RELATIONSHIP BETWEEN PIGMENTS PRODUCTION AND BIOFILM FORMATION FROM LOCAL *PSEUDOMONAS AERUGINOSA* ISOLATES

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ABSTRACT

The current study was designed to explore the association between the pigments production and biofilm construction in local *Pseudomonas aeruginosa* isolates. Out of 143 patients suffering from burns, urinary tract infections (UTI), respiratory tract infections and cystic fibrosis obtained from previous study by Mahmood (2015), twenty two isolates (15.38%) were identified from (11) hospitals in Iraq, splitted into three provinces, Baghdad, Al-Anbar and Karbala for the duration of June 2017 to April 2018. Characterization was carried out by using microscopical, morphological and biochemical methods which showed that all these isolates belong to *P. aeruginosa*. Screening of biofilm production isolates was carried out by using nutrient broth supplemented with glucose (0.25%) production medium which encourage this biofilm production. The percentage of pigmented isolates were collected from a total of 143 samples, 2.8% of the isolates from burns, 2.1% isolates from cystic fibrosis and 0.7% isolates from UTI. Quantitative assays for biofilm formation were conducted using ELIZA technique. The results showed that all (22) isolates produced biofilm except one (B1 isolate). Biofilm quantities were varied from strong to medium production in comparison with control (0.0663). Statistical analysis results using Fischer's Exact test ($p<0.05$) were non-significant, therefore the pigment production has no association with biofilm formation for all of them.

Key words: pyomelanin, pyocyanin pigments, *bacteria,* biofilm construction.

مجلة العلوم الزراعية العراقية -2020 :51 (5):1413-1419 محمود وآخرون

العلاقة بين انتاج الصبغات وتكوين الغشاء الحيوي لعزلات من الزوائف الزنجارية المحلية

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المستخلص

صممت الدراسة الحالية لاستكشاف العلاقة بين إنتاج الصبغة وتكوين الغشاء الحيوي في عزلات الزوائف الزنجارية المحلية. من بين 143 مريضاً يعانون من الحروق والتهابات المسالك البولية والتهابات الجهاز التنفسي والتليف الرئوي الكيسي تم الحصول عليها من الباحث (محمود واخرون ،2015)، تم تحديد 22 عزلة (15.38٪) في احدى عشر مستشفى في العراق ، انقسمت على ثلاث محافظات هي بغداد والأنبار وكربلاء للفترة من حزيران 2017 إلى نيسان 2018. تم إجراء توصيف للعينات باستخدام الطرق المجهرية والمظهرية والكيميائية الحيوية التي أظهرت أن جميع هذه العزلات تنتمي إلى الزوائف الزنجارية. اجري فحص لعزل إنتاج الأغشية الحيوية باستخدام مرق المغذيات المضاف له الجلوكوز (0.25٪) والتي تشجع على إنتاج الغشاء الحيوي. وجد ان 2.8% من العينات كانت منتجة للصبغات و الناتجة عن اصابات الحروق و 2.1 ٪ من التليف الكيسي و 0.7 ٪ من التهاب المسالك البولية. أجري التحليل الكمي لانتاج الغشاء الحيوي باستخدام تقنية ELIZA. أظهرت النتائج أن (22) عزلة أنتجت الغشاء الحيوي ما عدا عزلة واحدة (B1). تباينت كميات الغشاء الحيوي من إنتاج قوي إلى متوسط بالمقارنة مع عينة السيطرة (0.0663). كانت نتائج التحليل الإحصائي باستخدام اختبار Fischer's Exact عند مستوى معنوية ($p<0.05$) ليس لها تأثير معنوي ، وبالتالي فإن إنتاج الصبغات ليس له علاقة بتكوين الأغشية الحيوية للعينات قيد الدراسة.

