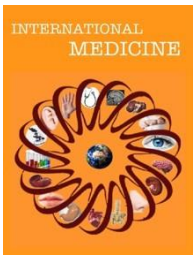
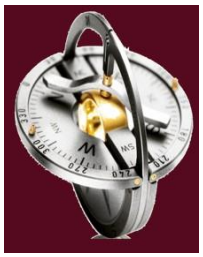
International Medicine 2024; 7(1): 12-15



International Medicine

https://www.theinternationalmedicine.com



Original Research

The Role of Biofilm Formation in Antibiotic Resistance of Multidrug-Resistant Pseudomonas aeruginosa

Maqsood Ali[1], Navid Iqbal[1], Md. Abdur Rakib[1], Kyung-Ah Lee[2], Mi-Hwa Lee[3], and Yong-Sik Kim[1*]

[1]*Department of Microbiology, College of Medicine, Soonchunhyang University, Cheonan 31151, Republic of Korea*
[2]*Saelounbio, Seoul, Republic of Korea*
[3]*Nakdonggang National Institute of Biological Resources, Sangju 37242, Republic of Korea*

Received: 28 Feb 2025 / Accepted: 20 Apr 2025

Abstract

Introduction The biofilm production retards the antimicrobial therapy against bacteria because the biofilm develops a barrier which reduces the drug penetration leading to treatment failure as well as hindering the recognition of the microorganisms by immune system. In view of the above mentioned facts, the objective of the current study was to phenotypically and genotypically evaluate the biofilm production ability of multi drug resistant Pseudomonas aeruginosa from clinical samples MATERIALS AND METHODS In this cross sectional study, 100 isolates of P. aeruginosa have been gathered from wound infections of burn patients admitted to Hospital. Each isolate has been determined due to the standard bacteriological methods including Gram staining, growth at 42°C in cetrimide agar, oxidation fermentation (OF), TSI, and oxidase tests. Negative stain of capsule Mucoid strains have been determined by the use of Anthony's capsule staining as the qualitative method. Briefly, for each of 100 isolates, a thin film of skim milk suspension has been prepared and air dried; the film has been flooded with crystal violet for 60 s. RESULTS Resistance levels detected in environmental PA isolates were as follows (in decreasing order): ciprofloxacin 50.25% (n = 82), levofloxacin 47.85% (n = 78), ceftazidime 34.57% (n = 56), cefepime 32.10% (n = 52), gentamicin 28.40% (n = 46), amikacin 22.84% (n = 38), meropenem 12.35% (n = 20), imipenem 11.11% (n = 18), and colistin 1.23% (n = 2; MIC > 2 mg/L). The resistotype distribution among environmental PA isolates is presented in Table 1. Overall, nineteen (I-XIX) different resistotypes were identified, with the most numerous resistotypes being IX (resistant to ciprofloxacin, levofloxacin, ceftazidime, and cefepime; n = 18; 10.98%) and X (resistant to ciprofloxacin, levofloxacin, gentamicin, and amikacin; n = 17; 10.37%). Of these respective isolates, 22.56% (n = 37) met the criteria to be MDR. CONCLUSION Pseudomonas aeruginosa is an opportunistic pathogen isolated in the patients admitted in hospitals. Formation of biofilm in Pseudomonas aeruginosa, which is often related with antibiotic resistance is a great concern. Our findings showed that there is a possibility of connection between drug resistance and biofilm development..

