

Antibiotic Sensitivity and Biofilm Formation in *Pseudomonas aeruginosa* Isolated from Wound Infections in Baghdad City

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Abstract:

Pseudomonas aeruginosa is considered as the most common gram-negative bacteria that infect hospitalized patients. One hundred sixty six specimens of wound discharge were collected during the period from October to December 2022. Thirty isolates of *Pseudomonas aeruginosa* have been identified via the cultural, morphological, and biochemical tests. Vitek-2 compact system used to determine the most accurate identification for *Pseudomonas aeruginosa*, and thirty isolates of *Pseudomonas aeruginosa* were recognized. In the present study, *Pseudomonas aeruginosa* was detected in 32.25% of the isolates. All isolates were subjected to antibiotic susceptibility tests to determine their susceptibility to 14 different antibiotics. The results showed total resistance to piperacillin (100%), high resistance rates to ceftazidime (96.7%), tobramycin (96.7%), gentamicin (80%), piperacillin-tazobactam (70%), aztreonam (66.7%), cefoperazone (66.7%), and amikacin (60%). Low resistance rates were found for imipenem (30%), meropenem (43.3%), and gatifloxacin (46.7%), but there was moderate resistance to ciprofloxacin (53.3%), cefotaxime (50%) and colistin (50%). The current investigation determined that among 30 isolates of *Pseudomonas aeruginosa*, six (20%) developed a weak biofilm, nine (30%) formed a moderate biofilm, and fifteen (50%) formed a strong biofilm. The findings of the present investigation revealed that the vast majority of the isolates with significant antibiotic-resistance can create biofilms.

Keywords: *Pseudomonas aeruginosa*, Antibiotic sensitivity, Biofilm formation, Wounds

Note: The research is based on an M.A thesis .

Introduction:

A wound is a break in the integrity of the skin's epithelium and may lead to additional disruption of the skin structure, physiology and functions of the skin (acute tissue disruption) via an outside force that tears, cuts, or punctures the skin (an open wound) or induces a contusion (a closed wound) [1]. Wound infections including acute and chronic have come to be a significant global healthcare problem that contributes extremely to high levels of mortality and morbidity [2,3]. The majority of chronic wounds are colonized with polymicrobial communities which can create biofilms, that can cause heightened inflammation and increased susceptibility to infection, delaying wound healing significantly [4]. This study aimed to investigate the antibiotic sensitivity and biofilm patterns of *Pseudomonas aeruginosa* from wound infection patients

Previous Studies:

A different investigation found that *Staphylococcus* spp and *P. aeruginosa* were the most common pathogens in wounds from both surface swabs and tissue biopsies in 71.2% of subjects [5]. Among the microorganisms causing nosocomial illnesses in hospitals is *P. aeruginosa*, which also causes 50% wound infections, 30% pneumonia, 19% UTI and 10% potentially fatal bloodstream infections [6]. *P. aeruginosa* is the main reason behind wound infections and a frequent source of nosocomial diseases in Iraq [7]. Sepsis resulting from an invasive burn wound infection is the main cause of mortality for 75% of those who have had serious burns [8,9,10]. Antibiotic resistance refers to the ability of a microorganism to resist the effects of an antimicrobial agent where it used to be susceptible to [11]. *P. aeruginosa* poses an important medical problem in the management of nosocomial illnesses and in selecting the most suitable antibiotic therapy because of its capacity to acquire resistance to several classes of antimicrobial agents rapidly [12]. In the field of medicine worldwide, antimicrobial resistance is becoming increasingly concerning since bacteria can develop resistance to both conventional and novel therapies in a number of different ways [13,14]. One of the major issues in treating infections is the production of biofilms [17,18]. Biofilms are communities of bacterial cells or it can be defined as specific aggregated types of bacteria that are embedded within self-produced extracellular polymeric substances (EPS) matrix, it can increase bacterial persistence on abiotic and biotic surfaces and assist pathogenic bacteria to withstand adverse circumstances such as fluctuating temperatures, low-level

