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Research Article

Uncovering the Enolase Gene (*eno*) and Its Role in Biofilm Formation in Clinical Isolates of *Staphylococcus aureus*

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ABSTRACT

Background: Enolase is an enzyme potentially possessed by *Staphylococcus aureus* (*S.aureus*) bacteria, which holds essential virulence factors in human infections. The eno gene that encodes enolase is important in attachment to host cells, leading to biofilm formation, evasion of host immune response, and bacterial central metabolism. This biofilm formation might complicate the therapy. **Purposes:** This study aimed to assess the prevalence of the enolase gene, namely eno, in clinical isolates of *S.aureus* and its association with biofilm production. **Methods:** The research was conducted from December 1, 2023, to February 29, 2024, at the Faculty of Medicine and Health Science Research Laboratory, Warmadewa University. This study employed an analytical approach with a cross-sectional design. **Result:** The collected samples comprised 18 isolates of *S.aureus*, 66.6% of which produced biofilm. Most of the *S.aureus* clinical isolates 17 (94.4%) were detected to have the eno gene. Six samples (33.3%) formed weak biofilm followed by strong and moderate, with the same number of 3 isolates each (16.7%). No correlation between the enolase gene and biofilm production in this study suggested phenotypic heterogeneity, environment and time forming biofilm in vivo differences, and various other genes that influence biofilm formation. **Conclusion:** The high prevalence of the enolase gene in these clinical isolates indicates the potential for more severe infections in patients related to its adherence, which leads to biofilm and resistance problems and metabolic function.

Keywords: biofilm, eno gene, *Staphylococcus aureus*

INTRODUCTION

Enolase enzyme is known as a critical component in the bacterial attachment to host cells, evasion of host immune responses, and biofilm formation (1). Enolase has been shown to bind to various host proteins, including plasminogen and laminin, which promotes bacterial invasion and spread. Other than that, enolase also plays a crucial role in glycolytic pathway of bacterial metabolism and evasion of host immune response (1,2). This enzyme is encoded by *eno* gene. The *eno* gene emerged as one of the most frequently detected Microbial Surface Components

Recognizing Adhesive Matrix Molecules (MSCRAMMs) gene that are pivotal factors in influencing both colonization and the development of antibacterial resistance (3). This *eno* gene prevalence in *S.aureus* varied across similar studies ranging from 29.6% to 85.6% and even 95.3% (4–6).

Staphylococcus aureus especially Methicillin-resistant *Staphylococcus aureus* (MRSA) strains, which are gram-positive coccus bacteria, are recognized as the primary culprits behind numerous human infectious ailments, spanning skin and soft tissue, respiratory tract, bone, joint, and endovascular infections, often linked to medical devices or implants (7–10). *S. aureus* colonize about 20-30% of people without causing disease (10). However, the presence of numerous virulence genes might convert commensal *Staphylococcus* into invasive pathogens (11).

The majority of *Staphylococcus spp.* are biofilm formers (12–14). This ability can lead to severe and chronic infections. Biofilms are characterized as a consortium of microbial cells that adhere permanently to a surface—whether inanimate or living—through an extracellular matrix composed of polymers (EPSs) (9,15). Biofilms form because microorganisms or bacteria tend to create a safe and comfortable environment for themselves (16).

S. aureus encodes many proteins that act as virulence factors including numerous crucial genes play a significant role in enabling *S. aureus* to adhere and penetrate host cells while evading the host's immune defenses (3). Twelve genes, namely *eno, icaA, icaB, icaC, icaD, fnbA, fnbB, fib, clfA, clfB, ebps,* and *cna*, have been identified as linked to the adhesion process and biofilm formation in *S. aureus*,. Alongside *eno* as one of the most common adhesin genes, the *ica* gene, specifically *icaD*, was identified in up to 90 % of *S. aureus* isolates and all biofilm-forming isolates, showing a significant correlation with biofilm formation (P = 0.0001) (4,17). We have done previous study regarding the *ica* A/B/C/D gene detection and correlation with biofilm production (17). The prevalence of genes suggests a multifaceted approach to biofilm production.