Keywords: *Pseudomonas aeruginosa; Antimicrobial resistance; Biofilm; Crystal violet.*

Introduction

Pseudomonas aeruginosa is a threatening and emerging public health problem throughout the world particularly in developing countries. It is still a main cause of mortality and morbidity in humans. It is ranked 4th among the nosocomial pathogens and difficult to treat due to its resistant behavior against different antibiotic drugs. [1] Pseudomonas aeruginosa is a foremost cause of nosocomial infections, including urinary tract infections, pneumonia and bacteremia. It is also found in patients having burns, surgical, pus and accidental wounds. These infections become severe when the patients have impaired immune system. Biofilms are structurally complex surface connected populations in which bacterial cells are enclosed by extra cellular polymeric substances (EPS) produced by their own self. These EPS are mostly exopolysaccharides, extracellular deoxyribonucleic acid and proteins. [2]

Biofilm formation contributes to pathogenesis of P. aeruginosa both in acute as well as chronic infection in clinical settings. [3]The bacteria residing in biofilms are much more resistant to antimicrobial agents and host immune response compared to their planktonic counterparts, leading to prolonged or chronic infections which are difficult to eradicate.[4] Biofilm forming bacteria produce couple of extracellular polymeric matrices which attached the bacterial community together within the biofilm.[5] The key components of biofilm matrix are polysaccharides because they contribute towards overall architecture of biofilm and make the bacterial population resistant to antibacterials. There are about three exopolysaccharides reported to be involved in P. aeruginosa biofilm formation which includes alginate, Psl, and Pel. Among these exopolysaccharides, Psl is a mannose-rich polymer having an essential role in preliminary steps of biofilm formation.[6].

Address for Correspondence Maqsood Ali, 1Department of Microbiology, College of Medicine, Soonchunhyang University, Cheonan 31151, Republic of Korea

.

DOI: 10.5455/im.98575

The biofilm production retards the antimicrobial therapy against bacteria because the biofilm develops a barrier which reduces the drug penetration leading to treatment failure as well as hindering the recognition of the microorganisms by immune system. In view of the above mentioned facts, the objective of the current study was to phenotypically and genotypically evaluate the biofilm production ability of multi drug resistant Pseudomonas aeruginosa from clinical samples.[7]

The most extreme case of decreased metabolism, also found in the biofilm, is represented by the persisters. [8,9]This is a special growth state that means less than 0.1% of the biofilm population, refractory to antibiotics, a kind of spore-like cell state activity that can become active after finishing the treatment. [10]

Materials and Methods

In this cross-sectional study, 100 isolates of P. aeruginosa have been gathered from wound infections of burn patients admitted to Hospital. Each isolate has been determined due to the standard bacteriological methods including Gram staining, growth at 42°C in cetrimide agar, oxidation fermentation (OF), TSI, and oxidase tests.

Furthermore, polymerase chain reaction (PCR) of exoA gene has been carried out to confirm the bacteriologic identification. A 397 bp fragment of the exoA gene has been selected with specified primers (forward: 5' GACAACGCCCTCAGCATCACCAGC 3,'reverse: 5' CGCTGGCCCATTCGCTCCAGCGCT 3').[23] Each PCR reaction was prepared in 20 µL volume include 10 µL the commercial Master Mix (containing Taq DNA polymerase, dNTPs, and MgCl2) (Ampliqon Denmark), 1 µL DNA sample, 0.5 µL of each primer (Metabion, Germany), and 8 µL distilled. Samples were then subjected to one cycle of 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 68°C for 30 s, and 72°C for 45 s and one final cycle of 72°C for 5 min. P. aeruginosa strain ATCC27853 (American Type Culture Collection) was included as the control.

Determination of mucoid strain

Negative stain of capsule Mucoid strains have been determined by the use of Anthony's capsule staining as the qualitative method. Briefly, for each of 100 isolates, a thin film of skim milk suspension has been prepared and air dried; the film has been flooded with crystal violet for 60 s. The slide has been gently rinsed with a 20% copper sulfate solution for capsule decolonization. The cell and background have been stained purple, and the capsule appears as a faint blue halo Quantification of alginate/biofilm production by microtiter method The P. aeruginosa isolates have been analyzed to quantify biofilm production using microtiter dish method. In this method, each strain has been grown overnight at 37°C in tryptic soy broth (TSB) including 0.25% glucose.