الكلمات المفتاحية: صبغة البايوميلانين، البايوسيانين، البكتريا ، تكوين الغشاء الحيوي

*Received:1/10/2019, Accepted:30/1/2020

INTRODUCTION

Pseudomonas genus is a Gram negative rod belonging to Pseudomonadaceae family, motile by polar flagella (6). *Pseudomonas aeruginosa* is an extremely common opportunistic pathogen and it has the ability of producing various bioactive molecules such pigments. It has great genomic content (~6.5 Mbp) for variations in metabolism and adaptation for several environmental roles and mismatch repair system (2, 12,13 and 16). Biofilm mean microbial community which inhabit on exteriors and covered the extracellular matrix (9). Biofilm of prokaryotes is an extracellular polymeric substance (EPS) that permits bacteria to bond to several substances located on the surfaces and facilitate the interconnection to each other which contains polysaccharides, and nucleic acids. Biofilms of *Pseudomonas aeruginosa* have been known as challenge in medical surroundings. On the other hand, it has the ability of infectivity to different hosts (1). The high-density of bacterial assemblages associated with chronic infections, for instance biofilm formation, which favors the development of variants, which accomplishment from the homologous recombination and DNA mismatch repair system (2, 12, and 15). Approximately 90% of the biomass of biofilm is due to the EPS which can provide significant contribution to the structural qualities and characterization of biofilms (5,13). The study of biofilms has been gotten greater attention than before after it was valued the biofilms complicated significantly to infective disease (13). Once the biofilms recognized as the source of disease, management becomes very problematic. Typically, instant controller by extra of high-dose for antibiotic required for long-term administration. Though, those radical actions are ineffective and lead to significant morbidity and mortality (7). Because of the significant importance of biofilms formation, pigments production, the role of motility and the antimicrobial resistance strategies resulted from biofilms formation, with the conformation of the biofilm surrounding substance information and the limitation of reports in regard with biofilm formation and pigment production. Therefore, the current study designed to estimate the association between pigment and biofilm production from local *P. aeruginosa* isolates and to achieve such goal the following steps were suggested, isolation and identification of local *P. aeruginosa* isolates from different pathogenic sources ,screening of local isolates capability of pigment and biofilm production from previous study, measurement of quantity of biofilm by using ELIZA technique and estimate the relationship between biofilm and pigment production using chi- square analysis.

MATERIALS AND METHODS

Out of 143 local *P. aeruginosa* isolates isolated from patients suffering from burns, urinary tract infections , respiratory tract infections and cystic fibrosis, twenty two isolates were identified from (11) hospitals in Iraq, splitted into three provinces, Al-Anbar , Baghdad, and Karbala for the period of June 2017 to April, 2018. These isolated samples were obtained from previous study by Mahmood, et al (12). Microscopical , morphological , cultural and genetic characterization were achieved for these isolates according to Bergy's manual . Twenty-two of these isolates had been exposed to produce biofilm *in vitro* using ELISA technique. Then these isolates were cultured on brain heart infusion broth and king and king B for distinguishing and enhancing different types of pigments.

Detection of biofilm

All procedures were prepared with aerobic environments to improve biofilm production.

A –Tube adherence method

The test microorganisms were cultured using trypticase soy broth (TSB) medium (10 ml) accompanied with 1% glucose. Then incubated at 37˚C for 24 h. Then, the tubes were washed with PBS (pH 7.3) then dried, the staining carried out using

crystal violet stain (0.1%). Washing the extra stain with distilled water, and then dried out the tubes in upside-down position. Biofilm production was positive when a visible film lined the wall and bottom of the tube. Biofilm quantity produced was scored as 1-weak/none, 2-moderate and 3-high/strong (3) (Fig 1-A).

(B)- Biofilm Formation Assay using ELISA technique

A microtiter plate method using ELISA technique was followed as previously described by Mirzee et al (13). Briefly, 180 µl of nutrient broth (N.B) supplemented with 1% glucose were filled the wells of the microtiter plate. 20 µl of previously prepared bacterial suspensions were supplemented to the well, while the negative control wells were filled with 200 µl of N.B with 1% glucose, then incubated at 37°C for 24 h before the elimination of the cultures. Then, the cells were poured, and the washing was carried out using sterile PBS (3-times). The fixation by methanol 20 min were achieved, drying at room temperature 25°C, and stained with 0.1% safranin. The safranin dye bound to the adherent cells was dissolved with 1ml of 99% methanol solution per well, finally the microtiter plates were read at 630nm (A630) using ELISA reader (Fig 1-B).

