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Biofilm Formation and Planktonic Antimicrobial Susceptibility of *Pseudomonas aeruginosa* Isolates from Ventilator-associated Pneumonia Patients: A Pilot Study

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ABSTRACT

Background and Aim: Ventilator-associated pneumonia (VAP) is a severe infection occurring in mechanically ventilated ICU patients with *Pseudomonas (P.) aeruginosa* being the leading etiology. This pathogen frequently develops resistance to multiple antibiotics through biofilm formation. Hence, this study aims to evaluate the association between *in-vitro* biofilm production and antimicrobial susceptibility of *P. aeruginosa* in the planktonic state.

Materials and Methods: This pilot study analyzed seven *P. aeruginosa* strains isolated from endotracheal aspirates of VAP patients at Dr. Soetomo General Hospital, Surabaya, Indonesia using Gram staining, oxidase testing, and VITEK® 2 system. Biofilm production was assessed via crystal violet staining, and antimicrobial susceptibility was determined by broth microdilution method. Statistical analysis was performed with the Mann-Whitney nonparametric test using GraphPad Prism 9 software.

Results & Conclusion: Significant differences were observed in biofilm production and antimicrobial susceptibility of *P. aeruginosa* isolates from VAP patients against ceftazidime, piperacillin-tazobactam, cefepime, aztreonam, levofloxacin, and piperacillin. Strong biofilm producers showed higher resistance, especially to ceftazidime, piperacillin-tazobactam, and piperacillin, while weak biofilm producers had minimal resistance, except to ciprofloxacin, ticarcillin-clavulanate, and levofloxacin. Colistin showed the MIC values of ≤2 µg/mL against all isolates. Our findings indicated biofilm formation as a critical factor influencing resistance, emphasizing the need to consider biofilm-producing capacity when selecting treatment strategies for VAP caused by *P. aeruginosa*.

Keywords: Antimicrobial Resistance, Biofilm, Indonesia, *Pseudomonas aeruginosa*, Ventilator-associated Pneumonia

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1. Introduction

Ventilator-associated pneumonia (VAP) is the most common infection among patients undergoing mechanical ventilation in the intensive care unit (ICU) for at least 48 hr after intubation **(1)**. The incidence rate of VAP ranged between 15-60%, with notably high rates being reported in Europe (42.7% in ICU patients) and USA (39% of all pneumonia cases) **(2, 3)**.

VAP increases the risk of death in patients on mechanical ventilation by eightfold, making it a critical cause of mortality in intensive care units **(4-6)**. Although Indonesia lacks national surveillance data, single-center reports have revealed high mortality rates, such as 33% in Surabaya **(7)**, 57.2% in Jakarta **(8)**, and up to 76.6% in Bandung **(9)**.

A major cause of VAP infections is *Pseudomonas (P.) aeruginosa*, often found in medical devices such as ventilators, due to its ability to thrive on moist surfaces **(10)**. A systematic review on studies conducted during the COVID-19 pandemic revealed that *P. aeruginosa* was the most frequent causative pathogen of VAP (7.5-72.5%), followed by *Klebsiella pneumoniae* (6.9-43.7%) **(11, 12)**. *Pseudomonas aeruginosa* can develop resistance to multiple antibiotic classes, with a meta-analysis revealing the overall prevalence of multidrug-resistant (MDR) strains at 33% **(2)**. The highest prevalence has been reported in Iran at 87.5% **(13)**, while the lowest was in the United States at 19.7% **(14, 15)**. Therefore, continuous evaluation and careful selection of the antibiotic therapy are essential, as they significantly impact patient outcomes.

Additionally, *P. aeruginosa* is known to be a highly resilient organism, even in extreme conditions, due to its ability to form biofilms—a complex three-dimensional structure of bacteria within an extracellular matrix or polymeric substance composed of polysaccharides, nucleic acids, and proteins. Biofilms possess unique properties like cell-cell communication, genetic exchange, and a protective barrier that shields bacteria from desiccation, immune responses, and antimicrobial agents; making biofilm-associated infections difficult to treat due to enhanced resistance and reduced antibiotic penetration **(16)**.

