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Detection of Resistant and Biofilm Forming *Pseudomonas aeruginosa* Isolated from Burn Patients in Ismailia Governorate.

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Abstract

Pseudomonas aeruginosa (*P. aeruginosa*) is considered as the foremost cause of hospital -acquired infections due to its innate and plasmid mediated resistance to multiple antibiotics making it a multi-drug resistant (MDR) pathogen. In our cross–sectional study, one hundred and twenty-six (126) non-duplicate clinical *P. aeruginosa* isolates were recovered from 450 clinical specimens from burn units in Ismailia Hospitals. The antibiotic sensitivity of strong and moderate biofilm producers isolates was investigated using the disc diffusion method. The isolated bacteria were tested for their ability to form biofilm using a microtiter plate assay. The MPA detected 80% (95 /126) isolates as biofilm producers, 18% (22/126) were strong biofilm producers, 34% (43/126) were moderate biofilm producers, 28% (35/126) were weak biofilm producers and 20% (31/126) non biofilm producers. Susceptibility pattern analysis of biofilm forming *P. aeruginosa* isolates (95) detected that 60% (68/ 95) were multi-drug resistant isolates (MDR). Resistance to all used antibiotics and multidrug resistance was higher among biofilm producing than non-biofilm producing strains, but the difference was statistically non-significant. The present study confirmed that antimicrobial resistance was more prominent in biofilm-producing *P. aeruginosa* than in non-biofilm-producers.

Keywords: Multi drug resistant; Biofilm formation; *Pseudomonas aeruginosa*.

1. Introduction

Pseudomonas aeruginosa is an opportunistic, Gram-negative, non-fermenting bacterium that is a common cause of human infections. This pathogen causes a wide range of infections, including urinary tract infection, respiratory infection, dermatitis, soft tissue infection, bacteremia, and a variety of systemic infections, especially in hospitalized patients and immunocompromised individuals. Patients with severe burns are particularly susceptible to *P. aeruginosa* infection during hospitalization, often resulting in significant morbidity and mortality **(Morshedi *et al.*, 2022).**

The high mortality rate of *P. aeruginosa* infection is due to the ability of the bacterium to easily adapt to environmental conditions, to rapidly develop resistance to antimicrobials and to produce of a variety of virulence factors **(Jurado-Martín *et al.*, 2021).**

In addition to the low permeability of the *P. aeruginosa* cell wall to anti-pseudomonal agents, this bacterium has a high genetic capacity to quickly acquire drug resistance **(Pachori *et al.*, 2019).** Multidrug-resistant (MDR) *P. aeruginosa* isolates can cause life-threatening and, in some cases, untreatable infections and are considered to be a

major problem in infection control in recent years **(Abd El-Aziz *et al*., 2019).**

P. aeruginosa also has a large number of cell-associated and extracellular virulence factors. Exotoxin A, a major virulence factor of *P. aeruginosa* encoded by the *toxA* gene, inhibits protein synthesis. Exoenzyme S, encoded by the *exoS* gene, is a major virulence factor involved in burn infections. This cytotoxic effect changes the function of the cytoskeleton of the host cell, resulting in bacterial colonization, invasion and dissemination during infection **(Veetilvalappil *et al*., 2022).**

In the biofilm matrix, diverse biomolecules, including polysaccharides and proteins, protect bacteria from the host's immune response and from antimicrobials. Alginate, encoded by the *algD* gene, is a common type of polysaccharide and found in the biofilm structure. In addition, the *pslA* gene encodes a neutral-charge exopolysaccharide providing structural support during the primary stage of biofilm formation and facilitating cell-to-cell and cell-to-substrate attachment **(Asadpour, 2018).**

Because of this, infections related to biofilm-forming strains are difficult to treat and can create serious problems in burn hospitals **(Frieri *et al*., 2017).**

This study aimed to determine the biofilm formation ability and testing their antibiotic resistance against different antibiotic groups among biofilm-forming strains of *P. aeruginosa* clinical isolates from burn units in Ismailia Hospitals, Egypt.

