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Relationship between Multidrug Resistance and Biofilm Formation in *Pseudomonas aeruginosa* Isolated from Burn wound Patients

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A B S T R A C T

Pseudomonas aeruginosa (P. aeruginosa) is a common nosocomial pathogenic bacterium found in hospitals and burn clinics and is a major cause of morbidity, and death. The current study aims to investigate the correlation among antibiotic resistance, pigment synthesis, and biofilm formation by *P. aeruginosa*. One hundred eighty samples were obtained from burn wounds at Azadi Teaching Hospitals/Burn Center. Only 48 isolates of *P. aeruginosa* are obtained from various sources of burns. A microtiter plate approach is used to detect biofilm formation. Results show that bacteria are most prevalent in flame burns, followed by hot liquid burns, gas explosions, and electrical burns, i.e., 41.7%, 35.4%, 16.7%, and 6.2%, respectively. Pyoverdine and pyocyanin are more frequently seen on cetrimide agar compared to other pigments. Antibiotic resistance rates vary across different antibiotics, such as Piperacillin (83.3%), Aztreonam (79.2%), Ceftazidime and Cefepim (62.5%), and Amikacin (52.1%). Levofloxacin, Gentamicin, and Tobramycin show moderate resistance rates of 41.7%, 37.5%, and 35.4%, respectively; however, Imipenem and Ciprofloxacin exhibited minimal resistance and are considered the most effective against *P. aeruginosa*, and 33.3% of isolates are positive for ESBL. Biofilm formation is positive in 95.8% of isolates, with 33.3% classified as strong producers, 45.8% as moderate producers, 16.7% as weak producers, and 4.2% as non-biofilm producers. An examination of the susceptibility test of biofilm-forming bacterial isolates reveals that 82.6% are multi-drug resistant (MDR). In conclusion, most *P. aeruginosa* isolates are potential biofilm producers and are significantly associated with antibiotic resistance. The development of multidrug resistance is found to be significantly correlated with pigment production.

Introduction

A burn is a severe damage of the skin or other biological tissues caused by heat, electrical discharge, chemicals or radiation (1). Burn injuries destroy the skin's protective layers and weaken the immune systems function, increase the susceptibility of burn victims to hospital-acquired infections (2). Patients with severe burn injuries are vulnerable to healthcare-associated infections (HAIs), and those patients are more likely to develop multidrug-resistant (MDR) bacterial infections as their hospitalization duration increases (3). *P. aeruginosa* is a Gram-negative aerobic bacillus that causes opportunistic or nosocomial infections in burn patients, peopole with cystic fibrosis as well as immunocompromised patients (4). Various extracellular pigments generated by *P. aeruginosa* produce water-soluble blue-green phenazine pigment pyocyanin (5). Several strains have the ability to create additional colors such as yellow pyoverdin, dark red pyorubin and dark black pyomelanin (6). In addition, the pathogen has the ability to form biofilms which promote colonization and ehnce the bacterial virulence causing treatment more challenging. Usually, immediate management with high-dose antibiotics is necessary for prolonged treatment. Biofilm allows cluster of bacteria to adhere to the mucosa and that is surrounded by extracellular polymeric substances (EPS). Moreover, biofilm protects the bacteria from the action of the immune cells and antibiotics, making it difficult to be eradicated, leading to treatment failure (7). Biofilm development is a fundamental and crucial virulence property, enhancing bacterial survival in challenging conditions, such as dryness or exposure to antiseptics (8). Various mechanisms contribute to *Pseudomonas* antibiotic resistance, including intrinsic resistance by limiting membrane permeability to antimicrobials, efflux systems that expel harmful compounds and antibiotic-inactivating enzymes. Moreover, many isolates include beta-lactamases and extended-spectrum beta-lactamases (ESBLs) (9). Multidrug-resistant *P. aeruginosa* is a significant issue due to its ability to induce life-threatening infections and prolong hospital stays, resulting in higher treatment expenses (10). The presence of both β-lactamase-producing phenotype and virulence characteristics makes *P. aeruginosa* a very dangerous pathogen, particularly in burn victims (11).