The cultures have been diluted 1:100 in TSB medium. The bacteria suspensions (125 µL) have been aliquoted into a 96 well polystyrene microtiter plate and inoculated for 24 h at 37°C without agitation. The wells have been washed three times with 300 µL distilled water; the attached bacteria have been fixed with absolute methanol for 10 min and finally stained with 125 µL of 0.1% crystal violet solution in water for about 10-15 min. After staining, the wells have been washed three times with distilled water to remove all nonadherent cells. The wells were destained with 125 µL of 30% acetic acid in water. A new sterile flat bottomed 96 well polystyrene microtiter plate was inoculated with 125 µL destaining solution in each well.

The absorbance of the destaining solution has been measured at 570 nm using an ELISA reader. Every experiment has been carried out in triplicate. As the control, the uninoculated medium was used. According to the optical density of each sample (ODi) and the negative control (ODc), the isolates have been categorized as strong (4× ODc < ODi), moderate(2×ODc< ODc).

Antibiotic susceptibility tests

Agar diffusion methods (Kirby-Bauer method) have been applied to determine the antibiotic susceptibility of isolated bacteria against TN (10 µg), aztreonam (ATM, 30 µg), imipenem (IMI, 10 µg), ceftazidime (CAZ, 30 µg), levofloxacin (LEV, 5 µg), piperacillin tazobactam (PTZ, 110 µg), and polymyxin B (PB, 300U). MDR, XDR, and PDR strains have been detected according to a new standardized international document.[26] P. aeruginosa (ATCC 27853) was used as a control strain.

Statistical analyses

Statistical Package for Social Sciences software (SPSS Inc. No. 23, Version 23.0. Armonk, NY: IBM Corp.) was used for statistical analyses. Fisher's exact test or Chi square test was used for the categorical data analysis. $P < 0.05$ was considered statistically significant.

Results

Resistance levels detected in environmental PA isolates were as follows (in decreasing order): ciprofloxacin 50.25% (n = 82), levofloxacin 47.85% (n = 78), ceftazidime 34.57% (n = 56), cefepime 32.10% (n = 52), gentamicin 28.40% (n = 46), amikacin 22.84% (n = 38), meropenem 12.35% (n = 20), imipenem 11.11% (n = 18), and colistin 1.23% (n = 2; MIC > 2 mg/L). The resistotype distribution among environmental PA isolates is presented in Table 1. Overall, nineteen (I-XIX) different resistotypes were identified, with the most numerous resistotypes being IX (resistant to ciprofloxacin, levofloxacin, ceftazidime, and cefepime; n = 18; 10.98%) and X (resistant to ciprofloxacin, levofloxacin, gentamicin, and amikacin; n = 17; 10.37%). Of these respective isolates, 22.56% (n = 37) met the criteria to be MDR.

Table 1. Resistotype distribution and MAR indices of environmental PA isolates.

Resistotype	Resistance Pattern	No. of Isolates	Percentage (%)
I	Cip, Lev	8	4.88
II	Cip, Lev, Gen	10	6.10
III	Cip, Lev, Caz	12	7.32
IV	Cip, Lev, Fep	9	5.49
V	Cip, Lev, Ami	11	6.71
VI	Cip, Lev, Mem	7	4.27
VII	Cip, Lev, Imp	6	3.66
VIII	Cip, Lev, Col	4	2.44
IX	Cip, Lev, Caz, Fep	18	10.98
X	Cip, Lev, Gen, Ami	17	10.37

AmpC-Overexpression, Carbapenemase-Production, and Overexpression of Efflux Pumps in Environmental PA Isolates

AmpC overexpression was detected in 58.49% (n = 31 out of 53 isolates; 19.21% overall) of ceftazidime-resistant isolates. Carbapenemase production was detected using the modified Hodge test: the test was positive in 30.00% (n = 7, out of 23 isolates meeting inclusion criteria; 4.32% overall) of cases. Overexpression of RND-type efflux pumps was noted in 63.41% (n = 52, out of 82 isolates; 29.26% overall). Simultaneous detection of resistance mechanisms was as follows: efflux pump and AmpC overexpression in n = 16 isolates, efflux pump overexpression and cloverleaf-test positivity in n = 2 isolates, cloverleaf-test positivity and AmpC overexpression in n = 2 isolates, while detection of all three mechanisms was seen in n = 3 isolates, respectively.

