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ORIGINAL RESEARCH

In vitro and in vivo Effect of Antimicrobial Agent Combinations Against Carbapenem-Resistant *Klebsiella pneumoniae* with Different Resistance Mechanisms in China

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Objective: This study aimed to evaluate the in vitro and in vivo effects of different combinations of antimicrobial agents against carbapenemase-producing and non-producing *Klebsiella pneumoniae* from China.

Methods: A checkerboard assay of meropenem (MEM), amikacin (AK), tigecycline (TGC), colistin (COL) and their combinations was carried out against 58 clinical carbapenem-resistant *K. pneumoniae* (CRKp) isolates, including 11 carbapenemase-non-producing *K. pneumoniae* isolates and 21 isolates producing KPC-2 enzyme, 11 NDM-1, 13 IMP, one VIM-1 and one OXA-48. The checkerboard assay was analyzed by the fractional inhibitory concentration index (FICI). A time–kill assay and *Galleria mellonella* infection model were conducted to evaluate the in vitro and in vivo effects of the four drugs alone and in combination.

Results: In the checkerboard assay, TGC+AK and MEM+AK combinations showed the highest synergistic effect against KPC-2 and NDM-1 carbapenemase-producing isolates, with synergy+partial synergy (defined as FICI <1) rates of 76.2% and 71.4% against KPC-2 producers, and 54.5% and 81.8% against NDM-1 producers. TGC+AK and MEM+COL combinations showed the highest rate of synergistic effect against IMP-producing isolates. Against carbapenemase-non-producing isolates, TGC+COL and TGC+AK combinations showed the highest rate of synergy effect (63.6% and 54.5%). MEM+AK showed a synergistic effect against one VIM-1 producer (FICI=0.31) and an additivite effect (FICI=1) against one OXA-48 producer. In the time–kill assay, COL+AK, COL+TGC, COL+MEM and AK+TGC showed good synergistic effects against the KPC-2-producing isolate D16. COL+MEM and COL+TGC combinations showed good effects against the NDM-1-producing isolate L13 and IMP-4-producing isolate L34. Against the carbapenemase-non-producing isolate Y105, MEM+TGC and COL+AK showed high synergistic effects, with $\log_{10}$CFU/mL decreases of 6.2 and 5.5 compared to the most active single drug. In the *G. mellonella* survival assay, MEM-based combinations had relatively high survival rates, especially when combined with colistin, against KPC-2 producers (90% survival rate) and with amikacin against metallo-beta-lactamase producers (95–100% survival rate).

Conclusion: Our study suggests that different antimicrobial agent combinations should be considered against CRKp infections with different resistance mechanisms.

Keywords: resistance mechanisms, time–kill curve assay, carbapenem-resistant *Klebsiella pneumoniae*, CRKP, antimicrobial agent combinations, *Galleria mellonella* infection model

Introduction

Carbapenem-resistant Enterobacteriaceae (CRE), including carbapenem-resistant *Klebsiella pneumoniae* (CRKp), are classified by the US Centers for Disease Control and Prevention (CDC) as urgent antimicrobial-resistant bacteria.[1] CRKp has been increasingly reported in the past decade.[2] The production of carbapenemases, especially KPC-2, NDM-1 and IMP-type carbapenemases, is the most important resistance mechanism of CRKp in China.[3] Some studies have also reported that the loss of outer membrane proteins, such as OmpK35 and OmpK36, could contribute to carbapenem resistance in CRKp.

Infections with CRKp bacteria, especially coexisting virulence factors, are associated with high mortality rates and the treatments are extremely limited.[4,5] Antimicrobial combination therapy is reported as an effective method against CRE infection.[6–9] Although some studies have evaluated the effects of combination against KPC-producing isolates,[10–12] it is still not clear whether the different types of carbapenemase affect the efficacy of combination therapy using in vitro and in vivo assays.

In order to identify the best combination regimen that could provide alternative treatments for carbapenemase-producing *K. pneumoniae*, we investigated the effectiveness of single drug and combinations of meropenem, colistin, amikacin and tigecycline against CRKp with different resistance mechanisms (carbapenemase-producing *K. pneumoniae* and carbapenemase-non-producing *K. pneumoniae*) using an in vitro checkerboard test and time–kill assays, and an in vivo *Galleria mellonella* infection model.[13,14]

Materials and Methods

Bacterial Isolates

A total of 58 non-repetitive carbapenem-resistant clinical *K. pneumoniae* isolates were collected from 11 teaching hospitals in China from 2015 to 2018. Carbapenemase genes were detected by polymerase chain reaction and sequenced as previously described.[15–17] All strains were identified by the Vitek 2 system (bioMérieux, Marcy-l'Étoile, France), and MALDI-TOF MS apparatus (Bruker Biotyper; Bruker Daltonik, Bremen, Germany) (Supplementary Table 1). All the *K. pneumoniae* isolates were further characterized by multilocus sequence typing (MLST) (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). We prefer to collect isolates with different ST types to avoid outbreak strains.

Antimicrobial Susceptibility Testing

Meropenem (MEM), amikacin (AK) and colistin (COL) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Tigecycline (TGC) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Drugs were diluted in sterile cation-adjusted Mueller–Hinton broth (BD BBL, Sparks, MD, USA) and the concentration ranges of each drug were as follows: meropenem (0.25–32 mg/L), amikacin (0.5–64 mg/L), colistin (0.06–8 mg/L), tigecycline (0.03–4 mg/L). The bacterial inoculum was 5×10^5 CFU/mL. *Escherichia coli* strain ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as the quality control strains in each batch.