Aims:

The current study aimed to investigate the correlations among antibiotic resistance, pigment synthesis and biofilm formation by *P. aeruginosa*.

Materials and Methods

1. Study design and period.

The cross-sectional study was conducted at Azadi Teaching Hospital in Kirkuk city, especially in the burn department and nursing clinic, from September 2023 through to February 2024.

2. Patients and sample collection.

One hundred eighty clinical sterile cotton swab transport media (Amies) were used to collected samples from burn wound infections. Patient information, such as name, age, gender, cause of burn, rate and degree of burning and treatment, was recorded using a questionnaire form. The swabs were cultured immediately after collection.

3. Bacterial isolation and identification

All samples were inoculated on blood agar and MacConkey agar, then incubated at 37˚C for 18-24 hours.The isolated colony was characterized using gram stain, colony morphology, oxidase production test, lack of lactose fermentation on MacConkey agar and hemolytic activity on blood agar.

4. Pigment production

The specimens were cultivated on cetrimide agar with 0.1% cetrimide (acetyl trimethyl ammonium bromide) to inhibit organisms other than *P. aeruginosa*, then incubated at 37˚C for 24-48 hours. Examination of pigment production was conducted.

5. Antibiotic susceptibility test (disc diffusion method)

Pure growth was utilized to prepare bacterial suspensions using 5ml of normal saline. The suspension density was adjusted to meet 0.5 McFarland criteria. 2µL portion of bacterial solution was streaked on Mueller Hinton agar using a cotton swab, rotating the plate in three separate directions. The plate was inverted at room temperature for a brief period. Ten antibiotics were applied to the plates, then incubated at 37˚C for 24 hours. The diameter of the growth inhibition zones surrounding the antibiotic discs was measured using a graduated ruler. The outcome was categorized as sensitive, intermediate resistance or resistant and analyzed using the guidelines of the National Committee for Clinical Laboratory Standards 2023

6. Phenotypic detection (Extended spectrum beta lactamase) production.

A total of forty-eight *P. aeruginosa* isolates were examined for the presence of ESBL production utilizing the double-disc synergy test. The inoculum was evenly distributed on Mueller Hinton agar using sterile cotton swabs adjusted to a turbidity level of 0.5 McFarland. The amoxicillin clavulanic acid (AMC) core disks were spread on the plate with 16 mm to 20 mm apart from the ceftazidime and cefepime disk. The plate was placed in an incubator and kept at 37˚C overnight. The result is considered positive if the inhibition zone measurement greater than 5 mm.

7. Identification of biofilm by microtitter plate method

A single bacterial colony was cultured in 5ml of Brain Heart Infusion (BHI) broth and then incubated for 24 hours at 37˚C. The bacterial culture was diluted with 1000 µl brain heart infusion broth enriched with 1% glucose. The initial three wells were filled with TSB as a control. Subsequently, 200µl of bacterial suspension was added to each well, followed by incubation at 37˚C for 24 hours. To get rid of any detached cells, the plate was washed three times with phosphate-buffered saline (PBS). 200µL of 0.1% crystal violet was added to each well and incubated at room temperature for 10 to 15 minutes. The crystal violet was then removed by washing the plate three times with distilled water. The microtiter plate was inverted and let to dry for a few hours. To resolubilize the dye, 200 µL of 95% ethanol solution was added to each well and the plates were allowed to rest at room temperature for 10 to 15 minutes. The optical density was measured at a wavelength of 630 nm. To get optical results, perform this method three times.