Typically, antimicrobial susceptibility testing is conducted with bacteria in their planktonic form, overlooking the biofilm characteristics that may lead to treatment failure in VAP cases **(17)**. Determination of minimum inhibitory concentration (MIC) is the standard method for assessing a compound ability to inhibit microbial growth, which is effective in treating many acute infections. However, therapies based solely on MIC may be ineffective for the chronic or device-associated infections involving biofilms. This study aimed to investigate whether the antimicrobial susceptibility of planktonic *P. aeruginosa* is associated with its capacity for *in-vitro* biofilm formation. We hypothesize that stronger biofilm-producing isolates exhibit greater antimicrobial resistance in their planktonic form.

2. Materials and Methods

2.1 Study Design and Data Collection

Endotracheal aspirate specimens were collected from patients presenting with the clinical signs of VAP across the study period (November 2023 to January 2024), in which the samples were transported to the Clinical Microbiology Laboratory of Dr. Soetomo General Hospital. VAP is defined according to the 2016 American Thoracic Society (ATS) Guideline as pneumonia case that occurs following endotracheal intubation for ≥48 hr **(18)**.

2.2 Isolation and Identification of Bacteria

Clinical endotracheal aspirate specimens were collected in sterile containers and immediately transported to the laboratory under refrigerated conditions. Specimens were firstly assessed for adequacy of the quantity and quality before further processing. Samples were processed within 2 hr of collection to ensure viability. Each sample was processed using the BD BACTEC™ semiautomated system (Becton, Dickinson, and Company, USA), which allows for rapid bacterial isolation and identification. Following incubation at 37°C for 24–48 hr, colonies exhibiting characteristic morphology, such as green pigmentation and grape-like odor, were confirmed as *P. aeruginosa* using Gram staining, oxidase testing, and biochemical profiling with VITEK® 2 automated identification system (bioMérieux, France).

2.3 Biofilm Quantification

Biofilm formation by the isolated *P. aeruginosa* strains was assessed using crystal violet (CV) staining method in a 96-well microtiter plate format. Overnight bacterial cultures were diluted in tryptic soy broth (TSB) supplemented with 1% glucose to an optical density (OD) of 0.1 at 600 nm. Aliquots of 200 µL were inoculated into the wells and incubated at 37°C for 24 hr. Wells were washed three times with phosphate-buffered saline (PBS) to remove non-adherent cells, fixed with methanol, and stained with 0.1% CV. Excess stain was removed by washing, and the dye retained by the biofilm was solubilized with 95% ethanol. The OD of each well was measured at 570 nm using a

microplate reader. Biofilm production was categorized as weak, moderate, and strong based on the optical density cut-off value (ODc), calculated as the sum of the average OD of the negative control and three times its standard deviation. The final OD value of each strain was presented as the average OD value of each sample reduced by ODc value. Based on the results obtained, biofilm producing capacity was divided into several categories: no biofilm producer if true OD <0.5; weak biofilm producer if 0.5≤ OD<2, and strong biofilm producer if OD≥2.

2.4 Antimicrobial Susceptibility Testing

The antimicrobial susceptibility of planktonic *P. aeruginosa* isolates was determined using broth microdilution method to establish MIC by adhering to the 2020 M100 Clinical and Laboratory Standards Institute (CLSI) guideline **(19)**. The following antibiotics were tested: ceftazidime, piperacillin-tazobactam, tobramycin, cefepime, meropenem, aztreonam, ciprofloxacin, levofloxacin, piperacillin, ticarcillin-clavulanate, and colistin. Inocula were prepared by suspending bacterial colonies in Mueller-Hinton broth to achieve a turbidity of 0.5 McFarland as standard. Serial dilutions of each antibiotic were prepared in a 96-well microtiter plate, and 100 µL of the bacterial suspension was added to each well. Plates were incubated at 37°C for 16–20 hours, and MICs were defined as the lowest concentration of the antibiotic that inhibited visible growth.

2.5 Statistical Analysis

OD of biofilms and MIC results of planktonic strains were extracted to Microsoft Excel for Mac version 16.89.1 (©Microsoft 2024, WA) and analyzed with Prism 9 for Mac version 9.5.1 (© 1994-2023 GraphPad Software, LLC). Clinical characteristics, antimicrobial susceptibility, and biofilm producing capacity of isolates were presented in frequency tables. Differences between biofilm production, represented as optical density values, and antimicrobial susceptibility was conducted using the Mann-Whitney nonparametric test. Statistical significance was determined at P-value less than 0.05.

