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The potential of meropenem and piperacillin-tazobactam combination to *Acinetobacter spp* clinical isolates *in vitro*

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ABSTRACT

Acinetobacter spp is one of the most common causes of nosocomial infection, especially sepsis. A lot of antibiotics resistance happen related to *Acinetobacter*-related sepsis treatment. This study aimed to evaluate the potential of meropenem and piperacillin-tazobactam combination against *Acinetobacter spp* in *in vitro* by using paper strip test. This was experimental study conducted in September to December 2015 at Departement of Microbiology, Faculty of Medicine, Public health, and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia. Clinical isolates of *Acinetobacter* samples were obtained from collections of the Department of Microbiology. The data were analyzed using post-test analysis which was conducted by observation over 24 h after the paper strip test was applied in bacterial culture. The MIC value of the antibiotic combination was recorded based on observation. The result showed 12 of 17 clinical isolates were synergistic potential (70.59%) and 5 others were indifferent potential (29.41%). Two of five clinical isolates that show indifferent potential were *A. baumannii* and all of the clinical isolates that show synergistic potential were *Acinetobacter spp*. It can be concluded that the combination of meropenem and piperacillin-tazobactam showed more synergistic dominantly than the single use of each of them.

ABSTRAK

Acinetobacter spp adalah salah satu penyebab paling umum infeksi nosokomial, terutama sepsis. Banyak resistensi antibiotik terjadi terkait dengan pengobatan sepsis yang berhubungan dengan *Acinetobacter*. Penelitian ini bertujuan untuk mengkaji potensi kombinasi meropenem dan piperacillin-tazobactam terhadap *Acinetobacter spp in vitro* dengan menggunakan uji strip kertas. Penelitian eksperimental ini dilakukan pada bulan September hingga Desember 2015 di Departemen Mikrobiologi, Fakultas Kedokteran, Kesehatan Masyarakat, dan Keperawatan, Universitas Gadjah Mada, Yogyakarta. Isolat klinis sampel *Acinetobacter* diperoleh dari koleksi milik Departemen Mikrobiologi tersebut. Data dianalisis menggunakan analisis *post-test* yang dilakukan setelah pengamatan selama 24 jam setelah uji strip kertas pada kultur bakteri. Selanjutnya nilai MIC kombinasi antibiotik ditetapkan. Hasil penelitian menunjukkan 12 dari 17 isolat klinis berpotensi sinergis (70,59%) dan 5 isolat tidak ada potensial (29,41%). Dua dari lima isolat klinis yang menunjukkan tidak potensi sinergis adalah *A. baumannii* dan semua isolat klinis yang menunjukkan potensi sinergis adalah *Acinetobacter spp*. Dapat disimpulkan bahwa kombinasi meropenem dan piperacillin-tazobactam menunjukkan lebih sinergis dominan daripada penggunaan tunggal masing-masing.

Keywords:
sepsis;
Acinetobacter spp;
paper strip test;
meropenem;
piperacillin;
tazobactam;

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INTRODUCTION

Acinetobacter spp. is the second most commonly found cause of sepsis after *P. aeruginosa* and before *Stenotrophomonas maltophilia*. Among *Acinetobacter spp*., the most familiar species to cause sepsis is *Acinetobacter baumannii.*[1] Sepsis itself requires systematic and comprehensive treatment. Before specific bacteria are identified as the cause, therapy of empiric antibiotics will be given to reduce the effect of the infection. However, not only have to identify the bacteria of the cause, but the treatment of sepsis should also consider the type of empiric antibiotics given as it should be specified according to the source of infection. Moreover, the sensitivity of the antibiotics should fit the possible resistance pattern. If those considerations are not taken into account, they will increase the resistance risk of infection-causing bacteria against given antibiotics.[2]