Biofilm is interpreted as
ODi ≤ ODc = Non biofilm producer
ODc < ODi ≤ 2 ODc = Weak biofilm producer
2 ODc < ODi ≤ 4 ODc = Moderate biofilm producer
4 ODc < OD = Strong biofilm producer

Results and discussion

Forming biofilms is a key component contributing to antibiotic resistance. Consequently, efforts have been undertaken to address these significant issues by discovering novel medications capable of inhibiting biofilm development (12). Forty-eight bacterial isolates were obtained from 180 samples collected from various burn injuries. *P. aeruginosa* isolates were most commonly found in flame burns, followed by hot liquid burns, gas explosions and electrical burns,with 41.7%, 35.4%, 16.7%, and 6.2% respectively, as seen in Figure 1. Rashad *et al*., 2022 recorded that 33 isolates of *P. aeruginosa* out of 220 samples taken from different clinical specimens. *P. aeruginosa* isolates were most frequently found in burn swabs, followed by urine samples, wound swabs, ear swabs, sputum and bronchial washes,with 42.4%, 21.2%, 15.2%, 12.1%, 6.1% and 3.0% respectively (13). Yet, a significant number of *P.aeruginosa* isolates were found in burn , as patients were more vulnerable to infections compared to others. The compromised skin due to burns damage could potentially leading to extended hospital stays for patients, increasing their vulnerability to hospital-acquired infections (14). The findings of our study were consistent with those of Fakhry *et al.*, 2024. They found that the diagnosed cases of injuries were distributed in different proportions based on the type of burn. The highest percentage of burn injuries, 50%, was caused by liquid burns, followed by burns caused by fire at 46.7%. The lowest percentage of injuries, 3.3%, was attributed to electric shock burns, with a statistically significant association ($P \leq 0.002$) (15).

On blood agar, the colonies exhibited a spreading form with flat, uneven boundaries. A metallic sheen pigment was recorded ranging from bluish to green, with a mucoid appearance. Many of them had beta-hemolytic properties with a grapelike or corn tortilla-like aroma. On MacConkey agar, low convex, colorless colonies were seen,that are non-lactose fermenters. A positive result was recorded for the oxidase test, due to the presence of cytochrome *c* oxidasein its electron transport system. Cytochrome oxidase is an enzyme present in a limited number of bacteria that transfers electrons to oxygen, the last supporter in some electron transport chains. It is a key distinguishing factor between *Pseudomonads* and enteric bacteria, and may be recognized using biochemical tests such as API 20E (Figures 2 and 3). Recent investigations obtained 126 isolates that exhibited pale yellow colonies on MacConkey agar and blue-green colonies on Nutrient agar and Cetrimide agar (16).

