BRIEF REPORT

REVISED Growth kinetics of multiple *Acinetobacter baumannii* resistotype after meropenem-based antibiotic combination exposure [version 2; peer review: 2 approved]

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Abstract

Background: Carbapenems are the treatment of choice for multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Acinetobacter baumannii* infections, but the emergence of carbapenem-resistant *A. baumannii* (CRAB) has rendered it ineffective in the vast majority of cases. Combination therapy has grown in popularity over the last decade; this study aims to analyze *A.baumannii* growth kinetics after exposure to meropenem and ampicillin-sulbactam compared with meropenem and amikacin antibiotic combinations in clinically relevant concentrations.
Methods: This experimental laboratory study was conducted on the *A. baumannii* ATCC 19606 isolate and three clinical isolates that were intermediate or resistant to tested antibiotics. Meropenem and ampicillin-sulbactam, as well as meropenem and amikacin, were tested at four different concentrations against isolates. Turbidity measurements were taken at predetermined time points of 0, 1, 2, 4, 6, 8, and 24 hours following exposure; bacterial concentration was enumerated using the agar plate method, with the results plotted in a time-kill curve.
Results: A bactericidal effect was achieved in isolates that were intermediate to ampicillin-sulbactam and resistant to meropenem after the administration of meropenem and ampicillin-sulbactam combination with a concentration of 4 µg/ml and 16/8 µg/ml, respectively. The combination of meropenem and ampicillin-sulbactam demonstrated bacteriostatic activity against isolates that were resistant to both antibiotics. Isolates treated with resistant antibiotics showed an increased growth rate compared to the growth control.
Conclusion: The combination of meropenem and ampicillin-sulbactam could be a promising combination therapy in treating CRAB infections. The mechanism and degree of antibiotic resistance in the

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Any reports and responses or comments on the article can be found at the end of the article.

isolates affect the efficacy of antibiotic combinations; further research is needed to corroborate the findings of this study.

Keywords
Acinetobacter baumannii, antibiotic combinations, time-kill, meropenem, ampicillin-sulbactam, amikacin

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REVISED **Amendments from Version 1**

In accordance with the reviewer's recommendations, we made several adjustments. The Figure and Table have been altered to convey the content better. On the underlying data in FigShare, tables have been newly constructed. The writing of the manuscript has undergone a few minor adjustments.

Any further responses from the reviewers can be found at the end of the article

Introduction

Acinetobacter baumannii is a Gram-negative rod that garners attention due to its role as a primary pathogen in healthcare-associated infections with a broad spectrum of antibiotic resistance[1,2]. Carbapenems are the preferred treatment for multidrug-resistant (MDR) *A. baumannii* infections. However, treatment options have dwindled due to high isolation rates of extensively drug-resistant (XDR) *A. baumannii* with concurrent carbapenem resistance[3,4].

The discovery of new antibiotics is critical for treating MDR and XDR *A. baumannii* infections. Nevertheless, antibiotic studies take a long time to complete and are difficult to implement in developing countries with limited access to the latest antibiotics. The alternative strategy that has gathered the most interest is antibiotic combination therapy, which is theoretically supposed to boost antibiotic effectiveness compared to single antibiotics[5–7].

In studies evaluating antibiotic combinations, isolates that are susceptible to at least one of the regimens are frequently used, whereas many *A. baumannii* clinical isolates frequently lack susceptibility to any antibiotic[5,8]. Additionally, because the antibiotic concentrations used in studies are typically multiple times of minimum inhibitory concentration (MIC) and are difficult to achieve during the administration of therapeutic antibiotic doses, the clinical application of study results is complicated[5,9–11].

Meropenem is one of the few remaining low-toxicity treatment options for MDR and XDR *A. baumannii* infections[12,13]. Sulbactam is a beta-lactamase inhibitor with intrinsic activity against *A. baumannii*, whilst amikacin is an aminoglycoside with relatively maintained efficacy against multidrug-resistant Gram-negative bacteria, including *A. baumannii*[14–17]. Ampicillin-sulbactam and amikacin are two antibiotics that are available and easy to obtain in Indonesia. A sole sulbactam regimen is not available; it is marketed in conjunction with ampicillin or cefoperazone. Ampicillin-sulbactam formulations were chosen because of the availability of breakpoints in CLSI M100 2022 and technical considerations such as affordability and convenience of access to the antibiotics.

Numerous *in vitro* studies have demonstrated synergy between meropenem and ampicillin-sulbactam as well as meropenem and amikacin; thus, this study aimed to compare the growth kinetics of various *A. baumannii* strains exposed to these two antibiotic combinations at clinically relevant concentrations[18–23].

Methods

Study design

Experiments were conducted on two MDR, one XDR clinical isolates from Clinical Microbiology Laboratorium Dr. Soetomo General Academic Hospital, and one standard reference isolate (ATCC *A. baumannii* 19606 KWIK-STIK™ Microbiologics). All clinical isolates are meropenem resistant, conforming to the Clinical and Laboratory Standard Institute (CLSI) 2022 breakpoint for *A. baumannii* (MIC >8 µg/ml as determined by an automatic susceptibility test using BD Phoenix® ID/AST instrument). MDR-1 is resistant to meropenem and amikacin (MIC >32 µg/ml) but is intermediate to ampicillin-sulbactam (MIC 16/8 µg/ml); MDR-2 is resistant to meropenem and ampicillin-sulbactam (MIC >16/8 µg/ml) but is intermediate to amikacin (MIC 32 µg/ml). XDR exhibited resistance to all antibiotics tested.

