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N-Acetyl Cysteine in combination with vancomycin or linezolid for biofilm eradication of methicillin-resistant *Staphylococcus aureus* (MRSA)

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***Abstract*---**Antibiotics are challenging to deliver because MRSA can build biofilms. In Indonesia, the major parenteral antibiotics for treating MRSA infections are Vancomycin and Linezolid. Because NAC can degrade the biofilm matrix, it is believed that combining it with antibiotics will improve its capacity to destroy MRSA biofilms. An experiment was done at RSUD Dr. Soetomo Surabaya's Clinical Microbiology Laboratory in June 2022. The biofilm-forming bacteria studied were MRSA clinical isolates. The antibiotics utilized were Vancomycin (2 g/mL), Linezolid (4 g/mL), and NAC (8 mg/mL). A biofilm assay microtiter test was also carried out to see how drug exposure affected the biofilm that had grown. Biofilm eradication was observed when comparing the mass yield and biofilm metabolism after treatment to the control. According to the study's findings, using a combination of NAC with Vancomycin or Linezolid was superior to using NAC, Vancomycin, or Linezolid alone. There was no apparent difference between the two combinations, however. The combination of NAC and Vancomycin had the same effect on eliminating MRSA biofilms as the combination of NAC and Linezolid.

***Keywords*---**biofilm, eradication, MRSA.

Introduction

A biofilm is a bacterial colony embedded in a matrix of extracellular polymeric substances linked to the surface. Biofilms let bacteria protect themselves from harmful environments and are essential to the bacterial disease cycle. Even within the same bacterial strain, mature biofilm-forming bacterial cells have biological features that differ from planktonic cells. As a result, biofilm is a chronic infection mediator that contributes significantly to the spread of infection in hospitals. Biofilms are believed to be responsible for 65–80% of all illnesses in humans (Yu, 2019).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a strain of S. aureus with the mecA gene. This gene produces Penicillin Binding Protein 2a (PBP2a), a beta-lactam antibiotic binding protein with a low affinity (Lee, 2018; Lowy, 2003; Mukerjee, 2021). The virulence of *Staphylococcus aureus*, both MSSA, and MRSA, is based on the production of biofilms and a few additional virulence factors (Rahimi, 2016). Bacterial infections are responsible for a significant amount of illness and mortality worldwide. Antibiotics are currently used to treat infections, but most bacterial diseases can create biofilms, which prevent illnesses from being effectively treated.

Several antibiotics' effective therapeutic concentrations against bacteria in biofilms are 10 to 1000 times higher than those against planktonic bacteria (Haney, 2021). Vancomycin and Linezolid have long been the medications of choice for most MRSA infections (Yang, 2018), yet they have no impact when used together as an anti-biofilm. N-acetyl cysteine (NAC) can cleave disulfide bonds required for extracellular protein stability. NAC can penetrate bacterial cell membranes, raise oxidative stress, and halt protein production. In EPS, acetyl and carboxylate groups disrupt proteins and DNA, resulting in biofilm disassembly (Li, 2020). According to one study, NAC doses greater than 7.5 mg/mL can reduce biofilms by 37-95 percent, similar to other studies claiming NAC doses of 8 mg/mL can reduce biofilms (Li, 2020; Leite, 2013). The combination study of NAC 10xMIC and Vancomycin revealed a significant reduction in biofilm (5-6 log 10 CFU) (Leite, 2013).

Extracellular Polymeric Substance is a barrier to reduce antimicrobial penetration, whereas inactivated persister cells make the biofilm antimicrobial resistant. These confounding factors can result in inaccurate antibiotic sensitivity tests of planktonic bacteria in clinical bacteriology, resulting in inappropriate clinical dosing and infection eradication. In addition, the exopolymer matrix protects biofilm cells from phagocytosis and aids in immune evasion of the host immune system. As a result, there is a critical need to develop combination anti-biofilms aimed at biofilm eradication. The matrix degrades when the biofilm is disrupted, forcing the embedded biofilm cells to detach. Without the protection of the biofilm matrix, the released bacterial cells are vulnerable to antibiotics used to treat the biofilm infection.