nutrients, and antimicrobial killing. Extracellular DNA (eDNA), lipids, proteins, and three exopolysaccharides Psl, Pel, and alginate, all make up the majority of the *P. aeruginosa* biofilm matrix and play important roles in biofilm structure stability, surface adhesion, and formation. *P. aeruginosa* ability to produce biofilm-mediated infections leads to life-threatening cystic fibrosis infections and results in persistent infections. *P. aeruginosa* cultures in biofilm show intrinsic resistance to immune system responses, antimicrobial treatments, as well as protection from adverse environmental conditions. Biofilm is a key factor in the pathogenesis of *P. aeruginosa* that produces fatal infections. There are strong signs that the microbe creates a multicellular aggregation where cells attach to one another and to surfaces, creating an extracellular polymeric matrix where the infection is present [19,20]. Even in healthy people, the persistence of infections caused by biofilm-forming *P. aeruginosa* variants has caused major complications in wound hospitals [15]. The development of biofilms demonstrates a protective growth pattern that enables the microbe to survive in a variety of settings [16,21]. According to certain research, strains of *P. aeruginosa* that can develop biofilm and can survive several antimicrobial drugs at doses higher than those required for killing planktonic cells [22]. Antibiotic resistance among bacteria in biofilm forms can be up to 1,000 times greater than that of bacteria in individual cells.

2. Methodology

2.1. Sample Collection

One hundred sixty-six clinical samples from patients wounds were collected during the period between October to December 2022, from different hospitals in Baghdad city (Ghazi AL-Hariri Hospital in Medical city, AL-Kindy Teaching Hospital and Imamein Kadhimein Medical city in Baghdad city). The samples were obtained from patients by sterilized cotton swabs and transported immediately to the laboratory under aseptic conditions for the isolation of *Pseudomonas aeruginosa*.

2.2 Isolation and Identification of *P. aeruginosa* from Wounds

All samples were cultured on nutrient agar and blood agar plates, then the colonies in these media were subcultured on MacConkey agar. Non-lactose fermenting colonies further subcultured on Cetrimide agar for preliminary selection of *P. aeruginosa* isolates. The recovered isolates were subjected to different biochemical and morphological tests for the identification to the species level as represented by Bergey's Manual for Systemic Bacteriology (2001) and confirmed by vitek-2 compact system [23].

2.3. Antibiotic Susceptibility Test

The Kirby-Bauer method was used to test the antibiotic susceptibility using 15 different antibiotics as described by WHO (2003).

1- Bacterial suspension was prepared by picking 1-2 isolated colonies of *P. aeruginosa* from the original culture and introducing it into a test tube containing 5 mL of normal saline to produce a bacterial suspension of moderate turbidity compared to McFarland the standard turbidity solution which equals to 1.5×10^8 CFU/ml.

2- A sterile cotton swab was dipped into the bacterial suspension and excess fluid were removed by pressing the swab against the tube wall.

3- A portion of bacterial suspension was transferred carefully and evenly spread on Mueller-Hinton agar medium and left to dry for 10 min.

4- Then antibiotic discs were placed on the surface of the agar medium using sterilized steel forceps and pushed firmly to ensure contact with the agar.

5- After that the plates were inverted and incubated at 37°C for 18-24 hr.

6- After incubation, the inhibition zones developed around the antibiotic discs and measured via millimeter (mm) using a metric ruler. The results obtained were compared with the Clinical Laboratories Standards Institute (CLSI) [24].

2.4. Biofilm Formation Assay

In the present investigation, the microtiter plate technique, as described by Zhang et al (2016) with some changes, was employed for assessing the potential of 30 clinical isolates of *P. aeruginosa* to create biofilm [25].

1- *P. aeruginosa* was isolated from fresh agar plate cultures and resuspended into 5 ml of brain heart infusion broth (BHI) containing sucrose (2%), which was subsequently incubated for 24 hours at 37°C [26].

2- Each microtiter well on the microtiter plate contained 180 µl of brain heart infusion broth (BHI), which was added along with bacterial suspension (20 µl) from each isolate (equivalent to 0.5 McFarland standard). The microtiter plate was then sealed and incubated for 24 hrs. at 37°C.

3- After incubation, the plates were rinsed three times using normal saline solution to remove any unattached cells.

4- To fix the adherent cells, 200 µl of 99% methanol was applied to each well for 15 minutes. The plates were left to dry for 30 minutes at room temperature.

5- After that, 200 µl of 1% crystal violet stain was applied for 15 minutes.

6- Removing stain solution and rinsing with sterilized distilled water, the remaining stain was dissolved in 96% ethanol, the optical density was calculated at 630 nm in an ELISA reader (see Table 1).

