Study of Antibiotics Resistance and Biofilm Formation of *Pseudomonas Aeruginosa*

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Abstract

The aim of study is to verify biofilm forming capacity of P. aeruginosa isolates from different clinical sources and its association with antibiotics resistance. In the current study, fifty (50) P. aeruginosa isolates were recovered from 250 clinical specimens collected from three main hospitals in Diyala Province. The results revealed that 64% of isolates were MDR and 18% XDR towards many antibiotics used 7 antibiotics including Piperacillin-tazobactam, Ceftazidim, Cefepem, Azetronam, Meropenem, Amikacin and Ciprofloxacin. The rates of resistance toward these antibiotics were 66%, 80%, 88%, 72%, 54%, 52% and 40% respectively. The (MIC) for Polymyxin B ranged from 2- 16 mg/ml. Biofilm-production was assessed the Micro-titer plate quantitative method showed that all of isolates were biofilm producers. There were differences in the biofilm-production distributed into 16 (32%) were strong biofilm producer 19 (38%) moderate, and 15 (30%) were weak. The capability of P. aeruginosa to form biofilms on expired human blood and serum was also detected, and the results showed that all isolates were 100% biofilm producers. The separated red blood corpuscles (RBCs) had a higher rate of biofilm formation than serum, and the results also varied between blood groups where blood group AB was the highest in productivity, followed by O, B and A group respectively. Biofilm formation results on blood serum revealed that blood group O and AB, were the highest biofilm forming among the blood groups, followed by serum A and B, respectively.

Keywords: *Pseudomonas aeruginosa; biofilm formation; multidrug resistance.*

دراسة مقاومة المضادات الحيوية وتكوين الغشاء الحيوي للزائفة الزنجارية

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الخلاصة

تهدف هذه الدراسة إلى تحديد إمكانية تكوين الأغشية الحيوية لبكتيريا الزائفة الزنجارية المعزولة من مصادر سريرية مختلفة وارتباطها بمقاومة المضادات الحيوية. في دراستنا الحالية تم عزل خمسين (50) عزلة من الزائفة الزنجارية من 250 عينة سريرية تم جمعها من ثلاثة مستشفيات رئيسية في محافظة ديالى. تم إجراء اختبار الحساسية للمضادات الحيوية باستخدام 7 مضادات حيوية وهي بيبيراسيلين- تازوباكتام، سيفتازيديم، سيفيبم، أزيترونام، ميروبينيم، أميكاسين وسيبروفلوكساسين. وكانت معدلات المقاومة لهذه

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المضادات الحيوية 66%، 80%، 88%، 72%، 54%، 52% و40% على التوالي. تراوحت نسبة الـ (MIC) للبوليميكسين ب بين 2 – 16 ملغم/مل. أظهرت النتائج أن 64% من العزلات كانت مقاومة للأدوية المتعددة و18% كانت ذات مقاومة واسعة (XDR) تجاه العديد من المضادات الحيوية المستخدمة في هذه الدراسة. تم تقييم إنتاج الأغشية الحيوية باستخدام طريقة اطباق المعايرة الدقيقة حيث ظهر أن جميع العزلات كانت منتجة للأغشية الحيوية و كانت هناك فروق في إنتاج الأغشية الحيوية توزعت على 16 (32%) منتجة قوية للأغشية الحيوية و 19 (38%) متوسطة الانتاج و15 (30%) ضعيفة الانتاج. كما تم الكشف عن قدرة الزائفة الزنجارية على تكوين الأغشية الحيوية على الدم ومصل الدم البشرية منتهية الصلاحية، وأظهرت النتائج أن جميع العزلات كانت منتجة للأغشية الحيوية بنسبة 100%. كان لخلايا الدم الحمراء المنفصلة معدل تكوين الأغشية الحيوية أعلى من مصل الدم، كما تباينت النتائج بين فصائل الدم حيث كانت فصيلة الدم AB هي الأعلى إنتاجية على خلايا الدم الحمر تليها كل من O، و B وA على التوالي. ايضاً تباينت النتائج بين فصائل الدم في المصل حيث كانت فصيلتي الدم O وAB الأعلى إنتاجية على مصل الدم تليها كل من B وA على التوالي.

