



Sinergicity test of silver nanoparticles and clindamycin against *Staphylococcus aureus*

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ABSTRACT

Acne is a chronic inflammatory disease. The pathogenesis of acne is multifactorial; one of them is caused by an overgrowth of microbes like *S. aureus*. Clindamycin is one of the antibiotics recommended for acne therapy. Still, the use of clindamycin causes various side effects such as changes in intestinal function associated with intestinal flora, pseudomembranous colitis, and increases the risk of resistance. Silver nanoparticles are potent antimicrobials, have broad-spectrum activity, and can reduce the development of resistance. Despite having potent activity, the long-term use of silver nanoparticles is reported to have side effects argyria. The use of antimicrobial combinations is a strategy to reduce side effects and increase the effectiveness of therapy. Antimicrobial combinations can use low concentrations but still have the potential to inhibit microbial growth. This research aim is to determine the antibacterial synergism of a combination of silver nanoparticles and clindamycin against *S. aureus*. Determination of the minimum inhibitory concentration (MIC) of silver nanoparticles, clindamycin, and synergism test was carried out by microdilution using 96-well microplate. Synergy test is carried out using the *Checkerboard* method by calculating the value of the *Fractional Index Concentration* (FIC). The results showed the MIC values of silver nanoparticles and clindamycin were 16 $\mu\text{g/ml}$ and 64 $\mu\text{g/ml}$, respectively. The synergy test of the combination of silver nanoparticles yielding an FIC value of 0.75. Based on the result of the study, it was concluded that the combination of silver nanoparticles and clindamycin had partial synergy properties. The Minimum bactericidal Concentration (MBC) value clindamycin alone compared in combination with silver nanoparticles 16 $\mu\text{g/ml}$ reduces from 256 $\mu\text{g/ml}$ to 128 $\mu\text{g/ml}$. Combinations of silver nanoparticle and clindamycin are the potential to reduce side effects and overcome resistance.

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INTRODUCTION

Acne is the most common skin disease. Acne is a chronic inflammatory disease that affects the sebaceous glands. *Staphylococcus aureus* is a common cause of inflammatory response in the sebaceous glands, and other bacteria are one of the factors causing acne (Kumar *et al.*, 2016). The use of antibiotics is one way to combat the overgrowth of microorganisms. Clindamycin is one of the antibiotics recommended for acne therapy (Nast *et al.*, 2012) which has a bactericidal effect on acne-

causing bacteria. The use of topical clindamycin causes various side effects, such as causing changes in intestinal function associated with intestinal flora and pseudomembranous colitis (Midtvedt *et al.*, 1986). Clindamycin 1% monotherapy or a combination of 0.1% adapalene is reported to cause local side effects such as erythema, burning, scaling, pruritus, or papules, and pustules. Another risk that can occur in the use of antibiotics is the emergence of resistance (Zhang *et al.*, 2004).

The development of antimicrobial resistance causes the need to find alternative treatments, one of which is by using nanotechnology. Particles in nanosize more easily enter cells and interact with cellular components (Cameron *et al.*, 2018). Compared with conventional antibiotics, antimicrobial in form nanoparticles has many advantages, such as reducing therapy cost, toxicity, and overcoming resistance (Weir *et al.*, 2008).

Metal nanoparticles that have been widely studied and are promising as antimicrobials are silver nanoparticles. Silver nanoparticles (AgNPs) are very small particles size between 1-100 nm. Silver nanoparticles are potent antimicrobials, have broad-spectrum activity, and can avoid the development of resistance (Mordorski and Friedman, 2017). Silver nanoparticles have bactericidal properties by damaging bacterial cell walls and causing reactive oxygen species (ROS) in bacterial cells (Huh and Kwon, 2011). High levels of ROS cause cell death due to the oxidation of proteins and DNA (Blanco *et al.*, 2018). Despite having potent activity against bacteria, the long-term use of silver nanoparticles is reported to have side effects of local argyria (Hadrup *et al.*, 2018). Argyria is a condition characterized by changes in pigment secondary to exposure to silver salt and its buildup on the skin and mucous membranes, usually in the form of blue or gray spots (Molina-Hernandez *et al.*, 2015).

The use of antimicrobial combinations is a strategy to reduce side effects and increase the effectiveness of therapy. Antimicrobial combinations can use low concentrations but still have the potential to inhibit or combat microbes. The antibacterial synergy test aims to assess the *in vitro* interactions of an antimicrobial combination to determine the potential use of two antimicrobials compared to the potential of each antimicrobial (Christoper, 2014). Combination therapy has the potential to eliminate resistant strains, delay the evolution of drug resistance, reduce individual drug doses, and reduce side effects (Worthington and Melander, 2013).

