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ORIGINAL

Relationship between algD gene and biofilm density in clinical isolates of Pseudomonas Aeruginosa

Relación entre el gen algD y la densidad de biopelícula en aislamientos clínicos de Pseudomonas Aeruginosa

Desi Wulandari[1], Puspa Wardhani[2], Aryati[1]

[1]Master of Basic Medical Sciences Student, Faculty of Medicine, Airlangga University, Surabaya, Indonesia.
[2]Department of Clinical Pathology, Faculty of Medicine, Airlangga University, Surabaya, Indonesia.

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Corresponding author: Puspa Wardhani

ABSTRACT

Introduction: infection due to Pseudomonas aeruginosa is a cause of nosocomial infections that are acquired when patients are hospitalized. The incidence of bacterial infections is 80 % related to biofilm formation, which is the main mediator of infection. Pseudomonas aeruginosa genetically produces at least three polysaccharides that help the biofilm formation process and maintain the stability of the biofilm structure, one of which is the algD gene.
Objective: analyze the relationship between the presence of the algD gene and biofilm density in clinical isolates of Pseudomonas aeruginosa.
Method: analytical observational research, consecutive sampling technique with a total sample of 33 clinical isolates of Pseudomonas aeruginosa. The biofilm formation test uses the microtiter plate assay method to determine the presence of the algD gene in the conventional PCR method.
Results: the results of the biofilm development process showed that 4 isolates (12,1 %) did not produce biofilm and 29 isolates (87,9 %) produced biofilm, including 10 isolates (30,3 %) produced weak biofilm, 13 isolates (39,4 %) produces moderate biofilm. And 6 isolates (18,2 %) produced strong biofilms. Based on PCR amplification, 31 isolates (93,9) carried the algD gene and 2 isolates (6,1 %) did not carry the algD gene. The statistical analysis results using the chi-square test obtained a p-value = 0,011.
Conclusion: There is a significant relationship between the presence of the algD gene and biofilm density in clinical isolates of Pseudomonas aeruginosa.

Keywords: Pseudomonas Aeruginosa; Biofilm Density; algD Gene.

RESUMEN

Introducción: la infección por Pseudomonas aeruginosa es una causa de infecciones nosocomiales que se adquieren durante la hospitalización de los pacientes. La incidencia de infecciones bacterianas está relacionada en un 80 % con la formación de biopelículas, que es el principal mediador de la infección. Pseudomonas aeruginosa produce genéticamente al menos tres polisacáridos que ayudan en el proceso de formación de biopelículas y mantienen la estabilidad de la estructura de la biopelícula, uno de los cuales es el gen algD.
Objetivo: analizar la relación entre la presencia del gen algD y la densidad de biopelículas en aislados clínicos de Pseudomonas aeruginosa.
Método: estudio observacional analítico mediante técnica de muestreo consecutivo con una muestra total de 33 aislados clínicos de Pseudomonas aeruginosa. La prueba de formación de biopelículas utiliza el método

de ensayo en placa de microtitulación y para determinar la presencia del gen algD utiliza el método de PCR convencional.
Resultados: los resultados del proceso de desarrollo de biopelículas mostraron que 4 aislados (12,1 %) no produjeron biopelícula y 29 aislamientos (87,9 %) produjeron biopelícula, de los cuales 10 aislados (30,3 %) produjeron biopelícula débil, 13 aislados (39,4 %) producen biopelícula moderada. biopelícula. biopelícula y 6 aislados (18,2 %) produjeron biopelículas fuertes. Según la amplificación por PCR, 31 aislados (93,9) portaban el gen algD y 2 aislados (6,1 %) no portaban el gen algD. Los resultados del análisis estadístico mediante la prueba de chi-cuadrado obtuvieron valor de p = 0,011.
Conclusión: existe una relación significativa entre la presencia del gen algD y la densidad de biopelículas en aislados clínicos de Pseudomonas aeruginosa.

Palabras clave: Pseudomonas Aeruginosa; Densidad de Biopelícula; Gen algD.

