مجلة دجلة • المجلد ٦ ،العدد ٤ (٢٠٢٣)، ص: ١٨ ـ ٢٥
ISSN: 2222-6583

Association of Multidrug Resistance with Biofilm Formation in Pseudomonas aeruginosa

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Keywords: *Pseudomonas aeruginosa*, Biofilm, Antimicrobial resistance, MDR.

Abstract

Pseudomonas aeruginosa is an opportunistic pathogen known for its multi-drug resistance (MDR) characteristics. When existing as biofilms, *P. aeruginosa* can endure in oxygen-deprived settings and other challenging conditions. Moreover, managing *P. aeruginosa* infections is notably challenging due to its rapid mutation rate and ability to adapt, ultimately leading to antibiotic resistance. A total of 25 *P. aeruginosa* isolates were obtained from CAC center/ Baghdad. The VITEK-2 system was used for confirmative diagnosis, and it revealed that all 25 isolates (100%) were identified as *P. aeruginosa* .The study focused on the isolates' ability to form biofilms, and they were categorized into two groups based on their biofilm-forming capabilities: strong biofilm-producers (18 isolates, 72%) and moderate biofilm-producers (7 isolates, 28%). All the 25 *P. aeruginosa* isolates were examined against (17) antibiotics by used antibiotic sensitivity test and the results showed that *P. aeruginosa* isolates were highly sensitive to Imipenem (IPM) (100%) and Piperacillin-tazobactam (TPZ) (100%), while it's showed a complete resistance (100%) to Erythromycin, Gentamicin and Ampicillin . Also, revealed a high level of resistant to Amoxicillin-clavulanate (90%), Ciprofloxacin (95%), Tetracycline (98%) and Levofloxacin (85%). Moreover, the results showed that all 25 (100%) of *P. aeruginosa* isolates have Multidrug resistance to many antibiotics.

Introduction

Pseudomonas aeruginosa is one of the most important clinical and epidemiological microorganisms. It is the leading cause of hospital infections among non-fermenting gram-negative bacilli and the leading cause of opportunist infections in disorder patients (Rocha *et al*., 2019). *P. aeruginosa* is widely recognized for its ability to form robust biofilms, which makes it an ideal model for researching biofilm development. Within the

context of diseases like cystic fibrosis, where the respiratory system hosts a complex mixture of microorganisms, *P. aeruginosa's* capacity to establish resilient biofilms is a key survival strategy. This bacterium also exhibits the ability to colonize various surfaces, including medical devices like urinary catheters and implants, as well as equipment in the food industry such as mixing tanks and tubing. Therefore, gaining a deeper understanding of the biofilm's composition, structure, and the molecular mechanisms responsible for the bacteria's enhanced resistance to antimicrobial agents when growing within a biofilm is of utmost importance. This knowledge is crucial for the development of effective strategies to manage, prevent, and, notably, eliminate infections associated with biofilms (Thi *et al*., 2020). This matrix consists of various polysaccharides, including PsI and Pel polysaccharides found in all strains, as well as alginates present in mucoid *P. aeruginosa* isolates. Additionally, proteins, lipids, extracellular outer membrane vesicles (OMVs), and extracellular DNA (eDNA) are part of this matrix (Esoda and Kuehna, 2019). The regulation of biofilm production in *P. aeruginosa* hinges on a single molecule known as cyclic di-GMP, which is encoded by a 15-gene operon responsible for controlling both cell-cell and cell-surface interactions (Whitney, 2015). When cyclic di-GMP concentration is low, *P. aeruginosa* exists in a free-swimming planktonic form. However, when the intracellular cyclic di-GMP concentration rises, it triggers the production of adhesive pili that act as "anchors," thereby stabilizing the attachment of *P. aeruginosa* to surfaces such as plastic, rock, or host tissues (Almblad, 2019). Simultaneously, cyclic di-GMP suppresses the synthesis of the flagella machinery, preventing *P. aeruginosa* from engaging in swimming motility (Huszczynski, 2020). Biofilms are formed through four primary stages: the initial surface attachment, microcolony development, maturation, and dispersion. These stages collectively play a crucial role in promoting strong antibiotic resistance and facilitating extended host colonization, contributing to the pathogen's adaptability (Rashad *et al*., 2022). As a result, we've observed the emergence of consequences such as increased biofilm formation and the growing prevalence of both intrinsic and acquired antibiotic resistance in *P. aeruginosa* in recent times. This situation has led to a scarcity of viable antibiotics for combating this bacterium. Furthermore, it's worth noting that the minimum inhibitory concentrations (MICs) of bacteria within biofilms can be 10 to 10,000 times higher compared to those of free-floating planktonic cells (Tuon *et al*., 2022). One of the most notable characteristics of *P. aeruginosa,* which is inherently resistant to a variety of antibiotics, is its ability to acquired resistance to almost all existing antibiotics. This resistance can occur through mutations in its chromosomal genes and the acquisition of resistance markers via horizontal gene transfer. This phenomenon has contributed to the global dissemination of a select few highly concerning multidrug-resistant (MDR) and extensively drug-resistant (XDR) high-risk clones (Fernández-Billón *et al.,* 2023). The term Multi Drug Resistant *P. aeruginosa* (MDRPA) is used to describe isolates that exhibit resistance to a minimum of three distinct antibiotic classes, which can include aminoglycosides, carbapenems, quinolones, and cephalosporins (Hadadi-Fishani *et al*., 2020). In 2023, Prinzi and Rohde demonstrated that a biofilm resistance involves a combination of various components working together to reduce or completely hinder antibiotic effectiveness, leading to what is known as recalcitrance. Three key mechanisms stand out as crucial contributors to antibiotic resistance

