In Vitro Synergism of Sulbactam-Cefoperazone and Fosfomycin Against *Escherichia Coli* and *Klebsiella Aeromobilis* from Indonesia

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Abstract

Introduction: *There is no susceptibility data of E. coli and K. aeromobilis in Indonesia, even data regarding minimal inhibitory concentration (MIC)-based susceptibility of E. coli and K. aeromobilis towards single antibiotic or combination of fosfomycin (FOS) and sulbactam-cepoferazone (SUL-CPZ) is very scarce, even though the data is required by clinicians.*
Methods*: A descriptive observational study was carried out at the Microbiology Clinical Laboratory of the Faculty of Medicine, Universitas Indonesia. Thirty strains each of clinical isolates of E. coli and K. aeromobilis were subjected to MIC determination against FOS and SUL-CPZ. For susceptibility criteria, we adopted the Eucast guideline. The synergism of the combined antibiotics was determined by checkerboard titration. One strain of E. coli and K. aeromobilis showing a synergistic and independent effect against the combined antibiotics was subjected to a time-kill assay. The post-antibiotic effect (PAE) was determined on a strain of E. coli showing synergism against the combined antibiotics.*
Results*: The MIC level of all strains decreased when the bacteria were exposed to the combined antibiotics. Synergism was observed in 53.3% of E. coli and 56.8% of K. aeromobilis. No antagonism was observed. Higher bacterial death during the first four hours occurred with the isolate, showing synergism compared to the isolate showing an independent effect. The PAE of E. coli was longer when exposed to combined antibiotics.*
Conclusion*: In vitro synergism of FOS and SUL-CPZ was observed in the majority of isolates and could be used as the basis for further research on empirical treatment.*

Keywords*: Escherichia coli, Klebsiella aeromobilis, fosfomysin, sulbactam-cefoperazone, in vitro antibacterial effects*

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Sinergisme *In Vitro Sulbactam-Cefoperazone* dan *Fosfomycin* Terhadap *Escherichia Coli* dan *Klebsiella Aeromobilis* Di Indonesia

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Abstrak

Pendahuluan: *Belum ada data kepekaan in vitro kombinasi Fosfomycin (FOS) dan Sulbactam-Cepoferazone (SUL-CPZ) di Indonesia. Bahkan data kepekaan berdasarkan Konsentrasi Hambat Minimum (KHM) masing-masing obat untuk Escherichia coli (E. coli) dan Klebsiella aeromobilis (K. aeromobilis) atau kombinasi keduanya sangat jarang, disisi lain data tersebut diperlukan oleh klinisi.*
Metode: *Penelitian ini merupakan penelitian deskriptif observasional. Masing-masing tiga puluh galur isolat klinis E. coli dan K. aerobilis diuji kepekaannya dengan penentuan KHM FOS dan SUL-CPZ menggunakan pedoman EUCAST. Fenomena sinergisme, indepedensi, dan antagonisme antibiotika kombinasi ditentukan dengan cara titrasi "checkerboard". Uji "time-kill" dikerjakan pada masing-masing satu isolat E. coli dan K. aeromobilis yang menunjukkan sinergisme dan indepedensi terhadap kombinasi antibiotika. Uji efek pasca paparan antibiotika dikerjakan pada satu galur E. coli yang menunjukkan efek sinergisme.*
Hasil: *KHM semua galur E. coli dan K. aeromobilis lebih rendah pada masing-masing antibiotika saat dalam kombinasi dibandingkan dengan KHM obat tunggal. Sinergisme pada E. coli dan K. aeromobilis teramati berturut-turut pada 53,3% dan 56,8%. Fenomena antagonisme tidak ditemukan Jumlah bakteri mati yang terpapar antibiotika kombinasi lebih banyak dibanding yang terpapar antibiotika tunggal, khususnya pada empat jam pertama. Efek pasca paparan antibiotika pada E. coli juga lebih panjang pada yang terpapar antbiotika kombinasi.*
Kesimpulan: *Efek sinergisme FOS dan SUL-CPZ teramati pada lebih dari separuh jumlah bakteri dan tidak ada antagonisme. Temuan ini dapat dipertimbangkan sebagai dasar untuk penelitian lebih lanjut terapi empirik infeksi E. coli dan K. aeromobilis.*