الكلمات المفتاحية *: الزائفة الزنجارية ، الأغشية الحيوية ، المقاومة المتعددة للمضادات .*

Introduction

Pseudomonas aeruginosa is an important opportunistic pathogen primarily causing nosocomial infections. It can cause sepsis, and chronic wound and lung infections, especially in immunocompromised and cystic fibrosis patients and is responsible for high mortality rates in burn units [1،2]. Antimicrobials resistance (AMR) became a problem in dealing with infections caused by *P. aeruginosa* as this pathogen began to demonstrate resistance to a variety of antibiotics classes, thus becoming harder to treat regarding morbidity and mortality [3]. The development of (AMR) has become a serious issue for healthcare professionals worldwide, as their therapeutic has become severely limited to address infections caused by multidrug-resistant (MDR) bacteria [4،5]. As a result, infections caused by MDR pathogens are related with increased mortality rates and hospitalization costs, and decreased quality of life (QoL) in affected patients [6]. In 2017, the World Health Organization identified *P. aeruginosa* as life-threatening due to the adaptableness and strong intrinsic antibiotic resistance, common antimicrobial treatments, such as antibiotics, exhibit poor effectiveness leading to a higher rate of mortality [7]. In addition, *P. aeruginosa* delays the treatment of severe infections since it can produce biofilms, which protect it from environmental stressors, prevents phagocytosis, and helps colonization and long-term perseverance [8]. Biofilm is the most important virulence factor of bacteria that establish on the surfaces. *P. aeruginosa*, biofilms are prearranged groups of bacterial cells implanted to an inactive or living surface and surrounded by an extracellular polysaccharide matrix, that defend the bacterial community from strict environment conditions such as host immune system and antibiotics [9].

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Methods

Collection of specimens

Two hundred fifty different clinical specimens were collected from three main hospitals in Diyala province for the period from September 2022 to January 2023, from different sources including: sputum, urine, blood, swabs (burn, wound, and ear). Specimens were transferred and inoculated directly in appropriate previously prepared culture media. Then they were numbered along the study as P_1, P_2, P_3,…etc.

Isolation and identification

Specimens were streaked on Blood agar, MacConkey agar and Cetrimide pseudomonas selective agar, over-night incubation at 37◦C, then examined biochemically and for oxidase production. The identification was confirmed by detection of *16SrRNA* gene.

16SrRNA detection

The identification was confirmed by detection of *16SrRNA*. Briefly, total DNA was extracted by using ABIOpure/ USA extraction kit according to the manufacture instruction, and the primers were obtained from Macrogen/ Korea, table (1).

Table (1): Sequence and molecular size of PCR products of *16s rRNA*.

Gene	Primer Sequence	Size of product (bp)	Annealing temp. ºC	Ref.
16SrRNA – F	GGGGGATCTTCGGACCTCA	956	58	[10]
16SrRNA – R	TCCTTAGAGTGCCCACCCG			

The PCR program and reaction condition explained in table (2).

Table (2): The conditions used for the amplifcation of 16s rRNA gene.

Initial denaturation	Denaturation in each cycle	Annealing	Primers extension	Final extension
95 °C, 5 min	95°C, 30s	55°C,30s	72°C, 30s	72 °C,7 min
1 cycle	30 cycles			1 cycle

Antibiotic Sensitivity Test

According to Kirby-Bauer method, AST test was conducted after lawn streaking on Mueller-Hinton agar. Explanation of results based on clinical and laboratory standard institute (CLSI) guidelines. Antibiotic discs that used were revealed in table (3). Isolates were resistant to at least three different antimicrobials classes counted as MDR.

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Table (3): Antibiotic discs used in the study.

Antibiotics name	Concentrations (µg)
Piperacillin-tazobactam (PRL)	100
Ceftazidim (CAZ)	30
Cefepem (CPM)	30
Azetronam (ATM)	30
Meropenem (MEM)	10
Amikacin (AK)	10
Ciprofloxacin (CIP)	5
Polymyxin B (PB)	MIC

Minimum inhibitory concentrations (MIC) for Polymyxin B

Serial micro-dilution method was used to determine the (MIC) of Polymyxin B using the 96-well micro-titer plate according to [11]. Briefly, 100 µl of Muller Hinton broth (MHb) was added to each well and 100µl of antibiotic prepared solution was added into the first wells in row A to obtain a concentration of 1024 mg/ml. serial dilutions using a micropipette were done steadily along the columns (from row A-H) to obtain the required dilutions (2, 4, 8, 16, 32, 64, 128, 256, 512, 1024), mixed well, and repeated up to the last row (H). 100µl of bacterial inoculum was transferred into all the wells except the negative control. The plates were incubated at 37ºC for 18-20 hrs. The results were read using an ELISA reader at a wavelength of 630 nm, where the (MIC) is the lowest concentration that prevents bacterial growth after incubation.