The minimum inhibitory concentrations (MICs) of the four antimicrobials were determined by the broth microdilution method according to CLSI recommendations.[18] CLSI criteria were used to interpret the antimicrobial susceptibility of meropenem, amikacin and colistin, whereas the FDA breakpoint was used for tigecycline.

Checkerboard Test

Six different combinations of antimicrobials were selected and tested by the checkerboard test: MEM plus AK, MEM plus COL, MEM plus TGC, AK plus COL, AK plus TGC and COL plus TGC. The information on the six isolates is shown in Table 1. The MICs of drug combinations were determined after incubation at 35°C for 18–24 h in ambient air.

The interaction between antimicrobial agents was determined based on the calculated fractional inhibitory concentration index (FICI). The FIC of each drug was calculated as a ratio of the MIC of drug A (or B) when used in the combination (combo) and the MIC of drug A (or B) when tested alone, according to the following formula: $FICI=FIC_A+FIC_B=MIC_{(A-combo)}/MIC_{(A-alone)}+MIC_{(B-combo)}/MIC_{(B-alone)}$.[19] The results were interpreted as follows: synergism, FICI $\leq$0.5; partial synergism, 0.5<FICI<1; additivity, FICI=1; indifference, 1<FICI<4; and antagonism, FICI $\geq$4.[20]

Time–Kill Assay

The time–kill activities of antimicrobial drugs against six clinical isolates were assessed. The selection criteria were based on different resistance mechanisms (Table 1). Drug concentrations were selected based on clinically achievable serum levels of each drug as defined by a literature

Table 1 Isolates Used in the Time–Kill Assay and *G. mellonella* Survival Assay

Strain No.	Organism	Specimen	Carbapenemase	MIC (mg/L) [Interpretation]			
				MEM	TGC	COL	AK
D16	*K. pneumoniae*	Blood	KPC-2	64 [R]	0.5 [S]	1 [S]	8 [S]
L13	*K. pneumoniae*	Bile	NDM-1	64 [R]	0.25 [S]	0.5 [S]	4 [S]
L34	*K. pneumoniae*	Blood	IMP-4	8 [R]	1 [S]	0.5 [S]	128 [R]
P5	*K. pneumoniae*	Sputum	VIM-1	8 [R]	8 [R]	0.5 [S]	4 [S]
P13	*K. pneumoniae*	Sputum	OXA-48	4 [R]	2 [S]	0.25 [S]	2 [S]
Y105	*K. pneumoniae*	Blood	Negative	16 [R]	0.25 [S]	0.5 [S]	16 [R]

Abbreviations: MEM, meropenem; TGC, tigecycline; COL, colistin; AK, amikacin; R, resistant; S, susceptible.

review.[21–25] All drugs were tested alone and in combination at fixed concentrations of meropenem 8 mg/L, colistin 2 mg/L, tigecycline 0.25 mg/L and amikacin 16 mg/L. Experiments were carried out with a starting inoculum of 5×10^5 CFU/mL. Tubes were incubated at 35°C with shaking. Samples were taken out at 0, 2, 4, 8, 16 and 24 h, serially diluted, plated and counted. The lower limit of accurately quantifiable CFU using was 1 $\log_{10}$ CFU/mL of viable bacteria per mL.[25]

Synergy was defined as ≥2 $\log_{10}$ CFU/mL decrease at 24 h for the antimicrobial combination compared with the most active single agent. No interaction was defined as <2 $\log_{10}$ CFU/mL increase or decrease at 24 h for the drug combination in comparison with the most active antibiotic alone. Antagonism was defined as ≥2 $\log_{10}$ CFU/mL increase between the combination and the most active single drug alone. Bactericidal activities of single drug or combinations were defined as a decrease of ≥3 $\log_{10}$CFU/mL compared with the untreated control at the start of each assay from the original inocula, whereas bacteriostatic activity was defined as <3 $\log_{10}$ CFU/mL decrease.[26]

In vivo *G. mellonella* Survival Assay

The in vivo *G. mellonella* survival assay was conducted as previously described.[22,27] Six clinical *K. pneumoniae* isolates with different resistance mechanisms were selected (Table 1). Standardized larvae were purchased from KaideRuixin Co. (Tianjin, China). The larvae are 5–6 instars, about 2–3 cm long, weighing about 250 mg, and have good activity and a creamy color. Caterpillars were inoculated with 10 μL of *K. pneumoniae* at a concentration of 1×10^7–1×10^8 CFU/mL (optimal infection dose of each strain causing approximately 80% lethality within 3 days) into the last left proleg using a 50 μL Hamilton syringe. Antibiotics were given as 10 μL injections either alone or in combination, into another proleg within half an hour after infection. The following dosages were based on human doses: meropenem 15 mg/kg, colistin 2.5 mg/kg, tigecycline 1 mg/kg, amikacin 15 mg/kg.[28,29] The larvae were observed for survival every 12 h for 3 days. Each treatment group had 20 caterpillars. Mock-inoculated (sterile saline) larvae were used as controls. Experiments were performed in triplicate and the results of any experiment in which two or more larvae died in any control group were discarded. The time–kill assay and *G. mellonella* survival assay were performed in triplicate.

Statistics

The differences in MIC values between different type of carbapenemase were estimated by Kruskal-Wallis test for one-way analysis of variance (ANOVA) followed by Dunn's multiple comparisons tests. Statistical significance was defined for an overall error at the 0.05 level (95% confidence interval). Survival rates were calculated and represented using GraphPad Prism 7 (GraphPad, La Jolla, USA). The log-rank (Mantel–Cox) test was used to compare survival rates between different treatment groups. *P* values of <0.05 were considered statistically significant.

