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Study of pyocyanin production and biofilm formation in clinical *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen involved in many infections in respiratory system, urinary tract, pneumonia, wounds, and bacteremia. It has many mechanisms and virulence factors such as adaptation and survival in extreme environments. In this study, 32 isolates of *P. aeruginosa* were collected from Teaching Laboratories in Baqubah General Teaching Hospital and Al-Batool Teaching Hospital, Iraq. The isolates were identified based on morphological and biochemical tests and confirmed by Vitek 2 system. The isolates varied in their antibiotic resistance towered ceftazidime, ceftriaxone, ticarcillin/clavulanic acid, meropenem, aztreonam, imipenem by 100%,100%,100%, 68.75%, 28.12%, and 18.75%, respectively. The biofilm formation test revealed that 43.75% of *P. aeruginosa* isolates were strongly biofilm forming, 46.8% moderate, and 6.25% were non-biofilm forming. All the tested isolates were able to produce pyocyanin with varied concentration ranged between 17 µg/ml (isolate P2) to 31.5 µg/ml (isolate P7). This study revealed the ability of the clinical *P. aeruginosa* to produce high amount of pyocyanin which could be used in many applications.

Keywords: *P. aeruginosa*, pyocyanin, biofilm, antibiotic susceptibility.

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دراسة انتاج صبغة البايوسيانين وتكوين الغشاء الحيوي في بكتريا الزائفة الزنجارية *Pseudomonas aeruginosa* السريرية

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الخلاصة

بكتريا الزائفة الزنجارية (*Pseudomonas aeruginosa*) هي من مسببات الأمراض الانتهازية التي تشارك في احداث العديد من الاصابات في الجهاز التنفسي والمسالك البولية وذات الرئة والجروح وتجرثم الدم. تمتلك البكتريا العديد من الآليات وعوامل الضراوة مثل التكيف والبقاء في البيئات المتطرفة. في هذه الدراسة، تم جمع 32 عزلة من *P. aeruginosa* من المختبرات التعليمية في مستشفى بعقوبة التعليمي العام ومستشفى البتول التعليمي، العراق. جرى تشخيص العزلات بناءً على الاختبارات المظهرية والكيموحيوية وتأكيدها باستعمال Vitek 2 compact system . تباينت العزلات في مقاومتها للمضادات الحياتية ceftazidime، ceftriaxone، ticarcillin/clavulanic acid، meropenem، aztreonam، و imipenem وبنسب مقاومة بلغت 100%، 100%، 100%، 68.75%، 28.12%، و 18.75% على التوالي. كشف اختبار تكوين الغشاء الحيوي أن 43.75% من عزلات *P. aeruginosa* امتازت بكونها قوية في تكوين الغشاء الحيوي، و 46.8% كانت متوسطة التكوين، و 6.25% كانت غير مكونة للغشاء الحيوي. كانت جميع العزلات التي تم اختبارها قادرة على إنتاج صبغة pyocyanin بتركيز تراوح بين 17 مايكروغرام/مللتر للعزلة رقم P2 و31.5 مايكروغرام/مللتر للعزلة رقمP7 . كشفت هذه الدراسة عن قدرة *P. aeruginosa* السريرية على إنتاج كمية عالية من صبغةpyocyanin التي يمكن استخدامها في العديد من التطبيقات.

الكلمات المفتاحية: بكتريا الزائفة الزنجارية، صبغة البايوسيانين، الغشاء الحيوي، الحساسية للمضادات الحياتية.

