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# **Vancomycin for methicillin-resistant staphylococcus aureus biofilm eradication is associated with the emergence of heterogeneous vancomycin intermediate staphylococcus aureus**

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**Abstract**--Vancomycin is the first-line therapy for MRSA infections, even though the standard dose is inadequate for biofilm eradication. This study aimed to assess the efficacy of vancomycin in eradicating biofilms and the influence of exposure on the emergence of hVISA isolates. The biofilm formed by MRSA isolates was exposed to vancomycin concentrations of 1 times the MIC, 1,000 times the MIC, and 10,000 times the MIC; exposed continuously for 24 hours vs intermittently for 6 hours/day for 3 days. Measurement of the optical density of the biofilm was carried out to determine the percentage of biofilm eradication. Biofilm specimens exposed to vancomycin were subcultured onto BHIA-VC selective media to isolate hVISA. The highest biofilm eradication effect was found in isolates exposed to vancomycin at a concentration of 10,000 times the MIC. Vancomycin exposure correlated with the emergence of hVISA isolates, especially after exposure to low concentrations of vancomycin. For optimum eradication of MRSA biofilms, vancomycin concentrations exceeding 1,000 times the MIC are required. Exposure to vancomycin at a dose

equal to one-times the MIC had no effect on biofilm eradication and was associated with the emergence of MRSA isolates with decreased susceptibility to vancomycin.

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

## Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the leading causes of persistent bacterial infections in humans (Silva et al., 2021). MRSA is a pathogen that causes mild community-related infections such as skin and soft tissue infections as well as serious infections in hospitals, such as pneumonia, osteomyelitis, brain abscess, and sepsis, with significant morbidity and mortality rates (Cascioferro et al., 2020). In addition to antibiotic resistance, which complicates the treatment of MRSA infections, MRSA's ability to form biofilms makes it one of the major causes of nosocomial and device-related infections (El-Hamid et al., 2020).

Biofilm-forming bacteria have such a tolerance to antibiotics, the body's immune system, and environmental stress due to extracellular matrix protective mechanisms and changes in metabolic rate and growth rate, which makes it challenging to treat biofilm-caused infections (Boakye et al., 2018; Sharma et al., 2019). The release of the device in an attempt to eliminate the source of infection is the primary action in cases of device-related biofilm infection, but this is not always possible, therefore antibiotics are sometimes the only treatment option (Silva et al., 2021).

Vancomycin is a glycopeptide antibiotic that is the main choice for treating MRSA infections. Experiments in vitro and in vivo have demonstrated that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of sessile cells in biofilms are 10-1,000 times greater than those of planktonic cells (Algburi et al., 2017; Cascioferro et al., 2021). Vancomycin has a complex molecular structure and a large size; in vitro studies reveal that its biofilm penetration ability is 64%, which is relatively lower than that of other antibiotics with activity against MRSA (Kaneko et al., 2021). Local administration of antibiotics can increase concentrations of antibiotics at the site of infection with minimal serum antibiotic concentrations and be developed as a therapeutic modality for biofilm-associated infections (Ciofu et al., 2017).

The efficacy of local antibiotics in treating MRSA infections has been extensively studied, yet their clinical application remains limited. One issue impeding its clinical implementation is the concern of increasing resistance rates due to the use of high doses of local antibiotics (Haas and Schultz, 2010). In this study, the authors attempted to eradicate MRSA biofilms by subjecting them to vancomycin and evaluating the effectiveness of eradication as well as the effect of in vitro exposure on the emergence of hVISA/VISA isolates.

## Method

### Study design

Experiments were conducted on ten MRSA isolates from Clinical Microbiology Laboratorium Dr. Soetomo General Academic Hospital and two standard reference isolates (ATCC MRSA 43300 and ATCC *S. aureus* 25923 KWIK-STIK® Microbiologics). The experiment included the identification of MRSA isolates that produce biofilms and proved that they were not hVISA isolates as evidenced by no growth on brain-heart infusion agar with 4 µg/ml vancomycin and 16 mg/ml casein (BHIA-VC) selective media, followed by a biofilm eradication test with vancomycin utilising a microtiter plate biofilm assay, and the detection of hVISA isolates from biofilm specimens after vancomycin exposure.

