Current Microbiology (2026) 83:202
https://doi.org/10.1007/s00284-026-04795-4

In Vitro Antimicrobial Activities of Carbapenem-beta Lactamase Inhibitor Combinations Against Carbapenem-resistant Gram-negative Pathogens Isolated from Türkiye

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Received: 5 July 2025 / Accepted: 13 February 2026
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Abstract

The global incidence of carbapenem resistance is increasing due to the presence of beta-lactamases such as carbapenemases. This study evaluated the in vitro antimicrobial activities and metabolic activities of biofilm cells of two carbapenem beta-lactamase inhibitor combinations, imipenem-cilastatin+relebactam and meropenem+vaborbactam, against imipenem and/or meropenem resistant, metallo-beta-lactamase negative clinical isolates of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Enterobacter* spp. Both combinations demonstrated antibacterial activity, particularly against *P. aeruginosa* and *E. coli*. MBCs were found higher than MICs against all microorganisms. Imipenem-cilastatin+relebactam was the most effective agent against *K. pneumoniae* and *Enterobacter* spp. biofilms, while meropenem-vaborbactam showed greater activity against *P. aeruginosa*, *A. baumannii*, and *E. coli* biofilms. This study presents new results by characterizing the antibiofilm activity of these combinations for the first time in clinical isolates from Türkiye. Synergy with colistin was observed in selected isolates, and no antagonist effect was detected in any of the strains studied. Therefore, the study results suggest that imipenem-cilastatin+relebactam and meropenem+vaborbactam combinations, alone or in combination with colistin, could play an important role in the treatment of carbapenem-resistant Gram-negative bacteria, including biofilm-associated diseases.

Introduction

Antibiotics are crucial drugs for treating infectious diseases. However, the widespread use of existing antibiotics in both medicine and agriculture has led to antibiotic resistance. In addition, since the discovery of new antibiotics has decreased, treatment options for serious infections have diminished due to multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria. This situation has led to a significant rise in disease burden, mortality, and economic losses on health systems all over the world [1, 2]. *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Enterobacter* species, members of the ESKAPE group, constitute the majority of the causes of hospital-acquired infections. After the World Health Organization published the "List of bacteria that urgently need new antibiotics" and declared ESKAPE microorganisms as priority pathogens for pharmaceutical companies, the importance of both these pathogens and antimicrobial resistance has been further emphasized [3]. For these reasons, it is necessary to establish resistance surveillance systems, implement resistance management practices, and control the use of antibiotics in both medicine and agriculture in order to combat this important health threat. One of the most important strategies is the development of new and effective antibiotics. However, in recent years, very few new antibiotics have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of infectious diseases.

Although carbapenems are frequently used in the treatment of these bacteria, the global incidence of carbapenem resistance is increasing due to the presence of beta-lactamases

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Published online: 21 February 2026

such as carbapenemases. Due to the insufficient activity of beta-lactamase inhibitors such as tazobactam and clavulanic acid against carbapenemases, relebactam and vaborbactam were synthesized as the first beta-lactamase inhibitors for use in combination with carbapenems [4]. Imipenem-cilastatin+relebactam and meropenem-vaborbactam have been approved and stand out as important combinations, particularly effective against Gram-negative bacteria [5]. These agents demonstrate potency exclusively against serine beta-lactamases, but they are inactive against metallo-beta-lactamases (MBL) [6].

Bacteria can exist in nature both as single, independent cells (planktonic) and as part of microbial communities (biofilms). Biofilms can form when bacteria adhere to a non-living surface such as an implant, or it can cause chronic infections by adhering to living tissues. A biofilm is defined as a community formed by various types of bacteria and fungi adhering to a surface, interface, or to each other. Biofilm formation causes the infection to become chronic and makes treatment difficult. Therefore, when investigating the potential of antibiotics in treating infections, it may be important to evaluate their activity not only against planktonic bacteria but also against biofilms [7, 8].

Due to the prevalence of MDR bacteria, colistin has been reintroduced as a last resort antibiotic and has become a widely used. However, it has some disadvantages such as dosing problems and nephrotoxicity [9]. In addition, reports indicate that colistin resistance is also becoming widespread [10, 11]. Therefore, colistin combinations can be used in the treatment of Gram-negative infections both to prevent resistance and to reduce toxicity [10, 12]. Despite the limited availability of robust evidence, colistin-carbapenem combination therapies are often preferred in clinical practice for the treatment of carbapenem-resistant Gram-negative bacterial infections [13].

