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Association of Multidrug Resistance With Biofilm Formation in Pseudomonas Aeruginosa Isolated from Clinical Samples in Kirkuk City

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Abstract. *Pseudomonas aeruginosa* is one of the most prevalent nosocomial pathogenic microorganisms that cause life-threatening infections. The formation of biofilm is one of the important features that lead resistance to antibiotics. The study aimed to clarify the relationship between biofilm formation, antibiotic resistance, and pigment production *P. aeruginosa.* A total of 33 isolates have been obtained from 220 different clinical samples, 42% of isolates obtained from burn swabs,15% wound swabs,12% from urine samples, 12% ear swabs ,6% sputum samples, and 3% from bronchial washes. The highest prevalence of these bacteria in our study was found in isolates obtained from burn swabs. Pyocyanin and pyoverdine are frequently seen on Cetrimide agar. *P. aeruginosa* can be completely isolated on Cetrimide agar. The production of biofilms detected by microtiter plate method and the result distributed as, 22% of strongly, 50% moderately, 27% weakly, and 4% were non biofilm producer. The Kirby-Bauer method (disc diffusion susceptibility method) was used to measure antibiotic resistance, and the results revealed a correlation as 86% multidrug resistance were strongly biofilm former, 75% multidrug resistance were moderately biofilm former and 11% multidrug resistance were weakly biofilm producers.
Conclusion, *P. aeruginosa* from clinical isolates has highly proportional capability to form biofilm and recorded a positive correlation with multidrug resistance, while this relation was not significant with pigment production.

Key word: *pseudomonas aeruginosa*, biofilm, multidrug resistance, pigments,

Introduction

Pseudomonas aeruginosa belongs to Pseudomonadaceae that is ubiquitous gram-negative bacilli, aerobic, and non-fermentative bacterium, able to life in a wide range of environments (1). *P. aeruginosa* is a foremost cause of nosocomial infections, including urinary tract infections, pneumonia and bacteremia. It is also found in patients having burns, surgical, pus and accidental wounds (2). These infections become sever especially in immune compromised individuals (3). It also produces a number of virulence factors which after colonization can cause extensive tissue damage, bloodstream invasion, and dissemination (4).

This opportunistic bacterium is a biofilm-forming producing various bioactive molecules such as pigments (5, 6). Prokaryotic biofilm is an extracellular polymeric material that allows bacteria to attach to a variety of substances on surfaces and enable interconnectivity, including polysaccharides and nucleic acids. Biofilms have been recognized as a problem in medical settings (7). Biofilm produced by four major steps: surface attachment, microcolony formation, maturation, and dispersion (8). Which contribute to high (adaptive) antibiotic resistance and mediate long-term host colonization (9). As a result, emerging antimicrobial techniques that target bacterial pathogenicity factors (such as those that regulate biofilm formation) or host factors (such as immunological activation) are attractive antibiotic alternatives, especially given the global problem of antibiotic resistance (10). The bacterium is naturally resistant to many antibiotics due to permeability barriers provided by cell wall

lipopolysaccharide, as well as its ability to colonize surfaces in a biofilm form, which yields the cells impervious to therapeutic concentration antibiotics (11).

Infections caused by multidrug resistant (MDR) *P.aeruginosa* have been associated with increased morbidity, mortality, and costs among patients suffering from nosocomial infection particularly those receiving intensive care treatments (12). A variety of extra-cellular pigments were produced. The characteristic feature of *P. aeruginosa* is the production of the water-soluble blue-green phenazine pigment pyocyanin (13). Also produce, yellow-green pigment (pyoverdin), bright red pigment (pyorubin) and brown-black pigment (pyomelanin) (14).

The study focused the relationship between biofilm formation, antibiotic resistance, and pigment production in *P. aeruginosa* isolates from different clinical sample in Kirkuk city.

Materials and Methods

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients verbal and analytical approval before sample was taken. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee according to the document number 42471 (including the number and the date in 9/12/2021) to get this approval.

