ABSTRACT

The current study was designed to explore the association between the pigment 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.
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, 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).

![Image](image1.png)

**Fig 1.** A-Tube adherence method

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 42°C 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).
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)(11,10 and 16).
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 *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      |            |

| Pigment*Biofilm Crosstabulation | NO | YES | TOTAL |
|---------------------------------|----|-----|-------|
| 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%|
| 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%|
| 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%|

REFERENCES
1. Aujoulat F, F. Roger, A. Bourdier, A. Lotthe, B. Lamy, H. Marchandin and E Jumas- Bilak. 2012. From environment to man: genome evolution and adaptation of human opportunistic bacterial pathogens. Genes (Basel) 3:191–2323
2. Boles BR, M. Thoendel, and PK Singh . 2004. Self-generated diversity produces “insurance effects” in biofilm
A. in sample of burn

– T.

Programmed bacterial life for fitness

Pseudomonas aeruginosa

11

Activity of AGNPS biosynthesized by (2019).

10.

Sciences

Infection.

Pseudomonas aeruginosa

Wilkins. 

Bacteriology. 9th ed. Williams and

Bergey's Manual of Determinative

J.T.

8

Tobramycin. Treatment with meropenem and

Pseudomonas aeruginosa

Left

L.R.

7

Springer; 

Gammaproteobacteria. New York:

Proteobacteria, Part B: The

Systematic Bacteriology: Volume 2: The

S

6.

Amsterdam, pp 19–34.

Garrity G, D.J. Brenner, N.R. Krieg 

, and J.R. Staley, 2005 Bergey’s manual of systematic bacteriology: volume 2: the proteobacteria, part B: the gammaproteobacteria. New York: Springer;

7. Gavin P.J., M.T. Suseno , F.V. Cook, L.R. Peterson, and R.B. Thomson. 2003. Left-sided endocarditis caused by Pseudomonas aeruginosa: successful treatment with meropenem and tobramycin. Microbial Infect Dis 47:427–430.

8. Holt, J. G., N.R. Krieg, P.H.A., Sheath, J.T. Staley, and S.T. Williams . 1994. Bergey’s manual of determinative bacteriology. 9th ed. Williams and Wilkins. Baltimore, Maryland, USA

9. Hanoon, R.A., I.G. Auda, and I.H. Aziz. 2017. Detection of ExoTene in local of Pseudomonas eruginosa in sample of burn infection. Iraqi Journal of Agricultural Sciences, 15(4):5-8

10. Hussein, N.N. and A.H. Muslim .(2019). Detection of the antibacterial activity of AGNPS biosynthesized by Pseudomonas eruginosa. Iraqi Journal of Agricultural Sciences, 50(2); 617-625

11- Keehoon, L. and S.Y. Sang. 2017. Pseudomonas aeruginosa Biofilm, a programmed bacterial life for fitness. J. Microbiol. Biotechnol. 27(6):1053–1064.

12. Mahmood, H.M., M.K. Mohammed, and M.T. Fleih. 2015. Purification and physicochemical characterization of pyomelanin pigment produced from local Pseudomonas aeruginosa isolates. WJPR .10:289-299.

13. Mirzaee, M., M.S.N. Peerayeh, and A.M. Ghasemian., 2014. Detection of icaABCD genes and biofilm formation in clinical isolates of methicillin resistant Staphylococcus aureus. Iranian Journal of Pathology . 9 (4): 257 – 262

14. Muhaidi, M.J., L.M. Aziz and M.N. Ahmed 2018. Genetic evaluation of phenazine synthsized by Pseudomonas aeruginosa isolated genital tract of farm animals. Iraqi Journal of Agricultural Sciences 49 (2): 151 -157

15. Tawfiq, S.M. 2018. Bacteriological and genetic study of Pseudomonas aeruginosa isolates. Iraqi Journal of Agricultural Sciences. 49(1): 27-35.

16. Oliver A, R. Canton, P. Campo, F.Baquer, and J. Blazquez . 2000. High frequency of hypermutable Pseudomonas aeruginosa in cystic fibrosis lung infection. Science. 88:1251–1254

17. Potera, C. 1999 Forging a link between biofilms and disease. Science 283:1837–1839.

18- Ramsey DM, and D.J.Wozniak., 2005. Understanding the control of Pseudomonas aeruginosa alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. Mol.Microbiol 56:309–322.

19. Saleh, G.M., S.A. Alash, H.Y .Fadil, and H.B. Ali. 2020. The effect of termites extract on inhibition of growth of some pathogenic bacteria and synthesis of biofilm. Iraqi Journal of Agricultural Sciences;51 (Special Issue): 176 -183.

20. Stoodley P, K.Sauer, D.G. Davies, and J.W.Costerton. 2002. Biofilms as complex differentiated communities. Annu Rev Microbiol. .56:187–209