Table 1: Microtiter plate evaluation for biofilm formation

Optical density	Adherence
OD < OD _c	Non-adherent
2 OD _c > OD > OD _c	Weak
4 OD _c > OD > 2 OD _c	Moderate
OD > 4 OD _c	Strong
Cut off value (OD_c) = average OD of negative control + (3 *Standard Deviation).	

2.5. Statistical Analysis

Data were analyzed using Statistical Program for Social Science (SPSS) version 20.0 as well as Data Analysis via Microsoft Excel 2016. Qualitative data were expressed as frequency and percentage. Chi-square (χ^2) was used to accept or reject the statistical hypotheses.

3. Results and Discussion

3.1. Between October and December 2022, 166 clinical samples were collected from the wounds of male and female patients at various hospitals in Baghdad city. Following the identification of 166 samples, *P. aeruginosa* was suspected in 30 isolates. On MacConkey agar, *Pseudomonas aeruginosa* formed pale, non-lactose-fermenting colonies with a unique fruity odor. On blood agar, colonies appear black in color and a translucent area around them, indicating that they may hemolyze blood (type β -hemolysis) because they produce hemolysin. The development of *P. aeruginosa* with a bright green color, raised colonies, and inhibition of other bacteria are all enhanced by the selective media for *P. aeruginosa* identification known as cetrimide agar [27] as shown in Figure 1. *P. aeruginosa* isolates appeared under microscope as reddish pink rods, do not produce spores, and appeared single, in pairs or in short chains. For additional identification, typical biochemical tests were also performed. All *P. aeruginosa* isolates tested positive for oxidase developing a purple shade indicates that the *P. aeruginosa* have cytochrome c oxidase and can utilize oxygen to produce energy via the conversion of O_2 to H_2O or H_2O_2 through the electron transfer chain [28]. In catalase test, all of the isolates produced bubbles indicating (+) results. Because the isolates of *P. aeruginosa* possessed flagella, they were able to move (motile) and produced a (+) motility result [29]. The ability of *P.*

P. aeruginosa isolates for growth at 42°C is regarded as a crucial diagnostic feature for *P. aeruginosa* to distinguish it from various Pseudomadaeae species such as *P. fluorescences* and *P. putida* that thrive at 4°C, whereas it fails to grow at 42°C [30]. In the present study, *P. aeruginosa* was detected in 30/30 (32.25%) of the isolates, followed by *E. coli* 27/30 (29.4%), *P. mirabilis* 17/30 (18.28%), *A. baumannii* 11/30 (11.83%), and *K. pneumoniae* 8/30 (8.6%) (see Table 2). *P. aeruginosa* was the most frequently identified Gram-negative bacterium in wounds, according to a previous investigation [31].

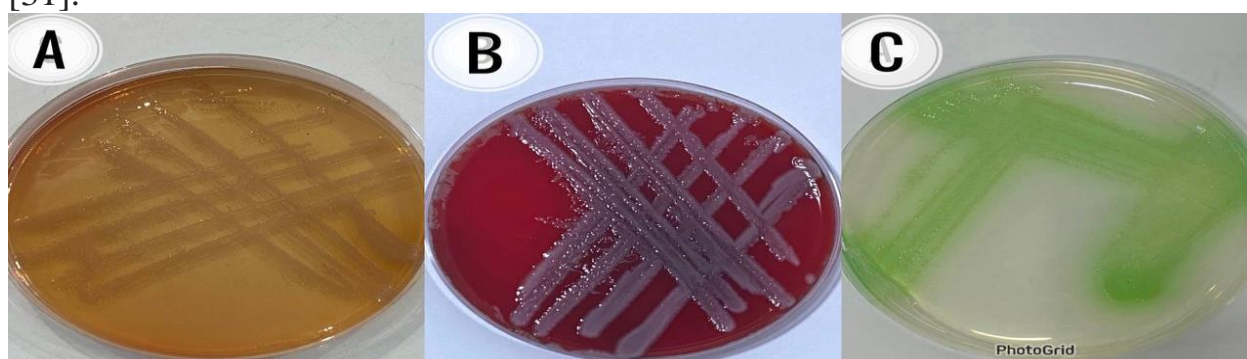


Figure 1: *P. aeruginosa* colonies on A- MacConkey agar, B- blood agar, C- Cetrimide agar