In this context, our study aimed to determine the effectiveness of imipenem-cilastatin+relebactam and meropenem+vaborbactam on Gram-negative bacteria, to obtain antibiotic resistance data, and to investigate their combinations with colistin as well as their effectiveness against biofilms.

Materials and Methods

Antimicrobial Agents

Imipenem-cilastatin (Koçak Farma İlaç ve Kimya Sanayi A.Ş., Türkiye), meropenem (Sigma Aldrich, USA), relebactam (Selleck Biotechnology GmbH, Germany), vaborbactam (Cayman Chemical Company, USA) and colistin (Sigma Aldrich, USA) were purchased from their manufacturers. All agents, with the exception of colistin, were prepared on the day of the experiment using the appropriate solvents. Stock solutions of colistin were prepared from dry powder at a concentration of 2560 mg/L in water and stored frozen at −80 °C, and the frozen solutions were used within 6 months.

Clinical Isolates

A total of 96 carbapenem-resistant Gram-negative bacterial isolates, resistant to imipenem and/or meropenem -including 36 *A. baumannii*, 33 *K. pneumoniae*, 13 *P. aeruginosa*, 8 *Escherichia coli* and 6 *Enterobacter* spp. were collected from various specimens including blood, urine, sputum, abscess, tracheal aspirate, and bronchoalveolar lavage fluid at Synevo Laboratories Ankara Central Laboratory in Türkiye (2020–2021). The sources of the isolates and their antibiotic susceptibilities by disk diffusion test are given as supplementary material (SM-1). Identification of the strains was performed using the Vitek 2 (BioMérieux, France), API 20 E, and API 20 NE (BioMerieux, France) systems. Before analyses, each isolate was cultured on Tryptic Soy agar (TSA, Difco Sparks, MD, USA) plates to ensure viability. A flowchart showing the entire study process is attached as supplementary material (SM-2).

Detection of MBL Production

"Meropenem-EDTA double-disk synergy test" was applied to determine the presence of MBL enzyme. Bacterial suspensions were prepared by selecting similar colonies from an overnight culture to the density of a McFarland 0.5 standard, approximately corresponding to 1–2 × 10^8 colony forming units (cfu)/mL and these suspensions were applied on Mueller-Hinton agar (Difco Sparks, MD, USA). Two meropenem disks were placed on the inoculated agar surface, one of which was combined with 0.5 M EDTA solution. Plates were incubated at 37 °C for 18–24 h. After incubation, a difference of 7 mm or more in the zone diameters of the meropenem disk and the meropenem-EDTA disk was considered MBL positive [14]. MBL positive strains were excluded from the study and experiments were continued with MBL negative strains (n = 44). The experiments were repeated twice.

The Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) of Carbapenem+Beta Lactamase Inhibitors

The susceptibilities of carbapenem-beta lactamase inhibitor combinations (imipenem-cilastatin+relebactam and meropenem+vaborbactam) against 44 MBL negative strains

and two reference strains, *E. coli* ATCC 25,922 and *P. aeruginosa* ATCC 27,853, were determined using the broth microdilution method recommended by EUCAST [15]. The inoculum was prepared using overnight cultures of the bacteria in a final concentration of 1×10^6 cfu/mL. Two fold serial dilutions of the antimicrobials, with a range of concentrations of 64-0.03.03 mg/L were tested in antimicrobial activity studies. Relebactam concentration of 4 mg/L and vaborbactam concentration of 8 mg/L were kept constant in all wells. Plates were incubated at 37 °C for 24 h. The MIC was defined as the lowest antimicrobial concentration that completely inhibited the visible growth of microorganisms.

In order to determine the MBC values of carbapenem-beta lactamase inhibitor combinations, 10 µl samples from each well that demonstrated no visible growth were applied to the surface of TSA and incubated at 37 °C for 24 h. MBC was defined as the lowest antimicrobial concentration that killed 99.9% of the inoculum [16]. The experiments were repeated at least twice. The study was continued with strains with imipenem-cilastatin+relebactam and/or meropenem+vaborbactam MIC values < 64/4 mg/L and < 64/8 mg/L, respectively ($n = 28$).

