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Research Article

Evaluation of in-vitro activity of Ceftazidime-Avibactam and Aztreonam combination therapy against MBL and Non-MBL producing strains of *Pseudomonas aeruginosa*

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ABSTRACT

Background: Pseudomonas aeruginosa is a significant contributor to healthcare-associated infections, and the rise of carbapenem resistance—often mediated by metallo-β-lactamases (MBLs)—presents considerable challenges for treatment. The combination of ceftazidime–avibactam (CZA) and aztreonam (ATM) has been proposed as a potential therapeutic strategy.

Materials and Methods: This prospective study included 100 non-duplicate *P. aeruginosa* isolates collected over a period of one year. Carbapenem resistance was assessed using disk diffusion and minimum inhibitory concentration (MIC) testing. MBL production was detected through the Imipenem–EDTA (Ethylenediaminetetraacetic Acid) double-disk synergy test, while the presence of carbapenemase genes ($_{blaVIM}$, $_{blaIMP}$ *and* $_{blaNDM}$) was identified via polymerase chain reaction (PCR).

Results: Among the 100 isolates studied, 21% were identified as carbapenem-resistant, comprising 14 MBL producers and seven non-MBL producers. None of the 21 carbapenem-resistant isolates demonstrated an increased inhibition zone with the CZA–ATM combination. PCR analysis of ten carbapenem-resistant isolates detected the bla_{VIM} gene in nine of these isolates.

Conclusion: In this study, both MBL- and non-MBL-producing *P. aeruginosa* demonstrated in vitro resistance to the CZA–ATM combination. These results suggest limited therapeutic potential in the local context and highlight the need for multi-center studies and advanced susceptibility testing to optimize treatment strategies against multidrug-resistant *P. aeruginosa.*

Keywords: Antimicrobial resistance, Carbapenem resistance, CZA-ATM testing, MBL+ non-MBL producers, synergy testing

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Introduction

Pseudomonas aeruginosa is a Gram-negative pathogen that ranks among the leading causes of healthcare-associated infections, especially in immunocompromised individuals.[1] This bacterium is a major contributor to nosocomial infections, with high prevalence in patients with burn wounds, cystic fibrosis, acute leukaemia, organ transplants, and those with intravenous drug addiction. The most severe infections associated with *P. aeruginosa* include malignant external otitis, endophthalmitis, endocarditis, meningitis, pneumonia, and septicaemia.[2]

This bacterium presents significant challenges within clinical environments due to its inherent resistance to a wide range of antibiotics, as well as its ability to swiftly acquire additional resistance mechanisms.[3] There are three main mechanisms of resistance to carbapenems: carbapenemase production, overexpression of the efflux pump, and membrane porin mutation. Among them, the production of carbapenemase is the major resistance mechanism. Three groups of carbapenemases are responsible for carbapenem resistance: KPC (Ambler class A), MBLs (metallo-β-lactamases, Ambler class B) including NDM, VIM, IMP etc, and OXA (Ambler class D) such as OXA-48. All of these enzymes are plasmid-mediated, which facilitates the horizontal transfer and global spread of the strains.[4]

Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) refers to a group of *P. aeruginosa* strains that have developed resistance to carbapenem antibiotics. Recognized as a significant public health concern, CRPA was classified as "a serious threat" by the US Centers for Disease Control and Prevention (CDC) in 2019 and designated as a "priority 1 – critical pathogen" by the World Health Organization (WHO) in 2017.[5,6] It is also a member of the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species) group, comprising multidrug-resistant bacteria responsible for the majority of healthcare-associated infections (HAIs).

The limited availability of effective antibiotics has made CRPA an exceptionally challenging pathogen. Infections caused by CRPA are difficult to treat, often requiring prolonged antibiotic courses and extended hospital stays. These infections are associated with increased morbidity and mortality, further underscoring their clinical significance. The increasing burden of antimicrobial resistance is further driven by suboptimal antimicrobial stewardship practices, underscoring the need for microbiology-guided therapeutic strategies to combat carbapenem-resistant *Pseudomonas aeruginosa*.[7]

Initially, *P. aeruginosa* was not inherently resistant to carbapenems. However, over time, repeated exposure to these antibiotics and the horizontal transfer of resistance genes from other bacteria have driven its genetic evolution. This has enabled *P. aeruginosa* to withstand the inhibitory and bactericidal effects of carbapenems, making it a formidable pathogen in healthcare settings.