Initial treatment of empiric antibiotics, if given appropriately, will be able to reduce mortality rate, hospitalization duration, and health cost. On the contrary, the inappropriate treatment can cause multi-drugs resistance (MDR) and increase mortality rate.[2] Bacteria resistance cases against antibiotics are often found during single antibiotic treatments. In India, the combination of two antibiotics of cephalosporin and aminoglycoside class is more effective to treat sepsis caused by *Acinetobacter spp*. compared to single antibiotics treatment.[3] In one of the hospital in Indonesia, 25 out of 342 blood specimens or patient's sputum that diagnosed as sepsis showed resistant of 14 antibiotics (>50%) and <50% for 9 antibiotics. The antibiotics that used are penicillin, cephalosporine, carbapenem, quinolone, aminoglycoside, macrolide, glycopeptide, sulfonamide, polymyxin, and antituberculosis.[2]

Single antibiotics treatment using antibiotics of aminoglycoside, amikacin, carbapenem, and β-Lactam class is often chosen to treat sepsis. However, this type of treatment has not been able to reduce the prevalence of death by sepsis especially in children patients, also when MDR take place.[4] Antibiotics in carbapenem class that is commonly used to treat sepsis is meropenem. Meropenem is a specific medicine for infections that caused by bacteria, therefore cannot be used for infections caused by fungi or viruses. Besides antibiotics in carbapenem class, other commonly used antibiotics for infections caused by bacteria are those from penicillin class or combination of penicillin and β-lactamase inhibitor. This type of medicine, for example in the form of piperacillin-tazobactam, is known for its ability to treat the infection from both gram-positive and gram-negative bacteria. However, piperacillin-tazobactam is still reported to cause resistance effect in treatments of heavy infections.[5]

Single-use of tazobactam has lower antibacterial properties than a combination with β-lactam, especially for *Staphylococcus aureus, Haemophilus influenzae, Neisseria gonorrhoeae, Escherichia coli,* and *Acinetobacter spp.* Piperacillin-tazobactam is the most active combination of therapy against infections caused by the bacillus genus, both aerobic and anaerobic, gram-negative bacteria. This combination does not affect the pharmacokinetics system of each other.[6] Ninety percent of *E. coli* and *Enterobacteriaceae* infection are sensitive to piperacillin-tazobactam. It also shows sensitivity up to 90-100% in *Streptococcus* or *Staphylococcus* infections. Although these antibiotics show lower potential than meropenem and imipenem to treat *Enterobacteriaceae* and *Acinetobacter spp.* Infection, its have higher potential for treating *Pseudomonas aeruginosa* infection.[7]

In various researches about

antibiotics combination test, some methods appear more often than the others, such as paper strip test, time kill assay, antibiotic susceptibility test, and checkerboard test. From those methods, paper strip test is characterized as a simpler method with shorter time.[8] In this study, we aimed to evaluate the potential of meropenem and piperacillin-tazobactam combination against *Acinetobacter spp* in *in vitro* by using paper strip test.

MATERIALS AND METHODS

MATERIALS

Apparatus used in this research were sterile cotton swabs, inoculating loops, 5 mL and 15 mL tubes, micropipettes, oxidase test strips, Petri plates, object glass, McFarland standard, ruler, ©Liofilchem® (Italy) test strips containing meropenem and piperacillin-tazobactam, bacteria growth incubator, documenting camera, and *Acinetobacter spp*. clinical isolates. Meanwhile the media were brain hearth infusion (BHI) medium, MacConkey agar medium, and Mueller-Hinton agar medium.

***Acinetobacter spp*.**

These bacteria belong to ubiquitous, free-living Gram negative saprophytic bacilli class. This type of bacteria is commonly found in soil, water, human skin, also home/hospital furniture such as mattresses or rubber coatings. *Acinetobacter spp.* also often find in human skin as its infection medium. *Acinetobacter spp.*, especially *A. baumannii* has a higher resistance to the hospital environment compared to other bacteria. These bacteria can stand a minimum of 10 days in the hospital environment and dry places.[9] Patients with the long-term stay or post-surgery condition, invasive procedure, or lack of maintenance in surrounding objects can trigger *Acinetobacter* infection, such as on curtains, mattresses, or door handles.