Kata kunci: *E. coli, K. aeromobilis, fosfomysin, sulbactam-cefoperazone, efek anti bakteri in vitro*

Introduction

E. coli and *K. aeromobilis* play a major role as human pathogens in community- and healthcare-associated infections and may affect many organs.[1] *K. aeromobilis*, or *Enterobacter aerogenes* in older taxonomy, is an opportunistic bacteria and had been described as a causative agent of several healthcare-associated infection outbreaks.[2] Although both bacteria have a large impact on medical services in Indonesia,[3,4] the MIC data of SUL-CFP and FOS against both pathogens is scarce and in many areas there is none. Most of the published data on the susceptibility of *E. coli* and *K. aeromobilis* is derived from the disc diffusion method.[5,6] In addition, there is no published data on the effects of combined SUL-CFP plus FOS on clinical isolates. On the other hand, the MIC level of each antibiotic and proportion of isolate that shows synergism against combined antibiotics is very important for patient treatment as well as for controlling antibiotic resistance. It has also been reported that FOS and cepoferazone act on bacteria in sequential steps. The initial step and a later step during cell wall synthesis were inhibited by FOS and SUL-CFP, respectively.[7] Based on this, we report the effect of combined SUL-CFP plus FOS on *E. coli* and *K. aeromobilis* from Jakarta, Indonesia.

Methods

Bacteria

Study was conducted at the Department of Clinical Microbiology, Faculty of Medicine, Universitas Indonesia. Thirty strains each of *E. coli* and *K. aeromobilis*

clinical isolates were randomly selected from stock cultures in our department. Ethical exemption was provided by the Ethical Committee for Health Research Faculty of Medicine Universitas Indonesia-Cipto Mangunkusumo Hospital. All strains were recultured using Mueller-Hinton broth at 37ºC overnight. The bacteria were harvested in the logarithmic phase. Each strain was standardized to have 0.5 McFarland turbidity. Thereafter, the bacterial suspension was diluted with Mueller-Hinton broth to achieve 3–5 x 10 5 colony forming units per milliliter (CFU/mL) of bacteria before conducting the MIC determination.

Determination of MIC for each antibiotic

The MIC of SUL-CFP and FOS was determined by the broth macro-dilution method in cation-adjusted Mueller-Hinton broth (CAMHB).[8] Briefly, 1 mL of the antibiotic solution was added to 9 mL of CAMHB, followed by vortexing to homogenize the solution. A two-fold dilution of the antibiotic was serially performed, and 1 mL of the bacterial suspension, as mentioned above, was added to each tube and incubated at 37ºC overnight. The MIC was determined as the lowest antibiotic concentration that inhibited visible growth of the bacteria. The determination of MIC was done in duplicate. An MIC of 32 µg/mL was used as the breakpoint level to differentiate susceptible and resistant strains.[9]

Determination of the synergism of combined FOS and SUL-CFP

The MIC of the antibiotic combination was determined by a checkerboard titration method using CAMHB in tubes. We adopted previously described procedures.[10] Briefly, an array panel consisting of 8 × 8 tubes was arranged. Tube number 1 contained 2 times the MIC of FOS, and tube number 64 contained 2 times the MIC of CPZ-SUL. Both antibiotics were two-fold serially diluted in a horizontal and vertical manner. An additional tube containing only CAMHB was used as the growth control. One milliliter of the bacterial suspension was then put into each tube, which was incubated at 37°C overnight. The fraction inhibition concentration index (FICI) was calculated accordingly. The MIC of the antibiotic combination was defined as synergism whenever the FICI of each strain is maximum (0.5), independent (between 0,5-4), antagonism (> 4). An independent effect implies that antibiotic combinations act independently.