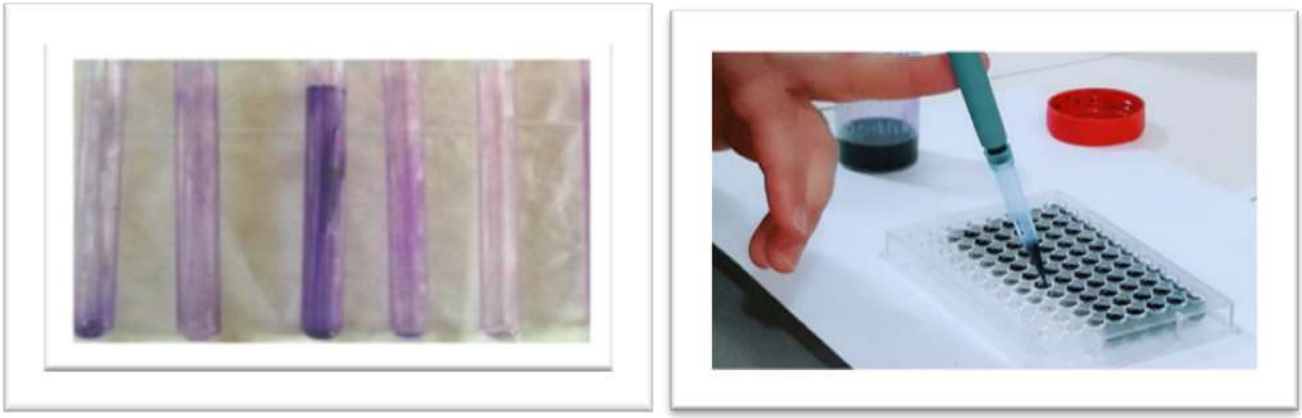

Fig 1. A-Tube adherence method B- Microtiter plate method using ELISA technique

Statistical analysis

Chi-square of association was performed to test the association between biofilm formation and pigment productions. Statistical analysis was performed by using SPSS version 23

RESULTS AND DISCUSSION

Characterization of *P. aeruginosa*

The current study has been carried out on twenty two clinical samples from burns, urinary tract infections (UTI), respiratory tract infections (RTI) and cystic fibrosis

B- Microtiter plate method using ELISA technique

(CF); at 11 hospitals in Iraq in three provinces namely, Baghdad, Al-Anbar and Karbala. *Pseudomonas aeruginosa* were isolated and identified by using Bergy's manual (8). All isolates were characterized using macroscopically characterization showed that the bacteria were Gram negative single rods, morphological characterization of isolates showed that all isolates appeared pale and translucent on MacConkey agar which was lactose non-fermenters. Isolates were able to grow on selective media 0.3 % Cetrimide agar at 42ºC as described by Holt et al. (8), also biochemical characterization were done such as oxidase test, catalase , sugar fermentation. Different pigments were produced and screened from these isolates such as pyocyanin, pyomelanin and fluorescence production (Table 1).

Table 1. Production of pigments and biofilm formation by local *P. aeruginosa* isolates

Sample	Pyocyanin	Pyomelanin	Fluorescence	Biofilm formation
B 1		+		+
B 2	+	+		+
B 4	+			+
B 5	+			+
B 6	+	+		+
B 7			+	+
B 8		+		+
B 9	+	+		+
B 10		+	+ +	
B 11	+			+
B 12	+			+
B 13	+			+
B 14	+	+		+
B 16	+			+
B 17	+	+		+
B 20	+			+
B 23	+			+
B 45	+			+
B 47		+		+
B 48	+	+		+
R1				+
R2	+	+		

