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Investigation of the Synergic Effect of the Colistin/Sulbactam Combination in Carbapenem-Resistant *Acinetobacter baumannii* complex Strains with Time-Kill and Checkerboard Methods

Karbapeneme Dirençli *Acinetobacter baumannii* complex Suşlarında Kolistin/Sulbaktam Kombinasyonunun Sinerjik Etkinliğinin Time-Kill ve Checkerboard Yöntemi ile Araştırılması

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ABSTRACT

Introduction: *Infections caused by carbapenem-resistant Acinetobacter strains have become very common in recent years, and the most frequently used medicinal treatment is colistin. Combination treatments should also be applied to prevent development of resistance to colistin. This study examines the in vitro synergic effect of the colistin/sulbactam combination in carbapenem-resistant Acinetobacter strains with the time-kill and checkerboard methods.*

Materials and Methods: *Twenty carbapenem-resistant Acinetobacter baumannii-calcoaceticus complex strains, which were isolated from various clinical samples, were included in this study. Strains were identified with mass spectrometry, and antibiotic sensitivity results were determined with the VITEK 2® system. The in vitro effect and synergic activity of the colistin, sulbactam, and colistin/sulbactam combination on the carbapenem-resistant strains were determined using the time-kill and checkerboard methods. Seventeen strains were examined with the time-kill method, and twenty strains were examined using the checkerboard method. The fractional inhibitory concentration index of strains was calculated for detection of synergic effect.*

Results: *Using the time-kill method applied on the colistin/sulbactam combination showed that the combination had a synergic effect on all 17 strains, while sulbactam alone did not have a bactericidal effect in the studied concentrations. When applying the checkerboard method, it was determined that the colistin/sulbactam combination had a synergic effect on 17 of the strains (85%) and an additive effect on 3 strains (15%), sulbactam had a low effect alone (15%), and colistin was effective on all strains.*

Conclusion: *Study results indicated that the colistin/sulbactam combination had a high level of synergic effect on all studied strains using both methods.*

Key Words: *Acinetobacter baumannii, Colistin, Sulbactam, Synergy, Time-kill, Checkerboard*

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ÖZ

Karbapeneme Dirençli *Acinetobacter baumannii* complex Suşlarında Kolistin/Sulbaktam Kombinasyonunun Sinerjik Etkinliğinin Time-Kill ve Checkerboard Yöntemi ile Araştırılması

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Giriş: *Karbapenem dirençli Acinetobacter suşlarının neden olduğu infeksiyonlar son yıllarda çok yaygın hale gelmiştir ve en sık kullanılan tıbbi tedavi kolistindir. Kolistin direncinin gelişmesini önlemek için kombinasyon tedavileri de uygulanmalıdır. Bu çalışma karbapenem dirençli Acinetobacter suşlarındaki kolistin/sulbaktam kombinasyonunun in vitro sinerjik etkisini zaman öldürme ve dama tahtası yöntemleriyle incelemektedir.*

Materyal ve Metod: *Çeşitli klinik örneklerden izole edilen yirmi karbapenem dirençli Acinetobacter baumannii-calcoaceticus kompleks suşu bu çalışmaya dahil edilmiştir. Suşlar kütle spektrometrisi ile tanımlanmış ve antibiyotik duyarlılık sonuçları VITEK 2® sistemi ile belirlenmiştir. Karbapenem dirençli suşlar üzerinde kolistin, sulbaktam ve kolistin/sulbaktam kombinasyonunun in vitro etkisi ve sinerjik aktivitesi, zaman öldürme ve dama tahtası yöntemleri kullanılarak belirlenmiştir. 17 suş zaman öldürme yöntemi ile incelendi ve yirmi suş da dama tahtası yöntemi kullanılarak incelendi. Suşların fraksiyonel inhibitör konsantrasyon indeksi sinerjik etkinin saptanması için hesaplanmıştır.*

Bulgular: *Kolistin/sulbaktam kombinasyonu üzerine uygulanan zaman öldürme yönteminin kullanılması, kombinasyonun 17 suşun tümü üzerinde sinerjik bir etkiye sahip olduğunu, ancak sulbaktamın sadece incelenen konsantrasyonlarda bakterisidal bir etkiye sahip olmadığını gösterdi. Dama tahtası yöntemini uygularken, kolistin/sulbaktam kombinasyonunun suşların 17'sinde sinerjik bir etki (%85) ve 3 suşta (%15) ilave bir etkiye sahip olduğu, sulbaktamın tek başına düşük bir etkiye (%15) sahip olduğu belirlenmiştir ve kolistin tüm suşlarda etkiliydi.*

Sonuç: *Çalışma sonuçları, kolistin/sulbaktam kombinasyonunun her iki yöntem kullanılarak incelenen tüm suşlar üzerinde yüksek düzeyde sinerjik etkiye sahip olduğunu göstermiştir.*

Anahtar Kelimeler: *Acinetobacter baumannii; Kolistin; Sulbaktam; Sinerji; Time-kill; Checkerboard*

INTRODUCTION

Infections caused by the *Acinetobacter* spp. species, referred to as opportunistic infections, have become more widespread in recent years. *Acinetobacter* species cause various infections, including ventilator-associated pneumonia, bacteremia, meningitis, catheter-related bloodstream infections, urinary tract infections, and surgical area infections[1-3]. The ability of *Acinetobacter* species to survive in contained areas for long periods depends on its resistance to heat and pH fluctuations, as well as external environmental conditions[2,4,5]. These characteristics pave the way for outbreaks through intensive care personnel or materials of common use.

