



## The Combination Effect of Ceftriaxone and Chloramphenicol on *Staphylococcus aureus* Isolate of Diabetic Gangrene

(Efek Kombinasi Seftriakson dan Kloramfenikol pada Isolat *Staphylococcus aureus* dari Gangren Diabetik)

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### ABSTRACT

**Background:** Diabetic gangrene is a complication of Diabetes mellitus caused by *Staphylococcus aureus*. The combination of Ceftriaxone and Chloramphenicol is often used to cure gangrene infection, even though, they produce antagonist interaction based on theory. **Objectives:** To evaluate the potency of Ceftriaxone, Chloramphenicol and its combination on *Staphylococcus aureus* isolate of Diabetic gangrene. **Material and Methods:** The research was done by using disc diffusion methods with Muller Hinton media. Ceftriaxone, Chloramphenicol and its combination dose of 7,5 µg/ml, 15 µg/ml and 30 µg/ml, respectively were tested on *Staphylococcus aureus* culture taken from the diabetic gangrene patients. Antibacterial effect was observed by measuring inhibition zone on bacteria culture. Type of interaction was analyzed by Ameri-Ziaei Double Antibiotic Synergism Test (AZDAST) method. The results of study were tested statistically with One Way ANOVA (p=0.05) followed by Least Significant Difference (LSD) test. **Results:** The combination of Ceftriaxone and Chloramphenicol showed an antibacterial effect lower than Ceftriaxone. β-lactam antibiotic like Ceftriaxone require the cell be growing and dividing in order to have a bactericidal action. Meanwhile, Chloramphenicol causes a slow growth of *Staphylococcus aureus* and impairs bactericidal effect of Ceftriaxone if they are combined. **Conclusions:** Ceftriaxone and Chloramphenicol combination has lower antibacterial effect than the single antibiotic groups on *Staphylococcus aureus* isolate of Gangrene diabetic and the type of interaction is antagonistic.



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## ABSTRAK

Latar Belakang: *Gangrene diabetik* merupakan salah satu komplikasi Diabetes melitus yang disebabkan oleh *S. aureus*. Kombinasi seftriakson dan kloramfenikol sering digunakan untuk pengobatan infeksi gangrene, meskipun interaksinya belum diketahui secara pasti. Tujuan: membuktikan potensi seftriakson, kloramfenikol dan kombinasinya pada isolat *S. aureus* dari gangrene diabetik. Bahan dan Metode: Penelitian dilakukan dengan metode difusi cakram dengan media Muller Hinton. Seftriakson, kloramfenikol dan kombinasinya dengan dosis 7,5 g/mL, 15 g/mL dan 30 g/mL, masing-masing diuji pada kultur *S. aureus* isolat gangrene diabetes. Efek antibakteri diamati dengan mengukur zona hambat pada kultur bakteri. Tipe interaksi dianalisa dengan metode Ameri-Ziaei Double Antibiotic Synergism Test (AZDAST). Hasil penelitian diuji secara statistik dengan One Way ANOVA ( $p=0,05$ ) dilanjutkan dengan uji Beda Nyata Terkecil (BNT). Hasil: Kombinasi seftriakson dan kloramfenikol menunjukkan efek antibakteri lebih rendah dibandingkan antibiotik tunggal seftriakson. Interaksi antagonis dihasilkan dari kombinasi antibiotik ini. Seftriakson memerlukan kondisi sel tumbuh dan membelah agar berfungsi sebagai agen bakterisida. Sementara kloramfenikol menghambat pertumbuhan *S.aureus* dan mengganggu efek bakterisidal dari seftriakson bila dikombinasi. Kesimpulan: Kombinasi seftriakson dan kloramfenikol memiliki efek antibakteri yang lebih rendah dibandingkan dengan antibiotik seftriakson tunggal pada isolat *S. aureus* diabetes Gangrene. Tipe interaksi antibiotik bersifat antagonis.