INTRODUCTION

Humans are susceptible to opportunistic infections caused by Pseudomonas aeruginosa. is a Gram-negative bacterium, invasive and toxogenic, can cause infections, and is the cause of nosocomial infections acquired when hospitalized.[1] The incidence of infection due to Pseudomonas aeruginosa in Indonesia reaches around 30 %.[2] Studies conducted in 11 DKI Jakarta hospitals revealed that 9,8 % of inpatients experienced nosocomial infections.[3] Nosocomial infections usually occur in patients with pus wound infections, burns, cystic fibrosis, and septicemia.[4] Pseudomonas aeruginosa pathogenesis is dependent on virulence factors, which are essential for both host tissue invasion and bacterial colonization, both of which can exacerbate infections. Biofilm is one of Pseudomonas aeruginosa's key virulence factors.[5]

Biofilm is a collection of bacteria that have attached to tissue and are coated with extracellular matrix and polysaccharides produced by the bacteria themselves.This makes the bacteria resistant to antibiotics and makes treating infections more difficult.[6] The incidence of bacterial infections is approximately 80 % associated with the formation of biofilms, making biofilms the main mediator causing infection.[7] Matrix components in biofilm formation include nucleic acids, lipids, polysaccharides, and proteins.[8] The stability of the biofilm structure depends on the genetic production of at least three polysaccharides by Pseudomonas aeruginosa: alginate, pel, and psl.[9] The Alginate gene is a polysaccharide that is produced in response to stress linked to chronic infection, whereas the Psl and Pel genes are crucial for the early phases of biofilm formation, namely during the acute phase of infection.[10] Only non-mucoid bacteria can create pel and PSL, although mucoid bacteria can produce alginate.

Alginate's advantages are that it protects against biofilm architecture and facilitates the colonization process. Overproduction of alginate boosts the biofilm-forming capability and increases the pathogenicity of Pseudomonas aeruginosa.[11,12] α-L-guluronic acid and ß-D-mannuronic acid make up the polymer alginate. It contributes to both biofilm stability and cell defense and is crucial for the biofilm's ability to retain water and nutrients.[13] AlgD is the first gene involved in alginate manufacture, and most alginate-producing genes are found in vast operons. algD transcriptional activation is a key point in regulating the alginate synthesis pathway.[14] The algD gene has an important role for Pseudomonas aeruginosa bacteria, namely encoding GDP mannose dehydrogenase, an important enzyme of the Alginate and polymer biosynthetic pathway that represents an important protection mechanism for bacteria. The algD gene is essential in biofilm structuring and regulates Alginate capsule polysaccharides and virulence factors.[15]

Biofilm density refers to the total number of bacterial cells trapped in the matrix that forms the biofilm and will form a density. The biofilm is at the edge of the dissolved microtiter plate. The microtiter plate is a quantitative method that is the gold standard for testing Bacteria that have the ability to stick to the plastic surface of the microtiter plate. The advantages of using the microtiter plate method are the ease of use of basic laboratory materials and adaptation to small or large samples that can be tested in a single measurement.[16] The disadvantage of using this method is the use of crystal violet dye which raises concerns due to its environmental toxicity.[17]

The testing method for detecting the algD gene in Pseudomonas aeruginosa is Polymerase Chain Reaction (PCR). PCR is an enzymatic method for amplifying DNA in vitro, Has advantages in terms of speed, specificity, and sensitivity in detecting a microorganism, making PCR the "method of choice".[18] The disadvantage of the PCR method is the possibility of false positives or negatives. The limitation of PCR is that it requires special equipment and is not cheap.[19] Previous research focused on the presence or absence of genes in biofilm producing bacteria. Research on the relationship between the algD gene and biofilm density in clinical isolates of Pseudomonas aeruginosa is very rarely carried out in Indonesia, especially in Surabaya. The research aims to determine the ability of various clinical isolates of Pseudomonas aeruginosa to produce biofilms and their relationship with the algD gene.

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METHOD

This study design used observational analysis using clinical isolates of Pseudomonas aeruginosa in the Clinical Microbiology Unit of Airlangga University Hospital, Surabaya. This study received approval from the Health Research Ethics Committee at Airlangga University Hospital. Through a letter with the number: 097/KEP/2024.

Sample

Clinical isolates that have been identified as Pseudomonas aeruginosa with a sample probability percentage of ≥ 93 % using the VITEK 2 Compact tool were collected and stored in cryotubes containing TSB liquid media and 20 % glycerol, then stored in a freezer at a temperature of -80℃ in the Clinical Microbiology Unit of Airlangga University Hospital. The sample collection process was carried out in July 2024 - September 2024. The collected samples were continued with biofilm density examination using an ELISA Reader (BioRad iMark Microplate Reader) and detection of the algD gene with PCR (SimpliAmp™Thermal Cycler) carried out at the Dengue Institute of Tropical Disease (ITD) Laboratory, Airlangga University. We used Pseudomonas aeruginosa PAO1 as a positive control for the examination of the algD gene and biofilm density.

