Infection and Drug Resistance

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ORIGINAL RESEARCH

In vitro Synergistic and Bactericidal Effects of Aztreonam in Combination with Ceftazidime/Avibactam, Meropenem/Vaborbactam and Imipenem/Relebactam Against Dual-Carbapenemase-Producing *Enterobacterales*

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Objective: Our aim was to elucidate the resistance mechanisms and assess the combined synergistic and bactericidal activities of aztreonam in combination with ceftazidime/avibactam (CZA), meropenem/vaborbactam (MEV), and imipenem/relebactam (IMR) against Enterobacterales strains producing dual carbapenemases.

Methods: Species identification, antimicrobial susceptibility testing and determination of carbapenemase type were performed for these strains. Plasmid sizes, plasmid conjugation abilities and the localization of carbapenemase genes were investigated. Whole-genome sequencing was performed for all strains and their molecular characteristics were analyzed. In vitro synergistic and bactericidal activities of the combination of aztreonam with CZA, MEV and IMR against these strains were determined using checkerboard assay and time-kill curve assay.

Results: A total of 12 *Enterobacterales* strains producing dual-carbapenemases were collected, including nine *K. pneumoniae*, two *P. rettgeri*, and one *E. hormaechei*. The most common dual-carbapenemase gene pattern observed was $bla_{(KPC\text{-}2+NDM\text{-}5)}$ (n=4), followed by $bla_{KPC\text{-}2+IMP\text{-}26}$ (n=3), $bla_{(KPC\text{-}2+NDM\text{-}1)}$ (n=2), $bla_{(KPC\text{-}2+IMP\text{-}4)}$ (n=1), $bla_{(NDM\text{-}1+IMP\text{-}4)}$ (n=1) and $bla_{(KPC\text{-}2+KPC\text{-}2)}$ (n=1). In each strain, the carbapenemase genes were found to be located on two distinct plasmids which were capable of conjugating from the original strain to the receipt strain *E. coli* J53. The results of the checkerboard synergy analysis consistently revealed good synergistic effects of the combination of ATM with CZA, MEV and IMR. Except for one strain, all strains exhibited significant synergistic activity and bactericidal activity between 2 and 8 hours.

Conclusion: Dual-carbapenemase-producing *Enterobacterales* posed a significant threat to clinical anti-infection treatment. However, the combination of ATM with innovative *β*-lactam/β-lactamase inhibitor compounds had proven to be an effective treatment option.

Keywords: dual-carbapenemase-producing *enterobacterales*, *β*-lactam/*β*-lactamase inhibitor combinations, aztreonam, checkerboard assay, time-kill curve

Introduction

In recent decades, the increasing resistance of *Enterobacterales* to carbapenems has been attributed to the global spread of carbapenemase-producing *Enterobacterales* (CPE), raising serious concerns for public-health.[1] Carbapenems, which belong to the *β*-lactam class of antibiotics, exhibit the broadest spectrum of activity and highest potency against both

Received: 3 July 2024
Accepted: 27 August 2024
Published: 4 September 2024

Gram-positive and Gram-negative bacteria.[2] Carbapenemases are *β*-lactamases with versatile hydrolytic capabilities against penicillins, cephalosporins, monobactams and carbapenems.[3] According to the Ambler classification system, carbapenemases associated with carbapenem resistance in *Enterobacterales* are categorized as class A, B or D. Class-B *β*-lactamases are metallic ion-dependent enzymes which possess divalent cations (usually zinc) in the active site, which are essential for hydrolysis of the *β*-lactam ring, whereas class A and D *β*-lactamases have serine at their active sites.[1,3] *Klebsiella pneumoniae* carbapenemases (KPCs) are the most prevalent transmissible class A carbapenemases circulating in Enterobacteriaceae worldwide.[4] Furthermore, metallo-*β*-lactamases (MBLs), such as imipenemase (IMP), Verona integron-mediated metallo-β-lactamase (VIM), and New Delhi metallo-β-lactamase (NDM), have emerged as significant issues in *Enterobacterales*.[5]

Due to their significant public-health threat, CPE have been classified as one of the most critical antibiotic-resistant pathogens by the World Health Organization (WHO).[6] In response to the emerging resistance posed by CPE and the resulting epidemics, novel *β*-lactam/*β*-lactamase inhibitor (BL/BLI) combinations, such as ceftazidime / avibactam (CZA), meropenem / vaborbactam (MEV), and imipenem / relebactam (IMR) have demonstrated high efficacy against CPE. However, these new antimicrobial agents usually lack efficacy against CPE strains producing MBLs.[7] Consequently, strains that produce dual carbapenemases, such as MBL plus KPC, MBL plus OXA-48-like, or dual MBLs, are often resistant to CZA, MEV, and IMR.[8,9] Given the current antimicrobial landscape, it is crucial to explore effective treatments for those infections, with combination therapy serving as a valuable alternative for addressing these resistant strains.