Kata kunci: Seftriakson; Kloramfenikol; *Staphylococcus aureus*, gangrene diabetik.

## INTRODUCTION

Diabetic foot infection is a major cause of hospitalized patient in developing country (Złoch et al., 2021). In 2009, research at Makassar Hospital Indonesia indicated about 20,61% hospitalized patients were caused by diabetic foot infection (Rohmah, 2019). Severe diabetic foot infections and late handled can lead to gangrene diabetik (Aulia et al., 2019). Variety of bacteria causing infection of diabetic gangrene is a gram positive, gram negative and anaerobic bacteria (Prasetya et al., 2019). Research in the New England Deaconess Hospital indicated gangrene diabetik infection always caused more than 2 groups of bacteria (Caroline, 2016). *Staphylococcus aureus*, *Streptococcus sp*, *Pseudomonas sp* are the major cause of gangrene infection (Yutaka, 2020).

One of pharmacological therapy for gangrene diabetik is antibiotics. The administration of antibiotics should be appropriate with the culture of gangrene bacteria on the infected wound. On the worsening of diabetic foot infections should be given antibiotics while waiting culture results. In cases of diabetic foot infection, combination of antibiotic is used to cure the infection (Caroline, 2016; Otoupal et al., 2021) Cephalosporin group are often used for such cases is Ceftriaxone (CTX) or Ceftazidim (Caroline, 2016; Jahani et al., 2017). The combination of antibiotics which include fixed combination has been proven and recognized as a fixed drug combination. Several combinations of antibiotics having bactericidal and bacteriostatic effect are not known exactly due to they are influenced by the dose (Navarro-Pérez et al., 2021). Theoretically, the combination of antibiotics is confirmed antagonist, however, it is still used because it is considered not clinically meaningful (Ocampo et al., 2014; Otoupal et al., 2021).

Treatment of diabetic foot infection using the combination of bactericidal antibiotic like Ceftriaxone (CTX) and bacteriostatic antibiotic like Chloramphenicol (CML) can produce antagonistic interactions

based on theory (Navarro-Pérez et al., 2021; Zloch et al., 2021). Their interaction will reduce the efficacy of antibiotic. The study can be performed using diffusion method and type interaction with Ameri-Ziaei Double Antibiotic Synergism Test (AZDAST) (Ziaei-Daroukalei et al., 2016). Based on explanation above, the objective of the study was to evaluate the combination effect of CTX and CML against *S. aureus* isolate of gangrene diabetic including the type of interaction.

## **MATERIAL AND METHODS**

### **MATERIALS**

*S. aureus* isolate of gangrene diabetic was obtained from Dr. Saiful Anwar Hospital, Malang, Muller Hinton media (Sigma-Aldrich), Ceftriaxone (Orchid Pharma), Chloramphenicol (Kimia Farma), Paper disk (Merck), and Mc-Farland turbidity standard (Sigma-Aldrich).

### **METHODS**

#### **Bacteria media preparation**

Muller Hinton media 3.8% was made by adding sterile distilled water and then boiled until complete dissolution. Muller Hinton media was poured into empty petri dish and left until hardened. Muller Hinton media has hardened put in an incubator with a temperature of 37°C for  $\pm$  24 hour to evaluate bacterial contaminants in the media that has been made. If a bacterial contaminant does not found therefore the media is ready to use.

#### **Antibiotic Preparation**

Both CTX and CML were each dissolved with sterile distilled water and then they were further dissolved to obtain 30  $\mu\text{g/ml}$ , 15  $\mu\text{g/ml}$ , 7.5  $\mu\text{g/ml}$ . Furthermore, the combination of CTX and CML were made to obtain three composition, the ratio CTX:CML are (C1) 30 : 7.5  $\mu\text{g/mL}$ , (C2) 15 : 15  $\mu\text{g/mL}$ , and (C3) 7.5 : 30  $\mu\text{g/mL}$ . Paper disc was inserted to the antibiotic solution for preparation of antibacterial assay.

