

Biofilm–Antibiotic Resistance Interplay in Chronic Wound Gram-Negative Pathogens: Insights From an Indonesian Tertiary Hospital

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Abstract

Biofilm-forming bacteria hinder wound healing and increase antibiotic resistance, complicating the diagnosis and treatment of chronic wound infections. This study aimed to investigate the antimicrobial resistance profiles of biofilm-forming Gram-negative bacteria isolated from chronic wound infections in patients at an Indonesian tertiary hospital. A total of 75 bacterial isolates from 60 patients with chronic wound infections were identified and tested for antimicrobial susceptibility. Biofilm formation was assessed using microtiter plate assays. Spearman's correlation test was used to analyze the relationship between antimicrobial resistance and biofilm production. Gram-negative bacteria accounted for 80% of the isolates. Surgical site infections (SSIs) were the most common type of wound. The biofilm-forming ability varied, with 45%, 38.3%, and 16.7% of isolates being weak, moderate and strong producers, respectively. *Escherichia coli*, the dominant species that is mainly linked to SSIs, showed weak or no biofilm formation. *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were also prevalent. A weak positive correlation was observed between biofilm formation and resistance to amikacin, gentamicin, and meropenem. An inverse correlation was observed with ceftazidime, while no correlation was found with aztreonam, cefepime, or ciprofloxacin. In conclusion, a weak but significant correlation exists between biofilm production and resistance to certain antibiotics in Gram-negative bacteria.

Keywords: Antibiotic resistance; biofilm; chronic wounds; Gram-negative bacteria; tertiary hospital

Introduction

The prevalence of chronic wounds worldwide is rising significantly. A systematic review of nine studies involving 185 chronic wounds found that biofilms were present in approximately 80% of chronic wounds. Biofilm-related infections are increasing and often demonstrate resistance to common antibiotics, which can prolong healing times and necessitate more invasive treatments to manage infections (Grooters *et al.*, 2024; M. Malone *et al.*, 2017). Specific data on chronic wound infections associated with bacterial

biofilms in Indonesia remain limited, primarily because routine diagnosis still depends on conventional culture methods that poorly detect sessile biofilm bacteria, while surveillance systems classify wounds by clinical diagnosis without recording biofilm involvement as a distinct microbiological parameter (Syaiful *et al.*, 2023).

Chronic wound infections present significant management challenges, particularly when bacterial biofilms are involved. Biofilms form a protective extracellular polymeric substance (EPS) matrix that shields microorganisms from host immune responses and antimicrobial agents,

promoting persistent inflammation, recurrent infection, tissue damage, and delayed wound healing. Globally, biofilms are estimated to be present in approximately 68–78% of chronic wounds, underscoring their major contribution to wound chronicity (Cavallo *et al.*, 2024a; Diban *et al.*, 2023; Ding *et al.*, 2022; Grooters *et al.*, 2024; Lichtenberg *et al.*, 2024). This matrix can increase bacterial tolerance to antibiotics by 100–800 times compared with planktonic bacteria, while metabolic and genetic adaptations within biofilms further enhance antimicrobial resistance, making biofilm-associated infections particularly difficult to treat with conventional antibiotics; accordingly, bacteria with strong biofilm-forming ability tend to exhibit higher antibiotic resistance (Grooters *et al.*, 2024; Jacqueline & Caillon, 2014).

Antibiotic resistance testing in tertiary hospitals mainly relies on phenotypic susceptibility methods targeting planktonic bacteria, while routine biofilm-specific diagnostics remain limited despite increasing recognition of biofilms in antimicrobial resistance (Coenye, 2023; Macias-Valcayo *et al.*, 2022). This study investigated antimicrobial resistance among biofilm-forming Gram-negative bacteria in chronic wound infections at an Indonesian tertiary hospital to demonstrate the limitations of conventional testing and support biofilm-oriented diagnostic approaches.