Results

Bacterial Isolates

From 2015 to 2018, a total of 58 non-repetitive carbapenem-resistant clinical *K. pneumoniae* isolates were collected from 11 teaching hospitals in China. The CRKP isolates were recovered from various clinical specimens, including sputum (n=17), blood (n=11), urine (n=10), abscesses (n=6), drainage (n=6), bile (n=4), bronchoalveolar lavage fluid (n=3) and cerebrospinal fluid (n=1).

Antimicrobial Susceptibility of Tested Strains

All strains were non-susceptible to meropenem (MIC ranged from 2 to >256 mg/L). Twenty-six isolates (44.8%) were resistant to amikacin (MIC ranged from 1 to

Table 2 Susceptibility of 58 Clinical CRKps to Four Antimicrobial Agents

Antimicrobial	MIC (mg/L)			Susceptibility [n (%)]		
	Range	MIC_{50}	MIC_{90}	S	I	R
Meropenem	2 to >256	16	64	0 (0)	4 (6.9)	54 (93.1)
Tigecycline	0.12 to 8	0.5	2	53 (91.3)	4 (6.9)	1 (1.7)
Colistin	0.06 to 128	0.5	1	54 (93.1)	–	4 (6.9)
Amikacin	1 to >256	32	>256	29 (50)	3 (5.2)	26 (44.8)

Abbreviations: MIC, minimum inhibitory concentration; S, susceptible; I, intermediate; R, resistant.

>256 mg/L), whereas only four isolates (6.9%) were resistant to colistin and five isolates were non-susceptible to tigecycline. The 50% MIC (MIC_{50}) values of MEM, TGC, COL and AK were 16, 0.5, 0.5 and 32 mg/L, respectively. The 90% MIC (MIC_{90}) values of MEM, TGC, COL and AK were 64, 2, 1 and >256 mg/L, respectively (Table 2).

KPC-2-producing isolates and NDM-producing isolates were distributed with higher MIC values for meropenem than IMP-producing isolates and carbapenemase-non-producing isolates, although there was no difference in susceptibility (Figure 1).

Resistance Mechanisms of Tested Isolates

Fifty-eight clinical isolates were screened for their resistance mechanisms and it was confirmed that 21 (36.2%) isolates carried *bla*KPC-2, 11 (19.0%) isolates carried *bla*NDM-1, 13 (22.4%) isolates carried *bla*IMP (eight isolates carried *bla*IMP-4, four isolates carried *bla*IMP-8 and one isolate carried *bla*IMP-26), one (1.7%) isolate carried *bla*VIM-1, one (1.7%) isolate carried *bla*OXA-48 and 11 (19.0%) isolates carried no carbapenemase genes but had porin (ompK35 and/or ompK36) loss.

MLST

Among the 58 clinical CRKP isolates, the ST types of enrolled isolates were diverse (35 distinct STs were observed). ST11 (n=9, 16%) was the most predominant clone, followed by ST17 (n=4, 7%), ST48 (n=3, 5%), ST4928 (n=3), ST4930 (n=3) and another 30 STs (n=36).

Twenty-one KPC-2-producing isolates belonged to 17 ST types, while 11 NDM producers belonged to six ST types, 13 IMP producers belonged to eight ST types and 11 carbapenemase non-producers belonged to seven ST types. One VIM-1 producer was ST54 type and one OXA-48-producer was ST353 type.

Checkuerboard Test

The results of the checkerboard synergy are shown in Table 3. Detailed FICI values are shown in Supplementary Table 2. Different combinations of antimicrobial agents had different effects on various carbapenemase types.

Against KPC-2 or NDM-1 carbapenemase-producing isolates, TGC+AK and MEM+AK combinations showed the highest synergistic effect. TGC+AK combination showed a synergy+partial synergy rate of 76.2% against KPC-2-producing isolates and 54.5% against NDM-1-producing isolates. The MEM+AK regimen showed a synergy+partial synergy rate of 71.4% against KPC-2-producing isolates and 81.8% against NDM-1-producing isolates. The other four combination regimens mostly exhibited additive or indifferent effects. No combination exhibited an antagonistic effect.

Against IMP-carbapenemase-producing isolates, TGC +AK and MEM+COL combinations showed the highest rate of synergy effect, with both synergy+partial synergy rate of 61.5%, followed by MEM+AK combination (46.2%), AK +COL (46.2%), TGC+COL (30.8%) and MEM+TGC (15.4%). No combination exhibited an antagonistic effect. Against VIM-1-producing isolates, TGC+COL (FICI=0.5) and MEM+AK (FICI=0.31) combinations showed synergistic effects, while the other four combinations showed indifference. Against OXA-48-producing isolated, two combinations, MEM+TGC and MEM+AK, showed additivity (FICI=1), while the others showed indifference. Against carbapenemase-non-producing isolates, TGC+COL and TGC+AK combinations showed the highest rate of synergy effect, with synergy +partial synergy rates of 63.6% and 54.5%, followed by MEM +TGC combination (45.5%), MEM+AK (45.5%), MEM +COL (36.4%) and AK+COL (9.1%). No combination exhibited an antagonistic effect.

Time–Kill Assay

The time–kill assays for six clinical isolates treated with antimicrobials at clinically achievable serum levels are shown in Figure 2.

