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ORIGINAL RESEARCH

In vitro Screening for Synergistic Polymyxin B-Based Combinations Against KPC- Producing Carbapenem-Resistant *Klebsiella pneumoniae*

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Objective: Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infections often necessitate combination therapy, yet effective treatment options remain limited. This study aimed to evaluate the bactericidal effects of polymyxin B–based combination therapy with ten representative antibiotics against KPC-producing CRKP.

Methods: Phenotypic and genotypic analyses were performed on clinically CRKP isolates. Susceptibility of KPC-producing strains was assessed via broth microdilution. Ten heterogeneous isolates were selected for 24-hour static time-kill assays to evaluate the bactericidal activity of polymyxin B and ten antibiotics (tigecycline, minocycline, meropenem, imipenem, doripenem, amikacin, fosfomycin, aztreonam, ceftazidime, and cefepime)—both as monotherapies and in combination with polymyxin B. Additionally, 24-hour time-course kill studies were conducted for a representative polymyxin B–based combination against a polymyxin B–resistant strain using clinically relevant concentration matrices.

Results: Among 22 CRKP isolates, 16 consistently produced KPC enzymes and carried the bla_{KPC-2} gene. Of the 10 selected KPC-producing strains, polymyxin B susceptibility was classified as 2 susceptible, 4 intermediate, and 4 resistant. All monotherapies, including polymyxin B, showed limited efficacy. Notable synergistic activity was observed when polymyxin B was combined with tigecycline (8/10), imipenem (7/10), ceftazidime (9/10), or cefepime (9/10), while other combinations were largely ineffective. A 24-hour time-course kill assay using polymyxin B plus ceftazidime as a representative demonstrated that synergy was concentration-dependent and could be rapidly achieved at clinical concentration combinations.

Conclusion: This study demonstrated that combinations of polymyxin B with tigecycline, imipenem, ceftazidime, or cefepime show promising therapeutic potential against KPC-producing CRKP. Further studies are warranted to evaluate their in vivo efficacy.

Keywords: carbapenem-resistant *Klebsiella pneumoniae*, time-kill assay, polymyxin B, combination therapy

Introduction

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infections are increasing rapidly worldwide, prompting the World Health Organization (WHO) to classify CRKP as a critical public health threat.[1,2] The resistance of CRKP to most clinically available antibiotics presents significant therapeutic challenges. Polymyxins—including colistin and polymyxin B—represent a last-line defense against multidrug-resistant Gram-negative pathogens such as CRKP.[3] Polymyxin B, a cationic polypeptide, disrupts lipopolysaccharides in the outer membrane of Gram-negative bacteria, increasing membrane permeability.[4] However, resistance—most commonly via activation of the PhoPQ and PmrAB systems—

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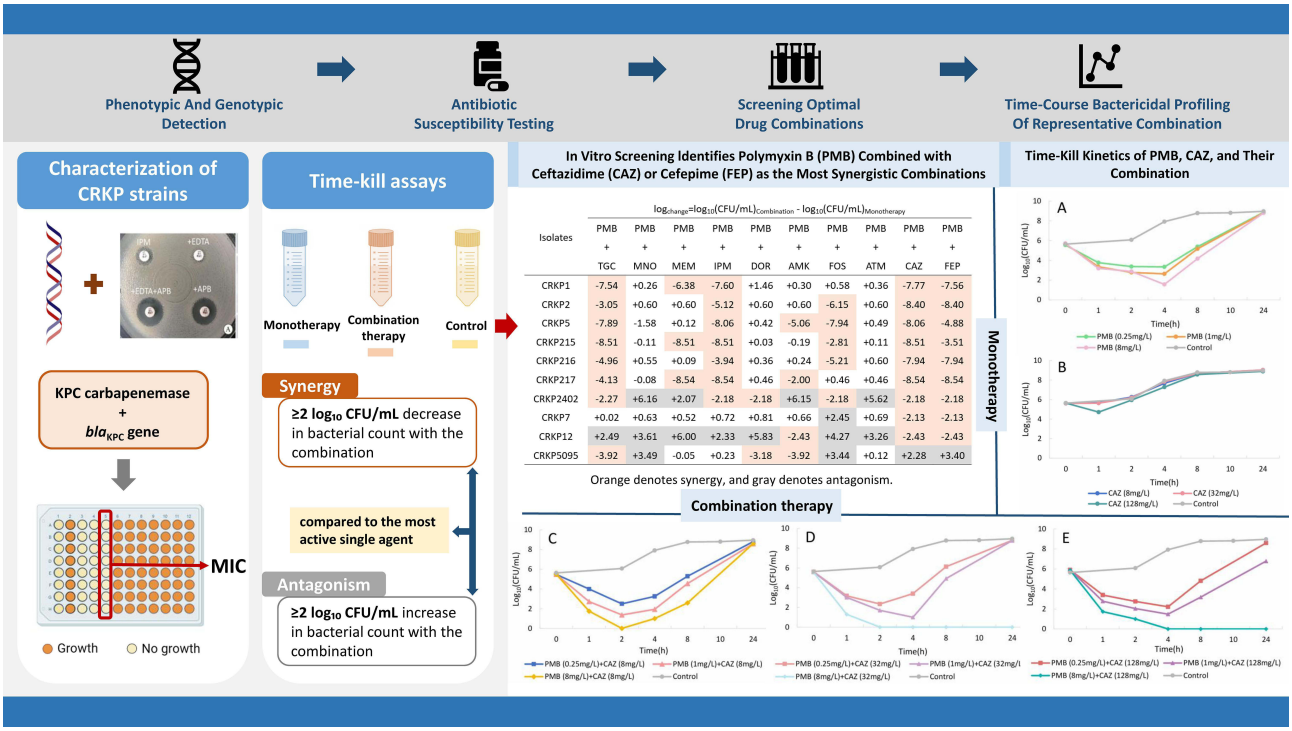
Graphical Abstract