Biofilm-Forming Capacity and the Relationship with Phenotypic Expression of Virulence Factors

The biofilm-forming capacity was assessed using a microplate-based assay. Based on the CV-assay, 24.07% (n = 40) of isolates were weak/non-biofilm producers, 29.63% (n = 49) were moderate biofilm producers, while 46.30% (n = 77) were strong biofilm producers,

respectively. No significant differences in biofilm-production were shown among environmental PA isolates (OD570 values non-MDR [mean ± SD]: 0.432 ± 0.172 vs. MDR: 0.410 ± 0.189; p > 0.05).

Table 2. Relationship between biofilm-formation and motility in environmental PA isolates.

Motility Type	Non-MDR (Mean ± SD)	MDR (Mean ± SD)	p-Value
Swimming	25.12 ± 7.41	23.67 ± 8.09	>0.05
Swarming	29.01 ± 6.32	27.44 ± 6.15	>0.05
Twitching	11.02 ± 2.50	10.21 ± 2.91	>0.05

Table 3. Relationship between biofilm-formation and siderophore production in environmental PA isolates.

Biofilm Production Level	Siderophore Positive (n)	Siderophore Negative (n)	Percentage Positive (%)
Weak/Non-Biofilm	32	8	80.00
Moderate Biofilm	41	8	83.67
Strong Biofilm	69	8	89.61

The majority of isolates (n = 142; 85.18%) tested were positive for siderophore production. No statistical association was shown between biofilm-production levels and siderophore production (p > 0.05).

Discussion

Development of resistance by P. aeruginosa to many antimicrobial agents is a great challenge in controlling its infections [11]. Comprehensive surveillance of antimicrobial resistance in European countries for 2017 demonstrated a range of combined resistance (resistance to three or more antimicrobial groups, including piperacillin ± tazobactam, ceftazidime, fluoroquinolones, aminoglycosides and carbapenems) from 0% (Iceland) to 59.1% (Romania) [12]. The prevalence of MDR P. aeruginosa in Iran has been estimated at 58%, with a variation in geographical areas: the highest and lowest rates were observed in Tehran (100%) and Zahedan (16%), respectively [13]. In a recent study, Bavasheh et al. [14] found that 27.8% of clinical P. aeruginosa isolates were MDR. Similarly, the rate of isolates with resistance to at least three antimicrobial groups in our study was 20% that was lower than that reported from other studies [15]. Although the rate of multi-resistance in the present study was relatively low, this may be an alarming situation that reflects a threat limiting treatment options in therapeutic centers studied.

Similar to the results of other studies [16], a significant number of included isolates (83.75%) formed biofilm. The present study revealed a high prevalence of algD, pslD, and pelF genes, being presented simultaneously in a considerable proportion (87.5%) of P. aeruginosa isolates, a finding that are similar to those found by Banar et al. [17]. Other genes associated with biofilm formation, such as pslA and pelA were detected by Ghadaksaz et al. [18] with a frequency of 83.7% and 45.2%, respectively, and Pournajaf et al. [19] with a frequency of 89.5% and 57.3%, respectively among P. aeruginosa clinical isolates. However, little data is available about the prevalence rate of pslD and pelF genes in different regions of the world.