Acinetobacter spp. isolates are resistant to many antibiotics, as they have plasmids, transposons, and integrons, which include genes resistant to different antibiotics, low outer membrane permeability for some antibiotics, and efflux pumps[6]. Another concern about *Acinetobacter* spp. is they rapidly develop resistance and, thus, lead to resistance to multiple drug- resistant strains. Resistance can also develop to carbapenem group antibiotics, one of the most important treatment alternatives for these infections. These issues present challenges in the treatment of *Acinetobacter* infections[2,7,8]. The most common, most important, and last alternative drug in treatment for these infections in recent years has been colistin (Polymyxin E)[9]. Despite its side effects, colistin is used in treatment today due to the lack of alternative options[10]. However, it is recommended to avoid using colistin alone to treat these infections; combination treatments are preferred to prevent development of resistance[11,12]. The presence of *Acinetobacter* strains, which are also resistant to colistin as observed in recent years, points to the importance of these combination treatments[13,14].

Various studies in the literature documented research on the synergic effect of various antibiotic combinations with colistin against *Acinetobacter* strains[10,15-18]; a few in vitro studies have examined the synergic effect of the colistin/sulbactam combination using various methods (time-kill, checkerboard, prediffusion, and E-test, to name a few)[14,18-20]. This study involves an examination of the synergic effect of the colistin/sulbactam combination on *Acinetobacter baumannii/calcoaceticus* complex strains, isolated from various clinical samples, which are also resistant to carbapenem group antibiotics, using the time-kill and checkerboard methods.

MATERIALS and METHODS

Identification and Antibiotic Sensitivity of Strains

Twenty *Acinetobacter baumannii/calcoaceticus* complex strains resistant to carbapenem group antibiotics, isolated from various clinical samples sent to Sakarya University Training and Research Hospital Medical Microbiology Laboratory, were included in this study. Samples were collected between January 2016 and May 2017. 8 samples were obtained from chest diseases, 6 from internal medicine, 1 from infectious diseases, 1 from surgery and 4 from the intensive care unit. The identification testing of the isolates was carried out using matrix-assisted laser desorption ionisation time of flight mass spectrometry (VITEK MS, bioMerieux, Marcy l'Etoile, France). Antimicrobial susceptibility tests were analysed using the VITEK® 2 automated system (bioMerieux, Marcy l'Etoile, France) (Table 1).

Time-kill method

Seventeen carbapenem-resistant *Acinetobacter baumannii/calcoaceticus* complex strains were studied with the time-kill method. Mueller Hinton Broth (MHB) was used for antibiotic dilution in tubes, and Mueller Hinton Agar (MHA) was used

Table 1. Antibiotic sensitivity of strains used in this study identified using the VITEK 2® automated system

Strain	CS	IPM	MEM	FEP	TET	TZP	GM	SXT	AMP	AMC	AMİ	CAZ	TGC	CIP	LEV	SAM	SFP
1	S	R	R	R	R	R	S	R	R	R	R	R	_	R	R	_	_
2	S	R	R	_	R	R	R	R	R	R	R	R	S	R	R	R	R
3	S	R	R	R	R	R	S	R	R	R	S	R	I	R	R	R	R
4	S	R	R	R	_	R	S	R	R	R	S	R	S	R	_	R	R
5	S	R	R	R	R	R	R	R	_	_	R	R	I	R	R	R	R
6	S	R	R	R	R	R	S	S	_	_	R	R	_	R	I	R	R
7	S	R	R	R	R	R	S	S	_	_	S	R	I	R	R	R	R
8	S	R	R	R	R	R	R	R	_	_	R	R	_	R	R	_	_
9	S	R	R	R	R	R	R	R	R	R	I	R	_	R	_	_	_
10	S	R	R	R	_	R	R	R	R	R	S	R	_	R	_	_	_
11	S	R	R	R	I	R	S	R	_	_	S	R	S	R	R	R	R
12	S	R	R	R	R	R	R	R	_	_	S	R	S	R	R	R	R
13	S	R	R	R	S	R	R	S	_	_	R	R	I	R	I	R	R
14	S	R	R	R	S	R	S	R	R	R	R	R	_	R	_	_	_
15	S	R	R	R	I	R	S	R	_	R	R	R	I	R	R	R	R
16	S	R	R	R	R	R	S	R	_	_	S	R	S	R	R	R	R
17	S	R	R	R	R	R	R	R	_	_	S	R	S	R	R	R	R
18	S	R	I	I	R	R	R	R	_	_	R	R	S	R	R	R	S
19	S	I	R	S	I	I	S	S	_	_	S	S	S	R	I	R	S
20	S	R	R	R	R	R	R	R	_	_	R	R	S	R	R	R	I

