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Antibiotic Resistance and Biofilm Formation in *Pseudomonas aeruginosa* from Different Clinical Samples in Nasiriya, Iraq

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Abstract*—Background: Pseudomonas aeruginosa is recognized as the main cause of nosocomial infections. The study aimed to investigate biofilm formation and antibiotic resistance patterns in clinical isolates of P. aeruginosa from various clinical samples from 12 to 70 ages. Methodology: This study included 120 samples obtained from Al-Hussein Teaching Hospital, Al-Nasiriya Teaching Hospital, Al-Haboubi Teaching Hospital, and Mohammed Al-Mousawi Children's Hospital in Nasiriya from January 2023 to June 2023. The samples included 56 wound swabs from cancer patients, 27 burn swabs from burn patients, 18 sputa, 14 ear swabs, and 5 bronchial-aspirate samples. The ages of the patients ranged between 12 and 70 years, and both sexes were represented. The drug susceptibility pattern and biofilm formation were performed for all the isolates. Results: After the final diagnosis, 80 samples (67%) were confirmed to be Pseudomonas aeruginosa. These isolates were obtained from 61 males (76.25%) and 19 females (23.75%). The highest resistance of P. aeruginosa to antibiotics was against the antibiotics Cefepime (96.25%), Amikacin (91.25%), Gentamicin (81.25%), and resistance to Ciprofloxacin was (76.25%), Ceftazidime (66.25%), Piperacillin (48.8%), and Aztreonam (47.5%). On the other hand, the lowest resistance reported in the present study was towards Colistin Sulphate (1.25%), followed by Imipenem (3.75%) and Meropenem (5%). In addition, the results of biofilm formation showed that 57 (71.25%) were non-biofilm producers, 10 (12.5%) were weak biofilm producers, 11 (13.75%) were moderate biofilm producers, and 2 (2.5%) were strong biofilm producers. Conclusion: no correlation was found between antibiotic resistance and biofilm formation.

***Keywords*—Pseudomonas aeruginosa, Multidrug resistance, Biofilm formation, Antibiotic resistance.**

I. INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is an aerobic, Gram-negative, opportunistic bacterium widely recognized for its remarkable adaptability and resistance to antibiotics. This versatile organism is commonly found in various environments, including soil, water, and even within the human body, where it can be part of the normal flora. The opportunistic nature of *P. aeruginosa* often associates it with serious infections, including pneumonia, urinary tract infections, and bloodstream infections. The pathogenicity and resistance mechanisms of *P. aeruginosa* are essentialfor developing effective treatment plans and improving the health [1]. *P. aeruginosa* has many virulence factors, such as flagella, pili, and lipopolysaccharides (LPS), which help it stick to and colonize its host; proteases and toxins that break down tissue; secretion systems that bring effectors and toxins into the host; and quorum sensing and biofilm formation[2]. The pathogenicity of *P. aeruginosa* is contingent upon its capacity to generate several virulence factors and its resistance to phagocytosis [3]. *P. aeruginosa* was classified as multidrug-resistant if it became resistant to at least one compound from three or more different antibiotic classes. A pathogen resistant to one agent in all categories except two or fewer is referred to as extensive drug-resistance (XDR). In comparison, a pathogen resistant to one agent in all categories is referred to as pan drug-resistant (PDR) [4]. *P. aeruginosa* demonstrates resistance due to its intrinsic multi-drug class capabilities, rapid acquisition of resistance to current therapies, and ability to produce biofilms. There are three main types of *P. aeruginosa* resistance: intrinsic resistance, acquired

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resistance, and adaptive resistance. Intrinsic resistance is caused by outer membrane permeability, and overexpression of efflux systems, besides antibiotic-inactivating enzymes. Acquired resistance is caused by horizontal gene transfer and mutations in genes that code for efflux pumps, porins, penicillin-binding proteins, and enzymes. Adaptive resistance is caused by long-term antibiotic exposure and excessive environmental stress. Conventional drugs targeting *P. aeruginosa* have varying mechanisms of action. Beta-lactams stop bacteria from building cell walls, fluoroquinolones stop DNA, and aminoglycosides stop protein from being made [5]. Polypeptides have the potential to serve as alternative agents for combating multi-drug-resistant *P. aeruginosa*, as they interact with the lipopolysaccharides and phospholipids found in the outer cell membrane of gram-negative bacteria. It replaces divalent cations from the phosphate groups of membrane lipids, which breaks down the outer cell membrane, allowing contents inside the cell to leak out and killing the bacteria [6]. Biofilm is characterized as an aggregate of microbial cells that adhere to a surface and are encapsulated in an extracellular matrix mainly composed of polysaccharide substances. Biofilm is a vital virulence component and significantly contributes to antibiotic resistance. Microorganisms utilize biofilm to adapt and over severe under challenging environments, enhancing their resistance to antimicrobial agents [7]. This study aimed to investigate the antibiogram pattern and biofilms-forming ability of clinical isolates of *P. aeruginosa* obtained from patients in Nasiriya.

