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The impact of biofilm on cefotaxime killing effect against Escherichia coli

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***Abstract*---**Biofilms cause chronic infections that are difficult to treat because of the resistance of microorganisms inside them. The goal is to assess the killing potential of cefotaxime against *Escherichia coli* in biofilms obtained from clinical isolates kept in the clinical microbiology laboratory of Dr. Soetomo Surabaya Hospital. The study was conducted by laboratory experimental design. Two steps challenged the bacterial isolates by applying cefotaxime to the planktonic bacterial state and the biofilm state. Minimum inhibitory concentration (MIC), minimum biofilm inhibitory concentration (MBIC), minimum bactericidal concentration (MBC) and minimum biofilm eradication concentration (MBEC) were used as an indicator for comparison. Nineteen isolates of *E coli* were used for this experiment, 7 isolates with MIC of 0.125-0.25 µg/ml (36,84%), 6 isolates with MBC value 0.25-0.5 µg/ml (31,57%). The MBIC > 128 µg/ml is 6 isolates (31,57%), MBEC > 128 µg/ml is 14 isolates (73,68%). Cefotaxime had lower killing efficacy against *E. coli* in biofilm than in the planktonic phase. MBIC of *E. coli* requires cefotaxime at minimal 5 times two-fold increase from MIC, with an average 7-8 times. MBEC of *E. coli* requires cefotaxime concentration minimal 5 times two-fold dilution increase from MBC, with an average 8 - 9 times.

***Keywords*---**biofilm, cefotaxime, E. coli, MIC, MBIC, infection.

Introduction

Cefotaxime is a third-generation cephalosporine antimicrobial widely used as empiric therapy in gram-negative bacterial infections, such as *Escherichia coli, Klebsiella pneumoniae*, and the Enterobacteriaceae group (Grayson et al., 2010). Antimicrobial susceptibility tests for bacteria, which are performed frequently in the Clinical Microbiology Laboratory using a disk diffusion test or MIC test against planktonic bacteria, are used to determine the efficiency of antimicrobials in the treatment of diseases (CLSI, 2006, 2021).

Planktonic bacteria, often known as free-living bacteria, are bacteria that live without attaching themselves to a surface. In liquid media, planktonic bacteria tend to become sessil, or bacteria that build biofilms (Brady et al., 2008). Biofilm development is a bacteria's adaptive response to changing environmental conditions. A biofilm is a bacteria colony adhered to an abiotic or biotic surface in an extracellular matrix substance (Vickery et al., 2013).

According to the National Institutes of Health (NIH), biofilm formation is responsible for 65% to 80% of bacterial infections and chronic illnesses, including infections caused by medical devices and infections caused by other causes (Anwar et al., 1989; Jamal et al., 2018). Medical devices attached to the patient are constantly in contact with the patient's body fluids and irrigation fluids, making them an excellent surface for biofilm formation (Rodney M. Donlan, 2001). Depending on the type of device used and the duration of usage, biofilms generated on the surface of medical devices can include one or more bacterial colonies (Ripolles-avila et al., 2018). Chronic wound infection and medical devices can also present bacterial biofilm (R. M. Donlan, 2001; Rodney M. Donlan, 2001). Forming biofilms, which increase microorganism resistance to antimicrobials, patient morbidity, death, and hospital cost burden, is challenging in dealing with hospital infections (Percival et al., 2015; Tutulan, 2015; Vickery et al., 2013). Biofilms can serve as bacterial cell reservoirs, posing a danger of chronic and persistent infection, and reinfection in bacteria-infested locations (Bueno, 2011; Trafny, 2008). The persistence of infectious organisms despite effective antibiotics characterizes chronic illness, and the host's immune response is also good (Chen et al., 2018; Macià et al., 2014b). Biofilms can protect bacterial cells from host immune system attacks and antimicrobial medications, leading to treatment failure, reinfections, and higher mortality (Thieme et al., 2019). Biofilms formed on the surface of medical devices can release bacteria into the bloodstream, causing secondary infections (Macià et al., 2014a).