Method

MRSA isolates that produce biofilms were identified using a microtiter plate biofilm assay with biofilm staining. Isolates shown to generate biofilms will be used as stock isolates until the study is finished. At the start of the investigation, stock isolates were revitalized. Next, the bacterial suspension inoculum's turbidity was adjusted with TSB until the suspension reached 0.5 McFarland (equivalent to $3x10^6$ CFU/ml). Creating a drug solution began with determining the drug concentration in the test solution: NAC at 8 mg/mL, Vancomycin at 2 µg/mL, and Linezolid at 4 µg/mL with the appropriate amount of drug working solution. Microtiter plates are classified into two types: those for crystal violet (CV) staining and those for triphenyl *tetrazolium chloride (*TTC) coloring. A 595 nm spectrophotometer was used to detect the biofilm mass at the bottom of the microplate well, and a 500 nm spectrophotometer was used to evaluate the biofilm metabolic activity, represented in optical density units. The study used six replications. The proportion is then used to compare the size and metabolism of the remaining biofilms to the control.

Results

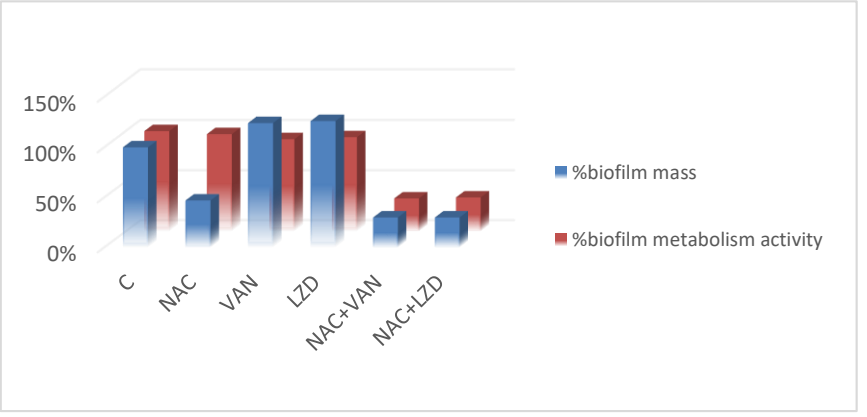

Figure 1. Biofilm eradication test results

Figure 1 depicts the percentage of biofilms that remain after a biofilm eradication test has been performed. The blue figure depicts biofilm mass using CV staining, while the red chart depicts biofilm metabolism activity using TTC staining. The treatment groups in the research are control with no current medicine; exposure to NAC; exposure to Vancomycin; exposure to Linezolid; exposure to both NAC and Vancomycin, and exposure to both NAC and Linezolid.

The Kruskal-Wallis test is used as a non-parametric statistical test to compare biofilms' mass and metabolism activity. The Kruskal-Wallis test results for biofilm mass show a significant difference ($p = 0.00$). Likewise, the results of the Kruskal-Wallis test for biofilm metabolism show a significant difference ($p = 0.00$). The Mann-Whitney test assesses biofilm mass and metabolism (Figure 2). The Mann-Whitney test findings on biofilm mass demonstrate the most significant differences in the groups NAC-Vancomycin combination ($p = 0.032$) and NAC-

Linezolid combination (p = 0.032). Nonetheless, the difference between the two combinations is statistically significant (p = 1.00).

