

Effect Biofilm Formation in *Pseudomonas aeruginosa* Resistance To Antibiotic

Hanan Hameed Shatti¹, Wisam M. Al-Saeed², Mohammed I. Nader³

¹Department of Laboratory, Iraqi Ministry of Health, Baghdad, Iraq, ²Department of Microbiology, College of Medicine, Mustansiriyah University, Baghdad, Iraq,

³Institute Genetic Engineering and Biotechnology for Post Graduated Studies, University of Baghdad, Baghdad, Iraq

Abstract

Objective: Due *Pseudomonas aeruginosa* is the most common type of bacteria that causes hospital-acquired infections because it has multiple resistance mechanisms to antibiotics such as biofilm formation. It is important to understand to investigate biofilm production in multidrug resistant clinical isolates of *P. aeruginosa* from different sites of infection. **Materials and Methods:** This work was done on 114 patients were collected from different specimens From hospitals Baghdad. The collected samples were cultured on different media (blood agar, MacConkey agar, nutrient agar, and Cetrimide agar) for isolation of *P. aeruginosa* bacteria as well as isolates from all patient were tested for antimicrobial susceptibility using disk diffusion method ,in vitro formation of biofilm in microtiter plates containing Muller – Hinton broth. **Results:** Forty specimens (35.1%) were observed to have bacterial growth (positive samples) for *P. aeruginosa*, the results of *P. aeruginosa* isolates were tested for antibiotic susceptibility showed that among forty positive results, the most isolates were potentially resistant to different antibiotics presented that the isolates resistant to piperacillin (34, 85%), gentamicin (26, 65%), tobramycin (30, 82.5%), amikacin (27, 67.5%), cefepime (25, 62.5%), meropenem (31, 77.5%), carbenicillin (31, 77.5%), ceftriaxone (30, 75%), ciprofloxacin (27, 67.5%), imipenem (22, 55%), ceftazidime 26 (65%), and norfloxacin 24 (60%). The results showed the ability of *P. aeruginosa* isolates to produce biofilm were 40 (100%) has the ability to produce biofilm, these were the result divided in to strong 19 (47.5%), moderate 12 (30%), and weak 9 (22.5%). **Conclusion:** There were 40 isolates biofilm producers in divers levels of biofilm strength.

Keywords: Antimicrobiogram biofilm, *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium belonging to the family Pseudomonadaceae that is able to survive in a wide range of environments. The opportunistic pathogen *P. aeruginosa* can infect a variety of organisms, including plants, animals, and most importantly, humans, in particular, immunocompromised, burn-wound patients and individuals suffering from cystic fibrosis.^[1] *P. aeruginosa* has many virulence factors such as pili, cilia, large polysaccharide (LPS), extracellular enzymes, exotoxins, and flagella. These bacteria contain a single polar flagellum, which is essential for movement, chemotaxis, and adhesion.^[2] One of the major virulence factors for this pathogen is its ability to form biofilms. This biological development protects the pathogen from host immunity and contributes to its antimicrobial resistance. It is estimated that about 80% of infectious diseases are due to biofilm formation. Biofilm-forming ability and

antimicrobial resistance of this pathogen lead to many persistent and chronic bacterial infections.^[3] A biofilm can be described as a microbially derived sessile community characterized by cells. These cells are irreversibly attached to a surface or interface or to each other, are inserted in a matrix of extracellular polymeric substances (EPSs) that they have produced, and exhibit an altered phenotype in terms of growth rate and gene transcription^[4] Biofilm formation is an important virulence factor increase the pathogenicity infectious of *P. aeruginosa*.^[5] The infection instigated by biofilm former *P. aeruginosa* can

Address for correspondence: Miss. Hanan Hameed Shatti,
Department of Laboratory, Iraqi Ministry of Health, Baghdad, Iraq.
E-mail: hanan1986microbiology@gmail.com

Submitted: 28-Jul-2021 Revised: 21-Aug-2021 Accepted: 28-Aug-2021 Published: 30-Jun-2022

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: WKHLRPMedknow_reprints@wolterskluwer.com

How to cite this article: Shatti HH, Al-Saeed WM, Nader MI. Effect biofilm formation in *Pseudomonas aeruginosa* resistance to antibiotic. Mustansiriyah Med J 2022;21:13-7.