Paper strip test

Paper strip test belongs to the antimicrobial susceptibility test (AST), which is a method used to examine the sensitivity rate of an antibiotic against gram-positive and gram-negative bacteria. In doing so, this method uses minimum inhibitory concentration (MIC) value as the indicator with a quantitative evaluation technique.

Sample collection

Acinetobacter spp. and *A. baumannii* samples were obtained from the collection of the Microbiology department, Faculty of Medicine, Public Health, and Nursing, UGM, and stored in glycerol solution at -80^{o}C.

***Acinetobacter spp*. growth process**

Bacteria growth processes were started from 17 samples collection. *Acinetobacter* clinical isolate (isolated by semi-automatic Microbact® method) goes through an oxidase test to confirm that the bacteria is oxidase-negative, proven by the absence of purple color in the result. Using a heated inoculating loop, the clinical isolate is streaked upon MacConkey agar medium and incubated for 20-24 h at 35±2 ^{o}C.

Inoculum preparation

Acinetobacter samples which had growth on MacConkey agar medium were collected using the inoculating loop at separate colonies. It was diluted inside NaCl-contained tube to 10^{-8} CFU/mL. The tube was compared with McFarland standard solution 0.5 and adjusted until the turbidity is visually similar.

Antibiotic sensitivity test

A sterile cotton swab was inserted into the inoculum tube and then streaked on Mueller-Hinton agar medium evenly. After 10-15 sec, meropenem paper strip was picked up with heated anatomic tweezers and placed on the agar

medium. The same action was done with piperacillin-tazobactam paper strip. Mueller-Hinton agar mediums with paper strips on were covered and incubated at 37°C for 18-24 h to obtain the MIC value of each antibiotic.

Antibiotic synergy test using paper strip

Acinetobacter was streaked on another Mueller-Hinton agar medium using a sterile cotton swab after MIC value was obtained. After 10-15 sec, meropenem paper strip and piperacillin-tazobactam paper strip were picked up with heated anatomic tweezers and placed on the agar medium with the intersection point each at MIC 2 µg/mL and 4 µg/mL, forming 90° in the center of Mueller-Hinton agar medium. The medium was covered and incubated at 37°C for 18-24 h. The MIC value of the combined antibiotics was determined from the value of inhibitor zone intersection at the scale of the paper strip. After obtaining the MIC value, fractional inhibitory concentration (FIC) of the combined antibiotics was calculated (FIC_{a+b}).[10] The FIC_{a+b} = FIC_a + $FIC_{b,}$ where FIC_a = (MIC A combine B/MIC A) and FIC_b = (MIC B combine A/MIC B). If, FIC_{a+b} value < 0.5 it was considered as synergistic effect, if FIC_{a+b} value between 0.5 – 1.0 it was considered as additive effect, if FIC_{a+b} value >1.0 – 4.0 it was considered as indifferent effect and if FIC_{a+b} value > 4.0 it was considered as antagonistic effect. FIC value is influenced by antibiotic diffusion level on Mueller-Hinton agar medium, bacteria colony suspension on Mueller-Hinton agar media, sensitiveness of the tested bacteria towards both meropenem and piperacillin-tazobactam, and bacteria growth level.

Data analysis

Obtained data were analyzed with a post-test analysis which was conducted 24 h after paper strips were put on bacteria culture medium. From the observation, MIC value of each antibiotics combination was recorded according to their sensitivity level.