### Ethical considerations

This study was reviewed by the Ethics Committee of the Faculty of Medicine, Airlangga University (0926/LOE/301.4.2/VI/2022).

### Procedure

Twelve isolates were subcultured onto Nutrient Agar to recover bacteria from frozen stocks. The growing colonies were suspended in normal saline with a turbidity of 0.5 McFarland. The biofilm formation procedure was carried out on a microtiter plate by adding 160 µl of TSB medium, 20 µl of glucose, and 20 µl of bacterial suspension to each well, followed by a 48-hour incubation period.

After the formation of the biofilm, the biofilm eradication test was carried out with vancomycin. The media in the wells was discarded and new TSB medium was added with vancomycin solutions to achieve a concentration of 2 µg/ml, 2 mg/ml, and 20 mg/ml in the test solution/well, respectively. Vancomycin was administered for either 24 hours or 6 hours per day for three consecutive days.

After the incubation procedure complete, microtiter plates are dyed with crystal violet and their optical density was evaluated using a spectrophotometer. To identify bacterial growth from biofilms, sterile swabs were used to collect biofilm samples, which were then plated on Nutrient Agar media. Colonies grown on Nutrient Agar were subcultured into BHIA-VC media to determine the presence of hVISA/VISA isolates. The experiment was conducted for four times.

Table 1. Characteristic of research isolates

Isolate	Specimen source	Biofilm production characteristic <sup>a</sup>
Klinis 1	Blood	<i>Strong-biofilm producer</i>
Klinis 2	Blood	<i>Strong-biofilm producer</i>
Klinis 3	Blood	<i>Strong-biofilm producer</i>
Klinis 4	Urine	<i>Strong-biofilm producer</i>
Klinis 5	Blood	<i>Strong-biofilm producer</i>
Klinis 6	Pus	<i>Strong-biofilm producer</i>
Klinis 7	Pus	<i>Moderate-biofilm producer</i>
Klinis 8	Pus	<i>Weak-biofilm producer</i>
Klinis 9	Blood	<i>Weak-biofilm producer</i>

Klinis 10	Sputum	<i>Moderate-biofilm producer</i>
MRSA 43300	ATCC	<i>Weak-biofilm producer</i>
<i>S.aureus</i> 25923	ATCC	<i>Weak-biofilm producer</i>

ATCC: *American Type Culture Collection*, MRSA: *methicillin-resistant Staphylococcus aureus*. Biofilm production characteristic is assessed from biofilm formation assay. Isolates were classified as strong biofilm producers if the optical density (OD) of the biofilm produced was four times of the negative control, moderate biofilm producers if the OD falls within the range 2-4 times of the negative control, weak biofilm producers if the OD was two times of the negative control, and non-biofilm producers if the OD was less than 0.5 times of the negative control.

## Discussion

The data of optical density value and growth on the BHIA-VC media did not follow a normal distribution (Shapiro-Wilk value 0.000). In terms of percentage, the biofilm density of the test isolate was compared to the biofilm density of the positive control. The OD of isolates exposed to 2 µg/ml vancomycin was greater than the OD of the positive control (without vancomycin exposure).

The eradication of biofilm by vancomycin was determined by the following formula:

$$\% \text{ eradication} = \frac{\text{Mean OD control} - \text{Mean OD treatment}}{\text{Mean OD control}} \times 100 \%$$

Vancomycin exposure at 2 µg/ml had no effect on biofilm eradication. Biofilm eradication rates ranged from 21 to 63.9% in the continuously exposed 2 mg/ml concentration group. Biofilm eradication in the group treated intermittently to 2 mg/ml for six hours per day for three days ranged from 32.8% to 69.2%. Biofilm eradication ranged between 60 and 87.6% in the group that was continuously exposed to 20 mg/ml. In the group that was intermittently exposed to 20 mg/ml, biofilm eradication ranged from 64 to 86.1%. The mean optical density varied significantly between exposure to vancomycin concentrations of 2 µg/ml, 2 mg/ml, and 20 mg/ml ( $p < 0.05$ ; Mann-Whitney; 95% confidence interval). The optical density measurements did not differ significantly between continuous and intermittent exposure groups ( $p = 0.220$ ; Mann-Whitney; 95% confidence interval). Vancomycin did not completely eliminate biofilm at any concentration nor incubation period.