Isolates	PMB + TGC	PMB + MNO	PMB + MEM	PMB + IPM	PMB + DOR	PMB + AMK	PMB + FOS	PMB + ATM	PMB + CAZ	PMB + FEP
CRKP1	-7.54	+0.26	-6.38	-7.60	+1.46	+0.30	+0.58	+0.36	-7.77	-7.56
CRKP2	-3.05	+0.60	+0.60	-5.12	+0.60	+0.60	-6.15	+0.60	-8.40	-8.40
CRKP5	-7.89	-1.58	+0.12	-8.06	+0.42	-5.06	-7.94	+0.49	-8.06	-4.88
CRKP215	-8.51	-0.11	-8.51	-8.51	+0.03	-0.19	-2.81	+0.11	-8.51	-3.51
CRKP216	-4.96	+0.55	+0.09	-3.94	+0.36	+0.24	-5.21	+0.60	-7.94	-7.94
CRKP217	-4.13	-0.08	-8.54	-8.54	+0.46	-2.00	+0.46	+0.46	-8.54	-8.54
CRKP2402	-2.27	+6.16	+2.07	-2.18	-2.18	+6.15	-2.18	+5.62	-2.18	-2.18
CRKP7	+0.02	+0.63	+0.52	+0.72	+0.81	+0.66	+2.45	+0.69	-2.13	-2.13
CRKP12	+2.49	+3.61	+6.00	+2.33	+5.83	-2.43	+4.27	+3.26	-2.43	-2.43
CRKP5095	-3.92	+3.49	-0.05	+0.23	-3.18	-3.92	+3.44	+0.12	+2.28	+3.40

neutralizes the negative charge of lipopolysaccharides, reducing polymyxin B binding and often leading to monotherapy failure.[5] To overcome treatment failure and resistance associated with polymyxin B monotherapy, combination therapy is commonly used for CRKP infections. Rational drug synergy can enhance clinical efficacy and reduce selection pressure, helping to delay the emergence of resistance.[6] In this context, while newer β-lactam/β-lactamase inhibitor combinations such as ceftazidime–avibactam have emerged as important therapeutic options for CRKP, their utility is increasingly challenged by the development of resistance. Therefore, polymyxin-based regimens remain a critical therapeutic alternative, especially for infections caused by isolates resistant to agents like ceftazidime-avibactam, or in settings where these newer agents are unavailable.

In clinical practice, the combination of polymyxin B with either tigecycline or meropenem is a commonly used regimen for treating CRKP infections. However, multiple retrospective clinical studies evaluating the efficacy of these combinations have yielded conflicting results, with some demonstrating efficacy and others showing no significant benefit.[7–9] To address this clinical uncertainty, in vitro screening is essential to identify effective combinations and provide experimental evidence to guide treatment decisions. Although previous studies have evaluated the synergistic activity of polymyxin B combined with various antimicrobials against CRKP using static time-kill or checkerboard assays, their results have been inconsistent,[10–13] likely due to heterogeneous resistance mechanisms among CRKP strains. Carbapenemase production—particularly KPC (most prevalent carbapenemase globally) and NDM genotypes—plays a central role in resistance, with each genotype influencing drug response differently.[14] However, current in vitro studies have key limitations: most do not stratify CRKP strains by carbapenemase genotype, often testing only 1–2 combinations against a small number of strains. As a result, these studies lack both mechanistic insight and clinical representativeness.

Given the dominance of KPC among clinical CRKP isolates, systematic drug combination screening targeting KPC-producing strains is urgently needed. This study aimed to explore genotype-guided polymyxin B–based combination

therapies against KPC-producing CRKP. By integrating phenotypic and genotypic analyses with in vitro time-kill assays, we sought to identify effective therapeutic strategies for clinical management of CRKP infections.

Methods

Strains and Carbapenemase Phenotype

We collected 22 clinical CRKP strains from The Second Xiangya Hospital of Central South University. Carbapenemase production in these isolates was detected by carbapenemase inhibitor enhancement test using phenylboronic acid (PBA) and ethylenediaminetetraacetic acid (EDTA). Carbapenemase classification was performed through comparative analysis of inhibition zone diameters on Mueller Hinton agar (MHA) plates.

Whole-Genome Sequencing

To characterize resistance genes, we conducted whole-genome sequencing using the Illumina Novaseq Xplus platform (2×150 bp). Genome assembly was conducted using the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) version 3.44.4 to generate contigs. Sequence types (STs) were determined through the Multi Locus Sequencing Typing (MLST) platform of the Center for Genomic Epidemiology. Acquired antibiotic resistance genes were identified using the ResFinder tool within the same platform.[15] For subsequent experiments, we selected KPC-producing CRKP isolates that demonstrated concordance between phenotypic and genotypic detection results.

Antibiotic Susceptibility Testing

The minimum inhibitory concentration (MIC) of polymyxin B was determined by broth microdilution method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines, using a concentration range of 0.125–4 mg/L. Escherichia coli ATCC25922 was used as the quality control strain.[16] Susceptibility results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) criteria (sensitive: ≤1 mg/L; intermediate: 2 mg/L; resistant: ≥4 mg/L).