in biofilm bacteria: 1. Resistance at the Biofilm Surface: The initial mechanism involves the resistance occurring at the surface levels of the biofilm when antibiotics attempt to penetrate the sticky and slimy matrix. The complexity of the biofilm structure, consisting of exopolysaccharides, DNA, and proteins, creates significant barriers for antibiotics. 2. Resistance within Biofilm Micro-environments: Once an antibiotic manages to penetrate the first surface layer of the biofilm, it encounters a challenging microenvironment in the deeper regions of the biofilm. Furthermore, oxygen levels may be severely reduced, resulting in an anaerobic environment. The combination of these factors has varying effects on antibiotics, depending on the specific structure and mechanism of action of each antibiotic. 3. Resistance of Bacterial "Persister" Cells: Deeper within the biofilm layers where bacteria reside, additional mechanisms to evade antibiotic therapy become evident. In their quest for survival, certain small subsets of bacteria manage to evade the effects of antibiotics and enter a "spore-like" state, rendering them highly resistant to extreme conditions, including chemical treatment and antibiotic action. These cells are called persister cells. In their dormant state, persister cells do not undergo division in the presence of antibiotics.

2. Methods

A total of (25) *P. aeruginosa* isolates were obtained from (Chemistry Analysis Center (CAC)/ Baghdad) and reidentified by VITEK-2 system. The system's fundamental concept relies on the use of a growth-based technology, employing colorimetric reagent cards that are automatically incubated and interpreted. These reagent cards vary in their capacity and level of automation. Additionally, the system offers the convenience of automatic pipetting and dilution for conducting antimicrobial susceptibility testing.

The Microplate Assay (MtP) is a quantitative method employed to assess biofilm production utilizing a microplate reader (Kırmusaoğlu, 2019). The procedure involves culturing bacteria obtained from fresh agar in Muller-Hinton broth supplemented with 1% glucose, adjusting the bacterial suspension to achieve a McFarland standard of 0.5 (equivalent to 1.108 CFU/ml) using a Dens Check machine, and subsequently diluting bacterial suspensions to 5.106 CFU/ml through a tenfold dilution (1/10) using a mixture of 180 µl of broth and 20 µl of bacterial suspensions. These diluted suspensions (200 µl each) were then deposited in triplicate within a 96-well flat-bottomed polystyrene microplate, with one well serving as a negative control containing only broth. After incubation for 24 hours at 37°C, biofilms were fixed by drying for 1 hour. Subsequently, the biofilm-forming isolates were stained with 200 µl of crystal violet for 30 minutes, followed by removal of excess dye through two washes with normal saline at pH 7.2. The microplate was then dried for an additional hour, and 150 µl of 95% ethanol was added to eliminate the dye from biofilms adhering to the microplate walls. Finally, the microplate was read using a microplate reader (ELISA) at 630 nm, and the optical density (OD) was measured to assess biofilm formation. The OD values were compared to differentiate between the samples and the negative control, categorizing the ability of bacteria in biofilm formation into four groups:

- ODc (control) < ODs (sample): Weak biofilm formation.
- 2*ODc (control) < ODs (sample) < 4*ODc (control): Moderate biofilm formation.
- 4*ODc (control) < ODs (sample): Strong biofilm formation.