Preparation of Microbial Samples

Samples of clinical isolates of Pseudomonas aeruginosa obtained from the Clinical Microbiology of Airlangga University Hospital were inserted into a cryotube containing Tryptic Soy Broth (TSB) medium and 20 % glycerol which was stored at a temperature of 37 ℃ for 24 hours and then stored in a freezer of -80 ℃. Bacterial isolates in Tryptic Soy Broth (TSB) medium and 20 % glycerol were taken using a sterile ose needle inserted into MacConkey agar media for bacterial growth and kept in an incubator for 24 hours at 37 ˚C. Bacterial isolates grown in MacConkey agar media were then subcultured on MacConkey agar media and thereafter incubated for 24 hours at 37 ˚C.

Biofilm Assay

After subculture for 48 hours, bacterial colonies were taken using sterile cotton swabs and then inserted into a test tube containing 0,45 % NaCl solution as a standard McFarland 0,5 solution. The tube containing 1,980 µl of Tryptic Soy Broth (TSB) was added with 20 µl of Pseudomonas aeruginosa bacterial suspension, then inserted into each well of the microtiter plate as much as 200 µl. The microtiter plate was closed and incubated at 37℃ for 24 hours. The wells were washed using Phosphate Buffer Saline (pH 7,2) three times and then dried by turning them upside down to dry in the open air. The bacterial biofilm bound to the well was fixed with 150 µl of 96 % methanol for 20 minutes. The microtiter plate was tapped at each washing process to remove the solution from the well. Biofilm with 1 % Crystal violet for 5 minutes and rinse gently 3 times with distilled water. Biofilm attached to the walls and bottom of the tube that has been stained with crystal violet will be dissolved with 150 µl of 96 % ethanol in each well. The preparation was left at room temperature for 5 minutes. The thickness of the biofilm attached to the bottom of the microtiter plate well was read using an ELISA reader with an Optical Density value read at λ 595 nm. This examination was repeated 3 times. The optical density value of each isolate (ODi) was taken and averaged over the triple wells, then the results were compared with the negative control optical density (ODc). Isolates were categorized into four categories, based on the average optical density (OD) associated with the ODc values. The four Categories are based as follows: ODi ≤ ODc indicates non-compliance; ODc < ODi ≤ 2 x ODc indicates weak compliance (+); 2 x ODc < ODi ≤ 4 x ODc indicates moderate compliance (++); and 4x ODc < ODi indicates very compliant (+++). As a negative control, TSB broth devoid of bacterial inoculum was employed. [27]

algD Gene Detection

DNA Extraction

DNA was extracted using the Biospin Bacteria Genomic DNA Extraction Kit (BIOER, China) depending on the DNA extraction procedure. To be used in PCR, the isolated DNA was kept at -20˚C. Primary: In this study, specific primers of the algD gene were designed based on the NCBI gene sequence information base. The primers were prepared by Costum oligo Macrogen, South Korea, as shown in (table 1).

Table 1. algD gene primers (July 07, 2024, Surabaya)

Gene	Primary	Primary Sequence (5'-3')	Amplicon size (bp)
algD	F R	AGAAGTCCGAACGCCACACC CGCATCAACGAACCGAGCATC	550bp

The reagent used is Master mix (NEXproTM e PCR 2X Master Mix), Composition of materials examined in PCR: Master mix as much as 12,5 µl, 1 µl primer forward gene algD, 1 µl primer reverse gene algD, 7,5 µl ddH2O. The

total final volume is 20 µl/sample tube. DNA template (sample) is pipetted as much as 3 µl which has been filled with 20 µl PCR mix so that the total final volume of each tube is 25 µl. PCR amplification with PCR SimpliAmpTM Thermal Cycler with PCR program settings, namely, predenaturation 95 ºC 5 minutes, denaturation 95 ºC 2 minutes, annealing 63ºC 30 seconds, extension 72 ºC 1 minute, the number of cycles is 35 times. Final Extension 72 ºC 5 minutes. The PCR results were then electrophoresed on a 2 % agarose gel for 30 minutes at 100 Volts and visualized using a UV transumilator. Positive PCR results of the algD gene and positive control (Pseudomonas aeruginosa PAO1) were indicated by the appearance of DNA fragments of 550 bp each.[11]

Statistical Methods

Statistical data analysis was conducted by correlation analysis using Fisher's Exact Test to examine the association between the algD gene and biofilm density in clinical isolates of Pseudomonas aeruginosa using the SPSS version 25 software. Fisher's exact test was employed with a p-value of 0,05 to assess significance.