Bacteria in biofilms are highly resistant to various antimicrobial drugs due to increased production of extracellular matrix, multi-layered colonies, decreased metabolic rate, reduced multiplication and polymicrobial colonization (Macià et al., 2014a; Mulla et al., 2016). Biofilms can tolerate antimicrobial drugs, even up to 100 - 1000 times the concentration of antimicrobials and disinfectants that can kill planktonic bacterial cells (Macià et al., 2014b). This resistance would be the cause of therapeutic failure in patients who have medical devices and prosthetics installed on their body, which form a biofilm (Mulla et al., 2016).

Antimicrobial resistance is a worldwide challenge leading to high morbidity and mortality in clinical settings (Akova, 2016). More than 2.8 million antibiotic-resistant infections occur in the U.S. each year. More than 35,000 people die as a result, according to CDC's 2019 Antibiotic Resistance (AR) Threats Report (CDC, 2019). Antimicrobial resistance (AMR) means the ability of microorganisms to survive and be viable under the influence of antimicrobial agents. Modifications on cell wall synthesis such as beta-lactams and glycopeptides, protein synthesis inhibition such as macrolides and tetracyclines, metabolic pathway inhibition such as sulfonamides, interference with DNA replication and translation such as fluoroquinolones, and biofilm formation are all examples of mechanisms that can cause significant metabolic and physiological changes (Anwar et al., 1989; Rodney M. Donlan, 2000).

The susceptibility of bacteria in biofilms to antimicrobials cannot be evaluated using methods routinely performed in the clinical microbiology laboratory. The sensitivity of bacteria in biofilms requires a special testing system. It is because of the presenting biofilm that inhibits the entry of antimicrobial to the cell (Azeredo et al., 2017; Mandakhalikar et al., 2018; Mulla et al., 2016). Thus, the results of antibiotic sensitivity tests against planktonic bacteria cannot function as a guideline in performing therapy in patients with infections due to the presence of biofilms in patients (Macià et al., 2014a). This study aims to analyze in vitro the killing potency of cefotaxime against *E. coli* in the planktonic phase and compare it with the killing power of cefotaxime against *E. coli* in the sessile phase from clinical isolates at Dr. Soetomo Surabaya hospital.

Method

The study was an experimental laboratory design, used *E. coli* isolates from the Clinical Microbiology Laboratory Dr. Soetomo Surabaya hospital, non-ESBL producing and sensitive to cefotaxime with MIC less than 1 µg/ml by PhoenixTM automated identification and susceptibility testing. The experiment was conducted by applying *E. coli* against cefotaxime in the planktonic state and again in the biofilm state. We used the test tube (TT) method to determine biofilm production. Biofilm formation was visualized using 1% crystal violet staining and observed by three people. A biofilm formation showed a blue layer on the tube wall (Mulla et al., 2016).

The MIC is determined by the standard protocol macro dilution method. The MBC is determined after a Minimum Inhibitory Concentration (MIC) test has been completed by inoculating the broth into plate (CLSI, 2006, 2021). We determine the MBIC and MBEC values of *E. coli* in biofilms using the test tube and plate counting/CFU assay methods (Fawzy et al., 2016; Mulla et al., 2016; Wilson et al., 2017). MBIC is the lowest concentration of an antimicrobial at which there is no increase in the number of viable biofilm cells when an early exposure time is compared with a later exposure time. The MBEC is the lowest concentration of cefotaxime that eradicates 99.9% of biofilm-embedded bacteria (^{3}log10 reductions in CFU/mL) compared to growth controls (Macià et al., 2014a). After determining the value of MIC and MBC, *E. coli* with turbidity 0,5 McFarland in tryptic soy broth incubated for 24 hours until they form biofilm. Two control groups were not treated with cefotaxime. Colony counting was performed before and after

exposure to cefotaxime in each control group. Colony counting was carried out after each biofilm was sonicated with a frequency of 42 kHz for 2 minutes. The treatment group was exposed to cefotaxime with certain concentrations, starting from 2 µg/ml to 128 µg/ml, then incubated for 24 hours. After incubation, the biofilm was dispersed by sonication. Then, a series of dilutions were carried out then inoculated on solid media. The solid media was incubated for 24 hours; then, the colonies were counted and data analyzed using the Wilcoxon and Spearman correlation tests. This study was approved by Health Research Ethical Committee Dr. Soetomo Surabaya Hospital (No. 0493/LOE/301.4.2/VI/2021).