Ethical considerations

This study was reviewed by the Ethics Committee of the Faculty of Medicine, Airlangga University (0758/LOE/301.4.2/I/2022).

Procedure

Drug concentrations were selected based on the CLSI breakpoint value for the susceptible category of tested antibiotics as it represents clinically achievable concentrations of drugs in human plasma following standard dosing. Fresh stocks of each antibacterial were prepared on the day of the experiment to achieve 0.5 MIC + 0.5 MIC, 1 MIC + 1 MIC, 2 MIC + 2 MIC, and 2 MIC + 0.5 MIC of meropenem + ampicillin-sulbactam and meropenem + amikacin (Sigma). Prior to the time-kill assay experiment, strains were subcultured onto blood agar (Oxoid CM0055 Blood Agar Base supplemented with 5% sheep blood) and incubated for 24 hours at 35°C. Mid-log phase growth suspension was obtained by inoculating isolated colony into cation-adjusted Mueller-Hinton broth (Oxoid CM0405 Mueller-Hinton Broth base) followed by 4 hours of incubation at 35°C. Static time-kill experiments were performed in sextuplicates on separate days at an initial inoculum of 6×10^5 CFU/ml with the combined antibiotic concentrations in the glass tube, incubated at 35°C. Samples were collected at 0, 1, 2, 4, 6, 8, and 24 h, measured for turbidity by nephelometer (BD PhoenixSpec™ Nephelometer), serially diluted in saline, plated on Mueller-Hinton agar (Oxoid CM 0337 Muelle-Hinton Agar base), and counted after 24 h of incubation for viable-cell counting. Enumeration was performed manually after 24 hours of incubation at 35°C. The limit of detection (LOD) was 10^2 CFU/ml. In the meantime, a control experiment was carried out simultaneously with the same procedure without antibiotic addition. Bactericidal activity was assessed as a ≥ 3 $\log_{10}$ reduction in a colony-forming unit (CFU)/mL over the period measured. Regrowth was defined as an initial decrease of turbidity or colony count followed by an escalation in the subsequent measurement hour.

Results

The turbidity and colony count data did not follow a normal distribution (Shapiro-Wilk value 0.000). There were significant differences in mean turbidity between isolates of ATCC 19606, MDR-1, MDR-2, and XDR at 2, 4, 6, 8, and 24 hours

following antibiotic exposure ($p<0.05$; Wilcoxon; CI 95%). There were significant differences in the mean colony count between isolates of ATCC 19606, MDR-1, MDR-2, and XDR at 6, 8, and 24 hours following exposure, ($p = 0.001$, $p = 0.01$, and $p = 0.000$; Wilcoxon; CI 95%). The full turbidity and colony count data can be found under *Underlying Data*[24].

Exposures to meropenem and ampicillin-sulbactam yield encouraging results. In the MDR-1 isolate, which was resistant to carbapenem and intermediate to ampicillin-sulbactam, the bactericidal effect of meropenem and ampicillin-sulbactam was achieved at a 2 MIC + 2 MIC concentration, respectively (Figure 1). During 0–24 hours, concentrations of