S: Sensitive, I: Intermediate, R: Resistance, CS: Colistin, IPM: Imipenem, MEM: Meropenem, FEP: Sefepim, GM: Gentamicin, SXT: Trimethoprim/sulfamethoxazole, TET: Tetracycline, TZP: Piperacilin/Tazobactam, Amp: Ampicilin, AMC: Amoxicillin/Clavulanic Acid, AMI: Amikacin, CAZ: Ceftazidime, CIP: Ciprofloxacin, LEV: Levofloxacin, SAM: Ampicilin/Sulbactam, SFP: Cefoperazone/Sulbactam, TGC: Tigecycline.

for viable count. Bacterial suspension was configured to the 0.5 McFarland standard with the photometric method for every strain, so the final bacterial count was adjusted to $1x10^5$cfu/mL.

Antibiotic (colistin and sulbactam) MHB was prepared in concentrations two times high and two times low than the MIC value in current Clinical & Laboratory Standards Institute (CLSI) standards for colistin and sulbactam. For the final concentration, the first tubes were prepared to include 8 µg/mL colistin (4x-MIC), 16 µg/mL sulbactam (4x-MIC), and 8 µg/mL colsitin+16 µg/mL sulbactam, and the tubes were held subject to serial dilution. Every tube received a transfer of 100 µL bacteria from the bacteria suspension and left for incubation at 35-37°C. Right before the start of incubation (0 hour), 100 µL from each concentration of antibiotics and the control tube was transferred to the first tube, which included 900 µL 0.09% saline solution, and the other tubes (remaining 5) were held subject to serial dilution. Bacteria+antibiotic suspension was taken with 0.001 mL single use standard loop from every tube after serial dilution and cultured in the MHA. The same process was repeated at 3, 6, 12, and 24 hours. Reproduction in the passages in the MHA was evaluated after 16-18 hours of incubation at 35-37°C. If reproduction was present, the number of colonies was recorded. Taken into account were 30-300 with colony reproduction of the plates from six dilutions. When less than 30 reproductions were identified in all six plates, the number of colonies in the dilution free plate or the number of colonies in the plate from the first dilution was taken into account[21,22].

If there was 3 $\log_{10}$ and higher reduction in the bacteria count in the reading periods compared to the initial dilution ($1x10^5$), it was the bactericidal effective concentration of the antibiotic in the respective reading period. The numbers of colonies in every antibiotic concentration and reading period for every strain were logarithmically recorded.

Checkerboard Method

As a result of the calculations made according to the MICs of antibiotics for the study; 1 mg colistin, 62.5 mL and 1 mg sulbactam were diluted with 7.8 mL MHB (16 µg/mL, 128 µg/mL). 1 mL of each dilution was taken and subjected to serial dilution in tubes containing 1 ml MHB. 100 µL of each of these prepared dilution tubes were pipetted into the wells in the microplate. Later; The bacterial suspension prepared in MHB

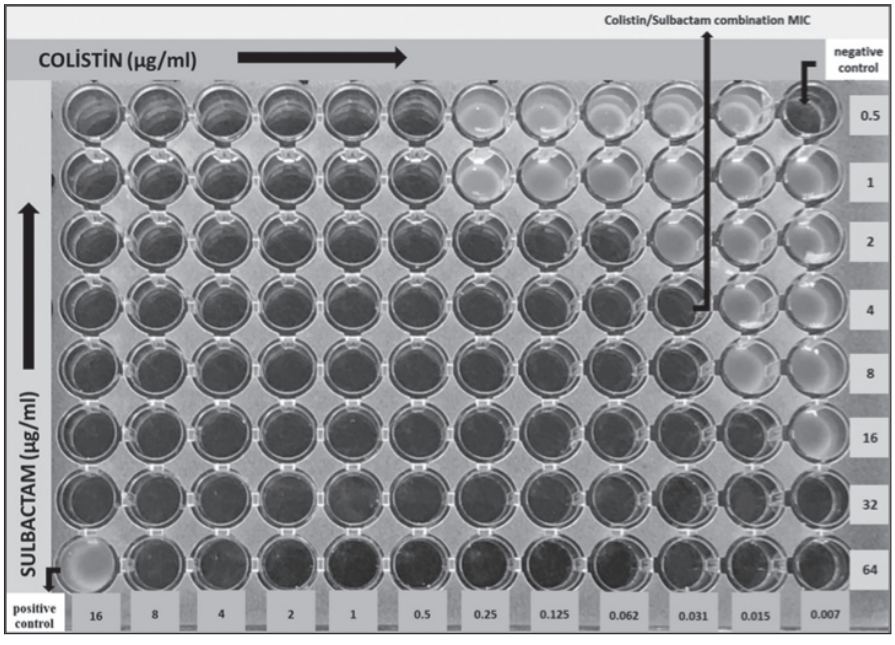


Figure 1. Colistin/sulbactam combination synergy study with checkerboard method.

was adjusted to 0.5 McFarland standard by photometric method, diluted 1/30 and 10 µL of this bacterial suspension was added to all wells in the microplate[37]. The results were evaluated after 18-20 hours of incubation at 35-37°C. MIC values of each strain were determined for colistin and sulbactam. MIC studies of 8 strains were performed in each microplate.