Biofilm production assays

Microtiter plate method (MTP) was used according to [12]. The results were calculated according to their optical densities as follows: (1) strong biofilm producer ($4 \times ODc < OD$); (2) moderate ($2 \times ODc < OD \leq 4 \times ODc$); (3) weak ($ODc < OD \leq 2 \times ODc$); (4) non-biofilm producer ($OD \leq ODc$).

Biofilm formation on expired human blood components (RBCs and Plasma)

Five isolates from different isolation sources were inoculated in BHI broth and incubated at 37°C for 24 hours. About 10-15 ml of expired blood bags were taken from each blood group (A, B, O and AB) and transferred into three opaque tubes, each tube containing 2.5-3 ml. The blood components (red blood cells from plasma) were separated by a centrifugation (3000 r/s for 5 min). The separated plasma was transferred to opaque tubes for each blood group, and the sediment RBCs were washed with PBS to get rid of the remaining plasma. The grown bacteria were diluted to 1:100 in BHI broth and mixed well. Next, 100 µl of plasma from each blood group was transferred to flat microtiter plates in two replicates for each isolate. Then each well was inoculated with 50 µl of diluted bacteria and incubated. After incubation, the components were disposed and washed three times with PBS, fixed with 99% methanol for 15 min, then removed and left to dry. 100 µl Crystal violet (1%) was added

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to each well, for 15 min, then washed 3-4 times with distilled water and leave it to dry. Finally, 100 µl of ethanol was added and measured the ODs as mentioned before.

Dendogram analysis

The genetic relationship between all bacterial isolates under study was found using a Dendogram by converting the results that appeared into a characterization table. When the result is positive, the number 1 is placed, and when the result is negative, the number is 0, and then enters this data into Past software using the Dic option in order to obtain a Dendogram.

Results and discussion

Isolation and Identification

P. aeruginosa bacterial colonies appeared pale, colorless, with irregular rims on MacConkey agar due to the non-fermentation of lactose sugar, and were characterized by their odor resembling the smell of fermented grapes. On the blood agar appeared gray in color, completely β-hemolysis, the isolates also showed clear growth on selective Cetrimide agar medium, which contains Cetrimide (0.03%). This percentage does not affect the growth of bacteria, but it inhibits the growth of the other bacterial species, as the colonies appeared colored, most of them blue-green, due to their production yellow-green pigment (pyoverdine). These pigments are characterized by being soluble in water, and sources indicate that most isolates of this species produce pigments [13]. The results of biochemical tests presented in table (4).

Table (4): Biochemical tests for *P. aeruginosa* identification.

Tests	Results
Catalase	+
Oxidase	+
Motility	Motile
Blood haemolysis	β- hemolysis
Gram stain	-ve
Pigments production	V

+ = positive, - = negative

Fifty *P. aeruginosa* isolates were identified after confirming their diagnosis phenotypically, microscopically, biochemical tests and genetically, table (5) showed the distribution of isolates according to isolation source, and figure (1) that shoed the *16SrRNA* gene product for diagnosis conformation.

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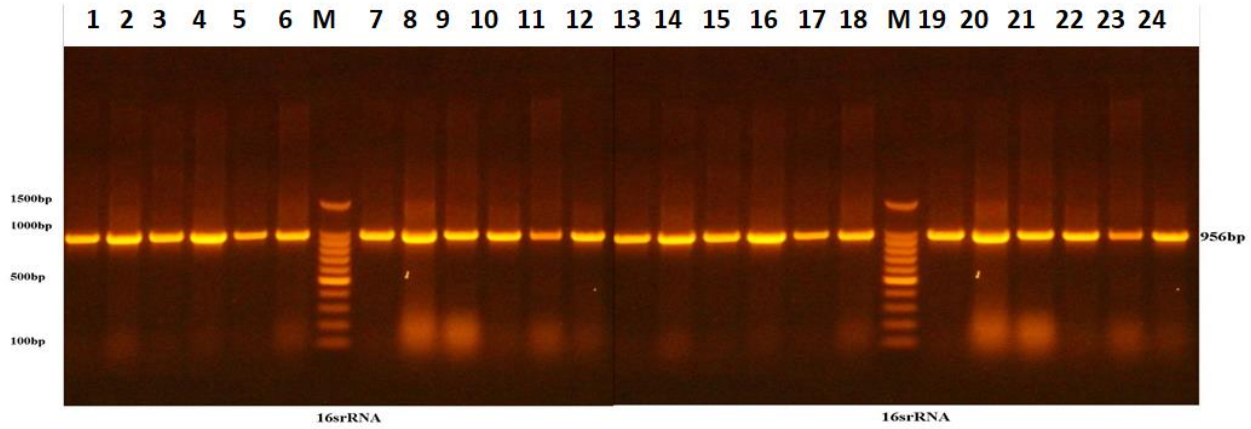

