In Vitro Synergistic Antimicrobial Activity of Combinations of Meropenem, Colistin, Tigecycline, Rifampin, and Ceftolozane/Tazobactam Against Carbapenem-Resistant *Acinetobacter Baumannii*

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Abstract

The aim of this study was to investigate the *in vitro* activity of various antimicrobial combinations against carbapenem-resistant *Acinetobacter baumannii* (CRAB) isolates producing OXA-23 carbapenemases.

In vitro activity of six two-drug combinations against CRAB isolates collected from patients with CRAB bacteremia was evaluated using the checkerboard method and time-kill assay [0.5 ×, 1 ×, 2 × minimum inhibitory concentrations (MIC)], to identify potential synergistic and bactericidal two-drug combinations against CRAB isolates, using meropenem, colistin, tigecycline, rifampin, and ceftolozane/tazobactam. All 10 CRAB isolates in our study carried the OXA-58-type and OXA-23-type carbapenem-hydrolyzing oxacillinase. The colistin-ceftolozane/tazobactam combination demonstrated a synergistic effect in both the time-kill assay (using an antibiotic concentration of 1 × MIC) and the checkerboard method, while simultaneously showing a bactericidal effect in the time-kill assay. For all 10 CRAB isolates, time-kill curves showed a significant synergistic bactericidal activity of the colistin-ceftolozane/tazobactam combination at 0.5 × MIC. Overall, there is substantial discordance of synergistic activity between the checkerboard microdilution and time-kill assay (with a concordance of 35%). Our study demonstrated that the two-drug combinations of colistin and ceftolozane/tazobactam can be a potential alternative for treating CRAB infections. The effect of these antibiotic combinations should be evaluated through clinical trials.

Introduction

Carbapenem-resistant *Acinetobacter baumannii* (CRAB), a leading nosocomial pathogen, poses a global threat to public health.¹ This pathogen is resistant to most of the available antibiotics in clinical practice and has extremely limited treatment options with a consequent increase in mortality.^{2–4} Furthermore, the spread of CRAB in a hospital environment is a thorny problem for infection control. They can colonize various body parts of hospitalized patients and survive for a long time on the surface of the hospital facilities.^{5,6}

In the Republic of Korea, the carbapenem resistance rate of *A. baumannii* isolated from patients hospitalized at intensive care units was 90%.^{7,8} According to the Korean part of the Global Antimicrobial Resistance Surveillance System, CRAB is the most common multidrug-resistant pathogen causing bloodstream infection at intensive care units, with an incidence of 6.3 cases per 10,000 patient-days.⁹

The World Health Organization has ranked CRAB as a pathogen of critical priority in the global priority list of multidrug-resistant bacteria urging the development of new antibiotics.¹⁰ Despite the relentless attempts to improve therapeutic approaches, there is no promising new antibiotic that can convincingly control CRAB infections.¹¹ Currently, only a few antibiotics of uncertain efficacy, such as colistin and tigecycline, are available for treating CRAB infections. The reduced susceptibility of CRAB, unfavorable pharmacokinetic properties, unclear optimal dosing, and potential adverse effects are barriers to the clinical use of existing drugs such as colistin and tigecycline.

Given the increasing multidrug-resistance rates and lack of effective antibiotics, combination therapy can be considered as an alternative interim strategy for effective management of CRAB infections. Antimicrobial combination therapy may broaden the spectrum of activity, minimize the development of antimicrobial resistance, and synergistically inactivate microorganisms. Several mechanisms proposed for synergistic antibacterial effects include enhanced bioavailability, inhibitor inhibition, sequential blockade, mutual stabilization, parallel pathway inhibition, and regulation modulation.¹² However, no standardized method has been established for the *in vitro* evaluation of combination therapies by the Clinical and Laboratory Standards Institute (CLSI).^{13–15} Nevertheless, several studies have addressed the therapeutic potential of combination therapy against CRAB infections.^{16–20}

The purpose of this study was to investigate the *in vitro* activity of antimicrobial combinations of meropenem, colistin, tigecycline, rifampin, and ceftolozane/tazobactam against CRAB isolates producing OXA-23 carbapenemases.

Methods

Study population

A total of 158 clinical isolates of *A. baumannii* were collected from nonduplicate patients with CRAB bacteremia in a 1,048-bed tertiary care hospital in Seoul, Republic of Korea from April, 2018 to January, 2020. Finally, 10 clinical isolates of CRAB exhibiting resistance to imipenem, meropenem, and ertapenem were randomly selected.²¹

The study protocol was approved prior to study initiation by the Institutional Review Board of Korea University Anam Hospital [No. 2020AN0157]. The study were performed in accordance with the ethical principles outlined in the Declaration of Helsinki. Informed consent was obtained from all subjects involved in this study.

Bacterial isolates and antimicrobial susceptibility testing

The identification and antimicrobial susceptibility testing of *A. baumannii* strains were performed initially using the MicroScan Pos Combo Panel Type 6 automated system (Baxter Diagnostics, West Sacramento, CA, USA) in a clinical microbiology laboratory. Confirmation of the identification of *A. baumannii* strains was performed using a matrix-assisted laser desorption/ionization-time of flight mass spectrometry (Bruker Daltonics, Bremen, Germany).

