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Biofilm detection of clinical isolates of *Klebsiella pneumoniae* from Pontianak, West Kalimantan

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ABSTRACT

Klebsiella pneumoniae (*K. pneumoniae*) belongs to the Enterobacteriaceae family, which is known as Gram-negative, encapsulated, and non-motile bacteria. One of its most important virulence factors of *K. pneumoniae* is the ability to form biofilms. A high percentage of *K. pneumoniae* as biofilm formation is associated with a high incidence of antibiotic resistance, leading to higher morbidity and mortality. This study aimed to evaluate biofilm formation of clinical isolates of *K. pneumonia* from Pontianak, West Kalimantan. A total of 24 *K. pneumoniae* clinical isolates from various specimens were subjected to biofilm formation detection. The biofilm formation was detected by crystal violet formation at wavelength of 570 nm as indicator using microtiter plate assay. As control of biofilm formation was *Staphylococcus aureus* ATCC 25923, and as control of non-biofilm formation was *Staphylococcus epidermidis* ATCC 12228. Data on biofilm formation of the isolates from various specimens were analyzed using Fisher's exact test with SPSS Ver. 26. All the *K. pneumoniae* clinical isolates were biofilm producer consisting of 19 isolates were strong biofilm producer, four were moderate biofilm producer, and one isolates was weak biofilm producer. No association between biofilm formation and specimen type was observed (p=0.541). In conclusion, all *K. pneumoniae* clinical isolates from Pontianak, West Kalimantan are identified as biofilm producer.

ABSTRAK

Klebsiella pneumoniae (*K. pneumoniae*) termasuk dalam famili Enterobacteriaceae, yang dikenal sebagai bakteri Gram-negatif, berkapsul, dan tidak motil. Salah satu faktor virulensi terpenting dari *K. pneumoniae* adalah kemampuannya untuk membentuk biofilm. Kemampuan yang tinggi *K. pneumoniae* membentuk biofilm dikaitkan dengan tingginya insiden resistensinya terhadap antibiotik, yang menyebabkan morbiditas dan mortalitasnya lebih tinggi. Penelitian ini bertujuan untuk mengevaluasi pembentukan biofilm isolat klinis *K. pneumoniae* dari Pontianak, Kalimantan Barat. Sebanyak 24 isolat klinis *K. pneumoniae* dari berbagai spesimen dideteksi kempuannya membentuk biofilm. Pembentukan biofilm dideteksi dengan pembentukan kristal violet pada panjang gelombang 570 nm sebagai indikator menggunakan uji pelat mikrotiter. Sebagai kontrol pembentukan biofilm adalah *Staphylococcus aureus* ATCC 25923, dan sebagai kontrol pembentukan non-biofilm adalah *Staphylococcus epidermidis* ATCC 12228. Data pembentukan biofilm isolat dari berbagai spesimen dianalisis menggunakan uji eksak Fisher dengan SPSS Ver. 26. Semua isolat klinis *K. pneumoniae* merupakan penghasil biofilm yang terdiri dari 19 isolat penghasil biofilm kuat, empat isolat penghasil biofilm sedang, dan satu isolat penghasil biofilm lemah. Tidak ada hubungan antara pembentukan biofilm dan jenis spesimen (p=0,541). Sebagai kesimpulan, semua isolat klinis *K. pneumoniae* dari Pontianak, Kalimantan Barat diidentifikasi sebagai penghasil biofilm.

Keywords:
antibiotic resistance; biofilm former; *Klebsiella pneumoniae;* clinical isolates

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INTRODUCTION

Klebsiella pneumoniae (*K. pneumoniae*) is a bacterium found in the environment and in humans. It is Gram-negative, encapsulated, and non-motile organism. *Klebsiella pneumonia* causes hospital and community-acquired infections, such as pneumonia, bacteremia, and urinary tract infections.[1,2] *Klebsiella pneumoniae* is classified as classical *K. pneumoniae* and hypervirulent *K. pneumoniae*.[3] Classical *K. pneumoniae* is the most common strain responsible for hospital-acquired infections, typically exhibiting low virulence and primarily affecting patients with low immunity.[4] Otherwise, hypervirulent *K. pneumoniae* often causes community-acquired infection in immunocompetent individuals. It can be found in the gastrointestinal tract and is a significant cause of pyogenic liver abscesses in Asia. Moreover, hypervirulent *K. pneumoniae* can spread to other parts of the body, such as the eyes, lungs, and central nervous system.[5]