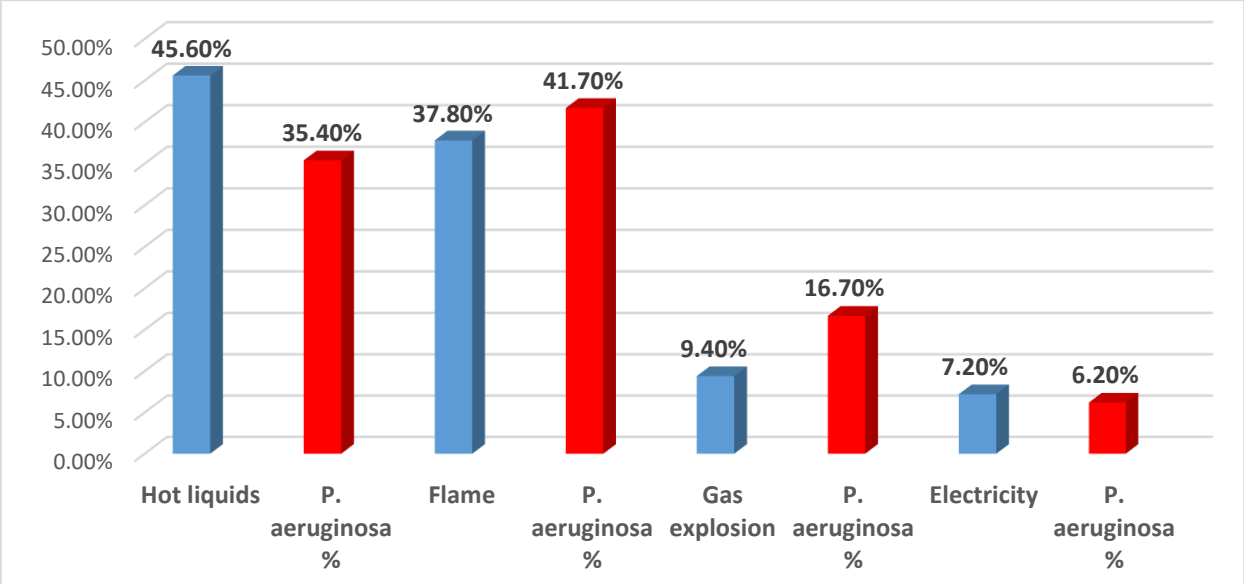
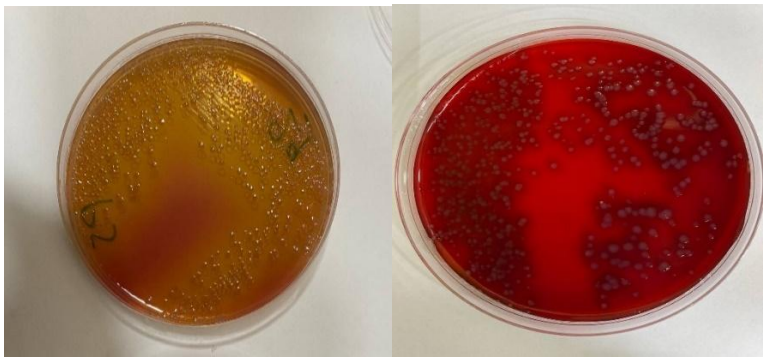
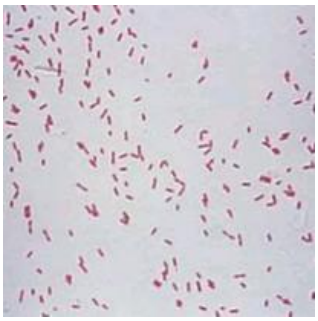


Figure: (1) Distribution of *P. aeruginosa* according to sample source.



A **B**

Figure (2): Cultural characteristics of *P. aeruginosa* on (A) MaCconkey agar and (B) Blood agar.



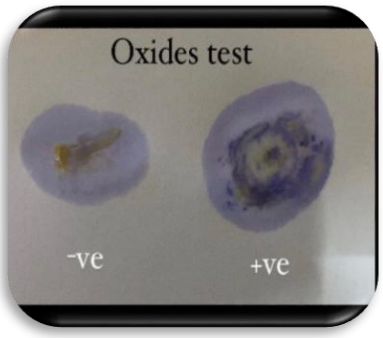


Figure (3) Gram stain for *P. aeruginosa* and Oxidase test

The percentage of pigments produced by *P. aeruginosa* on cetrimide agar

Cetrimide agar provides clear differentiation of *P. aeruginosa* and allows for the detection of pigments, such as pyocyanin and pyoverdine under UV light. 10 isolates (20.80%) exhibited the typical blue-green tint (pyocyanin pigment) as shown in Figure 4. The majority of isolates, 26 (54.20%), produced a yellow-fluorescent pigment, indicating pyoverdine pigment synthesis. In addition, pyomelanin was identified in 8 isolates (16.7%) and pyorubrin in 4 isolates (8.30%) of P. *aeruginosa*. Results were in line with those recorded by Ullah *et al*. (17), which identified 92.59% of pigment producers in China, with 64% producing pyoverdin pigment, which is compatible with the results for the current study. Phenotypic diversity in *P. aeruginosa* is common due to variations in biological conditions or iron levels, leading to changes in pigment synthesis (18).