The emergence of carbapenem resistance in *P. aeruginosa* is especially concerning and can be attributed to various mechanisms, including permeability defects, the production of carbapenemases, and the overexpression of efflux pump-encoding genes. Epidemiological analyses indicate the prevalence of high-risk clones of carbapenem-resistant *P. aeruginosa* (CRPA) that generate carbapenemases, harbor other beta-lactamase genes, and utilize multiple resistance strategies.[8] A notable aspect of *P. aeruginosa's* resistance profile is the *development* of multidrug resistance (MDR), which is primarily driven by the production of metallo-β-lactamases (MBLs) alongside other non-MBL-mediated resistance pathways.[9]

Strains of *P. aeruginosa* that produce MBLs are particularly concerning, as these enzymes can hydrolyse a wide spectrum of β-lactam antibiotics, including carbapenems, which are often employed as a last resort in treating resistant infections. The horizontal transfer of MBL genes via plasmids and additional mobile genetic elements exacerbates the global antimicrobial resistance crisis.[10] In India, the prevalence of MBL production in *P. aeruginosa* varies from one region to another, between 7% and 65%.[11] The widespread prevalence of VIM-, IMP-, and NDM-type MBLs among carbapenem-resistant *P. aeruginosa* (CRPA) strains underscores the urgent need for effective therapeutic interventions.

On the other hand, non-MBL-producing strains of *P. aeruginosa* rely on alternative resistance mechanisms, such as overexpression of efflux pumps, production of AmpC β-lactamases, and changes in porin channels. While these mechanisms differ from MBL-mediated resistance, they nonetheless contribute to significant reductions in antibiotic efficacy, complicating treatment strategies [12].

Infections caused by MBL-producing *P. aeruginosa* (MBL-PA) are particularly challenging, as these enzymes afford resistance to nearly all β-lactams and currently available β-lactamase inhibitor combinations, save for aztreonam (ATM) and the newly developed siderophore antibiotic, cefiderocol (FDC).[13] Nevertheless, MBL-PA strains often exhibit resistance to ATM as a result of the co-production of additional β-lactamases, such as Extended-Spectrum Beta-Lactamases (ESBLs), particularly GES-type enzymes, or through the overexpression of the intrinsic blaPDC gene, further limiting treatment options.[14]

To optimize antibiotic therapy for *P. aeruginosa* infections, it is imperative to comprehensively understand the

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differential susceptibility patterns between MBL and non-MBL-producing strains. Novel combination therapies, such as ceftazidime-avibactam (CZA) in conjunction with aztreonam (ATM), hold promise in addressing these resistant pathogens. Accurate antimicrobial susceptibility testing (AST) is essential to inform effective management strategies; however, there currently exists no practical or widely adopted AST method in clinical laboratories to evaluate the efficacy of the ATM-CZA combination.

However, in vitro testing of the ATM-CZA combination remains a significant laboratory challenge. While reference methods like checkerboard assays and time-kill studies provide accurate results, they are labor-intensive and impractical for routine diagnostics. Simpler alternatives, such as the double-disk synergy test (DDST), may offer a more feasible approach, particularly in resource-limited settings. Yet, their reliability and interpretative clarity remain underexplored.[15]

The present study aims to evaluate the in vitro efficacy of the aztreonam-ceftazidime-avibactam (ATM-CZA) combination against MBL and non-MBL-producing strains of *Pseudomonas aeruginosa* using a disk-based Double-Disk Synergy Test (DDST). The primary objective is to assess whether this method can serve as a simple, cost-effective, and reproducible tool for clinical laboratories to detect combination therapy efficacy, particularly in environments with limited access to advanced testing modalities.

By identifying reliable and accessible testing strategies, this study seeks to contribute to more informed antimicrobial stewardship and improved clinical management of infections caused by drug-resistant *P. aeruginosa* strains.

Materials and Methods

Study design and setting

A prospective study was conducted at a tertiary care hospital in South India from May to October 2023, with approval from the Institutional Ethics Committee (090/02/2024/PG/SRB/SMCH). The study involved 100 consecutive, non-duplicate isolates of *Pseudomonas aeruginosa* from clinical samples submitted to the microbiology laboratory for culture and sensitivity testing. These samples were obtained from patients admitted to medical and surgical wards and intensive care units (ICUs).