RESULTS

Distribution of Specimens in Clinical Isolates of Pseudomonas aeruginosa

During the research period from July to November 2024, 33 clinical isolates of Pseudomonas aeruginosa were obtained from patients at Airlangga University Hospital. Of these, the largest number of isolates came from sputum specimens with 22 isolates (66,67 %), followed by urine specimens with 6 isolates (18,18 %). In addition, 3 isolates (9,09 %) came from pus specimens, and 2 isolates (6,06 %) were obtained from blood specimens. Data on the distribution and characteristics of these isolates are based on the recording of Pseudomonas aeruginosa culture examination logbooks conducted in the Clinical Microbiology Laboratory of Airlangga University Hospital.

Table 2. Distribution of Pseudomonas aeruginosa Clinical Isolate Specimens (September 09, 2024, Surabaya)

Specimen	Number of Specimens	Percentage
Sputum	22	66,67 %
Urine	6	18,18 %
Blood	3	9,09 %
Pussy	2	6,06 %
Total	33	100 %

Biofilm Density Examination Results on Clinical Isolates of Pseudomonas aeruginosa

Table 3. Biofilm Density of Clinical Isolates of Pseudomonas aeruginosa (September 09, 2024, Surabaya)

Biofilm Density	Number of Isolates	Isolate percentage
Does Not Produce Biofilm (-)	4	12,1 %
Producing Weak Biofilm (+)	10	30,3 %
Producing Moderate Biofilm (++)	13	39,4 %
Producing Strong Biofilm (+++)	6	18,2 %
Total	33	100 %

Results of algD gene examination in clinical isolates of Pseudomonas aeruginosa

Table 4. The algD gene in clinical isolates of Pseudomonas aeruginosa (Airlangga University Hospital, Surabaya, 2024)

Number of Pseudomonas aeruginosa Isolates	algD gene (+)	algD gene (-)
33	31(93,9 %)	2 (6,1 %)

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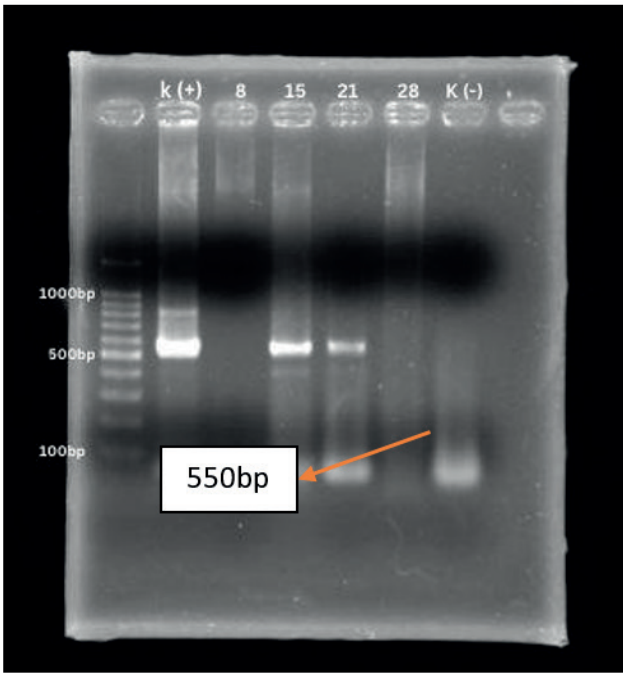

Figure 1. Elektroforesis gel agarosa (gen algD produk PCR,the band 550 bp) (Airlangga University Hospital, Surabaya, 2024)

Table 5. Relationship between algD gene and biofilm density in clinical isolates of Pseudomonas aeruginosa (Airlangga University Hospital, Surabaya, 2024)

Biofilm Density	algD gene (+)	algD gene (-)
Does Not Produce Biofilm	2 (50 %)	2 (50 %)
Producing Weak Biofilms	10 (100 %)	0 (0 %)
Producing Medium Biofilms	13 (100 %)	0 (0 %)
Producing Strong Biofilms	6 (100 %)	0 (0 %)
Total	31	2

A significant association (p-value of 0,011) was found between biofilm density and the presence of the algD gene in clinical isolates of pseudomonas aeruginosa, according to statistical data analysis using Fisher's exact test.

DISCUSSION

The distribution of clinical isolate specimens of Pseudomonas aeruginosa in this study showed a dominant pattern in the type of sputum specimen, namely 22 (66,67 %). This reflects that Pseudomonas aeruginosa is more often found in respiratory tract infections, where patients who have respiratory diseases can reduce their respiratory immunity and make them susceptible to Pseudomonas aeruginosa infection.[20] The percentage of isolates from urine specimens (18,18 %), supports the fact that this bacteria is often involved in urinary tract infections related to catheterization during surgical procedures in hospitals or in other urinary tract diseases that facilitate the colonization of Pseudomonas aeruginosa bacteria.[21] Pus isolates (9,09 %) indicate wound infection and blood (6,06 %) indicate bloodstream infection.

