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ORIGINAL RESEARCH

Aztreonam Acts as a Synergist for Ceftazidime/Avibactam Against Carbapenem-Resistant *Enterobacteriaceae* (CRE) of Various Carbapenemase Phenotypes in Southwestern China

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Background: Carbapenem-resistant Enterobacteriaceae (CRE) presents a significant challenge due to its role in severe and multidrug-resistant infections.

Purpose: This study aims to evaluate aztreonam (ATM) as a synergistic agent with ceftazidime/avibactam (CZA) for treating CRE strains with different carbapenemase phenotypes.

Methods: A total of 87 non-repeated clinical CRE strains were collected from various clinical specimens at The First Affiliated Hospital of Guangxi Medical University. Carbapenemase genotypes and phenotypes were identified using polymerase chain reaction (PCR) and NG-Test Carba 5 methods. The synergistic effect of CZA combined with ATM was assessed via the checkerboard MIC and disk stacking methods.

Results: The clinical analysis revealed that underlying pulmonary disease, pneumonia, urinary catheter, and central intravenous catheter were associated with poor prognosis in CRE infections ($p<0.05$). All 87 CRE strains showed high resistance to most antibiotics, especially cefazolin, ceftriaxone, piperacillin/tazobactam, ertapenem, and meropenem, with a rate of 100.00%. For strains with a single carbapenemase gene, NG-Test Carba 5 demonstrated 100.00% accuracy. Notably, The combination of CZA and ATM showed synergy in 95.40% (83/87) of the CRE strains overall, with specific rates of 100.00% (4/4) in strains lacking detectable carbapenemase genes, 94.29% (33/35) in those with $_{\text{blaNDM}}$, and 100.00% (3/3) in those with bla_{NDM} plus bla_{KPC-2} or bla_{KPC-2} plus $_{\text{blaIMP-4}}$.

Conclusion: In conclusion, ATM significantly enhances CZA's activity against CRE strains in Guangxi, achieving a high synergy rate across diverse isolates, regardless of the carbapenemase genes present.

Keywords: combination therapy, synergist, ceftazidime/avibactam, aztreonam, carbapenem-resistant *Enterobacteriaceae*

Introduction

Antibiotic resistance has emerged as a critical global threat to public health. Carbapenem-resistant Enterobacteriaceae (CRE) impose a heavy economic burden and are associated with a high mortality rate.[1] Recognizing the severity of this issue, the Centers for Disease Control and Prevention (CDC) listed CRE as one of the most urgent threats in 2017.[2] Before 2001, nearly 99.9% of clinically isolated *Enterobacteriaceae* worldwide were susceptible to carbapenem

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antibiotics.[3] However, data from China Antimicrobial Surveillance Network (CHINET, http://www.chinets.com) indicates a significant rise in carbapenem resistance from 3.0% in 2005 to 23.2% in 2020. This underscores the pressing need for the development of new antimicrobial drugs to address the escalating antibiotic resistance crisis.

The principal resistant mechanisms of *Enterobacterales* to carbapenems involve the production of class A (KPC) carbapenems, class B metallo-β-lactamases (MBLs, eg, NDM), Verona integron-encoded MBL (VIM), imipenemase (IMP), and class D OXA-48 family carbapenemases.[4] The prevalence of KPC-positive *Enterobacterales* was widespread in the Middle East, Europe, and China,[5,6] while NDM-positive *Enterobacterales* are globally disseminated, especially in Asia.[7] Thus, novel β-lactam-β-lactamase inhibitor combinations have been developed, including ceftazidime-avibactam (CZA), and meropenem-vaborbactam. Compared to other traditional enzyme inhibitors, avibactam possesses a broader spectrum in inhibition of diverse β-lactamases. Therefore, it has been widely used in clinical since it was launched in China in 2019.[8]

Nevertheless, the increased use of CZA has led to the emergence of more CZA-resistant CRE strains. Remarkably, a study identified that mutations in the bla_{KPC} sequence contribute to CRE-acquired resistance by weakening their ability to bind to avibactam under the pressure of CZA. However, the strains regained their susceptibility to carbapenems.[9] Knowing that ATM is able to withstand hydrolysis from metallo β-lactamases[10] The β-lactamase inhibitor avibactam exerts antimicrobial effects by inactivating the co-produced serine β-lactamase, allowing ATM to bypass the hydrolysis of these enzymes and safely reach their active site[11] We hypothesized that ATM could act as a synergist to CZA and enhance the antimicrobial spectrum of CZA. From an antibiotic use perspective, some scholars are against the use of CZA combined with ATM for CRE strains sensitive to either ATM or CZA alone.[12] However, from a cost-effectiveness standpoint, we contend that despite the efficacy ofCZA, its expense is notable ATM, being cost-effective, when used as a synergist, can significantly reduce the infection duration, alleviate economic burdens on patients, decrease the likelihood of nosocomial infections, and enhance overall patient benefits.