3. Results and Discussion

The clinical characteristics of the cohort (n = 7) indicate a predominance of males (71.4%) with a mean age of 49 ±10.79 years. Ventilator-associated pneumonia (VAP) onset was categorized as early (<5 days) in 42.9% and late (≥5 days) in 57.1% of cases. More than half of the patients had prior sepsis (57.1%), recent reintubation (57.1%), or a history of chronic diseases (42.9%). Recent surgical interventions were noted in 42.9% of the cohort. The average length of ICU stay was 13.29±3.35 days. Clinical outcomes included a mortality rate of 71.4%, with 28.6% of patients transferred to the general ward (Table 1).

The antimicrobial susceptibility profile of *P. aeruginosa* isolates from VAP patients demonstrate variable efficacy across different typical antipseudomonas agents. All isolates exhibited colistin MIC values of ≤2 µg/mL; while no susceptible category was defined by CLSI. These values fall below the threshold for resistance (≥4 µg/mL), suggesting preserved activity *in vitro*. High sensitivity rates were observed for tobramycin (5/7; 71.43%) and meropenem (5/7; 71.43%). Conversely, notable resistance was observed against ciprofloxacin and ticarcillin-clavulanate, with susceptibility rates of only 28.57% (2/7). Beta-lactam agents, including ceftazidime, piperacillin-tazobactam, and piperacillin, exhibited moderate susceptibility (4/7; 57.14%). Lower sensitivity percentage was observed among other typical antipseudomonas agent including cefepime, levofloxacin, and aztreonam at only 42.86% (3/7). These findings highlight the need for judicious antimicrobial selection guided by susceptibility testing to optimize treatment outcomes in VAP caused by *P. aeruginosa* (Table 2).

Distribution of antimicrobial resistance in *P. aeruginosa* isolates based on their biofilm-producing capacity (weak, moderate, and strong) is presented in Table 3. Resistance was most prevalent among the isolates with strong biofilm production, particularly for ceftazidime (CAZ), piperacillin-tazobactam (TZP), and piperacillin (PIP), where 66.7% (2/3) of resistant isolates were strong biofilm producers. Moderate biofilm producers accounted for 33.3–50% of resistance across several antimicrobials, while weak biofilm producers contributed minimally to resistance, except for ciprofloxacin (CIP), ticarcillin-clavulanate (TIC), and levofloxacin (LEV). Colistin (COL) exhibited no resistance across all biofilm capacities, underscoring its efficacy. These results suggest the association between biofilm production and antimicrobial resistance, emphasizing the challenge of treating biofilm-forming isolates.

The association between antimicrobial resistance and biofilm production in *P. aeruginosa* isolates quantified by optical density (OD) values are demonstrated in Table 4. Statistically significant differences of biofilm production among sensitive and intermediate/resistant isolates were observed for ceftazidime (CAZ), piperacillin-tazobactam (TZP), cefepime (FEP), aztreonam (AZM), levofloxacin (LEV), and piperacillin (PIP). These findings suggest that biofilm production capacity may be associated with specific changes of resistance profile of antipseudomonas antimicrobials.

The predominance of strong biofilm producers among resistant isolates highlights the challenge that biofilms pose in VAP management. While the link between biofilm formation and resistance has been established in

global studies, our data provides the first insight into Indonesian VAP isolates. Our results serves as pilot evidence of concerning prevalence in intermediate or resistant *P. aeruginosa* isolates to commonly used antipseudomonal agents in Indonesia, particularly ciprofloxacin, ticarcillin-clavulanate, cefepime, aztreonam, and levofloxacin. The Indonesian Ministry of Health 2023 regulation recommends using two antipseudomonal agents from different classes for empirical treatment of suspected VAP in patients with risk factors for drug resistance or those in units or ICUs where Gram-negative bacterial resistance to monotherapy exceeds 10%. For patients without resistance risk factors, a single antipseudomonal agent is advised if resistance among Gram-negative bacteria is below 10% **(20)**. A report from the ICU of Dharmais Cancer Hospital in Depok, Jakarta, Indonesia reflects these practices, reporting meropenem as the first-line empirical agent and a combination of meropenem and levofloxacin as the preferred regimen for the suspected VAP cases **(21)**.