Aztreonam(ATM), a monobactam class of antibiotic, exhibits effective antimicrobial activity against strains producing MBL, but is ineffective against isolates producing extended-spectrum β- lactamases(ESBLs), AmpC enzymes, and carbapenemases. When combined with avibactam or CZA, aztreonam has demonstrated high antimicrobial activity against MBL-producing *Enterobacterales* at approximately 80% efficacy,[9,10] because aztreonam is not hydrolised by MBLs. Compared to ATM alone, this combination resulted in a direct reduction of aztreonam's minimum inhibitory concentration (MIC) over 128-fold.[11] The use of ATM in combination with CZA is currently considered a reasonable option for clinical use in the management of infections caused by MBL producers. However, evidence of ATM in combination with MEV or IMR is relatively limited.[12]

The emergence of dual-carbapenemase-producing *Enterobacterales* has been reported sporadically worldwide. However, the in vitro efficacy of the combination of aztreonam with CZA, MEV or IMR against these strains was limited.[13] This study investigated 12 strains with different carbapenemase combination patterns. Alongside analyzing the molecular resistance characteristics of these strains, we evaluated the activity of the combinations of aztreonam with CZA, MEV and IMR.

Methods

Antimicrobial Susceptibility Testing and Carbapenemase Identification

A total of 12 carbapenem-resistant *Enterobacterales* strains were collected from clinical patients during routine clinical diagnostics in Zhejiang province. All isolates were identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Bruker Daltonik, Bremen, Germany). Antimicrobial susceptibility testing was conducted following the recommendations of the Clinical and Laboratory Standards Institute (CLSI). Susceptibility testing to ampicillin, ampicillin/Sulbactam, piperacillin/tazobactam, cefazolin, cefotetan, ceftazidime, ceftriaxone, cefepime, aztreonam, ertapenem, imipenem, amikacin, gentamicin, tobramycin, ciprofloxacin and levofloxacin was performed using the VITEK 2 automated system (bioḾ erieux, Marcy-l'´ Etoile, France). Susceptibility to tigecycline, colistin, ceftazidime-avibactam, meropenem-vaborbactam and imipenem-relebactam were further investigated using standard broth microdilution tests with Mueller–Hinton broth (cation-adjusted; Oxoid Ltd., England). Quality control (QC) strains *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were utilized for each experimental sitting. Carbapenem resistance was defined as resistance to any carbapenem. Main carbapenemase types were identified using a multiplex lateral flow immunoassay[14,15] (NG-Test CARBA 5, NG Biotech, France) including NDM-, KPC-, IMP- and VIM-type and OXA-48-like.

Plasmid Localization, Conjugation Assay and Stability Testing

The number and size of plasmids were determined using S1-pulsed-field gel electrophoresis (S1-PFGE) as previously described.[16] The localization of dual-carbapenemase genes were detected through southern blotting and hybridization with a DIG-labeled specific probe.

Conjugation experiments were performed using the broth mating method.[17] The carbapenem-resistance isolates served as the donor bacteria, while sodium azide-resistant *E. coli J53* was used as the recipient bacteria. Transconjugants were selected on MH agar supplemented with 1 μg/mL of meropenem and 100 μg/mL of sodium azide for 18 hours. PCR and antimicrobial susceptibility testing were performed to confirm the presence of the respective transconjugants.

The plasmid stability of transconjugants was assessed through serial passage over 10 days, with a 1:1000 dilution in antibiotic-free LB broth. Each day, isolates were collected and stored in glycerol at −80°C. The serially collected isolates were then analyzed using PCR to detect the presence of corresponding NDM-, KPC- and IMP- type carbapenemases.

Whole Genome Sequencing

DNA was extracted using the DNA extraction kit (Qiagen Valencia, CA). Dual-carbapenemase-producing *Enterobacterales* underwent whole genome sequencing using the HiSeq X ten (Illumina, San Diego, CA, USA) and MinION platforms (Nanopore, Oxford, UK) at Zhejiang Tianke (Hangzhou, China). Raven and Prokka 1.11 were employed to generate the complete genome sequence and for gene annotation, respectively.[18]

Detection of Antimicrobial Resistance Genes, Plasmids and MLST

Multi Locus Sequence Typing (MLST) of all strains was determined using MLST service at the Center for Genomic Epidemiology (http://genomicepidemiology.org/services/). Antimicrobial resistance genes were identified using ResFinder_v4.4.1 (http://genepi.food.dtu.dk/resfinder). Plasmid replicon types were investigated using PlasmidFinder and pMLST at the Center for Genomic Epidemiology (http://genomicepidemiology.org/services/) with a 95% threshold for identity and 100% coverage. The assembled sequences were deposited in GenBank bioproject as follows with accession number: no. PRJNA845008, no. PRJNA845066, no. PRJNA845067, no. PRJNA845069, no. PRJNA845070, no. PRJNA845071, no. PRJNA845073, no. PRJNA845075, no. PRJNA845076.

The Checkerboard Synergy Test

Strains that co-produced KPC and MBL, as well as strains producing dual MBL, were chosen for an in vitro checkerboard synergy test to evaluate the combined antibacterial efficacy of ATM with CZA, MEV and IMR. Before conducting the combined antibiotic synergy test, individual antibiotic susceptibility testing for ATM, CVZ, MEV, and IMR was performed using the standard microdilution method.

The standard broth microdilution checkerboard synergy test was performed in triplicate using 96-well microtiter plates to determine the combined antibacterial effects. The 96-well microtiter plates contained ATM concentrations ranging from 0.25 ug/mL to 1024 ug/mL on the Y-axis according to individual strain and with CZA, MEV and IMR concentrations ranging from 0.06/4 ug/mL to 64/4 ug/mL on the X-axis. This ensured that each concentration of ATM and BL/BLI combinations was cross-combined in the assay. The initial inoculum was approximately $5*10^5$ CFU/mL. Microtiter trays were incubated at 35°C for 24 h under aerobic conditions. One well with no antibiotic was used as a positive growth control on each plate. The MIC was determined as the well in the microtiter plate with the lowest drug concentration at which there was no visible growth.