The aim of this work is to determine the biofilm formation ability and testing their antibiotic resistance against different antibiotic groups among biofilm-forming strains of *P. aeruginosa* clinical isolates from burn units in Ismailia Hospitals, Egypt.

2.Materials and Methods

2.1 Experimental materials:

All antibiotic disks used in this study (Piperacillin, (PRL), Ceftazidime, (CAZ), Cefoxitin (FOX), Ceftriaxone (CRO), piperacillin/tazobactam (TZP), Imipenem (IPM), Meropenem (MEM) Aztreonam, (ATM), Cefepime (FEP), Cephradine (CE), Amox/Clav (AMC), Amikacin (AK), Sulpha/Trimethoprim (STX), Ciprofloxacin, (CIP), Cefuroxime, (CXO), Ampicillin/sulbactam (SAM) and Ertapenem (ETP) were purchased from (Oxoid Ltd., Basingstoke, and Hampshire, England). The 96- well flat-bottomed polystyrene plate and Mueller-Hinton broth were purchased from (Sigma-Aldrich, Poland), glacial acetic acid was purchased from (Zorka Pharma, Šabac, Serbia) and Crystal violet used for Gram staining was purchased from (Merck, Germany).

2.2 Specimens collection:

In our cross–sectional study, 126 of non-duplicate clinical *P. aeruginosa* isolates recovered from 450 clinical specimens, were collected over 14 months (November 2015 until April 2017). Samples were taken from clinically diagnosed infected burns, wounds sepsis and septicemia at inpatients and outpatients from burns unit and different departments in Suez Canal University Hospital and General Ismailia Hospital.

2.3. Specimens samples culture

All samples were cultured on Cetrimide agar media then the isolated organisms were identified by standard microbiological techniques as colony morphology , Gram staining (Gram-negative bacilli) and biochemical reactions (oxidase positivity, catalase positivity and oxidative-fermentative (OF) tests according to **(Cheesbrough, 2018).**

2.4 Detection of Biofilm Production

2.4.1. Using Congo red agar (CRA) medium (Freeman et al., 1989)

The CRA medium was prepared with 37 g/l BHI broth, 50 g/l sucrose, 10 g/l agar and 0.8 g/l Congo red. Congo red stain was prepared as a concentrated aqueous solution and autoclaved at 121˚C for 15 min separately from other medium constituents and was then added when the agar had cooled to 55 ˚C (Figure 2).

2.4.2 Using a microtiter plate test method

Biofilm formation was quantified with a using a microtiter plate test method described by **(Stepanović *et al.*, 2007).** Briefly, standard overnight cultures (1.5×108CFU/mL) were diluted 100-fold in brain–heart infusion broth. Bacterial suspension made of strong and moderate biofilm producer isolates. From each culture dilution, 200 µL [180 µL of Mueller-Hinton broth (MHB) and 20 µL of bacteria (5×105 CFU/mL)] was transferred into individual wells of a 96-well flat-bottomed polystyrene plate and was incubated at 37 ◦C for 48 h. Negative control wells contained broth only. The plates were incubated aerobically for 24 h at 35◦C. Thereafter, the content of each well was aspirated and the wells washed three times with 300 µl of sterile physiological saline. Biofilm was fixed with 200 µl of methanol per well, and after 20 min the plates were emptied and left to air dry. The plates were stained with 150 µl per well of Crystal violet used for Gram staining) for 5 min. After the plates were air dried, the dye bound to the adherent cells was resolubilized with 150 µl of 33% glacial acetic acid per well. The optical density of each well was measured at 570 nm by using an automated Multiscan EX reader (Lab systems, Helsinki, Finland). Based on the optical densities of bacterial biofilms, all strains were classified into the following categories: no biofilm producers (0), weak (+), moderate (++), or strong (+++) biofilm producers, as previously described **(Stepanović *et al*., 2007).**