In agreement with other studies [19], our results revealed a significant correlation between the biofilm forming capacity and the presence of relevant genes (p-value < 0.0001). About 88.06% of 67 biofilm producer isolates showed algD +/pslD +/pelF + genotypic pattern, while 11.94% were algD −/pslD −/pelF −. On the other hand, 84.61% of 13 non-biofilm producers carried biofilm genes. The capacity of biofilm production despite the absence of biofilm genes studied indicates other genetic determinants of biofilm participate in matrix formation in P. aeruginosa [20]. By contrast, the presence of genes without biofilm production may be due to chromosomal mutations in different regulatory systems, affecting the production of functional biofilm-associated proteins. Hou et al. [21] reported that no P. aeruginosa isolates were phenotypically positive for biofilm formation in Congo red agar and microtiter plate assays, whereas 31.03% of isolates contained the pslA gene. Conformational changes in quorum sensing proteins due to mutations in lasI/lasR and rhlI/rhlR systems were suggested in previous studies [22] as the reason why these isolates are unable to produce biofilm.

According to the results of this study, P. aeruginosa that produced biofilm and also those carried biofilm-associated genes were mainly considered as non-MDR. This may cause a misunderstanding in the first place that biofilm production is not related to antibiotic resistance. It is noteworthy that all isolates in our study were subjected to antimicrobial susceptibility testing as planktonic cells and not in biofilm form. Thus, multiple mechanisms of biofilm and its architectural features, including glycocalyx matrix, outer membrane structure, heterogeneity in metabolism and growth rate, persister cells formation, genetic adaptation, stress responses as well as quorum sensing conferring MDR phenotype were not involved [22].

Conclusion

Pseudomonas aeruginosa is an opportunistic pathogen isolated in the patients admitted in hospitals. Formation of biofilm in Pseudomonas aeruginosa, which is often related with antibiotic resistance is a great concern. Our findings showed that there is a possibility of connection between drug resistance and biofilm development. Since the isolates are antibiotic resistant, there is often shifting in choice of antibiotics which further increases the problem of global Antimicrobial resistance. Since, most of the drug-resistant isolates are shown to be biofilm formers, the combination therapy based on antibiotic treatment along with antibiofilm agents can be used in treating the biofilm associated Pseudomonal infections. There should be effective execution of infection control practices in hospitals to control the spread of nosocomial pathogens like P.aeruginosa. Prudent antibiotic usage, detection of biofilm formation and high standards of hospital infection control practices aid in combating the Pseudomonas aeruginosa infection as well as preventing the development of resistant strains.

References

1. Bukholm G, Tannaes T, Kjelsberg AB, Smith-Erichsen N. An outbreak of multidrug-resistant Pseudomonas aeruginosa associated with increased risk of patient death in an intensive care unit. Infect Control Hosp Epidemiol. 2002;23:441-6.
2. Colvin KM, Gordon VD, Murakami K, Borlee BR, Wozniak DJ, Wong GC, et al. The pel polysaccharide can serve a structural and protective role in the biofilm matrix of Pseudomonas aeruginosa. PLoS Pathog. 2011;7:e1001264.
3. Douraghi M, Ghasemi F, Dallal MMS, Rahbar M, Rahimiforoushani A. Molecular identification of Pseudomonas aeruginosa recovered from cystic fibrosis patients. J Prev Med Hyg. 2014;55:1-3.
4. Ghadaksaz A, Fooladi AAI, Hosseini HM, Amin M. The prevalence of some Pseudomonas virulence genes related to biofilm formation and alginate production among clinical isolates. J Appl Biomed. 2014;13:61-8.
5. Davies JC. Paediatric Respiratory Reviews: Pseudomonas aeruginosa in Cystic Fibrosis: Pathogenesis and Persistence. Paediatr Respir Rev. 2002; 3(2):128-34.
6. Boisvert AA, Cheng MP, Sheppard DC and Nguyen D. Microbial Biofilms in Pulmonary and Critical Care Diseases. Ann Am Thorac Soc. 2016; 13(9):1615- 1623.
7. Strohl WA, Rouse H, Fisher DB. Lippincott, s illustrated reviews: Microbiology. 3rd Ed. Baltimore, Lippincott Williams and Wilkins; 2013: 137-139.
8. Fraise AP, Lambert PA, Maillard JY. Principles and Practice of Disinfection, Preservation and Sterilization. USA, Blackwell; 2004: 556-619.
9. Aloush V, Navon-Venezia S, Seigman-Igra Y, Cabili S, Carmeli Y. Multidrugresistant Pseudomonas aeruginosa: risk factors and clinical impact. Antimicrob. Agents Chemother. 2006; 50: 43-48.
10. Dash M, Padhi S, Narasimham MV, Pattnaik S. Antimicrobial resistance pattern of Pseudomonas aeruginosa isolated from various clinical samples in a tertiary care hospital, South Odisha, India. Saudi J Health Sci. 2014; 3:15-9.
11. Sambrook J, Russel DW. Molecular Cloning: A Laboratory Manual. 3rd Ed. New York, Cold Spring Harbor; 2001: 1- 4.