3.2. Antibiotic Susceptibility Test

The sensitivity of 30 isolates of *Pseudomonas aeruginosa* isolated from wounds was examined using the Kirby-Bauer method (disc diffusion test) as described by WHO, 2003. Out of the 14 antibiotics tested in this study, 9 drugs showed high levels of resistance among *P. aeruginosa* isolates. Only three drugs showed intermediate resistance in *P. aeruginosa* isolates, whereas the remaining two showed lower resistance (higher sensitivity) as shown in Figure 2. Beta-lactams, aminoglycosides, and fluoroquinolones were the most commonly resistant drugs among *P. aeruginosa* isolates, piperacillin 30/30 (100%), ceftazidime 29/30 (96.7%), tobramycin 29/30 (96.7%), gentamycin 24/30 (80%), piperacillin-tazobactam 21/30 (70%), aztreonam 20/30 (66.7%), cefoperazone 20/30 (66.7%), amikacin 18/30 (60%), ciprofloxacin 16/30 (53.3%). The isolates exhibited moderate resistance to cefotaxime 15/30 (50%), colistin 15/30 (50%), and gatifloxacin 14/30 (46.7%). *P. aeruginosa* isolates were highly susceptible (low resistance) to meropenem 13/30 (43.3%) and imipenem 9/30 (30%) (see Table 3). The findings of this study demonstrated that all *P. aeruginosa* isolates were completely resistant 100% to piperacillin and it was consistent with the findings from a previous

investigation in Irbil, Iraq, found that all isolates of *P. aeruginosa* were piperacillin-resistant 100% [32]. According to the data from earlier studies found different results from ours, which were (32% and 37%), respectively [33,34]. Piperacillin-tazobactam, demonstrated great effect on *P. aeruginosa* isolates by decreasing their resistance to 70% and these findings concurred with the findings of Al-Muqati [35]. The resistance percentage for aztreonam in the current study was 66.7%, which is in accordance with the findings of Alhamdani and Al-Luaibi in Basrah, Iraq, which was 65.38% and [36]. Our results, however, are inconsistent with those of Younus in Erbil, who found that aztreonam resistance was 32.5% [37]. Colistin showed a 50% resistance rate in this study, which was in line with Khudair and Mahmood's findings who recorded 40.28% [38]. Meropenem and imipenem had resistance rates of 43.3% and 30%, respectively. With imipenem exhibiting the highest level of sensitivity (70%) and meropenem coming in second with 56.7%. Both drugs demonstrated decreased levels of resistance to *P. aeruginosa* isolates. Imipenem has proven to be the most effective drug in this study. Al-Kazrage findings which revealed that imipenem and meropenem showed resistance rates of 35% and 30%, respectively, agreed with our findings while Hu's findings indicated that the resistance rate for both was 75% [39,40].

Table 2: Testing *P. aeruginosa* susceptibility to several antibiotics

Antibiotic	No. of isolates	Resistant (%)	No. of isolates	Sensitive (%)
Amikacin	18	60%	12	40%
Gentamicin	24	80%	6	20%
Tobramycin	29	96.7%	1	3.30%
Piperacillin	30	100%	0	0%
Piperacillin-tazobactam	21	70%	9	30%
Aztreonam	20	66.7%	10	33.3%
Colistin	15	50%	15	50%
Ciprofloxacin	16	53.3%	14	46.7%
Gatifloxacin	14	46.7%	16	53.3%
Ceftazidime	29	96.7%	1	3.30%
Cefotaxime	15	50%	15	50%
Cefoperazone	20	66.7%	10	33.3%
Imipenem	9	30%	21	70%
Meropenem	13	43.3%	17	56.7%

