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Antibiotic Resistance Patterns and Biofilm Formation in Clinical Isolates of Pseudomonas aeruginosa from Three Hospitals in Diyala Province, Iraq

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ABSTRACT

Background and Objective: Pseudomonas aeruginosa is a highly resistant and dangerous bacterium that causes opportunistic infections, particularly in hospitals, where it causes pneumonia, sepsis, etc. The aim of this study is to investigate biofilm formation and its relationship with antibiotic resistance.

Methods: This cross-sectional study was conducted on 345 samples that were carefully collected from outpatients who had not taken antibiotics for at least one week. Special swabs containing transport medium were used to preserve the samples until they reached the laboratory. Samples were cultured directly on blood agar, pseudo-agar, and Mueller-Hinton agar. They were then incubated for 24 hours at 37°C. The initial diagnosis was determined based on phenotypic and biochemical characteristics. Final diagnoses and sensitivity tests were confirmed using the VETEC-2 system. The bacteria's ability to form biofilms was also determined.

Findings: The results showed that 93.2% of P. aeruginosa isolates were biofilm-forming, with a strong correlation to widespread resistance ($p<0.001$). High resistance was observed within the penicillin group, while resistance rates were low with imipenem, meropenem, amikacin, ciprofloxacin, moxifloxacin, and levofloxacin.

Conclusion: The results of the study demonstrated a strong correlation between biofilm formation and increased antibiotic resistance. In addition, resistance levels varied among the different drug groups.

Keywords: *Antibiotic Resistance, Resistance Mechanisms in Pseudomonas Aeruginosa, Biofilm Formation, Cephalosporin Group, Aminoglycosides Group*.

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Introduction

P. aeruginosa is an opportunistic bacterium commonly associated with hospital-acquired infections and one of the main reasons for the increased pathogenicity of P. aeruginosa believed to be its virulence factors, which are classified into cell-associated factors, including adhesions and lipopolysaccharides (1, 2). Moreover, there are secreted substances, such as exotoxin (A), proteases, exoenzymes, and phospholipases, as well as exotoxin C, pyocyanin, alginate, and DNase (3). In addition, it is characterized by its ability to adapt, so it poses a major challenge in clinical settings due to the multiplicity of its mechanisms of resistance to antibiotics, and its ability to form biofilms, which leads to persistent infection (4, 5). The occurrence of mutations also contributes to the development of resistance as well as the acquisition of additional resistance genes from other organisms through plasmids, transposable elements, and bacteria (6). Inappropriate use of antibiotics is the main reason for the spread of antibiotic resistance in humans (7, 8). This is due to their easy availability without a prescription, which has led to their overuse (9). Therefore, this descriptive study was designed to investigate biofilm formation and its relationship to antibiotic resistance.

Methods

Study Design and Ethical Approval: Ethical approval (Code No. 2025HRH972) was obtained on 12 October 2024 for this descriptive study, which aimed to determine the prevalence of antibiotic resistance in P. aeruginosa bacteria in major hospitals in the province and to assess its impact on patients and their chances of recovery.

Clinical Sample Collection: 345 samples were carefully collected using special swabs with transport medium, adhering to specific criteria including a minimum one-week antibiotic discontinuation period. These samples were then transported to the microbiology laboratory, where they were directly cultured on blood agar, Pseudomonas agar, and Mueller-Hinton agar, then incubated for 24 hours at 37°C. Pseudomonas agar served as a selective medium for P. aeruginosa bacterial isolates, and blue-green pigment indicated the presence of pyocyanin as a positive result. Bacteria were identified based on their color, size, morphology, hemolytic ability, and biochemical characteristics. Biochemical tests were performed using a VITEK 2 analyzer for each isolate, following the manufacturer's instructions (bioMérieux - France) and a GN cassette. Purified colonies were transferred from the plate to a tube containing 1 mL of 4.50–5.00% saline solution. The solution was thoroughly mixed to form a suspension, and turbidity was measured using a DensiChek. The inoculation card was then placed in the designated slot, and the card code was entered using a scanner (10).