The effects of antimicrobial combinations were evaluated based on the fractional inhibitory concentration index (FICI), which was calculated using the following formula: FICI= (MIC of agent A in combination/MIC of agent A alone) + (MIC of agent B in combination/ MIC of agent B alone). It was noted, when the MIC after combination was equal to or lower than the lowest concentration in the 96-well plate, the MIC of the next lower concentration was used for FICI index calculation. If the MIC was equal to or higher than the highest concentration in the 96-well plate, the MIC of the next higher concentration was utilized for FICI index calculation. The interpretation of FICIs was as follows: FIC <= 0.5

indicated synergism, 0.5 < FIC <= 1 denoted an additive effect, 1 < FIC <= 2 signified no effect, and FIC > 2 indicated an antagonistic effect.[19]

Time-Kill Curve Assay

This assay was used to confirm the synergistic effect of ATM with combined antibiotics. The time-kill curve assays were performed with some modifications according to a previously described method.[19] Specifically, 4 strains of *K. pneumoniae*(C, H, I, L), 1 strain of *E. hormaechei*(B) and 1 strain of *P. rettgeri*(Q) with different carbapenemases combinations were selected for time-kill assays. The antibacterial activities of ATM(16ug/mL), CZA(4/4ug/mL), IMR(4/4ug/mL) and MEV(4/4ug/mL) individually, as well as in combination as ATM-CZA, ATM-IMR and ATM-MEV, were determined. Briefly, a single clone was suspended in 5mL fresh LB broth and incubated overnight with shaking at 220 rmp/min. On the second day, the overnight culture was diluted 1:10 and cultured 3–4 hours, and then an appropriate amount of bacterial was prepared with a concentration of $5*10^5$ CFU/mL in a final volume of 10 mL LB broth containing antibiotics and the free-antibiotic LB serving as a growth control. All tubes were incubated at 37°C with shaking at 220 rmp/min and viability colony counts were determined by serially diluting aliquots in 0.9% saline and plating them on MH plates at intervals of 0 h, 30min, 1h, 2 h, 4 h, 6 h, 8 h and 24 h. The reproducibility of the kill-time assay results was confirmed through triplicate experiments.

Calculate the variation in CFU/mL between the combination tube at time 24h and the most effective single agent at time 24 hours, synergistic activity of a combination was defined as a decrease of ≥2 log10 CFU/mL between them. Then calculate the difference in CFU/mL between the combination tube at time 24 hours and at time 0 hour, bactericidal activity was defined as ≥3 log10 CFU/mL reduction in cell numbers compared to the initial inoculum after 24 hours. Time-kill curves were constructed by plotting mean colony counts versus time, using time point on the X-axis and bacterial amount on the Y-axis with GraphPad Prism5.0 software.

Results

Antimicrobial Susceptibility and Characteristics of Dual-Carbapenemase-Producing Enterobacterales Isolates

All those isolates were collected from four hospitals, comprising nine *Klebsiella pneumoniae* (*K. pneumoniae)*, two *Providencia rettgeri* (*P. rettgeri)* and one *Enterobacter hormaechei* (*E. hormaechei)* isolate. All strains demonstrated resistance to *β*-lactams including carbapenems. Additionally, they exhibited partial resistance to other antimicrobial agents such as amikacin, tobramycin, levofloxacin and ciprofloxacin. In contrast, tigecycline and colistin maintained relatively high susceptibility levels, positioning them as preferred options due to their effective in vitro activity, except for the intrinsic resistance observed in *P. rettgeri* (Table 1).

Among the 12 strains, most showed resistance to CZA, MEV, and IMR, while only a few strains were susceptible. Two strains of *P. rettgeri* were characterized as pan-drug resistant (PDR), demonstrating resistance to commonly used clinical antibiotics as well as to polymyxin B, CZA, MEV and IMR (Table 2).

The results of the NG-Test CARBA 5 rapid detection revealed that all 12 strains were positive for dual carbapenemases. Among them, 6 strains produced both KPC and NDM, 4 strains produced both KPC and IMP, one strain produced NDM and IMP, and one strain produced a duplicated KPC.

Localization of Dual-Carbapenemase and Plasmid Conjugation Ability

We conducted further investigations into the location of carbapenemase-encoding genes in these strains. S1-PFGE and Southern blot analysis confirmed that the carbapenem-resistant genes in each strain were situated on two plasmids of varying size (Supplemental Figure 1). Notably, none of the strains harbored dual-carbapenemase genes on the same plasmid simultaneously.