Results

Nineteen *E. coli* isolates matched the inclusion criteria, with details on five samples from male patients and 14 samples from female patients. The concentration of cefotaxime required to inhibit *E. coli* in the biofilm was minimally 4 - 8 µg/ml, whereas most isolates were inhibited at concentrations of more than 128 µg/ml. The concentration of cefotaxime required to eradicate *E. coli* in biofilms was at least 16 - 32 µg/ml, but most isolate concentrations needed more than 128 µg/ml.

Isolates 6, 9, and 10 have a MIC value to MBIC increases in two-fold dilution more than ten times. Isolates number 14 has a MIC value to MBIC increases in two-fold dilution more than nine times, isolates number 18 more than eight times, and isolates number 19 more than seven times. Some isolates with MBIC or MBEC higher than 128 µg/ml were collected in one group because the maximum concentration was 128 µg/ml. In 14 isolates, MBEC values were higher than 128 µg/ml. There is an increase in two-fold dilution > 6, > 7, > 8, > 9, and > 10.

The two-fold increase in dilution from MIC to MBIC is the lowest at 4 - 5 times, the highest is 11 - 12 times, and the average is 7 - 8 times. The smallest two-fold dilution increase from MBC to MBEC is 4 - 5 times, the highest is 11 - 12 times, and the average is 10 - 11 times, as shown in Figure 3.1.a. There was a significant difference in the concentration of cefotaxime needed to inhibit and eradicate the growth of *E. coli* in the planktonic phase compared to *E. coli* in the biofilm ($p < 0.05$). The statistical analysis showed $p = 0.215$ (MIC - MBIC) and $p = 0.816$ (MBC - MBEC); thus, the correlation was insignificant and weak.

The correlation test between MIC with an increase in two-fold dilution of MIC to MBIC (p value = 0.515) with a correlation coefficient of -0.159. That was not significant, with a weak correlation. The MBC has significant correlation with the increase of two-fold dilution of MBC to MBEC ($p < 0.05$), with a correlation coefficient of -0.606.

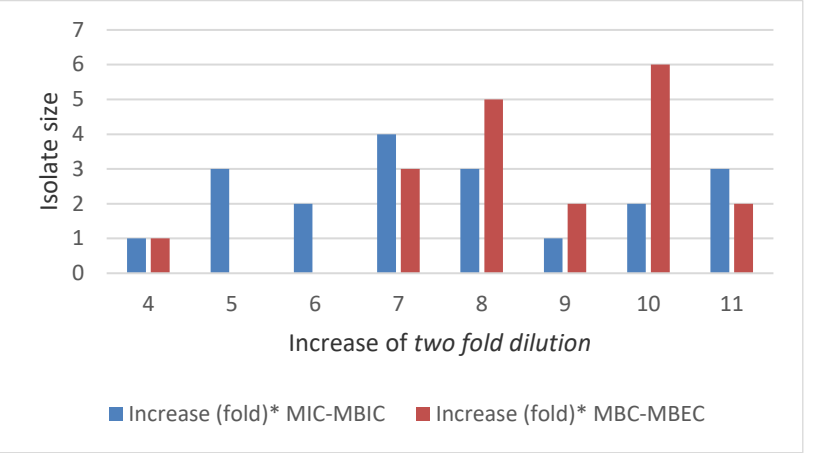

Figure 1. Frequency distribution of the two-fold increase in dilution from MIC to MBIC, and MBC to MBEC