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Introduction

P. aeruginosa (Family: Pseudomonadaceae) is Gram-negative bacilli bacterium, opportunistic pathogen classified as a facultative anaerobe. It can survive and spread in different environmental conditions such soil, water, plant and human [1]. Among opportunistic pathogenic bacteria, *P. aeruginosa*, which produces distinct virulence factors, is known to be an important human pathogen, responsible for various infections in immunocompromised people [2]. These virulence factors play a significant role in their pathogenesis, included cell-associated such as flagella, pili, lipopolysaccharide called endotoxins and alginate and extracellular factor such as hemolysin, alkaline protease, and elastase [3]. In addition, *P. aeruginosa* can adapt and survival in low nutrient environments and resist different types of antibiotics [4]. Therefore, World Health Organization (WHO) classified this bacterium as "critical" category in the priority list of bacterial pathogens [5]. This requires urgent research and the development of a new ways or generation of antibiotics to tackle their resistance trait [6]. *P. aeruginosa* formed a bacterial biomass called biofilm on biotic and abiotic surfaces. The most biofilms matrix embedding bacterial cells may account for over 90% of dry weight of whole biofilm mass. The biofilm matrix creates suitable environments for cells while shielding them from unfavorable conditions (e.g. antimicrobials and disinfectants/biocides) [7,8].

P. aeruginosa is characterised by producing secondary metabolites that support their maintenance and persistence. Phenazines are an example of these pigmented metabolites that produced at early stage of colonization to start the infection [9]. Phenazines play a number of negative roles in host cells. For instance, direct damage by producing reactive oxygen species, change releasing of cytokine, interference with cell signaling. From biotechnological prospective, this pigments were reported has applications in industry and agriculture in control of plant diseases [10]. *P. aeruginosa* produces group of phenazines pigments, including pyocyanin (bluish-green), fluorescein (yellowish pyoverdin), (dark red pyorubin), and (dark black pyomelanin) [11]. The aim of this article was to investigate some virulence factor in *P. aeruginosa* and extraction of pyocyanin pigment in supernatant of different clinical isolates from different sources in order to know the effect of pyocyanin on bacterial pathogenesis.

Materials and Methods

Samples collection and identification

Two hundred and twenty (220) clinical samples were collected from patients in Baqubah Teaching Hospital, Diyala Province, Iraq. The source of collection included sputum, wounds, burns, urine and ears. The isolates were cultured on Pseudomonas agar (selective differential medium), MacConkey agar, and Blood agar media. Thirty-two (32) samples were confirmed as P. *aeruginosa* based on microscopic examination through Gram stain technique. The biochemical testes were Voges-Proskauer, Methyl red, indole, and Triple Sugar Iron (TSI), citrate utilization, and two enzymes, catalase and oxidase [12]. VITEK-2 automated system was the final step to confirm the identification of the isolates [13] Pure cultures of all the isolates were stored for short term at 4 ^{o}C in sealed nutrient agar Petri plates.

Antibiotic susceptibility of *P. aeruginosa*

Antibiotic susceptibility of 32 isolates of *P. aeruginosa* were conducted using six standard disc antibiotics (supplied by Mast Group Ltd./UK). These antibiotics were Ceftazidime (30µg), Ceftriaxone (30 µg), Ticarcillin/clavulanic acid (75/10 µg), Meropenem (10 µg), Aztreonam (30 µg), and Imipenem (10 µg). The test was carried out by disc diffusion protocol (Kirby-Bauer testing). In brief, A Petri plates contain sterile Mueller–Hinton agar were inoculated with bacterial suspension adjusted to 0.5 McFarland turbidity standard (1.5 x 10^8 CFU/ml). Six discs were placed in each plate and then incubated at 37 ^{o}C for 24 hours. The results were recoded according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2020).

Biofilm formation test

Biofilm formation was tested *in vitro* at the stationary phase in 96-well flat-bottom polystyrene microtitre plate using 1 % crystal violet stain according to [14]. In brief, 200 ul of the overnight bacterial culture (10^6 CFU/ml) were distributed in triplicate into the wells and incubated for 24 hours. Then, the microplate was emptied and left to dry in air in the laminar air flow. The

biofilm formed was fixed using absolute ethyl alcohol. Aliquot of 150 µl crystal violet was added to the cultures and left for 5 minutes. The solution was throughout from and the wells were washed with distilled water and left to dry in the laminar air flow and 160 µl glacial acetic acid (33 %) were added. The optical density was taken using ELISA reader at 630 nm and interpret according to the criteria: OD ≤ $^{**}OD_c$ no biofilm formation, OD_c < OD ≤ 2 x OD_c (moderate), and 2 x OD_c < OD (strong).