In terms of conjugation ability, six strains successfully transferred a plasmid carrying a single carbapenemase gene to the recipient bacteria. However, all strains failed to transfer two plasmids simultaneously carrying carbapenemase genes to the recipient bacteria. The results indicated that in the successfully transferred plasmids, the plasmid harboring bla_{KPC}

Table 1 Antimicrobial Susceptibility Testing of Clinical Strains and Transconjugants (μg/mL)

Strain name	Species	Carbapenem Resistant genes	AMP	SAM	TZP	CZO	CTT	CAZ	CRO	FEP	ATM	ETP	IMP	AMK	GEN	TOB	CIP	LEV	TGC	CO	CZA
LSG-AR4324	*K. pneumoniae*	bla_{KPC-2}, bla_{KPC-2}	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	≥64	≥8	≥16	≥64	≥16	≥16	≥4	≥8	1	0.13	4
FYH-SY	*K. pneumoniae*	bla_{KPC-2}, bla_{IMP-4}	≥32	≥32	64	≥64	<=4	≥64	16	<=1	16	4	8	<=2	<=1	<=1	≥4	2	2	0.13	2
FSQ	*K. pneumoniae*	bla_{KPC-2}, bla_{NDM-5}	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	≥64	≥8	≥16	≥64	≥16	≥16	≥4	≥8	4	0.03	≥128
Kpn-96	*K. pneumoniae*	bla_{KPC-2}, bla_{IMP-26}	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	≥64	≥8	≥16	<=2	≥16	8	2	1	0.5	0.13	≥128
Kpn-98	*K. pneumoniae*	bla_{KPC-2}, bla_{IMP-26}	≥32	≥32	64	≥64	≥64	≥64	≥64	≥64	≥64	≥8	4	<=2	≥16	8	1	1	1	0.13	≥128
Kpn-103	*K. pneumoniae*	bla_{KPC-2}, bla_{IMP-26}	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	≥64	≥8	4	<=2	≥16	8	<=0.25	<=0.25	0.25	0.25	≥128
XL-200731214	*K. pneumoniae*	bla_{KPC-2}, bla_{NDM-5}	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	≥64	≥8	≥16	≥64	≥16	≥16	≥4	≥8	4	0.03	≥128
XL-20059286	*K. pneumoniae*	bla_{KPC-2}, bla_{NDM-5}	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	≥64	≥8	≥16	≥64	≥16	≥16	≥4	≥8	4	0.03	≥128
20040580	*K. pneumoniae*	bla_{KPC-2}, bla_{NDM-5}	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	≥64	≥8	≥16	≥64	≥16	≥16	≥4	≥8	0.5	2	≥128
21030615019	*P. rettgeri*	bla_{KPC-2}, bla_{NDM-1}	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	≥64	≥8	≥16	≥64	≥16	≥64	≥4	≥8	1	64	≥128
21030415031	*P. rettgeri*	bla_{KPC-2}, bla_{NDM-1}	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	≥64	≥8	≥16	≥64	≥16	≥16	≥4	≥8	1	>64	≥128
ZHH	*E. hormaechei*	bla_{NDM-1}, bla_{IMP-4}	ND	ND	≥128	≥64	≥64	≥64	≥64	≥64	≥64	≥8	≥16	16	8	≥16	1	1	1	0.5	≥128
E.coli J53	*E.coli*	/	8	4	<=4	<=4	<=1	<=1	<=1	<=1	<=0.5	<=1	<=2	<=1	<=1	<=0.25	<=0.25	<=0.25	0.25	0.13	0.25/4
J53T1	*E.coli*	bla_{KPC-2}	≥32	≥32	≥128	≥64	8	16	≥64	4	≥64	4	≥16	4	<=1	<=1	1	1	0.03	0.03	0.125
J53T2	*E.coli*	bla_{KPC-2}	≥32	≥32	64	≥64	<=4	2	8	<=1	16	≥8	8	<=2	<=1	<=1	2	1	0.25	2	0.25/4
J53T3	*E.coli*	bla_{KPC-2}	≥32	≥32	<=4	≥64	<=4	16	≥64	≥64	16	<=0.5	2	<=2	<=1	<=1	<=0.25	<=0.25	0.125	2	0.25/4
J53T4	*E.coli*	bla_{KPC-2}	≥32	≥32	≥128	≥64	<=4	16	≥64	2	≥64	4	≥16	<=2	<=1	<=1	1	1	0.25	2	0.5/4
J53T5	*E.coli*	bla_{NDM-5}	≥32	≥32	≥128	≥64	≥64	≥64	≥64	32	<=1	≥8	≥16	4	<=1	<=1	<=0.25	<=0.25	0.125	2	≥128
J53T6	*E.coli*	bla_{NDM-1}	≥32	≥32	≥128	≥64	≥64	≥64	≥64	8	<=1	4	≥16	<=2	<=1	<=1	0.5	<=0.25	0.03	0.13	≥128

Note: J53T1: *E.coli* J53 (pKPC-LSG-AR4324), J53T2: *E.coli* J53 (pKPC-FYH-SY), J53T3: *E.coli* J53 (pKPC-Kpn-96), J53T4: *E.coli* J53 (pKPC-Kpn-98), J53T5: *E.coli* J53 (pNDM-20040580), J53T6: *E.coli* J53 (pNDM-ZHH).
Abbreviation: AMP, ampicillin; SAM, ampicillin-sulbactam; TZP, piperacillin-tazobactam; CZO, cefazolin; CTT, cefotetan; CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; ATM, aztreonam; ETP, ertapenem; IMP, imipenem; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin; LEV, levofloxacin; TGC, tigecycline; CO, colistin; CZA, ceftazidime-avibactam. ND, not detected.

https://doi.org/10.2147/IDR.S474150

https://doi.org/10.2147/IDR.S474150 DovePress

Table 2 The Results of Checkerboard Synergy Test (μg/mL)