Access this article online

Quick Response Code:



Website:
<http://www.mmjonline.org>

DOI:
10.4103/MJ.MJ_11_21

proceed for chronic form due to traceability of antibiotic effects. The outstanding of biofilms to antibiotics is multifactorial including physic-physiological and genetic determinants.^[6]

MATERIALS AND METHODS

Specimens' collection

Through the period from November 2020 to February 2021, 114 isolates were collected from different specimens from different clinical sources from several hospitals in Baghdad governorate (Imam Kadhimi Medical City, Hospital, Al-Yarmouk Teaching Hospital, Al-Mahmoudia General Hospital) from both gender with ages ranged from 5 years to 75 years.

Bacterial isolation and identification

The isolates were identified based on^[7] the colony morphology on different culture media MacConkey agar, Nutrient agar, Blood agar, and Cetrimide agar, incubated for 24 h at 37°C. This isolates may belong to *Paeruginosa* growth on Cetrimide agar, in conjunction with traditional biochemical tests comprised gram staining, oxidase, catalase, oxidation/fermentation glucose, and growth and nongrowth at 42°C and 4°C, respectively, were performed to characterize *P. aeruginosa* colony morphologically, cell microscopically, and metabolically by ByAnalytical Profile Index (API) 20E Kit Biomerieux® company (France and USA), microtubules enzymatically tests to support strains identification, and the isolated *P.aeruginosa* which stored in brain heart infusion (BHI) broth contains 30% glycerol at refrigerator temperature and -20°C, respectively, for the next experiments.

Antibiogram test

The disk diffusion susceptibility method for antibiogram testing (Kirby–Bauer method) using Muller–Hinton agar plates was performed according to the Clinical and Laboratory Standards Institute.^[8] Antibiotic discs (Bioanalyse – Turkey) used in this study included tobramycin, ciprofloxacin, imipenem, meropenem–cefepime, ceftazidime, gentamicin, piperacillin, ceftazidime, carbenicillin, amikacin, and norfloxacin. After an overnight incubation at 37°C, zones of inhibition were measured and compared to the CLSI guidelines.

Quantitative biofilm formation assay

Quantification of biofilm formation by *P. aeruginosa* was assessed, as described by the previous study.^[9] In brief:

- All isolates were grown overnight in BHI broth at 37°C. Thereafter, it was adjusted to McFarland standard No. (0.5)
- Each isolate were transferred to Muller–Hinton broth containing 1% glucose and mixed well via pipetting. Thereafter, it was adjusted to McFarland standard No. (0.5)
- A volume (200 µl) for each isolate culture was added to duplicate of sterile 96-well U-shaped-bottom polystyrene microplates
- The plates were covered with their lids and incubated under aerobic conditions at 37°C for 24 h

- After the incubation period, the planktonic cells were rinsed twice with deionized water to remove the unattached bacteria and then shaken out the excess water by tapping the plate on paper towels (filter paper), dried, and fixed at 65°C for 1 h
- The adhering bacterial cells in each well were fixed with 200 µl of absolute methanol with a concentration of 99% for 20 min at room temperature
- The adhering cells were stained by adding 200 µl of 0.1% crystal violet to each well for 15 min
- Once the staining reaction has completed, the excess stain was removed and left to dry., then glacial acetic acid was added at a concentration of 33% and at a rate of 160
- Then, the optical density was read by enzyme-linked immunosorbent assay reader (ELISA reader) at a wavelength of 630 nm to determine the efficiency of the isolates in biofilm production and compared with the equations mentioned in Table 1.