#### **Antibacterial Assay**

*S. aureus* suspension was prepared and adjusted by comparison against 0.5 Mc-Farland turbidity standard ( $5 \times 10^7$  cells/ml) tubes. Next, it was diluted to obtain a final  $5 \times 10^6$  cells/ml. Bacteria was sub-cultured on Muller Hinton media for bacterial propagation (Solomon and Isaac, 2018). The broth was inoculated by the 0.2  $\mu\text{g /ml}$  by *S. aureus* and then added paper disk containing CTX, CML and its combination. Furthermore, the plates were incubated at  $37 \pm 1^\circ\text{C}$  for 24 h and observed for colony growth. Clear zone was measured by a caliper showing zone of inhibition (ZOI). Antibacterial activity is classified based on Greenwood, 1995 in Ramadheni et al., 2018: none (less than 10 mm), weak (11-15 mm), moderate (16-20 mm), potent (more than 20 mm). Meanwhile, percent of inhibition was calculated based on formula

$$\text{Percent of inhibition} = \frac{\text{ZOI sample} - \text{ZOI control}}{1.5 \text{ ZOI potent (30 mm)}} \times 100 \%$$

The IC<sub>50</sub> value was calculated by linear regression curve fit using SPSS version 16.0

### Analysis of antibacterial interaction

Interaction were analyzed using Ameri-Ziaei Double Antibiotic Synergism Test (AZDAST) method (Ziaei-Darounkalaei et al, 2016). Type of interaction are synergistic (AB >A&B and </> AA and/or BB), potentiation (one of A/B=0 and AB>A&B and >/< AA and/or BB), antagonistic (AB < A or B), additive (AB=AA and/or BB) and not distinguishable (AB = A or B).

### Statistical analysis

The experimental results were replicated four times and the data are expressed as the mean ± SD. The data were tested using One Way ANOVA test and followed by Least Significant Difference (LSD, p=0,05) test.

## RESULTS AND DISCUSSION

### Antibacterial activity

Antibacterial activity of compound was showed by inhibition of bacterial growth. Inhibition zone diameter of CTX, CML and their combination on *S. aureus* isolate of gangrene diabetic can be seen in the Table 1-2, and Figure 1-2.

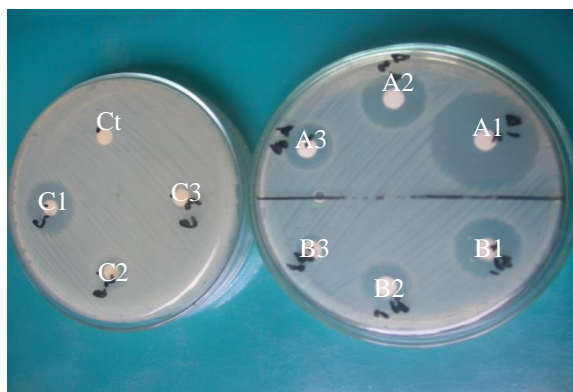


Figure 1. Inhibition zone diameter of Ceftriaxone, Chloramphenicol and their combination on *S. aureus* isolate of gangrene diabetic (Ct=control, A1=CTX-30, A2=CTX-15, A3=CTX-7.5, B1=CML-7.5, B2=CML-15, B3=30, C1=CTX30-CML7.5, C2= CTX15-CML15, C3= CTX7.5-CML30).