The distribution of CRE varies, exhibiting regional variations. To date, rare information on the susceptibility of CRE to the CZA combined with ATM therapy in the Guangxi region. Here, we explore the feasibility of CZA combined with ATM therapy in the treatment of CRE carried different carbapenemases, and hope to provide a feasible empirical drug strategy to clinicians. The objective was to assess whether ATM functions as a synergistic agent with CZA, thereby enhancing the susceptibility of CZA against CRE strains.

Methods

Bacterial Isolation and Identification

A total of 87 CRE isolates were obtained from various clinical specimens at The First Affiliated Hospital of Guangxi Medical University between April 2023 and October 2023. CRE was defined as clinical strains exhibiting resistance to carbapenems, including ertapenem, imipenem, and meropenem, in accordance with the guidelines set forth by the Clinical and Laboratory Standards Institute (CLSI). All CRE strains were identified using the Vitek-2 Compact system (bioMérieux, Marcy l'Etoile, France) or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Vitek MS, Marcy l'Etoile, France). The quality control strains used *Escherichia coli* (*E. coli)* ATCC25922 (National Center for Clinical Laboratories, Beijing, China). All strains were preserved at −80°C in strains store medium tube (Bkmamlab, Hunan, China). Freshly isolated underwent two subcultures on 5% sheep's blood agar plates (Autobio, Zhengzhou, China) for 24h at 35°C before each experiment. Our study complies with the Declaration of Helsinki and was approved by the Medical Ethics Committee of the First Affiliated Hospital of Guangxi Medical University (No. 2023-E422-01).

Antimicrobial Susceptibility Testing

In vitro, all isolates were tested for antimicrobial susceptibility tests using Vitek-2 Compact system or the Kirby-Bauer (KB) disk-diffusion method.

Checkerboard Method

The in vitro synergistic effects of CZA combined with ATM were assessed through a checkerboard assay following the manufacturer's instructions. The concentration range of CZA was 0.125/4-128/4μg/mL, and of ATM, it was 0.25–16μg/mL in susceptibility cards for CAZ combined with ATM (Bio-Kont, Zhejiang, China).

Disk-Stacking Method

A modified antimicrobial susceptibility disk-stacking method was used in this study. First, the ATM disk is placed on the center surface of the Mueller-Hinton (MH) agar (Autobio, Zhengzhou, China) and incubated at 35°C for 30 min.[12,13] The ATM disk was then removed, and a CZA disk was placed in the same position, followed by overnight incubation at 35°C. Besides, we placed an ATM disk alone and a CZA disk alone on the same plate, and the two disks were attached to the left and right sides 35cm from the center to provide separate information (The schematic diagram shown in Figure S1). After incubating at 35°C for 24h, the diameter was measured. The results were interpreted according to the guidelines provided by CLSI (version 2023) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2020).

Fractional Inhibitory Concentration Index (FICI) Calculate Method

The calculation and interpretation of the FICI was referred to the established standards.[14,15] FICI= (MIC of ATM in combined/MIC of ATM alone) + (MIC of CZA in combined/MIC of CZA alone). According to the reference, A FICI≤0.5 was indicated as a synergistic effect, 0.5<FICI≤1 was indicated as an additive effect, 1<FICI≤2 was indicated as an irrelevant effect, and FICI >2 was indicated as an antagonism.

Clinical Data Collection

We gathered comprehensive clinical data from the electronic medical records of patients. The information included basic data (Age, Sex, hospital days), departments, underlying diseases, infection type, invasive procedures and devices, as well as antibiotic exposure history.

