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ARTICLE

mrkA, mrkD, AND *fimH* BIOFILM GENES DETECTION: A STUDY ON *Klebsiella pneumoniae* CLINICAL ISOLATES

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ABSTRACT

Biofilm formation is a virulence factor contributing to the pathogenicity and antibiotic resistance of *Klebsiella pneumoniae.* This process occurs due to biofilm genes in clinical isolates, especially the *mrkA, mrkD,* and *fimH* genes. These three genes are related the quality of biofilms formed, which lead to chronic and prolonged infections. This study was conducted to determine the presence of *mrkA, mrkD,* and *fimH* genes as biofilm formers in *Klebsiella pneumoniae* clinical isolates. The study used a descriptive in vitro laboratory study, with total of 24 *Klebsiella pneumoniae* clinical isolates from the collection of the Department of Microbiology, Faculty of Medicine, Universitas Tanjungpura. Isolates were subculture in MacConkey Agar, Gram staining, followed by DNA extraction and gene detection using PCR. The results showed the presence of the *mrkA* gene in 91.6% of the isolates, while the *mrkD* and *fimH* genes were each in 87.5% of the isolates. The high percentage of biofilm gene detected, should increase health workers and stakeholder awarness about the impact.

Keywords: Biofilm; Clinical Isolate; Fimbriae; *Klebsiella pneumoniae*

АБСТРАКТ

Формирование биопленки является фактором вирулентности, способствующим патогенности и антибиотикорезистентности *Klebsiella pneumoniae.* Этот процесс происходит из-за генов биопленки в клинических изолятах, особенно генов *mrkA, mrkD* и *fimH*. Эти три гена связаны с качеством образующихся биопленок, что ведет к хроническим и длительным инфекциям. Исследование было проведено для определения присутствия генов *mrkA, mrkD* и *fimH* как образующих биопленку в клинических изолятах *Klebsiella pneumoniae.* В исследовании использовалось описательное *in vitro* лабораторное исследование с 24 клиническими изолятами *Klebsiella pneumoniae* из коллекции кафедры микробиологии медицинского факультета Университета Танджунгпуры. Изоляты были посеяны на агар МакКонки, затем проведены граммовое окрашивание, экстракция ДНК и детекция генов с помощью ПЦР. Результаты показали наличие гена *mrkA* в 91.6% изолятов, в то время как гены *mrkD* и *fimH* были обнаружены в 87.5% изолятов. Высокий процент обнаружения генов биопленки должен повысить осведомленность медицинских работников и заинтересованных сторон о возможных последствиях.

Ключевые слова: Биопленка; клинический изолят; фимбрии; *Klebsiella pneumon*

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INTRODUCTION

Klebsiella pneumoniae is a facultative anaerobic, bacillary bacterium that can be found in water, soil, plants, animals, or humans. The spread of *Klebsiella pneumoniae* can occur from one individual to another. *Klebsiella pneumoniae* causes infections in the respiratory system, urinary tract, and digestive tract, such as pneumonia, and sepsis.[1]

Klebsiella pneumoniae often causes nosocomial and community infections. *Klebsiella pneumoniae* is known as pneumonia etiology for 11.8% in worldwide. In Western countries, infections caused by *Klebsiella pneumoniae* range from 3-5%, which is different from developing countries, such as Africa, which 15%.[2] In West Kalimantan, *Klebsiella pneumoniae* as urinary tract infections (UTI) etiology in patients with type II diabetes mellitus was found 3%.[3,4]

Infection of *Klebsiella pneumoniae* could lead to mortality.[5] This occurs due to the virulence factors of *Klebsiella pneumoniae,* particularly its ability to form biofilms. Biofilm formation leads to increased resistance to exogenous stressors and antimicrobial factors. Biofilms possess a highly complex structure and can adhere to abiotic surfaces, contributing to bacterial pathogenesis.[6] Currently, more than 80% of bacterial infections are caused by biofilm formation.[7] In the context of nosocomial infections, biofilms are implicated in over 65% of infections associated with medical devices.[8]