The identification of *Pseudomonas aeruginosa* was conducted following standard laboratory protocols. This process involved performing Gram staining, biochemical tests, and culturing on Nutrient agar, 5% sheep blood agar, and MacConkey agar, all aimed at the phenotypic detection and isolation of *Pseudomonas aeruginosa*.

Phenotypic detection of Carbapenem resistance

Detection of carbapenem resistance was performed using Kirby-Bauer disk diffusion method using Imipenem and Meropenem disks, in accordance with CLSI 2023 guidelines. Also MIC determination of carbapenem resistance were detected by Vitek 2 systems.

Imipenem-EDTA double disk synergy test (DDST) was performed as per the procedure described by Yoeng et al[16]

Test strains were adjusted to the 0.5 McFarland standard and inoculated onto Mueller-Hinton agar plates. One disk containing Imipenem (10µg) and another containing Imipenem-EDTA (10µg of Imipenem combined with 750µg of EDTA) (Hi-media, Mumbai) were placed 20 mm apart (centre to centre) on Mueller–Hinton agar plates, as recommended in the original method described by Yong et al.[16] The plates were incubated aerobically at 37°C overnight. After incubation, the diameters of the zone of inhibition around the Imipenem and Imipenem-EDTA disks were measured and compared. If the zone of inhibition around the Imipenem-EDTA disk increased by 7 mm or more compared to the Imipenem disk alone, the strain was considered MBL positive.

Combination testing using the Disk diffusion method as described by Sreenivasan P et al[17]

Ceftazidime-avibactam disks (30/20 µg) (Hi-media, Mumbai) were placed on Mueller-Hinton agar (MHA) plates inoculated with the test organism and incubated at 37°C for 1 hour. After this incubation, the CZA disks were removed and replaced with aztreonam disks at the same location. Following disk placement, the plates were incubated overnight at 37°C. Interpretation of synergy was performed as described by Sreenivasan et al[17]. An increase in the zone diameter of ≥5 mm for aztreonam following replacement of the ceftazidime-avibactam disc, compared to aztreonam alone, was considered indicative of synergistic activity. An increase <5 mm or no change was interpreted as no synergy.

PCR for Carbapenemase gene detection

Conventional PCR using Petri 96-well Thermal Cycler (Applied Biosystems, USA) was performed for all the phenotypically carbapenem-resistant *Pseudomonas* isolates for detecting carbapenemase genes (bla_{VIM}, bla_{IMP} and bla_{NDM}). Amplicons to be visualized in a 2% agarose gel containing ethidium bromide. The primers used for detection of carbapenemases genes were given in the Table 1.

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Results

Age-wise Distribution

The study population comprised individuals across various age groups. The distribution was as follows: 11% were aged 0–20 years, 15% were aged 21–40 years, 39% were aged 41–60 years, and 35% were aged above 60 years.

Sex-wise Distribution

The majority of the participants were male (56%), while females accounted for 44% of the population.

Department-wise Distribution

Patients were distributed across multiple hospital departments. The highest representation was from General Surgery (33%), followed by the Intensive Care Unit (ICU) (13%) and Nephrology (7%). Other departments included General Medicine (11%), Pulmonology (6%), Plastic Surgery (6%), Neurosurgery (5%), ENT (4%), Emergency (4%), Pediatrics (4%), Orthopedics (3%), Obstetrics (3%), and Urology (1%).

Sample-wise Distribution

The clinical samples analyzed showed a predominance of exudates (56%), followed by respiratory samples (28%), blood (10%), and urine (6%). The data has been tabulated in Table 2.

Carbapenem Resistance

In this study, 21 out of 100 samples (21%) exhibited resistance to both Imipenem and Meropenem, indicating a 21% prevalence of Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) in the given clinical samples.

Imipenem-EDTA Double Disk Synergy Test (DDST)

Among the 21 Carbapenem-resistant *Pseudomonas aeruginosa* isolates, 14 (66.6%) were identified as MBL-producing strains, while the remaining 7 (33.3%) were identified as non-MBL-producing strains.