Biofilm Evaluation by Microtiter Plate Assay (Quantitative Assay): The isolated bacteria were inoculated in 5 mL of tryptone soy broth (TSB) supplemented with 4% sucrose and incubated for 24 hours at 37°C. A 96-well flat microtiter plate was used to inoculate 200 µL of the bacterial suspension, equivalent to 0.5 McFarland standard (1.5×10^8 CFU/mL), in three wells per isolate. Uninoculated tryptone soy broth supplemented with 4% sucrose was used as a negative control. The 96-well plate was covered and incubated at 37 °C for 24 hours. After the incubation period, the plate was washed with saline solution to remove any impurities. This process was repeated three times. To fix the attached cells, 200 µL of 99% methanol was added to each well and left for 20 minutes. The plate was then inverted and placed at room temperature overnight. The wells were then stained with 180 µL of 1% crystal violet dye at room temperature for 15 minutes. The microtiter plate was then washed and dried. Then, the attached dye was solubilized with 200

µL of 96% ethanol, and the optical density (OD) was measured by a microtiter plate reader at 570 nm. A higher absorbance value indicates a greater biofilm density, reflecting higher efficiency in biofilm formation (11, 12). The bacteria were classified according to the classification provided by Jasim et al. (13), as shown in Table 1.

Table 1. Biofilm Evaluation by Microtiter Plate Assay

OD values	Biofilm evaluation
OD ≤ ODc	No biofilm producer
ODc < OD ≤ 2 × ODc	Weak biofilm producer
2 × ODc < OD ≤ 4 × ODc	Moderate biofilm producer
4× ODc < OD	Strong biofilm producer

OD: The absorbance of wells containing cells. *ODc: The absorbance of wells containing only TSB

Antibiotic Susceptibility Testing: After confirming the bacterial diagnosis, antibiotic susceptibility testing was performed using the Vitek 2 system for different drug classes, such as penicillins and cephalosporins. This system measures the turbidity signal for each test well containing an antibiotic. The results are then used to generate bacterial growth curves, and by comparing them to a control sample, the minimum inhibitory concentration (MIC) for each antibiotic is estimated. The MIC results (µg/mL) were converted to clinical categories (sensitive, moderately sensitive, and resistant), then compared to determine the sensitivity category recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines (11).

Statistical analysis: Data were analyzed using SPSS (Statistical Package for the Social Sciences) version 26 software. One-way analysis of variance (ANOVA) and the least significant difference (LSD) test were performed to assess significant differences among means. The significance test was accepted when the *p-value was <0.05 ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

Results

Collection and identification of bacterial isolates: A total of 345 samples were collected from burn swabs, ear swabs, and wounds. Morphological identification and biochemical testing confirmed that 73 isolates (21.1%) were P. aeruginosa. VITEK 2 is an automated system that analyzes the metabolism of various sugars and enzyme activities. It then compares the pattern of positive and negative results against its database for accurate identification, using pre-filled cards containing multiple biochemical tests, as shown in Table 2.

Biofilm formation: The results in Table 3 showed that of the 73 positive P. aeruginosa isolates, most — 68 (93.2%) — were capable of forming biofilms, while only five (6.8%) were non-biofilm-forming isolates, all of which were obtained from ear samples. This indicates a discrepancy between the number of biofilm-forming bacteria and the source of isolation (Figure 1). However, a strong correlation was found between the number of positive samples and the assessment of biofilm production ($p<0.05$). Absorption values ranged between 0.034 and 0.036.