Time-kill test

Evaluation was done on one strain each that showing synergism and independent respectively adopted previous study[11] using a half MIC and estimated 6 × 107 CFU/mL bacteria. Briefly, 1 mL of the bacterial suspension was added to each 10 mL CAMHB tube containing SUL-CFP, FOS, or a combination of SUL-CFP and FOS, and the tubes were incubated at 37ºC. A tube without any antibiotic was used as a control. At hours 0, 4, 8, and 24, a portion of the inoculated broth was taken, homogenized in an ice bath, and serially diluted. One hundred microliters of each bacterial suspension were inoculated on an agar plate that was incubated at 37°C for overnight. The number of colonies that grew on the plate was counted and plotted on a curve diagram.

Determination of the PAE

One strain each of *E. coli* and *K. aeromobilis* showing synergism and an independent effect to the SUL-CFP and FOS combination, respectively, was inoculated into CAMHB at 37°C for 8 hours and then subjected to the PAE study according to a previously described method.[12] Briefly, 9 mL of a combination of SUL-CFP and FOS in CAMHB at an MIC of 4 and 1 mL of the bacterial suspension estimated to contain a final concentration of 106 to 107 CFU/mL were mixed, and the solution was incubated in a water bath at 37° C for 1 hour. Thereafter, the suspension was centrifuged, and the pellet was re-suspended in 10 mL of CAMHB. The removal of extracellular antibiotics by centrifugation of the bacterial suspension was repeated three times. Washed bacteria was then inoculated into Mueller-Hinton broth and incubated in a shaking water bath at 37°C. At 0 and 1 hour intervals, a portion of culture was taken, serially diluted, and plated on an agar plate. The number of colonies on the plate was counted and plotted on a curve diagram.

Results

Comparison of the MIC of SUL-CFP, FOS, and combination of SUL-CFP and FOS
The MIC of SUL-CFP and FOS for *K. aeromobilis* varies from 1 µg/mL to 32 µg/mL and from 8 µg/mL to 2048 µg/mL, respectively. The MIC of SUL-CFP and FOS in combined SUL-CFP plus FOS decreased to a range of 0.125 µg/mL to 16 µg/mL and to a range of 2 µg/mL to 512 µg/mL, respectively. The decreased MIC of SUL-CFP was observed in

all tested strains. All strains of *K. aeromobilis* were susceptible to SUL-CFP. On the contrary, only 16.7% of *K. aeromobilis* was susceptible to FOS. *K. aeromobilis* showed an increase in susceptibility to 30% in combined antibiotics when compared to FOS alone.

The MIC of SUL-CFP and FOS for *E. coli* ranged from 0.5 µg/mL to 16 µg/mL and from 4 µg/mL to 1024 µg/mL, respectively. The MIC of SUL-CFP and FOS in combined SUL-CFP plus FOS decreased from 0.125 µg/mL to 4 µg/mL and from 2 µg/mL to 128 µg/mL, respectively. All strains of *E. coli* were susceptible to SUL-CFP. In contrast, 56.7% of *E. coli* was susceptible to FOS, and a proportion of susceptible strains of *K. aeromobilis* against combined antibiotics increased to 86.7% when compared to FOS alone.

Compared to the MIC of each antibiotic, the MIC of each antibiotic in the combined antibiotic was lower in all tested strains. Two, fourth, eight sixteen-fold reductions in the MIC of SUL-CFP and FOS in combined antibiotics compared to the MIC of SUL-CFP and FOS alone are depicted in Table 2.

Table 1. MIC reduction of SUL-CFP and FOS in combined SUL-CFP and FOS

	K. aeromobilis		*E. coli*	
Fold Reduction	**SUL-CFP**	**FOS**	**SUL-CFP**	**FOS**
2	30%	16.7%	30%	20%
4	43.3%	40%	53.3%	63.3%
8	26.7%	36.7%	16.7%	16.7%
16	0%	6.6%	0%	0%

Effect of the SUL-CFP and FOS combination

The average FICI for *K. aeromobilis* and *E. coli* is 0.53 and 0.59, respectively. No antagonism of SUL-CFP and FOS was observed. The synergism of the SUL-CFP and FOS combination on both *K. aeromobilis* and *E. coli* was 63.3% and 56.7%, respectively. The synergism of combined SUL-CFP plus FOS on *E. coli* isolated from urine and *K. aeromobilis* isolated from sputum was detected in 50.0% and 63.6%, respectively.