Single-Timepoint (24 h) Bactericidal Assessment of Polymyxin B Combinations

Experimental CRKP strains were selected based on whole-genome sequencing confirmation of the bla_{KPC} gene and phenotypic verification of KPC carbapenemase production. Static single-timepoint (24-hour) time-kill assays were conducted to evaluate the bactericidal activity of polymyxin B and ten antibiotics (tigecycline, minocycline, meropenem, imipenem, doripenem, amikacin, fosfomycin, aztreonam, ceftazidime, and cefepime), both as monotherapies and in pairwise combinations with polymyxin B. The drugs were purchased from Macklin (Shanghai, China), except for meropenem, doripenem, ceftazidime, and cefepime, which were obtained from Aladdin (Shanghai, China). For all time-kill assays, cation-adjusted Mueller-Hinton broth (Ca-MHB) (Qingdao Hope Bio-Technology Co., Ltd.; calcium 50 mg/L, pH 7.3 ± 0.1 at 25°C) was used, which was prepared by dissolving 22 g of powder in 1000 mL of distilled water and autoclaving at 121°C for 10 minutes.

The experimental protocol was as follows: 160 μL of log-phase bacterial suspensions (1×10^7 CFU/mL) were added to 15 mL of Ca-MHB, followed by 1 mL of antibiotic solution, resulting in an initial inoculum of approximately 1×10^5 CFU/mL. Antibiotic concentrations in the suspension were set to the maximum clinically relevant unbound levels corresponding to standard therapeutic doses (Table 1). The suspensions were incubated at 35 °C for 24 hours, with samples collected for viable count determination at the endpoint. To minimize antibiotic carryover during bacterial quantification, 500 μL broth samples were centrifuged at 15,000 × g for 10 minutes. This condition was optimized to ensure complete bacterial pelleting. After careful aspiration and discard of the supernatant, the pellet was reconstituted in an equivalent volume of sterile normal saline, effectively reducing residual drug to sub-inhibitory levels. The resulting suspension was then serially diluted, plated on MHA plates, and incubated for colony enumeration. Viable counts (CFU/mL) were determined by counting the colony-forming units. Monotherapy was considered bactericidal if it resulted in a ≥3 $\log_{10}$ CFU/mL reduction from the initial inoculum, and bacteriostatic if the reduction was ≥2 $\log_{10}$ CFU/mL. For combination therapy, synergy was defined as a ≥2 $\log_{10}$ CFU/mL reduction in bacterial count compared to the most active

Table 1 Antibiotic Clinical Information and Concentration Settings for Static Time-Kill Studies

Antibiotics	Clinical Information			Experimental Concentration (mg/L)
	Clinical Dosing Regimen	Peak Concentration (mg/L)	Drug Unbound Fraction	
Polymyxin B	30,000 IU/kg/day	4.76	0.42	2
Tigecycline	100 mg every 12 h	6.98	0.29	2
Minocycline	200 mg every 12 h	3.125	0.24	0.75
Meropenem	2 g every 8 h (infused over 3 h)	65.3	0.98	64
Imipenem	1 g every 6 h (infused over 0.5 h)	40	0.80	32
Doripenem	1 g every 8 h (infused over 4 h)	14.44	0.90	13
Amikacin	20 mg/kg every 24 h	88.89	0.90	80
Fosfomycin	5 g every 8 h	930	1.00	930
Aztreonam	6 g every 24 h (infused over 24 h)	41.7	0.58	24
Ceftazidime	2 g every 8 h	124.4	0.80	100
Cefepime	2 g every 12 h	250	0.80	200

single agent, while an additive effect was defined as a 1–2 $\log_{10}$ CFU/mL reduction. Antagonism was defined as a ≥2 $\log_{10}$ CFU/mL increase in bacterial count with the combination compared to the most active single agent.

Time-Course Bactericidal Profiling of Selected Combination Across Multiple Concentrations

A 24-hour time-course bactericidal profiling was conducted for a representative polymyxin B–based combination against a polymyxin B–resistant CRKP strain. For single-drug treatments, three discrete concentrations encompassing the peak, trough, and an intermediate concentration were tested. Combination treatments employed a 3×3 concentration matrix design, whereby each drug in the pair was evaluated at its respective three concentrations. Bacterial samples were collected at 0, 1, 2, 4, 8, and 24 hours post-administration. Bacterial counts were determined as described in the previous section.

Statistical Analysis

All statistical analyses were performed using SPSS software (version 27.0). Given the paired design of the experiment (the same set of 10 bacterial isolates was tested against all 10 drug combinations), exploratory pairwise comparisons between combinations were performed. To assess the difference in synergy rates for each pair, McNemar's exact test was applied when the synergy rates of both combinations were greater than 0%. If the synergy rate of one combination in the pair was 0%, the exact P-value was calculated using the binomial probability formula $P = 2 \times (0.5)^b$, where b represents the number of isolates that showed synergy only for the combination with a synergy rate > 0%. A P-value < 0.05 was considered statistically significant.