Quantification of pyocyanin production

Production of pyocyanin was measured using a quantitative chemical assay in acidic solution as described by [15] by measuring the absorbance of pyocyanin solution at 520 nm. Briefly, 5 ml of *P. aeruginosa* liquid culture in Luria-Bertani broth was used to produce pyocyanin. The pigment was extracted with 3 ml of chloroform. One milliliter of 0.2 N HCl was added to the layer containing pyocyanin to give a pink to deep red solution which measured at 520 nm using microplate reader. The concentrations of pyocyanin in the solution was expressed as µg/ml and determined by multiplying the OD_{520} by 17.072 [15].

Statistical analysis

Data analysis was conducted by SPSS version 23 (Statistical Package for Social Science, Chicago, IL, USA). Data analysed by Chi-square for the comparison of categorical data and expressed as means ± standard deviation and considered significant different when *p* value ≤ 0.05.

Results and Discussion

Phenotypic detection of *P. aeruginosa*

The isolates of *P. aeruginosa* were identified in four ways included phenotypic traits, microscopic examination, biochemical tests and VITEK® 2 automated system. The isolates when cultured on MacConkey agar appears as small round convex colonies with the pale yellow color and as large flat bacterial colonies with a grape-like smell on blood agar (Figure 1a,b). The isolates were grown pseudomonas agar as selective differential medium which encourage

pigments production. The main pigment appear on agar medium is pyocyanin in blue-green color to (Figure 1c). The collected isolates were examined microscopically which displayed a small, pink-colored bacillus referred to negative bacteria in their interaction with the gram stains.

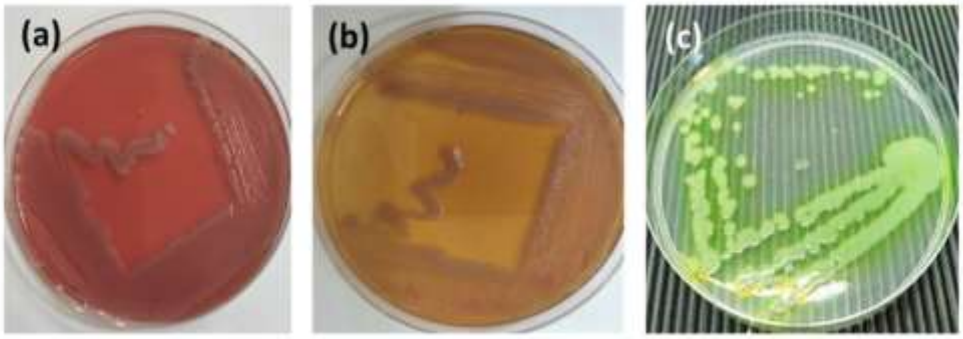

Figure 1: The appearance of *P. aeruginosa* colonies grown on (a) Blood agar; (b) MacConkey agar; (c) Pseudomonas agar after 24 hours of incubation at 37 ^{o}C

The essential biochemical tests were included some important enzymes such as oxidase, catalase, and citrate utilization. *P. aeruginosa* was identified by a positive oxidase test and the appearance of dark purple on the surface of colonies, indicating the formation of cytochrome oxidase. The presence of bubbles in the catalase test indicates a positive result, and this test is used to identify organisms that produce catalase which catalyze the breakdown of hydrogen peroxide into water and oxygen. Converting the culture medium into blue indicates a positive test for the citrate, since the blue color indicates the consumption of the citrate. In contrast, all isolates gave negative result to Voges-Proskauer, Methyl red, indole, and Triple Sugar Iron 32 (TSI). This indicates that all the studied isolates do not ferment glucose, lactose and sucrose, and do not produce CO_2 or H_2S [16] (Table1).

Table 1: Biochemical tests that applied to diagnose *P. aeruginosa* isolates.

BIOCHEMICAL TESTS	RESULT
Catalase	+
Oxidase	+
Indole production	-
Methyl red	-
Voges-Proskauer	-
Triple sugar iron	k/k*

*k/k alkaline test

Samples distribution according to the isolation source

The percentage of *P. aeruginosa* isolation from different clinical sources was changed from source to another as shown in Table (2).