One microplate was used for combination/synergy (colistin + sulbactam) study with each strain. For the colistin and sulbactam combination study, 50 µL of the colistin dilutions were placed in microplate wells before the 1 mL antibiotic tubes, dilutions of which were prepared as described above. Colistin concentration in the microplate was adjusted from left to right in such a way that the titers gradually decreased. Then, 50 µL was added with increasing sulbactam concentration from top to bottom (A-B-C-D direction). Except for the negative control (sterility control) well, 10 µL of the bacterial suspension was added to all the wells in the microplate and incubated. Colistin and sulbactam MIC values were determined under combination conditions after incubation. While evaluating the synergistic effect in the study, it was evaluated using the methods determined by the Clinical and Laboratory Standards Institute (CLSI)[23].

Determination of Synergy

Using the checkerboard method, the fractional inhibitory concentration index (FICI) of all strains was calculated as follows: $FICI_{A/B}$ = ($MIC_{A(combination)}$/$MIC_{A\ (alone)}$) + ($MIC_{B(combination)}$/$MIC_{B\ (alone)}$). According to accepted criteria, the result of FICIA/B was recorded for each strain as follows: ≤0.5, synergy; > 0.5-≤1, additivity; > 1-≤4, indifference; and > 4, antagonism[20,24].

Using the time-kill method, 3 $\log_{10}$ and/or more reduction in the bacteria count in the same dilution and at the same hour compared to the colistin/sulbactam combination and colistin alone was evaluated as synergic.

Ethical Assessment

Approval was obtained from Sakarya University Faculty of Medicine Deanery Non-invasive Ethics Committee for our study.

RESULTS

A bactericidal effect was identified in all 17 strains on which the colistin/sulbactam combination synergic effect was studied with the time-kill method. In addition to 17 isolates studied with the Time Kill method, 3 strains isolated later on were studied only with the Checkerboard method due to technical inadequacies. Using the time-kill method, synergy was determined in 15 isolates (88.3%) at 3 hours and in 13 isolates (76.4%) at 12 hours in MIC/2 dilution. Colistin was the most effective at 6 hours. Bactericidal effect was observed in some reading periods, even in the colistin MIC/2 concentration. The $\log_{10}$-based colony numbers of colistin, sulbactam and colistin sulbactam according to the incubation times of 17 strains in the time kill method are given in Table 3 and Figure 2.

The MIC values of antibiotics alone and in combination in the strains included in the study determined with the checkerboard method are provided in Table 2. The colistin MIC value was lower than the values determined in the VITEK 2® automated system in three strains. Using this method, sulbactam alone was only effective on the MIC level (8 µg/mL) in 3 strains (15%) with the checkerboard method. A synergistic effect was found in 17 (85%) of 20 strains with the colistin/sulbactam combination. An additive effect was detected in 15% of the 3 strains with this method. The MIC mean value was 0.05 ± 0.71 for colistin and 4.6 ± 3.11 for sulbactam in the combination. In addition, MIC values and synergy findings also obtained with the checkerboard method and VITEK 2® system are summarised in Table 2.

DISCUSSION

Acinetobacter species are important pathogens that cause ventilator-associated pneumonia, bloodstream infections, and wound infections in immunosuppressed patients. Hospital infections caused by *Acinetobacter* spp. have gradually increased in recent years. *Acinetobacter calcoaceticus-Acinetobacter baumannii* (ACB) complex is the most frequent factor in this type of infection among *Acinetobacter* species. The most important problem in the treatment of these infections is that

Table 2. MIC (µg/mL) and FICI values of colistin and sulbactam of strains with the checkerboard and time kill method synergy data

Strain	VITEK 2® Colistin MIC	Colistin MIC	Combination Colistin MIC	Sulbactam MIC	Combination Sulbactam MIC	Synergy		FICI	Comment
						Time Kill	Checkerboard		
1	0.5	0.25	0.062	16	4	+	+	0.5	Sinergy
2	0.5	0.5	0.125	16	4	+	+	0.5	Sinergy
3	0.5	0.5	0.062	8	2	+	+	0.37	Sinergy
4	0.5	0.5	0.015	32	4	+	-	0.56	Additive
5	0.5	0.5	0.125	16	2	+	+	0.33	Sinergy
6	0.5	0.25	0.031	16	2	+	+	0.13	Sinergy
7	0.5	0.5	0.031	32	4	+	+	0.18	Sinergy
8	0.5	0.5	0.015	8	4	+	+	0.5	Sinergy
9	0.5	0.25	0.031	16	4	+	+	0.37	Sinergy
10	0.5	0.5	0.031	32	4	+	+	0.18	Sinergy
11	0.5	0.5	0.031	32	4	+	+	0.18	Sinergy
12	0.5	0.5	0.062	16	4	+	+	0.37	Sinergy
13	0.5	0.5	0.015	32	8	+	+	0.28	Sinergy
14	0.5	0.5	0.031	16	4	+	+	0.31	Sinergy
15	0.5	0.5	0.062	16	2	+	+	0.25	Sinergy
16	0.5	0.5	0.31	8	4	+	-	0.56	Additive
17	0.5	0.5	0.031	32	8	+	+	0.31	Sinergy
18	0.5	0.5	0.015	32	16	Not tested	-	0.53	Additive
19	0.5	0.5	0.031	16	4	Not tested	+	0.31	Sinergy
20	0.5	0.5	0.031	16	4	Not tested	+	0.31	Sinergy