II. Materials and Methods

This study was conducted in four main hospitals in Nasiriyah; Al-Nasiriyah Teaching Hospital, Al-Haboubi Teaching Hospital, and Mohammed Al-Moussawi Children's Hospital, from January 2023 to June 2023. A total of 120 samples were obtained, including 56 wound swabs from patients at the Oncology Hospital with bedsores and surgical wounds resulting from immunodeficiency, 27 burn swabs from burn isolation wards, 18 sputum samples from pneumonia patients, 14 ear swabs from individuals with otitis media, and 5 bronchial-alveolar lavage samples from patients with persistent cough. Diagnostic tests were then performed on the samples. The study enrolled patients from different age groups from 12 to 70 years old, representing both sexes.

All samples were cultured on MacConkey agar and then identified and diagnosed as *P. aeruginosa* using the following tests: catalase, oxidase, indole, methyl red, and Voges-Proskauer and citrate utilization. Subsequently, the isolates were sub-cultured on a selective medium (cetrimide agar) for confirmation of diagnosis.

Antibiotic susceptibility was investigated using the Kirby Bauer method [8]. The following antibiotic discs (purchased from HiMedia, India) were used: Amikacin (AK 10 mg), Gentamicin (CN 10 mg/disc), Meropenem (MEM 10 mg), Imipenem (IMP 10 mg), Ceftazidime (CAZ 30mg), Cefepime (FEP 10mg), Ciprofloxacin (CIP 5 mg), Piperacillin (PRL100 mg), Aztreonem (ATM 30 mg), and Colistin Sulphate (CS 10 mg). Briefly, pure isolated colonies of *P. aeruginosa* were suspended in sterile saline and mixed to create a homogenous solution. The resulting suspension was adjusted to 0.5 McFarland standard, and the suspension was cultured within 15 minutes. A sterile swab was then inserted into the inoculum tube, and the dried surface of a Mueller Hinton agar plate was inoculated by streaking the swab three times across the surface. The antibiotic discs were positioned on the plates using sterile forceps, and the plates were incubated at a temperature range of 35°C ± 2°C. Zone diameters were measured visually while observing the backside of the Petri dish. The organisms were classified as being sensitive, intermediate, or resistant to each of the tested antibiotics using the Clinical and Laboratory Standards Institute (CLSI) guidelines 2023 [9].

Biofilm formation assay: The Biofilm formation was detected for all isolates of *P. aeruginosa* by using the microtiter plate assay [10]. The procedure includes: The ability of the isolated bacteria to form biofilms was evaluated by activating the isolates on a solid medium (Cetrimide agar) and incubating them for 18-24 hours at 37°C. After incubation was completed pure colonies were transferred into test tubes containing 5 mL of physiological saline to create a bacterial suspension, which was then compared to the standard tube (McFarland standard 0.5). Twenty microliters of the bacterial suspension, three replicates for each isolate, were transferred into 96-microtiter well plates, and 180 microliters of brain-heart infusion broth with 2% glucose were added. 180 microliters of glucose and BHI medium, free of bacterial suspension, were added to three wells as control points. Then, the microtiter plate was incubated at 37 °C for 24 hours. At the end of the incubation period, the wells were washed three times with phosphate-buffered saline (PBS) at pH 7.2 to remove non-adherent cells. Then 200 microliters of 1% crystal violet stain were applied after allowing the wells to dry at 25 °C for 15 minutes. The plate was maintained at a temperature of 25°C for 15 minutes to fix the dye in the cells. Upon completion of the fixation process, the dye was removed by washing the plate three times with PBS (phosphate-buffered saline) at pH 7. The plate was then left to dry at 25°C. Use 95% ethyl alcohol to sterilize the wells and leave them for 10 minutes, then measure the absorption at a wavelength of 630 nanometers using the ELISA reader. The isolates were considered weakly adherent according to the equation ($ODc<OD\leq 2\times ODc$), moderately adherent according to the equation ($2\times ODc<OD\leq 4\times ODc$) , and

strongly adherent according to the equation (OD>4×ODc) [11].