Materials and Methods

Materials. This cross-sectional study was conducted at Dr. Kariadi Hospital, Semarang, Central Java, Indonesia, from October to December 2022. Adult inpatients (≥ 18 years) with chronic wound infections meeting the inclusion criteria (infection ≥ 2 weeks and Gram stain Q-score ≥ 1) were enrolled through consecutive sampling in the infectious diseases ward. Based on the 2021 hospital records and an estimated 80% prevalence of biofilm-forming bacteria, the initial sample size was calculated to be 246 isolates. After adjusting for a limited population of 80 patients, the final minimum sample size was 60 patients.

Handling specimens. To obtain specimens from chronic wounds, the wound area was first cleaned using a sterile cotton swab (Onemed) soaked in physiological saline (B Braun) until the wound base was clearly visible. A sample

was collected by swabbing the wound with sterile gauze and applying pressure to the wound base and corners. The swab was placed in a transport medium and delivered to the laboratory at room temperature within two hours. For tissue or biopsy samples, the skin was cleansed with an antiseptic solution, allowed to dry, and an incision was made at the biopsy site to collect a sufficient amount of non-necrotic tissue, typically 3–4 mm in size. The tissue sample was placed in a sterile container and transported to the laboratory within two hours at room temperature (Nuryastuti *et al.*, 2023).

Identifications and Bacterial Culture. After Gram staining, Q-score analysis was performed. The Q-score was determined by assessing the number of leukocytes and epithelial cells under a 10x objective lens (low-power field), while bacterial morphology was observed under a 100x objective lens (high-power field). (Matkoski *et al.*, 2006) For aerobic bacterial culture and identification, specimens were streaked onto blood agar (Oxoid) and MacConkey (Oxoid) agar plates. Blood agar plates were incubated in a CO₂ incubator, whereas MacConkey agar plates were incubated in a standard incubator for 24 hours. The grown colonies were then observed, and Gram staining was performed on a single colony for bacterial identification. In cases where the colonies were piled up or unclear, subculturing was conducted to ensure purity, particularly if the colonies were suspected to be pathogenic. If Gram staining revealed Gram-negative rods or coccobacilli, an oxidase test was performed, followed by identification and AST using BD PhoenixTM. Gram-positive rods identified through Gram staining were considered contaminants (Kirmusaoğlu, 2019; Nuryastuti *et al.*, 2023).

Preparation of Bacterial Suspension. Bacterial strains were retrieved from cryogenic stocks and streaked onto solid agar plates. The plates were incubated at 37°C for 16–20 hours to obtain single colonies. Three to five colonies of similar size and morphology were selected and transferred into 5 mL of TSB liquid medium, followed by incubation at 37°C for 16–20 hours (Nuryastuti *et al.*, 2023).

Biofilm Growth Process. After 24 hours, the culture was checked for turbidity to confirm microbial growth. The 24-hour culture was centrifuged at 3000 rpm for 5 minutes, washed

with 2 mL of PBS, and the pellet was resuspended in 3 mL of NaCl, adjusted to a McFarland standard of 0.5 to achieve a concentration of 10^8 CFU/mL, or measured using a spectrophotometer. Ninety microliters of liquid medium was added to each well of a 96-well microtiter plate, followed by 10 microliters of the bacterial suspension at a concentration of 10^8 CFU/mL (culture-to-medium ratio of 1:10). Wells containing only the medium served as negative controls, whereas *S. epidermidis* ATCC 12228, a weak biofilm producer, was used as a positive control. The microtiter plates were covered, sealed with Parafilm, and incubated at 37°C for 24 hours to facilitate biofilm formation (Nuryastuti *et al.*, 2023).

Biofilm Staining with Crystal Violet and Interpretation. Crystal violet staining was performed following a standard microtiter plate assay (Kirmusaoğlu, 2019) with modifications. After incubation, the wells were gently aspirated, washed twice with PBS (pH 7.3), and stained with 125 µL of 0.1% crystal violet for 10–15 minutes. Plates were washed 3–4 times with water, air-dried, and the dye was resolubilized with 125 µL absolute isopropanol for 10–15 minutes. Absorbance was measured at 630 nm (BioTek Gen5™), and biofilm production was categorized based on the ODc values (Table 1).