Biofilm formation in *Klebsiella pneumoniae* is primarily facilitated by fimbriae, such as type I fimbriae, and type III fimbriae. By enhancing the bacterium's ability to adhere to surfaces, these fimbrial structures promote the establishment and development of biofilms.[9] *Klebsiella pneumoniae* has a high ability to form biofilms, with varying prevalence in various locations. In Kenya and Iran, the prevalance of *Klebsiella pneumoniae* biofilm former isolates were 83.3% and 93.6%, respectively. [10,11] Meanwhile, in Indonesia, specifically in Klaten, 85.65% of *Klebsiella pneumoniae* isolates were also identified as biofilm former.[12]

Biofilm play a crucial role in enhancing bacterial virulence and resistance. *Klebsiella pneumoniae* is a bacterium known for its high biofilm production, which suggests its ability to protect itself from the host immune system. Biofilms as bacteria protection mechanisms could lead to increasing the severity of the infection and difficulty of treatment, infection to persist for a longer period, and serious clinical complications.[13] The ability of *Klebsiella pneumoniae* to form biofilms significantly contributes to the severity of infections, particularly in patients with medical devices.[14] Biofilm formation facilitates colonization and results in persistent infections that are difficult to treat. Moreover, *Klebsiella pneumoniae* exhibits adaptability to various environmental stresses, including nutrient deprivation and physical changes, by forming biofilms as an adaptive response.[15] This suggests that biofilm formation functions not only as a defense mechanism but also as a survival strategy under unfavorable conditions.

Based on our knowledge, there is no established data about gene detection in *Klebsiella pneumoniae* clinical isolates at Pontianak. The study was conducted to determine the biofilm-associated genes in local isolates. Hopefully, our study could provide valuable insights into the biofilm formation potential of *Klebsiella pneumoniae* and contribute to the development of more effective treatment strategies, ultimately helping to mitigate the risk of antimicrobial resistance.[16]

MATERIAL AND METHODS

This research was conducted at the Microscopy Laboratory, Faculty of Medicine, Universitas Tanjungpura, and Microbiology Laboratory, Universitas Tanjungpura Hospital in February to October 2024.

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Sample

Of 24 clinical isolates of *Klebsiella pneumoniae* from the collection of the Department of Microbiology, Faculty of Medicine, Universitas Tanjungpura, which had been identified using API 20E (bioMérieux, France) were enrolled in the study. The isolates are collected from sputum, Bronchoalveolar Lavage, urine, feces, and pus. The isolates were subculture on MacConkey Agar (Merck, Germany), incubated at 37°C for 24 hours. The isolates then continued with Gram staining.

DNA Extraction

The subcultured isolates were transferred into 1.5 ml microcentrifuge tubes containing 200 µl of Nuclease-Free Water and homogenized using a vortex. The samples were centrifuged for 1 minute at 14.000 rpm, the supernatant was discarded, and the pellet was retained in the tube. The pellet was performed DNA extraction following the protocol of the Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan). The DNA elution was stored in a freezer at -80°C.

Detection of Biofilm Genes using PCR

Detection of biofilm genes using the ExcelTaqTM 2X Fast Q-PCR Master Mix vial PCR kit (Smobio, Taiwan). The aliquot process was performed in 1.5 ml microtubes. The 2X Fast QPCR Master Mix 270 µl, forward primer 16.2 µl, reverse primer 16.2 µl, and NFW 183.6 µl were mixed thoroughly. After mixing, the solution was homogenized, followed by a spindown process. The mixture was then transferred into PCR tubes in 18 µL aliquots. To each tube, 2 µL of template DNA was added, followed by homogenization and another spindown process.

The khe gene was utilized as a housekeeping gene in this investigation. After the khe gene was found by positive isolates, the presence of the mrkA, mrkD, and fimH genes was subsequently confirmed. The findings will be interpreted as either positive or negative results for the presence of the mrkA, mrkD, and fimH genes in the clinical isolates of Klebsiella pneumoniae. Klebsiella pneumoniae ATCC 13883 was also employed as a positive control for this investigation.