Table 2: The distribution of *P. aeruginosa* isolates based on their source.

SOURCES OF ISOLATION	TOTAL NUMBER OF SAMPLES	NUMBER OF POSITIVE ISOLATE	PERCENTAGES OF *P. AERUGINOSA* ISOLATES (%)
Wounds discharge swabs	60	9	28.1
Urine samples	53	8	25
Burns discharge swabs	48	7	21.8
Sputum samples	31	5	15.6
Ear swab discharge	28	3	7.5

Antibiotics susceptibility profile

The antibiotic susceptibility test was conducted for the 32 isolates of P. aeruginosa by Kirby-Bauer technique using six different antibiotics discs. These antibiotics are belonging to six different classes include ceftazidime, ceftriaxone, ticarcillin/clavulanic acid, meropenem, aztreonam, imipenem (Table 2). The assay showed that there was a significant rate of resistance to certain antibiotics. The percentages of the resistance profile of P. aeruginosa isolates were 100%, 100%, 100%, 68.75%, 28.12%, and 18.75%, respectively.

Table 2: The percentage of antibiotics susceptibility of *P. aeruginosa* isolates by Kirby-Bauer method.

Antibiotics		Resistant No. (%)	Intermediate No. (%)	Sensitive No. (%)	P. value
Class	Type				
Cephalosporins	Ceftazidime (CAZ)	32(100)	-	-	0.04
	Ceftriaxone (CRO)	32(100)	-	-	0.01
ß-lactam combination agent	Ticarcillin/ clavulanic acid (TIM)	32(100)	-	-	0.01
Carbapenems	Imipenem (IMP)	6(18.75)	1(3.12)	25(78.1)	0.05
	Meropenem (MEM)	22(68.75)	-	10(31.25)	0.05
Monobactams	Aztreonam (ATM)	10(31.25)	10(31.25)	12(37.5)	0.06

Ceftazidime (3rd generation cephalosporins) revealed 100% resistance in all 32 isolates which consistent with [17, 18] which was 91.2% and 90%, respectively. In contrast, the high resistance rate and did not correspond with 29% and 23.45% found by [19] and [20], respectively. The isolates were totally resistant to ceftriaxone (100%). A study by [21] and [22] reported 96% and 93.3%, respectively. Another study also recorded high rate with 85% of *P. aeruginosa* isolates were resistant to ceftriaxone [19]. *P. aeruginosa* was also totally resistant (100%) to Ticarcillin/clavulanic acid which did not correspond with the results of [23] which was 29.23%. In contrary, the current study agreed with [24] with 92%. In the current study, Aztreonam reported 31.25% resistant which is close to the values recorded by [23] which was 36.46%, but not corresponded with [25, 20, 22, 24] which were 26%, 50.61%, 86.7%, and 80%, respectively. Imipenem showed 18.75% resistance against *P. aeruginosa* isolates. This result did not agree with [26, 27] e whose results were (39.68% and 28%). Resistant against β-lactam antibiotic, Meropenem, was high in our study (68.75%) compare with 24% founded by [19]. [24] did not recorded any isolate of *P. aeruginosa* resist Meropenem antibiotic.

Antibiotic resistance can be attributable to a variety of factors, including increase of permeability cell membrane, sources of specimens, ecological and test conditions, changes in target site architecture [28]. Antibiotic resistance attributed to three main mechanisms: intrinsic, acquired, and adaptive. Intrinsic mechanism is stable, heritable resistance determinants. In *P. aeruginosa*, intrinsic mechanism is limited in outer membrane permeability, high efflux pump

activity, antibiotic inactivation by the β-lactamase AmpC. Bacteria can acquire resistant through genetic mutation and horizontal gene transfer (HGT). For example, many clinical isolates of *P. aeruginosa* have mutations in genes that encoding efflux pump repressors, resulting in constitutive overexpression. Adaptive resistance is non inheritable and dependent on environmental cues. Arguably, the most important environmental signal contributing to adaptive resistance is exposure to sub-inhibitory concentrations of antibiotics [29].