Table 2. Biochemical profile of P. aeruginosa obtained by the VITEK 2 system

No.	Type of test	Results	No.	Type of test	Results
1	Ala-Phe-Pro-Arylamidase (APPA)	+	22	Saccharose/Sucrose (SAC)	-
2	B-Xylosidase (BXYL)	-	23	Coumerate (CMT)	+
3	L-Lactate assimilation (ILATa)	+	24	D-glucose (dGLU)	+

4	Adonitol (ADO)	-	25	D-Tagatose (dTAG)	-
5	B-Alaninearylami-dasepNA (BAlap)	+	26	B-glucuronidase (BGUR)	-
6	L-Lactate alkalinization (ILATk)	+	27	y-Gutamyl-Transferase (GGT)	+
7	L-Pyrrolydonyl-Arylami-dase (PyrA)	-	28	D-Trehalose (dTRE)	-
8	L-Proline Arylamidase (ProA)	+	29	0/129 resistance (comp.vibrio) (0129R)	+
9	a-galactosidase (AGAL)	-	30	Fermentation Glucose (OFF)	-
10	L-arbitol (IARL)	-	31	Sodium Citrate (CIT)	+
11	Lipase (LIP)	+	32	Glu-Gly-Arg-Ary-lamidase (GGAA)	+
12	Phosphate (PHOS)	-	33	ß-Glucosidase (BGLU)	-
13	D-cellobiose (dCEL)	-	34	Malonate (MNT)	+
14	Palatinose (PLE)	-	35	L-Malate assimilation (IMILTa)	+
15	Glycine arylamidase (GlyA)	-	36	D-maltose (dMAL)	-
16	ß- Galactosidase (BGAL)	-	37	5-Keto-D-Gluconate (5KG)	-
17	Tyrosine Arylamidase (TyrA)	+	38	Ellman (ELLM)	-
18	Ornithine decar-boxylase (ODC)	-	39	D-mannitol (dMAN)	+
19	H2S production (H2S)	-	40	D-Sorbitol (dSOR)	-
20	Urease (URE)		41	L-histidine assimila-tion (IHISa)	-
21	Lysine decarboxy-lase (LDC)		42	GlutamylArylamidase-Transferase (AGLTP)	-
22	β-N-Acetyl-Glucosaminidase (βNAG)	-	43		

Table 3. Absorbency values and biofilm pattern based on MTP methods

Biofilm level compared to (ODc=0.038)	Absorbency at 570 nm	No. of biofilm pattern
Strong	(0.075- 0.149)	27
Moderate	(0.040- 0.065)	41
Non-biofilm producer	(0.034 -0.036)	5
Total		73

***p<0.05**

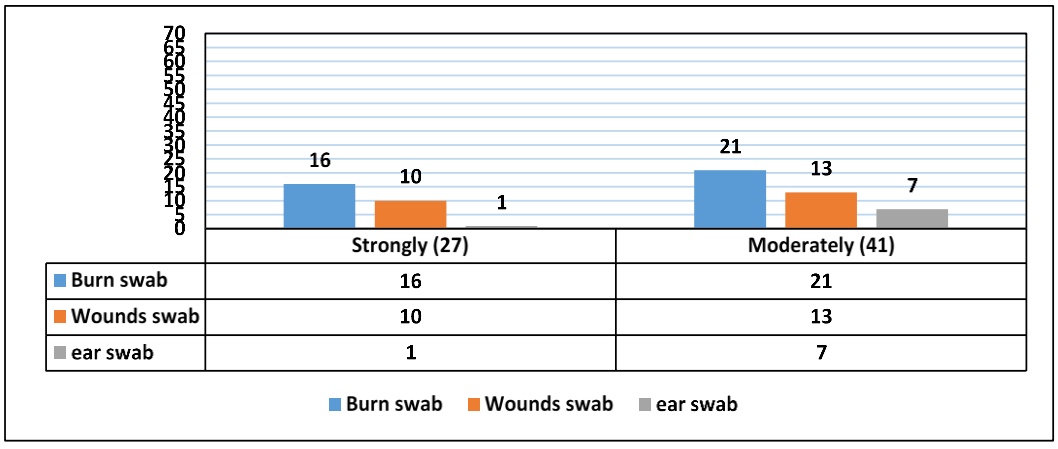


Figure 1. Distribution of biofilm-forming P. aeruginosa according to the source

Susceptibility of P. aeruginosa to different Antibiotics group: Seventy-three isolates of P. aeruginosa were tested for antibiotic susceptibility to 21 antibiotics using the Vitek-2 system (Table 4).