Results

Isolate Characteristics

A total of 22 clinical CRKP strains were collected and characterized. Phenotypic analysis (Table 2) demonstrated KPC carbapenemase production in 16 isolates. Genotypic profiling (Table 2) revealed ST11 as the predominant sequence type, with 18 isolates carrying $bla_{\text{KPC-2}}$. Extended-spectrum β-lactamase (ESBL) genes were detected in 21 strains, most commonly $bla_{\text{SHV-12}}$ and $bla_{\text{CTX-M-65}}$. Approximately 82% of strains harbored aminoglycoside resistance genes, predominantly *rmtB*, except for CRKP14, which carried *aac(6')-Ib-cr*. All strains except CRKP215 contained a fosfomycin resistance gene, with more than half harboring the *fosA6* variant, typically associated with *Klebsiella pneumoniae*.[17] Tetracycline resistance gene *tet(B)* was only detected in CRKP4.

Table 2 Genotype, Phenotype and Antimicrobial Susceptibility of the Studied CRKP Strains

Isolates	ST	Resistance Genotype										Phenotype	MIC (mg/L)
		Cationic Peptides	β-Lactams						Aminoglycosides	Fosfomycin	Tetracyclines			
		PMB	MEM	FEP	IPM	CAZ	ATM	DOR	AMK	FOS	TIG	MIN		
CRKP215	ST11	/	bla_{KPC-2}, bla_{SHV-12}						rmtB	ND	ND	ND	KPC	4(R)
CRKP216	ST11	/	bla_{KPC-2}, bla_{SHV-12}, $bla_{CTX-M-65}$						rmtB	fosA	ND	ND	KPC	8(R)
CRKP216034	ST11	/	bla_{KPC-2}, bla_{SHV-12}, $bla_{CTX-M-65}$						rmtB	fosA6	ND	ND	/	
CRKP217	ST11	/	bla_{KPC-2}, bla_{SHV-12}						rmtB	fosA	ND	ND	KPC	2(I)
CRKP1	ST11	/	bla_{KPC-2}, bla_{SHV-12}, $bla_{CTX-M-65}$						rmtB	fosA	ND	ND	KPC	2(I)
CRKP2	ST11	/	bla_{KPC-2}, bla_{SHV-12}						rmtB	fosA, fosA5	ND	ND	KPC	2(I)
CRKP3	ST307	/	bla_{OXA-1}, $bla_{SHV-100}$, $bla_{CTX-M-65}$						ND	fosA6	ND	ND	/	
CRKP4	ST11	/	bla_{KPC-2}, bla_{OXA-23}, bla_{OXA-66}, bla_{SHV-12}, $bla_{CTX-M-65}$						rmtB	fosA6	ND	tet(B)	/	
CRKP5	ST11	/	bla_{KPC-2}, bla_{SHV-12}						rmtB	fosA	ND	ND	KPC	4(R)
CRKP2402	ST11	/	bla_{KPC-2}, bla_{SHV-12}, $bla_{CTX-M-65}$						rmtB	fosA6	ND	ND	KPC	1(S)
CRKP6	ST273	/	$bla_{CTX-M-55}$						ND	fosA	ND	ND	/	
CRKP7	ST11	/	bla_{KPC-2}, bla_{SHV-12}, $bla_{CTX-M-65}$						rmtB	fosA6	ND	ND	KPC	16(R)
CRKP8	ST307	/	bla_{OXA-1}, $bla_{SHV-106}$, $bla_{CTX-M-15}$						ND	fosA6	ND	ND	/	
CRKP9	ST11	/	bla_{KPC-2}, bla_{SHV-12}, $bla_{CTX-M-65}$						rmtB	fosA6	ND	ND	KPC	2(I)
CRKP10	ST11	/	bla_{KPC-2}, bla_{SHV-12}						rmtB	fosA6	ND	ND	KPC	4(R)
CRKP11	ST11	/	bla_{KPC-2}, bla_{SHV-12}						rmtB	fosA6	ND	ND	KPC	2(I)
CRKP12	ST11	/	bla_{KPC-2}, $bla_{SHV-158}$, $bla_{SHV-159}$, $bla_{SHV-182}$, $bla_{CTX-M-65}$, bla_{SHV-99}, $bla_{SHV-147}$						rmtB	fosA6, fosA3	ND	ND	KPC	2(I)
CRKP14	ST273	/	bla_{NDM-1}, bla_{OXA-1}, $bla_{OXY-2-8}$, $bla_{CTX-M-55}$						aac(6')-Ib-cr	fosA	ND	ND	/	
CRKP04135	ST11	/	bla_{KPC-2}, bla_{SHV-12}, $bla_{CTX-M-65}$						rmtB	fosA6	ND	ND	KPC	2(I)
CRKP05029	ST11	/	bla_{KPC-2}, bla_{SHV-12}, $bla_{CTX-M-65}$						rmtB	fosA6	ND	ND	KPC	2(I)
CRKP05095	ST1582	/	bla_{KPC-2}						ND	fosA	ND	ND	KPC	1(S)
CRKP05225	ST11	/	bla_{KPC-2}, bla_{SHV-12}						rmtB	fosA6	ND	ND	KPC	2(I)

Abbreviations: PMB, polymyxin B; TIG, tigecycline; MIN, minocycline; MEM, meropenem; IPM, imipenem; DOR, doripenem; AMK, amikacin; FOS, fosfomycin; ATM, aztreonam; CAZ, ceftazidime; FEP, cefepime; ND, not detected; S, sensitive; I, intermediate; R, resistant.

Based on consistent phenotypic and genotypic findings, 16 KPC-producing CRKP strains were selected for polymyxin B susceptibility testing. The MIC distribution (Table 2) showed that 2 of 16 strains were susceptible (MIC ≤ 1 mg/L), 9 were intermediate (MIC = 2 mg/L), and 5 were resistant to polymyxin B (MIC ≥ 4 mg/L).