DNA Extraction and PCR

Total DNA extraction from the CRE strains was carried out following the guidelines provided by the Biospin Bacteria Genomic DNA Extraction Kit (Bioflux, Hangzhou, China). The DNA solution was stored at −20°C until further analysis. PCR was performed using Premix Taq (Takara, Japan) methodology to detect the expression of carbapenem resistance genes (KPC, NDM, IMP, VIM, and OVA-48) by Bio-Rad T100^{TM} Thermal Cycler System (Bio-Rad, USA). Primer sequences for KPC, NDM, IMP, VIM, and OXA-48 are detailed in Table S1. Subsequently, the PCR products underwent validation through 1% agarose gel electrophoresis, and the positive samples were sent to DNA sequencing (Sango Biotech, Shanghai, China). Nucleotide sequences were compared using the Basic Local Alignment Search Tool (BLAST) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

NG-Test Carba 5

Blood agar 24h cultures were tested with NG-Test Carba 5 kit (Fusun Diagnostics, Changsha, China) following the manufacturer's instructions. Using a 1-μL loop, one colony was touched and inoculated into a 1.5 mL EP tube containing 150μL extraction buffer. Then, vortexed for approximately 5 to 10s (Mucoid required a longer vortex of about 3min. Stand at room temperature for 10 min. Inoculate 100μL suspension into the NG-Test Carba 5 sample well with a pipette. After 15 min, the presence and absence of the control line and the test line were examined by eyes.

Statistical Analysis

The measurement data were evaluated as mean ± standard deviations, and the count data were evaluated as percentages. SPSS Statistics v25.0 (IBM, Chicago, IL, USA) was performed for data analyzed, including the 95% confidence interval of sensitivity and specificity of NG-Test Carba 5 and the Kappa index (κ index) for the evaluation of the agreement between NG-Test Carba 5 and PCR, the gold standard.

Results

Clinical Characteristics of CRE Strains

A total of 87 non-repetitive CRE strains were included in this study. *Klebsiella pneumoniae* (n=47, 54.0%), *E. coli* (n=25, 28.7%), and *Enterobacter cloacae* complex (n=10, 11.4%) were the most common species. For the infection type, the majority of CRE isolates were sourced from the respiratory tract (28, 32.18%), followed by urine (19, 21.8%), blood (11, 12.6%), wound (9, 10.3%), cerebrospinal fluid (CSF) (3, 3.4%), and tissue (3, 3.4%). The CRE strains were divided into positive outcomes and negative outcomes based on the final prognosis of patients. The positive outcome group comprised patients who were either cured or discharged from the hospital. Conversely, the negative outcome group included patients who died or opted out of treatment. We found that the rate of CRE isolates from the ICU was 10.00% in the positive outcome group, but in the negative outcome group was increased to 47.06% (p=0.001). For the underlying disease and infection type, the pulmonary disease and pneumonia showed a significant between the two groups (2.86% vs 17.65% and 30% vs 58.82 [positive vs negative groups], p=0.049 and 0.046, respectively). Besides, the invasive procedures included a urinary catheter and central intravenous catheter showed a significant between the two groups (65.71% vs 94.12% and 18.57% vs 58.82%, [positive vs negative groups], p=0.019 and 0.002, respectively). Furthermore, the negative outcome group was exposed to β-lactam-β-lactamase inhibitors and tetracycline antibiotics at a higher rate of 52.94% and 11.7%, p=0.041 and 0.036, respectively. Detailed information on the patients is shown in Table 1.

Table 1 Microbiological and Clinical Characteristics of 87 CRE Strains

Factor	Positive Outcomes (%)	Negative Outcomes (%)	*p*-value
Basic data			
Age	49.50±20.21	54.53±26.30	0.389
Male	51(72.86)	11(64.71)	0.556
Hospital days	42.37±36.01	30.18±23.97	0.189
Department			
ICU	7(10.00)	8(47.06)	**0.001**
Respiratory department	4(5.71)	2(11.77)	0.333
Other	59(84.29)	7(41.18)	**0.001**
Underlying diseases			
Diabetes	13(18.57)	6(35.29)	0.188
Hypertension	24(34.29)	7(41.18)	0.587
Pulmonary disease	2(2.86)	3(17.65)	**0.049**
Cardiovascular disease	6(8.57)	3(17.65)	0.369
Malignant tumors	7(10.00)	2(11.77)	1.000
Infection type			
Pneumonia	21(30.00)	10(58.82)	**0.046**
Urinary infection	17(24.29)	1(5.88)	0.178
Bloodstream infection	18(25.71)	7(41.18)	0.238
Intracranial infection	4(5.71)	2(11.77)	1.000
Invasive procedures and devices			
Tracheal intubation	19(27.14)	8(47.06)	0.145
Urinary catheter	46(65.71)	16(94.12)	**0.019**
Central intravenous catheter	13(18.57)	10(58.82)	**0.002**
Drainage tube	5(7.14)	1(5.88)	1.000
Surgery	30(42.86)	1(5.88)	**0.004**
Bone marrow biopsy	5(7.14)	4(23.53)	0.069
Bronchofiberscopy	15(21.43)	5(29.41)	0.526
Antibiotic exposure			
Cephalosporins	21(30.00)	7(41.18)	0.397
Carbapenem antibiotic	18(25.71)	8(47.06)	0.137