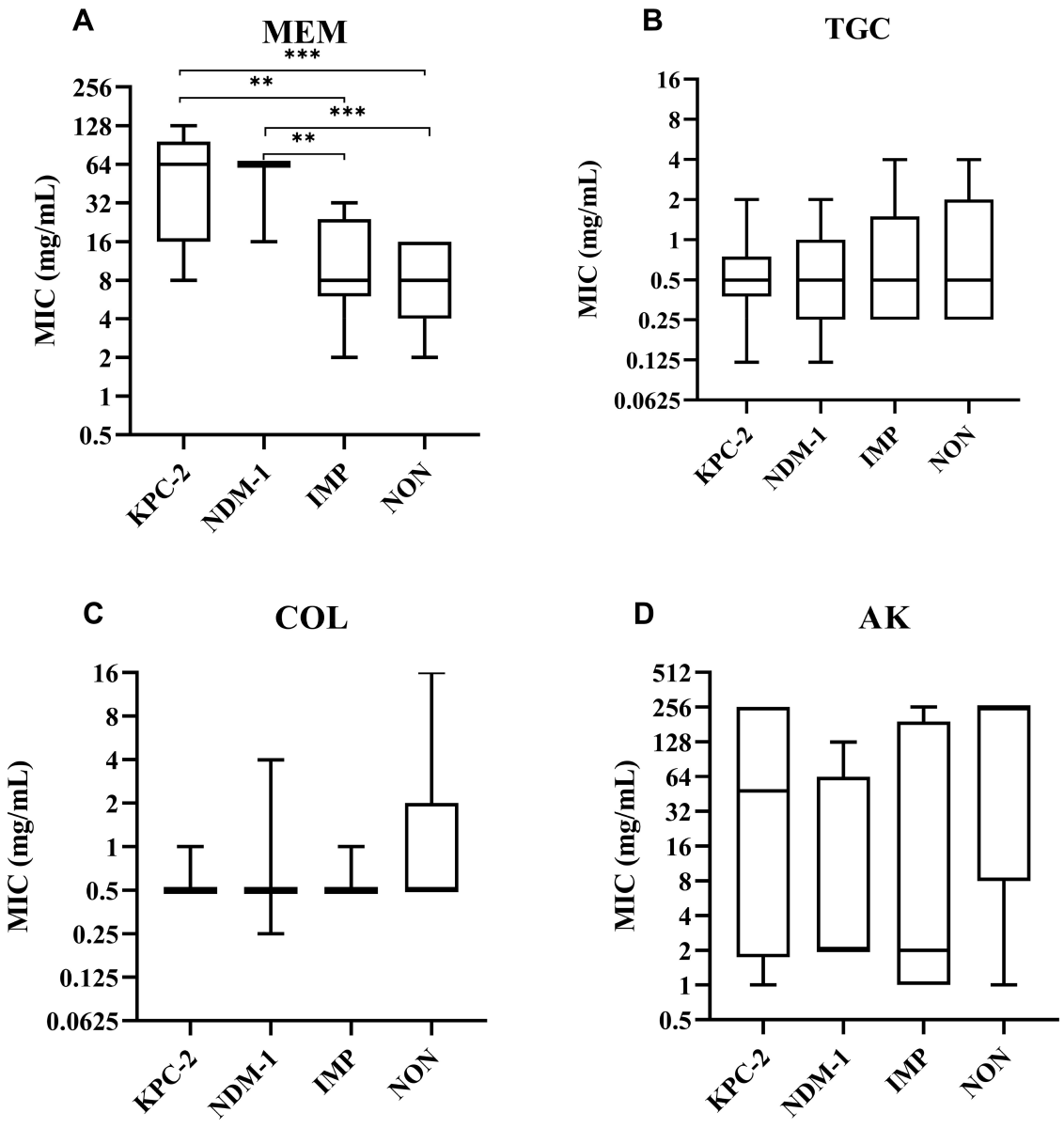

Figure 1 The difference in MIC values between different types of carbapenemase. (**A**) Meropenem (MEM); (**B**) tigecycline (TGC); (**C**) colistin (COL); (**D**) amikacin (AK).
Notes: ***p<0.001; **p<0.01.

Against KPC-2-producing isolate D16, colistin-based antimicrobial combinations, including COL+AK, COL +TGC and COL+MEM, showed a highly synergistic effect, with a $\log_{10}$ CFU/mL decrease of 7.4 at 24 h compared to the corresponding most active single drug. AK+TGC showed a 1.8 $\log_{10}$ CFU/mL decrease compared to single amikacin.

Against NDM-1-producing isolate L13, MEM+AK, COL+MEM and COL+TGC showed the highest synergistic effect, with $\log_{10}$ CFU/mL decreases of 6.5, 6.3 and 6.0 separately compared to the most active single drug.

Against IMP-4-producing isolate L34, MEM+COL and COL+TGC showed synergistic effects, with $\log_{10}$ CFU/mL decreases of 6.3 and 2.1 compared to the most active single drug.

Against VIM-1-producing isolate P5, COL+AK, MEM +TGC and AK+TGC showed synergistic effects, with $\log_{10}$ CFU/mL decreases of 6.4, 4.56 and 3.1 compared to the most active single drug.