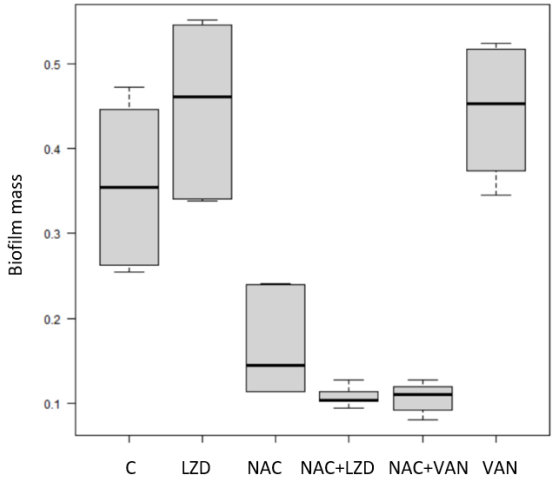

Figure 2. Mann-Whitney test for biofilm mass

The Mann-Whitney test findings on biofilm metabolism demonstrate the most significant differences in the groups NAC-Vancomycin combination (p = 0.032) and NAC-Linezolid combination (p = 0.032). Nonetheless, the difference between the two combinations is statistically significant (p = 1.00) (Figure 3).

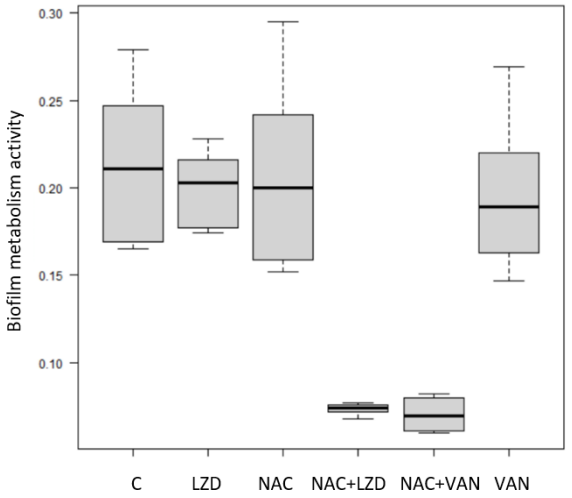

Figure 3. Mann-Whitney test for biofilm metabolism activity

Discussion

Methicillin-resistant *Staphylococcus aureus* (MRSA) can form a biofilm on either a biotic or an abiotic surface (Halim, 2018) via ica-dependent (i.e., encouraged by the ica operon) and ica-independent biofilm matrices (Doulgeraki, 2017). Biofilm

can be generated by species of bacteria, which produce monofilm, or by a variety of species participating in the structure, producing multifilm. The biocide or antibiotic diffusion rate varies throughout the biofilm surface and is influenced by cell temperature, molecule size, and concentration gradient molecules (Liaqat, 2019).

Vancomycin is a glycopeptide antibiotic used as a parenteral treatment for MRSA infections. Vancomycin is unquestionably the best antibiotic for MRSA infections. (Bal, 2017) Susceptible S. aureus strains had a MIC of 2 μg/mL, intermediate resistance strains had a MIC of 4-8 μg/mL, and resistant strains had a MIC of 16 μg/mL. (CLSI, 2021). Linezolid, an oxazolidinone, is the antibiotic of choice for MRSA infection. S. aureus susceptible strains showed MICs of 4 g/mL, while resistant strains had MICs of > 8 g/mL (CLSI, 2021). Several studies have demonstrated that Linezolid is more cost-effective than Vancomycin in treating MRSA infections because it allows for earlier hospital discharge. In general, as compared to Vancomycin, Linezolid can lower patient mortality. According to research, linezolid therapy is superior to Vancomycin for treating MRSA skin and soft tissue infections (Hashemian, 2018). However, there were no significant differences in overall mortality or adverse events between Vancomycin and Linezolid. (Kato, 2020).