FICI: Fractional inhibitory concentration index, MIC: Minimum inhibitory concentration.
If the total FIC index 0.5. it was evaluated as synergy, if 1 <FIC> 0.5 as additive. if> 1 as antagonist effect (Çıkman et al. 2013).

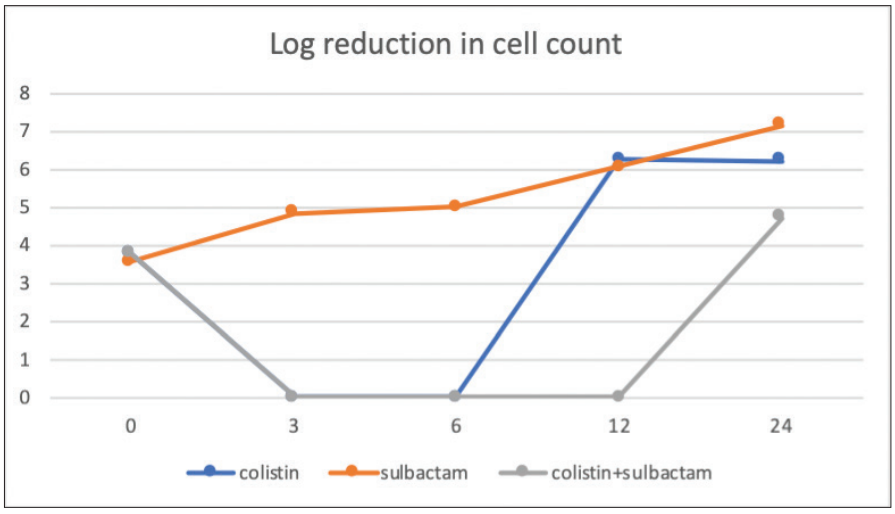


Figure 2. Change of $\log_{10}$-based colony numbers according to the incubation times of isolates in the time-killing method.

Table 3. For 17 strains, log_{10} based colony numbers of colistin, sulbactam and colistin + sulbactam in Time Kill method according to incubation periods

		Colistin				Sulbactam				Colistin + Sulbactam			
Isolate no	Time	4x	2x	x	x/2	4x	2x	x	x/2	4x	2x	x	x/2
1	**0.**	3.60	3.6	3.47	3.69	4.3	4.39	4.84	4.6	4	1.5	1.49	4
	3.	0	0	0	3.3	4.3	4.69	4.84	4.44	0	0	0	0
	6.	0	0	0	0	4.40	5	5.3	5.3	0	0	0	0
	12.	0	0	0	4.3	5	6.11	5.36	6.3	0	0	0	0
	24.	0	0	0	4.3	7.07	7.17	7.47	7.49	0	0	0	0
2	**0.**	4.07	4.07	4.07	4.07	4.17	4	3.95	4.07	4.07	4.25	4.32	4.39
	3.	0	0	0	0	5.3	4.9	4.84	4.5	0	0	0	0
	6.	0	0	0	0	5.2	5.23	6.17	5.9	0	0	0	0
	12.	0	0	0	3	6.17	5.9	5.9	5.6	0	0	0	0
	24.	0	0	3.2	3.39	6.77	7.07	7.07	6.9	0	0	4.07	3.77
3	**0.**	3.77	3.77	3.73	3.71	4.9	5.11	5.13	5.14	4.07	4.07	4.11	4.13
	3.	0	0	0	3.51	4.73	4.69	4.79	4.84	0	0	0	0
	6.	0	0	0	0	4.72	4.99	5.17	5.26	0	0	0	0
	12.	0	0	0	4.26	6.25	6.29	6.6	6.72	0	0	0	0
	24.	0	0	0	4.38	6.95	7.25	7.33	8.9	0	0	0	4.12
4	**0.**	3.9	3.92	3.91	3.9	4.17	4.3	5.09	4.89	4.25	4.25	4.26	4.28
	3.	0	0	0	3.27	5.04	5.11	5.14	5.25	0	0	0	0
	6.	0	0	0	0	5	5.03	5.17	5.14	0	0	0	0
	12.	0	0	0	4.2	5.25	5.5	5.6	6.43	0	0	0	0
	24.	0	0	0	4.32	6.9	7.14	8.28	7.79	0	0	0	5.14
5	**0.**	5.8	5.14	5.13	5.17	5.25	4.14	5.13	5.07	3.95	3.97	3.96	4.04
	3.	0	0	0	4.09	4.31	4.36	5.44	5.44	0	0	0	0
	6.	0	0	0	4.14	5.21	5.28	5.39	6.42	0	0	0	0
	12.	0	0	0	4.04	5.74	5.97	5.98	6.44	0	0	0	4.06
	24.	3.65	3.81	3.94	5.16	6.92	7.19	7.3	8.2	0	3.3	4.07	4.18
6	**0.**	3.39	3.47	3.6	3.6	3.69	3.65	3.65	3.6	3.47	3.5	3.54	3.6
	3.	0	0	0	2.9	3.65	3.77	4.14	4.07	0	0	0	0
	6.	0	0	0	0	4	4.06	4.25	4.3	0	0	0	0
	12.	0	0	0	3.3	4.95	4.95	6.9	6.96	0	0	0	3.65
	24.	0	0	3.9	5.4	5.3	5.68	6.86	7.79	0	4.39	6.07	7.26
7	**0.**	4	4.11	4.07	4.3	3.84	4.2	4.07	3.9	3.9	4	4	4.07
	3.	0	0	0	5.07	4.14	4.36	4.38	4.77	0	0	0	0
	6.	0	0	0	6.38	5.44	5.9	6.27	7.36	0	0	0	0
	12.	0	0	0	6.44	7.3	8.02	8.39	8.65	0	0	0	6.8
	24.	3.9	5.04	7.16	7.14	7.92	8.25	8.27	8.77	3.25	4.16	4.34	7.25
8	**0.**	3.6	3.61	3.77	4	3.65	3.65	3.57	3.74	3.65	3.66	3.77	3.55
	3.	0	0	0	3.81	4.25	4.34	4.84	5.4	0	0	0	0
	6.	0	0	0	3.95	6.3	6.27	5	5.97	0	0	0	0
	12.	0	0	6.25	6.3	6.35	6.4	6.07	7.13	0	0	0	5.21
	24.	5.3	6.02	6.2	6.4	6.68	6.99	7.15	8.07	0	4.39	4.7	5.3