The presence of the *eno* gene in *S. aureus* also varies across the studies alongside the expression of the gene as a biofilm producer. This difference in prevalence and its correlation makes researchers willing to know the numbers that are close to the samples taken in the research area hence in the future, it can be continued as a basis for further research in genetic modification, development of anti-adhesion therapies, enhanced infection control by disrupting biofilms production in clinical settings, and even designing antibiotics that penetrate biofilms or inhibit biofilm-specific bacterial functions. In addition, no other regional or national studies have addressed this species' eno gene. The aim of this study was to determine the prevalence of the eno gene in clinical isolates of *S.aureus* and its correlation with their ability to form biofilms.

METHODS

The research was conducted from December 15, 2023, to February 29, 2024, at the research laboratory of the Faculty of Medicine and Health Science, Warmadewa University. This study is an analytical study with a cross-sectional design to determine the prevalence of the eno gene in clinical isolates of *S. aureus* and its correlation with biofilm production ability.

The target population of this study was all patients with *S. aureus* infection. The target population was all patients with *S. aureus* infection who underwent treatment at Sanjiwani Hospital. Samples were taken consecutively. The study sample was patients with *S. aureus* infection who were hospitalized from June 2022 to December 2022. The inclusion criteria of this study were *S. aureus* isolates taken from cultures of infected patients. The exclusion criteria were incomplete patient medical record data and isolates that damaged during storage and transportation. Based on Slovin formula, with a degree of confidence of 1.96 an acceptable degree of error (d) of 0.10, and a P value based on previous research of 95.3% (3), the number of samples required is 17.2 samples (rounded to 18).

S. aureus Identification

Identification of *S. aureus* was carried out by culturing samples on Blood Agar (BA) media and then incubating for 24 hours at 37°C (8,9). *Staphylococcus* isolates showed positive results from the catalase test followed by the identification and resistance test including cefoxitin screen test for MRSA using the VITEK®2 biomerieux automatic machine (identification rate >90%).

Biofilm assay

The process continued with the biofilm test, as previously optimized and used during our study in 2021 and 2022 (17,18). The process begins with inoculation of bacteria from the culture in 1% glucose liquid and phosphate buffer saline (PBS) with PH 7 as much as 3 ml, measure turbidity with densicheck in the range of 0.50-0.63 Mac Farland, and transfer the suspension as much as 200 microliters to the microplate and store without shaking for 48 hours at 37˚C. Next, discard the remaining solution and rinse with aquabidest in each well. The biofilm cells adhered to the well were stained by adding 200 microliters of 0.1% crystal violet for 5 minutes. Following this, the plate underwent washing and drying. Subsequently, 200 microliters of 30% acetic acid were added to each well, and left to dissolve for 5-15 minutes, and the fluorescence intensity of crystal violet was measured using a microplate ELISA reader at a wavelength of 620-670 nm.

Gene Detection

DNA isolation was done by boiling method (19). Cultured colonies on BA media were taken and put into TE (Tris EDTA) solution pH 8. Bacterial stock in TE was vortexed and then boiled for 10 minutes at 100°C. After that, it was immersed in ice water for ± 3-5 minutes, then the sample was centrifuged at 8000 rpm for 1 minute. The supernatant was taken as much as 100 µl placed in an eppendorf® tube and stored in a refrigerator at -20°C. The centrifuged supernatant is the DNA that will be used for research and is ready to be detected using PCR.

PCR test was performed conventionally with a MiniAmp Thermal Cycler (Thermo Fisher Scientific®). The PCR process started by making a mixture of primers and master mix then adding DNA (0.5-1 ul) from the specimen to be examined to obtain a total of 25 ul of solution for PCR (20).

Primer sequences of *eno* gene are ACG TGC AGC AGC TGACT (F) and CAA CAG CAT YCT TCA GTA CCTTC (R). The expected amplification product sizes for PCR amplification

are 302 bp (21). The annealing TM of *eno* gene was obtained by adding 5°C to the average TM of the forward and reverse primers indicated in the primer instructions. To obtain the best annealing temperature, optimization was performed and the final reaction can be viewed in table 1.