Table 1. The calculation of cutoff value (ODc) of Biofilm

Biofilm formation Category	Optical Density
Non/weak	$\leq \text{ODc} / \text{ODc} < \sim 2 \times \text{ODc}$
Moderate	$2 \times \text{ODc} < \sim 4 \times \text{ODc}$
Strong	$> 4 \times \text{ODc}$

Note: *Optical density cut-off value* (ODc) = average of control negative OD + 3X SD control negative Absorbance was measured at $\lambda=630$ nm (Kirmusaoğlu, 2019; Nuryastuti *et al.*, 2023).

Analysis. Data analysis includes descriptive and bivariate analysis. In the descriptive variable analysis, the size of categories such as gender, age, type of wound and type of specimen is expressed in frequency distribution and percentage (n and %). Data with ordinal scale such as bacterial zone in culture media and density are expressed in frequency and percentage, optical mean, mean value and standard deviation. The Spearman Correlation Test is used for understanding the relationship between the optical density of biofilm formation and the MIC of antibiotics, with a 95%

confidence interval. Data analysis was performed using SPSS version 26.0 for windows.

Results and Discussion

Characterization of patients

Table 2 presents the characteristics of the patients, including age, gender, specimen type, and type of chronic wound. Patient age was categorized into three groups based on a descriptive analysis of the minimum, maximum and average values in this study. The types of chronic wounds are surgical site infections (SSIs) and nonsurgical site infections (non-SSIs, such as diabetic ulcers and decubitus ulcers), with SSIs being the most prevalent specimen type.

Chronic wounds create a unique environment conducive to biofilm formation due to moist conditions, a neutral-to-alkaline pH, and nutrient-rich exudates. Hypoxia, immune suppression, and necrotic tissue further promote microbial colonization and persistence (Thaarup *et al.*, 2022; Zou *et al.*, 2023). Biofilms, in turn, hinder healing and contribute to wound chronicity (Thaarup & Bjarnsholt, 2021). A review from low- and middle-income countries identified surgical site infections (SSIs) as a major contributor to hospital-acquired chronic wounds (De Simone *et al.*, 2020). In this study, most patients were middle-aged, aligning with data showing slower healing due to aging-related physiological changes (Csekes & Račková, 2021).

Table 2. Characteristics of the patients

Variables	Total (%) n=60
Age (years)	
18-44	19 (31.7)
45-64	36 (60.0)
65-70	5 (8.3)
Sex	
Female	34 (56.7)
Male	26 (43.3)
Chronic wound type	
SSIs	43 (71.7)
non-SSIs	17 (28.3)
Specimen type	
Wound swab	54 (90.0)
Tissue	6 (10.0)

The majority of samples were wound swabs, favored for convenience, but may underrepresent biofilm presence compared to tissue samples (Kumar *et al.*, 2023). SSIs were more common than non-SSIs, such as diabetic

and pressure ulcers, emphasizing their role in chronic wound development and susceptibility to biofilms due to delayed healing and exposure to pathogens (Hrynshyn et al., 2022).

Identification of Gram-negative bacteria

Gram-negative bacteria were identified in 60 out of the 75 isolates collected (80%). Therefore, this study focuses its analysis and discussion on the 60 Gram-negative bacteria. Table 3 outlines the characteristics of Gram-negative bacteria identified in chronic wound infections. In this

study, Gram-negative bacteria exhibited varying biofilm formation abilities, categorized as weak/non-biofilm producers, moderate, and strong. Figure 1 illustrates the distribution of optical density readings at a wavelength of 630 nm, based on biofilm formation categories. The majority (45%) of the bacteria were weak biofilm producers. Notably, the boxplot for the weak/non-biofilm producer category revealed one outlier, indicating a bacterium in this group did not produce any biofilm.