Strain Name	Species	ST Types	Carbapenem Resistant Genes	MIC Values of Mono-Antibiotic				Checkerboard Assay Result								
								ATM+CZA			ATM+MEV			ATM+IMR		
				ATM	CZA	MEV	IMR	ATM	CZA	FICI	ATM	MEV	FICI	ATM	IMR	FICI
FSQ	*K. pneumoniae*	11	bla_{KPC-2}, bla_{NDM-5}	2048	>1024	1024	512	64	<=0.13	0.031	512	64	0.313	64	1	0.033
Kpn-96	*K. pneumoniae*	476	bla_{KPC-2}, bla_{IMP-26}	512	>1024	32	2	2	<=0.13	0.004	8	<=0.06	0.017	/	/	/
Kpn-98	*K. pneumoniae*	476	bla_{KPC-2}, bla_{IMP-26}	128	≥128	2	2	2	<=0.13	0.004	/	/	/	/	/	/
Kpn-103	*K. pneumoniae*	/	bla_{KPC-2}, bla_{IMP-26}	256	>1024	32	2	4	<=0.13	0.016	4	<=0.06	0.017	/	/	/
XL-200731214	*K. pneumoniae*	11	bla_{KPC-2}, bla_{NDM-5}	1024	>1024	256	256	16	<=0.13	0.016	256	8	0.281	64	1	0.066
XL-20059286	*K. pneumoniae*	11	bla_{KPC-2}, bla_{NDM-5}	2048	>1024	1024	512	8	<=0.13	0.004	512	2	0.252	64	1	0.033
20040580	*K. pneumoniae*	11	bla_{KPC-2}, bla_{NDM-5}	1024	>1024	1024	512	128	<=0.13	0.125	1024	1	1.001	64	1	0.064
21030615019	*P. rettgeri*	/	bla_{KPC-2}, bla_{NDM-1}	256	>1024	1024	2048	<=0.25	<=0.13	0.001	<=0.25	<=0.06	0.001	0.5	1	0.002
21030415031	*P. rettgeri*	/	bla_{KPC-2}, bla_{NDM-1}	64	1024	1024	2048	8	<=0.13	0.125	<=0.25	<=0.06	0.002	0.5	1	0.008
ZHH	*E. hormaechei*	97	bla_{NDM-1}, bla_{IMP-4}	256	>1024	512	256	2	<=0.13	0.008	8	<=0.06	0.031	8	1	0.035
FYH-SY	*K. pneumoniae*	147	bla_{KPC-2}, bla_{IMP-4}	16	2	2	2	/	/	/	/	/	/	/	/	/
LAS-AR4324	*K. pneumoniae*	11	bla_{KPC-2}, bla_{KPC-2}	64	4	2	2	/	/	/	/	/	/	/	/	/

Notes: /, not applied. Since Kpn-96 and Kpn-103 were susceptible to IMR, the checkerboard synergy test of ATM with IMR was not applied. Kpn-98 and FYH-SY were susceptible to CZA, MEV and IMR, the checkerboard synergy test with ATM was not applied. Since LAS-AR4324 only carried KPC-2 and was susceptible to CZA, MEV and IMR, the checkerboard synergy test with ATM was not applied.

Abbreviation: ATM, aztreonam; CZA, Ceftazidime/avibactam; MEV, Meropenem/vaborbactam. IMR, Imipenem/relebactam.

was transferred when the combination was bla_{KPC} + bla_{IMP}, the plasmid harboring bla_{NDM} was transferred when the combination was bla_{NDM} + bla_{KPC}, and the plasmid harboring bla_{NDM} was transferred when the combination was bla_{NDM} + bla_{IMP}. Among the six transconjugants, three strains acquired the plasmid harboring bla_{KPC-2} and exhibited carbapenem resistance, except for one transconjugant that remained susceptible. Two transconjugants acquired the plasmid harboring bla_{NDM} and showed resistance to CZA (Table 1). None of the conjugates lost resistance genes for NDM-, KPC-, or IMP-type carbapenemases during 10 days of antibiotic-free passage.

MLST, Antimicrobial Resistance Genes and Plasmids

In order to enhance our understanding of the genomic characteristics and plasmid profiles of dual-carbapenemase-producing strains, we conducted whole-genome sequencing on nine strains. Among these strains, ST11 (n=5) emerged as the predominant clonal type of *K. pneumoniae*, accompanied by individual strains of ST147 and ST476. Furthermore, one strain of *E. hormaechei* was classified under ST97, while MLST typing for two *P. rettgeri* strains remained indeterminate due to the absence of established MLST standards.

Then, our analysis focused on the genetic determinants of carbapenem resistance and the multidrug-resistant patterns observed in the phenotypic susceptibility testing. The predominant carbapenemase gene identified was bla_{KPC-2} (n=11), followed by bla_{NDM-1} (n=4), bla_{NDM-5} (n=3), bla_{IPM-26} (n=3), and bla_{IPM-4} (n=2). The most common dual-carbapenemase gene patterns among CRE strains was $bla_{(KPC-2+NDM-5)}$ (n=4), followed by $bla_{KPC-2+IMP-26}$ (n=3), $bla_{(KPC-2+NDM-1)}$ (n=2), $bla_{(KPC-2+IMP-4)}$ (n=1), $bla_{(NDM-1+IMP-4)}$ (n=1) and $bla_{(KPC-2+KPC-2)}$ (n=1) which carried double copes of bla_{KPC-2}. The carbapenemase genes carried by each strain producing dual enzymes were situated on separate plasmids, and these strains also harbored other resistance genes, such as ESBL genes, aminoglycoside resistance genes, fluoroquinolone resistance genes, fosfomycin resistance genes, macrolide, lincosamide, and streptogramin B resistance genes, rifampicin resistance genes, sulfonamides resistance genes, tetracycline resistance genes, and trimethoprim resistance genes (Figure 1). Plasmids carrying bla_{KPC-2} were primarily of the IncFII(pHN7A8) type, whereas plasmids carrying bla_{NDM} encompassed various types, including IncT, IncX3, IncHI2, and IncI1-I (Alpha).