Table 1. Reactions were performed in a thermal cycler.

Gene	Step 1	Step 2				Step 3
	Initial denaturation	Denaturation	Annealing	Extension	Cycle	Final Extension
eno	94℃ 5 min	94℃ 60 sec	57℃ 60 sec	72℃ 60 sec	30	72℃ 10 min

The PCR products were analyzed using 1.5% agarose gel electrophoresis. Initially, 0.52 grams of agarose was weighed and dissolved in 35 ml of 1X TBE buffer. The solution was then heated to boiling in a microwave for 2 minutes, followed by allowing it to cool slightly. Finally, 1 µl of gel Red dye was added to the solution. Next, agarose was poured into a mold that had been given a comb to form wells. A total of 2 µl of PCR product was inserted into the well and then processed on an electrophoresis device for 45 minutes with a voltage of 100 Volts. The results of agarose gel electrophoresis were visualized using a UV Transilluminator. These results can also be read with Geldock for better visualization.

Data Analysis

The collected data were then analyzed in two stages. Initially, descriptive statistical analysis was performed to outline the characteristics of each variable. Data variables were presented as relative frequencies, denoted by both the number and percentage. The subsequent stage involved assessing the correlation between biofilm formation and the *eno* gene. The biofilm formation ability will be categorized into four groups. All data analysis was conducted using SPSS software for Windows, version 25.

This study was granted ethical approval from the ethics committee of the Faculty of Medicine and Health Sciences, Universitas Warmadewa with number: 86/Unwar/FKIK/EC-KEPK/I/2024 on 10 January 2024.

RESULTS

The study encompassed a total of 18 *S. aureus* isolates. Samples were collected from patients predominantly within the age range of 46-65 years, comprising 8 samples (44.4%). Additionally, female patients accounted for the majority, with 11 cases (61.1%). The most common specimen type was blood with 6 samples (33.3%) followed by wound swab 5 (27.8%) and sputum 4 (22.2%).

Biofilm formation was analyzed and categorized based on the absorbance of attached cells by crystal violet staining. Biofilm-producing *S. aureus* ATCC 35556 strain was used for strong biofilm production control (5).

Table 2. Subjects Characteristics based on Research Variables

Variable	Total (%) (n=18)
Age (Year)	
<17	6 (33.3)
17-45	1 (5.6)
46-65	8 (44.4)
>65	3 (16.7)
Sex	
Male	7 (38.9)
Female	11 (61.1)
Staphylococcus aureus	
MSSA	14 (78)
MRSA	4 (22)
Specimen type	
Blood	6 (33.3)
Wound swab	5 (27.8)
Sputum	4 (22.2)
Pus	2 (11.1)
Tissue	1 (5.6)
Diagnosis	
Infected wound/ abscess/ cellulitis	5 (27.8)
Pneumonia or other respiratory infection	6 (33.3)
CKD	4 (22.2)
DM and the complications	2 (11.1)
Sepsis and Fever of unknown origin (FUO)	1 (5.6)

Results revealed that 66.6% of isolates produced biofilm. By measuring the optical density using an ELISA reader, the mean biofilm formed (OD) was 0.277. The lowest value was 0.02 and the highest was 0.664. The cut-off of the optical density limit (ODc) was determined from the quantitative value of biofilm. It is defined as the mean OD of the negative control +3× standard deviation (SD) of the negative control as seen in table 3.

Table 3. Biofilm producing category

Cut off value	ODc mean	Ability to form biofilm
OD > 4×ODc	OD > 0.500	Strong
2×ODc < OD ≤4×ODc	0.250 < OD ≤0.500	Moderate
ODc< OD ≤ 2×ODc	0.125 < OD ≤ 0.250	Weak
OD ≤ 0.08324	OD ≤ 0.125	None

The categorical distribution of biofilm values in this study was calculated and shown in table 4. Six samples (33.3%) formed weak biofilm followed by strong and moderate with the same

number of 3 isolates each (16.7%). There were 4 samples (22.2%) with a positive cefoxitin test (MRSA).