The available statistical tool, IBM SPSS-29 (IBM Statistical Packages for Social Sciences, version 29, Chicago, IL, USA), was used to analyse the data. Simple frequency and percentage measures were used to display the data. The Pearson Chi-square test (x2-test) or Fisher Exact test, as appropriate, were used to assess the significance of differences in various percentages (qualitative data). The comparison of significance p-value in any test was considered as:

A p-value of greater than 0.05 was regarded as non-statistically significant (NS); A p-value of less than 0.05 (*) was considered statistically significant (S), while A p-value of less than 0.01(**) and p-value less than 0.001 (***) was regarded as highly statistically significant (HS).

III. Results

A. *Isolation and Identification of Pseudomonas aeruginosa*

This study included 120 samples, including 56 wound swabs from cancer patients suffering from bedsores and surgical wounds due to immunodeficiency, 27 burn swabs from burn isolation units, 18 sputum samples from pneumonia patients, 14 ear swabs from individuals suffering from otitis media, and 5 bronchial-aspirate samples from chronic cough patients. The ages of the patients ranged between 12 and 70 years, and both sexes were included. After the final diagnosis, 80 samples were confirmed as *P aeruginosa*, accounting for 67% of the sample as shown in (Figure 1). Table 1 illustrates the distribution of *P. aeruginosa* according to sample type.

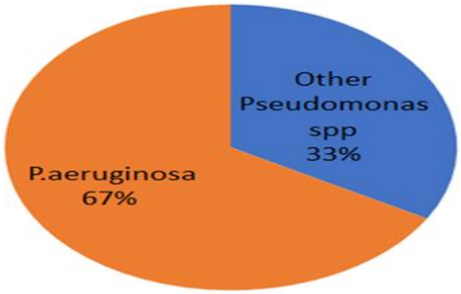

Figure 1: Distribution of isolates included in the study

Table (1) Distribution of the *P. aeruginosa* isolates according to clinical sample

Clinical isolates	Number	Percentages
Sputum	11	13.75
wound swab	32	40
Ear swab	7	8.75
burn swab	27	33.75
BAL	3	3.75
Total	80	100

B. *Demographic Characters of Patients from which P. aeruginosa were Isolated*

The percentage of infection with *P. aeruginosa* bacteria was higher in males 61 (76.25%) than in females 19 (23.75%), as shown in Table (2) and Figure (2).

Table (2) Demographic characters of patients included in the study

Characters			P-value
Age (Years)	M±SD	33.42±11.73	0.0001***
	Range	12-70	
Gender	Male	61 (76.25%)	
	Female	19 (23.75%)	

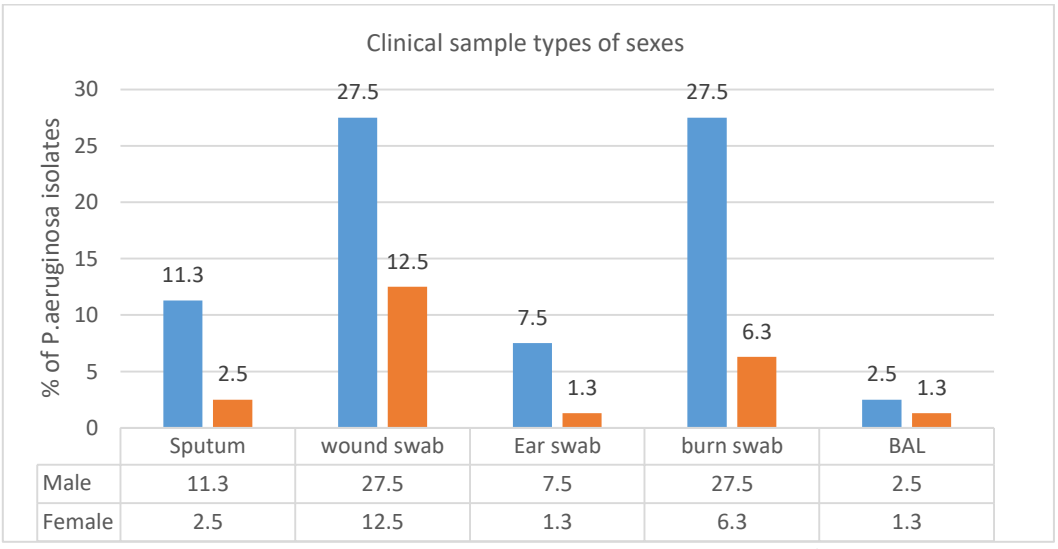

Fig (2): Distribution the P. aeruginosa isolated according to gender with the type of clinical isolates