In comparison, evidence from Nigeria reported that out of 40 *P. aeruginosa* isolates, 50% were resistant to gentamicin, while approximately 30% showed resistance to imipenem, aztreonam, and cefepime **(22)**. All isolates were also resistant to other cephalosporins, penicillin, ciprofloxacin, and nitrofurantoin. Another study validated the link between biofilm formation and carbapenem resistance. In a Korean hospital, 88% of carbapenem-resistant *P. aeruginosa* isolates exhibited strong biofilm formation and resistance to amikacin, ceftazidime, and cefepime, with biofilm formation identified as the primary resistance driver in 81% of isolates lacking plasmid-encoded carbapenemase genes, such as blaIMP or blaVIM **(23)**.

Our findings indicate interplay between the role of biofilm production in antimicrobial resistance, with 80% of ciprofloxacin- and ticarcillin-clavulanate-resistant isolates and 100% of cefepime- and aztreonam-resistant isolates demonstrating moderate to strong biofilm-forming capacities (Table 3). Previous research corroborates these findings, showing that biofilms exposed to sub-inhibitory ciprofloxacin concentrations develop higher resistance levels, while premature antibiotic discontinuation enables persistent populations to regrow and form biofilms **(24)**.

Interestingly, resistance to levofloxacin was associated with weaker biofilm production, with half of the isolates categorized as weak biofilm producers (Tables 3 and 4). Hence, despite levofloxacin-resistant isolates mostly demonstrating weak biofilm production, the biofilm itself is a significant factor for its resistance while indirectly suggest the involvement of alternative resistance mechanisms at play. Notably, a recent study showed that *P. aeruginosa* had the capability to yield heteroresistance against levofloxacin, a condition in which small subpopulations exhibit higher resistance levels than the main bacterial population **(25)**. The study suggests that *P. aeruginosa* heteroresistance to levofloxacin is not associated with biofilm formation, as levofloxacin down-regulates biofilm-related genes in certain strains (PAS71 and PAS81), reducing biofilm yield **(25)**. Instead, heteroresistance is linked to other mechanisms, including the up-regulation of essential genes involved in DNA repair, replication, homologous recombination, and virulence pathways. The study revealed that both strains exhibit similar resistance response, highlighting that heteroresistance is driven by these genetic and metabolic adaptations rather than biofilm production **(25)**.

Furthermore, a study from Greece demonstrated that levofloxacin at concentrations of 11–25 µg/mL achieved a time-kill effect within 4 hr of incubation in 53.2% of MDR *P. aeruginosa* derived from VAP patients, with MIC_{50}/MIC_{90} values of 16/64 µg/mL **(26)**. The authors challenged the notion that levofloxacin should be excluded from VAP treatment when the MIC exceeds 2 µg/mL, as they further observed synergy within 4 hr of incubation between levofloxacin and imipenem (58.6%) and levofloxacin and colistin (84.8%). Notably, these synergistic effects were independent of the MIC values **(26)**.

These findings collectively underscore the multifaceted nature of levofloxacin resistance, driven by both synergistic interactions and genetic adaptations beyond biofilm formation. However, future molecular analyses (e.g. biofilm gene expression, efflux pump profiling etc.) are warranted to clarify these mechanisms.

Despite valuable insights provided in this study, we acknowledge its inherent limitations. First, the small sample size of only seven patients significantly hampers the statistical power and generalizability, underscoring the need for larger-scale cohort studies. We therefore describe our results as pilot evidence that moderates our conclusions accordingly. Second, the evaluation of associations between biofilm production and antibiotic resistance was conducted using a bivariate nonparametric test. While this approach has been previously employed by Macias-Valcayo et al **(27)** in Gram-negative bacilli isolated from prosthetic joint infections, their study found no significant association between biofilm production and antibiotic resistance, although MDR isolates tended to form more substantial biofilms than non-resistant isolates **(27)**. Third, the study only lacks evidence indicating molecular insights into the mechanism linking biofilm production and resistance as well as not able to include other key resistance mechanisms, such as efflux pumps and genetic adaptations. Additionally, the *in vitro* biofilm quantification using optical density may not fully reflect clinical biofilm behavior, and complementary methods are recommended. The absence of correlations between

biofilm production, resistance profile, and clinical outcomes limits the practical applicability of the findings.