Table 3. For 17 strains, log_{10} based colony numbers of colistin, sulbactam and colistin + sulbactam in Time Kill method according to incubation periods (continue)

		Colistin				Sulbactam				Colistin + Sulbactam			
Isolate no	Time	4x	2x	x	x/2	4x	2x	x	x/2	4x	2x	x	x/2
9	**0.**	3.6	3.54	3.57	3.69	3.6	3.69	3.64	3.69	3.5	3.73	3.6	3.85
	3.	0	0	0	0	4.2	4.25	4.44	4.63	0	0	0	0
	6.	0	0	0	0	5	5.04	5.14	5.25	0	0	0	0
	12.	0	0	0	3.9	5.07	5.25	5.69	5.92	0	0	0	0
	24.	0	0	3.44	4.25	5.59	5.99	6.83	7.06	0	0	0	3.27
10	**0.**	4.44	4.43	4.38	4.38	4	4.07	3.9	4.07	4.07	4.07	3.95	3.84
	3.	0	0	0	3.15	4.34	4.39	4.45	4.91	0	0	0	0
	6.	0	0	0	4.14	5.25	5.36	5.39	5.39	0	0	0	0
	12.	0	0	0	5.22	5.6	5.81	6.07	6.84	0	0	0	0
	24.	0	0	5.3	6.34	5.95	6.84	6.97	8.14	0	0	4.17	5.25
11	**0.**	3.6	3.51	3.54	3.6	4.07	3.5	3.51	3.69	3.95	3.6	3.6	3.47
	3.	0	0	0	0	4.34	4.44	4.54	4.65	0	0	0	0
	6.	0	0	0	3.6	4.69	5.25	5.27	5.3	0	0	0	0
	12.	0	0	0	4.07	4.54	4.94	7.87	7.9	0	0	0	0
	24.	0	0	5.14	7.03	7.25	7.3	8.65	8.71	0	0	5.07	7.03
12	**0.**	4.17	4.25	4.29	4.3	4.07	4.09	4.11	4.16	3.84	3.81	3.85	3.84
	3.	0	0	0	3.65	4.16	4.3	5.39	5.99	0	0	0	0
	6.	0	0	0	4.19	5.9	6.27	6.95	7.11	0	0	0	0
	12.	0	0	0	4.26	5.95	7.25	6.92	7.04	0	0	0	0
	24.	0	0	4.86	5.65	6.95	6.96	7.01	7.3	0	0	3.54	4.07
13	**0.**	3.95	4	3.97	3.69	3.77	3.9	3.84	3.92	3.84	3.95	3.87	4
	3.	0	0	0	0	4.25	4.38	5.39	6.16	0	0	0	0
	6.	0	0	0	0	4.92	6.25	6.92	7.14	0	0	0	0
	12.	0	0	0	5.26	5.92	6.28	7	7.2	0	0	0	0
	24.	0	0	4.16	6.43	5.94	6.98	8.01	8	0	0	3.61	5.71
14	**0.**	3.68	3.65	3.7	3.57	3.94	4.06	4.14	4.12	3.57	3.69	3.72	3.77
	3.	0	0	0	0	4.14	4.38	4.82	5.04	0	0	0	0
	6.	0	0	0	0	5.09	6.3	6.39	6.73	0	0	0	0
	12.	0	0	0	0	5.17	6.14	6.31	6.27	0	0	0	0
	24.	0	0	0	3.97	6.18	6.14	6.99	7.03	0	0	3.27	4.94
15	**0.**	3.65	3.69	3.86	3.85	4.19	4.16	4.2	4.27	4.16	4.17	4.18	4.25
	3.	0	0	0	0	4.3	4.46	5.3	5.25	0	0	0	0
	6.	0	0	0	0	4.68	4.9	5	5.07	0	0	0	0
	12.	0	0	0	3.92	5.49	5.9	6	6.13	0	0	0	0
	24.	0	0	3.46	4.76	5.77	7.14	7.21	7.3	0	0	3.98	4.79
16	**0.**	3.99	4.07	4.12	4.15	4.07	3.89	4.03	3.94	4	3.5	3.49	3.69
	3.	0	0	0	4.07	4.34	4.44	4.54	4.65	0	0	0	0
	6.	0	0	0	3.54	4.69	5.25	5.27	5.34	0	0	0	0
	12.	0	0	0	4.99	4.54	5.97	6.69	6.9	0	0	0	0
	24.	0	3.23	4.65	5.27	7.25	7.3	7.65	8	0	0	3	3.74