Antibiofilm Effects of Carbapenem+Beta Lactamase Inhibitors

The biofilm-forming capacities of the 28 selected strains were evaluated using the crystal violet assay [17], and all isolates were identified as strong biofilm producers. Overnight cultures of the 28 bacterial isolates: *K. pneumoniae* (4), *P. aeruginosa* (9), *A. baumannii* (2), *E. coli* (8), *Enterobacter* spp. (5) were diluted to 1×10^6 cfu/mL in Brain Heart Infusion broth (BHIB, Thermo Scientific, Oxoid, USA) and transferred 100 µL each to 96-well flat bottom polystyrene microplates. The plates were incubated at 37 °C for 24 h to allow biofilm formation. After incubation, the non-adherent bacteria (planktonic cells) from the wells were removed and the wells were gently washed with sterile phosphate-buffered saline. Three different concentrations of imipenem-cilastatin (1, 2 and 4 mg/L) were combined with relebactam (4 mg/L), and three different concentrations of meropenem (4, 8 and 16 mg/L) were combined with vaborbactam (8 mg/L) and added to the wells. The plates were incubated for a further 24 h and after incubation the wells were washed again and assayed using the 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) solution to measure the metabolic activity of viable cells in biofilms. The MTT solution was prepared at a concentration of 0.5 mg/mL in PBS, added to each well and the plates were incubated at 37 °C in the dark for 3 h. After incubation, supernatant was removed and DMSO was added to the wells to dissolve the dye. The optical densities of the wells were determined at a wavelength of 550 nm in an ELISA microplate reader (EON, Biotek) and evaluated by comparison with the control [18].

Combination of Colistin and Carbapenem+Beta Lactamase Inhibitors

Interactions between colistin and carbapenem-beta lactamase inhibitor combinations (imipenem-cilastatin+relebactam and meropenem+vaborbactam) were tested with checkerboard technique against 28 bacterial isolates, which imipenem-cilastatin+relebactam and/or meropenem-vaborbactam MIC values were lower than 64/4 mg/L and 64/8 mg/L, respectively. Each microtiter well, containing a mixture of colistin and imipenem-cilastatin+relebactam or meropenem+vaborbactam was inoculated with fresh culture. Relebactam at a concentration of 4 mg/L, and vaborbactam and colistin at concentrations of 8 mg/L were kept constant in all wells, while carbapenems were tested at final concentrations ranging from 2xMIC to 1/8xMIC. Plates were incubated at 37 °C for 18–24 h. After incubation, the FIC (fractional inhibitory concentration) index was calculated using the following expressions: FICA = (MICA in combination)/(MICA alone), FICB = (MICB in combination)/(MICB alone), and the FIC index = FICA + FICB. The combination value was determined from the highest dilution of the antimicrobial combination that allowed no visible growth. Using this method, FIC index was interpreted as follows: ≤ 0.5 was synergy, > 0.5–4.5 was no interaction, and > 4.0 was antagonism [19, 20].

Time Kill Assays

Time kill assays were monitored to observe the dynamic killing kinetics of the antimicrobial agents at MIC values, according to the results of the microbroth checkerboard test, which showed significant synergy or low FIC index values. Suspensions of the selected bacterial isolates were incubated with antimicrobials alone or their combinations at 37 °C with gentle shaking. Samples were collected at 0, 2, 4, 6 and 24 h of incubation and the viable counts were determined. An antimicrobial-free control was included for each strain. The effects of the combination were analysed in comparison to the most active agent alone. Synergy was defined as a decrease of 2 log cfu/mL in colony counts after 24 h. The definition of bactericidal activity was based on a decrease of ≥ 3 log cfu/mL from the initial inoculum [21].

Statistical analysis

Data were statistically interpreted using GraphPad Prism 9.0 (GraphPad Software, San Diego, USA). Biofilm inhibition

Table 1 Distribution of MBL negative and positive strains

	MBL Negative	MBL Positive
K. pneumoniae	45% ($n=15$)	55% ($n=18$)
P. aeruginosa	69% ($n=9$)	31% ($n=4$)
A. baumannii	17% ($n=6$)	83% ($n=30$)
E. coli	100% ($n=8$)	0 ($n=0$)
Enterobacter spp.	100% ($n=6$)	0 ($n=0$)
Total	45.83% ($n=44$)	54.16% ($n=52$)

results were compared using either Kruskal-Wallis followed by Dunn post-hoc test or one-way ANOVA followed by Dunnett post-hoc test according to the normality of data distribution. The significance level was set at $p<0.05$.