2.5 Antimicrobial susceptibility testing

Susceptibility pattern analysis of strong and moderate biofilm forming of 65 *P. aeruginosa* isolates was carried out according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2014) against 17 different antimicrobial agents including: PRL (100 µg), CAZ (30 µg), FOX (30 µg), CRO (30 µg), TZP (100/10µg), IPM (10 µg), MEM (10 µg), ATM (30 µg), FEP (30 µg), CE (30µg), AMC (30 µg), AK (30µg), STX (25ug), CIP (5µg), CXO (30ug), SAM (10/10ug) and ETP (10ug). *P. aeruginosa* ATCC 27853 reference strain was used as a control. The turbidity of the suspension was matched to the turbidity of 0.5 McFarland standards. The isolates with resistance to at least 3 additional antibiotic classes were selected as MDR *P. aeruginosa*, as already explained **(Logan *et al*., 2017).**

3. Results

3.1 Demographic data

The present study was conducted with a total of one hundred and twenty-six (126) non-duplicate clinical *P. aeruginosa* Isolates recovered from 450 clinical specimens. (Table 1), (Figure 1).

3.2 Identification Tests

One hundred and twenty-six isolates showed pale yellow colonies on MacConkey agar and blue-green colonies on Nutrient agar and Cetrimide agar (Figure 2).

3.3 Biofilm formation

3.3.1 Using Congo red agar (CRA):

As shown in Figure 3.

3.3.2 Using a microtiter plate test method:

As shown in Figure 4.

Table (1): The different sources, numbers, and percentage of *P. aeruginosa* isolates.

Source	Number of clinical samples	*P.aeruginosa* isolates (n%)		Chi-square Sign
		No	Yes	
Burn unit at Suez Canal University Hospital	180	130 (62%)	50 (28%)	<0.001***
Suez Canal University Hospital Labs	200	140 (70%)	60 (30%)	<0.001***
Burn unit at Ismailia general Hospital	70	54 (67%)	16 (23%)	<0.001***
Total	450	126 (28%)	126 (28%)	<0.001***
Chi-square test	<0.001***			

*, **, *** significant at p<0.05, <0.01, <0.001, ns, non-significant at p>0.05

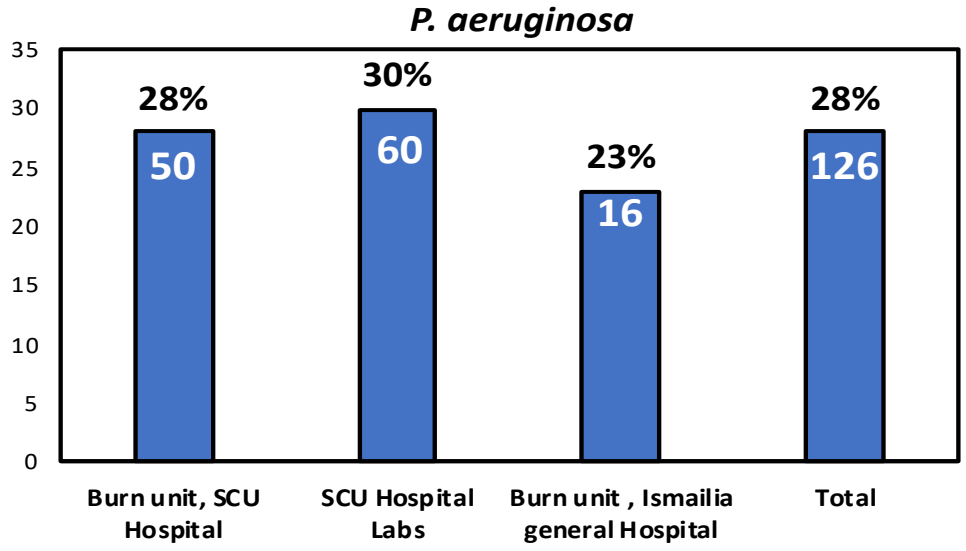

Figure 1: Bar chart presenting the prevalence of *P. aeruginosa* from different study sites.