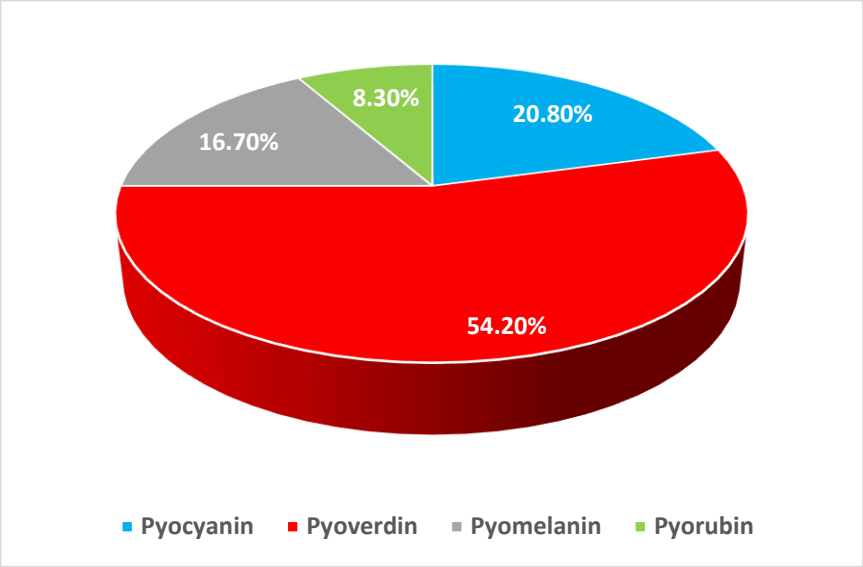


Figure (4): Pigments produced by *P. aeruginosa.*

Antibiotic susceptibility for *P. aeruginosa* isolates

Antibacterial-resistant strains have become a significant problem, leading to nosocomial infections and an increase in mortality and morbidity rates among hospitalized patients (19). *P. aeruginosa* have demonstrated significant levels of multi-drug resistance, being resistant to three or more kinds of anti-*Pseudomonal* antibiotics including carbapenems, fluoroquinolones, penicillin/cephalosporins and aminoglycosides (20). Figure 5 of the current study illustrated results of antibiotics sensitivity tests conducted on the isolated bacteria. The antibiotics utilized in the current study were Piperacillin, Cefepime, Ceftazidime, Aztreonam, Gentamicin, Amikacin, Tobramycin, Ciprofloxacin, Levofloxacin and Imipenem, of which multi-drug resistance (MDR) rate were 82.60%. All *P. aeruginosa* isolates in the current study exhibited various levels of resistance to different antibiotics. Specifically, 83.30%, 62.50%, 62.50%, 79.20%, 37.50%, 52.10%, 35.40%, 33.30%, 41.70%, and 20.80% were found to be resistant. Moreover, 4.20%, 0%, 4.20%, 0%, 0%, 0%, 0%, 0%, and 0% showed intermediate sensitivity. The remaining isolates demonstrated sensitivity to antibiotics at rates of 12.50%, 37.50%, 33.30%, 20.80%, 62.50%, 47.90%, 64.60%, 66.70%, 58.30%, and 79.20% respectively. The current findings were incompatible with those recorded by Almzil et al. (21), as Cefepime resistance rate recorded 1% in *P. aeruginosa* and a higher resistance rate of 64% for Cefazolin. Rashad *et al*. (13) found that antibiotic resistance in *P. aeruginosa* isolates was highest for Ceftazidime (10%), followed by Amikacin (10%) and Tobramycin (10%). A significant proportion of antibiotic-resistant microorganisms has been found in amoxicillin/clavulanic acid (100%). Results for the current study were in line with Corehtash et al findings, which reported a notably high prevalence of multidrug-resistant (MDR) isolates in Iran, as 93.1% of isolates were MDR, linking this phenomenon to extended hospital stays and inappropriate antibiotic usage (22). While *Heidari,et al* (23), found that 60% of the isolates were multidrug-resistant (MDR).