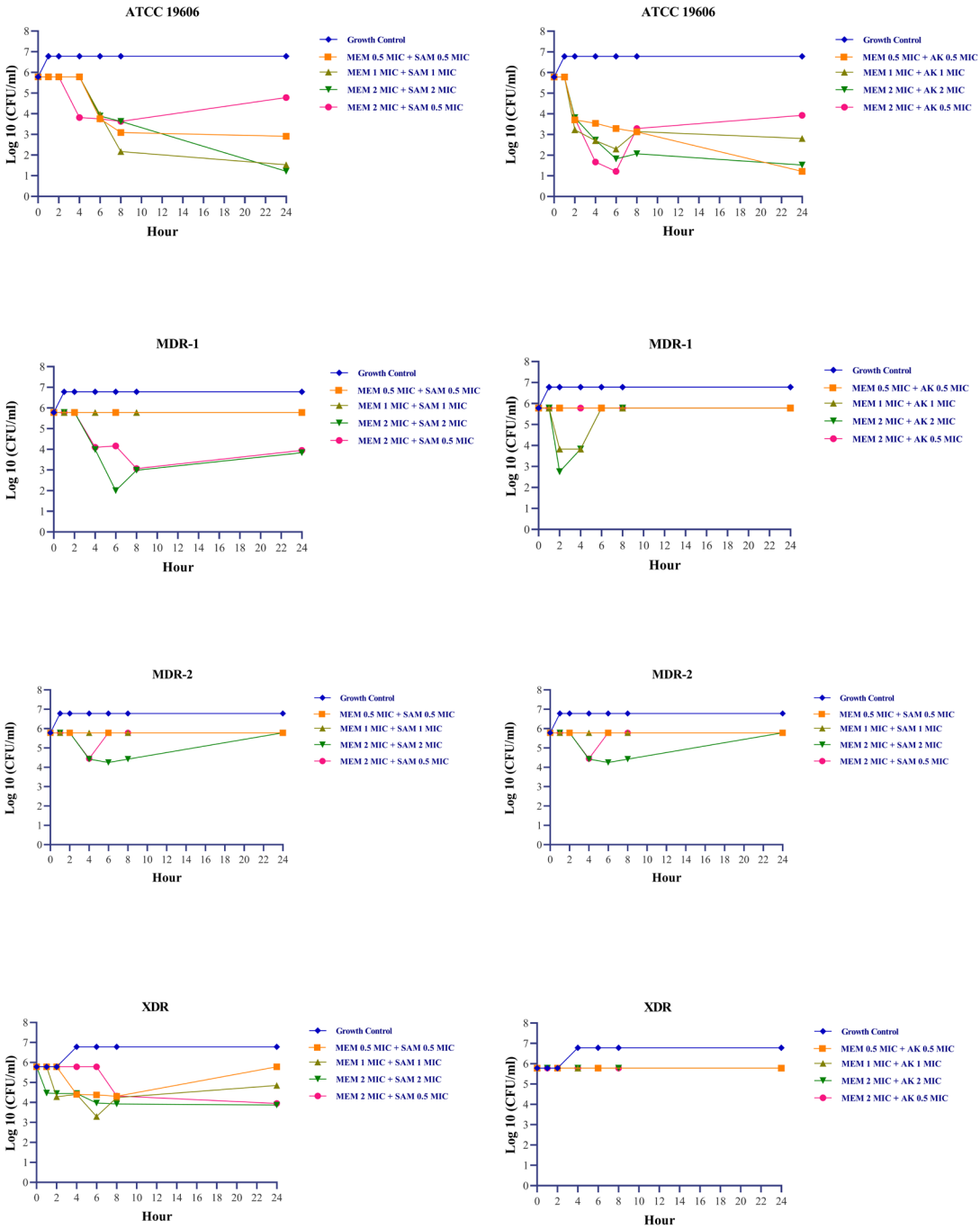

Figure 1. Colony count fluctuations of *Acinetobacter baumannii* following exposure with meropenem + ampicillin sulbactam and meropenem + amikacin combination[24]. MEM: *meropenem,* SAM: *ampicillin-sulbactam,* AK: *amikacin,* MIC: *minimum inhibitory concentration. Acinetobacter baumannii*'s MIC based on CLSI 2022 susceptible breakpoint: Meropenem 2 µg/ml, Ampicillin-Sulbactam 8/4 µg/ml, Amikacin 16 µg/ml.

0.5 MIC + 0.5 MIC, 1 MIC + 1 MIC, and 2 MIC + 0.5 MIC were able to sustain growth under the rate of growth control, as demonstrated by turbidity measurements. However, the turbidity was approximately indistinguishable at 48 hours (Figure 2). Changes in the number of colonies could not be observed at 0.5 MIC + 0.5 MIC and 1 MIC + 1 MIC concentration due to high colony count results. Exposure to a 2 MIC + 0.5 MIC concentration caused a transient inhibitory effect for up to 4 hours, but regrowth occurred at the hour of measurement thenceforth.

MDR-2 isolate (isolate resistant to meropenem and ampicillin-sulbactam) treated with meropenem and ampicillin-sulbactam