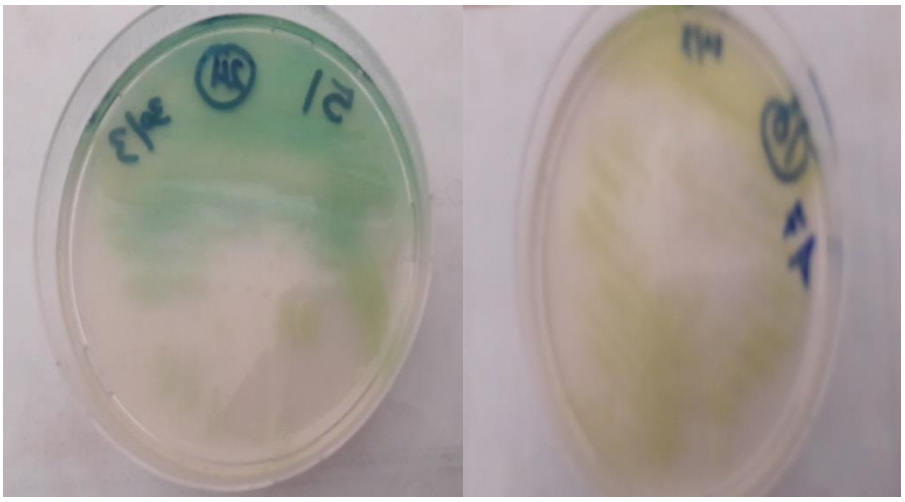

Figure (2): Culture characteristics of *P.aeruginosa* on cetrimide agar

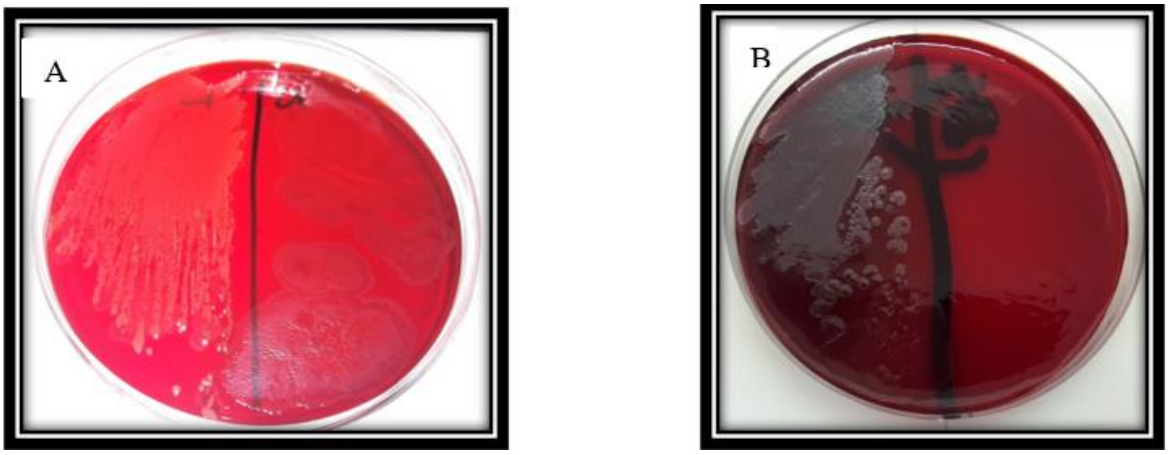

Figure 3: Biofilm production using Congo red agar.
A: Biofilm non-producing isolate with red colonies
B: Biofilm producing isolate with black colonies.