Discussion

The MIC value of *E. coli* against cefotaxime in this study was 0.0625 - 1 µg/ml. The MBC value was 0.125 - 2 µg/ml. Total of 78.94% isolates had an MIC value of 0.25 - 0.5 µg/ml, and 68.42% isolates had an MBC value of 0.5 - 1 µg/ml. The higher value of MBC than MIC of cefotaxime is consistent properties of bactericidal antibiotics (Grayson et al., 2010; Macià et al., 2014a).The lowest MBIC value is 4 - 8 µg/ml, and the lowest MBEC is 16 - 32 µg/ml. The highest MBIC and MBEC values were more than 128 µg/ml. Most isolates required more than 128 µg/ml concentrations to inhibit and eradicate *E. coli* biofilms. The lowest MBIC obtained is equivalent to a 6 - 7 times two-fold dilution increase from the minimum MIC value. Meanwhile, the lowest MBEC is equal to 7 - 8 times two-fold dilution increase from the MBC.

The increase in MBIC and MBEC compared to MIC and MBC is also under the results of research conducted by Poovendra et al, Chen et al, Dincer et al, and Fawzy et al, which conclude that inhibition and eradication of *E. coli* biofilms required a higher concentration of antibiotics than planktonic bacteria (Dincer et al., 2020; Fawzy et al., 2016; Poovendran & Ramanathan, 2014). Rafaque et al wrote that biofilms of Acinetobacter, Pseudomonas, *E. coli*, and Klebsiella, were generally resistant to third-generation cephalosporins (ceftazidime and cefotaxime) (Rafaque et al., 2020).

Fawzy et al found that killing bacteria in biofilms requires a concentration of antibiotics 100 times higher than killing bacteria in planktonic bacteria. Antimicrobial sensitivity variations between planktonic bacteria and bacteria in biofilms can be induced by discrepancies in antibiotic diffusion processes and complicated alterations in biofilm-forming bacteria's physiology (Fawzy et al., 2016).

EPS would influence MBIC and MBEC values that are higher than MIC and MBC. Also, as the changes in bacterial metabolism, changes in bacterial gene expression and microenvironment in the biofilm (Allison & Gilbert, 1992; Beloin et

al., 2008; Bryers, 2008; Gupta, 2015; Kharazmi et al., 1999; Singh et al., 2009). Water (97%), exopolysaccharide polymers, proteins, nucleic acids, lipids/phospholipids, different nutrients, and metabolic products make up matrix biofilm (Beloin et al., 2008). According to Donlan's research, the extra-polysacharide matrix generated in the biofilm had a significant role in bacterial resistance to antimicrobials in the biofilm (Rodney M. Donlan, 2001). Because it comprises numerous anion and cation molecules, such as proteins, glycoproteins, and glycolipids, which can bind to antimicrobial compounds, the biofilm matrix acts as a physical barrier against bacteria in the biofilm (Dincer et al., 2020). The biofilm matrix plays a crucial role in developing antibiotic resistance by blocking antimicrobial transport to biofilm cells and antimicrobial molecule binding to EPS (Beloin et al., 2008). By blocking antimicrobial transport to biofilm cells and antimicrobial molecule binding to EPS, the biofilm matrix plays a crucial role in developing antibiotic resistance (Ceri et al., 1999; Rodney M. Donlan, 2001; T. Salih & F. AL-Ani, 2013).

The glycocalyx layer of EPS can prevent antibiotics from dissolving and collect up to 25% of total antibiotic molecules (Dincer et al., 2020). The study by Suci et al. compared the penetration of ciprofloxacin into *Pseudomonas aeruginosa* biofilms. In this study, the diffusion of 100 µg/ml of ciprofloxacin from a sterile liquid medium to the surface took 40 seconds. Meanwhile, ciprofloxacin takes 21 minutes to diffuse in a liquid medium containing biofilm, and the level that reaches the surface is less than 100 µg/ml (R. M. Donlan, 2001).

The biofilm matrix contains a significant amount of extracellular DNA (eDNA). Because DNA can cause changes in cell membrane charge, eDNA can boost biofilm resistance to some antibiotics. Because DNA is an anionic molecule, it can attach to cations like magnesium ions and reduce their concentration in the membrane. The autolytic bacteria in the biofilm generate extracellular DNA, which functions as quorum sensing regulates. In the biofilm, this DNA release process results in horizontal gene transfer (Montanaro et al., 2011).