(*Continued*)

Table 1 (Continued).

Factor	Positive Outcomes (%)	Negative Outcomes (%)	*p*-value
β-lactam-β-lactamase inhibitors	18(25.71)	9(52.94)	**0.041**
Fluoroquinolones	11(15.71)	3(17.65)	1.000
Aminoglycosides	3(4.29)	3(17.65)	0.086
Tetracycline antibiotic	0(0.00)	2(11.77)	**0.036**
Glycopeptides	6(8.57)	2(11.77)	0.651

Notes: The bold font represents a statistically significant difference between the groups (*p*-value<0.05).
Abbreviation: ICU, Intensive Care Unit.

Antimicrobial Susceptibility Testing

The 87 CRE strains showed high resistance to most antibiotics, especially cefazolin, ceftriaxone, piperacillin/tazobactam, ertapenem, and meropenem, with a rate of 100.00%. Meanwhile, the resistance rates of ceftazidime and imipenem were 97.7%, and 96.55%, respectively. In contrast, the CRE strains showed low resistance to tigecycline and polymyxin B, with a rate of 10.34% and 4.60%, respectively (Table 2).

Carbapenemases Phenotype Experiment and Genotype Detection

Of the 87 CRE strains, 4 strains (4.60%) without detected carbapenemases, 42 strains (48.28%) carried the bla_{KPC-2} gene, and 18 strains (20.67%) carried bla_{NDM-5} gene, 16 strains carried bla_{NDM-1} gene, 2 strains carried bla_{OXA-48} gene, 2 strain carried bla_{NDM-1} plus bla_{KPC-2} (NG-Test Carba 5 results: one strain was NDM, another was NDM+KPC), 1 strain carried bla_{KPC-2} plus bla_{IMP-4} (NG-Test Carba 5 results: IMP) and 1 strain carried bla_{NDM-5} gene plus bla_{KPC-2} (NG-Test Carba 5 results: NDM+KPC). As shown in Table 3.

Regarding strains with one carbapenemase gene, the overall accuracy of the NG-Test Carba 5 was 100.00%. The sensitivity and specificity varied with different types of Carbapenemase showed in Table 4. For the most common carbapenemase of KPC, NG-Test Carba 5 with a sensitivity of 100.00% (95% CI, 89.56–100.00) and a specificity of

Table 2 Antimicrobial Susceptibility Results of 87 CRE Strains

Antibiotic	R (%)	I (%)	S (%); SDD(%)	NR (%)
Levofloxacin	74 (85.06)	5 (5.74)	8 (9.20)	0(0.00)
Tigecycline	9(10.34)	1(1.15)	58(66.67)	19(21.84)
Ceftriaxone	87(100.00)	0(0.00)	0(0.00)	0(0.00)
Amoxicillin-clavulanic acid	71(81.60)	1(1.15)	1(1.15)	14(16.10)
Piperacillin/tazobactam	87(100.00)	0(0.00)	0(0.00)	0(0.00)
Cefuroxime	82(94.25)	0(0.00)	0(0.00)	5(5.75)
Ceftazidime	85(97.70)	1(1.15)	0(0.00)	1(1.15)
Cefoperazone/sulbactam	82(94.25)	4(4.60)	1(1.15)	0(0.00)
Cefepime	81(93.10)	0(0.00)	2(2.30); 3(3.45)	1(1.15)
Ertapenem	87(100.00)	0(0.00)	0(0.00)	0(0.00)
Imipenem	84(96.55)	2(2.30)	0(0.00)	1(1.15)
Amikacin	51(58.62)	0(0.00)	34(39.08)	2(2.30)
TMP-SMZ	76(87.36)	0(0.00)	11(12.64)	0(0.00)
Aztreonam	80(91.95)	0(0.00)	7(8.05)	0(0.00)
Ciprofloxacin	75(86.21)	1(1.15)	6(6.90)	5(5.75)
Cefazolin	87(100.00)	0(0.00)	0(0.00)	0(0.00)
Gentamicin	71(81.60)	0(0.00)	12(13.80)	4(4.60)
Meropenem	87(100.00)	0(0.00)	0(0.00)	0(0.00)
Polymyxin B	4(4.60)	0(0.00)	28(32.18)	55(63.22)