The minimum inhibitory concentration (MIC) was determined for each of the following 15 antimicrobial agents using the broth microdilution method, with the Cation-Adjusted Mueller–Hinton Ⅱ broth (CA-MHB) (Becton Dickinson & Co., Sparks, MD, USA) and geometric 2-fold serial dilutions according to the CLSI recommendations:^{21,22} meropenem (Yuhan Co, Seoul, Korea), ertapenem (MSD-Chibret, France), colistin (SteriMax Inc., Ont, Canada), amikacin (shinpoong Co,

Seoul, Korea), tigecycline (Pfizer Pharmaceutical, Seoul, Korea), ceftolozane/tazobactam (Wyeth; Madison, NJ, USA), piperacillin/tazobactam (Chong kun dang pharmaceutical Co, Seoul, Korea), ampicillin/sulbactam (Keun-hwa pharm, Seoul, Korea), ceftazidime (Hanmi Co, Seoul, Korea), cefepime (Boryung Co, Seoul, Korea), astreonam (Crystal lifesciences, Cheonju, Korea), minocycline (Skchemical Co, Seongnam, Korea), fosfomycin (Pharmbio Co, Chungju, Korea), rifampin (Yuhan Co, Seoul, Korea), and ciprofloxacin (Bayer AG, Leverkusen, Germany). The MIC results were interpreted according to the CLSI breakpoint criteria.²¹ No breakpoints are available for tigecycline from the CLSI guidelines.²¹ Therefore, the criteria of the United States Food and Drug Administration for *Enterobacteriaceae* were used for tigecycline (susceptibility, 2 mg/L; resistance, 8 mg/L).²³ For interpretation of colistin and rifampin susceptibilities, the breakpoints proposed by Gales et al. (resistance, 4 mg/L) and Hogg et al. (resistance, 2 mg/L) were used respectively.^{24,25} *Pseudomonas aeruginosa* (ATCC 27853) was used as a quality control isolate.

All CRAB isolates from blood cultures were immediately stored in Brain Heart Infusion broth (Becton Dickinson & Co., Sparks, MD, USA) containing 20% glycerol and kept in a freezer at -70 °C until February 2020. The isolates were thawed, and primary and secondary cultures were inoculated in 5% sheep blood agar for the experiments.

Detection of carbapenem resistance determinants

For all CRAB isolates, Ambler class B metallo- β -lactamase genes (*bla*_{IMP-1}, *bla*_{IMP-2}, *bla*_{VIM-1}, and *bla*_{VIM-2}) and Ambler class D OXA-type carbapenemases-encoding genes (*bla*_{OXA-23}, *bla*_{OXA-24}, *bla*_{OXA-51}, and *bla*_{OXA-58}) were detected using the polymerase chain reaction (PCR) method or multiplex PCR technique. The primers used in this study are shown in Table 1.

Each reaction mixture (20 μ L) contained 1 μ L of genomic DNA, 10 pmol of each primer, 1 U of *Taq* DNA polymerase, 0.25 mM dNTP, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, and 1.5 mM MgCl₂. PCR reaction temperatures for the Ambler class B metallo- β -lactamase genes were 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, at each specific annealing temperature for 1 min and 72 °C for 1 min, followed by a final extension at 72 °C for 7 min (Table 1). PCR reaction temperatures for the Ambler class D OXA-type carbapenemase-encoding genes were 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, at each specific annealing temperature for 40 s and 72 °C for 50 s, followed by a final extension at 72 °C for 7 min (Table 1).

Checkerboard assays for synergy testing

The synergistic activities of various two-drug combinations against the 10 CRAB isolates were evaluated using the checkerboard assay, which was conducted in 96-well microtiter plates (Corning Inc., Kennebunk, ME, USA). In brief, panels of 96-well microtiter plates were prepared according to results obtained from the MIC of each antibiotic determined with broth microdilution. Dilution intervals were determined from 2–32 times higher and 1/8–1/64 below the MIC values obtained from the preliminary analysis. The antibiotic stock solutions were diluted with CA-MHB, and the concentrations of the upper left part of the plates were set to 0. The rows of the plates contained 50 μ L of two-fold serial dilutions of the first antibiotic in each well, and the columns contained 50 μ L of two-fold serial dilutions of the second antibiotic. The ranges of test concentrations of each antibiotic in combinations were as follows: colistin, 0–128 mg/L; meropenem, 0–128 mg/L; tigecycline, 0–8 mg/L; ceftolozane/tazobactam, 0–128 mg/L; and rifampin, 0–256 mg/L.

The *A. baumannii* inocula consisted of 100 μ L of a two-fold dilution of a 0.5 McFarland turbidity standard prepared in CA-MHB. The final inoculum concentration was 5×10^4 CFU/mL in each well. Except for the sterility control well, all the wells were inoculated and then incubated at 37 °C for 18–20 h, and then the wells were diluted to an OD 600 nm measured at the stationary phase with an absorbance microplate reader (SpectraMax Plus 384, Molecular Devices, Inc). The MIC was determined as the well in the microtiter plate with the lowest drug concentration at which there was no visible growth. The fractional inhibitory concentration index (FICI) was calculated using the formula below.

$$\text{FICI} = [(\text{MIC of drug A in combination})/(\text{MIC of drug A alone})] + [(\text{MIC of drug B in combination})/(\text{MIC of drug B alone})]$$

Interpretation of the FICI was as follows: FICI $\leq$ 0.5, synergistic; 0.5 < FICI $\leq$ 1, additive; 1 < FICI $\leq$ 4, indifferent; and FICI > 4, antagonistic.²⁶

Time-kill assay for synergy testing

Along with the checkerboard assay, time-kill assays were conducted on the 10 *A. baumannii* isolates. In brief, tubes containing freshly prepared CA-MH broth supplemented with the antibiotics, alone and in combination, were inoculated with CRAB isolates at a concentration of 10^4 CFU/mL. The tubes had a final volume of 10 mL and were incubated at 37 °C in a shaking incubator (200 rpm), in ambient air.