Table 4. Results of Susceptibility pattern in P. aeruginosa

Class	Antibiotic	No. R (%)	No. S (%)
Penicillin group	Amoxicillin	73 (100)	0 (0.0)
	Amoxicillin/ Clavulanic acid	73(100)	0 (0.0)
	Ticarcillin	72 (98.6)	1(1.4)
	Piperacillin	71(97.3)	2 (2.7)
	Ticarcillin/ Clavulanic acid	41 (56.2)	32 (43.8)
	Piperacillin/ Tazobactam	25(34.2)	48 (65.8)
Cephalosporin group	Cefuroxime	70(95.9)	3 (4.1)
	Cefepime	67(91.8)	6 (8.2)
	Ceftriaxone	59(80.8)	14 (19.2)
	Ceftazidime	57(78.1)	16 (21.9)
	Cefotaxime	56(76.7)	17 (23.3)
	Cefixime	11(15.1)	62 (84.9)
Carbapenem group	Imipenem	15(20.5)	58 (79.5)
	Meropenem	15(20.5)	58 (79.5)
Aminoglycosides group	Gentamicin	28(38.4)	45 (61.6)
	Tobramycin	23(31.5)	50 (68.5)
	Amikacin	12(16.4)	61 (83.6)
Fluoroquinolones group	Gemifloxacin	28(38.4)	45 (61.6)
	Ciprofloxacin	18(24.7)	55 (75.3)
	Moxifloxacin	16(21.9)	57 (78.1)
	Levofloxacin	15(20.5)	58 (79.5)

*****Penicillin group & resistance pattern – p<0.001, ***Cephalosporin group & resistance pattern –p<0.001, *Carbapenem group & resistance pattern – p<0.05, *Aminoglycosides group & resistance pattern – p<0.05, *Fluoroquinolones group & resistance pattern – p<0.05**

Discussion

Bacteria that produce more than one beta-lactamase form stronger biofilms capable of resisting antibiotics, compared with isolates that produce only one type of beta-lactamase (14–16). Our results showed that 93.2% of the total P. aeruginosa population produced biofilms. The results also showed a highly statistically significant difference ($p<0.001$) due to the emergence of broad resistance in the penicillin and cephalosporin groups, whereas the carbapenem, aminoglycoside, and fluoroquinolone groups showed statistical significance ($p<0.05$). This is consistent with the study by Ali et al. (17) in Iraq and Pajohesh et al. (18) in Iran, who showed a link between biofilm formation and high resistance rates. The results showed highly statistically significant differences ($p<0.001$) between the penicillin group and the other groups. High resistance was observed within the penicillin group, consistent with the findings of Jasim et al. (13). We found no local or international studies that contradict these results.

On the other hand, the results showed a statistically significant relationship ($p<0.05$) between the cephalosporin group and the carbapenem, aminoglycoside, and fluoroquinolone groups. Resistance rates were low for imipenem, meropenem, amikacin, ciprofloxacin, moxifloxacin, and levofloxacin. These results are consistent with those of Jameel et al. (19) and Ruh et al. (20), but contradict those of Ali et al. (17). The high rates of resistance can be explained by the fact that bacteria adapt quickly to different types of environments and spread rapidly due to their possession of various resistance mechanisms, through which they are able to overcome diverse conditions and cause pathogenicity. Inappropriate antibiotic use inadvertently promotes the selection of resistant strains and exacerbates the issue of antibiotic resistance.

There is a strong correlation between biofilm-forming bacteria and increased antibiotic resistance, as well as variations in resistance levels among different drug groups. Amikacin was the most effective antibiotic for eradicating the bacteria, followed by imipenem, meropenem, moxifloxacin, and levofloxacin.

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