Department-wise distribution of MBL strains of *P. aeruginosa*

Out of the total carbapenem-resistant *P. aeruginosa* (CRPA) isolates, MBL production was identified in several clinical units, with a notable concentration in high-dependency areas. The Intensive Care Unit (ICU) reported the highest number of MBL-positive isolates (n=5), followed by General Surgery (n=4) and other departments as shown in Figure 1.

Combination Testing Using Disk Diffusion Method

According to CLSI guidelines, individual testing of Ceftazidime-avibactam (CZA) using the Kirby-Bauer disk diffusion method revealed that 7 out of 21 isolates (33.3%) were susceptible to CZA, while 14 isolates (66.6%) were resistant. Similarly, testing for Aztreonam (AT) showed that 7 out of 21 strains (33.3%) were resistant, whereas 14 strains (66.6%) were susceptible. Subsequent testing involved the combination of CZA and AT by replacing the CZA disk with an AT disk after 1 hour of incubation, followed by overnight incubation at 35 ± 2°C. However, none of the 21 isolates exhibited a significant increase in the zone of inhibition when tested with the combination of CZA and AT as shown in Table 2 and Figure 2 & 3 respectively.

PCR for Carbapenemase Gene Detection

Conventional PCR analysis identified nine samples that displayed a band at approximately 390 bp on agarose gel electrophoresis, confirming the presence of the *blaVIM* gene. One sample, PA1, showed no detectable band and was deemed negative for the tested genes. The positive control produced satisfactory results, ensuring the reliability of the assay as shown in Table 4.

Table 1.Primers utilized for identifying carbapenemase genes

Gene	Primer Sequence (5'–3')	Amplicon Size (bp)	Reference
blaVIM	F: GATGGTGTTTGGTCGCATA	390 bp	Yong et al.[16]
	R: CGAATGCGCAGCACCAG		
blaIMP	F: GGAATAGAGTGGCTTAAYTCTC	232 bp	
	R: GGTTTAAYAAAACAACCACC		
blaNDM	F: GGTTTGGCGATCTGGTTTTC	621 bp	
	R: CGGAATGGCTCATCACGATC		

Table 2.Distribution of the clinical isolates of *Pseudomonas aeruginosa*

-	Frequency (n)	Percentage (%)
Age-wise Distribution (years)		
0-20	11	11
21-40	15	15

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41-60	39	39
>60	35	35
Total	100	100
Sex-wise Distribution		
Male	56	56
Female	44	44
Total	100	100
Department-wise Distribution		
General Medicine	11	11
General surgery	33	33
Urology	1	1
pulmonology	6	6
Orthopedics	3	3
Pediatrics	4	4
Obstetrics	3	3
Neurosurgery	5	5
ENT	4	4
Emergency	4	4
Nephrology	7	7
ICU	13	13
Plastic surgery	6	6
Total	100	100
Sample-wise Distribution		
Blood	10	10
Urine	6	6
Respiratory	28	28
Exudate	56	56
Total	100	100

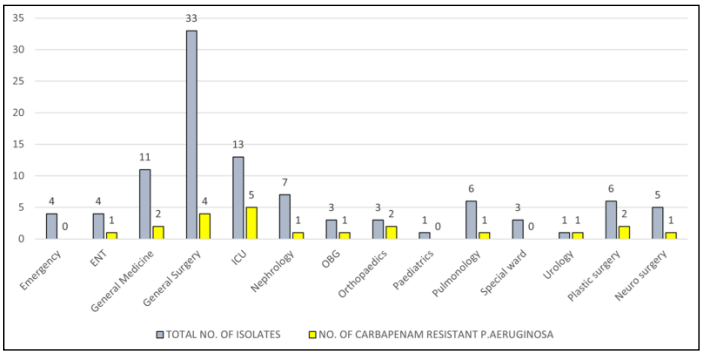

Figure 1.Department wise distribution of *Pseudomonas aeruginosa* and carbapenem-resistant strains.