Table 3. Characteristics of Biofilm-producing Gram-negative bacteria during the study period

Bacteria	n=60 (%)	Chronic wound type n (%)		Biofilm category n (%)		
		SSI	Non-SSI	Strong	Moderate	Weak/Non
<i>Escherichia coli</i>	29(48.3)	22(75.9)	7(24.1)	3(10.3)	8(27.6)	18(62.1)
<i>Pseudomonas aeruginosa</i>	9(15.0)	8(88.9)	1(11.1)	2(22.2)	3(33.3)	4(44.5)
<i>Klebsiella pneumoniae</i>	6(10.0)	3(50.0)	3(50.0)	1(16.7)	3(50.0)	2(33.3)
<i>Proteus mirabilis</i>	5(8.3)	2(40.0)	3(60.0)	2(40.0)	3(60.0)	0(0.0)
<i>Morganella morganii</i>	3(5.0)	2(66.7)	1(33.3)	1(33.3)	2(66.7)	0(0.0)
<i>Enterobacter cloacae</i>	2(3.3)	1(50.0)	1(50.0)	1(50.0)	1(50.0)	0(0.0)
<i>Klebsiella aerogenes</i>	1(1.7)	1(100.0)	0(0.0)	0(0.0)	1(100.0)	0(0.0)
<i>Klebsiella oxytoca</i>	1(1.7)	1(100.0)	0(0.0)	0(0.0)	1(100.0)	0(0.0)
<i>Citrobacter werkmanii</i>	1(1.7)	1(100.0)	0(0.0)	0(0.0)	0(0.0)	1(100.0)
<i>Serratia marcescens</i>	1(1.7)	1(100.0)	0(0.0)	0(0.0)	0(0.0)	1(100.0)
<i>Salmonella</i> sp.	1(1.7)	1(100.0)	0(0.0)	0(0.0)	0(0.0)	1(100.0)
<i>Pantoea agglomerans</i>	1(1.7)	1(100.0)	0(0.0)	0(0.0)	1(100.0)	0(0.0)
Total	60(100.0)	44(73.3)	16(26.7)	10(16.7)	23(38.3)	27(45.0)

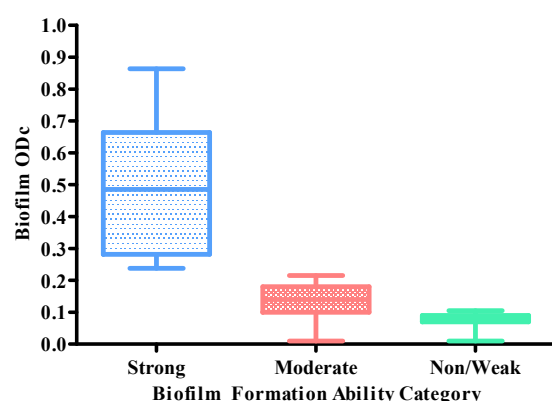


Figure 1. Optical density (OD630) from the biofilm formation assay on Gram-negative bacteria ($p < 0.001$).

The bacterial isolates exhibited varying biofilm-forming abilities. He *et al.* (2021) suggested up to 78.2% of chronic wounds are biofilm-positive, unlike acute wounds, (He *et al.*, 2021) with biofilm presence influencing microbial composition and healing outcomes (Percival, 2017). Diverse species were isolated, including *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, all known for biofilm-associated virulence and resistance (Cavallo *et al.*, 2024; Shan *et al.*, 2019). The detection of rare

pathogens such as *Salmonella* sp. and *P. agglomerans* reflects the polymicrobial complexity of these infections (Harrell *et al.*, 2021; Alrufaie *et al.*, 2023).

Distribution of Antimicrobial Resistance

The data are presented in Table 4 provides valuable insight into the distribution of antimicrobial resistance types based on biofilm formation ability among different bacterial isolates. This table highlights the different biofilm-forming abilities (strong, moderate, and weak/non-biofilm-forming) in relation to the resistance profile, namely, carbapenem-resistant organisms (CRO), extended-spectrum beta-lactamase producers (ESBLs), a combination of ESBLs-CRO and non-multidrug-resistant organisms (NonMDRO). These categories were specifically designed to distinguish between different bacterial resistance mechanisms. CRO refers to bacteria that are resistant to carbapenems alone, ESBL represents those resistant to extended-spectrum beta-lactamases only, and ESBL-CRO includes bacteria resistant to both ESBL and carbapenems.