Table 3. For 17 strains, log_{10} based colony numbers of colistin, sulbactam and colistin + sulbactam in Time Kill method according to incubation periods (continue)

		Colistin				Sulbactam				Colistin + Sulbactam			
Isolate no	Time	4x	2x	x	x/2	4x	2x	x	x/2	4x	2x	x	x/2
17	**0.**	3.95	4	3.97	4.04	3.77	3.91	3.84	3.96	3.84	3.95	3.87	4
	3.	0	0	0	5.65	4.25	5.3	5.39	6.43	0	0	0	0
	6.	0	0	0	6.26	5.9	7.25	6.9	7.11	0	0	0	0
	12.	0	0	0	7.43	6	7.27	6.92	7.14	0	0	0	0
	24.	0	0	7.16	8.02	6.94	6.96	8.01	8	0	0	4	5.3

X: MIC value of the drug (colistin: 2 µg/mL, sulbactam: 2 µg/mL), X/2: Half of MIC value, 2X: 2 times MIC value, 4X: 4 times MIC value.

most strains are resistant to many antibiotics, including carbapenem antibiotics, and do not provide adequate treatment as a result of decreased sensitivity to existing antibiotics[25]. Emergence and dissemination of infectious resistant bacteria have become a huge concern for clinicians. As a result, clinicians are seeking new treatment options. Combined antibiotic use is recommended to succeed in treatment of MDR ACB complex infections and prevent development of resistance[26,27]. Kengkla et al. reported in a review article that colistin/sulbactam combination treatment was superior to, and, in terms of side effects, similar to, colistin monotherapy. They also stated this combination could be used in treatment of MDR and XDR-B infections[28].

In the literature, the time-kill and checkerboard methods were generally used in combination or alone in a few in vitro studies on the synergic effect of colistin/sulbactam on carbapenem-resistant ACB complex isolates[29,30]. In this study, synergic effect on the same strains was studied using these two methods also.

The E-test method (gradient antibiotic strips) was used in many of the studies[20]. The reason for using the E-test method is possibly the methodological ease of use. Diffusion tests are not recommended, and the need for MIC control is emphasised in studies conducted with colistin[23,32]. The prediffusion method was used in the studies conducted with the E-test method. No matter how standardised this method is, problems may occur with commercial gradient tests and the respective method.

The fact that colistin does not remain stable for long should also be noted. In this study, colistin lost its effect after 12 hours with the time-kill method. It is already supported by half-life and treatment doses. Another important consideration is the form of colistin to use in in vitro studies. Current guidelines emphasise colistin sulphate use should be taken as reference, and colistimethate sodium (also called colistin methylsulphonate, pentasodium colistimethan sulphate, and colistin sulphonylmethate) should not be used in in vitro studies[23,32].

In some synergy studies, sulbactam was not studied alone, and an ampicillin/sulbactam combination was used for the same purpose[33,34]. It is obvious such use of sulbactam will not be suitable for colistin/sulbactam synergy studies. Because ampicillin is also used in combination with colistin, there will only be a threefold combination.