RESULTS

Isolation and identification of *P. aeruginosa* by traditional methods

A total of 40 (35%) isolates developed a growth on cetrimide agar blue-greenish color, mucoid colony, smooth in shape, and have a fruity odor showed in Figure 1, pale colonies on MacConkey agar. Moreover, they were oxidase and catalase positive. Hence these isolates were primarily identification as *p aeruginosa*, the results of media growth and biochemical tests are listed in Table 2.

For more accurate detection, by using the strip of API 20E metabolically and enzymatically assay, in Figure 2.

Table 1: Classification of bacterial adherence by microtiter plate method^[9]

Mean OD630	Biofilm intense
OD ≤ ODc*	Nonbiofilm producer
ODc < OD ≤ 2ODc	Weak
2ODc < OD ≤ 4ODc	Moderate
OD > 4ODc	Strong

*Classification of Bacterial Adherence by Microtiter plate Method. Cutoff value (ODc): Mean OD of negative controls +3 (SD of control). SD: Standard deviation

Table 2: Results of biochemical test for identification *Pseudomonas aeruginosa* isolates from different clinical samples

Biochemical test	Result
Catalase test	+
Oxidase test	+
Gram stain	–
Hemolysis production	+
Lactose fermentation	–
Growth at 42°C	+
Growth on cetrimide agar and pigments production	+

+: Positive test, -: Negative test

The API 20E results revealed that 40 isolates tested positive for belonging to the *P.aeruginosa* species.

Antimicrobiogram susceptibility test

Forty of *P. aeruginosa* were subjected to an antibiogram examination according to the Clinical and Laboratory Standards Institute to determine possible resistance to 12 antibiotics items from 5 separate groups The results were interpreted according to the recommendation of CLSI (2019). *P. aeruginosa* exhibited diversity in resistance phenomena, the isolates were the highest rate of resistance against to piperacillin (34, 85%), meropenem (31, 77.5%), carbenicillin (31, 77.5%), tobramycin (30, 75%), ceftriaxone (30, 75%), ciprofloxacin (27, 67.5%), gentamicin (26, 65%), and ceftazidime (26, 65%), then percentage resistance of isolates began gradually to decline with cefepime (25, 62.5%), norfloxacin (24, 60%), and imipenem with percentages as 22 (55%). These isolates showed different susceptibility toward these antibiotics, as shown in Table 3.

Biofilm formation

The capacity of all (40) *P. aeruginosa* isolates to generate biofilm was determined in this study using standard microtiter

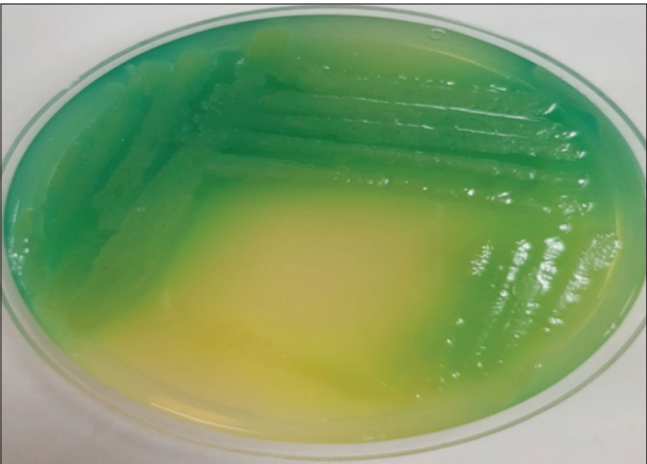


Figure 1: Colonies of *Pseudomonas aeruginosa* on cetrimide agar plate at 37°C for 24 h

plates and reading in an automated ELISA reader, using a 630 nm wavelength, the absorbance of stained biofilms was measured. The results revealed that 40 (100%) isolates were adhered and capable of forming a positive biofilm. These positive results were divided into there were 47.5% strong biofilm formation, 30.0% moderate and 22.5% weak. The weak biofilm formation is shown in Figure 3.