Fig. (1): Electrophoresis of *16SrRNA* gene product (956 bp) on an agarose gel using PCR technology for *P. aeruginosa* at a voltage of 100 V, a current of 100 mA, and a time of 60 min.

Table (5): distribution of isolates according to isolation source.

Source	No. of specimens	No. of isolates (%)
Burn	69	16 (6.4)
Wound	65	16 (6.4)
Urine	28	10 (4)
Blood	28	3 (1.2)
Sputum	25	3 (1.2)
Ear	35	2 (0.8)
Total	250	50 (20)

The results showed that the highest rates of isolation were from burns and wounds, in equal rates. In the study of [14] in Baghdad, they confirmed that the increase in infection rates in burn patients in particular was the result of damage to the tissues hosting the bacteria and the disruption of the patients' defense mechanisms, while [15] in South Africa explained that repeated scraping at the burn site and failure to practice preventive actions lead to an increased risk of infection and the spread of infection, and sometimes the reason is seasonal, as [16] showed that Burn infections caused by *P. aeruginosa* are four times more common in the winter than in the summer. The difference in isolation rates among the studies is due to many reasons, such as the virulence factors of the pathogen or due to the difference in sources of isolation and the number of samples that may vary according to the location of the infection and the difference in geographical location, and some other reasons, such as the type of sterilization and disinfectants used in hospitals and the methods used in sterilization.

Antibiotics susceptibility test

P. aeruginosa isolates showed different levels of resistance to each antibiotic, as shown in Figure (2). The highest rate of resistance to Cefepem was 88% and the lowest resistance rate was to Ciprofloxacin 40%.

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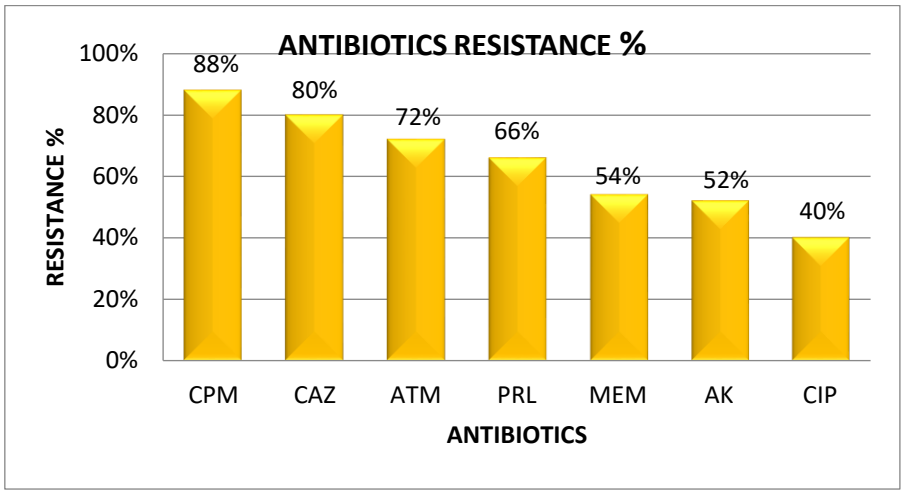

Fig. (2): Antibiotic resistance pattern of *P. aeruginosa.*

The results of the study showed high resistance of *P. aeruginosa* isolates to Cephalosporins, Monobactam, Penicillins, Fluuroquinolones, and Cephemes. This is due to the ability of bacteria to produce beta-lactam enzymes such as broad-spectrum β-lactamase enzymes (ESβLs), which work to degrade Penicillin and Cephalosporins in particular, and whose genes are either on chromosomes or on plasmids in many types of bacteria, which leads to multiple resistance to target proteins to different antibiotics [17].