Another theory proposes that chemical changes in the biofilm microenvironment cause antibiotic resistance. There are known changes in the concentration of nutrients in biofilms on a micro scale (He et al., 2015; Zhao et al., 2020). According to a study employing a small electrode, the oxygen in the biofilm is consumed at the surface layer, resulting in anaerobic conditions in the deeper biofilm layer. The metabolic product concentration gradient will match the nutrition gradient. pH differential of more than one between the EPS fluid and the cells in the biofilm will result from the accumulation of metabolic products. The antibiotic's performance will be affected by the pH differential. Because of the decrease in food concentrations and the accumulation of metabolic products, some bacteria will become non-growing. Antibiotics are less effective against bacteria in this non-growing state (Crabbé et al., 2019). Beta-lactam antibiotics work on bacteria actively developing and replicating by suppressing cell wall synthesis.The bacterial cells that are not in the form of growth or reproduction, the synthesis of bacterial cell walls will not be inhibited (Katzung, 2018). Osmotic alterations occur in the biofilm, triggering an osmotic stress response. By altering the relative porosity of the porin, this response can contribute to the development of antibiotic resistance by lowering antibiotic permeability. The biofilm's age

influences antimicrobial resistance. The older the biofilm is, the more EPS is produced, decreasing the entry of nutrients, oxygen, and antibiotics into the biofilm matrix (R. M. Donlan, 2001).

Another factor hypothesized to contribute to antibiotic resistance in biofilms is the lower growth rate of bacteria in biofilms compared to planktonic bacteria, which reduces antibiotic uptake. DuGuid et al. found similar results when they looked at the influence of ciprofloxacin on *S. epidermidis* biofilms. According to DuGuid, increasing growth rate led to enhanced antimicrobial susceptibility. Other research has found that the sensitivity of bacteria in biofilms is determined not only by their growth rate but also by their growth phase (Duguid et al., 1992). The study of Desai et al. on *Burkholderia cepacia* susceptibility to ciprofloxacin and ceftazidime, found that biofilm bacteria improved their resistance during the exponential phase. In contrast, the highest resistance was found in the stationary phase in both sessile and planktonic bacteria (CDC, 2019; Desai et al., 1998).

The MBIC and MBEC values reported from clinical *E. coli* isolates in this study differed a lot. These variances could be attributable to differences in the properties of the *E. coli* isolates collected, resulting in a wide range of biofilm thickness. Our study matches the findings of Naves et al who investigated the MIC and MBEC values of UPEC isolates, finding that MIC results ranged from 0.12 µg/ml to 4 µg/ml, with MBIC values ranging from 8 µg/ml to 256 µg/ml (Naves et al., 2010) The MIC value for *E. coli* against cefotaxim was 12 - 25 µg/ml, and the MBEC value was 96 - 200 µg/ml, according to Poovendran et al's research (Poovendran & Ramanathan, 2014).

Differential bacterial growth patterns, inoculum size, number of resistant mutants, dense cell density, transporting antibiotics to bacterial cells, the presence of pump efflux and persistent cells, and a small number of bacteria in the bacteria all influence the effect of antibiotics on biofilm-forming microorganisms. Biofilms can differentiate into a phenotypic form that is highly protected (Dincer et al., 2020; Naves et al., 2010). The physiological heterogeneity of bacteria in biofilms develops during biofilm growth due to biofilm nutrition and oxygen concentration changes. Because of the difference in nutritional and oxygen concentrations, bacteria cells closer to the surface consume more nutrients and oxygen than bacterium cells in the biofilm's deeper layers. This variation causes variations in bacteria's growth rate and physiology in the biofilm and differences in antibiotic effects in the biofilm (Dincer et al., 2020). Because of the variability in bacterial proliferation, the antibiotic used was only effective against some of the biofilm's microorganisms (Stewart & Costerton, 2001).