Patients and sample collection

The study was carried out in Azadi teaching hospital, pediatric hospital, Kirkuk general hospital, Women and children's hospital, and Public health laboratory, in Kirkuk city during the period from November 2021 to May 2022. Different clinical sample (Sputum, urine, bronchial wash, and swabs from burns, wounds, and ears) obtained from 220 clinical specimens the specimens were cultivated on blood agar, MacConkey agar, ceterimide agar, and brain heart infusion broth (BHI) broth. After growth, the colonies that formed were evaluated for oxidase production and biochemical responses. Further for API 20E used for accurate identification.

Bacteriological isolation and identification

Blood agar, MacConkey agar, and BHI broth were used to culture all of the samples, which were subsequently incubated at 370°C for 24-48 hours. The isolated colony was recognized by gram stain, colony shape, oxidase production test, non lactose fermentation on MacConkey agar, hemolytic activity on blood agar, and pigment formation on Cetrimide agar.

Antibiotic susceptibility testing

Kirby-Bauer method (disc diffusion susceptibility method), which is a practical, sensible, and well-standardized method. Bacterial growth (1-2x 108 CFU/ml) was placed on the surface of a Mueller-Hinton agar plate and incubated at 37 °C for 24 hours. The zone of growth inhibition around the antibiotic discs was measured to the nearest millimeter with a graduated ruler. The result was recorded as sensitive, intermediate resistance or resistant and compared to a standard strain.

Evaluation of biofilm formation by Microtiter plate method

The biofilm was estimated by the microtiter dish method (15). In this technique each isolate was cultured overnight at 37°C in tryptic soy broth (TSB) containing 0.25 percent glucose. The cultures were diluted to 1:100. The bacteria suspensions (125µL) were added to a 96-well polystyrene microtiter plate and incubated at 37°C without agitation for 24 hours. The wells were cleansed three times with 300 µL of distilled water each time; the adhering bacteria were fixed for 10 minutes in absolute methanol before being stained with 125 liters of 0.1 percent crystal violet solution. About 10–15 minutes in the water the wells were rinsed three times with distilled water after staining to eliminate all nonadherent cells. The wells were cleaned with 125 µL of water and 30 percent acetic acid. In each well of a new sterile flatbottomed 96-well polystyrene microtiter plate, 125 µL destaining solution was inoculated. Using an ELISA reader, the absorbance of the destaining solution was measured at 570 nm as showed in (figure 1). Every experiment was carried out three times. The un inoculated media was utilized as a control. The isolates were classified as strong (4 ODc ODi), moderate (2 ODc ODi 4 ODc), weak (ODc ODi 2 ODc), or nonproducer of biofilm (ODi ODc) based on the optical density of each sample (ODi) and the negative control (ODc) (16).

* The negative control was TSB broth without bacterial inoculums.