Results

MBLs Production Rates

According to "Meropenem-EDTA double-disk synergy test", 44 of the 96 strains were found to be MBL negative (Table 1 and Supplementary material (SM-3)). Further experiments were continued with MBL negative strains.

MIC and MBC Results of Carbapenem + Beta-Lactamase Inhibitors

The MIC values (mg/L) of imipenem-cilastatin combined with a fixed relebactam concentration of 4 mg/L were 0.125–8.125 against *P. aeruginosa*, 0.25 -< 64 against *A. baumannii*, 1 -< 64 against *K. pneumoniae*, 0.06–1.06 against *E. coli* and 4-<64 against *Enterobacter* spp. The MIC values of meropenem combined with a fixed vaborbactam concentration of 8 mg/L were found in the range of 0.125–8.125, 0.5 -<64, 4-<64, 1–32 mg/L against *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*, and *Enterobacter* spp., respectively. *E. coli* strains were found to be more sensitive to meropenem-vaborbactam combination and MIC ranges were 0.03/8−1/8 mg/L (Table 2). While the MIC values (mg/L) of imipenem-cilastatin+relebactam combination against quality control strains were determined as 0.25/4 against *E. coli* and 0.5/4 against *P. aeruginosa*, meropenem+vaborbactam combination MIC values (mg/L) was determined as 0.03/8 for *E. coli* and 0.5/8 for *P. aeruginosa*. Number of strains according to MIC values were also shown as supplementary material (SM-4).

According to the MBC results, imipenem-cilastatin+relebactam combination did not demonstrate bactericidal effect against *P. aeruginosa* and *K. pneumoniae* strains at any concentration studied and the MBC values were found higher than the MIC values against all microorganisms. The MBC values of the combinations were shown in Table 2. The study was continued with strains that had imipenem-cilastatin+relebactam and/or meropenem+vaborbactam MIC values below 64/4 mg/L and 64/8 mg/L, respectively.

Antibiofilm Activity Results of Carbapenem + Beta-Lactamase Inhibitors

The combinations of imipenem and meropenem with beta lactamase inhibitors were tested against 28 isolates by the MTT method and the results were shown in Fig. 1. To provide a more detailed and transparent presentation of the biofilm absorbance data, the complete quantitative results derived from three independent biological replicates are presented in Supplementary Table S5 (SM-5). According to the results, imipenem-cilastatin+relebactam combination was statistically significant for *K. pneumoniae* ($p<0.05$). Imipenem-cilastatin+relebactam decreased the viability of all bacteria compared to the control, mostly at the concentration 4/4 mg/L. In contrast, meropenem+vaborbactam combination was found to be effective only for meropenem at the concentration of 16/8 mg/L, with the expectation of *Enterobacter* spp.

Checkerboard Results

The efficacy of colistin in combination with carbapenem+beta lactamase inhibitors against 28 strains was investigated using the checkerboard technique, and the FIC indices were shown in Table 3. According to our results, both colistin+imipenem-cilastatin+relebactam and colistin+meropenem+vaborbactam combinations showed synergistic effect against one *K. pneumoniae* (**KP3**) strain. An additional synergistic effect was observed with

Table 2 MIC and MBC values of carbapenem+beta-lactamase inhibitors (mg/L)

	Imipenem-cilastatin+relebactam (relebactam fixed at 4 mg/L)		Meropenem+vaborbactam (vaborbactam fixed at 8 mg/L)	
	MIC	MBC	MIC	MBC
P. aeruginosa	0.125–8	< 64	0.125–8	1 - < 64
A. baumannii	0.25 - < 64	1 - < 64	0.5 - < 64	2 - < 64
K. pneumoniae	1 - < 64	< 64	4 - < 64	32 - < 64
E. coli	0.06–1	0.25- < 64	0.03–1	0.125-16
Enterobacter spp.	4 - < 64	16 - < 64	1–32	4 - < 64