Then, 100 μ L aliquots were obtained from each tube at 0, 2, 4, 8, 12, and 24 h of incubation and serially diluted in saline for determination of viable counts. Diluted samples (10 μ L) were plated on CA-MHA plates using a spreader (SPL life Science, Co) and incubated at 37 °C for 18–24 h, and then the number of colonies formed was counted. The antibiotic carry-over effect was minimized by washing the aliquots in sterile phosphate-buffered saline (PBS) and centrifuging for 5 min at 1,300 rpm before a 10-fold serial dilution in sterile PBS. The initial bacterial density from the original sample was calculated based on the dilution factor. The lower limit of detection for the colony counts was $2 \log_{10}$ CFU/mL. The concentrations of antibiotics applied were 0.5 $\times$ MIC, 1 $\times$ MIC, and 2 $\times$ MIC alone or in combination.

The bactericidal activity of single antibiotics or combinations was defined as a decrease of $\geq 4 \log_{10}$ in 24 h compared with the number of viable cells at the initial time point.²⁷ A synergistic effect was considered as a decrease of $\geq 2 \log_{10}$ CFU/mL in 6 or 24 h when comparing the antibiotics in combination with the most active drug alone at the different time points, whilst an increase of $> 2 \log_{10}$ was indicative of antagonism. Indifference was considered as any other outcome that did not meet the criteria for either synergy or antagonism.²⁸

Results

Characteristics of *A. baumannii* clinical isolates

Clinical isolates involved in this study are listed in Table 2. Out of 10 patients with CRAB bacteremia, 50% had in-hospital mortality. All 10 CRAB isolates carried the OXA-58-type and OXA-23-type carbapenem-hydrolyzing oxacillinase, whereas they did not harbor class B metallo-carbapenemases or other class D carbapenemases. Ten clinical isolates were resistant to meropenem with MICs of 64 mg/L. Among them, the susceptibility rate to each antibiotic is as follows: tigecycline 90%, minocycline 100%, rifampin 30%, colistin 10%, ceftolozane/tazobactam 0%, ciprofloxacin 0%, fosfomycin 0%, aztreonam 0%, ceftazidime 0%, ampicillin/sulbactam 0%, piperacillin/tazobactam 0%, and amikacin 0% (Table 2).

Checkerboard assay against *A. baumannii* clinical isolates

The results of the *in vitro* synergism and MIC values of the individual antibiotics in combination against the 10 CRAB isolates using the checkerboard assay are summarized in Table 3. In terms of checkerboard assay, *in vitro* synergistic activities ($\sum \text{FICI} \leq 0.5$) against CRAB isolates were most frequently observed for meropenem-tigecycline combination (90%), followed by ceftolozane/tazobactam-meropenem combination (70%), meropenem-rifampin combination (70%), ceftolozane/tazobactam-colistin combination (60%), colistin-tigecycline combination (30%), and meropenem-colistin combination (30%) (Table 3). All combinations displayed synergism to a certain extent.

Notably, antagonistic interactions were not observed in our study. The MICs of the antibiotics in combination were lower than the MICs of the antibiotics used as a single agent.

Time-kill assay against *A. baumannii* clinical isolates

All 10 CRAB isolates were evaluated using the time-kill method. When using an antibiotic concentration of $0.5 \times \text{MIC}$, $1 \times \text{MIC}$, and $2 \times \text{MIC}$, bactericidal activity of antibiotic monotherapy on the 10 CRAB isolates was found in meropenem ($0.5 \times \text{MIC}$, 0%, 0/10; $1 \times \text{MIC}$, 40%, 4/10; $2 \times \text{MIC}$, 70%, 7/10), ceftolozane/tazobactam ($0.5 \times \text{MIC}$, 0%, 0/10; $1 \times \text{MIC}$, 10%, 1/10; $2 \times \text{MIC}$, 70%, 7/10), colistin ($0.5 \times \text{MIC}$, 0%, 0/10; $1 \times \text{MIC}$, 50%, 5/10; $2 \times \text{MIC}$, 70%, 7/10), rifampin ($0.5 \times \text{MIC}$, 0%, 0/10; $1 \times \text{MIC}$, 10%, 1/10; $2 \times \text{MIC}$, 40%, 4/10) and tigecycline ($0.5 \times \text{MIC}$, 10%, 1/10; $1 \times \text{MIC}$, 10%, 1/10; $2 \times \text{MIC}$, 40%, 4/10) within 24 h (Supplementary file).

For the time-kill assay, using an antibiotic concentration of $1 \times \text{MIC}$, the *in vitro* synergistic activities against the 10 CRAB isolates were most frequently observed for the meropenem-colistin combination (100%), ceftolozane/tazobactam-colistin (100%), and tigecycline-colistin combination (100%), followed by the meropenem-tigecycline combination (70%), ceftolozane/tazobactam-meropenem combination (60%), and meropenem-rifampin combination (30%) within 24 h (Table 4).

