012) program was used to effect of different factors in study parameters. Least significant difference –LSD was used to significant compare between means in this study.

RESULTS AND DISCUSSION

Isolation and Diagnosis of Bacterial Isolates

Fifty-two samples of different ear infection discharges have been collected and isolated on different selective media (Table-1). The number and percentage of bacterial isolates from ear infections were distributed as: Acute Otitis Media, 12 (23.1%); Chronic Otitis Media, 16 (30.8%); Acute Otitis Externa, 10 (19.2%); and Chronic Otitis Externa, 14 (26.9%).

Table-1: Number of Bacterial Isolates of Ear Infections

Source of Infection	Number of Bacterial Isolates (%)
Acute Otitis Media	12 (23.1)
Chronic Otitis Media	16(30.8)
Acute Otitis Externa	10(19.2)
Chronic Otitis Externa	14(26.9)
Total	52(100)

Staphylococcus aureus colonies surrounding yellow zones after being incubated for 24 hours at 37 °c on mannitol agar. Mannitol fermentation is due to release of acidic byproduct formed that causes phenol red in the agar to turn yellow (Han *et al*, 2007). which is unique to *S. aureus.*

Isolates of *K. pneumoniae* appeared mucoid and convex along with lactose fermentation on MacConkey agar when cultivated at 37°C for 24 hours. Identification of *K. pneumoniae* isolates was performed using capsule test where it was tested positive, oxidase negative, catalase and citrate which were both positive also.

Pseudomonas aeruginosa isolates were circular mucoid smooth colonies are emitting sweat grape odor in nutrient agar. When put on blood agar it had a β-hemolysis, while it grew on MacConkey agar but with no lactose fermentation. Pyocin pigment was produced by *P. aeruginosa* on Mueller-Hinton agar.

Confirmation the identification of bacteria

Isolated bacteria had been confirmed the diagnosis by using the Vitek 2 system and the result is shown in figure-1.

Identification Information	Analysis Time:	4.75 hours	Status:	Final
Selected Organism	98% Probability	Klebsiella pneumoniae ssp pneumoniae		
	Bionumber:	6607734553564211		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	+	4	PyrA	+	5	IARL	-	7	dCEL	+	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	+	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	+	29	TyrA	+	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	-	47	ODC	-	48	LDC	+	53	IHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	(+)	64	ILATa	-			

Figure-1: Vitek 2 System Results for *K. pneumoniae*

Biofilm Production Assays Results

Congo Red Test

Nine of the samples were found to form biofilm phenotypically (figure-2) and were identified to be one isolate belong to *S. aureus* which its weak production, one isolate to *K. pneumoniae* with moderate production, and 7 to *P. aeruginosa* with strong production of biofilms (Table-2).

1

Figure-2: Biofilm Formation (Black colonies) on Congo red Agar

Table-2: Biofilm producer isolates on Congo red agar (CRA)

Bacterial spp.	Biofilm formation		
	Strong biofilm	Moderate biofilm	Weak biofilm
S. aureus	-	-	+
K. pneumoniae	-	++	-
P. aeruginosa	+++	-	-

Microtiter Plate Method Results

Results showed high absorbance values in *K. pneumoniae, P. aeruginosa* 1, 2, 4, 5 and 7 samples under the same conditions of experimentation (Figure-3).

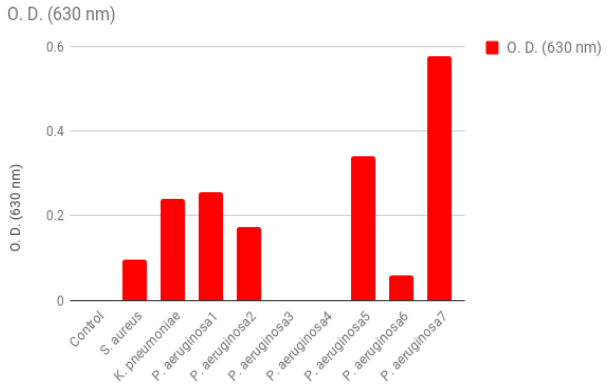


Figure-3: O.D.of Biofilm Production by Bacterial isolates

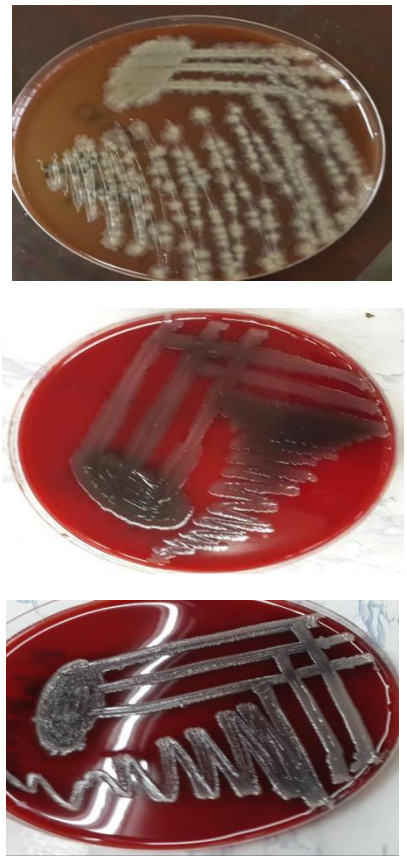


Figure-4: α-hemolysis by *S. aureus*, ƛ hemolysis by *K. pneumoniae*, ß-hemolysis by *P. aeruginosa*

The differences in biofilm strength can be explained by differences in the isolates capacity to form biofilm and probably the quality and quantity of auto-inducers (quorum sensing signaling molecules) for example produced from each isolate (Beenken et al., 2010).