Against OXA-48-producing isolate P13, COL+TGC and MEM+AK showed synergistic effects, with $\log_{10}$

Table 3 In vitro Combination Effect of Different Regimens Against CRKps with Different Resistance Mechanisms Using the Checkerboard Assay

Resistance Mechanism	Drug Combination	Synergy	Partial Synergy	Additivity	Indifference	Antagonism	Synergy+Partial Synergy
		FICI ≤0.5	0.5<FICI<1	FICI=1	1<FICI<4	FICI ≥4	
*bla*KPC-2 (n=21)	MEM+TGC	0 (0)	6 (28.6)	10 (47.6)*	5 (23.8)	0 (0)	6 (28.6)
	TGC+COL	1 (4.8)	7 (33.3)	3 (14.3)	10 (47.6)*	0 (0)	8 (38.1)
	MEM+AK	9 (42.9)*	6 (28.6)	4 (19.0)	2 (9.5)	0 (0)	15 (71.4)
	TGC+AK	6 (28.6)	10 (47.6)*	5 (23.8)	0 (0)	0 (0)	16 (76.2)
	MEM+COL	1 (4.8)	4 (19.0)	5 (23.8)	11 (52.4)*	0 (0)	5 (23.8)
	AK+COL	3 (14.3)	2 (9.5)	13 (61.9)*	3 (14.3)	0 (0)	5 (23.8)
*bla*NDM-1 (n=11)	MEM+TGC	0 (0)	4 (36.4)	5 (45.5)*	2 (18.2)	0 (0)	4 (36.4)
	TGC+COL	0 (0)	1 (9.0)	2 (18.1)	8 (72.7)*	0 (0)	1 (9.1)
	MEM+AK	3 (27.3)	6 (54.5)*	1 (9.0)	1 (9.0)	0 (0)	9 (81.8)
	TGC+AK	1 (9.0)	5 (45.5)*	2 (18.1)	3 (27.3)	0 (0)	6 (54.5)
	MEM+COL	1 (9.0)	3 (27.3)	1 (9.0)	6 (54.5)*	0 (0)	4 (36.4)
	AK+COL	0 (0)	3 (27.3)	4 (36.4)*	4 (36.4)*	0 (0)	3 (27.3)
*bla*IMP (n=13)	MEM+TGC	1 (7.7)	1 (7.7)	4 (30.8)	7 (53.8)*	0 (0)	2 (15.4)
	TGC+COL	0 (0)	4 (30.8)	1 (7.7)	8 (61.5)*	0 (0)	4 (30.8)
	MEM+AK	0 (0)	6 (46.2)*	1 (7.7)	6 (46.2)*	0 (0)	6 (46.2)
	TGC+AK	2 (15.4)	6 (46.2)*	4 (30.8)	1 (7.7)	0 (0)	8 (61.5)
	MEM+COL	2 (15.4)	6 (46.2)*	4 (30.8)	1 (7.7)	0 (0)	8 (61.5)
	AK+COL	1 (7.7)	5 (38.5)*	2 (15.4)	5 (38.5)*	0 (0)	6 (46.2)
*bla*VIM-1 (n=1)	MEM+TGC	0 (0)	0 (0)	0 (0)	1 (100.0)	0 (0)	0 (0.0)
	TGC+COL	1 (100.0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100.0)
	MEM+AK	1 (100.0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100.0)
	TGC+AK	0 (0)	0 (0)	0 (0)	1 (100.0)	0 (0)	0 (0.0)
	MEM+COL	0 (0)	0 (0)	0 (0)	1 (100.0)	0 (0)	0 (0.0)
	AK+COL	0 (0)	0 (0)	0 (0)	1 (100.0)	0 (0)	0 (0.0)
*bla*OXA-48 (n=1)	MEM+TGC	0 (0)	0 (0)	1 (100.0)	0 (0)	0 (0)	0 (0.0)
	TGC+COL	0 (0)	0 (0)	0 (0)	1 (100.0)	0 (0)	0 (0.0)
	MEM+AK	0 (0)	0 (0)	1 (100.0)	0 (0)	0 (0)	0 (0.0)
	TGC+AK	0 (0)	0 (0)	0 (0)	1 (100.0)	0 (0)	0 (0.0)
	MEM+COL	0 (0)	0 (0)	0 (0)	1 (100.0)	0 (0)	0 (0.0)
	AK+COL	0 (0)	0 (0)	0 (0)	1 (100.0)	0 (0)	0 (0.0)
Porin loss (n=11)	MEM+TGC	2 (18.2)	3 (27.3)*	3 (27.3)*	3 (27.3)*	0 (0)	5 (45.5)
	TGC+COL	2 (18.2)	5 (45.5)*	0 (0)	4 (36.4)	0 (0)	7 (63.6)
	MEM+AK	2 (18.1)	3 (27.3)	4 (36.4)*	2 (18.1)	0 (0)	5 (45.5)
	TGC+AK	0 (0)	6 (54.5)*	5 (45.5)	0 (0)	0 (0)	6 (54.5)
	MEM+COL	1 (9.0)	3 (27.3)	2 (18.1)	5 (45.5)*	0 (0)	4 (36.4)
	AK+COL	0 (0)	1 (9.0)	7 (63.6)*	3 (27.3)	0 (0)	1 (9.1)

Note: Date are presented as numbers (percentage %). *The maximum part in whole data in corresponding group.
Abbreviations: MEM, meropenem; TGC, tigecycline; COL, colistin; AK, amikacin.

CFU/mL decreases of 4.2 and 2.1 compared to the most active single drug.

Against carbapenemase-non-producing isolate Y105, MEM+TGC and COL+AK showed highly synergistic effects, with $\log_{10}$ CFU/mL decreases of 6.2 and 5.5 compared to the most active single drug. COL+TGC, MEM +AK and MEM+COL also showed slight synergistic effects.