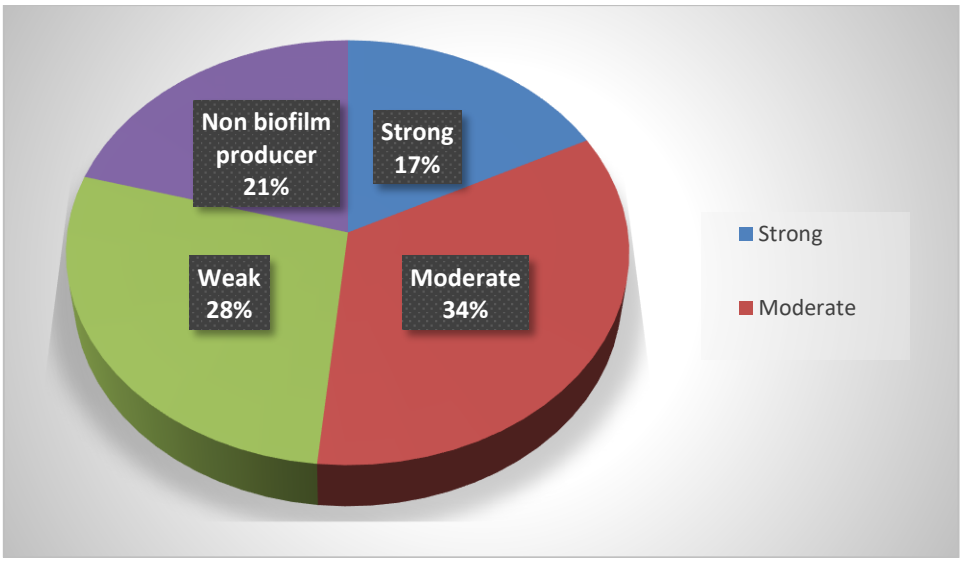

Figure 4: Biofilm Production assay using Microtiter Plate Method.

3.4 Antimicrobial susceptibility testing

In this technique, the concentration of antibiotics used is aimed at inhibiting the planktonic cell, which differs from cells in the biofilm state. The bacterial biofilm is 10-1,000 times more resistant to antimicrobial agents than the planktonic cell. Therefore, the conventional antibiotic susceptibility test cannot predict the bacteria involved in biofilm production. This can be one explanation as to why there is a higher failure rate in the eradication of biofilm-related infections. Antibiotic Susceptibility testing for the biofilm forming *P. aeruginosa* isolates (100) under the standard CLSI guidelines for different antimicrobial agents showed that 68% (68/ 100) were multi-drug resistant isolates (MDR) Pattern. The results of the susceptibility testing were categorized as sensitive, intermediate and resistant as shown in Table 2 and (Figures 5,6).

Table 2: Percentage of resistance of biofilm forming *P. aeruginosa* to 68 tested antibiotics samples.

Antimicrobial Agent(s) (µg)	Conc.	Resistant NO	%	Intermediate NO	%	Sensitive NO	%	Chi-square sign.
Cephradine	30	68	100.0	0	0.0	0	0.0	>0.999ns
Ampicillin/Sulbactam	10/10	68	100.0	0	0.0	0	0.0	>0.999ns
Cefuroxime	30	68	100.0	0	0.0	0	0.0	>0.999ns
Sulpha/Trimethoprim	19:1	64	94.1	1	1.5	1	1.5	<.001
Amoxycillin/clavulanic acid	30	64	94.1	2	2.9	0	0.0	<.001
Cefoxitin	30	64	94.1	2	2.9	0	0.0	<.001
Ertapenem	10	47	69.1	12	17.6	7	10.3	<.001
Ceftriaxone	30	34	50.0	29	42.6	0	0.0	0.225
Ceftazidime	30	25	36.8	10	14.7	31	45.6	0.004
Piperacillin	100	19	27.9	0	0.0	47	69.1	<.001
Cefepime	30	17	25.0	3	4.4	46	67.6	<.001
Piperacillin/Tazobactam	100/10	14	20.6	6	8.8	46	67.6	<.001
Ciprofloxacin	5	14	20.6	1	1.5	51	75.0	<.001
Aztreonam	30	13	19.1	14	20.6	39	57.4	<.001
Amikacin	30	10	14.7	2	2.9	54	79.4	<.001
Meropenem	10	0	0.0	0	0.0	68	100.0	>0.999ns
Imipenem	10	0	0.0	0	0.0	68	100.0	>0.999ns

*, **, *** significant at p<0.05, <0.01, <0.001, ns, non-significant at p>0.05.