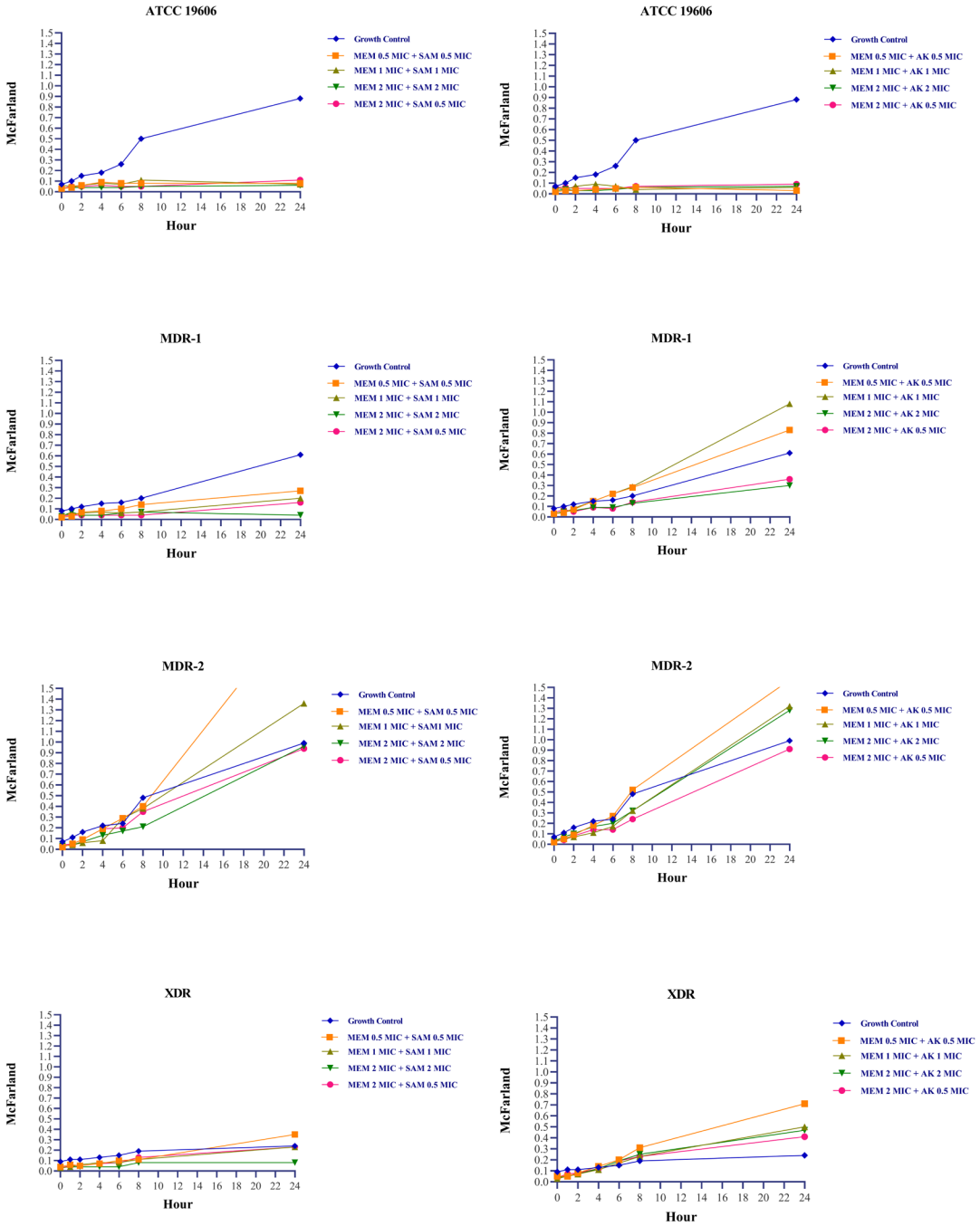

Figure 2. Turbidity fluctuations in *Acinetobacter baumannii* suspension following exposure with meropenem + ampicillin-sulbactam and meropenem + amikacin combination[24]. MEM: *meropenem,* SAM: *ampicillin-sulbactam,* AK: *amikacin,* MIC: *minimum inhibitory concentration. Acinetobacter baumannii*'s MIC based on CLSI 2022 susceptible breakpoint: Meropenem 2 µg/ml, Ampicillin-Sulbactam 8/4 µg/ml, Amikacin 16 µg/ml.

combination at concentration equal to or less than the MIC demonstrated higher turbidity compared to positive growth control after 24 and 48 hours. At a concentration twice the MIC, there is a reduction in colony count after four hours, followed by regrowth. During post-exposure monitoring, XDR isolate exposed to meropenem and ampicillin-sulbactam did not show any signs of regrowth, except at a concentration of 1 MIC + 1 MIC, where regrowth occurred at 8 and 24 hours (Table 1).

Meropenem and amikacin had no bactericidal impact on intermediate and drug-resistant isolates; hence on all clinical

Table 1. Antibiotic combination activity against *Acinetobacter baumannii* isolates.

Isolate	Antibiotic	Concentration[a]	Activity[b]	Δ Log 10[c]	*Regrowth*[d]	Turbidity measurement higher than growth control[e]
ATCC 19606	MEM + SAM	½ MIC + ½ MIC	Bacteriostatic	2.87	No	No
		1 MIC + 1 MIC	Bactericidal	4.25	No	No
		2 MIC + 2 MIC	Bactericidal	4.56	No	No
		2 MIC + ½ MIC	Bacteriostatic	2.15	Yes	No
	MEM + AK	½ MIC + ½ MIC	Bactericidal	4.56	Yes	No
		1 MIC + 1 MIC	Bactericidal	3.48	Yes	No
		2 MIC + 2 MIC	Bactericidal	4.26	Yes	No
		2 MIC + ½ MIC	Bactericidal	4.56	Yes	No
MDR 1	MEM + SAM	½ MIC + ½ MIC	Bacteriostatic	0	Yes	No
		1 MIC + 1 MIC	Bacteriostatic	0	Yes	No
		2 MIC + 2 MIC	Bactericidal	3.78	Yes	No
		2 MIC + ½ MIC	Bacteriostatic	2.71	Yes	No
	MEM + AK	½ MIC + ½ MIC	Bacteriostatic	0	Yes	Yes, since hour 6 after exposure
		1 MIC + 1 MIC	Bacteriostatic	1.95	Yes	Yes, since hour 6 after exposure
		2 MIC + 2 MIC	Bacteriostatic	2.83	Yes	No
		2 MIC + ½ MIC	Bacteriostatic	0	Yes	No
MDR 2	MEM + SAM	½ MIC + ½ MIC	Bacteriostatic	0	Yes	Yes, at hour 24 after exposure
		1 MIC + 1 MIC	Bacteriostatic	0	Yes	Yes, at hour 24 after exposure
		2 MIC + 2 MIC	Bacteriostatic	1.53	Yes	No
		2 MIC + ½ MIC	Bacteriostatic	1.33	Yes	No
	MEM + AK	½ MIC + ½ MIC	Bacteriostatic	0	Yes	Yes, since hour 8 after exposure
		1 MIC + 1 MIC	Bacteriostatic	0	Yes	Yes, at hour 24 after exposure
		2 MIC + 2 MIC	Bacteriostatic	1.79	Yes	Yes, at hour 24 after exposure
		2 MIC + ½ MIC	Bacteriostatic	0	Yes	No
XDR	MEM + SAM	½ MIC + ½ MIC	Bacteriostatic	1.47	No	Yes, at hour 24 after exposure
		1 MIC + 1 MIC	Bacteriostatic	2.48	Yes	No
		2 MIC + 2 MIC	Bacteriostatic	1.91	No	No
		2 MIC + ½ MIC	Bacteriostatic	1.83	No	No
	MEM + AK	½ MIC + ½ MIC	Bacteriostatic	0	Yes	Yes, since hour 6 after exposure
		1 MIC + 1 MIC	Bacteriostatic	0	Yes	Yes, since hour 6 after exposure
		2 MIC + 2 MIC	Bacteriostatic	0	Yes	Yes, since hour 6 after exposure
		2 MIC + ½ MIC	Bacteriostatic	0	Yes	Yes, since hour 6 after exposure