For the colistin-ceftolozane/tazobactam combination (both at a concentration of $1 \times \text{MIC}$), the 10 CRAB isolates yielded synergy rates of 60% in 12 h and 50% in 24 h, respectively (Figure 1). For the colistin-meropenem combination (at $1 \times \text{MIC}$), the 10 CRAB isolates showed synergistic rates of 50% in 12 h and 40% in 24 h, respectively (Figure 1). For the meropenem-ceftolozane/tazobactam combination (at $1 \times \text{MIC}$), the 10 CRAB isolates showed synergistic rates of 20% in 12 h and 50% in 24 h, respectively (Figure 1). For the meropenem-tigecycline combination (at $1 \times \text{MIC}$), the 10 CRAB isolates showed synergistic rates of 40% in 12 h and 50% in 24 h, respectively (Figure 1). However, the meropenem-rifampin and colistin-tigecycline combinations (at $1 \times \text{MIC}$) did not show synergy rates of more than 50% (Figure 1). Overall, for six combinations of antibiotics, doubling the antibiotic concentration did not improve synergy rates (Figure 1). Unlike synergistic inhibitory activity for other antibiotic combinations sustained until 24 h, the CRAB 34 isolates demonstrated a regrowth at 4 h for the colistin-ceftolozane/tazobactam combination (both at a concentration of $1 \times \text{MIC}$) (Figure 2).

For the time-kill assay, using an antibiotic concentration of $1 \times \text{MIC}$, *in vitro* bactericidal activities for the 10 CRAB isolates were most frequently observed for the meropenem-colistin combination (100%), ceftolozane/tazobactam-colistin combination (100%), and tigecycline-colistin combination (100%), followed by the meropenem-tigecycline combination (50%), ceftolozane/tazobactam-meropenem combination (50%), and meropenem-rifampin combination (30%) within 24 h (Table 4).

At $1 \times$ and $2 \times \text{MIC}$ concentrations, the combinations of colistin-ceftolozane/tazobactam ($1 \times \text{MIC}$, 100%, 10/10; $2 \times \text{MIC}$, 100%, 10/10), colistin-tigecycline ($1 \times \text{MIC}$, 70%, 7/10; $2 \times \text{MIC}$, 100%, 10/10), and colistin-meropenem ($1 \times \text{MIC}$, 100%, 10/10; $2 \times \text{MIC}$, 100%, 10/10) against the 10 CRAB isolates, showed bactericidal activity usually beginning at 12 h after inoculation (Figure 1).

With respect to the time-kill assay test, at $1 \times \text{MIC}$ concentrations, antibiotic combinations that showed both bactericidal activity and synergistic effect within 24 h are as follows: colistin-ceftolozane/tazobactam (70%), colistin-tigecycline (70%), colistin-meropenem (50%), meropenem-ceftolozane/tazobactam (50%), meropenem-tigecycline (30%), and rifampin-meropenem (30%) (Table 5). However, the frequency of simultaneously showing both effects at the same time-point was relatively low (Figure 1). In more than 50% of the 10 clinical isolates, antibiotic combinations showing both bactericidal activity and synergistic effect at the same time-point, were colistin-ceftolozane/tazobactam (60% and 50% in 12 h and 24 h, respectively, after inoculation) and colistin-meropenem (50% at 12 h after inoculation) (Figure 1).

Regarding the synergistic effect of antibiotic combinations, 35% (21/60) of the results of the time-kill assay were consistent with the checkerboard results (Table 5). Both the time-kill assay and the checkerboard assay showed synergistic effects, while simultaneously showing bactericidal effects in the time-kill assay as follows: colistin-ceftolozane/tazobactam (40%), meropenem-tigecycline (30%), meropenem-ceftolozane/tazobactam (30%), colistin-meropenem (30%), colistin-tigecycline (20%), and rifampin-meropenem (20%) within 24 h (Table 5).

The time-kill curves of six different combinations of antibiotics with 0.5 ×, 1 ×, 2 × MIC concentrations against the CRAB isolates are shown in Figure 3. No antagonism was observed in the time-kill assay for the antibiotic combinations (Supplementary file).

Discussion

Our study was performed to determine which antibiotic combinations might be potentially suitable options for treating CRAB infections. This is the first study, to the best of our knowledge, to evaluate the *in vitro* synergistic activity of ceftolozane/tazobactam with other antibiotics against CRAB isolates. Our findings revealed that the combination of colistin and ceftolozane/tazobactam was supported *in vitro* by synergistic and bactericidal effects against OXA-23-type carbapenemase-producing CRAB isolates.

All 10 CRAB isolates in our study carried the OXA-58-type and OXA-23-type carbapenem-hydrolyzing oxacillinase. Majority of the CRAB isolates in the Republic of Korea were identified to be carrying *bla*_{OXA-23}.²⁹ All 10 CRAB isolates had MIC of 64 mg/L to meropenem, and 90% of the CRAB isolates had MIC of ≤ 1 mg/L to tigecycline. Particularly, MICs to colistin ranged from 2 to 8 mg/L. Notably, for the time-kill method, using an antibiotic concentration of 1 × MIC in our study failed to show a stable bactericidal effect of antibiotic monotherapy against the 10 CRAB isolates. In this scenario, combination antibiotic therapy can become the ultimate resource for treating CRAB infections. In addition, it is predicted that the difference in susceptibilities to each antibiotic would have various effects on the antibiotic combination effect.