N-acetyl cysteine (NAC) is a disruptive biofilm agent that can reduce the biofilm matrix, allowing biofilm cells to exit with minimum physiological impact. NAC with passive ability dispersion results in reduced biofilm biomass, and the remaining biofilm cells are more susceptible to antibiotics than cells in non-dispersed biofilms because they allow more compound antimicrobial penetration through biofilm (Yu,2019). The composition of EPS changes depending on time, location, nutrition availability, stress, mechanical and bacterial species, and the function of the significant EPS barrier to effector host or antimicrobials (Leite, 2013). The mechanism underlying NAC antimicrobial and antibiofilm activity is still unknown. However, it includes (1) inhibition of cysteine utilization in bacteria, (2) reaction between group NAC thiols and cell proteins bacteria, (3) reduction substance polymer extracellular bacteria responsible for adhesion and pathogenicity, and (4) interference balance redox intracellular with potency effect not directly on metabolism cells and transduction signal. NAC is hypothesized to be capable of operating on a level driving matrix, increasing treatment antibacterial efficacy but having no effect on cells in biofilms. NAC activity can cause cell dissociation (Leite, 2013).

Infection with the target pathogen is traditionally treated immediately. On the other hand, some antibiotics' therapeutic concentrations for bacteria in biofilm maybe 100 or even 1000 times higher than bacteria in planktonic culture (Rabin, 2019). To limit antibiotic penetration, biofilm cells can assemble and wrap themselves with matrix exopolymer-produced defenders (alone). Disruption of the biofilm produces matrix deterioration, necessitating the release of embedded biofilm cells. Without the protection of the biofilm matrix, cells discharge bacteria into easily exposed antimicrobials to eradicate biofilm infection.

Regarding research findings of the biofilm eradication test on MRSA using NAC at a concentration of 8 mg/mL revealed that the average biofilm mass was still as

high as 47%, and the average biofilm metabolism was as high as 97% in this study. The biofilm eradication test on MRSA using Vancomycin MIC dose of 2 μg/mL reveals that the average biofilm mass is 124%, and the average biofilm metabolism is 92%. The biofilm eradication test on MRSA using NAC at a dose of 8 mg/mL and Vancomycin MIC dose of 2 μg/mL reveal that the average biofilm mass is still up to 30%, and the average biofilm metabolism is still up to 34%. This shows that killing biofilm with NAC and Vancomycin significantly differed from killing biofilm with NAC and Vancomycin alone. According to the findings, NAC doses greater than 7.5 mg/mL can reduce biofilm by 37-95%, which is consistent with another study that found NAC at a dose of 8 mg/mL can reduce biofilm by 37-95%. (Li, 2020; Leite, 2013). In addition, biofilm is reduced by 5-6 log10 CFU when NAC 10xMIC and Vancomycin are used together (Leite, 2013).

Regarding the biofilm eradication test on MRSA using NAC at a concentration of 8 mg/mL, it was revealed that the average biofilm mass was still as much as 47%, and the average biofilm metabolism was still as much as 97% in this study. On the other hand, the findings of the biofilm eradication test on MRSA using Linezolid MIC dosage of g/mL show that the average biofilm mass is 126%, and the average biofilm metabolism activity is still as high as 94%. Whereas biofilm eradication test findings on MRSA using NAC at a dose of 8 mg/mL and Linezolid at a MIC level of 4 μg/mL reveal that average biofilm mass is still up to 30% and average biofilm metabolism activity is up to 34%. This demonstrates that biofilm eradication utilizing a combination of NAC and Linezolid was significantly different compared to NAC and Vancomycin.

MRSA eradication studies with NAC at 8 mg/mL and Vancomycin MIC at 2 g/mL demonstrate that average biofilm mass is still up to 30%, and moderate biofilm metabolism activity is up to 34%. The biofilm eradication tests on MRSA using NAC at a dose of 8 mg/mL and Linezolid at a MIC level of 4 μg/mL show that average biofilm mass is still up to 30%, and average biofilm metabolism is up to 34%. The statistical test shows no statistically significant difference between the two combinations.

Conclusion

There is a difference between providing a combination of NAC and Vancomycin and using NAC and Vancomycin only for MRSA biofilm eradication. A combination of NAC and Linezolid is more effective than either NAC or Linezolid alone. When it comes to removing MRSA biofilm, there is no difference between the combinations of NAC and Vancomycin and NAC and Linezolid.

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