RESULTS

Gram staining and fermentation from the existing supply of clinical isolates were done to identify the bacteria type. After the clinical isolates identified as *Acinetobacter spp.*, the bacteria were re-cultured on MacConkey agar medium. Afterward, the MIC values of single antibiotics previously tested (meropenem and piperacillin-tazobactam) against *Acinetobacter spp.* were determined based on the MIC value standard on performance standards for antimicrobial susceptibility testing-CLSI 2014. The result is showed in TABLE 1.

TABLE 1. MIC value of meropenem and piperacillin-tazobactam monotherapy to *Acinetobacter* (CLSI, 2014)

Antibiotic	Antimicrobial susceptibility		
	Sensitive	Intermediate	Resistant
Meropenem	≤ 2	4	≥ 8
Piperacillin-tazobactam	≤ 4	8 - 16	≥ 32

After the sensitivity test on the combination of meropenem and piperacillin-tazobactam against *Acinetobacter spp.* was conducted, the result can be used to determine the potential of the combination of the antibiotics into the categories of synergistic, additive, indifferent, or antagonistic.

According to the identification table of meropenem and piperacillin-tazobactam combination MIC value against *Acinetobacter spp*. (TABLE 2) there were 12 synergistic bacteria isolates (70.59%) and 5 indifferent bacteria isolates (29.41%). Meropenem and piperacillin-tazobactam combination average MIC value that showed the potential of synergy was 0.13, while the average MIC value of isolates showing the potential of indifference was 2. This result is presented in FIGURE 1 and 2.

TABLE 2. The MIC value of meropenem and piperacillin-tazobactam to *Acinetobacter spp*. (Source: primary data)

Bacterial Code	MIC (Minimum Inhibitory Concentration)				FIC (Fractional Inhibitory Concentration)			Notes
	Monotherapy		Combination					
	Meropenem	Piperacillin-tazobactam	Meropenem	Piperacillin-tazobactam	Meropenem	Piperacillin-tazobactam	Meropenam and Piperacillin-tazobactam	
A1	32	256	32	256	1	1	2	Indifferent
A2	2	4	0.38	0.016	0.19	0.004	0.194	Synergistic
A3	2	4	0.38	0.023	0.19	0.00575	0.19575	Synergistic
A4	32	256	32	256	1	1	2	Indifferent
A5	32	256	32	256	1	1	2	Indifferent
A6	2	4	0.25	0.016	0.125	0.004	0.129	Synergistic
A7	2	4	0.094	0.064	0.047	0.016	0.063	Synergistic
A8	2	4	0.38	0,.016	0.190	0.004	0.194	Synergistic
A9	32	256	32	256	1	1	2	Indifferent
A10	32	256	32	256	1	1	2	Indifferent
A11	2	4	0.25	0.125	0.125	0.03125	0.15625	Synergistic
A12	2	4	0.19	0.016	0.095	0.004	0.099	Synergistic
A13	2	4	0.19	0.016	0.095	0.004	0.099	Synergistic
A14	2	4	0.19	0.016	0.095	0.004	0.099	Synergistic
A15	2	4	0.19	0.016	0.095	0.004	0.099	Synergistic
A16	2	4	0.19	0.016	0.095	0.004	0.099	Synergistic
A17	2	4	0.19	0.125	0.095	0.03125	0.12625	Synergistic