The antibiotic sensitivity test for *P. aeruginosa* isolates was conducted using the Kirby-Bauer disk diffusion method. The *P. aeruginosa* isolates, which had previously been identified using the VITEK 2 System, were cultured in 5 ml of nutrient broth. These cultures were then incubated at a temperature of 37ؚC for duration of 24 hours. The sample was diluted to a concentration (1.5×10^8). CFU/ml in compared with Macfarland turbidity standard tube No. (0.5). A sterilized cotton swap was dipped in bacterial suspension and removed the excess liquid by press on the side of the tube. A sterilized forceps was used for pressed each antibiotic disc gently on Muller Hinton agar surface. Only three discs were placed in each plate, and then plates were incubated at (37oC) for (24 hr). Following incubation, the diameter of the zone of inhibition, defined as the area with no apparent development surrounding the disc, was measured with a ruler (mm) and compared to a standard value of each drug based on CLSI, (2022) published data.

3. Results

3.1. Identification of *P. aeruginosa* by VITEK-2 system

The identification confirmation test of *P. aeruginosa* isolates was done by VITEK-2 system. According to the results obtained from the VITEK-2 system, out of the total isolates tested, 100% (25 isolates) were identified as *P. aeruginosa*.

3.2. Phenotype detection of the biofilm formation for *P. aeruginosa* isolates.

The ability of bacteria to form a biofilm was measured using a 96- well flat bottom plate Polystyrene microtiter plates, this method was assumed as a quantitative biofilm assay based on exopolysaccharide production using enriched media supplemented with 1% glucose and an ELISA reader were optical density (OD) value measurement. Is easier to diagnose with it than by Congo Red Agar (CRA) (Jabber and Aal Owaif, 2020). Results revealed that 100% (25/25) was biofilm producers in figure (1).

Additionally, these 25 isolates were (18) of (25) isolates have the ability to form strong biofilm (72%) and (7) isolates moderate biofilm producers (28%) and no weak biofilm producers under same experimental conditions as in table (3-1).

Table (3-1): Biofilm formation by *P. aeruginosa* isolates.

Biofilm	No. of isolates	The percentage (%)
Strong biofilm	18	74
Moderate biofilm	7	26
Weak biofilm	0	0
Total	25	100