Table 4. Biofilm production and presence of *eno* gene

Biofilm production	Total (percentage)	Presence of *eno* gene	MRSA
Strong	3 (16.7)	2	1
Moderate	3 (16.7)	3	1
Weak	6 (33)	6	2
None	6 (33)	6	0

The *eno* gene was identified in 17 out of the 18 *Staphylococcus aureus* isolates, indicating a detection rate of 94.4%. One of the strong biofilm producers didn't have the *eno* gene.

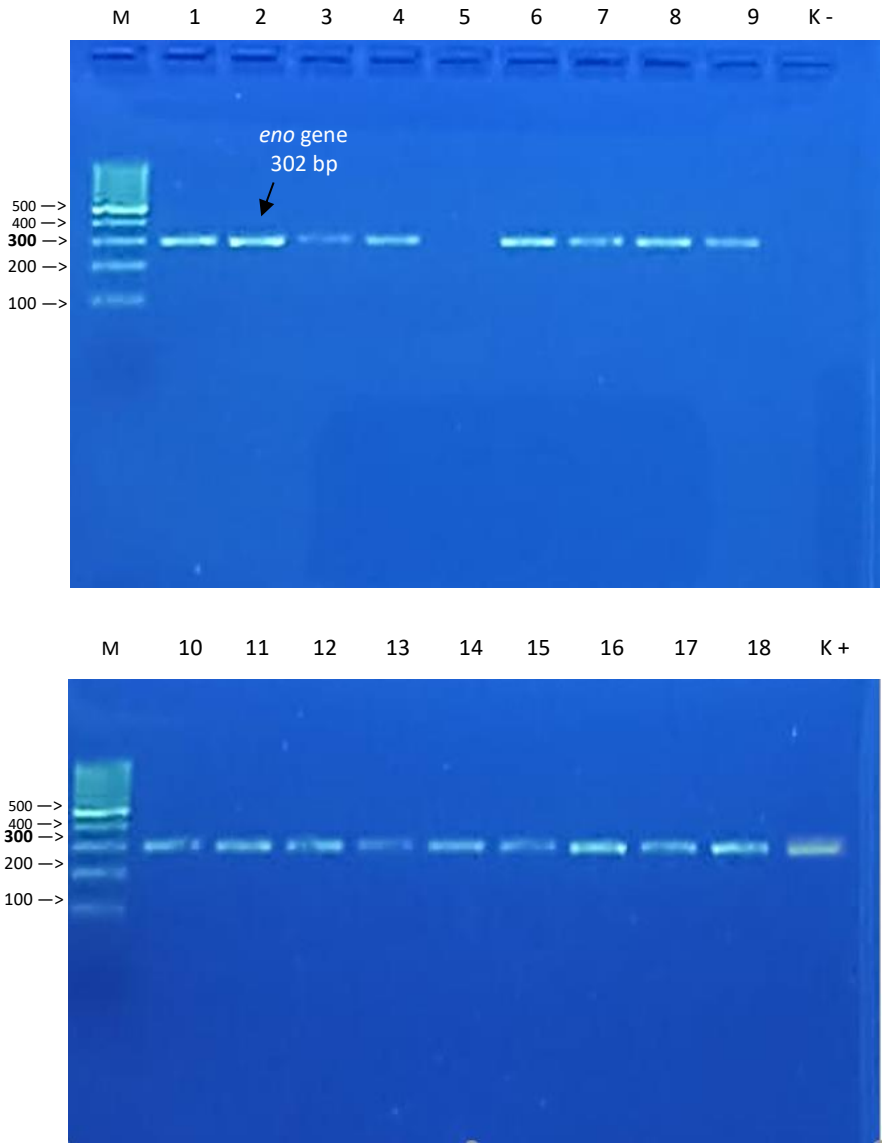

Figure 2. Result of *eno* gene detection by PCR

DISCUSSION

Patient's data revealed the most dominant diagnoses among the samples were skin/ soft tissue infection and lung infection. This was consistent with the predilection of infection caused by *S. aureus* as commensal bacteria on the skin and respiratory tract (22–24).