The colistin-ceftolozane/tazobactam combination demonstrated a synergistic effect in both the time-kill assay and the checkerboard method, while simultaneously showing a bactericidal effect in the time-kill assay. In contrast, the meropenem-tigecycline, meropenem-ceftolozane/tazobactam, and rifampin-meropenem combinations showed antagonistic effects for some CRAB isolates.

Ceftolozane/tazobactam, a novel beta-lactam/beta-lactamase inhibitor, has demonstrated potent *in vitro* activity against *Pseudomonas aeruginosa*, including carbapenem-resistant isolates, except for class B carbapenemase producers, but poor activity against *A. baumannii* isolates.³⁰ The susceptibility of CRAB isolates to ceftolozane/tazobactam was poor with a MIC range of 16–128 mg/L, which was in accordance with previous results.³¹ However, our findings identified the potential to induce synergistic interaction in combination with different antibiotics.

For all 10 CRAB isolates, the time-kill curves showed a significant synergistic bactericidal activity of the colistin-ceftolozane/tazobactam combination at 0.5 × MIC (Figure 3). This finding has promising implications for using lower doses of colistin in treatment, thereby reducing its potential nephrotoxic effect.

In our findings, for the meropenem-tigecycline combination, *in vitro* synergistic activities were found in 90% and 50% of the 10 CRAB isolates in terms of the checkerboard and time-kill assays, respectively. However, *in vitro* antagonistic activities were found in 20% of the 10 CRAB isolates in terms of the time-kill assay. A previous meta-analysis revealed a synergistic rate of 24.5% and 20.0% for CRAB isolates using the checkerboard (36 studies) and the time-kill method (35 studies), respectively.¹⁹ Probably, the high susceptibility rate (90%) of the CRAB isolates to tigecycline in our study may have contributed to these results. A recent clinical study reported that the tigecycline-colistin combination was associated with a higher mortality rate when the MIC of tigecycline was > 2 mg/L, which was achieved with the combination of a carbapenem and colistin.³² Therefore, it is important to know how to better select from the existing antibiotic treatment regimens according to the phenotype of antibiotic resistance (hospital antibiogram) to achieve an improved clinical outcome. However, non-colistin-based combination regimens may have an important role in the treatment of CRAB infections, for those who are concerned about the nephrotoxic side effects and the emergence of hetero-resistance or resistance to colistin of CRAB isolates.

In our study, we incubated CRAB in the presence of antibiotics for 24 h, as a result, the regrowth phenomenon was observed in the time-kill assay of the CRAB 34 isolate at 4 h after inoculation for the colistin-ceftolozane/tazobactam combination (both at a concentration of 1 × MIC) (Figure 2). Although most of the time-kill analyses incubated bacteria in the presence of antibiotics for 24 h, incubation for 48 h may be better to detect the regrowth phenomenon considering the selective amplification of the resistant subpopulation.³³ A previous study reported the regrowth phenomenon commonly detected in time-kill assays using colistin, despite the *in vitro* antimicrobial activity of colistin against the CRAB isolates.¹⁶

In more than 50% of the 10 clinical isolates, the antibiotic combinations showing both bactericidal activity and synergistic effect at the same time-point, were colistin-ceftolozane/tazobactam and colistin-meropenem (Figure 1). In our study, the meropenem-colistin combination provided 30% and 50% synergistic activities in the checkerboard and time-kill assays, respectively. The meropenem-colistin combination remains an area of active research for the treatment of CRAB infections. Based on previous studies, a time-kill investigation of antibiotic combinations against 12 CRAB isolates at 5 × 10⁵ CFU/mL identified the meropenem-colistin combination as the most synergistic combination.³⁴ Regarding synergy rates, the checkerboard and time-kill assays yielded 60–73.3% and 60–96.1% synergistic effects, respectively.^{19,35–37} According to a previous meta-analysis, the synergistic rates shown by time-kill methods were significantly higher than those obtained using checkerboard, which is similar to our results.^{20,38} Overall, there is great discordance between the checkerboard microdilution and time-kill assay with a concordance of 35% in our study. In contrast, a previous meta-analysis showed a higher synergistic rate for CRAB isolates in a combination of meropenem and colistin than a combination of imipenem and colistin.^{20,39}

The present study has several limitations. First, the results do not apply to CRAB isolates that produce metallo-beta-lactamase. Notably, the CRAB isolates in our study are highly resistant to meropenem and may behave differently to the combinations if MICs to meropenem are lower. In addition, this study included a small number of CRAB isolates assessed in the checkerboard and time-kill assays. The MIC values of colistin in many of the CRAB isolates were remarkably high, because only several isolates were evaluated for the *in vitro* synergistic and bactericidal activities of antibiotic combinations. However, it is meaningful to collect isolates from patients with CRAB bacteremia in a clinical setting. Finally, we acknowledge that *in vitro* studies do not always lead to similar results in clinical practice; therefore, caution is required when applying these results in clinical practice.

Conclusions

In conclusion, the present study demonstrated that the combination of colistin and ceftolozane/tazobactam may be a promising alternative to colistin for treating CRAB infections. These *in vitro* synergy studies can provide preliminary guidance for optimal drug combination use in treating patients with CRAB infections. However, the benefits of these antibiotic combinations should be validated through multicenter randomized clinical trials.