Considering the possibility of side effects and bacterial resistance to clindamycin, it is necessary to find

alternative treatments, one of them is using a combination of clindamycin and silver nanoparticles. The combination of clindamycin and silver nanoparticles is expected can reduce the MIC value of clindamycin and silver nanoparticles, produce a synergistic and effective bactericidal effect, reduce the possible side effects and be able to avoid antibiotic resistance.

MATERIALS AND METHODS

Chemicals

Silver nanoparticles (Sigma St. Louis, USA), Clindamycin phosphate (Zhejiang Hisoar, India), the culture of *S. aureus* ATCC 25923 (Faculty of Medicine, Universitas Indonesia) were used in this study.

In order to produce an accurate result, the purity of bacterial test isolates is important to confirm. Before the study begins, we conducted identification of the culture by gram staining and biochemical test using Vitek.

Culture preparation

One loop of bacterial isolates was taken by the Koch screening quadrant method and inoculates on nutrient agar, then incubated at a temperature of 37°C for 24 hours. After the incubation period, the growing colonies were observed and identified by Gram staining and biochemical tests using Vitek.

Gram staining

Bacteria that have been prepared are dropped with a drop of NaCl solution on the object-glass and then dry. After drying, dropped with a gentian violet solution for 1 minute. After 1 min, washed with water. Furthermore, Lugol liquid is dropped for 1 min then washed with water. The preparations then washed with alcohol for 3-30 seconds or until the dye is clean, then rinse with water. Safranin is dropped into preparations for 1 min, then washed with water and dried. Preparations can be evaluated under a microscope after dropped with emersion oil.

Biochemical tests for bacterial identification

In this test, fresh bacteria and pure colonies are used. A number of colonies suspended in 3 ml of 0.45% NaCl solution pH 5.0 in a test tube. The turbidity of Gram-positive bacteria inoculum is adjusted for 0.50-0.63 McFarland by checked the turbidity with DensiChek™. The Vitek cards inoculated with bacteria suspensions using an integrated vacuum apparatus, then placed into a cassette, and the identification card is placed in the Vitek device.

The Minimum Inhibitory Concentration (MIC) testing

The MIC test was conducted to determine the smallest concentration that could inhibit bacterial growth

from silver nanoparticles (AgNP) and clindamycin. The MIC of AgNP and clindamycin was determined against *S. aureus* using a twofold broth microdilution method in 96-well microplates. The MIC test of AgNP used 8 series of concentrations ranging from 16 to 0.125 $\mu\text{g/mL}$ (16; 8; 4; 2; 1; 0.5; 0.25; 0.125 $\mu\text{g/mL}$). MIC test for clindamycin used 10 series of concentrations ranging from 254 to 0.5 $\mu\text{g/mL}$ (254; 128; 64; 32; 16; 8; 4; 2; 1; 0.5 $\mu\text{g/mL}$). A volume of 100 μl of *Brain Heart Infusion Broth* (BHIB) media was added to each well. A volume of 100 μL test solution of AgNP and clindamycin each added to first well and then make twofold broth microdilution until the last well. A volume of 10 μL of the microbes that have a concentration of 1.5×10^6 CFU/mL was inoculated to each well and incubated under aerobic conditions at 37°C for 24 hours. The MIC is determined as the lowest concentration of the compound that inhibits bacterial growth after the incubation period. The determination of the MIC is done by the appearance of turbidity that can be seen by the eye compared to the negative and positive control. The lowest concentration that does not show turbidity (clear) is the MIC value. The MIC test was done in triplicate.

Antibacterial synergy test

Antibacterial synergy in vitro test was carried out using a *checkerboard* method based on the value of the FIC (*Fractional Inhibitory Concentration*). The FIC value was calculated by comparing the MIC of each agent AgNP and clindamycin compared with the MIC combination AgNP-clindamycin. Clindamycin was added in concentrations between 64 and 8 mg/mL and AgNP in a range of 2–16 mg/mL .

To determine the MBC value, the test tube that didn't show any growth was streak in nutrient agar and incubated at 37°C for 24 h in aerobic conditions. The value of MBC was read as the lowest concentration that can inhibit 99,9% microbial growth. The MBC value was done in triplicate.