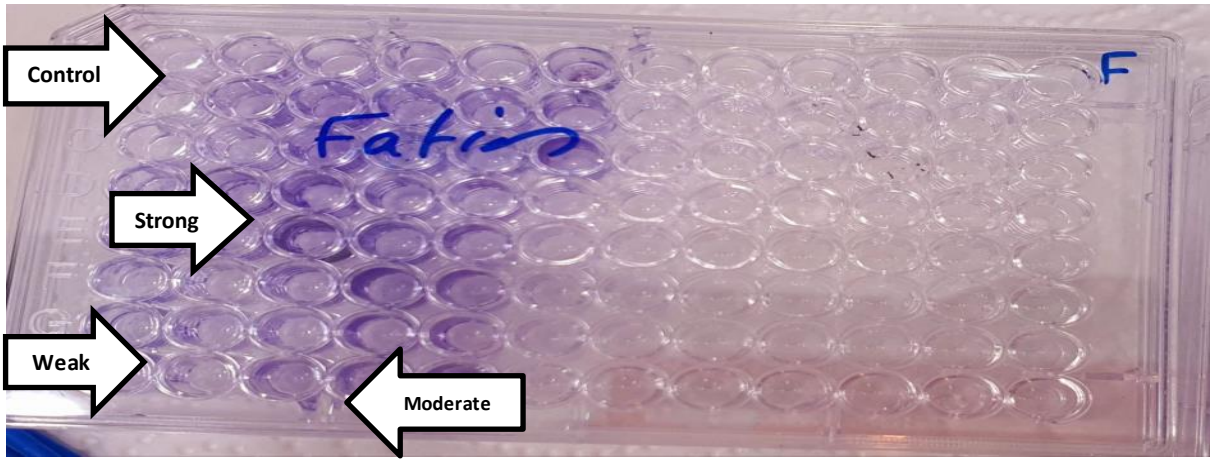

Figure (1): The biofilm production of P. aeruginosa.

Result and discussion

Isolates of *Pseudomonas aeruginosa* in various clinical samples:

Thirty three isolates of *P. aeruginosa* were isolated from 220 samples collected from various clinical specimens. The frequency of *P. aeruginosa* isolates were highest in the burn swabs followed by urine samples, wound swabs, ear swabs, sputum, and bronchial washing with percentages (42.4%, 21.2%,15.2%, 12.1%, 6.1%, 3.0%) respectively, (Figure 2).

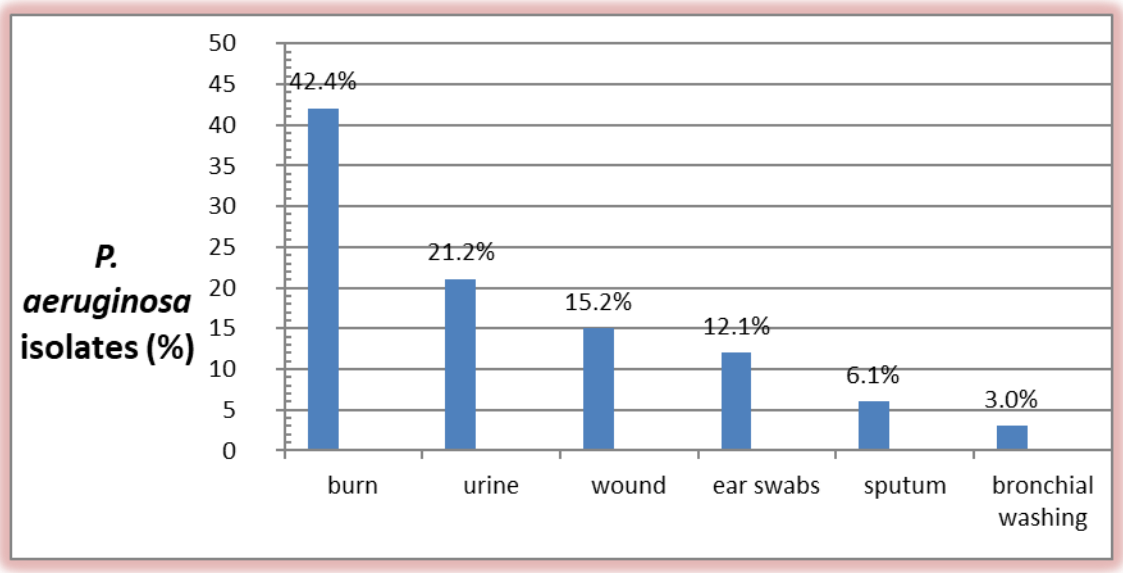

Figure (2): Frequency of *P. aeruginosa* isolates in different clinical specimens

These findings are in agreement with those of *Aljebory* (17) in Kirkuk, who reported (31 %) of isolates from burns and (13 %) from wound swabs. Also *Al-Kaaby* (18) in Al-Diwaniya recorded the highest percentage of isolation in burn and wound swabs (82.2%) and (51.4%), respectively.

However, a high prevalence of *P.aeruginosa* isolates were recorded from burns due to the fact that patients were more susceptible to infections than others. However the damaged skin barrier may have lost the first immune system, in addition to prolonged hospitalization of the patients, which exposes them to nosocomial infections (19). The findings are also compatible with the study conducted in Egypt, which found that *P.aeruginosa* was isolated from urine (22%), sputum (3%), ear discharge (16%), and wounds (11%). (18). The discrepancy in isolation frequency could be attributable to differences in virulence factors, infection location, and clinical specimen source (20).