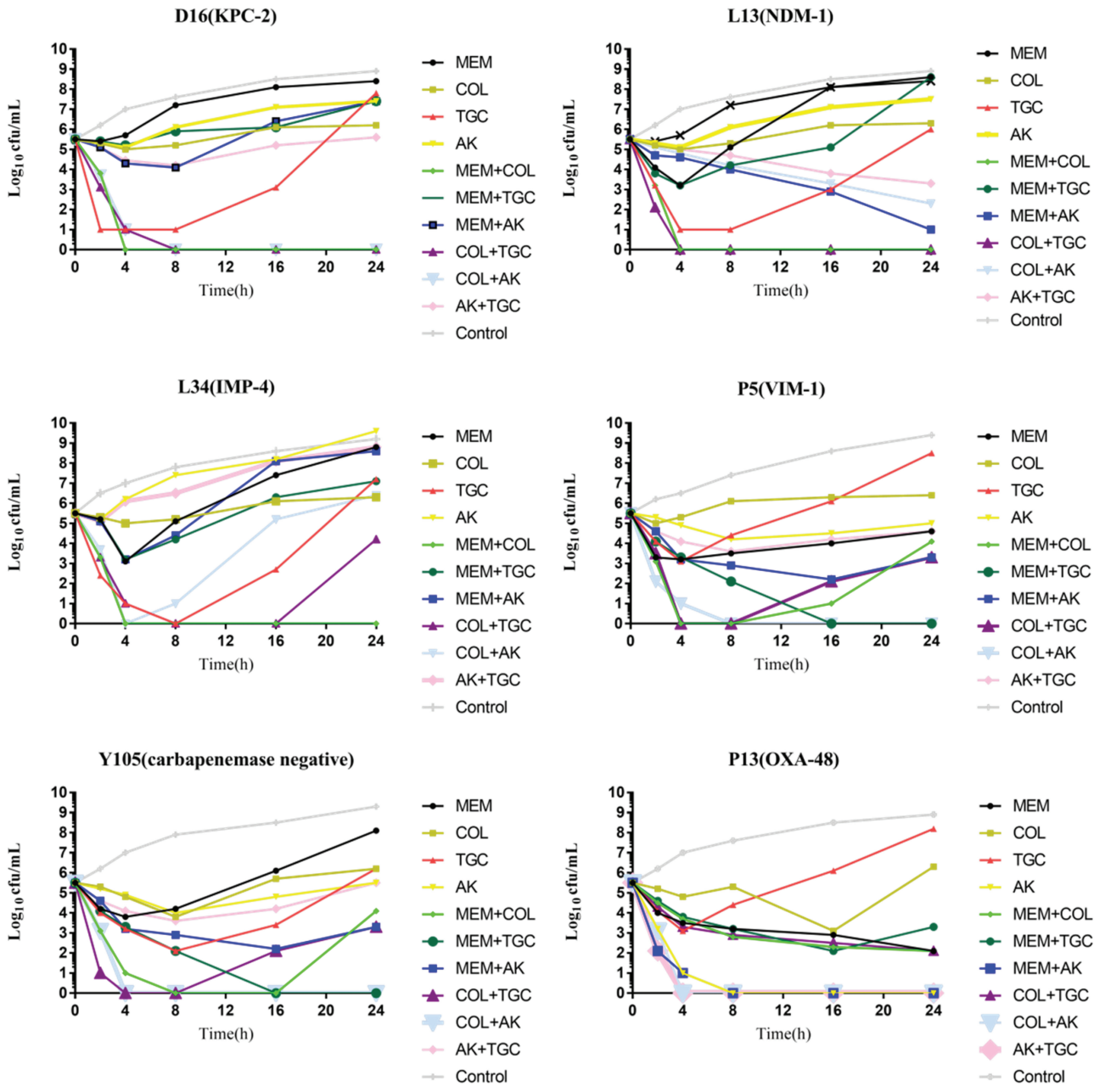

Figure 2 In vitro time–kill assay using meropenem (MEM), colistin (COL), tigecycline (TGC) and amikacin (AK), either alone or in combination, against six CRKps with different resistance mechanisms

In vivo Survival Tests in Larvae

Galleria mellonella larvae infected with six clinical strains were used to evaluate the in vivo interactions of drug combinations. Survival rates were used as the primary index to assess the in vivo interactions (Figure 3).

Against all the five isolates with different types of carbapenemases (KPC-2, NDM-1, IMP-4, VIM-1, OXA-48), MEM+AK, MEM+TGC and MEM+COL showed high survival rates (ranging from 70% to 100%) after 72 h of inoculation, compared to the control. And AK+TGC showed a high survival rate against IMP-4 (60%), VIM-1 (60%) and OXA-48 (85%). Against carbapenemase-non-producing isolate Y105, MEM+AK showed the highest survival rate (85%), followed by the MEM+TGC group (60%).

Against KPC-2-producing isolate D16, the control group without antimicrobial agents indicated that this strain a hypervirulent one. The MEM+COL group showed the highest survival rate (90%) after 72 h of inoculation, followed by the MEM+AK group (80%) and MEM+TGC