Single-Timepoint (24 h) Bactericidal Assessment of Polymyxin B Combinations

Of the 16 KPC-producing CRKP strains, 10 (2 susceptible, 4 intermediate, and 4 resistant) were selected for 24-hour single-timepoint bactericidal assessment to identify effective polymyxin B–based combinations. Polymyxin B monotherapy was largely ineffective, with 8 of 10 strains exceeding the initial inoculum at 24 hours (Table 3). Similarly, other single agents showed minimal activity, with only tigecycline, doripenem, and fosfomycin demonstrating limited effect against one strain each.

Polymyxin B–based combinations showed clear efficacy diversities against the 10 CRKP strains (Table 3). The least effective were minocycline and aztreonam, which exhibited no synergism and notable antagonism (30% and 20% of strains, respectively). Moderate activity was observed with meropenem (30% synergism, 20% antagonism), doripenem (20% synergism, 10% antagonism), amikacin (40% synergism, 10% antagonism), and fosfomycin (50% synergism, 30% antagonism). The strongest synergism occurred with tigecycline (80% synergism, 10% antagonism), imipenem (70% synergism, 10% antagonism), and the cephalosporins—ceftazidime and cefepime—each achieving 90% synergism with only 10% antagonism.

Time-Course Bactericidal Profiling of Selected Combination Across Multiple Concentrations

Based on the 24-hour single-timepoint assessment, the polymyxin B–ceftazidime combination was selected for 24-hour time-kill kinetics against the resistant CRKP5 strain (MIC = 4 mg/L). Different concentrations of polymyxin B (0.25, 1, and 8 mg/L)[18] and ceftazidime (8, 32, and 128 mg/L)[19] were tested as monotherapies and in combination.

Monotherapy with either polymyxin B or ceftazidime was ineffective at all clinically relevant concentrations, with marked bacterial regrowth by 24 hours (Figure 1A and B). Polymyxin B initially reduced counts by 2–4 $\log_{10}$ CFU/mL at 4 hours across all concentrations, but regrowth occurred thereafter. Ceftazidime monotherapy—except at 128 mg/L, which achieved ~1 $\log_{10}$ reduction at 1 hour—showed no inhibitory effect at any time point. In contrast, the combination therapy produced marked concentration-dependent inhibitory or bactericidal effects (Figure 1C–E). Notably, 8 mg/L polymyxin B combined with 32 or 128 mg/L ceftazidime achieved sustained ≥4 $\log_{10}$ CFU/mL reductions without regrowth over 24 hours. Other combinations showed early bactericidal activity (within 4 hours) but were followed by varying levels of regrowth.

Further evaluation of the polymyxin B–ceftazidime combination showed significant additive or synergistic effects at multiple time points, with these effects increasing as drug concentrations rose (Table 4). At 0.25 mg/L polymyxin B, no synergism was observed at any ceftazidime concentration. At 1 mg/L polymyxin B, synergism occurred with 128 mg/L ceftazidime after 8 hours, while at 8 mg/L polymyxin B, additive or synergistic effects were seen across all ceftazidime concentrations.

Statistical Analysis

The results of all 45 exploratory pairwise comparisons are summarized in a matrix format in Table 5, which displays the P-values for each pair. Among the 45 exploratory pairwise comparisons performed, the most pronounced differences in synergy rates were observed for several pairs involving the highest-performing combinations. For instance, polymyxin B–ceftazidime combination (90% synergy) showed a substantial difference compared to polymyxin B–doripenem (20% synergy) with an unadjusted P value of 0.039. Similarly, polymyxin B–ceftazidime (90% synergy) differed markedly from polymyxin B–meropenem (30% synergy) (P = 0.031).

Table 3 Overview of the Bactericidal Effects of Polymyxin B and 10 Representative Antibiotics, Evaluated as Monotherapies and in Combination Using 24-Hour Single Time-Point Time-Kill Experiments