Abbreviations: R, Resistant; I, Intermediate; S, Susceptible; SDD, Susceptible-dose dependent; NR, No-report; TMP-SMZ, Trimethoprim-sulfamethoxazol.

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Table 3 Resistance Genes of 87 CRE Strains

Resistance gene	Strains	Percentage (%)
KPC-2	42	48.28
NDM-1	16	18.39
NDM-4	1	1.15
NDM-5	18	20.69
OXA-48	2	2.30
KPC-2+IMP-4	1	1.15
KPC-2+NDM-1	2	2.30
KPC-2+NDM-5	1	1.15

Table 4 Comparison of the NG-Test Carba 5

Genes	NG-Test Carba 5						Kappa Index (κ index)
	TP	FP	FN	TN	Se% (95% CI)	Sp% (95% CI)	
bla_{KPC}	42	0	0	45	100.00(89.56–100.00)	100.00(90.20–100.00)	1.00(1.00–1.00)
bla_{NDM}	35	0	0	49	100.00(87.69–100.00)	100.00(90.94–100.00)	1.00(1.00–1.00)
bla_{OXA-48}	2	0	0	85	100.00(19.79–100.00)	100.00(94.61–100.00)	1.00(1.00–1.00)
bla_{KPC}+bla_{IMP}	0	0	1	86	0.00(0.00–94.54)	100.00(94.67–100.00)	0
bla_{KPC}+bla_{NDM}	2	0	1	84	66.67(12.53–98.24)	100.00(94.55–100.00)	0.79(0.40–1.19)

Abbreviations: TP, ture-positive; FP, false-positive; FN, false-negative; TN, Ture-negative; Se, Sensitivity; Sp, specificity. 95% confidence interval.

100.00% (95% CI, 90.20–100.00). The κ index was 1.00 (95% CI, 1.00–1.00). As for NDM, NG-Test Carba 5 with a sensitivity of 100.00% (95% CI, 87.69–100.00) and a specificity of 100.00% (95% CI, 90.94–100.00). For the strains with co-producing KPC and NDM, NG-Test Carba 5 missed one target, with one false-negative result, leading to a specificity of 66.67% (95% CI, 12.53–98.24).

Antimicrobial Susceptibility Testing and Interpretation

The results indicated that among 87 CRE strains, 91.95% were resistant to ATM. For these resistant strains, the MIC values were all over 16 μg/mL, and the diameter of the KB inhibition zone ranged from 6 to 17 mm. Additionally, 50.57% of the strains were resistant to CZA. Regarding these resistant strains, the MIC value ranged from 16/4 to over 128/4 μg/mL, and the diameter of the KB inhibition zone ranged from 6 to 20mm. We further analyzed all CRE strains in our study and calculated the MIC50 and MIC90 following ATM-alone, CZA-alone, and ATM+CZA combination therapy (Table 5). Our results demonstrated that the MIC50 and MIC90 of ATM alone were more than 16 μg/mL. For CZA alone, the MIC50 was 32/4 μg/mL, and the MIC90 was more than 128/4 μg/mL. However, in the combined treatment of ATM + CZA, the MIC50 of ATM dropped to less than 0.25 μg/mL, the MIC90 decreased to 1 μg/mL, and the MIC50 and MIC90 of CZA decreased

Table 5 MIC Distribution of CRE Strains

Strains Type	Number	Alone				In Combination			
		ATM (μg/mL)		CZA (μg/mL)		ATM (μg/mL)		CZA (μg/mL)	
		MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90
All strains	87	>16	>16	32/4	>128/4	<0.25	1	<0.125/4	<0.125/4
bla_{KPC}-only	42	>16	>16	2/4	32/4	0.5	1	<0.125/4	<0.125/4
bla_{NDM}-only	35	>16	>16	>128/4	>128/4	<0.25	0.5	<0.125/4	<0.125/4