P. aeruginosa identification and isolation:

A gram stain investigation revealed a gram negative, non-spore producing motile rod. On blood agar colonies developed in a spreading shape with flat, irregular borders, typically metallic sheen-bluish to green colored, colonies mucoid, and many of them were beta-hemolytic with grapelike or corn tortilla-like scent colonies (21) while on MacConkey agar appeared low convex, colorless colonies (non lactose) fermenter (22), the isolate was oxidase positive, because of containing cytochrome C oxidase in the electron transport system. Cytochrome oxidase is an enzyme found in a small number of bacteria that transports electrons to oxygen, the last acceptor in some electron transport chains; it is one of the primary differences between Pseudomonads and enteric bacteria, and biochemical tests identified by API 20E (Figure 3) (23).

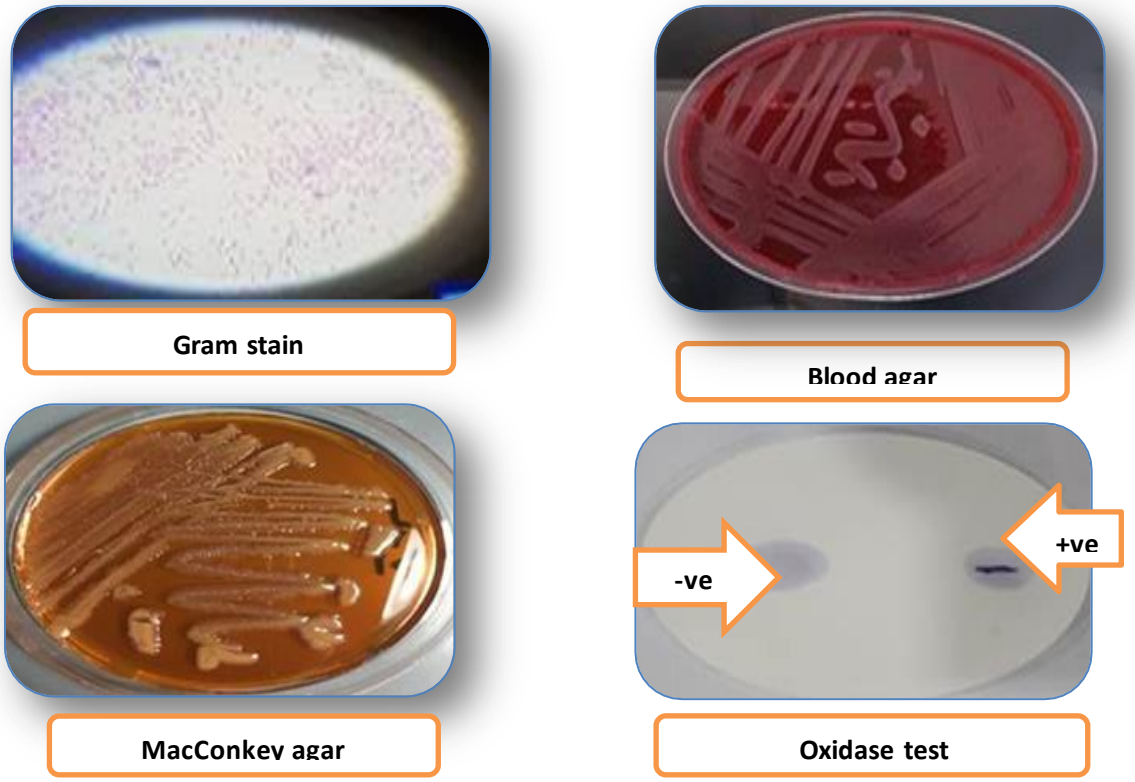

Figure (3): Identification of *P. aeruginosa* using API 20E biochemical assay

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

Cetrimide agar offers pure separation of *P. aeruginosa* and pigment visibility under UV light, where both pyocyanin and pyoverdine are commonly detected. The usual blue-green tint (pyocyanin pigment) was identified in 6 isolates (18.2%) in this investigation. As shown in, 20 isolates (60.6%) produced green-yellow pigment, whereas 7 isolates (21.2%) produced yellow-fluorescent pigment, indicating pyoverdine pigment synthesis (Figure 4,5).