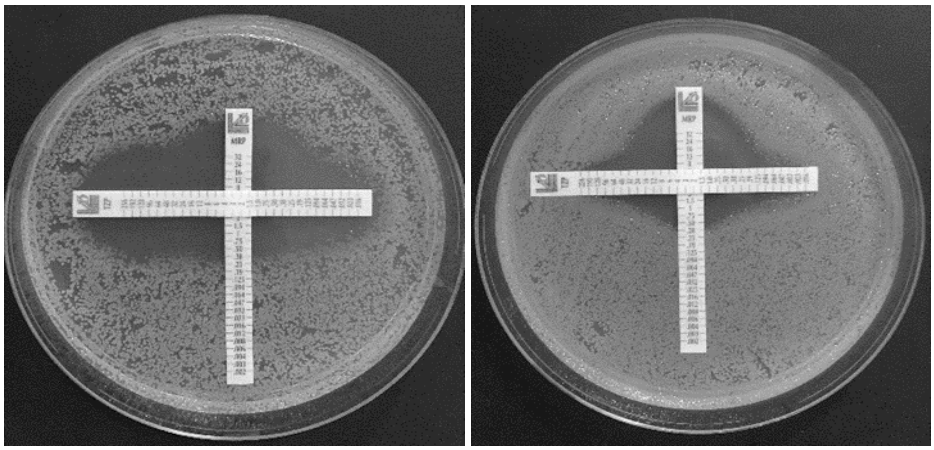

FIGURE. 1. Synergistic MIC result of meropenem and piperacillin-tazobactam on A2 (left) and A3 (right) clinical isolates.

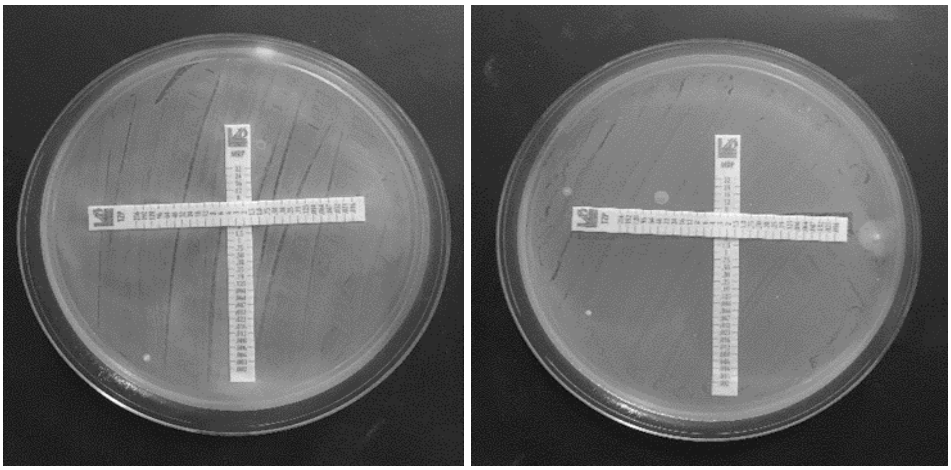

FIGURE. 2. Indifferent MIC result of meropenem and piperacillin-tazobactam on A9 (left) and A10 (right) clinical isolates.

The potential of indifference resulted from 5 clinical samples, 2 of them (40%) were *A. baumannii* (A1 and A9), the other 3 (60%) were *Acinetobacter spp* (A4, A5, and A10). The 5 isolates showed the FIC value of meropenem and piperacillin-tazobactam combination was 2. Based on the 2014 CLSI guideline, the FIC value belonged to the indifferent category. On the other hand, the 12 clinical isolates show the potential of synergy (A2, A3, A6, A7, A8, and A11-A17) identified as *Acinetobacter spp.* (100%). At the 12 isolates, the FIC value of meropenem and piperacillin-tazobactam combination was <0.5 (average value= 0.13). Based on the 2014 CLSI guideline, the FIC value belonged to the synergistic category.

DISCUSSION

The measurement of single antibiotics (meropenem and piperacillin-tazobactam) MIC value against A1 clinical isolate showed resistance on both meropenem and piperacillin-tazobactam. This consistent with study by Harris *et al.*[11] who reported that the use of single antibiotics like meropenem or piperacillin-tazobactam produces in resistance and increases morbidity and mortality by 10-20%.