Table 1. Inhibition zone diameter of CTX, CML and their combination on *S. aureus* isolate of gangrene diabetic

No.	Group	n	Mean ± SD (mm)	Antibacterial effect*
1	Control (Ct)	4	6.00±0.00 <sup>a</sup>	None
	CTX-30 (A1)	4	28.96±0.02 <sup>b</sup>	Potent
	CML-7,5 (B3)	4	7.97±0.24 <sup>c</sup>	None
	CTX-30:CML-7.5 (C1)	4	17.77±0.32 <sup>d</sup>	Moderate
2	Control (Ct)	4	6.00±0.00 <sup>a</sup>	None
	CTX-15 (A2)	4	21.00±0.05 <sup>b</sup>	Potent
	CML-15 (B2)	4	12.37±0.49 <sup>c</sup>	Weak
	CTX-15:CML-15 (C2)	4	14.03±0.22 <sup>c</sup>	Weak
3	Control (Ct)	4	6.00±0.00 <sup>a</sup>	None
	CTX-7,5 (A3)	4	11.88±0.00 <sup>b</sup>	Weak
	CML-30 (B1)	4	17.98±0.05 <sup>c</sup>	Moderate
	CTX-7,5:CML-30 (C3)	4	8.45±0.44 <sup>d</sup>	None

a,b,c : different letter show the different effect (LSD, p<0.05 %)

\* : antibacterial effect based on Greenwood classification, 1995 in Ramadheni et al (2018)

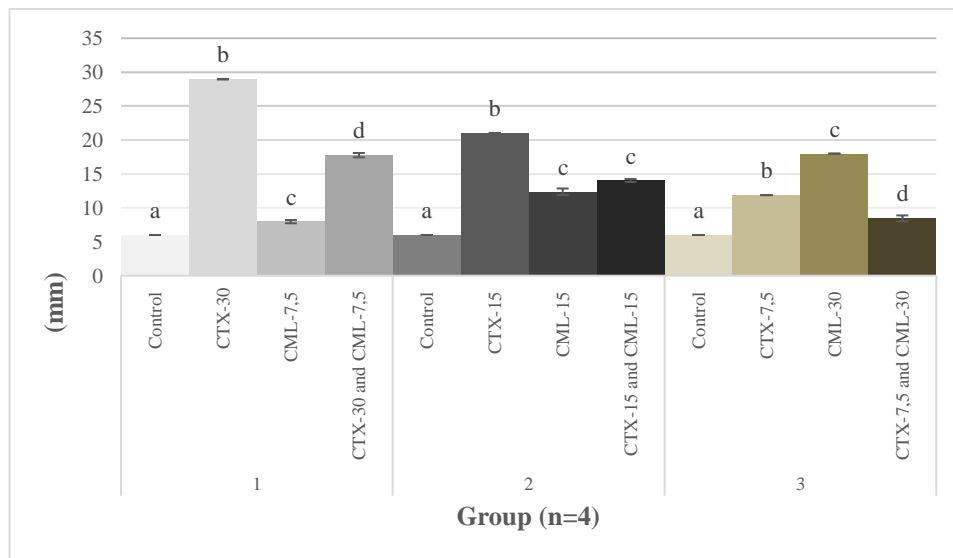


Figure 2. Inhibition zone diameter of CTX, CML and their combination on *S. aureus*

Table 2. Inhibitory concentration-50 (IC<sub>50</sub>) of antibiotic on *S.aureus* isolate of gangrene diabetic

Antibiotic	Concentration (µg/mL)	n	Mean ± SD (mm)	% Inhibition	IC <sub>50</sub> (µg/mL)
CTX	0	4	6.00±0.00 <sup>a</sup>	0.00	18.32
	7.5	4	11.88±0.00 <sup>b</sup>	19.60	
	15	4	21.00±0.05 <sup>c</sup>	50.00	
	30	4	28.96±0.02 <sup>d</sup>	76.53	
CML	0	4	6.00±0.00 <sup>a</sup>	0.00	37.17
	7.5	4	7.97±0.24 <sup>a</sup>	6.56	
	15	4	12.37±0.49 <sup>b</sup>	21.23	
	30	4	17.98±0.05 <sup>c</sup>	39.93	

a,b,c : different letter show the different effect (LSD, p<0.05 %)

Based on these result, the first composition indicated a significant decrease of inhibition zone diameter on combination CTX-CML (30:7.5 µg/mL) compare to CTX 30 µg/mL. However, inhibition zone diameter of the combination was higher than CML 7.5 µg/mL and it has a moderate activity. CML showed a low antibacterial effect due to the dose used and the less sensitivity of them to *S. aureus* (Yutaka, 2020; Alsherbiny et al., 2021).