One of the major virulence factors of *K. pneumoniae* is its ability to form biofilms.[6] Biofilms are highly structured microbial communities that act as a bacterial defense mechanism against the host immune response. Several factors contribute to biofilm formation in *K. pneumoniae*, including polysaccharide capsules, fimbriae, pili, iron metabolism, and the presence of other bacterial species. Biofilm can form on biotic surfaces, such as the mucosal tissues of the respiratory, urinary, and gastrointestinal tracts, as well as on abiotic surfaces, including medical devices and catheters.[6,7] Biofilm formation reduces the effectiveness of antibiotics and contribute to antibiotic resistance by impairing antibiotic penetration. Therefore, antibiotics may not reach or effectively kill the planktonic bacteria embedded within the biofilm layer. As an impact, biofilm-associated antibiotic resistance can significantly increase morbidity and mortality in infected patients.[8-10]

A study conducted in Rome demonstrated that *K. pneumoniae* strains with strong biofilm-forming capacity also exhibited carbapenem resistance.[11] Other studies have reported a high percentage of biofilm-forming *K. pneumoniae* strains, with a strong association with morbidity and mortality rates.[9,10] A study by Karimi *et al.*[9] reported that the level of biofilm formation is significantly higher in antibiotic-resistant strains compared to antibiotic-sensitive ones, suggesting a link between antibiotic resistance and biofilm production.[9] Previous studies reported that the percentage of biofilm-forming *K. pneumoniae* clinical isolates was 74.5% and 92.2% in West Irian and Jordan, respectively.[9,12] In Klaten, Indonesia, 85.63% of *K. pneumoniae* clinical isolates were reported as biofilm formers.[13]

Until now, the prevalence data of biofilm-forming *K. pneumoniae* clinical isolates in Pontianak have not been reported, yet. This study aimed to evaluate biofilm formation of clinical isolates of *K. pneumonia* from Pontianak, West Kalimantan.

MATERIAL AND METHODS

Design

It was a descriptive laboratory-based study conducted at the Microscopic Laboratory of the Faculty of Medicine, Universitas Tanjungpura, and the Provincial Animal Husbandry Service Laboratory between July and August 2024. The study was approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Tanjungpura, Pontianak (reff. 7924/UN22.9/PG/2024).

Samples

A total of 24 clinical isolates collected at the Department of Microbiology, Faculty of Medicine, Universitas

Tanjungpura, Pontianak that had been identified with API 20E (bioMérieux, France) as *K. pneumoniae* were subjected in this study. The biofilm-forming and non-biofilm-forming controls were *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228, respectively.[14,15]

All *K. pneumoniae* isolates were sub-cultured in MacConkey agar (Millipore, Germany). *S. aureus* ATCC 25923, as biofilm former control, and *S. epidermidis* ATCC 12228, as non-biofilm former control, were sub-cultured in blood agar (Millipore, Germany). All cultures were incubated for 18-24 hr at 37°C. Colony morphology of bacterial colonies formed on agar media was observed. Gram staining of the colony was observed under a microscope at 1000x magnification using immersion oil. All colonies appropriate with *K. pneumoniae* characteristics are included in the study. The catalase and coagulase tests were performed for *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228.[16,17]

Detection of biofilm formation

Biofilm formation was detected using microtiter plate assay test. Bacterial suspensions were prepared from an 18 hr old bacteria culture and dissolved in 0.9% NaCl, equivalent to 0.5 McFarland standard. A total of 2µL the bacteria suspension was inoculated into 198µL of trypticase soy broth (TSB) medium (Millipore, Germany). A volume of 200µL of TSB was used as a media control. Clinical isolates, biofilm former controls, non-biofilm former controls, and media controls were added to 96-well flat-bottom polystyrene microtiter plate (Abdos Labtech, China) in triplicate.[13,16,17]

The microtiter plates were incubated for 24 hr at 37°C. Then, each well was washed three times with sterile phosphate buffer saline (PBS) pH 7.2. After washing, 200 µL of 0.1% crystal violet was added to each well and incubated for 10–15 min, followed by rinsing three times with 200 µL of sterile distilled water. The plate was then inverted and allowed to dry completely. Once dry, 200 µL of 30% acetic acid was added to each well and incubated for 15 min. Finally, absorbance was measured using an ELISA reader (Thermo Scientific™, Finland) at a wavelength of 570 nm.[13,16,17] All experiments were performed in triplicate.

The *K. pneumoniae* biofilm formation characteristics were categorized into four classifications, namely: 1) non-biofilm formation (ODr ≤ ODc); 2) weak biofilm formation (ODc < ODr ≤ 2×ODc); 3) moderate biofilm formation (2×ODc < ODr ≤ 4×ODc); 4) strong biofilm formation (ODr > 4×ODc). Optical density values obtained in absorbance reading are counted as averaged (ODr). Optical density cut-off (ODc) values were obtained by the formula average non-biofilm former control + [3 × standard deviation (SD) average non-biofilm former control].[17]

Statistical analysis

Fisher's exact test using SPSS Ver. 26 was conducted to demonstrate the association between the specimen and biofilm forming. The results are considered as significant if p value < 0.05.