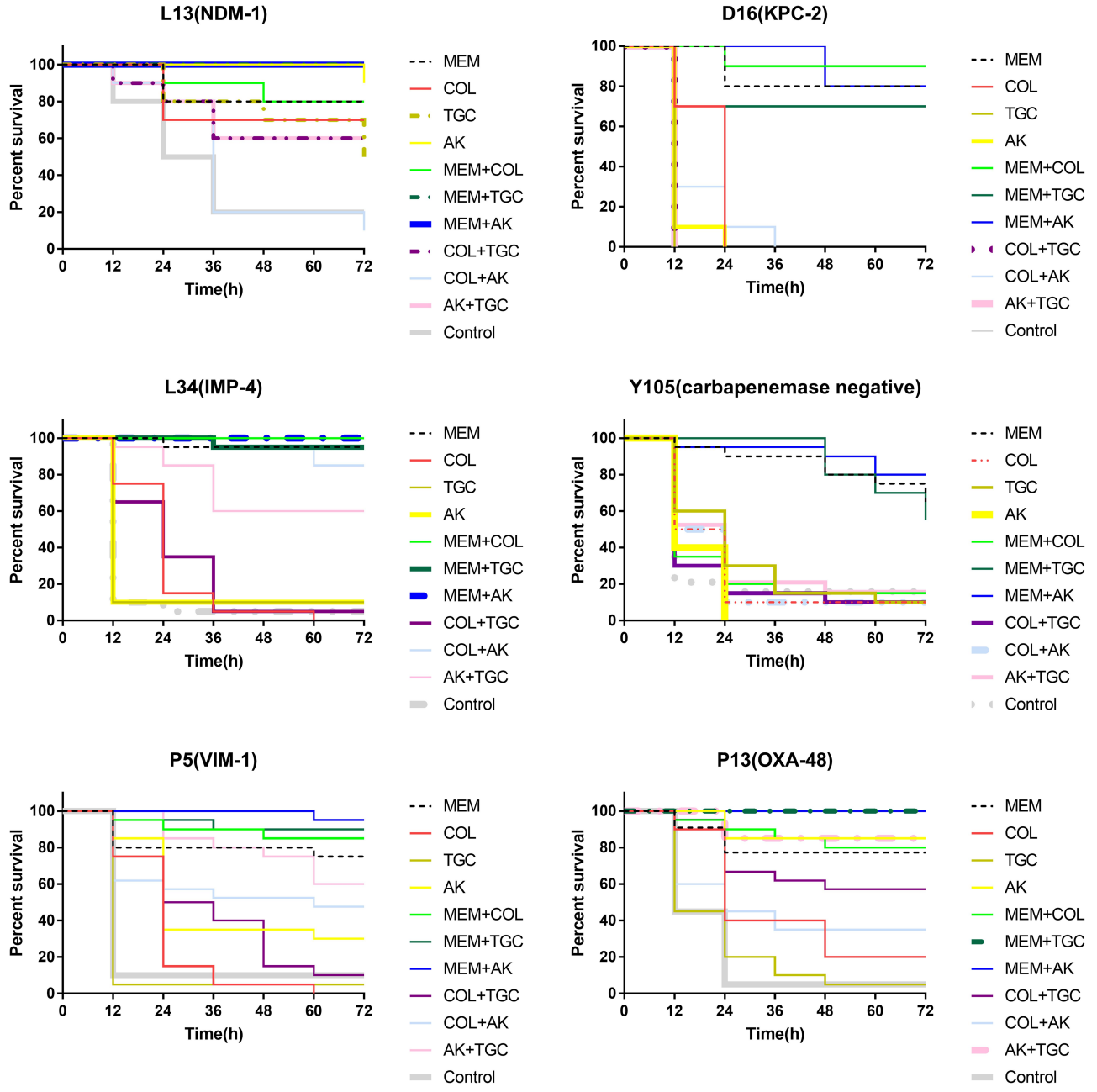

Figure 3 Survival rate of infected *Galleria mellonella* larvae treated with different drugs: meropenem (MEM), colistin (COL), tigecycline (TGC) and amikacin (AK), or mock-inoculated with sterile saline (controls).

group (70%). COL+TGC, COL+AK and AK+TGC groups showed inferior survival rates.

Against NDM-1-producing isolate L13, the MEM +TGC group and MEM+AK group showed the highest survival rate (100%) after 72 h of inoculation, followed by the MEM+COL group (80%).

Against IMP-4-producing isolate L34, the MEM+AK and MEM+COL group showed the highest survival rate (100%) after 72 h of inoculation, followed by the MEM +TGC group (95%) and AK+TGC group (60%).

Against VIM-1-producing isolate P5, the MEM+AK and MEM+TGC groups showed the highest survival rates (95% and 90% separately) after 72 h of inoculation, followed by the MEM+COL group (85%) and AK+TGC group (60%).

Against OXA-48-producing isolate P13, the MEM +AK and MEM+TGC groups showed the highest survival

rate (100%) after 72 h of inoculation, followed by the AK +TGC group (85%) and MEM+COL group (80%).

Against carbapenemase-non-producing isolate Y105, MEM+AK showed the highest survival rate (85%) after 72 h of inoculation, followed by the MEM+TGC group (60%).

Discussion

Infections caused by CRKp are severe and associated with limited treatment options; therefore, some researchers are looking for new future therapeutic weapons against strains of multidrug-resistant *K. pneumoniae*, such as essential oils from parts of or whole plants, poma inhibitor drugs and combinations of different agents.[30–33] Among them, combined antimicrobial therapy is increasingly used as the first-line treatment for CRKp to reduce the dosage of single antimicrobial agents,[34] and is associated with higher survival.[35] Although many studies have reported antimicrobial agent combinations against CRKP, there has been little research into therapy against CRKPs with different resistance mechanisms. In this study, we evaluated the synergistic effect of different combinations of clinically important antimicrobials used for CRKp infections by the checkerboard combination test, time–kill test and in vivo *G. mellonella* larvae infection model.