The second combination CTX-CML (15:15 µg/mL) indicated a significant reduce of inhibition zone diameter compare to CTX 15 µg/mL. On the other hand, inhibition zone diameter of the combination was higher than single CML 15 µg/mL and it has a weak activity. CTX more potent than CML at the same dose, it supported also with the percent inhibition and IC<sub>50</sub> value which is showed at table 2. CTX has IC<sub>50</sub> value lower than CML, it means CTX more potent to inhibit *S.aureus* growth compare to CML.

For third combination CTX-CML (7.5:30 µg/mL) showed a significant decrease of inhibition zone diameter compare to both of single CTX 7.5 µg/mL and CML 30 µg/mL. This combination has lower antibacterial activity than two combination mentioned previous and according to Ramadhani et al., 2018, it does not have antibacterial effect. Three combination of CTX and CML are disadvantage since the potency of antibacterial decreased until it has no activity. The first combination has antibacterial effect stronger than two combination others. It is caused by the difference of composition bactericidal and bacteriostatic which is used for each combination. CTX has bactericidal effect whereas CML has bacteriostatic effect. When two antibiotics above are combined, the activity of bactericidal antibiotic will be inhibited by bacteriostatic antibiotic meaningful (Ocampo et al., 2014; Navarro-Pérez et al., 2021). CTX works through inhibiting the formation of cell wall, meanwhile CML blocking protein synthesis especially at ribosome sub unit 50S (Eyler and Shvets, 2019; Inayah et al., 2020). The combination with high dose of CTX and low dose of CML results a strong antibacterial effect on *S. aureus*.

Mixed infection caused by several groups of bacteria like in gangrene diabetic infection results the use of combination antibiotic (Caroline, 2016; Złoch et al., 2021). Antibiotic combination of CTX and CML

is used to increase efficacy on gangrene diabetic infection. The combination was selected to expand the spectrum of bacteria also (Kothari et al., 2022). Actually, the aims of antibacterial combination to increase potency, extend spectrum, reduce resistance and toxicity (Alsherbiny et al., 2021; Kothari et al., 2022). However, incorrect selection of antibacterial combination and the dose produce therapy failure (Gilbert et al., 2010; Parkunan et al., 2019).

### Interaction of antibacterial CTX and CML

The interaction of CTX and CML is analyzed using AZDAST methods, the result can be seen at table 3

Table 3. Interaction of CTX and CML combination on *S. aureus*

Antibiotic	n	Mean $\pm$ SD (mm)	Antibacterial effect*	Type of interaction <sup>#</sup>
CTX-7.5	4	11.88 $\pm$ 0.00 <sup>a</sup>	Weak	
CML-7.5	4	7.97 $\pm$ 0.24 <sup>b</sup>	None	Antagonistic
CTX-7.5 + CML-7.5	4	9.02 $\pm$ 0.13 <sup>b</sup>	None	
CTX-7.5 + CTX-7.5	4	21.00 $\pm$ 0.05 <sup>c</sup>	Potent	
CML-7.5 + CML-7.5	4	12.37 $\pm$ 0.49 <sup>a</sup>	Weak	
CTX-15	4	21.00 $\pm$ 0.05 <sup>a</sup>	Potent	
CML-15	4	12.37 $\pm$ 0.49 <sup>b</sup>	Weak	Antagonistic
CTX-15 + CML-15	4	14.03 $\pm$ 0.22 <sup>b</sup>	Weak	
CTX-15 + CTX-15	4	28.96 $\pm$ 0.02 <sup>c</sup>	Potent	
CML-15 + CML-15	4	17.98 $\pm$ 0.05 <sup>d</sup>	Moderate	

a,b,c : different letter show the different effect (LSD,  $p < 0.05$  %)