Standardized MDR/XDR classifications for the isolates could be applied in future studies as it would strengthen the statistical analysis and clinical implication. Variability in local resistance trends and laboratory methods further restricts the reproducibility of the results. Lastly, while combination therapies involving levofloxacin and colistin were mentioned, their effects on biofilm-associated resistance were not explored, highlighting an area for future investigation. These limitations underscore the need for more comprehensive research to deepen our understanding of biofilm-associated resistance and its clinical implications.

Table 1. Baseline characteristics of the study cohort (N=7).

Characteristics		N (%)
Sex	Male	5 (71.4%)
	Female	2 (28.6%)
Age (y), mean±SD		49±10.79
Onset of VAP	Early onset (<5 d)	3 (42.9%)
	Late onset (≥5 d)	4 (57.1%)
	Prior sepsis	4 (57.1%)
	Recent surgery	3 (42.9%)
	Reintubation	4 (57.1%)
	Presence of other chronic disease	3 (42.9%)
Length of ICU stay (d), mean±SD		13.29±3.35
Clinical outcome	Death	5 (71.4%)
	Transfer to general ward	2 (28.6%)

Table 2. Antimicrobial susceptibility profile of *P. aeruginosa* isolates from VAP patients.

Antimicrobial	MIC	
	S	I/R
Ceftazidime (CAZ)	4 (57.14%)	3 (42.86%)
Piperacillin-tazobactam (TZP)	4 (57.14%)	3 (42.86%)
Tobramycin (TOB)	5 (71.43%)	2 (28.57%)

Cefepime (FEP)	3 (42.86%)	4 (57.14%)
Meropenem (MEM)	5 (71.43%)	2 (28.57%)
Aztreonam (AZM)	3 (42.86%)	4 (57.14%)
Ciprofloxacin (CIP)	2 (28.57%)	5 (71.43%)
Levofloxacin (LEV)	3 (42.86%)	4 (57.14%)
Piperacillin (PIP)	4 (57.14%)	3 (42.86%)
Ticarcillin-clavulanate (TIC)	2 (28.57%)	5 (71.43%)
Colistin (COL)†	7 (100%)	0

†Susceptibility breakpoints for colistin against *P. aeruginosa* are not defined in the CLSI M100 guidelines. Therefore, isolates denoted as "S" in this table represent those with MIC values ≤2 µg/mL, reported descriptively and not as categorical susceptibility. MIC: Minimum inhibitory concentration; S: sensitive; I: intermediate; R: resistant

Table 3. Distribution of antimicrobial resistant isolates across different biofilm producing capacity.

Antimicrobial	I/R	Biofilm Producing Capacity		
		Weak	Moderate	Strong
Ceftazidime (CAZ)	3	0	1 (33.3%)	2 (66.7%)
Piperacillin-tazobactam (TZP)	3	0	1 (33.3%)	2 (66.7%)
Tobramycin (TOB)	2	0	1 (50%)	1 (50%)
Cefepime (FEP)	4	0	2 (50%)	2 (50%)
Meropenem (MEM)	2	0	1 (50%)	1 (50%)
Aztreonam (AZM)	4	0	2 (50%)	2 (50%)
Ciprofloxacin (CIP)	5	1 (20%)	2 (40%)	2 (40%)
Levofloxacin (LEV)	4	2 (50%)	1 (25%)	1 (25%)
Piperacillin (PIP)	3	0	1 (33.3%)	2 (66.7%)
Ticarcillin-clavulanate (TIC)	5	1 (20%)	2 (40%)	2 (40%)
Colistin (COL)	0	N/A	N/A	N/A

N/A: not available

Table 4. Association between antimicrobial resistance and biofilm production represented in optical density values.