RESULTS AND DISCUSSION

Gram staining

This test is one of the important methods used in the microbiology laboratory. A gram stain is used for the initial evaluation of microorganism identification. Gram staining is the fastest and most effortless test to characterize microorganisms. Gram staining is the simplest test to determine the morphology of the cells (Beveridge, 2001). The difference between Gram-negative and Gram-positive bacteria is that the cell walls of Gram-positive bacteria consist of thick peptidoglycan, allowing bacteria to

coloured in purple on gram staining while the cell wall of gram-negative bacteria contains thin peptidoglycan without teichoic acid, it allows cell wall to be stained pink. The results of Gram's staining show that the bacterial colonies are round purple, clustered like grapes. *Staphylococcus aureus* is a Gram-positive bacterium so that when stained with Gram stain, it will absorb violet colors from the reagent because it has a cell wall with thick peptidoglycan compared with Gram-negative.

Biochemical identification test with Vitek

The Vitek is an automated microbiology system, and it can be used to identify microorganisms. The Vitek system is a useful tool to identify bacteria by miniaturized biochemical tests with special cards containing 64 fluorescent biochemical tests (Rave et al., 2018). The carbohydrate fermentation test is one of the biochemical tests to identify a microorganism. Each microbe can ferment carbohydrates that are very varied and different patterns of carbohydrate fermentation can be used to identify the microbes (Mahon et al., 2010), so that the characteristics of this carbohydrate fermentation can be used to distinguish bacterial species in one particular genus for identification purposes. The Vitek test results showed that the bacterial culture 94% was *S. aureus*, as seen in Figure 3.

Minimum Inhibition Concentration and antibacterial synergy test

The minimum inhibitory value for AgNP and clindamycin against *S. aureus* were 16 $\mu\text{g/mL}$ and 64 $\mu\text{g/mL}$, respectively, as presented in Table 1. The synergy test for the AgNP-clindamycin combination is based on the MIC obtained from combination compared with a single agent. The AgNP concentration range is set at 16–2 $\mu\text{g/mL}$ and clindamycin at 64–8 $\mu\text{g/mL}$. The observation results of microorganism growth can be seen in Figure 1.

After an incubation period at 37°C for 24 hours, it was observed that the MIC of AgNP alone compared with AgNP in combination with clindamycin reduced from 16 $\mu\text{g/mL}$ to 8 $\mu\text{g/mL}$, whereas the MIC of clindamycin alone compared clindamycin in combination with AgNP dropped from 64 $\mu\text{g/mL}$ to 16 $\mu\text{g/mL}$. The synergistic effect of the combination of AgNP-clindamycin can be evaluated from the calculation of the FIC value of the combination MIC results. Determination of the FIC value of the combination of AgNP and clindamycin using the following formula

$$FIC = \left[\frac{(MIC AB)}{MICA} + \frac{(MIC BA)}{MICB} \right]$$

Note,

Table 1: The MIC values of silver NPs and clindamycin against *S. aureus*; + cloudy/growth; - clear/no growth

Silver Nanoparticles Test Solution Series ($\mu\text{g/ml}$)									
16	8	4	2	1	0,5	0,25	0,125		
-	+	+	+	+	+	+	+		
Clindamycin Test Solution Series ($\mu\text{g/ml}$)									
256	128	64	32	16	8	4	2	1	0,5
-	-	-	+	+	+	+	+	+	+

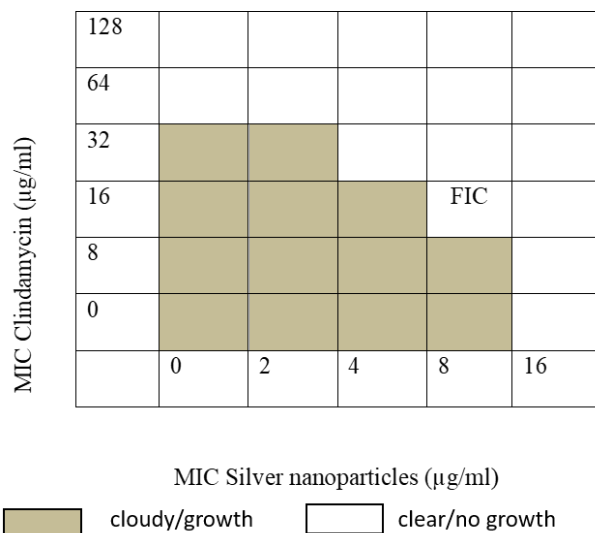


Figure 1: The results of microbial growth in a combination of AgNP and clindamycin

MIC AB = Minimum Inhibitory Level (MIC) clindamycin in combination with AgNP

MIC BA = MIC AgNP in combination with clindamycin

MIC A = MIC clindamycin

MIC B = MIC AgNP

FIC is interpreted as synergy with an FIC index < 0.5 , defined as a fourfold or greater decrease in MIC of both drugs in combination compared to individual drugs. Partial synergy with an FIC index $> 0.5 > \text{FIC} < 1$, is defined as a fourfold or greater decrease in MIC in one agent and a double decrease in another agent. An additive effect with an FIC index = 1, is defined as a doubling in MIC reduction with both agents. Indifference with a FIC index > 1 , but < 4 , when there is no change in MIC either tested alone or in combination or antagonist with an FIC index of 4, ie, when there is a fourfold increase in MIC for both agents when the drug is tested in combination compared to the respective results self-testing of drugs (Dawis, 2003). The test results showed an FIC value combination of AgNP with clindamycin was 0.75.