C. *The Antibiotic's Susceptibility of Pseudomonas aeruginosa*

The antibiotic sensitivity test was conducted for 10 antibiotics, and the current study results showed that all *P. aeruginosa* isolates exhibited a clear variation in resistance to the antibiotics used in this study. The highest level of resistance was towards Cefepime (96.25%), followed by Amikacin (91.25%), Gentamicin (81.25%), Ciprofloxacin (76.25%), Ceftazidime (66.25%), Piperacillin (48.8%) , and Aztreonam (47.5%). On the other hand, the lowest resistance reported in the present study was towards Colistin Sulphate (1.25%) , followed by Imipenem (3.75%) and Meropenem (5%), as shown in Table (3).

Table (3): Distribution of Antimicrobial Resistance in *P. aeruginosa* clinical isolates

Antimicrobiale agent	Class	R (N,%)	S (N,%)	I (N,%)	P-value
AK (10 mg/disc)	Aminoglycosides	73 (91.25%)	1 (1.25%)	6 (7.5%)	0.0001 *** H.S
CN (10 mg/disc)		65 (81.25%)	5 (6.25%)	10 (12.5%)	0.0001 *** H.S
MEM (10 mg/disc)	carbapenemes	4 (5%)	70 (87.5%)	6 (7.5%)	0.0001 H.S
IMP (10 mg/disc)		3 (3.75%)	71 (88.75%)	6 (7.5%)	0.0001 *** H.S
CAZ (30mg/disc)	Cephalosporin	53 (66.25%)	18 (22.5%)	9(11.25%)	0.0001 *** H.S
FEP (10mg/disc)		77(96.25%)	2(2.5%)	1(1.25%)	0.0001 *** H.S
CIP (5 mg/disc)	Fuoroquinolones	61 (76.25)	2 (2.5)	17 (21.25)	0.0001 *** H.S
PRL (100 mg/disc)	Penicillin	39 (48.8)	12 (15)	29 (36.2)	0.0001 *** H.S
ATM (30 mg/disc(	Monobactam	38 (47.5)	12 (15)	30 (37.5)	0.0001*** H.S
CS (10 mg/disc)	Polypeptide	-	80 (100%)	-	-

As seen in Table 4, of the 80 isolates that were part of the investigation, 95%, 3.75%, and 1.25% were multidrug resistant (MDR), extended drug resistant (XDR), and pan-drug resistant, respectively.

Table (4): Types of Drug resistance in *P. aeruginosa*

Type of resistance	No.of isolates	%
MDR	76	95
XDR	3	3.75
PDR	1	1.25
Total	80	100

MDR=Multiple drug resistance;XRD=Extensive Drug Resistance;PDR= Pan drug resistance

Biofilm formation was investigated using a semi-quantitative microtiter assay as shown in figure (3). Biofilm production was considered negative, weak, moderate, and strong as mentioned previously. The results showed that non-biofilm producing isolates were 57 (71.25%). Whereas weak, moderate and strong biofilm producing isolates were 10 (12.5%), 11 (13.75%), and 2 (2.5%) respectively as illustrated in Table 5 and Figure 4.

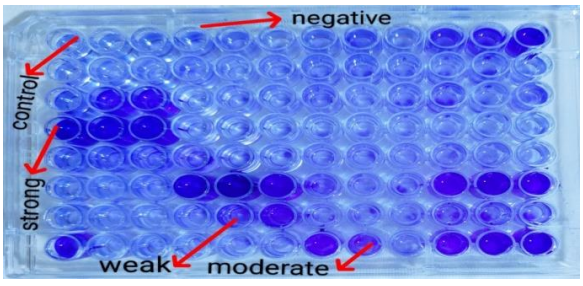

Fig (3): Detection of biofilm formation of *P. aeruginosa* by the microtitration assay

Table (5): Biofilm producing score of *P. aeruginosa* isolates

Biofilm score	Number	Percentage
Non-biofilm producer	57	71.25
Weak biofilm producer	10	12.5
Moderate biofilm producer	11	13.75
Strong biofilm producer	2	2.5
Total	80	100