All isolates of *P.aeruginosa* were pigment producers, with pyoverdin pigment producers accounting for 60.6 percent of green-yellow pigment, 21.2 percent yellow-fluorescent pigment, and pyocyanin producers accounting for the remaining 18.2 percent. The current findings are consistent with those of *Ullah et al* (24), who discovered (92.59 percent) pigment producers in China, with the majority of them (64 percent) producing pyoverdin pigment, which is also consistent with our findings. Because of differences in biological environments or iron availability, phenotypic variety is prevalent in *P. aeruginosa*; resulting in differences in pigment production (25).

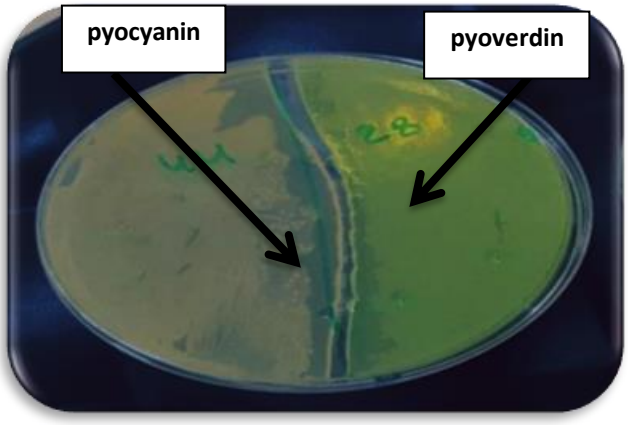

Figure (4): Pigments production of *P.aeruginosa* on Cetrimide agar.

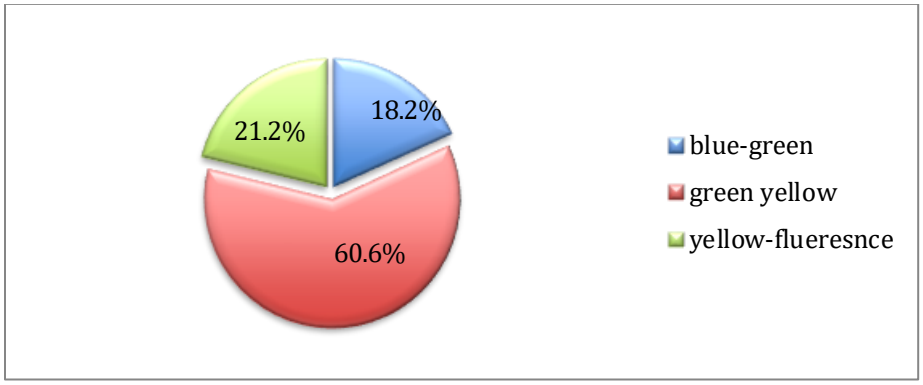

Figure (5): Pigments produced by *P. aeruginosa* on Cetrimide agar

Antibiotic susceptibility for *P. aeruginosa* isolates

All *P.aeruginosa* isolates were tested for antibiotic susceptibility in this study. 30.3%,30.3%,30.3%,30.3%, , 54.5%, 66.6%, 84.8%, , and 100%, Ceftazidime, ciprofloxacin, amikacin, tobramycin, imipenem, levofloxacin, trimethoprim/sulfamethoxazole, and amoxicillin/clavulanic acid were all resistant, whereas 0%, 0%, 6.0%, 6.0%,6.0%, 9.0%, 9.0%, and, 9.0%, Trimethoprim/sulfamethoxazole, amoxicillin/clavulanic acid, ciprofloxacin, imipenem, tobramycin, levofloxacin, amikacin, and ceftazidime are intermediately sensitive, and ciprofloxacin, imipenem, tobramycin, levofloxacin, levofloxacin, amikacin, and ceftazidime ,and 0%,12.1%, 24.2%,39.3%, 60.6%,63.6%, 63.6%, 63.6%,were they sensitive respectively as display in (table 1).