Comparative killing speed of antibiotics

The evaluation of killing speed due to the antibiotic tested was done on one strain, showing a synergistic and independent effect against combined antibiotics, respectively. Our study found different patterns of killing speed on *K. aeromobilis* and *E. coli*, revealing synergism and an indifferent effect of the antibiotics tested. However, rapid killing of K. aeromobilis and *E. coli* due to the SUL-CFP and FOS combination was observed during the first four hours of exposure to the antibiotic combination (Figure 1a, 1b and Figure 2a, 2b).

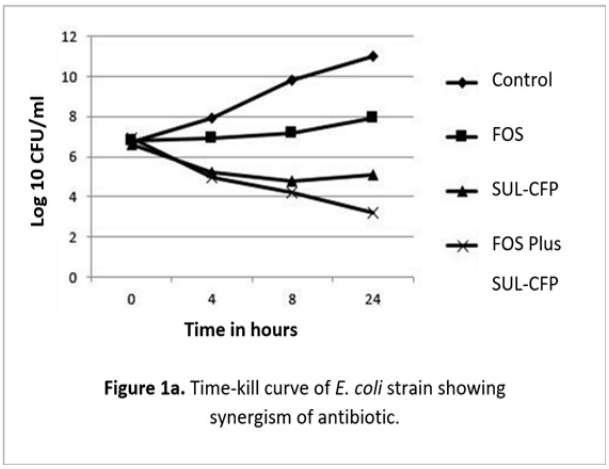
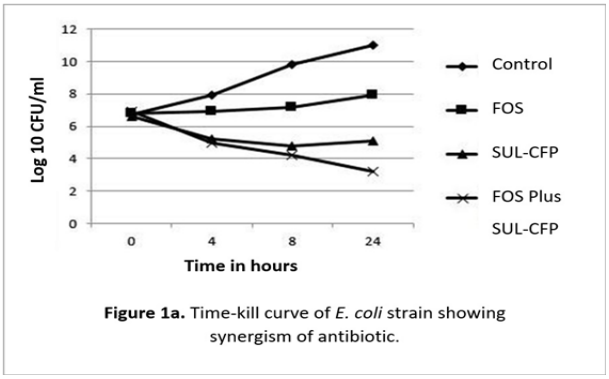


Figure 1a. Time-kill curve of *E. coli* strain showing synergism of antibiotic.



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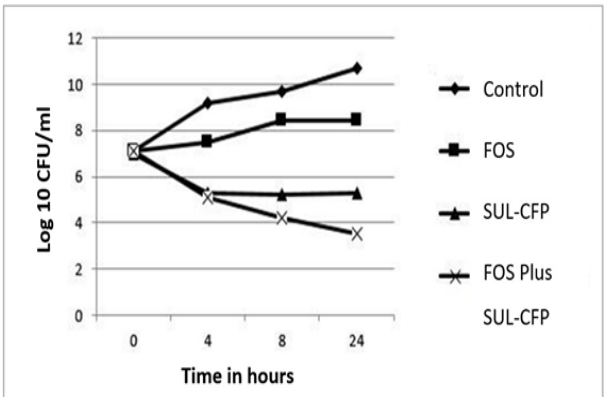

Figure 2a. Time-kill curve of *K. aeromobilis* showing synergism of antibiotic.

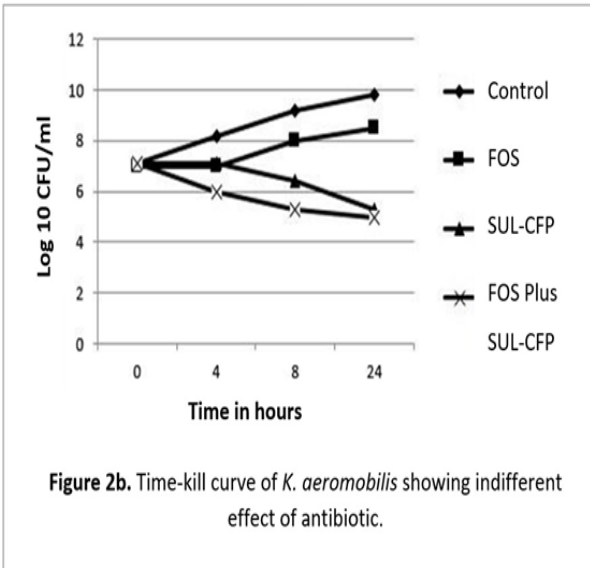

Figure 2b. Time-kill curve of *K. aeromobilis* showing indifferent effect of antibiotic.