Isolates	$log_{change}=log_{10}(CFU/mL)_{24h} - log_{10}(CFU/mL)_{0h}$											$log_{change}=log_{10}(CFU/mL)_{Combination} - log_{10}(CFU/mL)_{Monotherapy}$										
	Control	PMB	TIG	MNO	MEM	IPM	DOR	AMK	FOS	ATM	CAZ	FEP	PMB + TIG	PMB + MNO	PMB + MEM	PMB + IPM	PMB + DOR	PMB + AMK	PMB + FOS	PMB + ATM	PMB + CAZ	PMB + FEP
CRKP1	+3.62	+3.51	+3.05	+3.25	+3.25	+3.11	+3.00	+3.21	+0.93	+3.15	+3.28	+3.07	−7.54	+0.26	−6.38	−7.60	+1.46	+0.30	+0.58	+0.36	−7.77	−7.56
CRKP2	+3.12	+2.54	−0.85	+3.39	+3.18	+3.33	+3.16	+3.36	+0.29	+3.43	+3.52	+3.39	−3.05	+0.60	+0.60	−5.12	+0.60	+0.60	−6.15	+0.60	−8.40	−8.40
CRKP5	+3.43	+2.66	+2.49	+2.97	+3.08	+3.14	+3.09	+3.41	+2.54	+3.73	+3.50	+3.00	−7.89	−1.58	+0.12	−8.06	+0.42	−5.06	−7.94	+0.49	−8.06	−4.88
CRKP215	+3.31	+2.93	+3.00	+3.19	+3.30	+3.17	+3.13	+3.26	+2.23	+3.30	+3.29	+3.30	−8.51	−0.11	−8.51	−8.51	+0.03	−0.19	−2.81	+0.11	−8.51	−3.51
CRKP216	+3.52	+2.71	+3.12	+2.89	+3.25	+3.30	+3.05	+3.36	+1.48	+2.75	+3.30	+2.89	−4.96	+0.55	+0.09	−3.94	+0.36	+0.24	−5.21	+0.60	−7.94	−7.94
CRKP217	+3.47	+3.05	+3.35	+3.17	+3.51	+3.18	+3.10	+3.16	+3.35	+3.25	+3.38	+3.16	−4.13	−0.08	−8.54	−8.54	+0.46	−2.00	+0.46	+0.46	−8.54	−8.54
CRKP2402	+3.19	−3.27	−2.85	+3.08	+2.95	+3.10	+3.01	+3.03	+0.49	+3.16	+3.26	+3.05	−2.27	+6.16	+2.07	−2.18	−2.18	+6.15	−2.18	+5.62	−2.18	−2.18
CRKP7	+3.20	+2.50	+2.99	+3.18	+2.52	+3.15	+3.21	+3.40	−3.52	+3.35	+3.45	+3.22	+0.02	+0.63	+0.52	+0.72	+0.81	+0.66	+2.45	+0.69	−2.13	−2.13
CRKP12	+3.17	−3.33	+3.04	+3.09	+2.70	+3.14	+2.89	+3.16	+3.18	+3.15	+3.22	+3.07	+2.49	+3.61	+6.00	+2.33	+5.83	−2.43	+4.27	+3.26	−2.43	−2.43
CRKP5095	+3.25	−1.82	−0.69	+3.18	+2.92	+0.24	−2.29	+3.14	+3.29	+3.15	+2.85	+2.99	−3.92	+3.49	−0.05	+0.23	−3.18	−3.92	+3.44	+0.12	+2.28	+3.40

Notes: Green background indicates bactericidal activity, blue indicates bacteriostatic activity, orange denotes synergy, and gray denotes antagonism.
Abbreviations: PMB, polymyxin B; TIG, tigecycline; MIN, minocycline; MEM, meropenem; IPM, imipenem; DOR, doripenem; AMK, amikacin; FOS, fosfomycin; ATM, aztreonam; CAZ, ceftazidime; FEP, cefepime.

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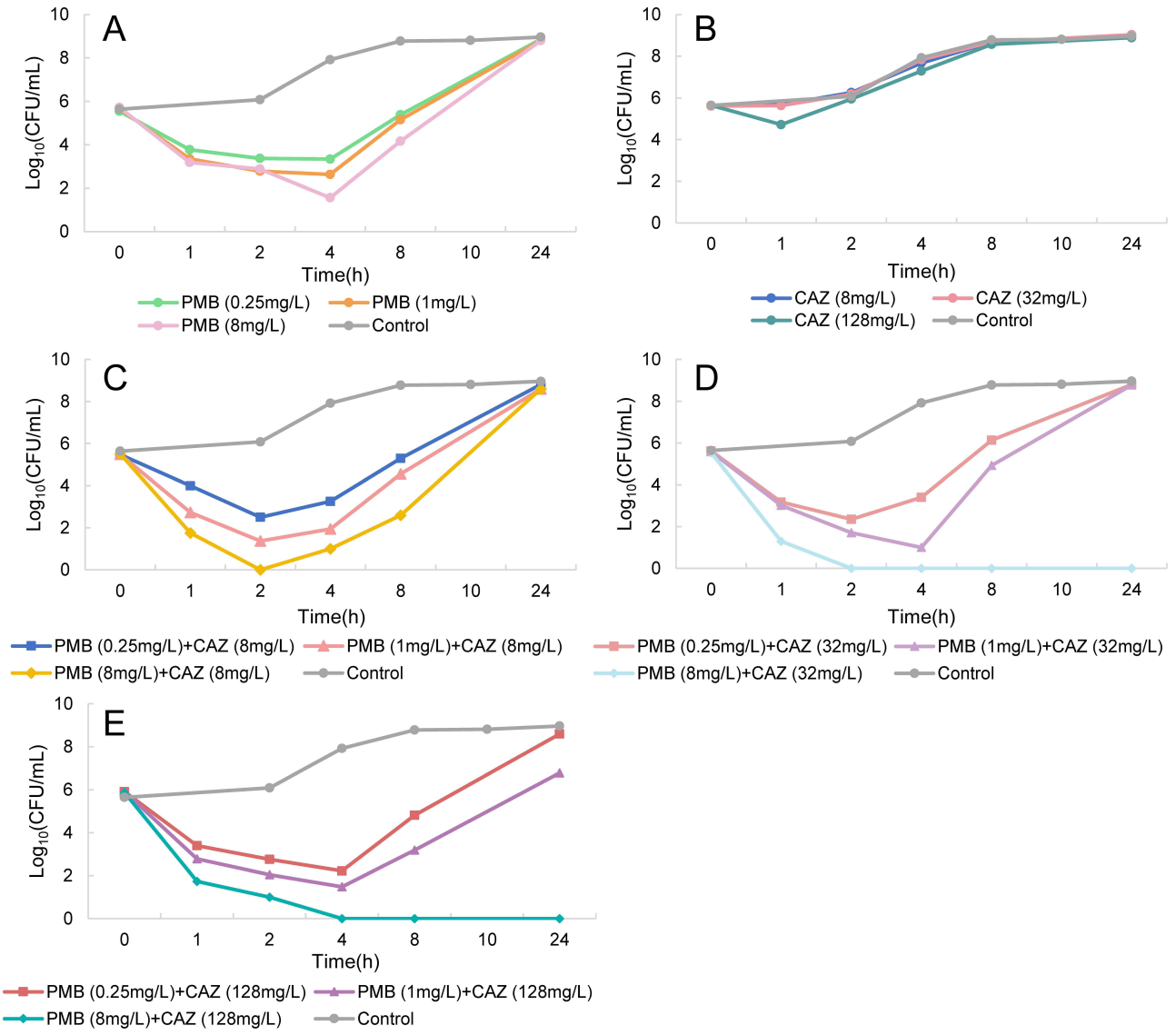