ATCC: *American Type Culture Collection,* MDR: *multidrug-resistant,* XDR: *extensively drug-resistant*, MEM: *meropenem*, SAM: *ampicillin-sulbactam,* AK: *amikacin,* MIC: *minimum inhibitory concentration*

[a]: Meropenem MIC = 2 μg/ml; Ampicillin-Sulbactam MIC: 8/4 μg/ml; Amikacin MIC: 16 μg/ml

[b]: Bactericidal: ≥ 3 $\log_{10}$ reduction in a colony-forming unit (CFU)/ml over the period measured. Bacteriostatic: < 3 $\log_{10}$ reduction in a colony-forming unit (CFU)/mL over the period measured (compared to initial measurement of tested isolate)

[c]: **Δ Log 10: Log 10 of the total colony-forming unit (CFU/ml) reduction over the measurement time** (compared to initial measurement of tested isolate)

[d]: Regrowth: initial decrease of turbidity or colony count followed by an escalation in the subsequent measurement hour

[e]: Comparison of the colony count between the treatment group and growth control group of isolate. Growth control: isolate without antibiotic combination exposure

isolates of *A. baumannii* in this study. The most significant reduction in the number of bacteria was observed following exposure to 2 MIC and 2 MIC; however, these concentrations had no effect on the number of colonies in XDR isolates when compared to the number of colonies at 0 hours measurement.

Discussion

This investigation discovered regrowth in clinical isolates from nearly all exposure groups. Regrowth is influenced by various factors related to the concentration of antibiotics and bacterial inoculum, as well as the susceptibility of bacteria[25]. Regrowth may occur when bacterial growth is not fully inhibited by exposure to antibiotics (due to insufficient antibiotic concentration or a resistant bacterial strain)[26]. Persistent/resistant bacterial subpopulations can also be inferred from time-kill curve regrowth[27–29]. Antibiotic degradation in the test suspension also plays a role; decreased active antibiotic amount during the final hours of testing may render inhibition ineffective, allowing regrowth to occur[30].

Meropenem and ampicillin-sulbactam are time-dependent beta-lactam antibiotics[31]. The synergism may be due to the distinct penicillin-binding proteins (PBP) binding mechanisms, hence enhancing the activity of beta-lactams in bacteria[32]. Meropenem has a high affinity for PBP 2, PBP 3, PBP 1a, and PBP 1b, ampicillin has a high affinity for PBP 4, and sulbactam has a high affinity for PBP 1 and PBP 3[33,34]. The downregulation of native and subsequent synthesis of altered PBPs is one of the mechanism behind *A. baumannii's* resistance to beta-lactam antibiotics[35–37]. In addition to its simultaneous action on PBP, sulbactam's beta-lactam inhibitory activity can boost meropenem's affinity and, consequently, activity[38,39]. Numerous investigations have demonstrated that subinhibitory concentrations of beta-lactam antibiotics can alter the shape of bacteria's cell walls[40]. In theory, it has the potential to augment the intake of other antibiotics[41].

Meropenem in combination with ampicillin-sulbactam at a concentration twice the MIC was bactericidal against isolates intermediate to ampicillin-sulbactam. Moreover, it had a lower rate of regrowth than the meropenem and amikacin exposure groups. Differences in resistance levels are believed to have an effect on the efficiency of antibiotic combinations[42–44]. It should be anticipated that the distinct resistance mechanisms held by various strains resulted in different responses to combination antibiotic exposure[20,45,46].

Additionally, this study found that isolates treated at sub-MIC concentrations of antibiotics had a higher colony count than the growth control group. This finding merits additional investigation to ascertain the underlying mechanism. Antibiotics have a selection and inducer effect on antibiotic resistance, which demonstrates the importance of using them prudently.

Conclusions

Meropenem in combination with ampicillin-sulbactam at a concentration twice the MIC was bactericidal against isolates resistant to meropenem and intermediate to ampicillin-sulbactam. Meropenem and ampicillin-sulbactam in combination demonstrated bacteriostatic activity against isolates resistant to both antibiotics. Meropenem and amikacin in combination had no bactericidal effect on isolates that were either intermediate or resistant to meropenem and amikacin. Combined administration of meropenem and ampicillin-sulbactam can be considered in cases of *A. baumannii* infection that is not susceptible to any antibiotics. Higher doses show better results and should be attempted when clinical circumstances allow.

Data availability

Underlying data

Figshare: Colony Count and Turbidity Data from Time-Kill Assay of Acinetobacter baumannii exposed to Meropenem-based Antibiotic Combinations. https://doi.org/10.6084/m9.figshare.20024270.v3[24].

This project contains the following underlying data:

- Colony Count Data.csv
- Turbidity Data.csv

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Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 05 December 2022

https://doi.org/10.5256/f1000research.140132.r156794

✔ **Sarunyou Chusri**
Division of Infectious Diseases, Prince of Songkla University, Songkhla, Thailand
Arnon Chukamnerd
Faculty of Medicine, Prince of Songkla Univiversity, Hat Yai, Thailand

The author responded to all comments.
No further requirement is needed.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbiology and epidemiology

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 07 October 2022

https://doi.org/10.5256/f1000research.134184.r149383

✔ **Ardiana Kusumaningrum**
Department of Microbiology, Faculty of Medicine, Clinical Microbiology Medicine Staff Group, Universitas Indonesia Hospital, Universitas Indonesia, Depok, Indonesia

In general, this article provides important in vitro data regarding the potential use of antibiotics combination in *Acinetobacter baumannii* infection management. The research method used is appropriate.