Antimicrobial	Biofilm Production (OD) Across Different Antimicrobial Susceptibility Profile, (Median [IQR])		P-value
	S	I/R	
Ceftazidime (CAZ)	0.94 (0.53-1.44)	2.58 (1.56-3.02)	0.034*
Piperacillin-tazobactam (TZP)	0.94 (0.53-1.44)	2.58 (1.56-3.02)	0.034*
Tobramycin (TOB)	1.34 (0.53-2.58)	2.23 (1.44-3.02)	0.245
Cefepime (FEP)	0.54 (0.53-1.34)	2.07 (1.44-3.02)	0.034*
Meropenem (MEM)	1.43 (0.53-2.58)	2.18 (1.34-3.02)	0.439
Aztreonam (AZM)	0.54 (0.53-1.34)	2.07 (1.44-3.02)	0.034*
Ciprofloxacin (CIP)	0.94 (0.53-1.34)	1.56 (0.54-3.02)	0.121
Levofloxacin (LEV)	1.56 (0.53-2.58)	1.39 (0.54-3.02)	0.034*
Piperacillin (PIP)	0.94 (0.53-1.44)	2.58 (1.56-3.02)	0.034*
Ticarcillin-clavulanate (TIC)	0.99 (0.54-1.44)	1.56 (0.53-3.02)	0.439
Colistin (COL)	1.44 (0.53-3.02)	N/A	N/A

***Statistical significance (*P*<0.05)**
N/A: not available

5. Conclusion

Our pilot study indicates that increased biofilm production by *P. aeruginosa* isolates from VAP patients is associated with higher resistance to several key antipseudomonal agents, particularly ceftazidime, piperacillin-tazobactam, and piperacillin, while weak biofilm formers showed resistance mainly to ciprofloxacin, ticarcillin-clavulanate, and levofloxacin. Colistin exhibited no resistance across all biofilm capacities. These findings suggest biofilm formation may be an important contributor to resistance phenotypes in VAP, and underscore the value of incorporating biofilm assessment into antimicrobial stewardship decisions. Future research should validate these observations in larger cohorts and explore molecular mechanisms to inform targeted therapeutic strategies against biofilm-associated resistance.

6. Declarations

6.1 Acknowledgment

The authors acknowledge the contributions of the laboratory analysts and research assistants for their diligent efforts during sample preparation and data collection. Their technical expertise and commitment were instrumental in ensuring the accuracy and reliability of the study findings.

6.2 Ethical Considerations

The protocol for this study was reviewed and approved by the Health Research Ethics Committee of Dr. Soetomo General Hospital, Surabaya, Indonesia, under reference number 1810/LOE/301.4.2/X/2024. In accordance with recommendation of the ethics committee, the study exclusively utilized isolates of *Pseudomonas aeruginosa* obtained from clinical samples of patients with ventilator-associated pneumonia (VAP). As the study did not involve direct interaction with human subjects or identifiable personal data, individual consent was not required.

6.3 Authors' Contributions

Conceptualization: MOS, SUK, IAM, PBD, LAI; Data curation: MOS, SUK, IAM, PBD; Formal data analysis: MOS, PBD; Funding acquisition: MOS, SUK, IAM, LAI; Investigation: MOS, SUK, IAM, PBD; Methodology: MOS, SUK; Project administration: MOS, SUK, LAI; Resources: MOS; Software: PBD; Supervision: SUK, IAM; Validation: PBD; Visualization: PBD; Writing-original draft: MOS, PBD; Writing-review & editing: SUK, IAM, PBD, LAI. All authors have read and approved the final manuscript.

6.4 Conflict of Interests

The authors declare no conflict of interest.

6.5 Financial Support and Sponsorship

The authors funded and conducted the research independently using personal or institutional resources. This indicates the research was conducted without external financial influence.

6.6 Using Artificial Intelligence Tools (AI Tools)

The authors would like to acknowledge the assistance of ChatGPT 4.0 by OpenAI in supporting the development of this manuscript. The tool was utilized for translation, paraphrasing, grammar checking, and improving the cohesiveness of the narration. Its contribution greatly facilitated the refinement of the manuscript language and structure. The authors affirm that intellectual content, data analysis, and conclusions presented in the manuscript are solely their own.

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