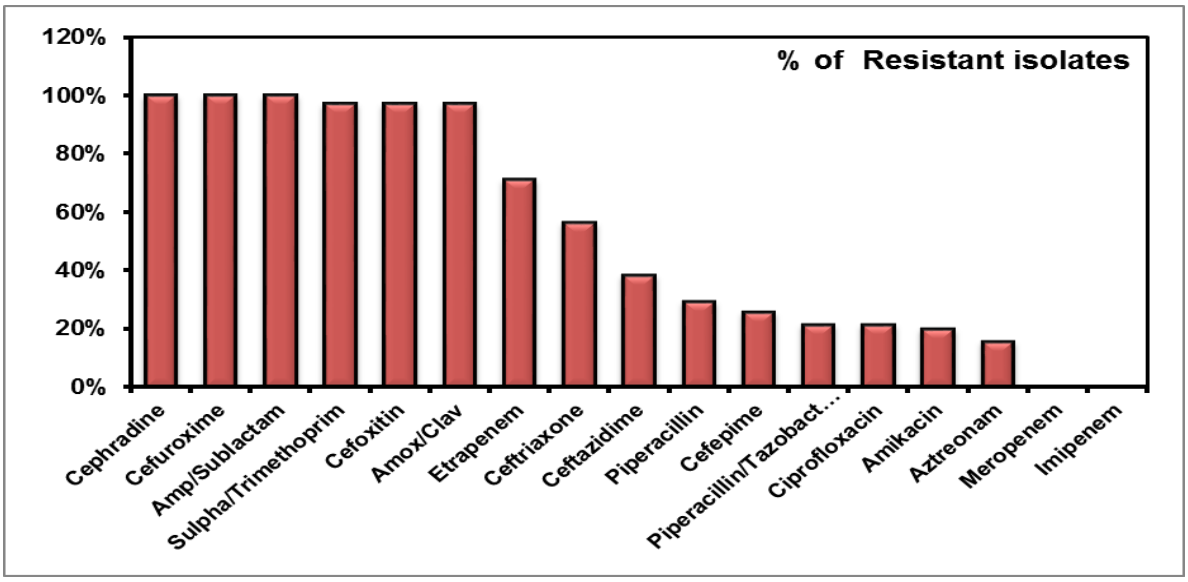

Figure 5. Histogram showing resistance of *P. aeruginosa* to tested antimicrobials.