However, there are several issues that need to be considered:

1. Is there any preliminary examination to ensure that the antibiotic concentration used is as expected, at the beginning and also the end of observation time?

2. How did the author confirm that the bacterial isolates being tested were in exponential growth period/log phase?

3. Is it possible to conduct duplo testing to increase the strength of study method?

4. Figure 1 --> is there any missing line on the picture? For example, at XDR colony count fluctuations, there are only 2 lines observed (growth control and MEM 0,5MIC + AK 0,5MIC)

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: antimicrobial resistance, MDRO

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 21 Oct 2022

Erizka Rivani, Sriwijaya University, Palembang, Indonesia

Dear Dr. Ardiana,

My co-authors and I were pleased to receive your response and the opportunity to resubmit a revised version of this manuscript. We attempt to respond to reviewer questions with relevant information obtained from our research.

1. Is there any preliminary examination to ensure that the antibiotic concentration used is as expected at the beginning and end of observation time?
- Thank you for the question.
- There were two preliminary trials conducted before this study. The first preliminary trial was carried out to determine the appropriate colony measurement method for the test isolates exposed to the selected antibiotics for this study. The second preliminary study was carried out to determine the time required by the test isolates to reach the log phase of growth.
- We did not conduct a preliminary trial to ascertain the concentration of the test antibiotic because antibiotic exposure was performed for 24 hours (additional measurements were taken at 48 hours to collect post-antibiotic exposure data), which is comparable to the duration of antibiotic susceptibility tests conducted in clinical microbiology laboratory with antibiotic powders that were subjected to routine quality control.

2. How did the author confirm that the bacterial isolates tested were in the exponential growth period/log phase?
- Thank you for drawing attention to this.
- In the preliminary test, isolates were grown without antibiotic treatment in liquid media. This test is designed to determine the time required for the test isolate to reach the log phase under identical conditions to the actual test. After transferring isolated colonies from solid to liquid media, turbidity measurements and colony growth calculations were undertaken every 30 minutes. The data obtained was therefore plotted on a growth curve. According to the preliminary test results, all isolates entered the log phase after two hours of incubation, and six hours later, they began to reach the stationary phase. Therefore, in the actual experiment, isolated colonies were cultured in liquid media for four hours prior to the time-kill test (at the mid-log phase).

3. Is it possible to conduct duplo testing to increase the strength of study method?
- Thank you for the comment.
- The tests were carried out six times over the course of two days. On the first day of the trial, three replications were performed. On the second day, three additional replications were conducted, bringing the total number of replications to six. This experiment was repeated six times with four test isolates treated with two types of combination antibiotics at four different concentrations on each combination.

4. Figure 1 --> is there any missing line on the picture? For example, at XDR colony count fluctuations, there are only 2 lines observed (growth control and MEM 0,5MIC + AK 0,5MIC)
- We thank you for bringing this to our attention.
- Several lines in the figure are joined because they have the same value, so the end

result gives the impression that some figures are missing lines (there are just two lines instead of five). We shall attempt to revise the graphic to ensure its meaning is more evident.

Competing Interests: N/A

Reviewer Report 19 August 2022

https://doi.org/10.5256/f1000research.134184.r147379

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Sarunyou Chusri
Division of Infectious Diseases, Prince of Songkla University, Songkhla, Thailand

The brief report by Erizka et al. demonstrated the bacteriostatic and bactericidal activities of meropenem in combination with ampicillin-sulbactam and amikacin. The finding showed that the meropenem + ampicillin-sulbactam provided the good activity against these isolates, which might be considered as an alternative treatment for *A. baumannii* infection, especially MDR and XDR strains. However, I have some comments to be addressed.

Major comments

1. Page 6, in the results section: "XDR isolates which were also resistant to meropenem and ampicillin-sulbactam, did not show any regrowth phenomena during post-exposure monitoring except at a concentration of 1 MIC + 1 MIC, where regrowth occurred at 8 and 24 hours" – please verify these results again. I found that regrowth occurred not only at a concentration of 1 MIC + 1 MIC but also at a concentration of 0.5 MIC + 0.5 MIC and other concentrations. In addition, only 1 isolate of XDR was included in the study. Why did the authors write "XDR isolates" in this sentence?

2. Page 6, in the results section: "Meropenem and amikacin had no bactericidal impact on intermediate and drug-resistant isolates; hence on all clinical isolates of *A. baumannii* in this study." – according to Figure 1 and Table 1, not all concentrations of MEM + AK have no bactericidal effect on the clinical isolates. At twice MIC of this combination against MDR-1, it seems to provide ≥ 3 $\log_{10}$ reductions, which was considered to have bactericidal activity. Please rephrase this sentence.

3. Please carefully verify the combination activity (bactericidal and bacteriostatic) in Table 1, because there are some incorrect results.
    - The authors reported the bacteriostatic activity in the MEM 0.5 MIC + SAM 0.5 MIC against ATCC 19606 isolate, the MEM 2 MIC + SAM 0.5 MIC against MDR-1 isolate, the MEM 2 MIC + AK 2 MIC against MDR-1, and the MEM 1 MIC + SAM 1 MIC against XDR,

whereas it seems to provide ≥ 3 $\log_{10}$ reductions (bactericidal), compared to the growth control (Figure 1).

- "Colony count higher than growth control[d]" – did the authors mean "Turbidity higher than growth control[d]"? Because the results seem to be received from Figure 2. If yes, please change the title and the description of this column.