RESULTS

Of the 24 *K. pneumoniae* clinical isolates, 17 were obtained from sputum, 2 from broncho alveolar lavage (BAL), 2 from pus, 2 from feces, and 1 from urine. All clinical isolates of *K. pneumoniae* were biofilm producers (TABLE 1). The majority of isolates were strong biofilm producers (79.16%), followed by moderate biofilm producers (16.66%), and weak biofilm producers (4.16%). No isolates were classified as non-biofilm producers.

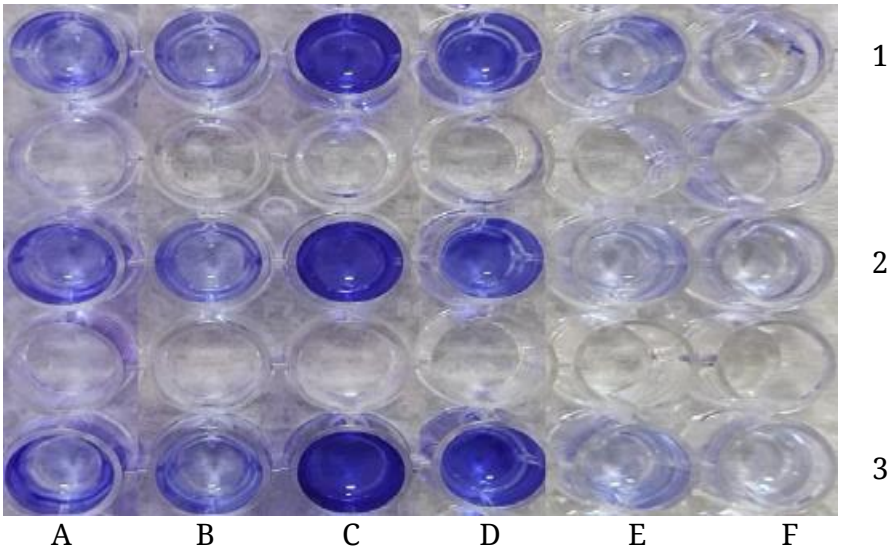

FIGURE 1. Top to bottom: detection of biofilm formation using microtiter plate assay (triplicate). Left to right: A: weak biofilm formation (sample); B: moderate biofilm formation (sample); C: strong biofilm formation (sample); D: control of biofilm formation (S. aureus ATCC 25923); E: control of non-biofilm formation (S. epidermidis ATCC 12228); and F: control of media.

TABLE 1. Biofilm formation detection

Specimen	Characteristic of biofilm formation			p
	Weak [n (%)]	Moderate [n (%)]	Strong [n (%)]	
Sputum	1 (5.88)	2 (11.76)	14 (82.35)	0.541
Broncho alveolar lavage	0 (0)	1 (50)	1 (50)	
Pus	0 (0)	0 (0)	2 (100)	
Urine	0 (0)	0 (0)	1 (100)	
Fesses	0 (0)	1 (50)	1 (50)	
Total	1 (4.16)	4 (16.66)	19 (79.16)	

DISCUSSION

Klebsiella pneumoniae is a normal flora found in the respiratory tract, which causes infection when the host's immune system is decreased or immunocompromised.[1,2] All *K. pneumoniae* clinical isolates were biofilm producers (TABLE 1). Previous studies also demonstrated a high percentage of clinical isolates of *K. pneumonia* as biofilm producers with 100%, 94.8%, and 92.2% in Egypt, Nepal, and Jordan, respectively.[12,18,19] Based on the classification of biofilm producers, our study revealed that 79.16% were

strong biofilm producers, 16.66% were moderate producers, and 4.16% were weak biofilm producers (TABLE 1). Other studies showed a variety of results. In Italy, it was reported that 55.8% of *K. pneumoniae* were strong biofilm producers.[11] In contrast, a study conducted by Shiman, *et al.* in Egypt demonstrated that among classical *K. pneumoniae* biofilm producers, 53.5% were strong, while among hypervirulent *K. pneumoniae* biofilm producers, 48.2% were strong.[19] It could not classify the biofilm based on the strain of *K. pneumonia* as a study limitation.