Considering studies conducted with the time-kill method only, Lee et al. reported synergism for the colistin/sulbactam combination[29]. Pongpech et al. determined synergy against 96.7% of MDR *A. baumannii* of the threefold combination of meropenem/sulbactam/colistin, while they obtained 70%, 73.3%, and 53.3% synergic effect for meropenem/sulbactam, meropenem/colistin, and colistin/sulbactam, respectively[30]. Laishram et al. reported 100% bactericidal effect in lower respiratory tract samples and 96% in blood samples in colistin/sulbactam combinations with the time-kill method and 36% synergy and 64% additive effect in colistin/sulbactam combination with the checkerboard method[11]. In this study results indicated

100% bactericidal effect and 88.2% synergy at 3 hours and 76.4% at 12 hours with the time-kill method in all clinical sample types. The reason for not observing synergy at 6 hours might be the colistin sensitivity in all isolates and very low MIC levels. The situation observed in two strains without synergy determined at three hours was evaluated similarly. Synergy was not determined in colistin concentrations higher than MIC/2, as the strains were sensitive to colistin and had low MIC values. In other words, whether sulbactam has any contribution cannot be determined, as colistin is effective against these strains, even in very low concentrations. Bactericidal effect was not determined in the studied concentrations of sulbactam alone with the time-kill method.

Thamlikitkul et al. did not determine synergy in the colistin sensitive strains in the colistin/sulbactam combination. However, they reported synergy in colistin resistant strains[35]. Deveci et al. in their study conducted with the checkerboard method obtained 50% synergy and 50% additive effect for the colistin/sulbactam combination in the *Acinetobacter baumannii* strains isolated from clinical samples (with no information reported on carbapenem sensitivity)[16]. Percin et al. reported 50% synergy in the colistin/sulbactam combination with the checkerboard method in a study they conducted on colistin-resistant *Acinetobacter baumannii* strains[17]. Marie et al. reported 29% synergy and 38.9% partial synergy in the colistin/sulbactam combination with the checkerboard method in a study they performed on MDR *Acinetobacter baumannii* strains[18]. Dong et al. did not report colistin/sulbactam combination synergy with the checkerboard method in MDR *Acinetobacter baumannii* strains[15]. In this study, in the checkerboard method, it was determined that the colistin/sulbactam combination was synergistic in 17 (85%) strains, additive in 3 strains (15%), and sulbactam alone (15%) was low. Colistin was effective in all strains. Considering the results obtained with the checkerboard method in our study, a higher synergy was observed compared to other studies. As there are limited publications in this field, with more isolates and multicentred, in vivo and in vitro studies, if possible, are needed.

Anandan et al. reported 96% bactericidal effect and 68% synergy for the colistin/sulbactam combination with the time-kill method and 16% synergy and 84% ineffective for the colistin/sulbactam combination with the checkerboard method in MDR *Acinetobacter baumannii* strains[24]. In the study of Yılmaz et al. (2015) in which patients undergoing VAP treatment for MDR and XDR *A. baumannii* were included, the results of colistin, sulbactam colistin and carbapenem colistin treatment were evaluated. A total of 17 patients (24.3%) were administered colistin alone, 20 patients (28.6%) were administered colistin and sulbactam, and 33 patients (47.1%) were administered colistin and carbapenem. Clinical and microbiological response rates were higher in the carbapenem combination group (63.6% and 63.6% in both) than in the sulbactam combination group, which registered 55.0% and 60.0%, respectively. As a result of the study, no significant difference was found between colistin alone and combination groups regarding clinical and microbiological efficacy and mortality[38]. In the study of Kalin et al. (2014), 89 patients diagnosed with VAP were worked. Colistin was given to 58.4% of them, while colistin combined with sulbactam was given to 41.6% patients. On the 5. day of treatment, the clinical reaction rate was 40.4% in the colistin group and 43.2% in the combined group. As a result of the treatment, the clinical response rate was 29.8% and 40%, and the microbiological response rate was 72.3% and 85.7%, respectively. It was reported that the clinical response and bacteriological cure rates were better in the sulbactam-colistin group, but the difference was not statistically significant[39].

Clinical studies researching the effect of combination therapy with according to clinical-microbiological response, and mortality have been limited, and there is no consensus. In our study, we investigated the effect of colistin and sulbactam combined as in vitro. One of the limitations of our study was the inability to perform in vivo synergy tests.

In conclusion, it was determined sulbactam was solely effective on a low (15%) MIC level against ACB complex strains and did not have

a bactericidal effect, while colistin was effective on all strains. It was observed that the colistin/sulbactam combination had a synergic effect on many of the evaluated strains using both methods (time-kill and checkerboard). Synergy studies on colistin in combination with other antibiotics should be conducted on antibiotic-resistant or high MIC strains. However, in vitro studies should be accompanied by in vivo studies. Current studies in the literature produced different results. Therefore, additional studies are needed in which a higher number of isolates and concurrency with in vivo studies are demonstrated.

ETHICS COMMITTEE APPROVAL

Approval was obtained from Sakarya University Faculty of Medicine Deanery Non-invasive Ethics Committee for our study (Decision no: 176).

CONFLICT of INTEREST

The authors declare that they have no conflict of interest.

AUTHORSHIP CONTRIBUTIONS

Conception/Design: MK

Data Acquisition: İK, MK

Analysis/ Interpretation: HH, ÜK, İK

Writing: EPKK, İK

Final Approval: MA, MK

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