4. The authors should recreate the supplementary tables (Colony Count Data and Turbidity Data) in data availability (underlying data). The information should be clearly and easily understood.

Minor comments

1. Page 1, in the abstract section, in a part of the results: "... that were intermediate to ampicillin sulbactam and ..." – a hyphen between ampicillin sulbactam was missed.

2. Page 2, in the keywords section: "Acinetobacter baumannii" should be italic.

3. Page 3, in the methods section, in a part of the study design: "... ATCC A.baumannii 19606 ..." – a space between genus and species was missed.

4. Page 3, in the methods section, in a part of the study design: "... (CLSI) 2022 breakpoint for A.baumannii ..." – again, a space between genus and species was missed, and please check this point throughout the manuscript.

5. Page 3, in the methods section, in a part of the procedure: "... in a colony-forming unit (CFU)/mL over ..." – "mL" should be replaced by "ml" as same as the other part of the manuscript.

6. Page 4, in the results section: "In MDR 1 isolates, sc. MDR isolates that ..." – what does the "sc." mean?

7. Page 4, in the results section: "In MDR 1 isolates, sc. MDR isolates that were both carbapenem-resistant and intermediate to ampicillin-sulbactam" – please rephrase this sentence.

8. Page 5, in the results section: "At 24 and 48 hours, MDR 2 isolates (isolates resistant to meropenem and ampicillin-sulbactam) ..." – did the authors mean the isolate that was coded as "MDR 2"? If yes, it might be better to change the code of "MDR 1" and "MDR 2" isolates. Because these codes make confusion between the code and the number of the isolates. As shown in this case, it also means 2 isolates of MDR bacteria. The authors could use other codes such as "MDR-1" and "MDR-2".

9. Pages 6 - 7, in the results section: "... these concentrations had no effect on the number of colonies in XDR isolates when compared to the number of colonies at 0 hours measurement." – according to Figure 1, the authors should also specify the type of antibiotic combination, because this phenomenon was only found in XDR against MEM + AK combination, but not XDR against MEM + SAM.

10. Page 7, in the discussion section: "... sulbactam has a high affinity for PBP 1 and 3" could be replaced by "... sulbactam has a high affinity for PBP 1 and PBP 3".

11. Page 7, in the discussion section: “Numerous investigations have demonstrated that subinhibitory concentrations of beta-lactam antibiotics can alter the shape of bacteria’s cell walls” – please provide some references for this sentence.

12. Figures 1 and 2: the color and the shape represented the results of “Growth Control” were similar to the results of “2 MIC + 0.5 MIC”. In the case of turbidity fluctuations in the MEM + AK combination against XDR isolate (Figure 2), the color and the shape represented the results of “Growth Control” were exactly the same as the results of “2 MIC + 0.5 MIC”. The authors probably use another color and shape (such as the star shape) to represent the results of “Growth Control”.

13. How many replications did the authors performed for time-kill assay?

14. According to the description in Figure 1, Figure 2, and Table 1, why did the MICs not represent the exact MICs, but it represents the MIC at the susceptible breakpoint from CLSI guideline?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbiology and epidemiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 21 Oct 2022

Erizka Rivani, Sriwijaya University, Palembang, Indonesia

Dear dr. Chusri,

My co-authors and I were pleased to receive your response and the opportunity to resubmit a revised version of this manuscript. We would like to thank you for providing your constructive and detailed review comments on our manuscript.
We have attempted to fully address comments in the revised manuscript; the reviewer's original comments are listed below, followed by our response to each comment. Edited text in the attached revised manuscript is visible as tracked changes under the markup mode of Microsoft Word that we've sent to Editor.
All authors have read and approved the revised manuscript. We hope our resubmission is now suitable for acceptance, and we look forward to hearing from you.

Major Revision
1. Page 6, Result
"XDR isolates which were also resistant to meropenem and ampicillin-sulbactam, did not show any regrowth phenomena during post-exposure monitoring except at a concentration of 1 MIC + 1 MIC, where regrowth occurred at 8 and 24 hours"
Please verify these results again. I found that regrowth occurred not only at a concentration of 1 MIC + 1 MIC but also at a concentration of 0.5 MIC + 0.5 MIC and other concentrations.

- Thank you for this observation. The second and third paragraphs of the Results section describe the measurement of the first antibiotic combination exposures, meropenem and ampicillin-sulbactam. The fourth paragraph discussed the results of meropenem and amikacin exposure. In XDR isolates exposed to meropenem and ampicillin-sulbactam, regrowth only occurred at a concentration of 1 MIC + 1 MIC. In XDR isolates exposed to meropenem and amikacin, regrowth did occur in all concentration groups.To clarify this conclusion, we attempt to rearrange the sentences.

Only 1 isolate of XDR was included in the study. Why did the authors write "XDR isolates" in this sentence?

- Thank you very much for the reminder. We revised the sentence accordingly.

2. Page 6, Result

"Meropenem and amikacin had no bactericidal impact on intermediate and drug-resistant isolates; hence on all clinical isolates of A. baumannii in this study."
According to Figure 1 and Table 1, not all concentrations of MEM + AK have no bactericidal effect on the clinical isolates. At twice MIC of this combination against MDR-1, it seems to provide $\geq 3 \log_{10}$ reductions, which was considered to have bactericidal activity. Please rephrase this sentence.