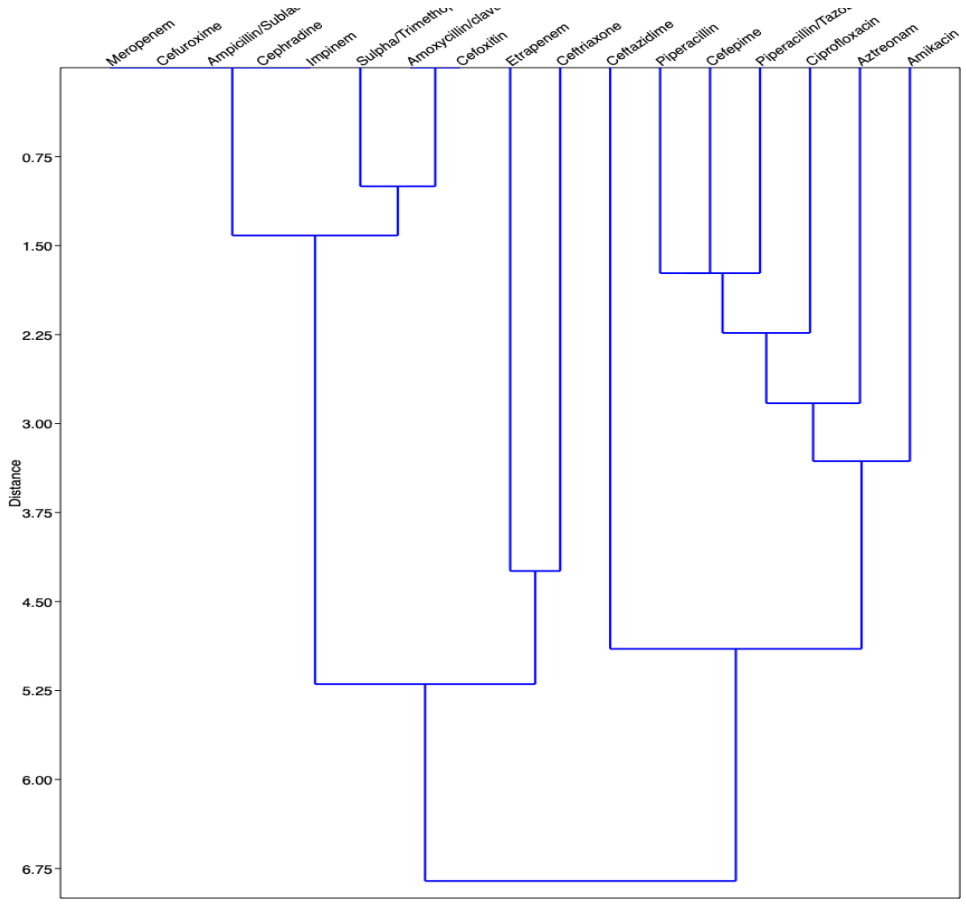

Figure 6: Clustering showing the resistance of *P. aeruginosa* to tested antimicrobials, Cluster constructed using PAST version 4.04.

4. Discussion

Antimicrobial resistance is one of the major problems in the treatment of infectious diseases worldwide. *P. aeruginosa* is inherently resistant to multiple antimicrobials owing to the low permeability of the outer membrane, constant expression of several efflux pumps and the production of various antimicrobial- inactivating enzymes. It also has a high biofilm production capacity that makes antimicrobial penetration and access to the bacteria difficult.

A number of 126 isolates of *P. aeruginosa* were tested for their ability to form biofilm using a microtiter plate test method. Our study results found that the phenotypic detection of biofilm formation revealed that 80% (100/126) of clinical isolates were positive biofilm producers; 18% (22/126) were strong biofilm producers, 34% (43/126) were moderate biofilm producers, 28% (35/126) were weak biofilm producers and 20% (31/126) non biofilm producers the results are shown in Table 4.

Several previous studies reported different rates of biofilm production by *P. aeruginosa* isolates. A previous study in Egypt on biofilm production reported that 27% (27/100) of clinical isolates were positive biofilm producers; 14% (14/100) produced strong biofilm, 7% (7/100) produced moderate biofilm and 6% (6/100) produced weak biofilm **(Abdelraheem *et al*., 2020).**

Another study in Egypt also reported that biofilm formation was detected in 32/35 (91.4%) *P. aeruginosa* isolates; 25.7%, 40%, 25.7% and 8.6% of isolates were strong, moderate, weak and non-biofilm producers, respectively **(Elnegery *et al*., 2021).**

Maita and Boonbumrung (2014) reported that 60% (82/136) of *P. aeruginosa* isolates obtained from different clinical samples were strong biofilm producers, 11% (14/136) were moderate biofilm producers and 7% (9/136) were weak biofilm producers.

Our results are in accordance with **Harika *et al.* (2020)** reported that 78.2% (72/92) of clinical isolates were positive biofilm producers; 69.5% (64 /92) produced strong biofilm, 8.7% (8/92) produced moderate biofilm and 21.7% (20/92) produced weak biofilm.

The variability in results between different studies may be attributed to many factors such as the difference in type and number of samples collected in each study and differences in isolates capacity to form a biofilm. Better understanding of the route of biofilm development and its control may constitute

a platform for the design of strategies that used to combat and eradicate the infection.

Multidrug resistance (MDR) (resistant to three or more antimicrobial classes) was higher among biofilm producing than non-biofilm producing strains but the difference between the two groups was not statistically significant. Furthermore, the results showed that all isolates were susceptible to Meropenem and Imipenem, sensitivity was absolute (100%) and the highest resistance rate was observed against (Cefuroxime), (Cefoxitin, Amox/Clav, Sulpha/Trimethoprim) and Ceftriaxone showed resistance rates of 100%, 97% and 71% and 56% respectively. Whereas the lowest resistance rate was to amikacin 15.2 % and moderate resistance rate was observed against Ceftazidime, Piperacillin, Cefepime, levofloxacin showing resistance rates of 38 %, 29%, 25.5 % and 21 %, respectively.