In this study, the ability to form biofilms in clinical isolates of Pseudomonas aeruginosa in 33 isolates obtained results, namely 4 (12,1 %) did not produce biofilms and 29 (87,9 %) were able to produce biofilms, including 10 (30,3 %) producing weak biofilms, 13 (39,4 %) producing moderate biofilms and 6 (12,1 %) producing strong biofilms.

Research conducted by Vasiljevic et al. involving 163 Pseudomonas aeruginosa isolates revealed that 97,55 % of these isolates were capable of producing biofilm. Among them, 39,26 % formed strong biofilms, 34,36 % medium biofilms, 23,93 % weak biofilms and 2,45 % of the isolates did not produce biofilms. According to Banar et al., Isolates that developed biofilms generated 21,8 % weak, 47,3 % medium, and 30,9 % robust biofilms. 2 isolates, representing 3,5 % did not produce any biofilm at all.[22]

The biofilm formed protects bacteria from the body's immune system and antibiotic therapy, resulting in infections that are difficult to treat and require longer treatment times. The spread of isolates that can produce biofilms is a problem for public health because it has the potential to increase morbidity rates, mortality, and

antimicrobial resistance, especially in immunocompetent patients.[23]

In this study, the results of PCR amplification showed that the algD gene was detected in as many as 31 (93,9 %) of 33 clinical isolates of Pseudomonas aeruginosa. This outcome is consistent with a research by Banar et al. that found that 100 % of the 57 isolates of Pseudomonas aeruginosa had the algD gene. In addition, another study conducted reported algD gene frequencies of 98 %, 87,5 %, and 78,9 %, respectively.[11,14,23]

The findings of the study demonstrated a significant relationship (p 0,011) between the biofilm density and the presence of the algD gene in clinical isolates of Pseudomonas aeruginosa. The research results are in line with Rajabi et al, who identified a significant association between the presence of the algD gene and the development of biofilms in Pseudomonas aeruginosa clinical isolates, with a p-value greater than 0,01.

Pseudomonas aeruginosa strains carrying biofilm-forming genes, such as algD, are known to have a higher capacity to cause severe infections.[24] The resulting biofilm protects the germs from the body's immune system and antibiotic therapy. resulting in infections that are difficult to treat and require a longer treatment time.[25]

The algD gene encodes the enzyme GDP-mannose dehydrogenase, a key step in alginate biosynthesis. The primary precursor for alginate production, GDP-mannose, is changed by this enzyme into GDP-mannuronate. Alginate is crucial for protecting biofilms and giving them structural stability. Alginate is the main exopolysaccharide in the biofilm matrix that protects against antibiotics and the immune system.[26] Consistent detection of the algD gene in most isolates indicates the importance of the algD gene to the virulence of Pseudomonas aeruginosa. High detection of the algD gene suggests that this gene is a relevant molecular target for the diagnosis or development of therapies aimed at inhibiting biofilm formation.

The spread of isolates with biofilm-forming ability is a serious challenge for public health because can potentially increase morbidity, mortality, and antimicrobial resistance. These results emphasize the importance of monitoring biofilm-forming genes in Pseudomonas aeruginosa isolates as a step to identify the risk of wider spread of infection and design strategies for more effective infection control. A limitation of the study was not testing Alginate levels produced by mucoid and non-mucoid strains so it could not verify whether the presence of the algD gene was directly correlated with Alginate production. The study did not test other genes (Psl and Pel) to determine the influence of other genes on biofilm formation.

CONCLUSION

According to the study, biofilm density and the presence of the algD gene in clinical isolates of Pseudomonas aeruginosa were significantly correlated.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORSHIP CONTRIBUTION

Conceptualization: Desi Wulandari.
Data curation: Desi Wulandari, Puspa Wardhani, Aryati.
Formal analysis: Desi Wulandari.
Research: Desi Wulandari.
Methodology: Desi Wulandari, Puspa Wardhani, Aryati.
Project management: Desi Wulandari, Puspa Wardhani, Aryati.
Resources: Desi Wulandari, Puspa Wardhani, Aryati.
Software: Desi Wulandari.
Supervision: Puspa Wardhani, Aryati.
Validation: Puspa Wardhani, Aryati.
Display: Puspa Wardhani, Aryati.
Drafting - original draft: Desi Wulandari.
Writing - proofreading and editing: Desi Wulandari, Puspa Wardhani.