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ISSN: 2222-6583

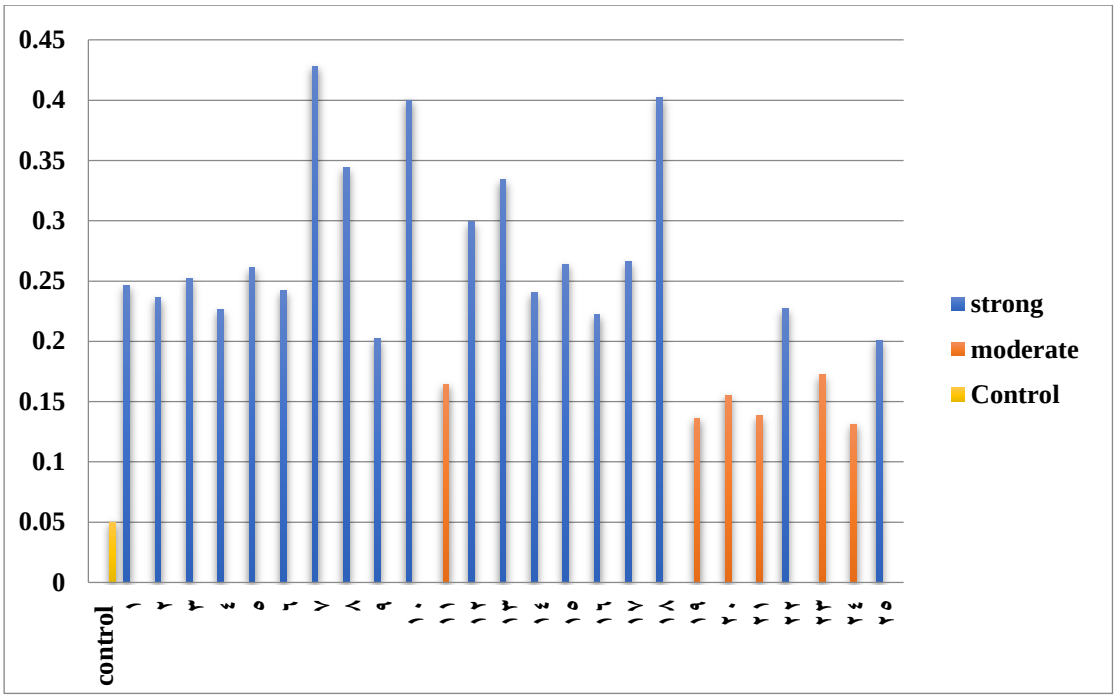

Figure 1: Biofilm formation O.D values of *P. aeruginosa*

3.3. Antibiotic sensitivity test

Antibiotics sensitivity test was performed for 25 *P. aeruginosa* isolates by using a disc diffusion method towards 9 antimicrobial agents. The results showed that *P. aeruginosa* isolates have different levels of sensitivity and resistance patterns toward these antibiotics. The diameter of the inhibition zone for *P. aeruginosa* isolates was compared with CLSI standard value, 2021 as shown in table (3-2).

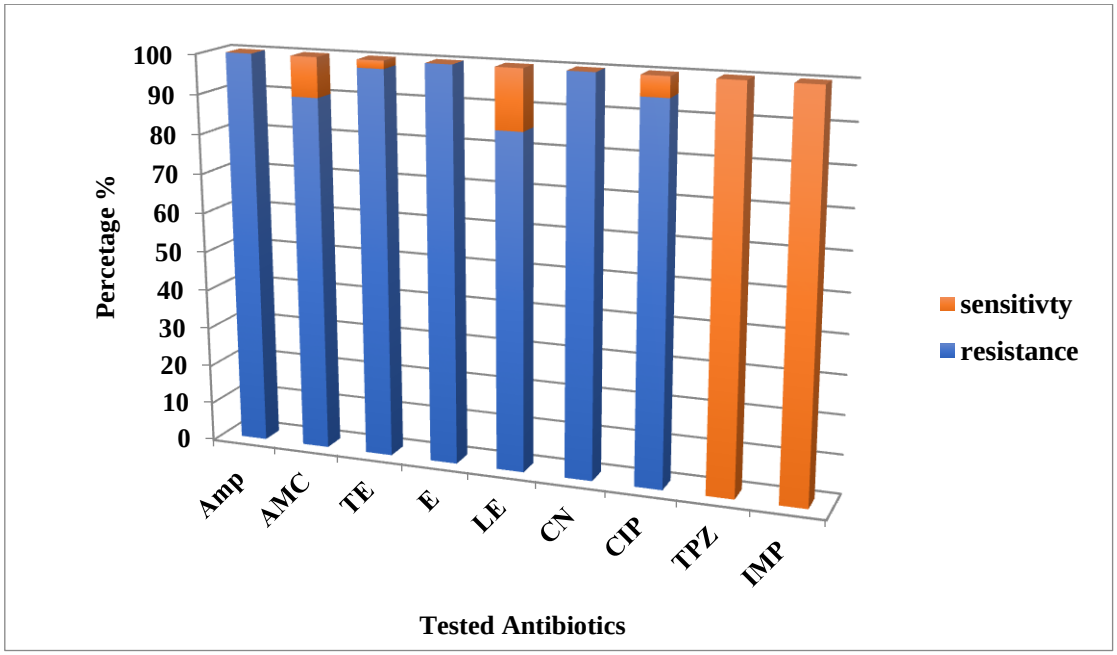

Figure 2: percentage of antibiotic resistance and sensitivity patterns of *P. aeruginosa*