Similar previously results of antibiotic Susceptibility testing were obtained by **Ijaz *et al*. (2019)** who reported that 58.6% were multi-drug resistant (MDR) for the biofilm forming *P.aeruginosa* isolates.

In addition, **Maita and Boonbumrung (2014)** reported results that 51 % MDR were multi- drug resistant (MDR) strains of *P.aeruginosa*. Furthermore, our results are nearly similar to the previous studies who's reported that resistance pattern against carbapenem group i.e., meropenem and imipenem was only 6.67% which correlates with other studies in India, Nepal, Spain and Italy **(Raza *et al*., 2013; Al Sanjee *et al*., 2018).**

All of those studies suggested meropenem and imipenem as the most effective anti-pseudomonal drugs. **Al-Jasser and Elkhizzi (2004)** showed sensitivity to meropenem (91.6%), imipenem (90.2%) and piperacillin/tazobactam (81.3%). **Raja and Singh (2007)** showed sensitivity to imipenem (90.1%) and piperacillin/tazobactam (90.6%). However, several reports indicated increasing resistance towards this antibiotic group day by day **(Yusuf *et al*., 2017; Woerther *et al.*, 2020).** In agreement with our study, **El Kholy *et al.* (2003)** further reported the highest resistance rate against ampicillin and chloramphenicol (100%) and the lowest against ceftazidime (38%).

In addition to, a previous study in Bangladesh reported 89.5% resistance against Ampicillin and 89.3% resistance against Amoxiclav **(Yasmin *et al*., 2015).** Our results are nearly similar to **Abdelraheem *et al.* (2020)** that reported lower incidence of amikacin resistance of 13.2% (18/136). Another study in Egypt reported nearly similar results of lower resistance to amikacin (12%) **(Hassan *et al*., 2015).** In addition to, **Kannan *et al.* (2017)** from Pakistan showed that 30% of *P. aeruginosa* strains were MDR with the highest resistance rate against cefuroxime and cefixime (each with 100%) and the lowest resistance rate against amikacin (10%). In contrast to our study, an Indian study which reported that imipenem and meropenem presented with resistant rates of 13.5%, and 21.6% respectively.

Also, our results were dissimilar to the results of the **Hakemi *et al.* (2013),** which shows that resistance of P. aeruginosa isolates to tested antibiotics in antibiogram test were 100% to cefpodoxime, 82.98% to ceftriaxone, 78.73% to imipenem, 75% to meropenem, 72.72% to gentamicin, 69.23% to ciprofloxacin and aztreonam, 67.57% to cefepime, 65.95% to ceftazidime, and 61.53% to piperacillin.

Furthermore, a study in Egypt reported dissimilar results, where 12/35 (34.3%) strains were resistant to ceftazidime, 9/35(25.7%) strains were resistant to levofloxacin and 7/35(20%) strains were resistant to imipenem but lower resistance 28.6% of *P. aeruginosa* isolates were resistant to amikacin **(Elnegery *et al*., 2021).** The European Antimicrobial Resistance Surveillance Net- work (EARS-Net) in 2015 reported an increasing trend for resistance against piperacillin/tazobactam during 2011–2015, with the highest resistance related to piperacillin/tazobactam (36.1%) and levofloxacin (36.6%), and the lowest (1%) was against colistin in European hospitals **(Weist and Högberg, 2016).** Similarly, resistance to piperacillin/tazobactam, levofloxacin, and colistin was reported as 27.1%, 29.5%, and 1.1%, respectively in the U.S. hospitals **(Karballaei Mirzahosseini *et al.*, 2020).** The variation in the level of resistance between different studies may be attributed to the difference in geographical distribution, type and number of samples collected in each study and the difference in antibiotic policies implemented in each country.

5. Conclusion

The increasing rate of resistance to β-lactam antimicrobials is considerable, limiting choices for suitable treatment of patients with severe burn infections.

6. Acknowledgments

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7. Conflict of interest

The authors declare that there is no conflict of interest.

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