Resistance to both tests of single antibiotics occurs due to the pathogenic properties of *Acinetobacter spp.*, which results in the β-lactamase excretion, PBP modification, and increase in β-lactam outer membrane permeability. Therefore, a single antibiotics therapy in the form of meropenem or piperacillin-tazobactam will result in resistance when it used to treat infection by *Acinetobacter spp.*

Another characteristic of *Acinetobacter* may also cause resistance against a certain antibiotic by increasing its active efflux, mutating the target area of the therapy, even inactivating the antibiotic.[12,13] Twelve out of 17 clinical bacteria isolates (70.59%) showed synergistic results to the combination of antibiotics, while the remaining five isolates showed indifferent results (29.41%). This also corresponded to research by Viswanathan *et al.*[3] in India, that reported from 50% non-fermenter gram-positive bacteria having MDR characteristics, 30% of them are resistant to carbapenem class antibiotics. Dewi *et al.*[14] reported that the use of meropenem starts to show ineffectiveness or resistance to several sepsis-causing gram-negative bacteria.

Synergistic potential of the antibiotics combination (FIGURE 1) shows evident symbiosis between those antibiotics in increasing bactericidal effect. In the case of meropenem and

piperacillin-tazobactam combination, the symbiosis works by blocking the formation of bacteria wall and restraining the synthesis of β-lactamase, then finally binding itself with the protein of the bacteria, thus preventing the bacteria to bind with its host. The synergistic potential of meropenem and piperacillin-tazobactam combination was shown by the existence of bacterial growth inhibitor zone intersection (MIC value <0.5). On the other hand, indifferent potential (FIGURE 2) does not show any change or benefit from the combination of meropenem and piperacillin-tazobactam. This demonstrated that the effects of using single and combine antibiotic usage are similar. The indifferent potential of meropenem and piperacillin-tazobactam combination was shown by the same MIC value of single antibiotic and combined antibiotic of 32 and 256. After combination, both antibiotic samples did not show any symbiosis, proven by the inexistence of bacterial growth inhibitor zone intersection.

From 5 clinical isolates having the potential of indifference, 2 of them are *A. baumannii*. This bacteria type has higher resistance in various conditions compared to other types of *Acinetobacter*.[1] Therefore, it is assumed that the resistant nature is the cause of indifferent results on meropenem and piperacillin-tazobactam combination test. Moreover, *Acinetobacter*, especially *A. baumannii,* has serine carbapenemase enzyme with higher effect against β-lactam antibiotics, especially carbapenem. The active efflux pump in *A. baumannii* also takes part in the resistance by exerting β-lactam from its cell membrane.

Although both meropenem and piperacillin-tazobactam had resistance possibility in single-use sensitivity test, there were synergistic and indifferent potentials when the two antibiotics were combined. This may due to the interaction of the antibiotics. Meropenem is a bactericidal antibiotic that kills bacteria by breaking down bacterial cell walls, while piperacillin-tazobactam is a bacteriostatic antibiotic that inhibits β-lactamase enzyme exerted by *Acinetobacter*. The bacteriostatic ability of piperacillin-tazobactam may be able to protect meropenem from the β-lactamase enzyme exerted by *Acinetobacter*, therefore creating synergistic potential. However, the interaction between bactericidal and bacteriostatic antibiotics may also cause a rather dominant antagonistic potential since bactericidal antibiotics work by killing bacteria cell and bacteriostatic works by inhibiting bacterial cell growth. Therefore, the occurring effect is indifferent instead of positive.[15]

In this research, the usage of paper strip test was proven to be easy, simple, and did not take a long time or special experts. White *et al*.[8] reported that paper strip test method can be examined by both qualitative and quantitative techniques. Compared to other methods such as time kill assay, antibiotic susceptible test or checkerboard test, paper strip test method is simpler and faster. However, the concentration/suspension of bacteria needed in the test will highly affect the final result. In appropriate of bacteria concentration may result in false results, both positive and negative. Therefore, spectrophotometer is needed to accurately measure the bacteria concentration to conduct the test.

CONCLUSION

The combination of meropenem and piperacillin-tazobactam generates more dominant synergistic potential, compared to the single-use of meropenem only or piperacillin-tazobactam only.

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