Hemolysin Production Test

Clinical isolates of *S.aureus* were had hemolytic activity and it produced type α-hemolysin, while *Klebsiella* strains produced ɣ-hemolysin and *P. aeruginosa* produced ß-hemolysin as shown in Figure-4. The production of biofilm and haemolysin by tested isolates in the study of suggest that they are pathogenic and may cause undesired health problems if consumed via contamination, where it has been found that hemolysin plays an important role essentially in cell-to-cell interaction during biofilm formation (Caiazza and O'Toole, 2003).

Production of ESBL

S. aureus, K. pneumoniae and P. aeruginosa showed inhibition zones to both Ceftazidime and Cefotaxime with 30mm, 37 mm; 32mm, 34 mm; and 18mm, 22mm respectively (Table-3).

Table-3: Diameter of Inhibition zones of bacterial Isolates

Bacterial isolates	Inhibition zone (mm) Ceftazidime	Cefotaxime
S. aureus	30 mm	37 mm
K. pneumoniae	32 mm	34 mm
P. aeruginosa	18 mm	22 mm

The present study showed increased prevalence of ESBL production among tested bacteria. Multidrug resistance is common among strong biofilm producers this probably is due to high rate of conjugation in biofilm, which facilitates transfer of drug resistant genes (Norouzi *et al*, 2010).

Protease Production

The enzyme protease was produced by *S. aureus* and *P. aeruginosa*, while it is devoid in *K. pneumoniae* (Figure- 5). The inhibition zone for protease production from *S. aureus* was the highest (32mm), and to *P. aeruginosa* was (25mm), while *K. pneumoniae* showed no production for protease (Table-4).

Table-4: Protease production of bacterial isolates

Bacterial isolates	Inhibition zones (mm) of Protease
S. aureus	32
K. pneumoniae	0
P. aeruginosa	25

Antibiotic Sensitivity Test Results

The results of antibiotic sensitivity test showed that *S. aureus* was resistant to Vancomycin and Ciprofloxacin, and sensitive to Gentamicin, Rifampicin, Erythromycin, Chloramphenicol, Tetracycline and Oxacillin. While *K. pneumoniae* was resisted to Ciprofloxacin and Erythromycin, and sensitive to Meropenem, Ofloxacin, Levofloxacin, Gatifloxacin and Cefuroxime. *Pseudomonas aeruginosa* (isolate no.4) was resisted to Vancomycin, Ceftriaxone, Ciprofloxacin, Amikacin and Rifampicin, and sensitive to Meropenem, Cefazolin and Linezolid as shown in Table -5.

Figure-5: Protease Production (From left to right: *S. aureus, K. pneumoniae, P. aeruginosa*)

Bacteria in biofilm exhibit increased resistance to antibiotic. Treatment of chronic ear infections is becoming difficult because of multidrug resistance. Co-resistance to other non beta lactam antibiotics like, Gentamicin, Norfloxacin, Co-trimoxazole was observed among ESBL producers (Nair et al., 2013).

Measurement of Bacterial Cell Surface Hydrophobicity

Salt Aggregation Test (SAT) results showed that *P.aeruginosa* aggregated with ammonium-sulfate solution of ≤1.0 M concentration, whereas, *Klebsiella pneumoniae* and *S.aureus* isolates had SAT value ≤1.5 M (Table-6). These results indicate that all tested bacterial isolates were hydrophobic (Fakruddin et.al., 2015).

(Korres et.al., 2013) showed that *K. pneumoniae subsp. Pneumoniae* isolates demonstrated surface hydrophobicity in aggregation tests in different concentrations of ammonium sulfate, aggregating in concentrations >3.0 M.

Bacterial Adhesion to Hydrocarbons

P. aeruginosa isolates have strong capability to adhesion (value >50%, about 65%), while *Klebsiella pneumoniae and Staphylococcus aureus* have the moderate capability (CHA value 21-50%) to adhere to hydrocarbons when exhibited 50% and 30% respectively (Table-7).

From the results of (Fakruddin et.al., 2015) ExPEC isolates vary significantly in terms of their ability to adhere to hydrocarbons. About 41.53% ExPEC isolates have the moderate capability (CHA value 21-50%) to adhere to hydrocarbons.