Synergy Testing with Combination Drugs

Based on the enzymatic properties of CZA, MEV, and IMR against β-lactamases, it is established that these antibiotics were ineffective in inhibiting MBLs in CPE. Conversely, ATM demonstrated efficacy in overcoming the hydrolysis by MBLs. Our study aimed to investigate the synergistic effects of ATM in combination with CZA, MEV, and IMR against strains that produced both MBLs and KPC-2 carbapenemase. Due to the presence of dual bla_{KPC-2} in LAS-AR4324, and the susceptibility of FYH-SY and Kpn-98 to CZA, MEV, and IMR, these strains were excluded from subsequent testing. Moreover, as Kpn96 and Kpn103 demonstrated susceptibility to IMR, they were not included in the synergistic assessment involving combinations with ATM and IMR.

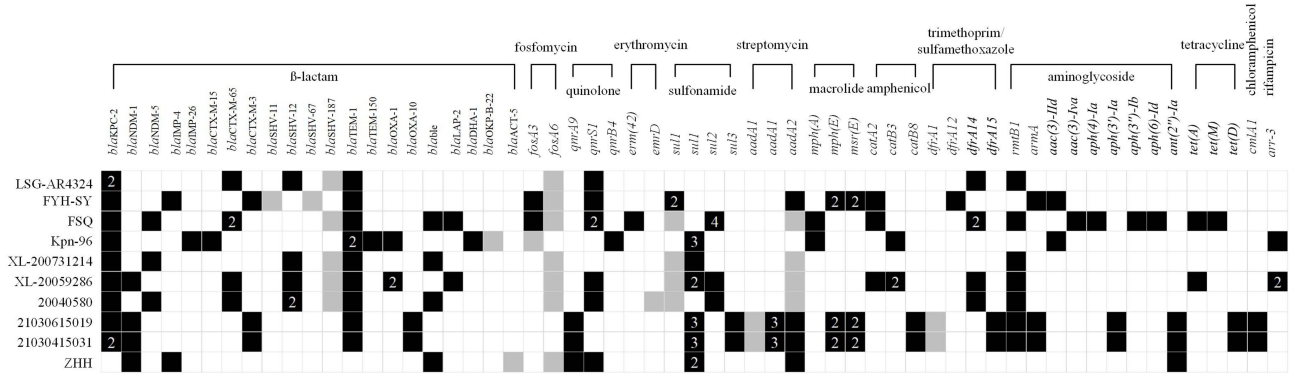

Figure 1 Resistance genes harboring in the representative strains. Black represents resistance genes on plasmids, while gray represents resistance genes on chromosomes.

The checkerboard synergy analysis consistently demonstrated favorable synergistic effects across most strains, with the exception of one strain exhibiting an FICI level of one when ATM was combined with MEV. (Table 2)

Kill-Time Curve Analysis for ATM in Combination with CZA, IMR and MEV

To further verify the synergistic effects of ATM in combination with CZA, MEV and IMR, kill-time curves were generated for these combinations against the respective strains. As showed in Figure 2, strains B, C, L and Q underwent kill-time curve analysis for the combination of ATM with CZA. The colony counts of these strains treated with combination agents showed a significantly decreased ($p < 0.0001$) compared to those treated with ATM or CZA alone between 2–6 hours. The results demonstrated significant bactericidal activity effects within this frame. It was noteworthy that the kill-time curve of strain C and L displayed remarkable bactericidal activity effects between 2 and 6 hours, whereas the synergistic effect of ATM and CZA gradually diminished after 8 hours, leading to regrowth. Conversely, B, and Q demonstrated sustained and prominent synergistic bactericidal effects throughout the observation period.

The kill-time curve analysis of ATM and MEV demonstrated substantial bactericidal activity during the initial 8 hours of the experiment. However, as time advanced, proliferation was observed in strain B, while the growth of strains C and Q was markedly inhibited over the observed 24-hour period. Notably, strong synergistic and bactericidal effects were observed in strains C and Q.

Strains B, Q, I, and H were subjected to kill-time curve analysis for ATM in combination with IMR. The combined treatment exhibited significant bactericidal activity effects from 2 to 8 hours for strains B and Q, leading to markedly lower bacterial counts compared to those treated with individual drugs. However, after 8 hours, strains B and Q displayed regrowth, and the bactericidal activity of ATM in combination with IMR gradually attenuated. Strain H demonstrated notable bactericidal activity effects from 2 to 6 hours, but bacterial growth became apparent after 6 hours. Although the FICI for strain I suggested a synergistic effect for the ATM plus IMR combination, this was not confirmed by the kill-time curve analysis.