Table 1. Target gene primers and PCR condition

Target Gene	Primer Sequence (5' to 3')	PCR Condition
khe[17]	F: TGATTGCATTCGCCACTGG	95°C 30 seconds, 55°C 45 seconds, 72°C 45 seconds (30 cycles)
	R: GGTCAACCCAACGATCCTGG	
mrkA[18]	F: ATGCGAACGTTTACCTGTCTCC	95°C 30 seconds, 58°C 30 seconds, 72°C 30 seconds (33 cycles)
	R: CCCGGGATGATTTTGTTGG	
mrkD[18]	F: GTCTTTTCGTCCCGGGTATATAAC	95°C 30 seconds, 58°C 30 seconds, 72°C 30 seconds (33 cycles)
	R: CCACATCGACATTCATATTTTTCC	
fimH[19]	F: GCCAACGTCTACGTTAACCTG	94°C 30 seconds, 43°C 30 seconds, 72°C 1 minutes (30 cycles)
	R: ATATTTCACGGTGCCTGAAAA	

RESULT

A total of 24 clinical isolate of *Klebsiella pneumoniae* were collected from sputum 17 (70.84%), Bronchoalveolar Lavage 2 (8.33%), pus 2 (8.33%), urine 1 (4.17%), and feces 2 (8.33%). The detection of biofilm genes is considered positive when there is an increase in the amplification curve in the PCR results, indicating the replication of the target DNA fragment during the reaction (Figure 1).

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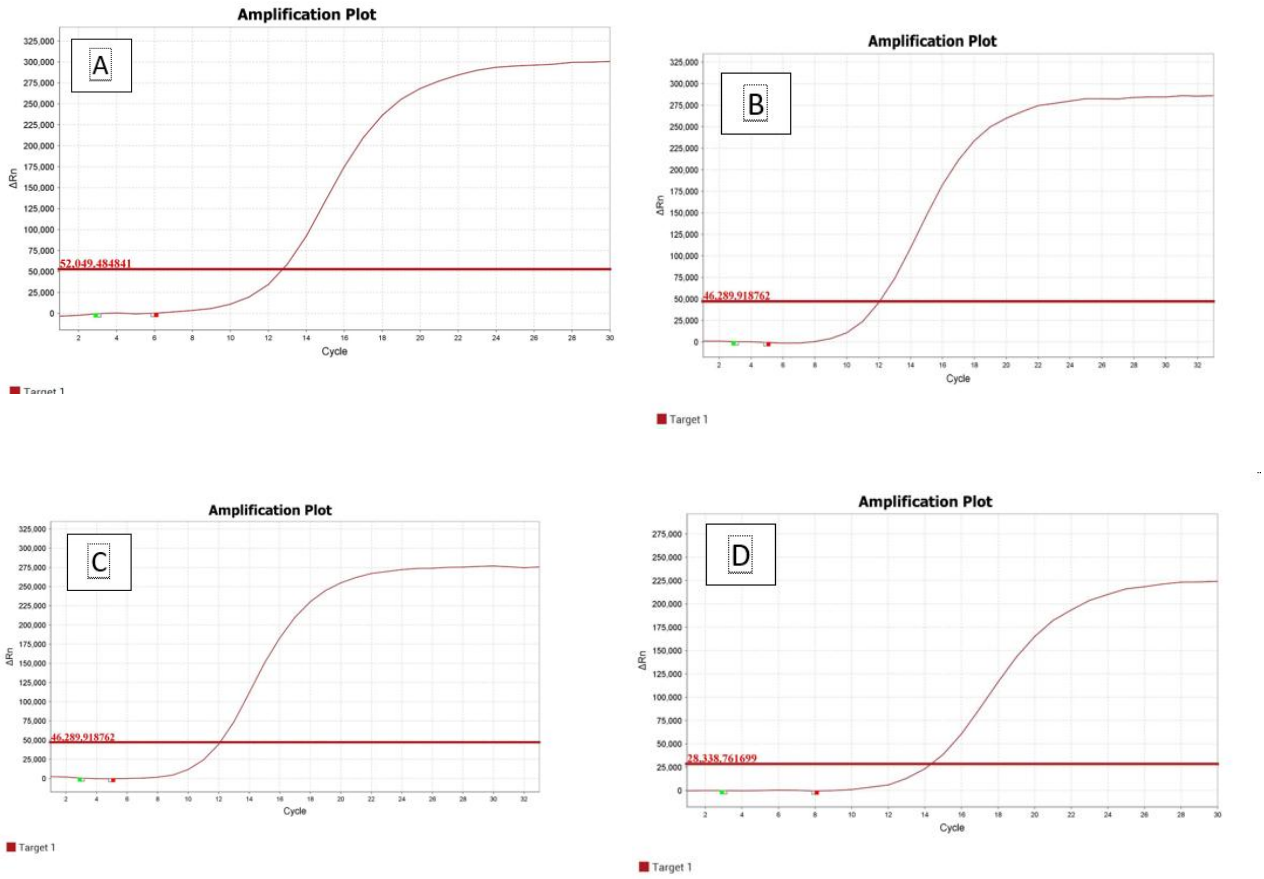