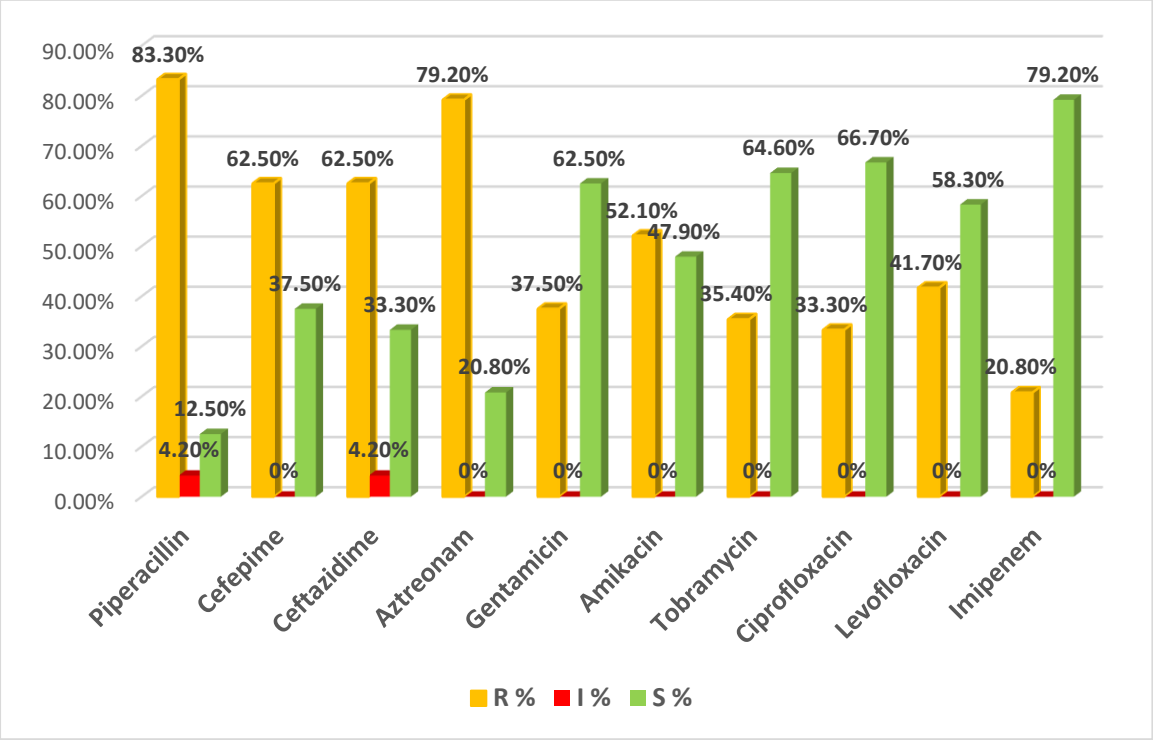


Figure (5) Antibiotic susceptibility test for *P. aeruginosa* isolates.

Extended-spectrum beta-lactamases (ESBL)production in *P. aeruginosa*

In the current study 48 samples analyzed, 16 (33.3%) isolates of *P. aeruginosa* were identified as ESBL-positive, whereas 32 were ESBL-negative as seen in Figure 6. Kaur and Singh (24) found that the prevalence of ESBL-mediated resistance in *P. aeruginosa* was 25% and that ESBL production from various clinical samples of *P. aeruginosa* was 17.7%. Another study indicated a notable occurrence of *P. aeruginosa* producing ESBL, which can lead to serious health issues in children, higher rates of illness and death, and limited treatment options due to the high level of resistance to multiple drugs in patients with existing medical conditions. Patients with predisposing conditions, such as prolonged hospitalization, severity of disease, compromised immunity and urine catheterization are at the highest risk for ESBL-producing bacteria (25).

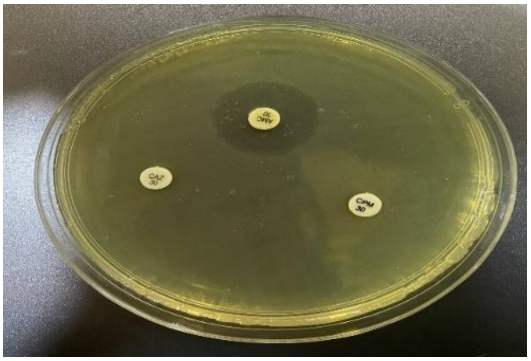


Figure (6): Production of ESBL by double-disc synergy test (DDST).