Abbreviations: MIC, minimal inhibitory concentration; ATM, aztreonam; CZA, ceftazidime/avibactam.

to less than 0.125/4 μg/mL. In addition, we also stratified the calculation of MIC50/MIC90 according to the carbapenemase genotype. Our results showed that for the strains with bla_{KPC} alone (n = 42), after the combination of ATM + CZA, the MIC50 of ATM decreased to 0.5 μg/mL, the MIC90 decreased to 1 μg/mL, and the MIC50 and MIC90 of CZA decreased to less than 0.125/4 μg/mL. For the strains with bla_{NDM} alone (n = 35), after the combination of ATM + CZA, the MIC50 of ATM decreased to less than 0.25 μg/mL, the MIC90 decreased to 0.5 μg/mL, and the MIC50 and MIC90 of CZA decreased to less than 0.125/4 μg/mL.

Overall, CZA combined with ATM showed synergistic effects to 95.40% (83/87), 4.60% (4/87) showed additive effects, with a minimum decrease of 2 dilution gradients (equivalent to a 4-fold reduction) and a maximum decrease of 10 dilution gradients (equivalent to a 1024-fold reduction) in the MIC of CZA. The results of the modified disk-stacking method revealed that the diameter of the inhibition zone of CZA increased by up to 21 mm after combining with ATM, compared to CZA alone. The strains without detected carbapenemases (100.00%, 4/4) are susceptible to CZA combined with ATM. For the strains with bla_{NDM}, CZA combined with ATM showed synergistic effects to 94.29% (33/35), and additive effects to 5.71% (2/35). Interestingly, CZA combined with ATM showed synergistic effects to 100.00% (3/3) of strains with bla_{NDM} plus bla_{KPC-2} and bla_{KPC-2} plus bla_{IMP-4}. No antagonism was found for CZA combined with ATM (Table S2).

Discussion

The emergency of multidrug-resistant (MDR) strains is attributed to various factors, including overuse and misuse of antibiotics such as unnecessary prescriptions, inappropriate dosages, incomplete or extended courses of treatment, lack of appropriate and rapid diagnostic measures, etc.[16] CRE infections have evolved into a global concern, presenting clinicians with serious challenges due to their resistance to nearly all available antibiotics. Due to the limited salvage therapy drugs and their toxic side effects (eg tigecycline and polymyxin), combination therapy is considered an effective treatment for CRE infection.[17,18] However, identifying suitable combinations presents a significant challenge. Moreover, Ding et al have proposed that CZA susceptibility testing should be performed concurrently with carbapenemase testing.[9] In light of this, our study aims to evaluate the detection performance of the lateral flow immunochromatographic assays, namely, NG-Test Carba 5, in comparison with the gold standard method PCR, for assessing carbapenemase phenotype and genotype. Additionally, we explore the in *vitro* evidence for the synergistic activity of the combination of ATM and CZA against different carbapenemase types.

Since the first case of KPC-positive *Klebsiella pneumoniae* was detected in 1996, different drug-resistant enzyme-resistant CRE strains have been reported around the world.[19–22] The horizontal transfer of KPC-positive plasmids from *Klebsiella pneumoniae* to *Escherichia coli* has been documented, indicating inter-species plasmid transfer.[23] A molecular epidemiological study of carbapenem-resistant *E. coli* (CREC) in ICU patients has highlighted the crucial role of plasmids in facilitating the transfer of carbapenemase resistance genes among CREC strains.[24] Similarly, our study found the negative outcome group with a higher percentage of ICU patients ($p<0.05$). Furthermore, our study revealed an increasing prevalence of bla_{NDM-5} in the Guangxi region, while bla_{NDM-1} demonstrated a declining trend. This phenomenon aligns with previous reports and may be attributed to the evolutionary dynamics of bla_{NDM-1} under antibiotic treatment.[24] Notably, a study identified that bla_{NDM-5} is situated on a stable 46-kb IncX3 plasmid, potentially explaining its extensive horizontal gene transfer capability.[25] In recent years, with the emergence and increasingly widespread application of new antibiotics such as CZA, *Enterobacteriaceae* with the coexistence of bla_{KPC} and bla_{NDM}, bla_{NDM} and bla_{OXA-48}, bla_{KPC} and bla_{IMP} have been reported, such as *Citrobacter freundii*,[26] *Proteus mirabilis*,[27] *Serratia marcescens*,[28] *Klebsiella michiganensis*,[29] *E. coli*[30] and so on. In our study, 4 strains of CRE carrying two carbapenemases were identified, including 1 strain carried bla_{KPC} and bla_{IMP}, and 3 strains carried bla_{KPC} and bla_{NDM}. Due to the presence of both carbapenemases, these bacteria are extensively resistant to existing antibiotics. However, the genes encoding two carbapenemases are rarely located in the same plasmid or the translocated genetic element, but recent research has identified a conjugative IncFII/IncR plasmid carrying both bla_{KPC-2} and bla_{NDM-1},[31] and Tn1696-related transposons in IncHI5 plasmid carrying both bla_{KPC-2} and bla_{NDM-1}.[32] The discovery of novel transposons harboring multiple carbapenemase genes may contribute to the further dissemination of carbapenem-resistant strains within hospital settings.