A total of 51.7% of ESBL-producing bacteria formed biofilms in this study (Tabel 4). Notably, biofilm development occurred across resistant and non-resistant strains, highlighting biofilms as an independent survival strategy (Grooters *et al.*, 2024). Resistance traits were also noted in moderate and strong biofilm producers. Mechanistically, biofilm formation in ESBL-producing bacteria, such as *K. oxytoca* and *K.*

pneumoniae, involves genetic and metabolic shifts that promote resistance (Shrief *et al.*, 2022). Strong biofilm-formers like *P. aeruginosa* often display multidrug resistance, enhanced by efflux pumps, porin loss, or beta-lactamase production (Lepe & Martínez-Martínez, 2022; Shukla *et al.*, 2022; Tompkins *et al.*, 2021).

Table 4. Distribution of bacterial resistance type based on biofilm formation ability

Biofilm formation ability	Type of Antimicrobial Resistance n (%)				Total
	CRO	ESBL	ESBL_CRO	Non-MDRO	
Strong	1(1.7)	5(8.3)	0(0.0)	4(6.7)	10(16.7)
Moderate	2(3.3)	11(18.3)	3(5.0)	7(11.7)	23(38.3)
Weak/Non	0(0.0)	15(25.0)	1(1.7)	11(18.3)	27(45.0)
Total	3(5.0)	31(51.7)	4(6.7)	22(36.7)	60(100.0)

Biofilm Formation Ability

Table 5 presents the correlation between biofilm formation ability and the effectiveness of various antibiotics tested on Gram-negative bacteria in this study. The Spearman correlation test revealed a weak positive relationship between increased biofilm optical density (OD) and higher minimum inhibitory concentrations (MICs) of amikacin, gentamicin, and meropenem.

Table 5. Correlation between biofilm optical density of Gram-negative bacteria and MIC of the antibiotics

Antibiotics	<i>p</i>	<i>r_s</i>
Amikacin	0.031*	0.279**
Aztreonam	0.955	0.007
Ceftazidime	0.526	-0.083
Cefepime	0.948	0.009
Ciprofloxacin	0.488	0.091
Gentamicin	0.047*	0.258**
Meropenem	0.040*	0.266**

A weak positive correlation between biofilm density and MICs of amikacin, gentamicin, and meropenem (Table 5), supporting the protective role of biofilms (Karami *et al.*, 2020; Oyari *et al.*, 2023). Interestingly, the inverse correlation observed with Ceftazidime may indicate that increased biofilm density was not accompanied by increased ceftazidime resistance, suggesting retained susceptibility in certain isolates rather than confirmed efficacy against biofilm-associated bacteria. This finding aligns with studies showing that *E. coli* and *K. pneumoniae* biofilms correlate with specific antibiotic resistance patterns (Syaiful *et al.*, 2023). These results emphasize that targeting biofilms,

regardless of antimicrobial resistance status, is vital in chronic wound management. Biofilm assessment should complement traditional susceptibility testing to guide more effective therapies.

Conclusions

Of the 60 Gram-negative bacteria identified, only one did not produce biofilm. Most of the bacteria exhibited weak biofilm formation, with fewer showing moderate or strong capabilities. A weakly significant correlation was observed between the optical density of biofilm-forming bacteria and the minimum inhibitory concentration (MIC) of amikacin, gentamicin, and meropenem. This study also highlights that antimicrobial resistance mechanisms in biofilm-associated bacteria differ from those in planktonic bacteria, indicating that adjustments in antibiotic resistance testing are necessary to better assess biofilms' role in antibiotic resistance.

Conflict of Interest

There is no conflict of interest.

Declaration of generative AI

During the preparation of this work the authors used Scite AI in order to check the language. After using this tool/service, the author(s) reviewed and edited the content as

needed and take(s) full responsibility for the content of the published article

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Ethical approval

Ethical approval for this study was granted by the Medical and Health Research Ethics Committee (MHREC) of the Faculty of Medicine, Public Health, and Nursing at Universitas Gadjah Mada and Dr. Sardjito General Hospital, under approval code Ref No KE/FK/0771/EC/2022.

Author contribution

DUA, RH, and TN designed the study. DUA and MM performed the laboratory work. DUA, HW, and TNK analyzed the data. DUA wrote the manuscript. All authors have read and approved the final version of the manuscript.

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