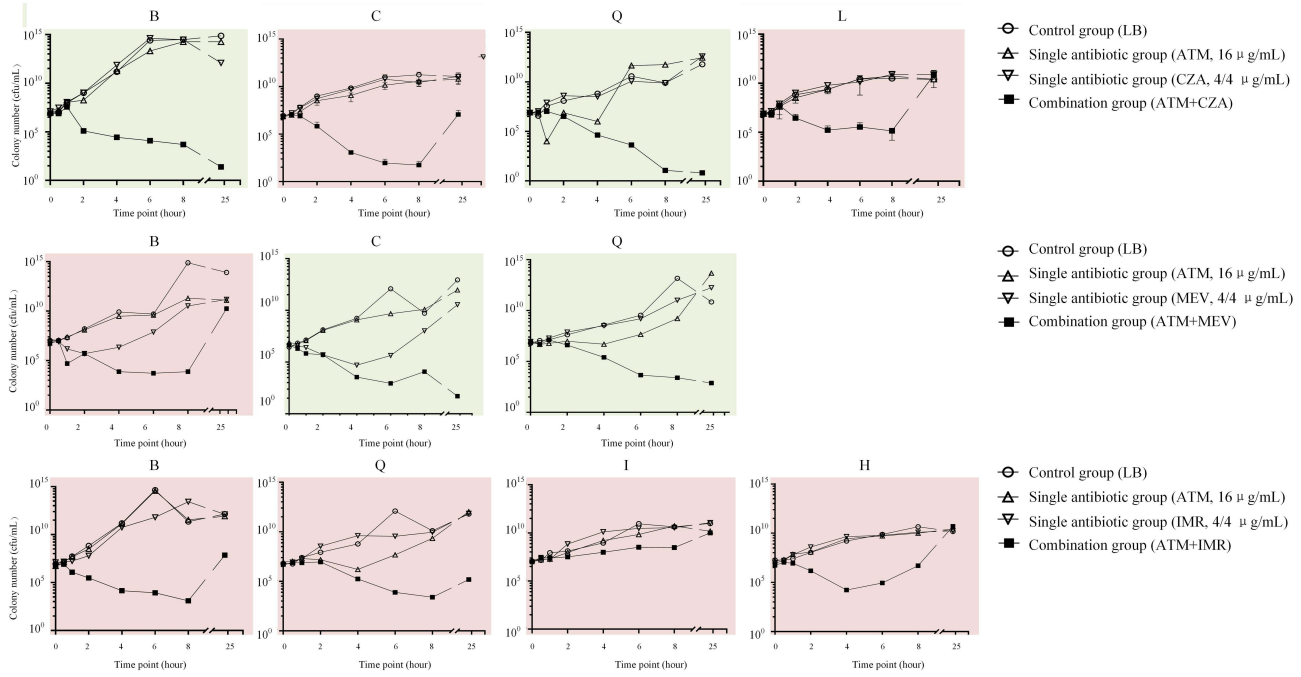

Figure 2 The result of time-kill experiment. Red indicated bacterial regrowth post single-dose combination therapy, denoting incomplete eradication and potentially requiring an increase in dosing frequency for clinical intervention; in contrast, green denoted complete eradication. We did not perform time-kill curve tests on all strains producing dual-carbapenemases. Representative strains were selected for time-kill curve assay based on the identification of bacterial species, STs and carbapenemases types. Consequently, strain B (strain ZHH with carbapenem resistant genes bla_{NDM-1} and bla_{IMP-4}), C (strain Kpn-96 with carbapenem resistant genes bla_{KPC-2} and bla_{IMP-26}), L (strain FSQ with carbapenem resistant genes bla_{KPC-2} and bla_{NDM-5}), Q (strain FSQ with carbapenem resistant genes bla_{KPC-2} and bla_{NDM-1}) were selected for the group of ATM combined with CZA, strain B, C, Q were selected for the group of ATM combined with MEV, strain B, Q, I (strain XL-20059286 with carbapenem resistant genes bla_{KPC-2} and bla_{NDM-5}), H (strain XL-200731214 with carbapenem resistant genes bla_{KPC-2} and bla_{NDM-5}) were selected for the group of ATM combined with IMR.

Discussion

Enterobacterales that produce single carbapenemase have been fully studied, covering various aspects such as epidemiology, resistant plasmids, molecular genetic evolution, and more. However, in recent years, clinically isolated strains producing dual carbapenemases have emerged and gained attention. Wang's study indicated that plasmids carrying dual carbapenemases in CRKP could be transferred through conjugation, and the trans-conjugant strains exhibited no significant fitness costs compared to the recipient strains.[20] Therefore, it could be predicted that these isolates were stable and had the potential for transmission among patients. Our research results suggested that the molecular genetic background of CRE strains producing dual carbapenemases was highly similar to that of strains producing a single carbapenemase. The plasmids carrying each of the dual carbapenemases all possessed mobile elements capable of conjugation and transfer. These findings indicated that, similar to strains producing single carbapenemases, strains producing dual carbapenemases also exhibited multidrug resistance and the ability to transfer resistance, posing a serious threat to public health.