Table (1): Antibiotic susceptibility of *P. aeruginosa* to different antibiotics.

Antibiotics	R NO./%	I NO. / %	S NO. / %
Ceftazidime (CAZ) (30µg)	10 (30.3%)	3 (9.0%)	20 (60.6%)
Levofloxacin (LEV) (5µg)	22 (66.6%)	3 (9.0%)	8 (24.2%)
Ciprofloxacin (CIP) (5µg)	10 (30.3%)	2 (6.0%)	21 (63.6%)
Imipenem (IMI) (10µg)	18 (54.5%)	2 (6.0%)	13 (39.3%)
Amikacin (AK) (30µg)	10 (30.3%)	3 (9.0%)	21 (63.6%)
trimethoprim/ sulfamethoxazole (SXT) (1.25µg)/ (23.7µg)	28 (84.8%)	0 (0%)	4 (12.1%)
Tobramycin (TN) (10µg)	10 (30.3%)	2 (6.0%)	21 (63.6%)
amoxicillin/ clavulanic acid (AMC) (20/10meg)	33 (100%)	0 (0%)	0 (0%)

***No. (Number), S (sensitive), I (intermediate), R (resistant).**

Pathogenic strains that are resistant to antibiotics have recently been a major concern, resulting in nosocomial infections and an increment in the ratio of mortality and morbidity among hospitalized patients *Pachori et al* (26). The *P. aeruginosa* pathogens showen to be highly multi-drug resistant that is resistant to three or more anti-Pseudomonal antibiotic classes (carbapenems, fluoroquinolones, penicillin/cephalosporins, and aminoglycosides) (27).

Antibiotic resistance detected in *P. aeruginosa* isolates were as follows Ceftazidime (10%), followed by Amikacin (10%) and Tobramycin (10%). However, a large percentage of antibiotic-resistant bacteria has been identified in amoxicillin/ clavulanic acid (100%). This result agrees with Farhan et al (28), *P. aeruginosa* isolates were found to be totally resistant to amoxicillin–clavulanic acid. In terms of MDR isolates, the findings of this study accord with those of *Unan et al* (29), (Turkey), who reported the same percentage of MDR isolates (60%), *Corehtash et al* (30) observed an extremely high rate of MDR in Iran, finding that (93.1%) of isolates were MDR and attributed this to prolonged hospital stays and incorrect antibiotic administration.

Quantification of biofilm formation in *P. aeruginosa* isolates using microtiter plate method

After culturing in Tryptone soy broth, biofilm formation of P. aeruginosa isolates was quantified using the microtiter plate method; 96 % isolates were biofilm formers, while only 4% were non-biofilm formers (non-adherent); 7 isolates 22 percent were strongly adherent, 16 isolates 50 % moderately adherent, 9 isolates 27 % weakly adherent, and 4% were non-adherent **(Figure 6)**.