Figure 1. Curves of the *khe* (A), *mrkA* (B), *mrkD* (C), and *fimH* (D) genes

All of the clinical isolates of Klebsiella pneumoniae were shown to include the biofilm gene; yet, the patterns of gene detection varied between the isolates. The mrkA was not found in isolates number 14 and 16; the mrkD was not found in isolates number 14, 16, and 24; and the fimH was not found in isolates number 3, 15, and 24. These differences indicate that there is genetic variety among the Klebsiella pneumoniae isolates, which may impact the mechanisms by which biofilms form and the bacteria's ability to adapt to environmental conditions, including resistance to antimicrobial medicines.

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Table 2. Biofilm gene detection in *Klebsiella pneumoniae* clinical isolates

Isolates	Specimen	Biofilm Genes		
		mrkA	***mrkD***	***fimH***
Klebsiella pneumoniae ATCC 13883	-	+	+	+
1	Sputum	+	+	+
2	Sputum	+	+	+
3	Sputum	+	+	-
4	BAL	+	+	+
5	BAL	+	+	+
6	Pus	+	+	+
7	Sputum	+	+	+
8	Sputum	+	+	+
9	Sputum	+	+	+
10	Sputum	+	+	+
11	Sputum	+	+	+
12	Sputum	+	+	+
13	Sputum	+	+	+
14	Sputum	-	-	+
15	Sputum	+	+	-
16	Sputum	-	-	+
17	Sputum	+	+	+
18	Urine	+	+	+
19	Sputum	+	+	+
20	Sputum	+	+	+
21	Pus	+	+	+
22	Feces	+	+	+
23	Feces	+	+	+
24	Sputum	+	-	-

Notes: - = Biofilm gene not detected
+ = Biofilm gene detected

Table 3. Biofilm gene detection in *Klebsiella pneumoniae* clinical isolates

Biofilm Genes	Quantity	Percentage
mrkA	22	91.67%
mrkD	21	87.5%
fimH	21	87.5%

DISCUSSION

Biofilms formed by *Klebsiella pneumoniae* contribute significantly to its pathogenicity, consisting of microbial communities on surfaces, protected by an extracellular matrix of DNA, proteins, and polysaccharides that provide mechanical strength, facilitate communication, and retain nutrients.[20] Biofilm formation is a complex process that involves various factors, including the expression of specific genes such as *mrkA, mrkD,* and *fimH.* The *mrkA* and *mrkD* genes, which are type III fimbriae, act as adhesins that enhance the ability of bacteria to attach to the host surface.[10] Biofilm formation also involves the *fimH* gene, a type I fimbriae, which is an adhesin involved in the binding of bacterial cells to the host surface, which is the first step in biofilm formation.[21]

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It was known that the majority of specimens were obtained from sputum samples (Table 2.), as sputum was a readily accessible sample for diagnosing respiratory tract infections. *Klebsiella pneumoniae* was a major pathogen responsible for lung infections, which was commonly identified through sputum analysis. Sputum was the preferred specimen for detecting *Klebsiella pneumoniae* due to the bacterium's tendency to colonize the respiratory tract and cause symptoms such as coughing, often accompanied by thick, bloody sputum.[22]

In the study, biofilm genes were detected in *Klebsiella pneumoniae* clinical isolates. The *mrkA* was detected in 91.6% of isolates, while *mrkD* and *fimH* were detected in 87.5% of isolates (Table 3.). Another study in line with ours, which the *mrkA, mrkD,* and *fimH* genes were detected in 88% of the clinical isolates used.[19] In another study, the *mrkA* gene was detected in 81.81% of isolates, the *mrkD* gene in 69.09%[18] of isolates, and the *fimH* gene in 72.7% of isolates.[23] The detection rates of these biofilm genes are relatively high, suggesting that biofilm formation is a prevalent and significant characteristic of this pathogen.[24]

In the study, some isolates did not detect all biofilm genes, that is isolates No. 3, 14, 15, 16, and 24 (Table 2.). The absence of biofilm gene detection in these isolates may indicate differences in the biofilm-forming capabilities among the tested isolates. This could be attributed to genetic variations or other factors influencing the expression of genes associated with biofilm formation.[23]