Abbreviations

Am, amikacin; An, antagonism; A/S, ampicillin/sulbactam; Az, aztreonam; B, bactericidal; C, colistin; CA-MHB, Cation-Adjusted Mueller–Hinton α broth; Cef, ceftazidime; Cip, ciprofloxacin; CLSI, Clinical and Laboratory Standards Institute; CRAB, carbapenem-resistant *Acinetobacter baumannii*; C/T, ceftolozane/tazobactam; F, female; FICI, fractional inhibitory concentration index; F, fosfomycin; I, indifference; M, male; Me, meropenem; Mi, minocycline; MIC, minimum inhibitory concentration; N, non-bactericidal; PCR, polymerase chain reaction; P/T, piperacillin-tazobactam; R, rifampin; Syn, synergy; T, tigecycline.

Declarations

Ethics committee approval: The study protocol was approved prior to study initiation by the Institutional Review Board of Korea University Anam Hospital [No. 2020AN0157].

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Author contributions: YKY conceived, designed, and performed the study; HSY, MGL, and YGJ contributed to the experiments and the analysis of the data. HJL, CKL, and JWS contributed to the collection of clinical isolates. YKY and YGJ wrote the manuscript.

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Conflict of interest: None

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Tables

Table 1. Polymerase chain reaction (PCR) primers, conditions, and product size for the detection of carbapenemases among *Acinetobacter baumannii* strains

Target gene	Primers (5' à 3')	Annealing temperature for multiplex PCR	Product size (bp)
<i>bla</i> _{IMP-1}	ACCGCAGCAGAGTCTTTGCC ACAACCAGTTTTGCCTTACC	55 °C	587
<i>bla</i> _{IMP-2}	GTTTTATGTGTATGCTTCC AGCCTGTTCCCATGTAC	55 °C	678
<i>bla</i> _{VIM-1}	GGGAGCCGAGTGGTGAGT GGCACAACCACCGTATAG	55 °C	519
<i>bla</i> _{VIM-2}	ATGTTCAAACCTTTTGAGTAAG CTACTCAACGACTGAGCG	55 °C	801
<i>bla</i> _{oxa-23}	GATCGGATTGGAGAACCAGA ATTTCTGACCGCATTTCCAT	52 °C	501
<i>bla</i> _{oxa-24}	GGTTAGTTGGCCCCCTTAAA AGTTGAGCGAAAAGGGGATT	52 °C	246
<i>bla</i> _{oxa-51}	TAATGCTTTGATCGGCCTTG TGGATTGCACTTCATCTTGG	52 °C	353
<i>bla</i> _{oxa-58}	AAGTATTGGGGCTTGTGCTG CCCCTCTGCGCTCTACATAC	52 °C	599

Table 2. Clinical and microbiological characteristics of *Acinetobacter baumannii* isolates

Isolate no.	Patient information				Carbapenemase genes		MICs of antimicrobial agents (mg/L)*												
	Age	Sex	Clinical specimen	In-hospital mortality	<i>bla</i> _{oxa-51}	<i>bla</i> _{oxa-23}	Me	C	T	C/T	R	Cip	F	Mi	Az	Cef	A/S	P/T	Am
ATCC27853	-	-	-	-	-	-	0.25	2	4	0.5	64	0.125	4	16	8	2	>128	4	1
CRAB 19	90	M	Blood	No	+	+	64	2	0.5	128	4	16	>128	0.25	128	>128	64	>128	128
CRAB 32	66	F	Blood	Yes	+	+	64	8	0.5	16	4	32	128	0.25	64	128	64	>128	>128
CRAB 33	95	F	Blood	Yes	+	+	64	8	0.5	32	4	64	128	0.25	64	128	64	>128	>128
CRAB 34	81	F	Blood	No	+	+	64	8	0.5	64	>128	64	128	0.25	128	>128	64	>128	>128
CRAB 35	75	F	Blood	No	+	+	64	4	0.5	64	>128	64	128	0.25	128	>128	64	>128	>128
CRAB 36	57	F	Blood	No	+	+	64	8	0.5	64	>128	128	128	0.25	128	>128	64	>128	>128
CRAB 37	66	M	Blood	No	+	+	64	8	0.5	32	>128	64	128	0.25	128	>128	64	>128	>128
CRAB 38	81	F	Blood	Yes	+	+	64	8	0.5	64	>128	64	128	0.25	128	>128	64	>128	>128
CRAB 39	68	M	Blood	Yes	+	+	64	4	1	32	>128	64	128	0.25	128	>128	64	>128	>128
CRAB 40	89	M	Blood	Yes	+	+	64	8	4	32	>128	128	64	0.5	64	>128	64	>128	>128

Am, amikacin; A/S, ampicillin/sulbactam; Az, aztreonam; Cef, ceftazidime; CRAB, carbapenem-resistant *Acinetobacter baumannii*; Cip, ciprofloxacin; C, colistin; C/T, ceftolozane/tazobactam; F, female; F, fosfomycin; M, male; Me, meropenem; Mi, minocycline; MIC, minimum inhibitory concentration; P/T; piperacillin-tazobactam; R, rifampin; T, tigecycline

*Gray cell, non-susceptible; white cell, susceptible

Table 3. Results of checkerboard assay for two-drug combinations against clinical isolates of *Acinetobacter baumannii*.