DISCUSSION

P. aeruginosa is a major pathogen in hospitalized patients, causing morbidity and mortality due to its multiple resistance mechanisms.^[10] The findings of this study revealed that 40 (35.1%) of the total 114 samples isolated from patients were positively identified as *P. aeruginosa*. The results were similar to this study (37.9%) from hospitalized burn patients in Karbala.^[11] Moreover, other results that showed 55 (37.93%) of the total 145 sample isolates from burn patients were positively diagnosis as *P. aeruginosa*^[12] and this may be attributed to the highest prevalence of this genus of bacteria *P. aeruginosa* in the contamination and nature. *P. aeruginosa* is distinguishable from other types of bacteria by its capability for growth at 42°C and formation of blue–greenish color pigments associated with *P.aeruginosa* on selective medium cetrimide agar and this result agreement with the previous study.^[13] Antibiotic sensitivity testing is an essential tool for treatment. *P. aeruginosa* isolates showed high prevalence of

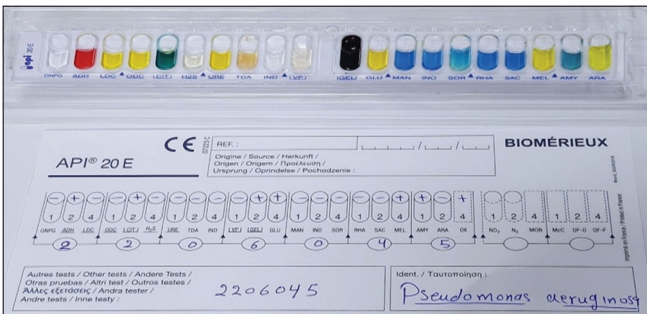


Figure 2: API 20E tests for identification of *Pseudomonas aeruginosa*

Table 3: Result of antibiotic susceptibility test (n=40)					
Number	Type of antibiotics	Resistance isolates, n (%)	Intermediate isolates, n (%)	Sensitive isolates, n (%)	P
1	Piperacillin	34 (85)	0	6 (15)	0.0001**
2	Carbenicillin	31 (77.5)	0	9 (22.5)	0.0001**
3	Amikacin	27 (67.5)	1 (2.5)	12 (30)	0.0001**
4	Gentamicin	26 (65)	0	14 (35)	0.0001**
5	Tobramycin	30 (75)	3 (7.5)	9 (22.5)	0.0001**
6	Norfloxacin	24 (60)	1 (2.5)	15 (37.5)	0.0001**
7	Ciprofloxacin	27 (67.5)	2 (5)	11 (27.5)	0.0001**
8	Ceftazidime	26 (65)	1 (2.5)	13 (32.5)	0.0001**
9	Cefepime	25 (62.5)	4 (10)	11 (27.5)	0.0001**
10	Ceftriaxone	30 (75)	3 (7.5)	7 (17.5)	0.0001**
11	Meropenem	31 (77.5)	1 (2.5)	8 (20)	0.0001**
12	Imipenem	22 (55)	2 (5)	16 (40)	0.0001**
P		0.0002**	0.042*	0.0014**	--