Biofilm formation in *P. aeruginosa* isolates

The unique nature of biofilm lifestyle presents significant obstacles for traditional antimicrobial treatments, which requires the development of multitargeted or combinatorial medicines. Burn injuries could cause extensive harm to the skin, with infection being responsible for 75% of burn-related fatalities. Opportunistic infections can grow and create biofilms in the secretions of burn wounds (26). Following incubation in brain heart infusion broth, the biofilm formation of *P. aeruginosa* isolates was measured using the microtiter plate technique. Results showed that 95.8% of isolates were able to form biofilms, while 4.2% were non-biofilm

formers. Among the biofilm formers, 33.30% were strongly adherent, 45.80% were moderately adherent and 16.70% were weakly adherent. The remaining 4.20% were non-adherent, as shown in Figure 7.

This proportion of production of biofilms in the current study was almost similar to that recorded by Divyashree *et al.* (27) who found 39% of strong biofilm and 57% for moderate biofilm producers, while the result of producing weak biofilms (16.7%) was nearly similar to that recorded by Al-Fridawy *et al.*, (28), as 13% resulted in producing weak biofilms. The current study revealed that the bacteria exhibited a high ability to create biofilms, consistent with the findings of a previous study by Alageedi *et al*. (29), which reported that *P. aeruginosa* formed 94.28% of biofilm formation and 5.71% did not. This explains how the quorum sensing system regulates gene expression in bacterial cells to enable biofilm development under certain conditions. It also influences the pathogenicity of bacteria by modulating biofilm formation and other virulence genes (30).

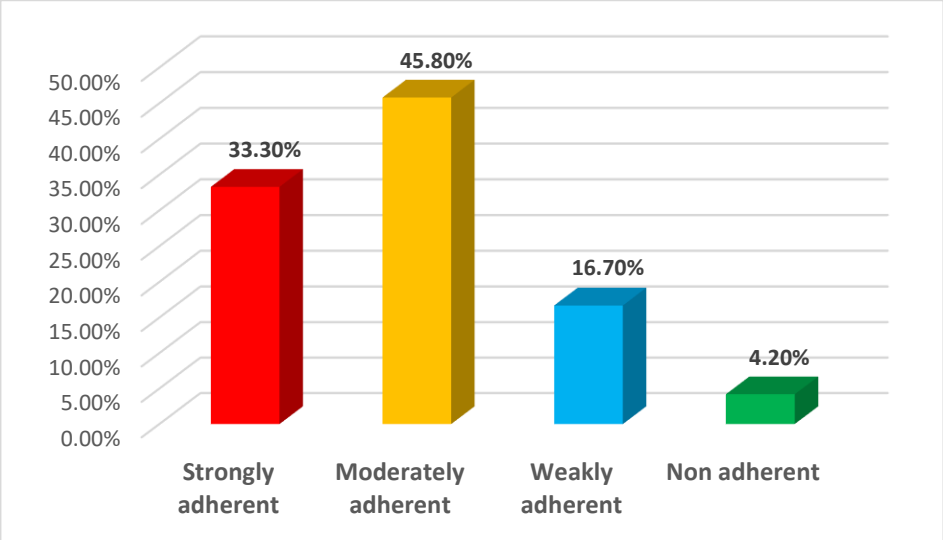


Figure (7): Results of biofilm formation in *P. aeruginosa.*

Correlation between biofilm formation and antibiotic resistance and pigment production in *P.aeruginosa*