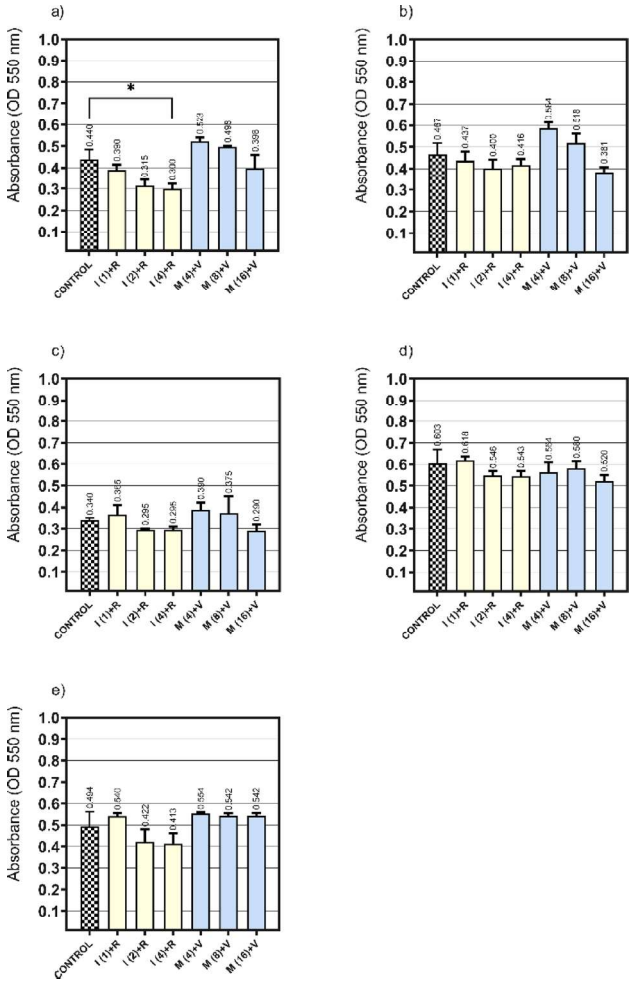

Fig. 1 Antibiofilm activities of carbapenem+beta-lactamase inhibitor combinations. Absorbance values were measured at OD 550 nm to assess the metabolic activities of bacteria within biofilms following exposure to three different concentrations of imipenem-cilastatin (1, 2, and 4 mg/L) combined with relebactam (4 mg/L) and three different concentrations of meropenem (4, 8, and 16 mg/L) combined with vaborbactam (8 mg/L). For each strain, biofilm assays were performed in three independent biological replicates, and mean absorbance values were calculated. The bars represent strain-level mean ± SD values. **a**) *K. pneumoniae* ($n=4$); **b**) *P. aeruginosa* ($n=9$); **c**) *A. baumannii* ($n=2$); **d**) *E. coli* ($n=8$); **e**) *Enterobacter sp.* ($n=5$) Statistical analysis was performed on a strain-by-strain basis using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test. Asterisks indicate levels of statistical significance as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. IR: imipenem-cilastatin+relebactam, MV: meropenem+vaborbactam

colistin+meropenem+vaborbactam combination against one *Enterobacter* sp. (**EB1**) strain. No antagonist effect was observed in any of the strains studied.

Time Kill Assay Results

The time kill assays were monitored against 4 bacteria (KP3, EB1, PA4, and EC3). According to the results obtained, colistin showed bactericidal activity against all four strains studied. The colistin+meropenem+vaborbactam combination showed early synergist activities against **KP3** and **EB1** strains at 4 and 6 h, and an early synergist activity against **PA4** strain at 4 h. Colistin+imipenem-cilastatin+relebactam combination showed an early synergist effect at the 6 h and an additive effect at 24 h against **EC3** strain. The results were shown in Fig. 2.

Table 3 FIC index obtained with combinations of carbapenem+beta lactamase inhibitors with colistin

Strain number	C+IR	C+MV
KP 1	2	1,125
KP 2	1,06	1,06
KP 3	**0,185**	**0,185**
KP 4	1,03	1,03
PA 1	1,06	1,06
PA 2	2,06	2,06
PA 3	1,125	1,25
PA 4	0,53	0,53
PA 5	1,125	1,06
PA 6	1,06	1,06
PA 7	1,06	1,06
PA 8	1,125	1,25
PA 9	1,03	1,03
AB 1	2	2
AB 2	1,06	1,06
EC 1	1	1,5
EC 2	1,03	1,03
EC 3	0,53	1,03
EC 4	0,53	1,03
EC 5	1,5	2
EC 6	1	1
EC 7	1	1
EC 8	1,5	2
EB 1	1,06	**0,12**
EB 2	2	1,125
EB 3	1,06	1,06
EB 4	1,25	1,25
EB 5	1	1,25

KP: *K. pneumoniae*; PA: *P. aeruginosa*; AB: *A. baumannii*; EC: *E. coli*; EB: *Enterobacter* spp