3.3 Biofilm Formation

Biofilm production is thought to be an indicator of virulence. In the present study, a 96-well polystyrene microtiter plates were used as a typical technique for identifying the presence of biofilm growth. The absorption values showed how thick a biofilm the investigated isolates had created on the microtiter well's surfaces. Based on limitations, the findings of this study were divided into four categories: non-biofilm producers, weak, moderate, and strong. The current investigation determined that among 30 isolates of *P. aeruginosa*, only six (20%) developed a weak biofilm, nine (30%) formed a moderate biofilm, and fifteen (50%) formed a strong biofilm (see Table 4). However, certain study disagreed with our results, showing that only four *P. aeruginosa* isolates out of 16 generated a mild biofilm, while the other twelve created weak biofilms [41]. Only 16% of *P. aeruginosa* isolates produced strong biofilms, according to a previous study, while 51% and 32% of the isolates produced moderate and weak biofilms [7]. The findings of the present investigation revealed that the vast majority of the isolates with significant antibiotic-resistance can create biofilms [42]. A study found that 14 out of 15 isolates of *P. aeruginosa* were positive for biofilm production using the microtiter plate method [43]. *P. aeruginosa* capacity to produce biofilm is an essential element of bacterial pathogenicity, which promotes survival of bacteria in a variety of conditions, including burn wounds, and ultimately leads to chronic infections [44]. Previous investigations revealed a link between *P. aeruginosa* capacity to produce biofilms and its multidrug resistance phenotypes [45].

Table 3: Biofilm thickness according to *P. aeruginosa* isolates cutoff values.

ID Biofilm	Biofilm productivity of isolates	OD630 Limits	Number of isolates	percentage (%)
1	Non-biofilm producer	< 0.056	0	0
2	Weak	0.056 - 0.112	6	20%
3	Moderate	0.112 - 0.224	9	30%
4	Strong	≥ 0.224	15	50%

Conclusions

Pathogenic bacteria were obtained from patient wounds in Iraq and their antimicrobial susceptibility testing revealed that all *P. aeruginosa* isolates were completely resistant to piperacillin. Additionally, it exhibited extremely

high gentamicin, tobramycin, and ceftazidime resistance. We suggest that strict guidelines should be set in place for the use of antibiotics in the management of wounds. Before recommending antibiotics for bacteria isolated from wounds, bacterial culture and antibiotic susceptibility testing must be conducted. Our results might help clinicians choose the best course of antibiotic therapy for individuals with chronic wounds.

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Pseudomonas aeruginosa حساسية المضادات الحيوية وانماط تكوين الأغشية الحيوية لبكتريا
المعزولة من إصابات الجروح في مدينة بغداد

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مستخلص البحث:

تعتبر البكتيريا سالبة الجرام *P. aeruginosa* من العوامل المسببة للأمراض الرئيسية لدى المرضى في المستشفيات. تم جمع مائة وستة وستين عينة خلال الفترة من أكتوبر إلى ديسمبر 2022. وتم التعرف على ثلاثين عينة من بكتيريا *P. aeruginosa* عن طريق الاختبارات الزرعية والمورفولوجية والكيميائية الحيوية. حددت نتائج نظام Vitek-2 المدمج التشخيص الأكثر دقة لبكتيريا *P. aeruginosa*، وتم التعرف على ثلاثين عينة من *P. aeruginosa*. في الدراسة الحالية، تم اكتشاف *P. aeruginosa* في (32.25%) من العزلات، تليها (*E. coli* (29.4%)، *P. mirabilis* (18.28%)، *A. baumannii* (11.83%)، و *K. pneumoniae* (8.6%). تم إخضاع جميع العزلات لاختبارات الحساسية للمضادات الحيوية لتحديد مدى حساسيتها لـ 14 مضاد حيوي مختلف. أظهرت النتائج مقاومة كلية للبيبراسيلين (100%)، معدلات مقاومة عالية للسيفتازيديم (96.7%)، التوبراميسين (96.7%)، الجنتاميسين (80%)، البيبراسيلين-تازوباكتام (70%)، الأزترينونام (66.7%)، سيفوبيرازون (66.7%)، وأميكاسين (60%). تم العثور على معدلات مقاومة منخفضة للإيميبينيم (30%)، والميروبينيم (43.3%)، والجاتيفلوكساسين (46.7%)، ولكن كانت هناك مقاومة معتدلة للسيفروفلوكساسين (53.3%)، والسيفوتاكسيم (50%)، والكوليسيتين (50%). حدد البحث الحالي أنه من بين 30 عينة من *P. aeruginosa*، طورت ستة (20%) غشاء حيوي ضعيف، و9 (30%) شكلت غشاء حيوي معتدل، وخمسة عشر (50%) شكلت غشاء حيوي قوي. كشفت نتائج البحث الحالي أن الغالبية العظمى من العزلات ذات المقاومة الكبيرة للمضادات الحيوية يمكن أن تخلق أغشية حيوية.

الكلمات المفتاحية: *P. aeruginosa*، حساسية المضادات الحيوية، تكوين الغشاء الحيوي، الجروح