In this study, the lowest two-fold dilution increase from MIC to MBIC was 4-5 times, the highest was 11-12 times, and the highest was 7-8 times. The smallest two-fold dilution increase from MBC to MBEC is 4-5 times, the highest is 11-12 times, and the highest is 10-11 times. MBIC and MBEC values at least increased by four times two-fold dilution of the MIC and MBC values. The MBEC value of third generation cephalosporin against Gram-negative rods increased by more than one-fold dilution of the MIC value, according to Macia et al (Macià et al., 2014a). Four isolates had a two-fold dilution increase from MBC to MBEC that was less than the two-fold increase from MIC to MBIC, namely isolates 8, 22, 4,

and 21. The isolate no 8, the increase in two-fold dilution MIC-MBIC was 7, while the two-fold increase in MBC-MBEC dilution was 4. This was due to the MBC value, which increased four times the MIC value, while the MBEC value only increased one-fold from MBIC. In isolates 22, 4, and 21, the MBIC and MBEC values obtained were the same, more than 128 µg/ml (maximum value). The MBC value was higher than the MIC value in these four isolates.

There is no correlation between MIC values and MBIC or MBC values and MBEC, as shown in graphs 4 and 5. The high and low MIC and MBC do not correlate to the high and low MBIC and MBEC. The structure and composition of the biofilm have a more significant impact on the high and low values of MBIC and MBEC. Fawzy et al found a positive correlation between MBEC imipenem and ceftazidime on biofilm formation. It showed that microbial resistance strongly correlates with biofilm formation (Fawzy et al., 2016). Antimicrobial resistance in biofilms is a complicated mechanism controlled by many factors, including the bacteria's intrinsic features and several adaptive mechanisms. Biofilm features such as gene expression variations, extracellular matrix heterogeneity, and subpopulation metabolic heterogeneity all play a role in developing antibiotic resistance (Fawzy et al., 2016).

There is no significant correlation between MIC with two-fold dilution increase of MIC to MBIC ($p>0.05$; correlation coefficient = -0.159), but significant between MBC and the two-fold dilution increase of MBC to MBEC ($p<0.05$ coefficient -0.606). In the clinical setting, to inhibit the growth of *E coli* in the biofilm state, cefotaxime needs 7-8 times the two-fold dilution of the MIC value. While to eradicate *E. coli* in biofilms, cefotaxime with a concentration of 10-11 times the value of MBC is required. The therapeutic dose of cefotaxime in adults is 1-2 g each time with an interval of 8 hours, while in children, the therapeutic dose required is 100 - 150 mg/kg BW with an interval of 4-6 hours (Grayson et al., 2010). The level of cefotaxime expected to reach the target tissue with this therapeutic dose will be effective against *E. coli*, with a MIC of 1 µg/ml (Bryers, 2008). If the needed level of cefotaxime is 7 - 8 times the MIC value, the dose must be 7 - 8 times higher than the current dose, or equivalent to 7 - 8 grams per 8 hours. Cefotaxime reaches its hazardous dose at 6 g/kg BW/day (Grayson et al., 2010). The failure of conventional therapy is caused by a combination of tolerance and microbial resistance in biofilms. Antimicrobials' effectiveness against microorganisms in biofilms has been studied using a variety of methodologies. One option is to use quorum sensing inhibitors and various approaches to disrupt the biofilm matrix, improving antimicrobial penetration (Beloin et al., 2008; Singh et al., 2009; T. Salih & F. AL-Ani, 2013).

Conclusion

The killing potency of cefotaxime against *E. coli* in the biofilm was lower than against *E. coli* in the planktonic phase. The minimal inhibitory concentration of cefotaxime against *E. coli* in the biofilm was five times two-fold dilution greater than the minimum inhibitory concentration of planktonic *E. coli*, with an average increase of 7-8 times two-fold dilution of MIC. Cefotaxime's minimal kill rate against *E. coli* in the biofilm was five times two-fold dilution greater than the

planktonic *E. coli* minimum kill rate, with an average 8 - 9 times two-fold dilution increase.

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