12. Da Costa Lima JL, Alves LR, Pereira da Paz JN, et al. Analysis of biofilm production by clinical isolates of Pseudomonas aeruginosa from patients with ventilator-associated pneumonia. Rev Bras Ter Intensiva. 2017; 29(3): 310-316.
13. Faraji F, Mahzounieh M, Ebrahimi AO, et al. Molecular detection of virulence genes in Pseudomonas aeruginosa isolated from children with Cystic Fibrosis and burn wounds in Iran. Microbial Pathogenesis. 2016; 99:1-4.
14. Saeed M, Rasheed F, Afzal RK et al. Pseudomonas aeruginosa: Evaluation of Pathogen Burden and Drug-Resistance Trends in a Tertiary Care Hospital. Journal of the College of Physicians and Surgeons Pakistan. 2018; 28(4): 279-283.
15. Unan D, Gnseren F. The resistance of P. aeruginosa strains isolated from nosocomial infections against various antibiotics. Mikrobiyol Bult. 2000; 34: 255-60.
16. Corehtash ZG, Khorshidi A, Firoozeh F, Akbari H, Aznaveh AM. Biofilm Formation and Virulence Factors Among Pseudomonas aeruginosa Isolated From Burn Patients. Jundishapur J Microbiol. 2015; 8(10): e22345.
17. Hoque MM, Ahmad M, Khisa S, Uddin MN, Jesmine R . Antibiotic Resistance Pattern in Pseudomonas Aeruginosa Isolated from Different Clinical Specimens. JAFMC Bangladesh. 2015; 11(1):45-49.
18. Direkel Ş, Uzunoglu E, Uzalp Ç, Findik E, Tontak S, and Ahmadli C. Determination of Piperacillin/Tazobactam and Ticarcillin/Clavulanate Susceptibilities in Pseudomonas aeruginosa Isolates in Hospitalised Patients by E-test Gradient Method and Comparison of Results with Disk Diffusion Tests. Clin Microbiol. 2017; 6:1.
19. Abidi SH, Sherwani SK, Siddiqui TR, Bashir A, and Kazmi SU. Drug resistance profile and biofilm forming potential of Pseudomonas aeruginosa isolated from contact lenses in Karachi-Pakistan. BMC Ophthalmol. 2013; 13: 57.
20. Yekani M, Memar MY, Alizadeh N, Safaei N, Ghotaslou R. Antibiotic Resistance Patterns of Biofilm-Forming Pseudomonas aeruginosa Isolates from Mechanically Ventilated Patients. International Journal of Scientific Study. 2017; 5(5): 1-5.
21. Costa Limaa JLD, Alvesb LR, Araújo Jacoméb PRL et al. Biofilm production by clinical isolates of Pseudomonas aeruginosa and structural changes in LasR protein of isolates non biofilm-producing. braz j infect dis. 2018;2 2(2):129-136.
22. Pournajaf A, Razavi S, Irajian G, et al. Integron types, antimicrobial resistance genes, virulence gene profile, alginate production and biofilm formation in Iranian cystic fibrosis Pseudomonas aeruginosa isolates. Le Infezioni in Medicina. 2018; 3: 226- 236