The main reason for resistance to aminoglycosides is the presence of modified aminoglycosidase enzymes (AMEs). The high rate of resistance to aminoglycosides is due to the indiscriminate use of these antibiotics [18]. Fluoroquinolones prevent the construction of bacterial DNA by inhibiting the enzyme DNA gyrase, and thus inhibiting the transcription of DNA. Nuclear and its reproduction. *P. aeruginosa* resistance to this antibiotic class is because of mutation in the target enzyme, DNA gyrase, or via the efflux pump system [19].

Determination of MIC for Polymyxin B

The results showed that the values of MIC of Polymyxin B ranged between 2-16 mg/ml, that 34 (86%) were sensitive and 14% of isolates were resistance. This result was close to the result of [20] in Baghdad by 18.5%.

Multi-drug resistance (MDR)

Forty-one isolates (82%) under study showed multiple resistance (MDR) that ranged between three to eight antibiotics according to Table (6). The results of the study close to the results of [21] that represent 73.3% MDR between their isolates.

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Table (6): Multidrug Resistance *P. aeruginosa* isolates.

No. of antibiotics	Resist. type	No. of isolates	%
3	MDR	5	**10%**
4	MDR	7	**14%**
5	MDR	10	**20%**
6	MDR	10	**20%**
7	MDR	7	**14%**
8	XDR	2	**4%**
Total		41	**82%**

The difference in results between studies is due to the different antibiotic use strategy related to each country, and increasing the catastrophe of misuse of antibiotics without suitable prescriptions [22].

Dendogram

The Dendogram cluster analysis included the results of the susceptibility test of *P. aeruginosa* bacteria to antibiotics in the current study, Figure (3) showed there were two main groups, A and B, that revealed 40% similarity. Group A included two isolates, P2 and P39, that similar in their resistance to the ATM sensitive to PRL, CAZ, CPM, and MEM. Group B, included 48 isolates and subdivided into two groups, the first B1 included isolate P31, which was characterized by its resistance to CAZ and MEM, and its sensitivity to PIPM, CPM, ATM, AK, CIP, and PB. The second group, B2, included 47 isolates and further divided into two parts. The first part includes isolate P19 that is resistant to PRL, AK, and PB. The second part includes 46 isolates, which were also divided into two parts. The first part included isolates P28 and P48, which showed similarity in resistance to CPM and ATM and in sensitivity to PRL, CAZ, AK, CIP, and PB, while the second part included isolates that were unique in their pattern of resistance and sensitivity to the antibiotics under study and included 18 isolates. It also included isolates similar in resistance and sensitivity, which were divided into 6 clones, each clone consist of number of isolates that similar in their resistance and sensitivity to the antibiotics under study.

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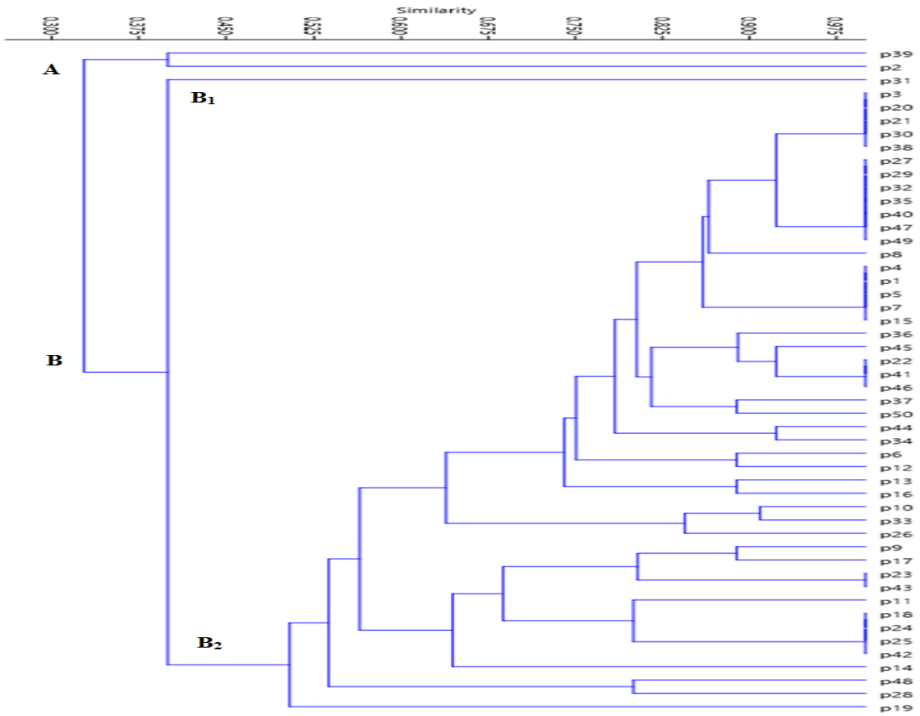

Fig. (3): Dendogram for AST results to *P. aeruginosa* isolates.