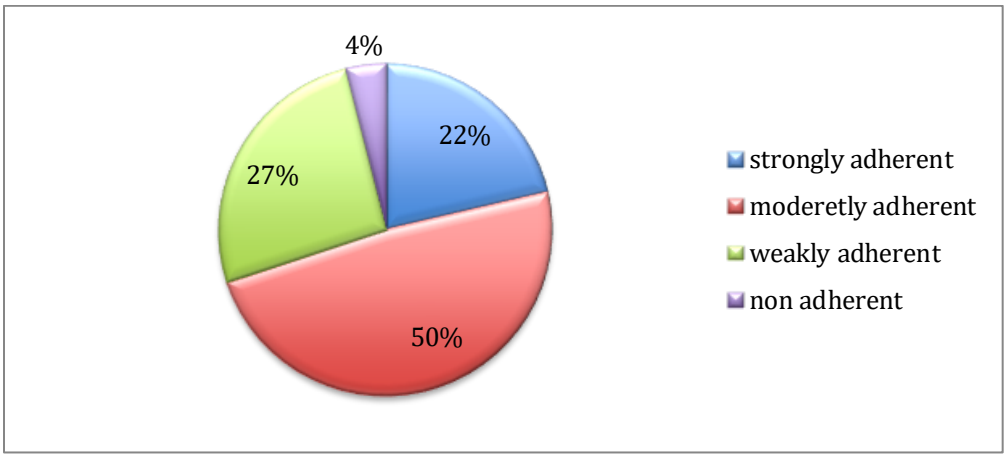

Figure (6): Biofilm development in *P. aeruginosa*

The findings are in agreement with those of *Namuq et al* (31), who discovered that 98% of isolates were biofilm formers (adherent), while only 2% were non biofilm formers(non-adherent). Biofilm formers included (22%) strongly adherent isolates, (50%) moderately adherent isolates, and (27%) weakly adherent isolates.

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

Positive isolates (33) samples of *P. aeruginosa* were categorized into 21.2 %, 48.4 percent, and 27.3 %, respectively, based on biofilm formation types that were strongly, moderately, and weakly adherent (Table 2). Multidrug resistant (MDR) isolates make up 86% of strongly adherent isolates, with moderately adherent isolates accounting for 75% and weakly adherent isolates accounting for 11%. On Cetrimide agar, 100% of strongly adherent isolates developed pyoverdine pigment, while none of the isolates produced pyocyanin pigment. Isolates with a moderate level of adhesion produced 75% pyoverdine and 25% pyocyanin. And 78 % of weakly adherent isolates produced pyoverdine and 32 % produced pyocyanin (Table 2).The relationship between biofilm formation and MDR is very significant, whereas the relationship between pigment production and MDR is not significant as showed in (Table 2).

(Table 2): Correlation between biofilm formation, antibiotic resistance, and pigment synthesis in *P. aeruginosa*.

Biofilm formation		Antibiotic susceptibility		Pigment types	
Types	No. %	MDR No. / %	Sensitive No. / %	Pyocyanin No. / %	Pyoverdine No. / %
Strongly adherent	7 21.2%	6/7 86%	1/7 14%	0/7 0%	7/7 100%
Moderately adherent	16 48.4%	12/16 75%	4/16 25%	4/16 25%	0/16 0%
Weakly adherent	9 27.3%	1/9 11%	8/9 89%	2/9 32%	0/9 0%
Total	32 96.9%				

The results of study were compatible with those of *Yekani et al* (32) who found that isolates of biofilm-forming *P. aeruginosa* were more resistant to antibiotics than non-biofilm-forming isolates, also a significant correlation between MDR and biofilm development. In comparison to non-biofilm generating colonies, the inhibition of different antibiotics increases 10-1000 fold in several microbial biofilms. Resistance can be caused by a variety of methods, including: (a) antibiotics fail to penetrate the dense matrix, (b) antimicrobial drug concentrations are below optimal, (c) antimicrobial agents are unable to inhibit microorganisms because the majority of them in the biofilm's deeper layers are metabolically inactive, and (d) antibiotics are removed from the biofilm via microbial communities “efflux action' (33). On Cetrimide agar, (33%) of biofilm formers produced pyocyanin and (21%) of biofilm formers produced pyoverdin. The relationship between biofilm formation and MDR is very significant, whereas the link between pigment production and MDR is not. The results of this investigation accord with those of *Kalaiarasan* (34) in India, who discovered pyocyanin synthesis (14%) and pyoverdine production (42%) in biofilm producer isolates. Despite the significance of pyoverdine in biofilm development, there was no link between biofilm formation and pigment synthesis in the current investigation due to the presence of pyoverdine in both biofilm formers and non-formers and pyocyanin in zero percent in strong biofilm formers.
So we concluded from the results of the study that most isolates of *P.aeruginosa* pathogens from different clinical samples showed a highly proportional ability to form biofilm with a positive relation to multidrug resistance, while this relation was not significant with pigment production.

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