*P≤0.05, **P≤0.01

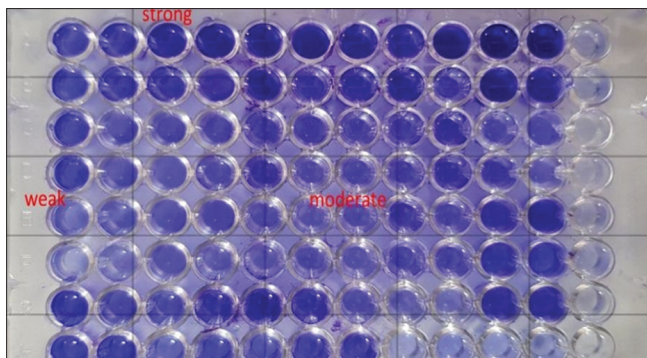


Figure 3: Screening of biofilm producers by MTP method

resistance against, β -Lactamase inhibitors class (piperacillin, carbenicillin) 85%, 77.5% respectively. Due to *P. aeruginosa* can produce β -lactamase enzymes is the most common and important mechanism of resistance in Gram-negative bacteria. β -lactamase diffuses through or directly traverse porin channels in the outer membrane of Gram-negative bacterial cell walls. *P. aeruginosa* either decreased porin production or increased efflux and reduced permeability.^[14] Hence, the result showed resistance to meropenem (31, 77.5%), tobramycin (30, 75%), ceftriaxone (30, 75%), ciprofloxacin (27, 67.5%), gentamicin (26, 65%), and ceftazidime (26, 65%), then percentage resistance of isolates began gradually to decline with cefepime (25, (62.5%), norfloxacin (24, 60%), and imipenem with percentages as 22 (55%). The study showed that the multidrug resistant (MDR) which is resistant to three classes of antimicrobials increased among these organisms making it difficult to choose appropriate suitable antimicrobial therapy, and the increased resistance of *P. aeruginosa* to numerous antibiotics, as a result of excessive antibiotic administration, is now leading to the accumulation of antibiotic resistance and cross-resistance between antibiotics and the appearance of MDR forms of *P. aeruginosa*.^[15] The method microtiter of the plate is very important in the early study due to determining the membrane configuration and classification of production to high, medium, and weak for biofilm formation are very important because of the factors of the survival and protection of bacteria in addition to its role in the adhesion on the surface, especially in the hospital environment and it causes acquired disease so.^[16] Regarding the biofilm, the result in this study showed that 100% of isolates produced biofilm formation, there were 47.5% strong biofilm formation, 30.0% moderate, and 22.5% weak biofilm formation. This result was compatible with other studies that showed 32 (91.4%) of *P. aeruginosa* isolates were biofilm producers and 25.7%, 40%, and 25.7% of isolates were strong, moderate, and weak biofilm producers, respectively.^[17] In Iranian investigations found that all isolates from burn patients (92.4%).^[18] In India, isolates forming the biofilm were resistant to all antibiotics, and this resistance increased in these isolates by 10–100 times compared to the isolates that did not form the biofilm. Thus, the formation of the biofilm is one of the most important virulence factors important in the treatment of infections caused by bacteria. In

addition, the grade and strength of biofilm development are determined by the location and form of infection, the length of bacterial invasion, host immune activity, the composition of EPS, and environmental adaptation.^[19] The high percentages in the current study of bacterial production of the biofilm may explain the high rate of bacterial resistance to all antibiotics, as the biofilm plays a major role in pathogenicity because it forms the mucous layer and the host's proteins, which provides the appropriate place for the growth of bacteria and their resistance to treatment.^[20] The discrepancy in results between different studies may be attributed to many factors such as the different countries from which the samples were collected, the number and the type of clinical specimens from which the isolates were obtained, and also the differences in isolates capability to form biofilm.

CONCLUSION

P. aeruginosa isolates differ in their ability to produce biofilm formation, bacteria that produce biofilm more resistant to the antibiotic.