	Clinical isolates	MIC of a single agent (mg/L)		MIC in combination (mg/L)		FICI	Result of checkerboard assay
		Drug A	Drug B	Drug A	Drug B		
Meropenem (A) + colistin (B)	CRAB 19	64	2	4	0.25	0.19	Synergistic
	CRAB 32	64	8	16	0.5	0.31	Synergistic
	CRAB 33	64	8	0.125	0.25	0.03	Synergistic
	CRAB 34	64	8	32	0.25	0.53	Additive
	CRAB 35	64	8	32	0.25	0.56	Additive
	CRAB 36	64	8	32	0.5	0.56	Additive
	CRAB 37	64	8	32	0.25	0.53	Additive
	CRAB 38	64	8	32	0.25	0.53	Additive
	CRAB 39	64	4	32	0.25	0.56	Additive
	CRAB 40	64	8	32	0.25	0.53	Additive
Meropenem (A) + tigecycline (B)	CRAB 19	64	0.5	0.125	0.03	0.06	Synergistic
	CRAB 32	64	0.5	8	0.125	0.38	Synergistic
	CRAB 33	64	0.5	32	0.5	1.5	Indifferent
	CRAB 34	64	0.5	16	0.125	0.5	Synergistic
	CRAB 35	64	0.5	16	0.125	0.5	Synergistic
	CRAB 36	64	0.5	0.125	0.125	0.25	Synergistic
	CRAB 37	64	0.5	0.125	0.125	0.25	Synergistic
	CRAB 38	64	0.5	16	0.125	0.5	Synergistic
	CRAB 39	64	1	16	0.125	0.38	Synergistic
	CRAB 40	64	4	8	0.125	0.16	Synergistic
Meropenem (A) + rifampin (B)	CRAB 19	64	4	32	0.25	0.56	Additive
	CRAB 32	64	4	32	4	1.5	Indifferent
	CRAB 33	64	4	16	2	0.75	Additive
	CRAB 34	64	256	4	8	0.09	Synergistic
	CRAB 35	64	256	16	4	0.27	Synergistic
	CRAB 36	64	256	16	8	0.28	Synergistic
	CRAB 37	64	256	16	4	0.27	Synergistic
	CRAB 38	64	256	16	4	0.27	Synergistic
	CRAB 39	64	256	16	4	0.27	Synergistic
	CRAB 40	64	256	8	8	0.16	Synergistic
Meropenem (A) + ceftolozane/tazobactam (B)	CRAB 19	64	128	32	16	0.63	Additive
	CRAB 32	64	16	8	4	0.38	Synergistic
	CRAB 33	64	32	8	16	0.63	Additive
	CRAB 34	64	64	8	4	0.19	Synergistic
	CRAB 35	64	64	2	2	0.06	Synergistic
	CRAB 36	64	64	32	32	1	Additive
	CRAB 37	64	32	4	2	0.13	Synergistic
	CRAB 38	64	64	16	2	0.28	Synergistic
	CRAB 39	64	32	2	4	0.16	Synergistic
	CRAB 40	64	32	8	8	0.38	Synergistic
Colistin (A) + tigecycline (B)	CRAB 19	2	0.5	0.006	0.03	0.06	Synergistic

	CRAB 32	8	0.5	4	0.25	1	Additive
	CRAB 33	8	0.5	0.5	0.25	0.56	Additive
	CRAB 34	8	0.5	8	0.5	2	Indifferent
	CRAB 35	8	0.5	4	0.25	1.5	Indifferent
	CRAB 36	8	0.5	2	0.125	0.5	Synergistic
	CRAB 37	8	0.5	8	0.5	2	Indifferent
	CRAB 38	8	0.5	8	0.5	2	Indifferent
	CRAB 39	4	1	4	1	2	Indifferent
	CRAB 40	8	4	0.125	1	0.27	Synergistic
Colistin (A) + ceftolozane/tazobactam (B)	CRAB 19	2	128	2	128	2	Indifferent
	CRAB 32	8	16	4	8	1	Additive
	CRAB 33	8	32	0.125	2	0.08	Synergistic
	CRAB 34	8	64	0.5	8	0.19	Synergistic
	CRAB 35	8	64	0.25	16	0.31	Synergistic
	CRAB 36	8	64	8	64	2	Indifferent
	CRAB 37	8	32	1	16	0.63	Additive
	CRAB 38	8	64	2	2	0.28	Synergistic
	CRAB 39	4	32	0.25	4	0.19	Synergistic
	CRAB 40	8	32	0.125	8	0.27	Synergistic

CRAB, carbapenem-resistant *Acinetobacter baumannii*; FICI, fractional inhibitory concentration index; MIC, minimum inhibitory concentration

Table 4. Results of time-kill assay and bactericidal activity of two-drug combinations (1 × MIC) against each *Acinetobacter baumannii* isolate