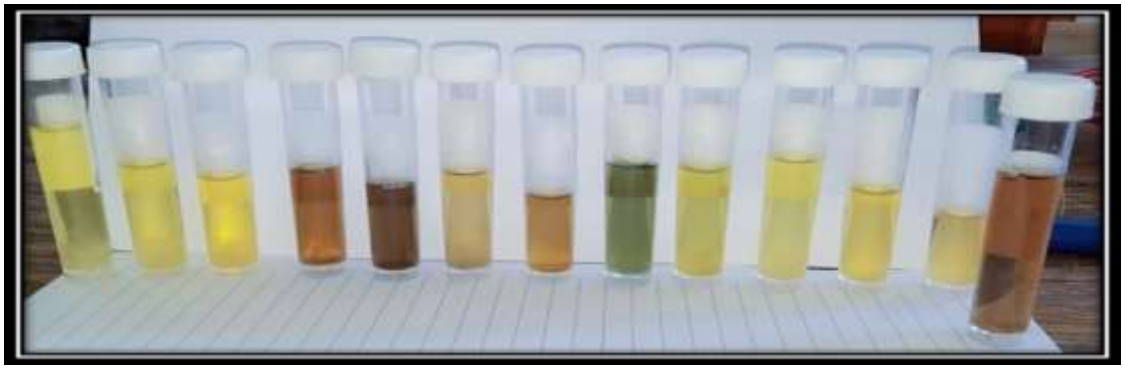

Figure 2. Different pigments produced from local *Pseudomonas aeruginosa* isolates

Twenty-two isolates which produced biofilm screened and cultured on brain heart infusion broth for activation different pigments pyocyanin, fluorescein and pyomelanin after 1-7 days.

Detection of Biofilm using ELISA technique: Microtiter plates were carefully chosen for assay of biofilm production and quantify attachment. The results showed all clinical isolates except one tested were able to produce biofilm on inert polystyrene surfaces and different pigments (Fig. 3). The biofilm formation and attachment which were located on the abiotic surfaces for instance, "catheters and implanted devices is unique of the main virulence factors in *P. aeruginosa*. The bacterial resistance to inappropriate conditions was caused by biofilm formation as, stress, host phagocytosis, antibiotics, and immune response (resistance to oxygen radicals and proteases)(11,10and16).

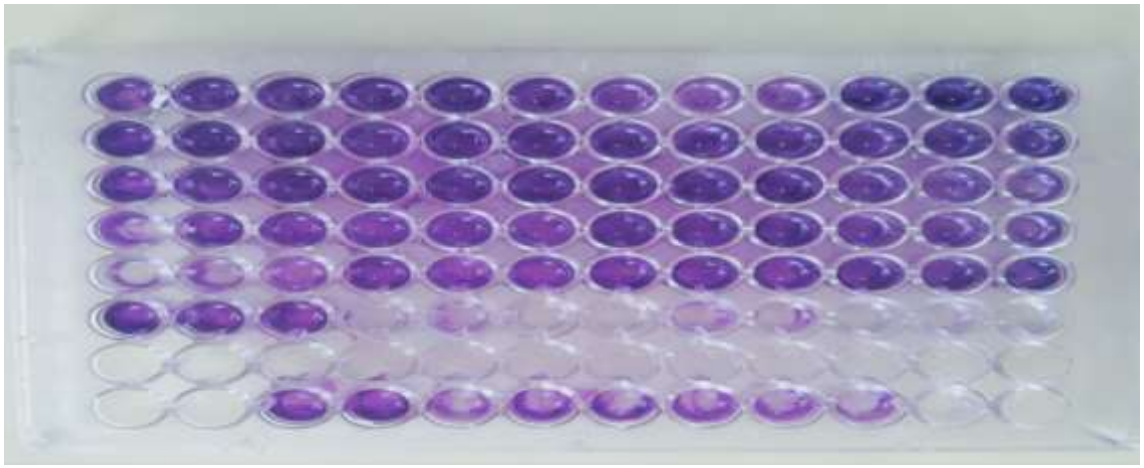

Figure 3. Positive and negative results in microtiter plate of biofilm production by local *Pseudomonas aeruginosa* isolates

Table 2. Biofilm absorbance at 630nm using ELISA technique of local *Pseudomonas aeruginosa* isolates

No.of isolate	Abs. at 630nm	No.of isolate	Abs. at 630nm
B1	0.552	B13	0.565
B2	0.732	B14	0.458
B4	0.605	B16	0.552
B5	0.685	B17	0.491
B6	0.565	B20	0.412
B7	0.612	B23	0.670
B8	0.680	B45	0.543
B9	0.544	B47	0.443
B10	0.556	B48	0.426
B11	0.662	R1	0.473
B12	0.713	R2	0.718

According to the absorbance report of ELISA technique (Table 2) at 630nm, the biofilm quantity of local *Pseudomonas aeruginosa* isolates varied from medium to heavy quantity in comparison with the control table (3).