Study Minimum Bacterial Concentration (MBC) of

Clindamycin against *S. aureus* showed that clindamycin at a concentration of 256 $\mu\text{g/ml}$ killed 99,9% of microbes, this can be seen from Figure 2. The MBC test at a concentration of 256 $\mu\text{g/ml}$ showed no bacterial growth. MBC value of clindamycin and after combined with AgNP 16 $\mu\text{g/ml}$ showed a decrease from 256 $\mu\text{g/ml}$ to 128 $\mu\text{g/ml}$.

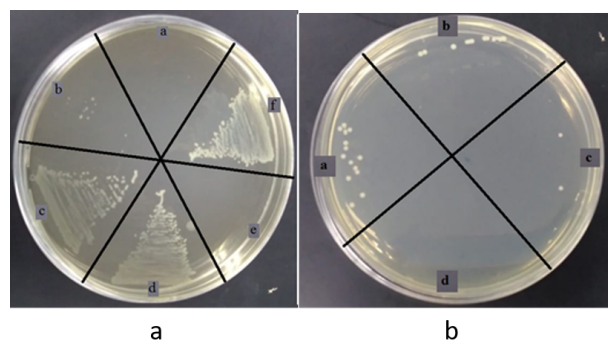


Figure 2: Observation of Minimum Bactericidal Concentration (MBC). (a). MBC of clindamycin against *S. aureus* at concentration, a. 256 $\mu\text{g/ml}$; b. 128 $\mu\text{g/ml}$; c. 64 $\mu\text{g/ml}$; d. 32 $\mu\text{g/ml}$; e. negative control; f. positive control. (b). MBC study of combination AgNP-clindamycin at concentrations, a. 2/128 $\mu\text{g/ml}$; b. 4/128 $\mu\text{g/ml}$; c. 8/128 $\mu\text{g/ml}$; d. 16/128 $\mu\text{g/ml}$

Reports of the emergence of resistance and side effects of the use of clindamycin and silver nanoparticles led to developing new effective antimicrobial agents, one of them is the use of a combination of antibiotics and silver nanoparticles. Studied the synergistic effect of AgNP with clindamycin conducted to find new alternative therapy, which more effective in reducing the adverse effect and overcome the antibiotic resistance problem. The efficacy of the use of antimicrobial combination can be evaluated by conducting a synergism test. Synergism study is done by measuring the MIC value of each agent, and in the combination, then calculated the FIC value.

The study showed that AgNP were more potent as antimicrobial compared to clindamycin. *Staphylococcus aureus* is more sensitive to the silver