All non-biofilm-producer *S. aureus* in this study were methicillin-sensitive isolates indicating a good sensitivity. MRSA was detected in 22.2% of isolates. This amount is more or less close to previous research in one tertiary hospital in Indonesia which was Dr. Hasan Sadikin General Hospital, in 2019 that revealed out of the total isolates, 75 (17%) were identified as MRSA, with 46 (53%) of these obtained from surgical patients (24).

Most *S. aureus* isolates in this study produced biofilm during biofilm assay (66.6%). *S. aureus* possesses a virulence factor in the form of biofilm formation, which complicates the treatment of bacterial infections, particularly in patients utilizing medical devices such as intravenous catheters, central venous catheters, implanted devices like pacemakers, and urinary catheters (25–27). In addition, the formation of biofilms on poorly cleaned wound surfaces will hinder the wound-healing process and cause chronic wounds, biofilm-related infections are hard to eradicate and have been the subject of intense scientific research (28).

The *eno* gene was identified in 94.4% of the samples. Even though it varies in previous studies (29.6-95.3%), *eno* gene is generally agreed to be one of the most commonly found MSCRAMMs gene (5,21,29). This study used clinical isolates, allowing for a high prevalence of virulent genes in the samples. Other than its metabolic function, the *eno* gene acts synergistically with other expressed biofilm genes such as *ica* genes to influence the initiation and advancement of bacterial colonization, subsequently leading to biofilm formation (17). Enolase involves in bacterial attachment to host cells and biofilm formation and shown to bind to various host proteins, including plasminogen and laminin, which promotes bacterial invasion and spread hence *eno* gene is called laminin-binding protein. This capability also enhances bacteria's resistance to antibacterial agents and contributes to the development of severe infections. The physical barrier of the biofilm matrix and the altered metabolic state of bacteria within biofilms contribute to this resistance. All MRSA isolates in this study belong to biofilm-producer group either weak, moderate, or strong.

In this study, only one of the samples had no *eno* gene and it was one of the strong biofilm-producer isolates. The variety genes in biofilm-producer bacteria suggests a multifaceted approach to biofilm production, indicating that various factors may influence different stages of biofilm development, not only *eno* gene (4,17).

This study also revealed that all non-biofilm-producer isolates here have the gene. The possibility is that those isolates did not or have not yet expressed the *eno* gene in the form of biofilm production. The expression of genes is often regulated in response to environmental conditions conducive to biofilm formation. Quorum sensing, a bacterial communication process, can coordinate the expression of the genes. Another study observed a significant increase in the expression levels of the genes encoding elastin-binding protein (*ebps*) and laminin-binding protein (*eno*) in biofilm formations at 3 hours, across both strongly and weakly adhering strains (30). Regulation of gene expression is often complex and involves multiple regulatory networks and signaling pathways, bacterial populations can exhibit phenotypic heterogeneity, where different subpopulations express genes at varying levels. The correlation

did not occur between *eno* gene and biofilm production in the study due to the above reasons but the role of the *eno* gene on the likelihood of virulence factors of *S. aureus* remains.

Limitations of this study were the natural genetic variation among bacteria, external factors such as nutrient, pH, temperature, and the presence of host factors (in vivo vs in vitro), the sensitivity and accuracy of techniques used to measure gene expression and biofilm assay. The method of research can be developed further to have more optimal results and closer resemblance to in vivo conditions.

CONCLUSION

The enolase gene (*eno*) is owned by 94.4% of *S. aureus* isolates. The high prevalence of *eno* gene in this study was because the samples used were clinical isolates. Biofilms were produced by 66.6% of the isolates. No direct correlation between the enolase gene and biofilm production in this study suggested gene expression differences (phenotypic heterogeneity), differences in biofilm production in vivo and other biofilm-producing genes influenced the biofilm development in *S. aureus*. The presence of the *eno* gene in these clinical isolates indicates the potential for more severe infections in patients related to its metabolic and adherence function leading to biofilm formation and antimicrobial resistance, especially in individuals with medical devices and immunocompromised conditions.

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None.

CONFLICT OF INTEREST

None.

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