Table 2. Optical density (OD)

Vancomycin Exposure		Isolates (OD)											
Incubation period	Concentration	1	2	3	4	5	6	7	8	9	10	11	12
Positive Control		0,49	0,43	0,28	0,25	0,24	0,19	0,14	0,13	0,12	0,15	0,10	0,08
24 hours	2 µg	0,91	0,50	0,37	0,24	0,29	0,243	0,15	0,135	0,12	0,165	0,13	0,09
	2 mg	0,18	0,25	0,13	0,13	0,19	0,07	0,06	0,07	0,08	0,08	0,06	0,06
	20 mg	0,08	0,10	0,08	0,08	0,08	0,06	0,02	0,04	0,04	0,02	0,02	0,03
Positive Control		0,89	0,84	0,92	0,65	0,71	0,46	0,42	0,40	0,45	0,52	0,76	0,32
6 hours/day for 3 consecutive days	2 µg	1,31	1,18	0,93	0,79	0,86	0,57	0,46	0,50	0,41	0,69	0,84	0,33
	2 mg	0,60	0,50	0,28	0,30	0,32	0,19	0,17	0,18	0,15	0,28	0,31	0,21
	20 mg	0,29	0,30	0,13	0,17	0,17	0,09	0,08	0,10	0,08	0,18	0,11	0,09

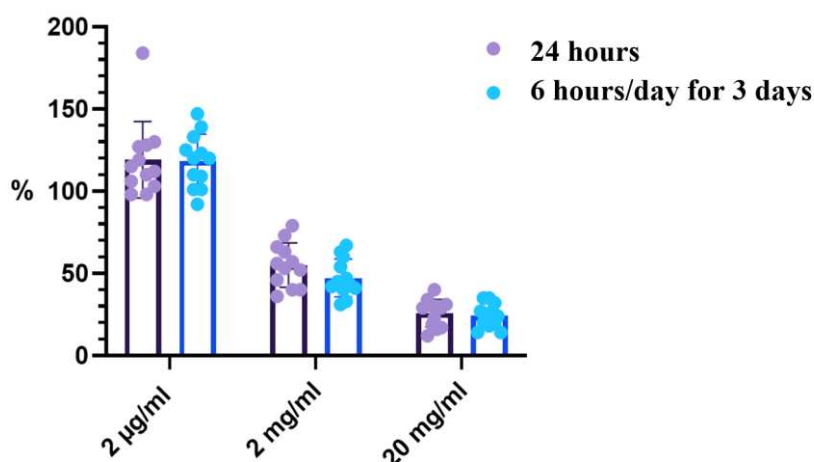


Figure 2. Percentage of biofilm density following exposure to vancomycin (compared to positive control)

After vancomycin exposure to its biofilm, three isolates grew on BHIA-VC medium. The highest incidence of hVISA isolation occurred in the group exposed to 2 µg/ml vancomycin, followed by the group exposed to 2 mg/ml vancomycin, and finally the group subjected to 20 mg/ml vancomycin. The concentration of vancomycin corresponded with the subsequent emergence of hVISA ( $p=0.046$ ; cross-tabulation; 95% confidence interval).

There was no statistically significant difference in the effect of the emergence of hVISA isolates based on time of exposure ( $p=0.333$ ; cross-tabulation; 95% confidence interval). However, growth in BHIA-VC media was more common in those exposed to vancomycin for 6 hours each day and incubated for 72 hours (intermittent exposure).