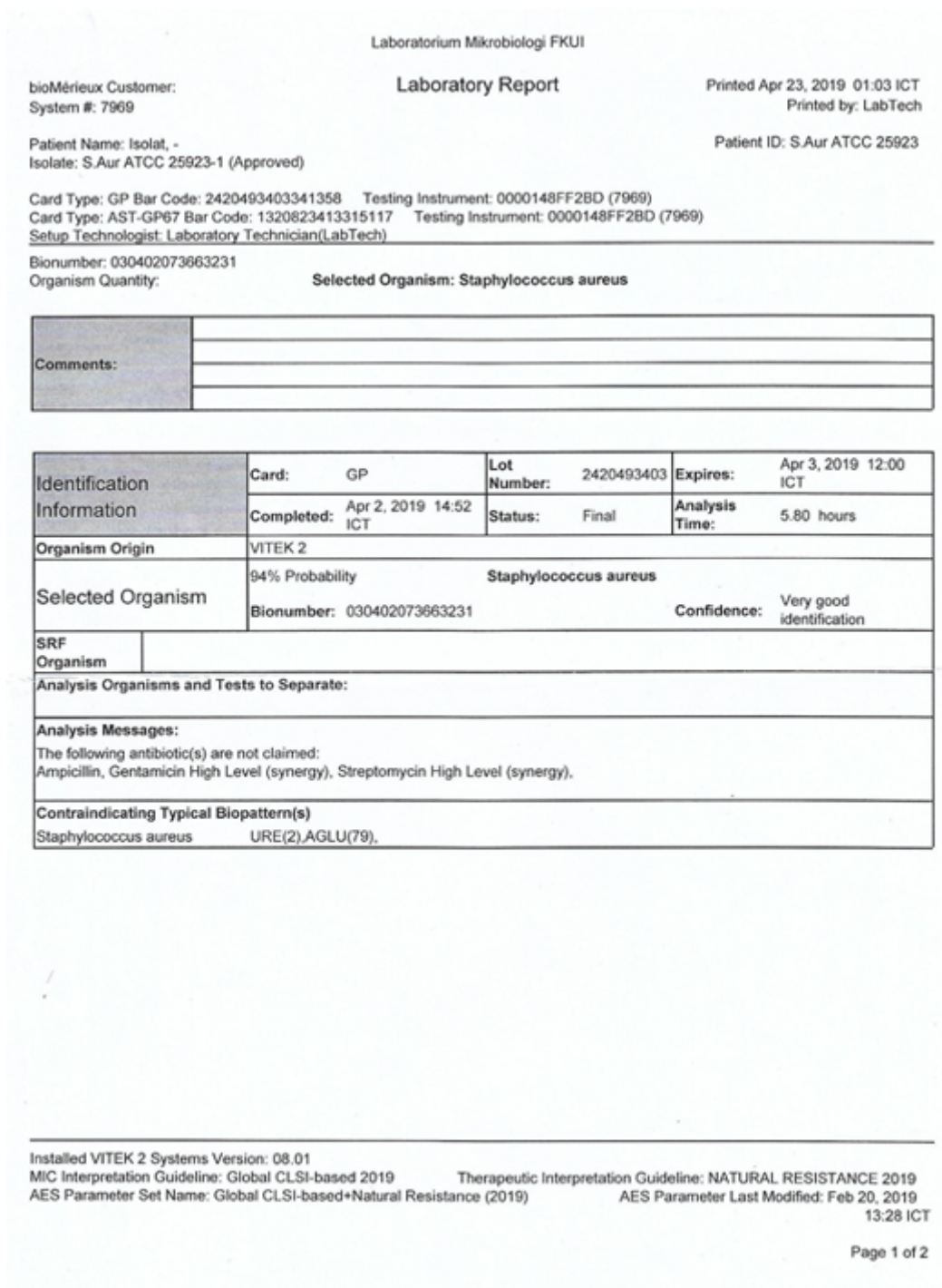


Figure 3: Attachment

nanoparticle, which can be seen from the MIC value shown by AgNP is significantly lower than clindamycin i.e., 16 µg/ml and 64 µg/ml, respectively. Silver nanoparticles that were used in this study were spherical, a stable morphology, and giving a high surface area to volume ratio, facilitated them to pass through bacterial cell membranes, damage, and killing the cell (Shameli et al., 2012). It was considered to enhance the potency and efficacy of the AgNP.

The FIC value obtained from this study was 0,75 means that a combination of silver nanoparticles and clindamycin showed a partial synergy. Study MBC value of clindamycin after combined with AgNP 16 µg/ml showed a decrease value from 256 µg/ml to 128 µg/ml. It was suspected because of the different mechanisms of action so it can strengthen the effect of inhibiting the growth of microorganisms. Clindamycin is a semisynthetic antibiotic that has mechanism action by inhibiting protein synthesis by

binding to the 50S bacterial ribosomal subunit (Carley *et al.*, 2018). The other hand mechanism action of silver is postulated in many ways. (Zaenglein *et al.*, 2016) demonstrated that AgNP-induced leakage of intracellular sugars and proteins, impairment of metabolic activity, and increased oxidative stress resulting in an imbalance in the antioxidant proteins. AgNP disrupts membrane stability, and activation of respiratory chain dehydrogenases triggers the generation of Reactive Oxygen Species (ROS), which inhibits respiration and growth of cells. The release of silver ions mainly causes the silver antimicrobial mechanism. Ionic silver has a broad spectrum of antimicrobial activity, and it is postulated that silver ions can interact with several target sites and proteins that result in structural and metabolic disfunction .

This study found that was partial synergistic action of AgNP and clindamycin resulted in enhanced antibacterial effect. *Staphylococcus aureus* was found to be susceptible to the two of the tested antimicrobial, exposure of bacteria in a combination of AgNP and clindamycin reduced the MIC and MBC significantly.

CONCLUSIONS

The results of this study indicate that the combination of silver nanoparticles and clindamycin has partial synergy with an FIC value of 0.75. Combinations of silver nanoparticle and clindamycin are the potential to reduce the adverse effect and prevent the development of bacterial resistance.

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