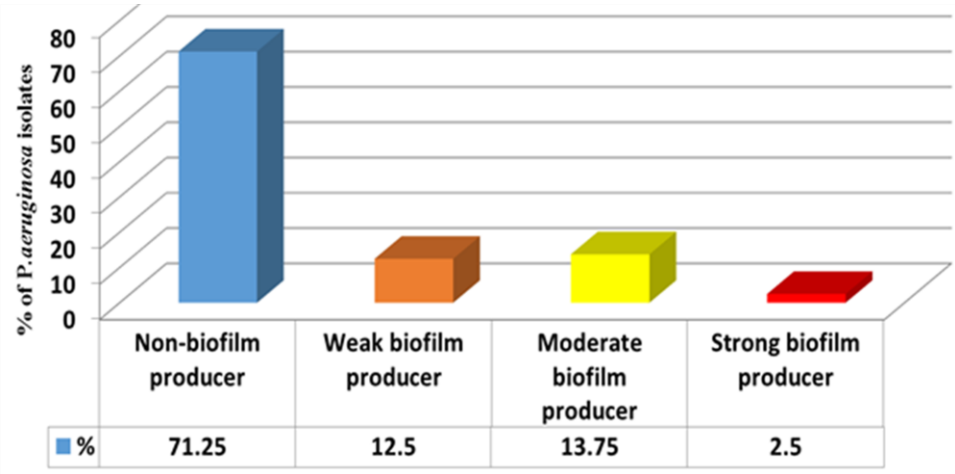

Fig (4): Biofilm producing score of *P. aeruginosa* isolates

The distribution of *P. aeruginosa* isolates according to their biofilm-forming ability and antibiotic susceptibility status to Monobactam and Polypeptide antibiotics showed no significant association ($P > 0.05$). However, statistical analysis showed a significant relation between biofilm-forming ability and the antibiotic susceptibility status to penicillin ($p \leq 0.05$) (illustrated in Table 6).

Table 6: Distribution *of P. aeruginosa* isolates according to susceptibility status and biofilm score under the actions of penicillin, Monobactam, and Polypeptideantibiotics.

Antibiotic	Biofilm score	R (N,%)	S (N,%)	I (N,%)	Total	P-value
PRL-Penicillin	Non-Biofilm	24 (30.0%)	10 (12.5%)	23 (28.8%)	57 (71.3%)	0.05 *
	Weak biofilm	4 (5.0%)	1 (1.3%)	5 (6.3%)	10 (12.5%)	
	Moderate biofilm	10 (12.5%)	0 (0.0%)	1 (1.3%)	11 (13.8%)	
	Strong biofilm	1 (1.3%)	1 (1.3%)	0 (0.0%)	2 (2.5%)	
Total		39 (48.8%)	12(15.0%)	29 (36.3%)	80(100.0%)	
ATM-Monobactam	Non-Biofilm	29 (36.3%)	7 (8.8%)	21 (26.3%)	57 (71.3%)	0.14 *
	Weak biofilm	2 (2.5%)	4 (5.0%)	4 (5.0%)	10 (12.5%)	
	Moderate biofilm	6 (7.5%)	0 (0.0%)	5 (6.3%)	11 (13.8%)	
	Strong biofilm	1 (1.3%)	1 (1.3%)	0 (0.0%)	2 (2.5%)	
Total		38 (47.5%)	12 (15.0%)	30 (37.5%)	80 (100.0%)	
CS-Polypeptide	Non-Biofilm	0 (0.0%)	57 (71.3%)	-	57 (71.3%)	0.06 *
	Weak biofilm	1 (1.3%)	9 (11.3%)	-	10 (12.5%)	
	Moderate biofilm	0 (0.0%)	11 (13.8%)	-	11 (13.8%)	
	Strong biofilm	0 (0.0%)	2(2.5%)	-	2 (2.5%)	
Total		1 (1.3%)	79 (98.8%)		80(100.0%)	

IV. Discussion

Pseudomonas genus, contributes significantly to hospital infections and isthe main cause of infections in burns and wounds, as well as in patients with weakened immune systems and those with otitis media and respiratory infections [12].The current study is consistent with many local and international studies showing that *P. aeruginosa* is the most commonly isolated bacteria from burns and wound infections, as indicated in the results of the study conducted in Baghdad hospitals with similar percentages of *P. aeruginosa* [13] and in Duhok [2]. Other studies in Egypt [14] and Saudi Arabia found similar findings[15].The majority of *P. aeruginosa* isolates were from male patients with a male-to-female ratio of 3.2:1. This may be due tooccupational reasons as males are more likely than females to work in jobs that expose them to risks of burns and wounds. Other studies in Baghdad have reached similar findings [16] and [17].