C+IR: colistin+imipenem-cilastatin+relebactam

C+MV: colistin+meropenem+vaborbactam

Discussion

The beta-lactamase inhibitors relebactam and vaborbactam both show activity against Ambler class A, C and D (for relebactam only) and no inhibition of class B beta-lactamases, like MBLs [22]. MBLs have been increasingly reported worldwide in recent years [23]. In a study involving 40 countries, 163 MBL-positive Enterobacterales isolates and 308 *P. aeruginosa* isolates were identified among 38,266

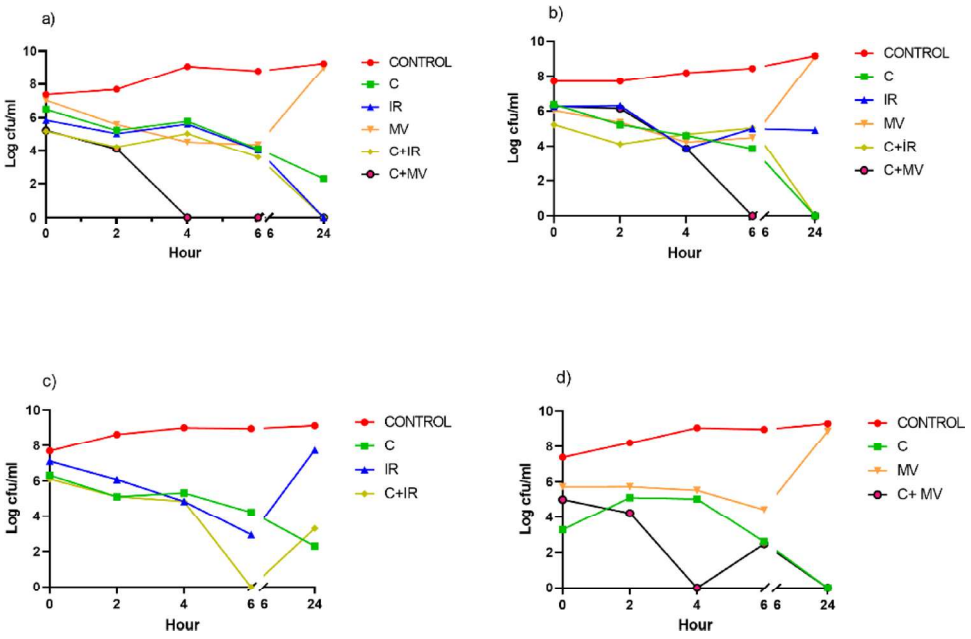

Fig. 2 Bactericidal activity of antimicrobial agents and their combinations against four strains determined by the time-kill curve method. A≥2 $\log_{10}$ reduction in viable counts compared to the most active single agent was considered indicative of synergistic activity. Each curve represents a single representative isolate for the indicated species. **a**) KP3: *K. pneumoniae* strain **b**) PA4: *P. aeruginosa* strain **c**) EC3: *E. coli* strain **d**) EB1: *Enterobacter sp.* strain C: colistin, IR: imipenem-cilastatin+relebactam, MV: meropenem+vaborbactam C+IR: colistin+imipenem-cilastatin+relebactam C+MV: colistin+meropenem+vaborbactam

Enterobacterales isolates and 8,010 *P. aeruginosa* isolates collected between 2012 and 2014 [24]. Another study identified 18.79% MBL producing isolates among 628 nonfermentative Gram-negative bacilli isolates [25].

In the present study, the "meropenem-EDTA double-disc synergy" test was used to determine the presence of MBL enzymes. 44 of the 96 Gram negative bacteria were found to be MBL negative and the experiments were continued with these MBL negative strains. As a limitation of the study, it should be noted that this phenotypic method specifically detects class B metallo-β-lactamases and does not identify class A (e.g., KPC) or class D (e.g., OXA-48) carbapenemases. Therefore, the absence of MBL activity does not completely exclude other carbapenemase-mediated resistance mechanisms. Molecular confirmation by PCR for carbapenemase genes such as KPC, OXA-48, or NDM would further strengthen the interpretation of resistance profiles.