Detection of Biofilm Formation

All isolates showed the ability to form biofilm at a rate of 100%, table (7).

Table (7): Biofilm formation levels using MTP.

Biofilm levels	No. of isolates	%
Strong	16	**32**
Moderate	19	**38**
Weak	15	**30**
Non-producers	0	**0**

Among the biofilm-producing isolates, 32% of the isolates were strongly biofilm producers, and this result is consistent with [23], which was 36.5%. The results also showed that 38% of the isolates had moderate biofilm production, and this result agreed with [24] that found 34% of his isolates were moderate biofilm producers.

Microtiter plate method, is accurate in distinguishing and classifying strong, moderately productive, weak and non-productive isolates, because this method uses constant conditions and can be effective in identifying and studying many virulence factors for biofilm formation (such as Cilia and Pilli, enzymes, etc.), and this method can be used with different bacterial species, as motile bacteria stick to the bottom walls while non-motile bacteria stick to the bottom of the holes [25].

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Biofilm formation on expired blood components

The results of the study showed the ability of *P. aeruginosa* to form biofilms on blood and plasma and blood components were suitable media for biofilm formation, and if they were not enhancing, they were not inhibitory, but the highest formation was on red blood cells (RBCs). The ability of *P. aeruginosa* of five isolates from different sources (one isolate from each source) to form a biofilm. The results showed that the ability of the isolates to form biofilms ranged from strong to weak productivity, as the separated red blood cells had a higher rate of biofilm formation than plasma. The results also varied between blood groups as follows: AB blood type was the highest in productivity, followed by blood group O and blood group B, respectively, while blood group A was the least productive of its counterparts table (8).

Table (8): Biofilm formation on expired human blood components.

Isolates no.	Isolates source	Biofilm on BHI	Serum				Blood			
			O	**AB**	**B**	**A**	**O**	**AB**	**B**	**A**
P22	Burn	S	S	S	M	S	S	S	S	**M**
P17	Ear	M	S	M	M	M	S	M	S	**S**
P42	Wound	S	S	M	W	M	S	S	S	**S**
P37	Sputum	S	S	S	M	M	S	S	S	**S**
P28	Urine	S	W	S	M	W	W	S	W	**W**

S = strong, M= moderate, W= weak

For the growth of biofilms on blood plasma: After comparing the groups, it was found that blood group O plasma, then blood group AB plasma, were the highest, followed by plasma A and blood plasma B, and they were the least biofilm forming among the blood groups. The results also showed that isolates from burns and sputum had the highest production, followed by isolates from ear and wounds, then isolates from urine, which were the least productive among the isolation sources.

Many factors control the production of biofilm by *P. aeruginosa* inside the host, especially the blood, including pH, glucose concentration, temperature, phenotypic range, and genotype. The quantity and type of nutrients present in the surrounding environment, such as glucose in body fluids, is an essential factor for membrane formation that bacteria benefit from by increasing their numbers and facilitating the sending and receiving of intercellular signals between them to form and develop the biofilm. The presence of glucose in the blood increases the production of bacteria for the membrane, as it is considered the main substrate for the polysaccharide EPS and is included in its composition through the EPS pathway, which is considered one of the basic factors that increases the adhesion of cells to each other and to biological surfaces. Generally, the study showed that all the biofilm producer *P. aeruginosa* isolates were MDR. This make the infections difficult to treat and leads to higher concentrations of antibiotics consumed in the treatment.

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Conclusion

Our findings exhibit that there is a strong relation between biofilm formation and antibiotic susceptibility profile among clinical *P. aeruginosa* isolates. The aptitude to form biofilms was noticeably more tending to have the resistance phenotype MDR. Furthermore, the highest biofilm formation in blood component was on red blood cells (RBCs) and AB blood type was the highest in production.

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