Regarding the results of antibiotic resistance, the findings of the current study agree with the results obtained in Duhok, by[18], in Nasiriyah [19], India,[20], Baghdad [21], , Bangladesh,[22] , Baghdad [23], Kirkuk, [24], Al-Najaf, [25] and in Iran [26].

On the other hand, the lowest resistance reported in the present study was towards colistin sulphate, followed by imipenem and meropenem. The current study agrees with studies in Duhok City, [2] and India, [27]. In Iran, researchers [28] found the the lowest resistance rates to the antibiotics were observed towards Imipenem and Meropenem. In another study in India, researchers [20] found that the lowest resistance rate was to the antibiotics Imipenem and Meropenem. The resistance rate was found to be (6.4%) and (8%). This aligns with the findings of the current study.

Statistical analysis of antibiotic resistance showed a highly significant difference between resistant and sensitive isolates for each one of the tested antibiotics.

In this study, 95% of isolates were multidrug-resistant (MDR), which is slightly greater than the findings of [24] who found 91.66% of MDR isolates reported in previous research. While, [29] showed 100% MDR. While 3.75% were extensively drug-resistant (XDR), and only 1.25% of *P. aeruginosa* bacterial isolates were pan-drug resistant (PDR). This study is in agreement with [30], which found XDR 9% and PDR 2%.

There are three types of resistance in *P. aeruginosa:* acquired, adaptive, and innate resistance. The permeability of the outer membrane, overexpression of efflux systems, and the presence of enzymes that deactivate antibiotics are the causes of intrinsic resistance; horizontal gene transfer and mutations in the genes encoding efflux pumps, porins, penicillin-binding proteins, and enzymes are the causes of acquired resistance*;* adaptive resistance is induced by prolonged antibiotic exposure and excessive environmental stress. Conventional antipseudomonal agents function via many mechanisms. For example, Aminoglycoside antibiotics inhibit protein translation by targeting ribosomal 30S subunits; Fluoroquinolone antibiotics impede DNA replication by interacting with DNA gyrase and topoisomerase IV; β-lactam antibiotics, including Penicillin, Carbapenems, Cephalosporins, and Monobactams, obstruct cell wall synthesis by acting on enzymes responsible for peptidoglycan formation; Polymyxin antibiotics engage with lipopolysaccharides (LPS) to enhance membrane permeability and induce hydroxyl-mediated cellular toxicity[31]. A variety of factors, such as the increased use of antimicrobial medications and improper prescription drug use linked to antimicrobial treatment, play a role in the growing problem of resistance. The regular application of various standard antimicrobial agents by physicians may result from the influence of factors like cost-effectiveness and low toxicity in drug selection. The incorrect medication of antimicrobial agents can manifest, as seen in the initial administration of a broad-spectrum medication that may be improper or ultimately ineffective against the pathogens responsible for the infection. The overuse of antibiotics in humans presents an important risk, as it may result in the development of resistant organisms. Moreover, prior use of antimicrobial medications increases the risk of a patient developing an infection due to a drug-resistant organism. Individuals with the highest levels of antimicrobial exposure often correspond to those infected with resistant bacterial strains [32].

The results of this study contradict previous studies , which concluded that biofilm formation is quite common in *P. aeruginosa* isolated from different clinical samples , with some studies reporting that most of their isolates were strong biofilm producers [33], [34], [35]. The current results coincide with that of [36], which found (75%) of the isolated *P. aeruginosa* bacteria were unable to produce biofilm and (25%) produced biofilm.

P. aeruginosa has inherent resistance to several antibiotic classes due to the continuous release of different enzymes. Additionally, chromosomal changes or the acquisition of extrachromosomal DNA enhance the bacterium's resistance to drugs. Moreover, biofilm significantly contributes to the augmentation of antibiotic resistance [37]. The connection between biofilm formation and MDR characteristics is often unclear.Our analysis shows no relationship between the development of biofilm and the multidrug-resistant features of isolates which is consistent with the findings of an Iranian study [38].

In conclusion, based on the findings of the current study, no significant association was found between antibiotic resistance in *P. aeruginosa* and biofilm formation.

V. Conclusions

At the most evident level, Cefepime had the strongest resistance, whilst Colistin Sulphate exhibited the lowest. No correlation was found between antibiotic resistance and the formation of biofilms.

Conflict of interest

Authors declare that they have no conflict of interest.

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