In this study, the MIC values (mg/L) of the imipenem-cilastatin+relebactam combination (with a fixed relebactam concentration of 4 mg/L) were determined as 0.125–8.125, 0.25-<64, 1-<64, 0.06–1.06 and 4-<64 against *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*, *E. coli* and *Enterobacter* spp., respectively. For the meropenem+vaborbactam combination (with a fixed vaborbactam concentration of 8 mg/L), the MIC values were found to be 0.125–8.125, 0.5 -<64, 4-<64, 0.03–1.03 and 1–32 mg/L against the same bacterial species, respectively. Both imipenem-cilastatin+relebactam and meropenem+vaborbactam demonstrated significant activity against *E. coli* isolates. Although carbapenems are bactericidal beta-lactam antibiotics, in our study imipenem-cilastatin+relebactam combination did not show bactericidal effect against *P. aeruginosa* and *K. pneumoniae* strains at any concentration studied. Additionally, MBC values were higher than MIC values against all microorganisms in both combinations.

The addition of relebactam (2- to 128-fold reduction in MIC) or vaborbactam significantly improves (2- to > 1024-fold reduction in MIC) the efficacy of imipenem or meropenem against most species of *Enterobacterales* depending on the presence or absence of β-lactamase enzymes. Nevertheless, the inactivation of the porin protein OmpK36 in *K. pneumoniae* has been shown to provide resistance to both imipenem-relebactam and meropenem-vaborbactam [4]. Consistent with these findings, our study demonstrated limited efficacy of relebactam or vaborbactam against *K. pneumoniae* strains.

In another study, researchers evaluated the antimicrobial activities of different antibiotics including meropenem, imipenem, imipenem+relebactam, and colistin against 96 non-MBL producing carbapenem-resistant *Enterobacterales* clinical isolates. They showed that the isolates were susceptible to imipenem+relebactam, with MIC_{50}, MIC_{90}, and MIC ranges of 0.5/4, 1/4 and 0.06–1/4 mg/l, respectively [26].

In a recent study, the in vitro antibacterial activity of imipenem+relebactam against imipenem-nonsusceptible *Enterobacterales* and *P. aeruginosa* isolates from Japan was evaluated. $MIC_{50/90}$ and MIC range of imipenem+relebactam against 24 *P. aeruginosa* strains were 2/128 mg/L and 0.25->128 mg/L, respectively [27].

According to previous studies against *P. aeruginosa*, the addition of relebactam improved the activity of imipenem [4, 28]. Similarly, in our study, the addition of relebactam

and especially vaborbactam significantly increased the activities of carbapenems against *P. aeruginosa.*

Based on the limited data available, the addition of relebactam or vaborbactam does not enhance the activity of imipenem or meropenem against *A. baumannii* [4]. Indeed, in our study, most *A. baumannii* strains were found to be resistant to both imipenem-cilastatin+relebactam and meropenem+vaborbactam combinations. According to all these results, our findings are consistent with the literature.

Beta-lactamase production is widespread among Gram-negative clinical isolates in Türkiye, with OXA-48 carbapenemases being predominant and a growing incidence of MBL such as New Delhi MBL. Co-production of multiple carbapenemase genes within the same isolates has also been documented [29, 30]. Considering this resistance pattern, our findings suggest that meropenem-vaborbactam and imipenem-cilastatin-relebactam may serve as clinically relevant alternatives for treating infections caused by carbapenem-resistant strains, particularly those producing class A and some class D beta-lactamases. However, their limited activity against MBL-producing isolates underscores the need for continued surveillance and rational antibiotic selection to optimize therapeutic outcomes in Türkiye.

Due to reduced antibiotic penetration, decreased growth rate of cells, decreased metabolism of cells, the presence of persistent cells, and the expression of specific resistance genes in biofilms, it is well known that microorganisms within biofilms exhibit high resistance to conventional treatment methods. Furthermore, the use of antibiotics alone to treat infections caused by biofilms is often ineffective [31]. Therefore, in present study, the combinations of imipenem-cilastatin and meropenem with beta lactamase inhibitors were tested against 28 bacteria using the MTT method. According to the results, imipenem (4 mg/L)-cilastatin+relebactam was the most effective antibiotic combination against *K. pneumoniae* and *Enterobacter* spp. biofilms, while meropenem (16 mg/L)+vaborbactam was the most effective antibiotic combination reduced the metabolic activity of biofilm-associated cells of *P. aeruginosa*, *A. baumannii* and *E. coli*. From a clinical viewpoint, these reductions in metabolic activity could be important for limiting biofilm persistence and enhancing the effectiveness of additional antimicrobial treatments, even when there is no direct disruption to the biofilm. To our knowledge, there are no biofilm studies of imipenem-cilastatin and meropenem with beta lactamase inhibitors (relebactam and vaborbactam); our results are the first data in this regard.