Table 3. The quantity of biofilm produced from selected *P. aeruginosa*

Isolates	Pigments	Quantity of biofilm
B 1	Pyomelanin	Heavy
B 2	Pyomelanin+ Pyocyanin	Heavy
B 4	Pyocyanin	Heavy
B 5	Pyomelanin + Pyocyanin	Heavy
B 6	Pyocyanin+ Pyomelanin	Heavy
B 7	Fluorescene	Medium
B 8	Pyomelanin	Heavy
B 9	Pyomelanin + Pyocyanin	Heavy
B10	Fluorescene+ Pyomelanin	Heavy
B11	Pyocyanin	Medium
B 12	Pyocyanin+ Pyomelanin	Heavy
B 13	Pyomelanin+Pyocyanin	Heavy
B 14	Pyocyanin	Heavy
B 16	Pyocyanin+Pyomelanin	Heavy
B 17	Pyocyanin+Pyomelanin	Heavy
B 20	Pyocyanin	Heavy
B 23	Pyocyanin	Heavy
B 45	Pyocyanin	Heavy
B 47	Pyocyanin	Heavy
B 48	Pyocyanin+Pyomelanin	Heavy
R1	Fluorescene+ Pyomelanin	Heavy
R2	Pyocyanin	Heavy

*** The results in comparison with negative control (0.0663).**

The results in Table (4) show the statistical analysis using Fishers Exact test which was non-significant at ($p<0.05$) this confirmed there were no association between pigment production and biofilm formation for all isolates. Information regarding the relationship between biofilm formation and pigment production of *P. aeruginosa* is inadequate and there are currently no reports documenting biofilm production capability with pigment production in Iraq. For that reason, the capability to produce biofilm could be an substantial virulence factor for some of *P.aeruginosa* isolates which show an significant effect in the pathogenesis and prognosis of infection and establishing of chronic, recurrent and stubborn infections (9,,10,14 and 20). Biofilms are known to increase pathogenicity and antibiotic resistance in different kinds of pathogenic bacteria such as *P.aeruginosa*, *E.coli*, *Klebsiella* And others (15 ,18 and 19).

Table 4. Statistical analysis using Fisher's exact test of association between biofilm and pigment production.

	Value	df	Asym. Sig.	Exact Sig.	Exact Sig.
Pearson Chi-square	**0.30**	**1**	**0.862**		
Continuity Correction	**.000**	**1**	**1.000**		
Likelihood Ratio	**.030**	**1**	**0.862**		
Fishers Exact Test				**1.000**	**0.691**
N of Valid Cases	**50**				

Pigment*Biofilm Crosstabulation

		NO	YES	TOTAL
NO	**Count**	**1**	**27**	**28**
	Expected count	**1.1**	**26.9**	**28.0**
	% within Pigment	**3.6%**	**96.4%**	**100.0%**
	% within Biofilm	**50.0%**	**56.3%**	**56.0%**
	% of Total	**2.0%**	**54.0%**	**56.0%**
YES	**Count**	**1**	**21**	**22**
	Expected count	**.9**	**21.1**	**22.0**
	% within Pigment	**4.5%**	**95.5%**	**100.0%**
	% within Biofilm	**50.0%**	**43.8%**	**44.0%**
	% of Total	**2.0%**	**42.0%**	**44.0%**
TOTAL	**Count**	**2**	**48**	**50**
	Expected count	**2.0**	**48.0**	**50.0**
	% within Pigment	**4.0%**	**96.0%**	**100.0%**
	% within Biofilm	**100.0%**	**100.0%**	**100.0%**
	% of Total	**4.0%**	**96.0%**	**100.0%**

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