Of the 24 isolates enrolled in the study, the majority were from sputum specimens, with as many as 17 clinical isolates. Among these, 14 (82.35%) isolates were strong biofilm producers, 2 (11.76%) isolates were moderate biofilm producers, and 1 (5.88%) isolate was a weak biofilm producers. Several factors, such as nutrient content and pH of sputum, could cause strong biofilm formation in sputum specimens.[20,21] Sputum contains lipids and proteins that play a favorable role in the formation of *K. pneumoniae* planktonic bacteria, leading to biofilm formation. The pH of sputum in infectious and inflammatory diseases ranges from 7.2-7.4, which also impacts increased biofilm formation.[20,21] A study demonstrated that under pH 7.5, biofilm formation increased by 113-177%.[20] Our result is in line with the study by Shadkam *et al.*,[22] which found that biofilm-forming clinical isolates from sputum specimens had the highest percentage compared to other specimens.[22] No significant differences between the specimens and the ability to perform biofilm was observed in this study (TABLE 1). It could be assumed in this study that specimens do not play a role in biofilm's producer ability. As our results demonstrated all specimens were biofilm producer with the majority of strong biofilm.

Biofilms are thin layers of microorganism communities encased in extracellular polymeric substances and attached to biotic or abiotic surfaces. This layer is highly structured and functional.[7] Bacterial biofilms are attached to biotic surfaces because the body provides an ideal environment for attachment and biofilm formation. Bacterial biofilms can also grow and form when bacteria attach to open internal wounds. Other than biotic surfaces, biofilm can also form on abiotic surfaces, that can lead to the occurrence of infectious diseases caused by biofilm-forming bacteria, such as endotracheal tubes (ETT), urine catheters, contact lenses, intrauterine devices, or prosthetic heart valves.[23,24] *Klebsiella pneumoniae* can cause colonization in the mucosal layer of the human body. Type 1 and 3 fimbriae mediate biofilm formation in *K. pneumoniae*. Type 1 fimbriae play a role in enhancing epithelial cell adhesion and leading to strong biofilm production. At the same time, type 3 fimbriae help form thick and dense biofilms and mediate attachment to damaged epithelial surfaces by binding the extracellular matrix to the urinary and respiratory epithelial tissues.[20]

Biofilms are known to be related to disease and to increase the occurrence of antibiotic resistance cases, posing a new challenge to prevent and control infectious diseases caused by biofilms. The formation of disease-causing biofilms can occur in several ways, namely: 1) blood and urinary tract infections and the formation of emboli can be caused by the release of biofilm-associated cell masses; 2) cells within the biofilm can exchange resistance plasmids; 3) cell sensitivity to antimicrobial agents decreased; 4) production of endotoxins by biofilm-forming bacteria; and 5) cell resistance to the host's immune system.[23,24]

Bacterial biofilm formation that leads to antibiotic resistance can be prevented through several mechanisms. Early-stage biofilm formation can be inhibited

with antibacterial drugs, supplements, and by adjusting environmental factors. Antimicrobial peptides (AMPs) are a type of cationic that can be used to down-regulate biofilm-forming genes, prevent bacterial attachment to cell-matrix surfaces, down-regulate Quorum Sensing (QS) signals between bacterial cells, and produce various antibacterial activities. AMPs can be used alone or in combination with antibiotics, effectively inhibiting biofilm formation, destroying biofilms, and enhancing their anti-microbial role. Enzymes can also remove specific components of biofilms by inhibiting extracellular matrix formation and inhibiting the QS system of bacteria. Enzymes are capable of hydrolyzing extracellular polymeric substance components with antibiofilm activity.[23] GH-B2 is one of the enzymes with high activity and stability that can function as a biofilm matrix exopolysaccharide degrader and prevent bacterial biofilm formation.[25]

A study by Karimi *et al.*[9] stated that biofilm-forming *K. pneumoniae* clinical isolates play a role in antibiotic resistance. Biofilm-forming clinical isolates with resistance to ampicillin-sulbactam were 44/47 (93.6%), piperacillin-tazobactam was 34/36 (94.4%), and tobramycin 33/35 (94.2%). Thus, biofilm formation in *K. pneumoniae* clinical isolates is a risk factor for antibiotic resistance.[26] Our study could not be described further in this regard, which is a limitation of the study.

All *K. pneumoniae* clinical isolates in this study were biofilm-forming, which should raise awareness among health workers. Notably, biofilm-forming *K. pneumoniae* can cause antibiotic resistance, impacting therapy selection, morbidity, and mortality. Even though our study sample was limited, the data may reflect conditions that could be encountered in the future, especially in Pontianak, West Kalimantan.

CONCLUSION

The study demonstrated that all *K. pneumoniae* clinical isolates were biofilm producers. These circumstances should be raise awareness among health workers. The correlation between biofilm formation and antibiotic resistance is well-established and could increase morbidity and mortality rates. Thus, detection of biofilm-forming strains could be a supplementary test in clinical microbiology for optimizing patient therapy.

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