Quantification of biofilm formation

This assay was carried out to detect and quantify formation of the biofilm by *P. aeruginosa* isolates. ELISA reader was used to determine the amount of biofilm created by adherence to the surface wells and expressed as a numerical value of absorbance at 630 nm. The absorption represents the thickness of the biofilm formed. In this study, 43.75% of *P. aeruginosa* isolates were strongly biofilm forming, 46.8% were moderate, and 6.25% were non-biofilm forming as shown in Figure (2,3).

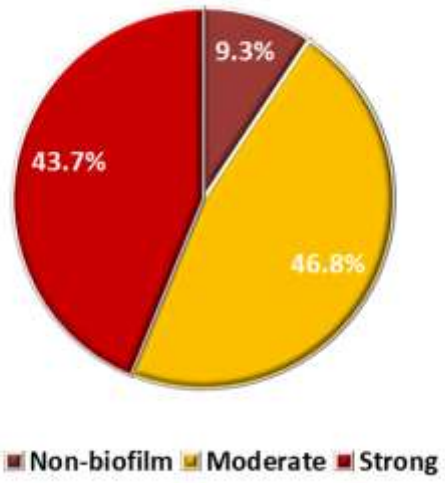

Figure 2: Percentage of biofilm formation type of *P. aeruginosa* isolates.

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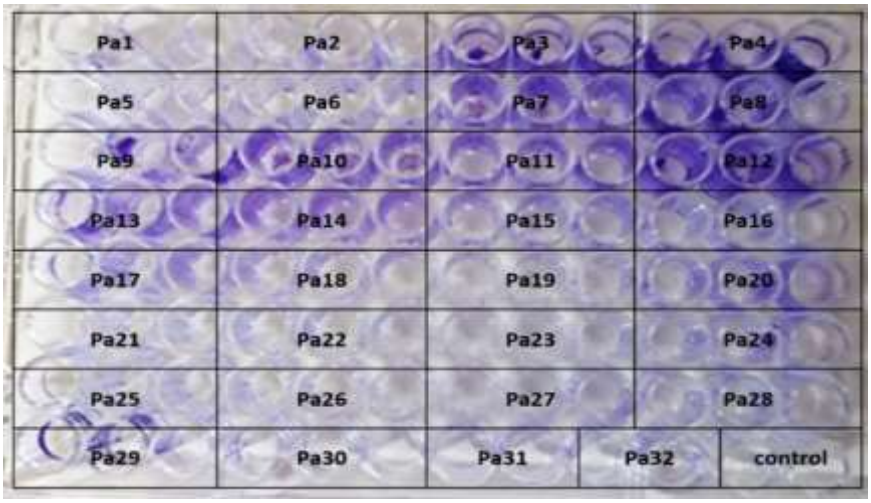

Figure 3: Microtitre plate screening of biofilm formation using crystal violet dye of *P. aeruginosa* isolates. The microplate shows different grades of the biofilm. The absorbance (ODs) of the stained adherent cells were measured with a microplate reader at 630 nm.

Biofilm formation was studied [30] on local isolates of *P. aeruginosa* and found that 45% were strongly biofilm-forming, 48% moderate, and 6.25% were non-biofilm-producing which agreed with current study. However, the percentages obtained in this study differ from [34] which was 100% strong biofilm-producing isolates. The results of our study are not agreeing with [26], where 46.03% of the isolates were weak biofilm producers, while 39.68% were moderate, and 14.28% of the isolates were produced strong biofilm, The reason behind the differences or similarities in biofilm formation may be due to the variation in culture medium components that used in biofilm formation test (e.g. Tryptic Soy Agar or Luria-Bertani broth), the concentration of bacterial cells in culture, or the difference in incubation periods [18]. Also, the cell density in biofilm increases with prolong the length of incubation period. The concentration of dye used as indicator in the test could also plays a role in the result differences since 5% (w/v) of dye gives better results compared with 1%. The results may also affect by the type of the microplate used in the assay. It was approved that 96-microplate is made of Polystyrene is more efficient in biofilm sticking than the microplate made of other materials such as glass, steel, or granite [18].