Figure 1 Time-kill curves of polymyxin B (PMB), ceftazidime (CAZ), and their combination against CRKP5 over 24 hours. (**A**) PMB monotherapy at 0.25, 1, and 8 mg/L. (**B**) CAZ monotherapy at 8, 32, and 128 mg/L. (**C**–**E**) PMB–CAZ combination therapy at various concentration combinations.

Discussion

Our study demonstrated that although polymyxin B monotherapy achieved substantial initial bactericidal activity, it failed to maintain this effect beyond 24 hours, even at the highest clinically achievable concentration. This finding aligns with clinical observations that polymyxin B monotherapy often leads to resistance development and treatment failure, supporting current guideline recommendations favoring combination therapy to enhance efficacy and suppress resistance.[20,21]

We evaluated the bactericidal activity of polymyxin B combined with 10 antibiotics against KPC-producing CRKP strains using time-kill assays. These antibiotics were selected for their diverse mechanisms of action, enabling dual-target strategies that enhance synergy and potentially limit resistance.[22] Tetracycline derivatives (tigecycline, minocycline) and the aminoglycoside amikacin inhibit protein synthesis by binding to the 30S ribosomal subunit.[23,24] Carbapenems (meropenem, imipenem, doripenem), the monobactam aztreonam, and cephalosporins (ceftazidime, cefepime) inhibit penicillin-binding proteins, disrupting peptidoglycan synthesis and cell wall integrity.[25] Fosfomycin acts uniquely by irreversibly inhibiting MurA, blocking early steps in peptidoglycan precursor synthesis.[26] As polymyxin B works by

Table 4 Summary of Bacterial Count Changes (Log_{10} CFU/mL) for the Polymyxin B–Ceftazidime Combination Over 24 Hours, Relative to the Most Effective Monotherapy at Each Time-Point

Antibiotic Regimen		Time (h)				
		1	2	4	8	24
CAZ 8 mg/L	PMB 0.25 mg/L	+0.30	−0.80	−0.01	−0.01	+0.02
	PMB 1 mg/L	−0.44	−1.23	−0.51	−0.42	−0.03
	PMB 8 mg/L	−1.23	−2.67	−0.35	−1.37	+0.01
CAZ 32 mg/L	PMB 0.25 mg/L	−0.66	−1.08	0.00	+0.70	−0.12
	PMB 1 mg/L	−0.24	−0.99	−1.54	−0.15	+0.06
	PMB 8 mg/L	−1.71	−2.70	−1.38	−3.99	−8.62
CAZ 128 mg/L	PMB 0.25 mg/L	−0.74	−0.98	−1.48	−0.93	−0.57
	PMB 1 mg/L	−0.81	−0.98	−1.39	−2.22	−2.27
	PMB 8 mg/L	−1.61	−2.03	−1.71	−4.32	−8.95

Notes: An orange background indicates synergy, while a yellow background indicates an additive effect.
Abbreviations: PMB, polymyxin B; CAZ, ceftazidime.

Table 5 Matrix of P-values from Pairwise Comparisons of Synergy Rates Between Ten Polymyxin B-Based Combinations

(Row) vs (Column))	PMB +TIG	PMB +MNO	PMB +MEM	PMB +IPM	PMB +DOR	PMB +AMK	PMB +FOS	PMB +ATM	PMB +CAZ	PMB +FEP
PMB+TIG	–	0.008**	0.063	1.000	0.031*	0.219	0.250	0.008**	1.000	1.000
PMB+MNO		–	0.250	0.016*	0.500	0.125	0.063	1.000	0.004**	0.004**
PMB+MEM			–	0.125	1.000	1.000	0.687	0.250	0.031*	0.031*
PMB+IPM				–	0.125	0.453	0.500	0.016*	0.500	0.500
PMB+DOR					–	0.625	0.375	0.500	0.039*	0.039*
PMB+AMK						–	1.000	0.125	0.125	0.125
PMB+FOS							–	0.063	0.125	0.125
PMB+ATM								–	0.004**	0.004**
PMB+CAZ									–	1.000
PMB+FEP										–

Notes: Values in parentheses indicate the difference in synergy rates (Row% – Column%). ** denotes P-value < 0.01, and * denotes P-value < 0.05 (and ≥ 0.01).
Abbreviations: PMB, polymyxin B; TIG, tigecycline; MIN, minocycline; MEM, meropenem; IPM, imipenem; DOR, doripenem; AMK, amikacin; FOS, fosfomycin; ATM, aztreonam; CAZ, ceftazidime; FEP, cefepime.