Thus, rapid detection of CRE producers might be the best way to prevent their spread. In developing countries, many laboratories do not have PCR instruments to detect resistance genes in CRE, so this study aimed to evaluate the detection performance between NG-Test-Carba 5 and standard method PCR. To the best of our knowledge, the NG-Test Carba 5 is the most simplified assay among the non-genotypic methods that can distinguish between the major carbapenemase families, capable of meeting the needs of clinical treatments. Our results show that the accuracy of NG Test Carba 5 detection is 100% in single carbapenemase gene, but only 98.85% in double carbapenemase gene, which is similar to the reported in the United States, France, the United Kingdom, and China.[33–36] Thus, if molecular testing is not available in the clinical laboratory, NG-Test-Carba5 can be used to confirm the presence of carbapenemase. We hypothesize that the absence of detection of bla_{KPC-2} in two CRE strains by NG Test Carba 5 may be attributed to the presence of multi-carbapenemase genes or the existence of a rare variant. However, further confirmation through whole-genome sequencing (WGS) is imperative. Our findings suggest that NG-Test Carba 5 can serve as a rapid and efficient method for discerning carbapenemase phenotypes within clinical laboratory settings.

Traditional antibiotics are becoming increasingly ineffective, and antibiotic adjuvants have emerged as a promising strategy to combat MDR pathogens and restore the efficacy of existing antibiotics. These adjuvants enhance the efficacy of existing antibiotics by reducing the MIC of the antibiotic, thereby preserving currently available treatment options.[37] As a class A antibiotic adjuvant,[38] avibactam forms a combination drug CZA with ceftazidime, which can be considered a front-line treatment option for treating CRE infections.[39] CZA inhibits class A, class C β-lactamase, and ESBLs. Our study showed that CZA is very active against KPC-positive, OXA-48-positive, and non-detected carbapenemase CRE strains, achieving 89.58% (43/48) susceptibility. However, our study has revealed that CZA lacks activity against NDM-positive CRE strains (38/38), consistent with the previous studies.[39–41] During empiric treatment, physicians typically prescribe two or more antibiotics to cover all potential bacterial infections. Evidence suggests that combination therapy exerts a stronger synergistic effect compared to monotherapy.[42] ATM is a monocyclic β-lactam antibiotic that is not affected by MBL-producers, but its use has been limited due to the prevalence of extended-spectrum β-lactamases (ESBLs).[43–45] In our study, we found that after the CZA combined with ATM, the MIC value reduced to their sensitive ranges, the MIC of CZA can drop from 128/4 (μg/mL) to 0.125/4 (μg/mL) by the maximum, and the FICI values were all below 0.51, indicating good synergistic or additive effects. Besides, a total of 40 CRE strains carrying bla_{NDM} or bla_{IMP} were included. Among these strains, 3 were sensitive to ATM (MIC < 4μg/mL). For these 3 strains, the synergistic effect produced by their combined use may be due to the inherent activity of ATM. The other 37 strains were all resistant to ATM (MIC>16μg/mL), indicating that although ATM is hydrolysis-resistant to MBLs, its inhibitory effect on most MBL strains is limited when used alone. These results indicate that the synergistic effect of ATM + CZA is not only due to the inherent activity of ATM. Instead, avibactam in CZA enhances the overall antibacterial effect by inhibiting ESBL/AmpC enzymes. The results of the modified disk stacking method were consistent with the checkerboard method, which is particularly valuable in low-resource healthcare settings due to its low cost, ease of operation, and straightforward easy visual interpretation of results. Similarly, Jayol et al[46] reported that the CAZ/AVI and ATM combination had a synergistic effect on 63 strains isolated from France, Colombia, and Turkey, and noted that this combination was effective against MBL-producing *Klebsiella pneumoniae*, especially against strains that produced multiple carbapenemase genes. Taha et al[47] reported that the CZA/AVI and ATM combination is an effective therapeutic against *Klebsiella species* and *E. coli* isolates producing more than one carbapenemase gene in Egypt. Lu et al[48] found the combination of CZA and ATM has a good synergistic bactericidal effect on NDM-, IMP-, KPC+IMP-, and KPC+NDM- producing strains in *vitro* and in *vivo*.