Screening of *P. aeruginosa* isolates for pyocyanin production

Thirty-two isolates of *P. aeruginosa* were subjected to a standard protocol for pyocyanin production screening to identify the potent isolate. All the isolates were positive for their ability to produce pyocyanin (Figure 4). The concentration of the pigment was ranged between 17 µg/ml (isolate P2) to 31.5 µg/ml (isolate P7). As reported by [32], approximately 90-95% of all *P. aeruginosa* isolates produce pyocyanin as deep blue in colour (referred to as "blue pus" from pyocyaneus). In Iraqi study, most of *P. aeruginosa* non-producer and producer pyocyanin isolates were obtained from burns sources by 34.92% and 23.81%, respectively. The concentration of the pigment in burn isolates was 10.85 µg/ml and the highest level in wound isolates reached to 6.163 µg/ml [26]. The enhancement of pyocyanin production is clearly influence by culture medium composition [32]. [33] investigate the effect of nutrients on pyocyanin production using King's medium and Nutrient medium.

The results showed the enhancement rate of the pigment increased by 2.56 µg/ml supplemented with soya bean in comparison with 1.702 µg/ml achieved nutrient medium with sweet potato. Pseudomonas agar was developed and recommended for the production of pyocyanin by *Pseudomonas* species. This medium promotes the production of pyocyanin while inhibiting the production of other pigments. Different substances were included in culture media and confirmed to produce high levels of pyocyanin such as glycerol and alanine (as a precursors), sulphur, and iron [32]. On the other hand, the differences in the amount of pyocyanin pigment within the isolates of *P. aeruginosa* can be due to presence of a regulators of the quorum sensing system named QteE. The overexpression in this regulator will reduce the accumulation of homoserine lactone signals affecting the production of pyocyanin [34].

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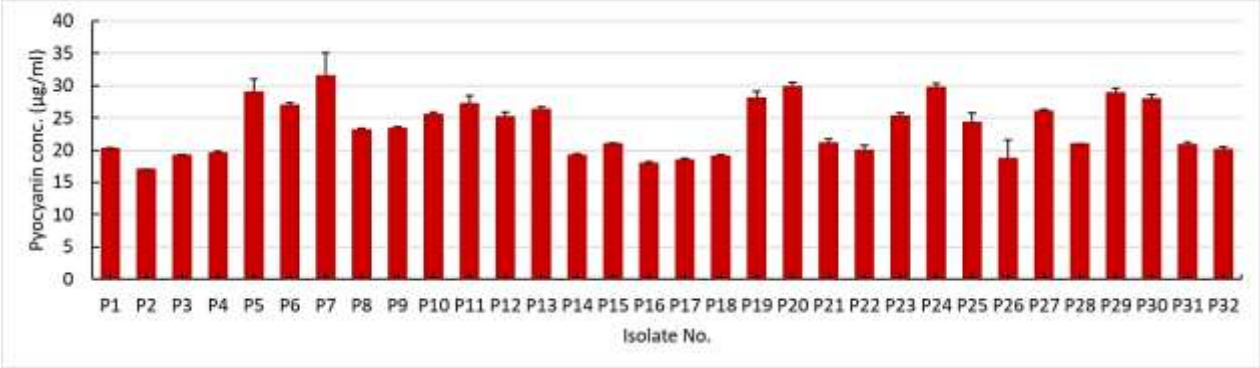

Figure 4: Screening of *P. aeruginosa* for pyocyanin production. The pigment concentration in liquid culture was measured as ug/ml (OD_{630} x 17.072).

Conclusion

The *P. aeruginosa* was resist most of tested antibiotics indicating a correlation between the multidrug resistance and the production of biofilm and pyocyanin. Most of the isolates were strong to moderate biofilm formation. Burn isolates were more potent in pyocyanin production. The isolate that gave strong biofilm formation produced high level of pyocyanin.

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