KPC-type carbapenemase is the most common transmissible class A carbapenemase in Enterobacteriaceae worldwide, while KPC-2 is common in China. In a report from the China CRE Network, KPC-2 enzyme was the most common carbapenemase and was identified in 78/155 isolates.[36] KPC carbapenemases are capable of hydrolyzing all β-lactams and always lead to high-level resistance to several antimicrobials. In our study, TGC+AK and MEM+AK combinations showed the highest synergistic effect against KPC-2-producing isolates (synergy+partial synergy rate of 76.2% and 71.4%), which is in agreement with previous studies.[37,38] At the same time, against selected isolate D16, we also found a synergistic effect of the COL+AK and COL+MEM combinations in the checkerboard test, which is also consistent with the time–kill assay and *G. mellonella* model. In our study, colistin and tigecycline showed high antimicrobial susceptibility against all CRKP isolates (93.1% and 91.3%, respectively), which was consistent with the high susceptibility rates of TGC (88.6%) and colistin (73.9%) to CRE in another survey.[39] However, inferior clinical outcomes and colistin resistance with colistin monotherapy were frequently observed in patients infected with KPC-producing *K. pneumoniae*.[40,41] Qureshi et al's study reported that combination therapy with colistin-polymyxin B or tigecycline and a carbapenem improved survival in bacteremia due to KPC-producing *K. pneumoniae*.[40] A clinical report published in 2019 showed that a TGC and MEM combination therapy failed in an infection caused by KPC-2-producing *K. pneumoniae*.[42] In this study, apart from the colistin and meropenem combination, AK combined with TGC or MEM showed in vitro synergistic effects as well, which may give physicians more therapeutic options.

The class B metallo-beta-lactamases are a complex group of enzymes that can hydrolyze all β-lactams except for the monobactams, including carbapenems, and are not inhibited by commercially available β-lactamase inhibitors. Notable transmissible MBL genes in Enterobacteriaceae include IMP-type, VIM-type and NDM-type.[43]

In our study, NDM-1-producing strains generally showed moderate to high levels of meropenem resistance (MICs of 16–64 mg/L). We found that amikacin showed a low resistance rate to NDM-1 producers, which was different from reports from India and the UK.[36,44] Regarding the best combination evaluation, we considered that the MEM+AK combination showed good in vitro and in vivo effects.

Klebsiella pneumoniae isolates producing IMP-type and VIM-type enzymes are rare in China. The 11 IMP-producing strains in this study showed low-level resistance to meropenem, in agreement with a previous study.[45] Against IMP-carbapenem-producing isolates, TGC+AK and MEM+COL combinations in the checkerboard test showed the highest rate of synergy effect, with a synergy+partial synergy rate of 61.5%. MEM+COL also showed good effects in the time–kill model and *G. mellonella* model.

Only one VIM-1-producing strain was enrolled in this study because this enzyme is very rare in *K. pneumoniae* in China. TGC+COL and MEM+AK combinations showed synergistic effects, while MEM+AK combination also had a good effect in the time–kill model and *G. mellonella* model.

OXA-48 enzymes hydrolyze penicillins at a high level and carbapenems at a low level, while sparing extended-spectrum cephalosporins. In China, OXA-48-producing *K. pneumoniae* strains are very rare. In our study, only one OXA-48-producing isolate was enrolled. MEM+TGC and MEM+AK showed additivite effects in the checkerboard test. MEM+AK showed good synergistic effects in the time–kill test and *the G. mellonella* model.

In addition to carbapenemase production, porin loss also plays an important role in carbapenem resistance. In *K. pneumoniae* isolates, the carbapenemase-non-producing

CRKp always lack their OmpK35 and OmpK36 porins. In our study, TGC+COL and TGC+AK combinations showed the highest rate of synergy effect, with synergy+partial synergy rates of 63.6% and 54.5%, which indicates that a tigecycline-based regimen may be effective against this type of CRKp.

In this study, we found that MEM+AK or MEM+COL combination always showed synergistic effects against CRKps, even though these strains had a low or high level of resistance to meropenem. Previous studies have indicated the mechanisms by which meropenem disrupts the formation of cell walls and helps amikacin to act on the bacteria. Carbapenem is a β-lactam antibiotic which binds to penicillin-binding proteins and prevents peptidoglycan synthesis, leading to lytic cell death.[46] Amikacin is an aminoglycoside antibiotic due to its polycationic nature, whereby aminoglycosides first bind to the anionic compounds found on the bacterial surface, then aberrant cytoplasmic membrane proteins cause damage to the integrity of the cytoplasmic membrane, facilitating the entry of aminoglycoside molecules in abundant quantities.[47] In our study, we also found a good effect of tigecycline plus amikacin, which may be useful in clinical guidance in the selection of combination drugs.

Conclusion

In summary, we have determined superior antimicrobial agent combinations against CRKp isolates by different resistance mechanisms. The combinations of MEM+AK, MEM+COL and TGC+AK showed synergistic effects and could provide alternative treatments for carbapenemase-producing *K. pneumoniae*, while TGC+COL performed better against carbapenemase-non-producing *K. pneumoniae*, which will be helpful in clinical settings where different CRKps are encountered. However, additional clinical studies are required to confirm the efficacy of these combinations.

Ethics Approval and Consent to Participate

The protocol has been reviewed by the human research ethics committee of the Institutional Review Board (IRB) of the Peking Union Medical College Hospital (Ethics Approval Number: S-K1167), and since all bacterial strains were from residual samples used in clinical diagnosis, it was determined that they met the criteria for exemption. This project did not involve any patient information, nor did it affect the normal diagnosis and treatment of patients. After consultation with the IRB, formal ethical approval was reviewed and waived, and written patient consent was not required.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work. The funders had no role in the study design, collection, and analysis of data, interpretation of results, or preparation of the manuscript.

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