During the period of 2018–2019, strains producing dual carbapenemases, mainly OXA-48-like and NDM, were reported in the United States, the United Kingdom, and France, respectively.[21–24] Wang H et al conducted a study on 2057 clinical CRKP strains from 65 hospitals in 25 provinces and cities in China from January 2012 to December 2017, and they found that 7 strains produced both KPC-2 and NDM-1 carbapenemases.[20] Hu F et al analyzed 186 clinical strains of CRKP from 32 hospitals in 22 provinces and cities in China from January 2016 to December 2018, and they identified 11 strains as dual-carbapenemase producers, with the predominant enzyme gene combination being $bla_{\text{KPC-2}}$ and bla_{NDMs} (n=8).[15] Chen L et al detected 16 strains of KPC and NDM co-producing isolates among 147 cases of bloodstream isolates of CRKP from multiple centers between April 2015 and November 2018.[25] Bianco G et al suggested that the occurrence of multi-carbapenemase producers was not uncommon in the Northern region of Italy during COVID-19 pandemics.[13] In our study, the combination of $bla_{(\text{KPC-2+NDMs})}$(n=6) was the most common pattern, followed by the combination of $bla_{(\text{KPC+IMP})}$(n=4). He F revealed a clear upward trend in the prevalence of CRKP carrying two carbapenemase genes over time, increasing from 0.40% before 2010 to 9.67% in 2021.[26] Additionally, the frequency of strains carrying both bla_{KPC} and bla_{NDM} also increased, rising from 0.00% before 2010 to 4.40% in 2021.[26]

Globally, the NDM and OXA-48-like combination emerged as the predominant enzymatic profile, with prevalent strain clones belonging to ST16, ST14, ST147, and capsular polysaccharide types (KL) mainly distributed as KL51, KL64, KL2. In China, the major dual-carbapenemase combination observed was KPC and NDM, with prevalent clonal types identified as ST11, and the dominant capsular polysaccharide types mainly represented by KL64.[26]

Currently, there are evidence-based therapeutic recommendations available only for infections caused by single carbapenemase-producing CRE. However, there are no recommended regimens for combination antimicrobial therapy for infections caused by dual carbapenemase-producing CRE. Our research results demonstrated that in vitro, the combinations of aztreonam with ceftazidime/avibactam, meropenem/vaborbactam, and imipenem/relebactam all exhibited good synergistic effects. The results from the combined kill-time curves showed that, following the initial administration, all strains, except for strain I(strain XL-20059286 with carbapenem resistant genes $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-5}}$), exhibited significant bactericidal activity between 2–8 hours, leading to a substantial reduction in bacterial counts compared to the initial time point (0 hours). In contrast, individual treatments with ATM, CZA, MEV, and IMR did not exhibit bactericidal effects. These findings suggested that, for these dual carbapenemase-producing strains, combinations of ATM with CZA, MEV, and IMR were appropriate treatment options.

In our experiments, without replenishing the antibiotic concentrations in the combination antibacterial system after the initiation of the experiment, bacterial growth began to occur gradually over the extended observation time due to the depletion of antibiotics. Therefore, the synergistic antibacterial effects of antibiotic combinations were concentration-dependent. According to the combined kill-time curves, replenishing the initial dose of combination antibiotics at 6–8 hours in the kill-time assay could lead to sustained bactericidal effects.

The study by Khan et al showed that combining ATM with CZA exhibited a synergistic effect against CRE and carbapenem-resistant *P. aeruginosa* (CRPA) strains that produced a single metallo-β-lactamase. Furthermore, it demonstrated a significant in vitro synergistic effect against *K. pneumoniae* producing both OXA-48 and NDM enzymes

(FIC=0.021).[27] Our results were consistent with previous studies, all suggesting that combination therapy of ATM with CZA, MEV, and IMR is an effective treatment option for dual carbapenemase-producing strains.[13] Additionally, Bianco G's study indicated that cefiderocol exhibited good antibacterial activity against dual-carbapenemase *Enterobacterales*, making it a viable alternative treatment option besides combination therapy.[13]

Our study had several limitations. First, it was conducted with a limited number of bacterial strains, focusing exclusively on *Enterobacteriaceae* and excluding non-fermenting Gram-negative species. Second, the study did not investigate the prevalence of dual-carbapenemase producers. Third, clinical studies were needed to evaluate the efficacy of the proposed combination therapies against infections caused by dual-carbapenemase producers.

Above all, the emergence of strains producing dual carbapenemases posed a significant challenge in treating CRE. According to our results, infections caused by carbapenemase-producing strains, including dual producers, required early identification of carbapenemases such as MBLs or combinations involving MBLs. This identification was crucial as it allowed for predicting resistance to new beta-lactamase/inhibitor combinations and potential combinations with aztreonam. Such insights were vital for guiding antimicrobial stewardship efforts and facilitating the timely use of newly approved drugs. Additionally, actively exploring effective combination treatment regimens was beneficial for saving patients' lives, slowing the spread and dissemination of dual carbapenemase-producing strains. For strains producing dual carbapenemases, particularly those with both KPC and MBL enzymes, ATM combined with novel β-lactam/β-lactamase inhibitor (BL/BLI) combinations proved to be an effective treatment option.

Institutional Review Board Statement

The study was approved by the Ethics Committee of Sir Run Shaw Hospital, Zhejiang University School of Medicine, Approval No.2022-0495.

Funding

This research was supported by Applications of High-throughput Sequencing Technology in Infectious Diseases (MTP2022B019), Medical Health Science and Technology Project of Zhejiang Province(2023KY974) and Xinjiang Production and Construction Corps First Division Alar City Science and Technology Plan Project(2023YL01).

Disclosure

The authors declare that there are no conflicts of interest.

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