Table 3. Growth on BHIA-VC media

Vancomycin Exposure		Isolat											
Incubation periode	Concentration	1	2	3	4	5	6	7	8	9	10	11	12
24 hours	2 µg	+	-	-	-	+	-	+	-	-	-	-	-
	2 mg	-	-	-	-	-	-	+	-	-	-	-	-
	20 mg	-	-	-	-	-	-	-	-	-	-	-	-
6 hours/day for 3 days	2 µg	+	-	-	-	+	-	+	-	-	-	-	-
	2 mg	+	-	-	-	+	-	+	-	-	-	-	-
	20 mg	-	-	-	-	-	-	+	-	-	-	-	-

(+) : growth on BHIA-VC media. (-) : no growth on BHIA-VC media

The eradication of biofilms by vancomycin was evaluated using a microtiter plate biofilm assay in accordance with the procedure published by Haney et al. in 2021. In this study, the percentage of MRSA biofilm eradication after exposure to vancomycin at concentrations of 1 times the MIC (2 µg/ml), 1,000 times the MIC (2 mg/ml), and 10,000 times the MIC (20 mg/ml) was determined. The rate of

eradication was evaluated by the optical density of the residual biofilm following vancomycin exposure. Optical density of MRSA biofilm between exposed group and positive control group (which was not exposed to vancomycin) was then measured.

Continuously or intermittently, the group exposed to 2 µg/ml vancomycin concentration of 1 MIC demonstrated a thicker biofilm than the positive control. MRSA isolates exposed to sub-MIC concentrations of vancomycin (1 µg/ml) showed an increase in biofilm formation through an autolysis-dependent mechanism that enhanced extracellular DNA synthesis and membrane vesicle secretion (Hsu et al., 2011; Mirani and Jamil, 2011; He et al., 2017). The biofilm penetration capacity of vancomycin is inadequate, resulting in a lower drug concentration in the biofilm layer than in the test solution (Singh et al., 2011).

The proportion of biofilm thickness varied significantly between groups treated to vancomycin concentrations of 2 g/ml, 2 mg/ml, and 20 mg/ml. The most significant difference was observed between groups exposed to one times the MIC concentration and those exposed to the other two concentrations, with the eradication effect only occurring in groups exposed to 1000 and 10,000 times the MIC. Biofilm elimination was higher when subjected to 10,000 times MIC than when subjected to 1,000 times MIC, although 100% eradication was not reached.

The percentage of biofilm thickness did not differ substantially between the groups that received continuous or intermittent vancomycin exposure for a total of 24 hours and 18 hours. Vancomycin demonstrated a partially time-dependent antibiotic profile in tests against planktonic bacteria and a time-dependent antibiotic profile in tests on mature biofilms, where concentrations of 0.2 mg/ml to 2 mg/ml were able to eradicate the total MRSA mature biofilm on metal implants after 28 days of exposure (Post et al., 2017). This study concludes that a concentration of at least 1,000 times the MIC and an exposure time of at least 18 hours, either continuously or intermittently, are required for effective biofilm eradication.

Detection of hVISA/VISA isolates employing the Satola technique in this investigation. Brain-heart infusion agar media containing 4 g/ml vancomycin and 16 mg/ml casein showed a 90% sensitivity and 95% specificity for detecting the growth of hVISA/VISA (Howden et al., 2010; Satola et al., 2011). Three of the twelve examined isolates grew in BHIA-VC selective media after vancomycin treatment, indicating the presence of the hVISA/VISA subpopulation. Through selective pressure mechanisms, exposure to glycopeptide antibiotics and beta-lactams induces hVISA by polymutations that result in alterations to the cell wall of *S. aureus* (Hanaki et al., 2005; Matsuo et al., 2013; Roch et al., 2014). Prior studies on the development of hVISA by in vitro vancomycin exposure used planktonic bacteria and subinhibitory concentrations. Our recent work demonstrated that exposure to extremely high doses above the MIC can still trigger the development of hVISA isolates in biofilms from sessile bacteria.

## Conclusion

This study concludes that the administration of vancomycin in the treatment of biofilm-associated MRSA infection poses the risk of selection and induction of isolates with decreased susceptibility to vancomycin. Vancomycin treatment with treatment of vancomycin at suboptimal doses increases the probability of hVISA isolation. To achieve a near-100 percent eradication efficacy with vancomycin, up to 10,000 times the MIC is necessary, hence local antibiotic therapy regimens for MRSA-associated biofilm infections must always be adjusted.

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