disrupting bacterial outer membranes, increasing permeability and facilitating antibiotic entry. This polymyxin B-based combination approach may enhance efficacy by simultaneously targeting multiple pathways—cell membrane integrity, cell wall synthesis, and protein synthesis—thereby potentially improving bacterial eradication and reducing the risk of resistance through multi-mechanistic inhibition.[27]

In this study, we identified 16 KPC-producing CRKP isolates through genotypic and phenotypic screening, and selected 10 representative isolates for time-kill experiments based on the following considerations. First, nine of the ten isolates belonged to ST11—the predominant CRKP sequence type in China[28]—which commonly carries KPC carbapenemases, particularly KPC-2, conferring carbapenem resistance.[29,30] Second, the selected isolates reflected diverse resistance profiles. All carried bla_{KPC-2} along with various ESBL genes, including combinations of bla_{SHV-12}, $bla_{SHV-158}$, $bla_{SHV-159}$, $bla_{SHV-182}$, $bla_{CTX-M-65}$, $bla_{CTX-M-99}$, and $bla_{CTX-M-147}$, representing clinically relevant multidrug resistance patterns.[31] Third, the isolates exhibited heterogeneous antimicrobial susceptibility profiles. Evaluating

polymyxin B–based combinations against strains with varying susceptibility may help identify broadly effective therapeutic strategies for real-world scenarios where heterogeneous resistant subpopulations coexist within patients.[32]

Our study demonstrated that polymyxin B combined with either ceftazidime or cefepime exhibits broad synergistic activity against KPC-producing CRKP—a finding not previously reported. Ma et al, previously described the bactericidal effect of polymyxin B combined with ceftazidime-avibactam against three clinical KPC-producing CRKP strains.[33] In their study, ceftazidime (128 mg/L for two strains and 256 mg/L for one strain) plus avibactam (4 mg/L) combined with polymyxin B (2 mg/L) still resulted in regrowth in two strains (including one exposed to 128 mg/L ceftazidime). Notably, although ceftazidime is the core component of ceftazidime-avibactam, our findings demonstrate that ceftazidime (100 mg/L) alone synergizes effectively with polymyxin B (2 mg/L) without requiring the β-lactamase inhibitor avibactam. Likewise, we observed similarly potent synergy when polymyxin B was combined with cefepime. The robust synergism of polymyxin B with either ceftazidime or cefepime against resistant CRKP underscores its therapeutic potential.

This study confirmed that the combination of polymyxin B and tigecycline exhibited strong synergistic effects against KPC-producing CRKP strains, consistent with previous reports and supporting its potential clinical utility.[34,35] For instance, Huang et al, evaluated this combination against four CRKP strains (two KPC-producing and two NDM-producing) using a similar time-kill assay. Their results demonstrated synergistic activity against both KPC-producing strains (one polymyxin B–susceptible and one resistant), aligning with our findings.[36] In contrast, no synergy was observed against the two NDM-producing strains (one susceptible and one resistant to polymyxin B). These results suggest that the synergistic effect may be genotype-dependent and primarily confined to KPC-producing CRKP strains.

We also observed that the combination of polymyxin B and imipenem produced synergistic activity against 7 of the 10 tested strains. In contrast, the synergy rate declined markedly when polymyxin B was combined with other carbapenems, such as meropenem. Previously, Sharma et al, reported strong synergy for the polymyxin B (2 mg/L) and meropenem (60 mg/L) combination in 8 of 10 KPC producing CRKP strains.[37] Under similar experimental conditions, we observed synergy in only 3 of 10 strains and even antagonism in 2 strains. This discrepancy may be attributed to differences in the susceptibility profiles of the tested isolates. In Sharma et al ‘s study, 8 of 10 strains were susceptible to polymyxin B (5 with MIC < 0.5 mg/L, 2 with MIC = 0.5 mg/L, and 1 with MIC = 1.0 mg/L), whereas in our study, only 2 strains were susceptible (MIC = 1 mg/L). These findings suggest that the therapeutic efficacy of the polymyxin B–meropenem combination might be strongly influenced by the susceptibility of the infecting CRKP strain. For clinical isolates resistant to polymyxin B, this strategy may lead to suboptimal or even unfavorable outcomes.

While our in vitro studies showed that polymyxin B in combination with tigecycline, imipenem, ceftazidime, or cefepime exhibited excellent synergistic activity against KPC-producing CRKP strains, this study has limitations. The use of ten strains for screening may not be fully representative, and the in vitro nature of the assays cannot guarantee in vivo effectiveness. Furthermore, the preliminary nature of our time-kill kinetics data, with technical replicates only (performed in duplicate), restricted the application of formal inferential statistics. The experiments were performed under static conditions using concentrations corresponding to peak in vivo levels, and these static exposures may overestimate the true synergistic effects achievable in clinical settings. Therefore, further evaluation using dynamic models (eg, hollow fiber infection systems) or animal studies, is warranted to better assess the in vivo efficacy of these polymyxin B–based combinations.

Conclusion

In this study, we identified four promising polymyxin B–based combinations that exhibited strong synergistic activity against heterogeneous KPC-producing CRKP strains. Notably, the synergistic effect of polymyxin B combined with either ceftazidime or cefepime was reported for the first time. A 24-hour time-kill experiment using polymyxin B plus ceftazidime as a representative combination further demonstrated that bactericidal synergy could be achieved shortly after administration.

Data Sharing Statement

All the relevant data are shown in the manuscript.

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Disclosure

The authors declare no competing interests.

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