Table (3-2): Susceptibility test of *P. aeruginosa* isolates to 9 antibiotics

Antibiotic	Susceptibility		
	S	I	R
	Isolate No. (%)	Isolate No. (%)	Isolate No. (%)
Amoxicillin- clavulanate	0 (0)	5 (10)	20 (90)
Ampicillin	0 (0)	0 (0)	25 (100)
Ciprofloxacin	0 (0)	4 (5)	21 (95)
Erythromycin	0 (0)	0 (0)	25 (100)
Gentamicin	0 (0)	0 (0)	25 (100)
Imipenem	25 (100)	0 (0)	0 (0)
Levofloxacin	0 (0)	7 (15)	18 (85)
Tetracycline	0 (0)	3 (2)	22 (98)
Piperacillin-tazobactam	25 (100)	0 (0)	0 (0)

4. Discussion

Pseudomonas species hold significant importance among opportunistic gram-negative bacteria responsible for nosocomial infections in individuals who are long-term hospitalized or immune-compromised. The prevalence of bacteria exhibiting resistance to multiple antibiotics is on the rise, primarily due to the indiscriminate use of antibiotics and the frequent utilization of broad-spectrum antibiotics in medical treatment. This form of resistance has become alarmingly common, particularly among gram- negative bacteria. Consequently, managing infections caused by biofilm-forming strains of *P. aeruginosa* has become increasingly challenging due to the heightened antibiotic resistance (Sezener *et al.,* 2022). In the research conducted by Manohar *et al.* (2018), it was discovered that out of the 24 *P. aeruginosa* strains isolated from different clinical cases, 8 were confirmed to exhibit biofilm production. Also Wannigama *et al.*, in (2020) among (127) biofilm producer isolates, 46% (56 isolates) were found to form a stronger biofilm. Biofilm helps bacteria can adapt to environmental hypoxia and nutrient deprivation by modulating the metabolism, protein production and gene expression, which can lead to a lower metabolic rate and reduced rate of cell division (Vestby *et al.,* 2020). Dolatshah and Tabatabaei, (2021) elucidated that out of the 69% of isolates that formed biofilm, only (9%) created a strong biofilm, while the remaining (13%) and (47%) produced only moderate or low levels.

Additionally, the results of the antibiogram test revealed that (63%) of the strains demonstrated resistance to gentamicin and while (71%) exhibited resistance to ciprofloxacin. The findings from the antimicrobial susceptibility pattern analysis revealed that *P. aeruginosa* isolates exhibited the highest resistance to the following antibiotics: Gentamicin (56.5%), Amikacin (52.9%), Amoxicillin clavulanic (47.6%), Ertapenem, and Imipenem (47.1%). Regarding the phenotypic detection of biofilm formation in the study, it was observed that 161 out of the isolates (84.3%) were identified as biofilm producers. Among these, 24 isolates (12.6%) were categorized as strong producers, 79 isolates (41.4%) as moderate producers, 58 isolates (30.4%) as weak producers, and 30 isolates (15.7%) as non-producers. Furthermore,

the study detected a high prevalence of a multi-drug resistant (MDR) pattern among *P. aeruginosa* isolates. Additionally, a significant association between MDR and biofilm formation was observed (El-sayed and Fahmy 2021). The findings of this study closely align with those of Chika (2017), who observed a high rate of multi-drug resistance (MDR) in clinical and environmental isolates of *P. aeruginosa*. Similarly, Pérez (2019) conducted a study involving 255 *P. aeruginosa* isolates from burn injuries, wounds, and otitis media cases across twelve hospitals in Spain, Italy, and Greece. The study reported MDR rates of 90.88% in Greece, 78.22% in Spain, and 83% in Italy. Furthermore, Gupta (2022) in India, while isolating *P. aeruginosa* from patients with wounds and burns, recorded that over 85% of these isolates exhibited MDR resistance patterns.

5. Conclusions

The development of a protective biofilm by *Pseudomonas* species is a critical factor in their ability to thrive, providing protection against antibiotics and immune cells within the body, as well as in challenging environmental settings. Therefore, the eradication of biofilms holds significance from both therapeutic and infection control standpoints. It was determined that the resistance profiles of *P. aeruginosa* were high. Biofilm, formations have been determined to be produced by all strains and it is known that produced biofilm plays an important role in escape of host defense mechanisms and resistance to antibiotics.

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