Forty six isolates of *P. aeruginosa* samples were classified into three categories based on biofilm formation: firmly adherent (33.3%), moderately (41.7%) and weak (20.8%), as shown in Table 1. All firmly adherent isolates are multidrug resistant (MDR), whereas moderately adherent isolates are also 100% MDR and weakly adherent isolates are 0% MDR. On Cetrimide agar, 62.50% of strongly adherent isolates created pyoverdine pigment, 50% produced pyocyanin pigment and 25% and 12.5% of isolates produced Pyomelanin and Pyorubin pigments respectively. Isolates with moderate adhesion levels produced 54.50% pyoverdine and 18.2% pyocyanin. Pyomelanin and Pyorubin were produced in 13.60% and 12.50%, respectively. Among weakly adherent isolates, 25% produced pyoverdine, none produced pyocyanin, 12.50% were Pyomelanin producers and none Pyorubin producer.

Table (1): Correlation between biofilm formation, antibiotic resistance and pigment synthesis in *P. aeruginosa.*

Biofilm formation		**Antibiotic resistance**		**Pigment types**			
Types	**No. %**	**MDR No.%**	**Sensitive No.%**	**Pyocyanin No.%**	**Pyoverdine No.%**	**Pyomelanin No.%**	**Pyorubin No.%**
Strongly adherent	**16 33.30%**	**16 100%**	**0 0%**	**6 50%**	**10 62.50%**	**4 25%**	**2 12.50%**

Biofilm formation		Antibiotic resistance		Pigment types		
Moderately adherent	**22**	**22**	**0**	**4**	**12**	**3**	**2**
	41.70%	**100%**	**0%**	**18.20%**	**54.50%**	**13.60%**	**12.50%**
Weakly adherent	**8**	**0**	**8**	**0**	**2**	**1**	**0**
	20.80%	**0%**	**100%**	**0%**	**25%**	**12.50%**	**0%**
Total	**46**	**38**	**8**	**10**	**24**	**8**	**4**
	95.80%	**82.60%**	**17.40%**	**21.70%**	**52.20%**	**17.4**	**8.70%**

Results in the current study were in line with that recorded by Yekani *et al* (32), as biofilm-forming *P. aeruginosa. aeruginosa* isolates exhibited high antibiotic resistance compared to non-biofilm-forming isolates. Additionally, a notable relationship between multidrug resistance (MDR) and biofilm formation was recorded. Antibiotic resistance in microbial biofilms is 10-1000 times higher than that compared to non-biofilm producing colonies. Resistance could result from various factors such as: (a) disability of antibiotics to penetrate the dense matrix, (b) antimicrobial drug levels being suboptimal, (c)) inability of antimicrobial agents to inhibit microorganisms due to the metabolic inactivity of most microorganisms in the deeper layers of the biofilm and (d) removing antibiotics from the biofilm through microbial communities' "efflux action"(32). A previous study re vealed that there was no relationship between pigment synthesis and biofilm development for any isolates. The potential of producing biofilm may be a key virulence factor for some *P. aeruginosa* isolates, impacting the pathogenesis, prognosis and development of chronic, recurring and recalcitrant infections (33). Accoding to pigment production and drug resistance, Kothari *et al.*, 2022 studies, was in agreement with results of the current study, in which yellow pigment-producing strains presented significant resistance to antibiotics groups, including β-lactam (91.5%), aminoglycosides (70.5%) and carbapenems (51.9%) compared to green and non-pigmented strains (34). This indicates the importance of pigment production in the environment which could promote the bacteria to mask antibiotics or neutralize antibiotics via interaction to the active site where the antibiotic agent may enter or peneterate the bacterial cell membrane.

Conclusion

P. aeruginosa , which is obtained from burn samples has a high tendency to build biofilms and is significantly associated with antibiotic resistance. The development of multidrug resistance was found to be significantly correlated with pigmant production, according to the results.

Ethical issue

The current study was conducted following the ethical guidelines derived from the Declaration of Helsinki. The procedure was conducted after obtaining verbal and analytical consent from the patients. The study protocol and the patient information and consent form were approved by a local ethics committee under document number 587 on the 18th of September 2023.

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