\* : antibacterial effect based on Greenwood classification, 1995 in Ramadhani et al (2018)

<sup>#</sup> : type of interaction based on AZDAST method

The combination produces antibacterial effect lower than single antibiotic due to antagonistic interaction between bactericidal antibiotic and bacteriostatic, therefore it reduces their potency as antibiotic.  $\beta$ -lactam antibiotic like CTX require the cell be growing and dividing in order to have a bactericidal action. CML cause slow growth of *S. aureus* furthermore it impairs bactericidal effect of CTX (Ocampo et al., 2014; Navarro-Pérez et al., 2021). The combination CTX and CML result antagonistic interaction based on AZDAST method.

Conditions of bacterial growth affect the activity of bactericidal agents also. Conditions at the site of infection that favor no growth or slow growth of bacteria will disrupt bactericidal action. Thus, whether a bactericidal or a bacteriostatic effect is achieved can vary depending on not only the antibiotic, however, the organisms and the growth conditions also (Eyler and Shvets, 2019; Navarro-Pérez et al., 2021). Antibiotic was classified as bacteriostatic and bactericidal sketchily, bacteriostatic inhibits replication and growth of bacteria. Meanwhile bactericidal kill bacteria and reduce of bacteria number lived (Sari et al., 2018). Even though the classification is simple, in some case antibiotic is bacteriostatic for one organisms and are bactericidal to other organisms. For example, CML is bacteriostatic to gram-negative rods but it is bactericidal to *Pneumococcus* sp (Sood, 2016; Lorenzo, 2019).



The experimental laboratory indicated the growth of bacteria was inhibited by bacteriostatic. Microorganisms can live although there were exposed by bacteriostatic drugs. On the other hand, giving of bactericidal drugs can kill bacteria and decrease of bacteria number lived (Otoupal et al., 2021). Mixed infection caused by several group bacterial such as gangrene diabetic induces the use of antibiotic combination (Caroline, 2016).

Antibiotics are effective drug in the therapy of infection diseases due to their potency to kill microorganisms. In many cases, antibiotic treatment requires controlling of dose to eradicate microorganisms furthermore the body can tolerate it (Paterson et al., 2016).

Selection of antibiotic for infection therapy requires the knowledge about microorganism identity and their sensitivity to antibiotic, place of infection, antibiotic safety, patient factor and cost (Gilbert et al., 2010). However, some seriously ill patient require empiric therapy that is giving antibiotic as soon as possible while waiting the culture results (Prasetya et al., 2019).

In addition, bactericidal activity is not an invariable property of an antibiotic, it can depend upon the organism and the growth conditions. For example, *S. aureus* is not killed by protein synthesis inhibitors like CML and Erythromycin (ERT), which are classical bacteriostatic agents having target at the ribosome (Eyler and Shvets, 2019; Schulte-Werning et al., 2021). The most of bacteria, *S. aureus* and *S. pneumoniae* are killed by cell wall inhibitors such as Penicillin and Vancomycin, as well as by the Fluoroquinolones, which are classical bactericidal agents (Navarro-Pérez et al., 2021). These agents *in vitro* do not produce 99.9% kill of Enterococci within the 24-hour time frame, therefore they are considered to be only bacteriostatic against this organism (Gilbert et al., 2010; Ocampo et al., 2014). The continue between bactericidal activity and bacteriostatic that varies depending on the antibiotic, the bacterial species or isolate, and growth conditions (Ocampo et al., 2014; Paolo et al., 2014).

## CONCLUSION

Combination of CTX and CML has antibacterial effect lower than the single antibiotic groups on *S. aureus* isolate Gangrene diabetic and the type of interaction is antagonistic. The combination of high dose of CTX and low dose of CML shows antibacterial effect in the moderate category.

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No declared.

## CONFLICT OF INTEREST

The author declared no conflict of interest



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