In isolates No. 3 and 15, the *fimH* gene was not detected (Table 2.), indicating the possibility that the adhesion mechanism associated with this gene does not play a role. The *fimH* gene is a structure that helps bacteria adhere to the surface of host epithelial cells. The absence of this gene could impact the ability of *Klebsiella pneumoniae* to attach to cell surfaces which rich in type 1 fimbriae receptor. This condition does not mean that biofilms will not be formed. Other factors, such as type III fimbriae, encoded by *mrkA* and *mrkD* genes, will lead to biofilm formation.[25]

In isolates No. 14 and 16, the *mrkA* and *mrkD* genes were not detected (Table 2.), which suggests that biofilm formation becomes more reliant on type I fimbriae. Type I fimbriae are critical for the initial adhesion to mannosyl receptor-rich surfaces, such as epithelial cells in the upper respiratory or urinary tract. The absence of type III fimbriae may impair the bacteria's ability to form and maintain a robust and mature biofilm. Isolates lacking type III fimbriae but possessing type I fimbriae may exhibit thinner biofilms and could be more vulnerable to external disturbances.[25]

In isolate No. 24, the *mrkD* and *fimH* genes were not detected (Table 2.), which may limit the bacteria's ability to form stable and complex biofilms. The absence of these genes could also impair the durability and quality of the biofilms formed. The *mrkA* gene plays a significant role in biofilm formation, and even in isolates where all adhesion genes are undetected, virulence factors may still be involved.[19] Despite the absence of *mrkD* and *fimH,* the presence of the *mrkA* gene can still support the adhesion process, which is essential for biofilm formation.[25]

In the study, biofilm genes were detected in various specimens, indicating the presence of isolates associated with hospital-acquired infections (HAIs) and community-acquired infections (CAIs). This finding necessitates careful consideration, as the presence of biofilm genes suggests the pathogen's potential to persist and thrive in diverse environments. The existence of these biofilm genes increases the risk of virulence and complicates infection management, highlighting the need for enhanced vigilance in controlling the spread of *Klebsiella pneumoniae* in the community and health facilities.[10]

Biofilm formation on isolates leads to increased antibiotic resistance because antibiotics cannot access bacteria.[18] *Klebsiella pneumoniae,* which is resistant to various antibiotics, is one of the leading causes of

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infection in multiple parts of the world. The mechanism of antibiotic resistance in *Klebsiella pneumoniae* is very diverse, one of which is related to gene expression, including biofilm genes. This will have an impact on morbidity and mortality.

The detection of biofilm genes in *Klebsiella pneumoniae* clinical isolates provides several advantages. One significant benefit is the identification of specific genes involved in biofilm formation, which is a crucial factor in virulence and antibiotic resistance.[18] Furthermore, biofilm gene detection facilitates a deeper understanding of the relationship between biofilm formation and antibiotic resistance. Isolates that produce biofilms have been shown to exhibit increased resistance to various antibiotics.[26] Therefore, beyond pathogen identification, biofilm gene detection is a valuable consideration in the management of *Klebsiella pneumoniae* infections.

CONCLUSION

Biofilm genes were detected in all *Klebsiella pneumoniae* clinical isolates, indicating the potential of these isolates to form biofilms that contribute to the pathogenesis of infection. Specific consideration is required in treating infections caused by *Klebsiella pneumoniae,* as biofilm formation may affect the success of therapy. Health workers and stakeholders should be aware of this issue, as biofilm-related resistance may complicate treatment outcomes.

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DECLARATIONS

Conceptualization and methodology: I.A.A. and M.D.; Investigation: I.A.A.; Data analysis and interpretation: I.A.A., M.D., D.F.L., and M.H; Visualization: I.A.A., M.D., D.F.L., and M.H.; Writing – Original draft: I.A.A.; Writing – Review and Editing: M.D., D.F.L.; Funding acquisition: M.D., D.F.L., and M.H.; Supervision: M.D. and D.F.L. The authors read and approved the final manuscript. This research received funding from the Department of Microbiology, Faculty of Medicine, Universitas Tanjungpura The authors declare no conflict of interest. This study was approved by Ethical Clearance Committee of Faculty of Medicine, Universitas Tanjungpura (No: 7924/UN22.9/PG/2024).

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