However, establishing the clinical efficacy of a drug solely based on in *vitro* findings poses challenges, particularly in the context of limited treatment alternatives for CRE infections and a dearth of clinical studies evaluating the effectiveness of ATM and CZA combination therapy. Based on a recent prospective observational study found that a standard dose of the combination of CZA (2.5g, q8h) and ATM (2g, q8h) was adequate to achieve a favorable clinical response.[49] A recent clinical study[49] found that the combination therapy of CZA and ATM was positively associated with lower 30 days mortality and shorter hospital stays compared to other antibiotics. These results suggest that ATM can significantly enhance the sensitivity of CZA to CRE strains in different regions as a synergist both in *vitro* and in *vivo*. Existing evidence indicates that CZA is ineffective as a salvage treatment for multidrug-resistant Gram-negative bacteria

infections.[50] Our study conducts a larger-scale strain analysis, explores the CRE resistance characteristics in our region, and confirms that the strain resistance background has no significant impact on the efficacy of the ATM+CZA combination therapy. Moreover, it systematically validates the feasibility of ATM as a CZA synergistic agent in southwest China. Our results practically complement the existing results, providing new laboratory evidence for further research and potential clinical translation of the ATM+CZA combination strategy.

This study is not without limitations. Firstly, these methods need to be further evaluated in a larger multicenter study with more isolated CRE strains, and different manufacturer brands of NG Test Carba 5 kit and disks to confirm the accuracy, sensitivity, and specificity. Secondly, in this study, patients were not enrolled for the clinical treatment regimen of ATM combined with CZA, and data on the treatment effect of patients were not obtained.

Conclusion

In conclusion, our study found that underlying pulmonary disease, pneumonia, urinary catheter, and central intravenous catheter may be factors for the poor prognosis of CRE infection ($p<0.05$). NG-Test Carba 5 can serve as a rapid and efficient method for discerning carbapenemase phenotypes within clinical laboratory settings. ATM serves as an effective synergist, significantly augmenting the activity of CZA against CRE strains in Guangxi, irrespective of the carbapenemase genes carried by CRE strains. Consequently, considering the low price, low side effects, and stable efficacy of ATM, CZA combined with ATM should be used as an early treatment for CRE infection in clinical practice.

Abbreviations

CRE, Carbapenem-resistant Enterobacteriaceae; CRKP, Carbapenem-resistant Klebsiella pneumoniae; CDC, Centers for Disease Control and Prevention; ATM, aztreonam; CZA, ceftazidime/avibactam; PCR, Polymerase chain reaction; CLSI, Clinical and Laboratory Standards Institute; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; KB, Kirby-Bauer disc-diffusion method; BLAST, Basic Local Alignment Search Tool; CRP, C-reactive protein; NT-pro-BNP, N-terminal-pro-brain natriuretic peptide; BALF, Bronchoalveolar lavage fluid; SCF, cefoperazone sulbactam; MXF, moxifloxacin; CAS, caspofungin; E. coli, Escherichia coli; CSF, cerebrospinal fluid; ICU, Intensive Care Unit; MDR, multidrug-resistant; MIC, minimum inhibitory concentration; ESBLs, extended-spectrum β-lactamases; FICI, fractional inhibitory concentration index.

Ethics Approval and Consent to Participate

We obtained consent from patients or their families, and the Medical Ethics Committee of the First Affiliated Hospital of Guangxi Medical University has approved the protocol (No. 2023-E422-01). Informed consent was obtained from all participants.

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Disclosure

No potential conflict of interest was reported by the authors.

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