PAE

The PAE was determined on one strain of *E. coli* having an FICI index of 0.5 representing synergism effect. The growth recovery of the strain exposed to the tested antibiotic is shown in Figure 3. The PAE of FOS, SUL-CFP, and the combined antibiotic was 0.4 hours, 0.2 hours, and 1.7 hours, respectively.

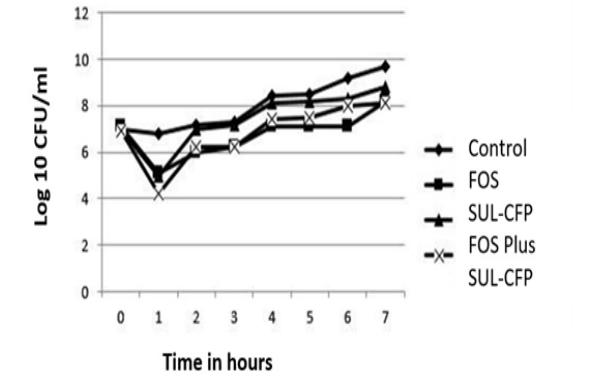

Figure 3. Growth curve of *E. coli* showing synergism of antibiotic in PAE determination test.

Discussion

Purpose of antibiotic combination usage includes: (i) an additive effect of the combined antibiotic, which leads to antibacterial spectrum expansion;[13] (ii) a lowered incidence of harmful adverse effects of the antimicrobial agent;[14] (iii) limited resistant bacteria to replicate;[16] and (iv) a beneficial effect of the synergistic action of the combined antibiotics.[17] Previous studies with extended-spectrum-beta-lactamase (ESBL) E. coli showed the synergism of FOS plus penem, FOS plus aztreonam, FOS plus colistin, FOS plus netilmicin, and FOS plus tigecycline.[18–20] Furthermore, FOS plus doripenem, FOS plus aztreonam, and FOS plus aztreonam plus amdinocillin combinations have the ability to reduce the drug-resistant *K. pneumoniae* population.[19,20] The synergistic effect on ESBL-producing *K. pneumoniae* of FOS plus imipenem was better than the synergistic effect of FOS plus colistin, netilmicin, or tigecyclin.[18] So far, there are no data available for *K. aeromobilis*.

Our data showed a decreased MIC of SUL-CFP and FOS when both drugs were combined against all strains of *E. coli* and *K. aeromobilis*; although, overall synergism was only observed in 63.3 and 56.7% of *K. aeromobilis* and *E. coli* strains, respectively. It was also observed that the proportion of *E. coli* and *K. aeromobilis* showing synergism differs slightly between isolates from urine and sputum. The later should be confirmed using more isolates. Moreover, the combined SUL-CFP and FOS showed a higher bactericidal effect, especially within the first four hours of exposure, and induced a longer PAE as compared to the effect of each antibiotic. The overall data indicate that combined SUL-CFP and FOS may at least limit the mutation window, which leads to a slower rate of emerging resistant bacteria. Considering that cross-resistance against FOS with other antibiotics has not yet been reported,[21] FOS modulates antibody-secreting cells and polymorphonuclear leucocyres in a positive sense,[22] and data from our study showed that combination of SUL-CFP and FOS is predicted to have a positive clinical implication. Further study is required to confirm the above predictions.

Conclusion

In vitro synergism of FOS and SUL-CPZ was observed in the majority of isolates and could be used as the basis for further research on empirical treatment.

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Conflict of interest disclosure

The authors stated that they have no other interests which might be perceived as posing a conflict or bias.

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