Colistin is often the final therapeutic option for the treatment of infections caused by multidrug-resistant Gram-negative bacteria. It is frequently used in combination with antibiotics such as rifampicin, azithromycin, gentamicin, imipenem and meropenem in order to improve its antibacterial activity, despite the consequent increase in toxicity [32]. In present study, the efficacy of colistin in combination with carbapenem+beta lactamase inhibitors were investigated using the checkerboard technique which is the most straightforward and high throughput method available for the assessment of antimicrobial combinations. The results demonstrated that both combinations exhibited a synergistic effect against one *K. pneumoniae* (KP3) strain and a colistin+meropenem+vaborbactam combination against an *Enterobacter* sp. (EB1) strain, among the 28 bacterial strains tested. No antagonist effect was observed in any of the strains studied. A limitation of this study is that clonal relationships among the isolates were not investigated. Therefore, potential genetic similarities between isolates could not be excluded. Assessing clonal relationships using PCR-based methods would provide additional molecular context and allow for a more meaningful interpretation of the observed phenotypic interactions between antibiotic combinations. This aspect should be addressed in future studies.

Although checkerboard technique is an efficient screening test for antibiotic combinations, it does not provide any information about the time course of antibiotic activities. Time kill assays method provide a dynamic killing kinetics of the antimicrobial agents. For this purpose, in the present study, the time kill assays were also monitored to observe the dynamic killing kinetics of the antimicrobial agents, which were significantly synergistic or with low FIC index according to checkerboard assays. Colistin alone showed bactericidal activity, while in combination with carbapenem+beta lactamase inhibitors it showed early synergistic activity against the four strains studied. This is the first study investigating colistin in combination with carbapenem+beta lactamase inhibitors against Gram negative bacteria, both with checkerboard technique and time kill assays. However, time kill assays were performed on a limited number of representative strains selected according to checkerboard synergy scores, and therefore the findings cannot be generalized to all isolates. Further molecular-level analyses and future studies involving a larger number of strains are needed to further confirm and expand these findings.

Asempa et al. (2019) performed time kill assays of imipenem-relebactam alone and in combination with amikacin or colistin against ten clinical *P. aeruginosa* isolates (9 imipenem resistant). Imipenem-relebactam in combination with colistin was rapidly bactericidal against all isolates at 6 h and met the definition of synergy at 24 h in 8 of the 10 isolates and colistin alone produced rapid killing over the first 3 to 6 h in all isolates [33].

In another study the in vitro activities of antimicrobial agents including imipenem+relebactam and meropenem+vaborbactam, against *Serratia marcescens*

enzyme producing, which is an Ambler class A carbapenemase, -clinical *S. marcescens* strains. Also, carbapenem was not bactericidal alone against any strain. The combination of relebactam with imipenem did not restore bactericidal activity or result in synergistic effects against any of the tested strains. In contrast, the combination of meropenem-vaborbactam demonstrated bactericidal activity and exhibited synergistic effects against all strains [34].

Conclusion

Consequently, the study results, consistent with existing literature, suggest that the beta-lactam-beta-lactamase inhibitors, imipenem-cilastatin+relebactam and meropenem+vaborbactam, may provide potential therapeutic benefits in treating resistant Gram-negative bacteria. Although synergistic effects with colistin were observed only in a few isolates, these preliminary findings highlight the need for further comprehensive studies with a larger number of strains to better define the clinical relevance of such combinations.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00284-026-04795-4.

Author contributions Ayse Seher Birteksoz Tan: Conceptualization, Supervision, Resources, Methodology, Writing- Reviewing and Editing. Mayram Hacioglu: Conceptualization, Methodology, Data curation, Writing- Original draft preparation. Ozlem Oyardi: Conceptualization, Visualization, Investigation, Writing- Original draft preparation. Fatima Nur Olcay: Visualization, Investigation, Writing-Reviewing and Editing. Nese Inan: Methodology, Investigation, Validation, Resources, Writing- Reviewing and Editing.

Funding This work was supported by Scientific Research Projects Coordination Unit of Istanbul University under Project number: TSA-2021-37825.

Data Availability The data and materials in the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors report there are no competing interests to declare.

Consent of participate Not applicable.

Ethics approval Not applicable.

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