Recommendations

1. Studying the other mechanisms of antibiotic resistance
2. Determine genes related to the formation of the biofilm.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Pachori P, Goyalwal R, Gandhi P. Emergence of antibiotic resistance *Pseudomonas aeruginosa* in intensive care unit; a critical review. *Genes Dis* 2019;6:109-19.
2. Garcia M, Morello E, Garnier J, Barrault C, Garnier M, Burucoa C, et al. *Pseudomonas aeruginosa* flagellum is critical for invasion, cutaneous persistence and induction of inflammatory response of skin epidermis. *Virulence* 2018;9:1163-75.
3. Elmanama AA, Al-Sheboul S, Abu-Dan RI. Antimicrobial resistance and biofilm formation of *Pseudomonas aeruginosa*. *Int Arab J Antimicrob Agents* 2020;10:3.
4. Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. Biofilms: An emergent form of bacterial life. *Nat Rev Microbiol* 2016;14:563-75.
5. Issa MA, Ghaima KK, Saleh MB, Nader MI. Antibiogram study and prevalence of *pslA* gene among biofilm *Pseudomonas aeruginosa* producers isolated from some clinical specimens in the qar province. *Pak J Biotechnol* 2018;15:681-8.
6. Ciofu O, Tolker-Nielsen T. Tolerance and resistance of *Pseudomonas aeruginosa* biofilms to antimicrobial agents-how *P. aeruginosa* can escape antibiotics. *Front Microbiol* 2019;10:913.
7. Gillespie S, Hawkey PM. Principles and Practice of Clinical Bacteriology. John Wiley and Sons; 2006.
8. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing, M100-S29. 29th ed. Clinical and Laboratory Standards Institute; 2019.
9. Al-Ouqaili MT, Al-Kubaisy SH. Crystalline biofilm produced by *Proteus mirabilis*: An overview on their formation assays and antimicrobial interaction. *Al Anbar J Med* 2008;6:33-42.

10. Al-Daraghi WA, Al-Badrwi MS. Molecular detection for nosocomial *Pseudomonas aeruginosa* and its relationship with multidrug resistance, isolated from hospitals environment. *Med Leg Updat* 2020;20:631-6.
11. Shilba AA, Al-Azzawi RH, Al-Awadi SJ. Dissemination of carbapenem resistant *Pseudomonas aeruginosa* among burn patients in Karbala Province/Iraq. *Iraqi J Sci* 2015;56:1850-7.
12. Ismail ST, Altaai MI. Study ndvB gene expression in *Pseudomonas aeruginosa* producing biofilm. *Med Leg Updat* 2021;21:961-5.
13. Al-Shamaa NF, Abu-Risha RA, Al-Faham MA. Virulence genes profile of *Pseudomonas aeruginosa* local isolates from burns and wounds. *Iraqi J Biotechnol* 2016;15:31-9.
14. Ammeter D, Idowu T, Zhanel GG, Schweizer F. Development of a nebramine-cyclam conjugate as an antibacterial adjuvant to potentiate β -lactam antibiotics against multidrug-resistant *Paeruginosa*. *J Antibiot (Tokyo)* 2019;72:816-26.
15. Yayan J, Ghebremedhin B, Rasche K. Antibiotic resistance of *Pseudomonas aeruginosa* in pneumonia at a single university hospital center in Germany over a 10-year period. *PLoS One* 2015;10:e0139836.
16. Pedersen SS, Høiby N, Espersen F, Koch C. Role of alginate in infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *Thorax* 1992;47:6-13.
17. El-Khashaah TH, Erfan DM, Kamal A, El-Moussely LM, Ismail DK. *Pseudomonas aeruginosa* biofilm formation and quorum sensing lasR gene in patients with wound infection. *Egypt J Med Microbiol* 2016;38:1-8.
18. Ghanbarzadeh Corehtash Z, Khorshidi A, Firoozeh F, Akbari H, Mahmoudi Aznavah A. Biofilm formation and virulence factors among *Pseudomonas aeruginosa* isolated from burn patients. *Jundishapur J Microbiol* 2015;8:e22345.
19. Hall-Stoodley L, Stoodley P. Evolving concepts in biofilm infections. *Cell Microbiol* 2009;11:1034-43.
20. Algburi A, Comito N, Kashtanov D, Dicks LM, Chikindas ML. Control of biofilm formation: Antibiotics and beyond. *Appl Environ Microbiol* 2017;83:e02508-16.