- Thank you for pointing this out. According to the colony count of the MDR-1 isolate (attached in the Underlying Data), the colony count decreased by 2.83 log 10 CFU/ml following exposure to 2 MIC + 2 MIC concentrations of meropenem and amikacin (from 5.78 log 10 CFU/ml to 2.95 log 10 CFU/ml). Because the reduction in colony counts did not surpass 3 log 10 CFU/ml over the period measured, we categorized the activity as bacteriostatic. We will attempt to add column in table with Δ Log information to ensure that the findings are more easily discernible.

3. Table 1
"Bacteriostatic activity in the MEM 0.5 MIC + SAM 0.5 MIC against ATCC 19606 isolate, the MEM 2 MIC + SAM 0.5 MIC against MDR-1 isolate, the MEM 2 MIC + AK 2 MIC against MDR-1, and the MEM 1 MIC + SAM 1 MIC against XDR"
Please carefully verify the combination activity (bactericidal and bacteriostatic) in Table 1, because there are some incorrect results. it seems to provide ≥ 3 log10 reductions (bactericidal), compared to the growth control.

- Thank you for the comment. As stated in Point 2, the reduction in colony count for the isolates mentioned did not reach 3 log 10 CFU/ml; therefore, it was classified as bacteriostatic (MEM 0.5 MIC + SAM 0.5 MIC against ATCC 19606 isolate: 2.87 log 10 CFU/ml, MEM 2 MIC + SAM 0.5 MIC against MDR-1 isolate: 2.71 log 10 CFU/ml, MEM 2 MIC + AK 2 MIC against MDR-1: 2.83 log 10 CFU/ml, MEM 1 MIC + SAM 1 MIC against XDR 2.48 log 10 CFU/ml). As it explains the growth of the isolates when exposed to antibiotics, the decrease in colony count of the isolates was compared to the time-to-time colony count of the isolates rather than to the growth control. We will attempt to add column in table with Δ Log information to ensure that the findings are more easily discernible.

Title and description of Table 1

- Thank you for the helpful reminder. We have made the necessary adjustments.

4. Data availability (Underlying Data)
Recreate the supplementary tables (Colony Count Data and Turbidity Data)

- Revised accordingly.

Minor Revision
1. Page 1, Abstract
Results: "..... that were intermediate to ampicillin sulbactam and ...". A hyphen between ampicillin sulbactam was missed.

- Revised accordingly.

2. Page 2, Keywords
"Acinetobacter baumannii" should be Italic

- We appreciate your pointing this out. Updated as required.

3. Page 3, Methods
"... ATCC A.baumannii 19606 ..." "... (CLSI) 2022 breakpoint for A.baumannii ...". A space between genus and species was missed.

- Thank you. Revised accordingly.

4. Page 3, Methods
"... in a colony-forming unit (CFU)/mL over ...". "mL" should be replaced by "ml" as same as the other part of the manuscript.

- We concur.

5. Page 4, Results
"In MDR 1 isolates, sc. MDR isolates that ...". What does the "sc" means?

- Thank you for your inquiry. The word "sc" in the sentence above is an abbreviation of *scilicet*, a contraction of Latin *scire licet*, meaning "it is permitted to know." Sc.

introduces additional information regarding something stated earlier, often in the form of a list to remove an ambiguity or supply a word omitted in the preceding text. We shall attempt to rephrase the sentence such that the meaning is more clearly apparent.

6. Page 4, Results
"In MDR 1 isolates, sc. MDR isolates that were both carbapenem-resistant and intermediate to ampicillin-sulbactam". Rephrase the sentence.
- We concur.

7. Page 5, Results
"At 24 and 48 hours, MDR 2 isolates (isolates resistant to meropenem and ampicillin-sulbactam) …". Use other codes such as "MDR-1" and "MDR-2"
- Thank you. Revised accordingly.

8. Page 6-7, Results
"… these concentrations had no effect on the number of colonies in XDR isolates when compared to the number of colonies at 0 hours measurement." According to Figure 1, the authors should also specify the type of antibiotic combination, because this phenomenon was only found in XDR against MEM + AK combination, but not XDR against MEM + SAM.
- We thank you for bringing this to our attention. As stated in Point 1.a, attempts are made to arrange the sentences.

9. Page 7, Discussion
"… sulbactam has a high affinity for PBP 1 and 3" . Replaced with "… sulbactam has a high affinity for PBP 1 and PBP 3".
- Revised accordingly.

10. Page 7, Discussion
"Numerous investigations have demonstrated that subinhibitory concentrations of beta-lactam antibiotics can alter the shape of bacteria's cell walls". Provide some references for this sentence.
- We have made adjustments in accordance with the revision.

11. Page 4 and 5, Figure 1 and Figure 2
Use another colour and shape to represent the results of "Growth Control"
- We have made adjustments in accordance with the revision.

12. How many replications did the authors performed for time-kill assay?
- Experiments were conducted in six replications.

13. According to the description in Figure 1, Figure 2, and Table 1, why did the MICs not represent the exact MICs, but it represents the MIC at the susceptible breakpoint from CLSI guideline?
- Thank you for the question. The antibiotic concentration was based on the CLSI breakpoint since the susceptible breakpoint value was based on the patient's clinically standard dosing regimen. This study aims to identify clinically relevant, effective antibiotic combinations for patients with MDR and XDR *A.baumannii* infections; consequently, it is necessary to utilize antibiotic concentrations achieved through a standard dosing regimen. MDR and XDR *A.baumannii* are frequently resistant to the tested antibiotic, with MIC typically being multiple times that of susceptible isolates, which is difficult to achieve during the administration of therapeutic antibiotic doses, thereby complicating the clinical application of those kinds of studies.

Competing Interests: N/A