Table-5 : Antibiotic susceptibility of the ear bacterial isolates

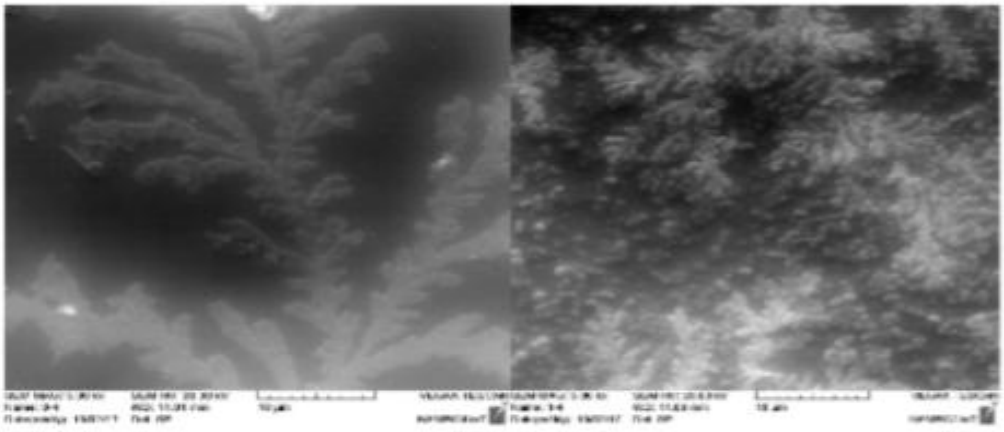
Antibiotic	Dose	Sensitivity to antibiotic		
		S.aureus	*K. pnumoniae*	*P. aerougenes*
Vancomycin	30 mg	R	-	R
Meropenem	10 mg	S	S	-
Ceftriaxone	30 mg	R	-	-
Ciprofloxacin	5 mg	R	R	R
Linezolid	30 mg	S	-	-
Cefazolin	30 mg	S	-	-
Amikacin	10 mg	R	-	-
Rifampicin	5 mg	R	-	S
Ofloxacin	5 mg	-	S	-
Levofloxacin	5 mg	-	S	-
Erythromycin	15 mg	-	R	S
Co-trimoxazole	25 mg	-	R	-
Gatifloxacin	5 mg	-	S	-
Cefuroxime	30 mg	-	S	-
Gentamicin	10 mg	-	-	S
Chloramphenicol	30 mg	-	-	S
Tetracycline	10 mg	-	-	S
Oxacillin	5 mg	-	-	S

Table-6 : Hydrophobicity capability of Ear isolates to hydrocarbon

Bacterial isolates	Hydrophobicity				
	0.5M	1M	1.5M	2M	2.5M
			3M		
P.aeruginosa	-	+	-	-	-
Klebsiella pneumoniae	-		+	-	-
S.aureus	-		+	-	-

Table-7: Adhesion capability of Ear isolates to hydrocarbon

Bacterial isolates	Weak (0-20%)	HA capability Moderate (21-50%)	Strong (>50%)
P.aeruginosa	-	-	*65%*
K. pneumoniae	-	50%	-
Saph. aureus	-	30%	-



a b

Figure-6 : SEM micrographs showing biofilm on glass (a) and on polyester (b)

Table-8 : Comparison of virulence phenotypes in ear bacterial isolates

Virulence property	*S.aureus*	*K.pneumoniae*	*P.aerugenese*
Biofilm formation	+	++	+++
Haemolysin	**A**	**Γ**	**ß**
ESBL	++	++	+++
Protease	+	_	++
Antibiotic resistance	+	++	+++
Hydrophobic	++	++	+++
Adhesion to Hydrocarbons	++	++	+++

Biofilm detection by Scanning Electron Microscope

Biofilm were monitored by SEM, and showed that *Pseudomonas aeruginosa* (isolate no. 4) was able to form a biofilm on both surfaces, glass (Figure- 6a) and polyester (Figure-6 b).

From this result Biofilm formation by *P.aeruginosa* was higher on polystyrene than on glass, and this similar to (Korres et al, 2013) how showed that cell aggregation test performed on pathogenic isolates of *Klebsiella pneumoniae*, and adhesion to polystyrene and glass. Biofilm may form on a variety of surfaces, including living tissue, indwelling medical devices, water pipes, etc. Biofilm protects the bacterium from host defense mechanisms and antibiotic action. Bacterial biofilms are often associated with long term persistence of bacteria in various environments (Nair et.al., 2013).

This study was intended to determine any possible correlation between biofilm forming capability of pathogenic ear bacteria and their virulence properties. A number of biofilm formation ability and associated properties and virulence characters of 9 isolates of *S.aureus*, *K.pneumoniae*, *P.aerugeno*sa has been evaluated (Table- 8).

Little evidences subsist to relating the correlation between biofilm formation, virulence properties and antibiotic resistance of pathogenic ear bacteria. This work can be viewed as a step in elucidating such correlation.

CONCLUSIONS

The findings of this work indicate that biofilm formation can be seen as an indication of virulence and drug resistance of pathogenic ear bacteria isolates and biofilm formation has strong correlation with these virulence properties. A more detailed work, including molecular level should be directed to see the correlation between biofilm formation and specific virulence genes to elucidate the underlying mechanisms.

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