Isolate	Time-kill assay						Bactericidal activity						
							(Timepoint, hours; 2/4/6/8/12/24)						
	Me+T	Me+C	Me+R	Me+C/T	C+T	C+C/T	Me+T	Me+C	Me+R	Me+C/T	C+T	C+C/T	
CRAB 19	Syn.	Syn.	Syn.	Syn.	Syn.	Syn.	N/N/N/N/B	N/N/B/B/B	N/N/B/B/B	N/N/N/B/B	N/B/B/B/B	N/N/B/B/B	
CRAB 32	Syn.	Syn.	I.	Syn.	Syn.	Syn.	N/N/N/B/B	N/N/B/B/B	N/N/N/N/N	N/N/N/N/B	N/N/N/B/B	N/N/B/B/B	
CRAB 33	Syn.	Syn.	I.	Syn.	Syn.	Syn.	N/N/N/N/N	N/N/N/B/B	N/N/N/N/N	N/N/N/N/N	N/N/N/N/B	N/N/N/B/B	
CRAB 34	Syn.	Syn.	Syn.	Syn.	Syn.	Syn.	N/N/N/N/B	N/N/N/B/B	N/N/N/B/B	N/N/N/N/B	N/B/B/B/B	B/N/B/B/B	
CRAB 35	Syn.	Syn.	I.	I.	Syn.	Syn.	N/N/N/N/N	N/N/N/B/B	N/N/N/N/N	N/N/N/N/N	N/N/N/N/B	N/N/N/B/B	
CRAB 36	Syn.	Syn.	I.	Syn.	Syn.	Syn.	N/N/N/B/B	N/N/N/B/B	N/N/N/N/N	N/N/N/N/B	N/N/B/B/B	N/N/N/B/B	
CRAB 37	Syn.	Syn.	Syn.	Syn.	Syn.	Syn.	N/N/N/B/B	N/N/N/B/B	N/N/N/B/B	N/N/N/N/B	N/B/B/B/B	N/N/N/B/B	
CRAB 38	I.	Syn.	An.	I.	Syn.	Syn.	N/N/N/N/N	N/N/N/B/B	N/N/N/N/N	N/N/N/N/N	N/N/N/N/B	N/N/N/B/B	
CRAB 39	An.	Syn.	An.	I.	Syn.	Syn.	N/N/N/N/N	N/N/N/B/B	N/N/N/N/N	N/N/N/N/N	N/N/N/B/B	N/N/N/B/B	
CRAB 40	I.	Syn.	An.	An.	Syn.	Syn.	N/N/N/N/N	N/N/N/B/B	N/N/N/N/N	N/N/N/N/N	N/N/N/B/B	N/N/N/B/B	

An, antagonism; B, bactericidal; C, colistin; C/T, ceftolozane/tazobactam; I, indifference; Me, meropenem; N, non-bactericidal; R, rifampin; Syn, synergy; T, tigecycline

Table 5. Comparison of checkerboard assay and time-kill assay associated with the bactericidal activity of two-drug combinations (1 × MIC) against each *Acinetobacter baumannii* isolate

Isolate	Checkerboard assay results						Time-kill assay results					
	M+T	M+C	M+R	M+C/T	C+T	C+C/T	M+T	M+C	M+R	M+C/T	C+T	C+C/T
CRAB 19	Syn.	Syn.	Additive	Additive	Syn.	I.	I./B.	Syn./B.	Syn./B.	Syn./B.	Syn./B.	Syn./B.
CRAB 32	Syn.	Syn.	I.	Syn.	Additive	Additive	I./B.	Syn./B.	I./N.	Syn./B.	I./B.	Syn./B.
CRAB 33	I.	Syn.	Additive	Additive	Additive	Syn.	Syn./N.	Syn./B.	I./N.	Syn./N.	Syn./B.	Syn./B.
CRAB 34	Syn.	Additive	Syn.	Syn.	I.	Syn.	Syn./B.	I./B.	Syn./B.	Syn./B.	Syn./B.	Syn./B.
CRAB 35	Syn.	Additive	Syn.	Syn.	I.	Syn.	Syn./N.	Syn./B.	I./N.	I./N.	Syn./B.	Syn./B.
CRAB 36	Syn.	Additive	Syn.	Additive	Syn.	I.	Syn./B.	I./B.	I./N.	Syn./B.	Syn./B.	I./B.
CRAB 37	Syn.	Additive	Syn.	Syn.	I.	Additive	Syn./B.	Syn./B.	Syn./B.	Syn./B.	Syn./B.	Syn./B.
CRAB 38	Syn.	Additive	Syn.	Syn.	I.	Syn.	An./N.	I./B.	An./N.	An./B.	I./B.	I./B.
CRAB 39	Syn.	Additive	Syn.	Syn.	I.	Syn.	An./N.	I./B.	An./N.	An./N.	Syn./B.	Syn./B.
CRAB 40	Syn.	Additive	Syn.	Syn.	Syn.	Syn.	I./N.	I./B.	An./N.	An./N.	I./B.	I./B.

An, antagonism; B, bactericidal; C, colistin; C/T, ceftolozane/tazobactam; I, indifference; M, meropenem; N, non-bactericidal; R, rifampin; Syn, synergy; T, tigecycline

*Gray cells mean that the results of the time-kill assay were consistent with the results of the checkerboard assays.

Figures

Figure 1

Results of time-kill assay and bactericidal activity of two-drug combinations against 10 *Acinetobacter baumannii* isolates. The percentage in the figure indicates the ratio of isolates showing bactericidal or synergistic effect among all 10 CRAB isolates.

C, colistin; C/T, ceftolozane/tazobactam; Me, meropenem; R, rifampin; T, tigecycline

Figure 2

Time-kill curves of *A. baumannii* isolates (CRAB 34) at 1 × MIC concentration for six combinations of antibiotics

Figure 3

Time-kill curves of the 10 *A. baumannii* isolates for six combinations of antibiotics with the different concentrations

C, colistin; C/T, ceftolozane/tazobactam; Me, meropenem; R, rifampin; T, tigecycline

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