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Table 3.Zone of Inhibition of CZA, AT and CZA+AT respectively

SAMPLE NO.	MBL/ NON-MBL	CEFTAZIDIME -AVIBACTAM (CZA) (ZI = ≥21mm)	AZTREONAM (AT) (ZI = ≥22mm)	CEFTAZIDIME-AVIBACTAM+ AZTREONAM (CZA+AT)	INFERENCE
PA1	Non MBL	6	20	16	No increase in ZI
PA 2	MBL	21	18	18	No increase in ZI
PA 3	MBL	21	23	21	No increase in ZI
PA 4	Non MBL	14	25	20	No increase in ZI
PA 5	MBL	22	20	20	No increase in ZI
PA 6	MBL	25	22	22	No increase in ZI
PA 7	MBL	21	18	18	No increase in ZI
PA 8	MBL	16	21	16	No increase in ZI
PA 9	MBL	20	24	22	No increase in ZI
PA 10	Non MBL	18	18	20	No increase in ZI
PA 11	MBL	8	18	20	No increase in ZI
PA 12	MBL	20	18	18	No increase in ZI
PA 13	Non MBL	21	21	21	No increase in ZI
PA 14	MBL	16	24	20	No increase in ZI
PA 15	MBL	21	20	20	No increase in ZI
PA 16	MBL	20	22	21	No increase in ZI
PA 17	MBL	18	20	21	No increase in ZI
PA 18	Non MBL	16	21	18	No increase in ZI
PA 19	Non MBL	20	22	22	No increase in ZI
PA 20	MBL	20	18	21	No increase in ZI
PA 21	Non MBL	18	20	20	No increase in ZI

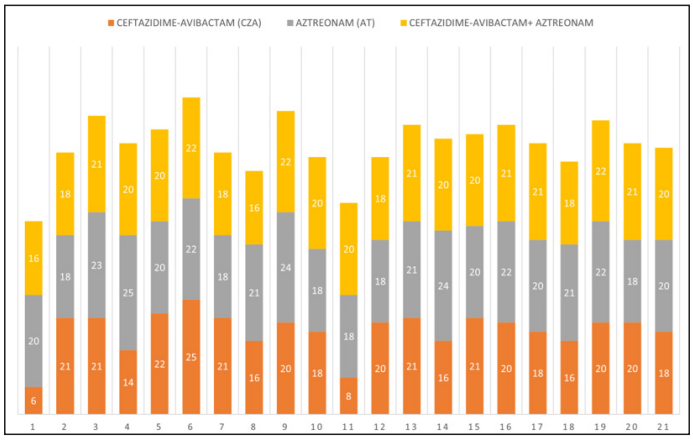

Figure 2.Zone of Inhibition of CZA, AT and CZA+AT respectively

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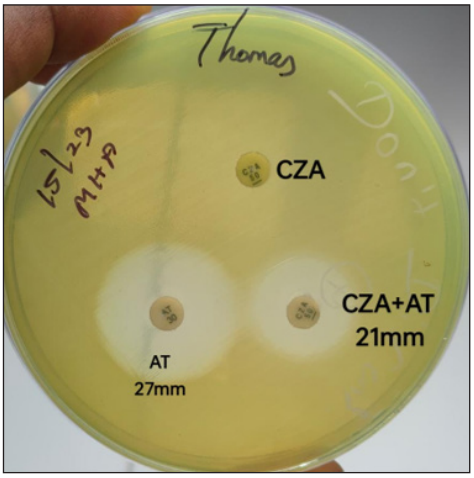

Figure 3.Combination testing of using Disk diffusion method

Table 4.Genes positive by multiplex PCR in *Pseudomonas aeruginosa* isolates

SAMPLE ID	GENES
PA1	Negative
PA2	bla_{VIM}
PA3	bla_{VIM}
PA4	bla_{VIM}
PA5	bla_{VIM}
PA6	bla_{VIM}
PA7	bla_{VIM}
PA8	bla_{VIM}
PA9	bla_{VIM}
PA10	bla_{VIM}

Discussion

The increasing prevalence of carbapenem-resistant organisms (CROs), particularly *Pseudomonas aeruginosa*, presents a growing clinical challenge. Despite recent advances in antimicrobial therapy, metallo-β-lactamase (MBL)-producing strains continue to limit therapeutic options. Although aztreonam is structurally stable against MBL-mediated hydrolysis, its efficacy is often compromised due to the co-production of serine β-lactamases, necessitating the use of combination therapies such as aztreonam with ceftazidime-avibactam (CZA). However, the clinical data supporting this combination remain limited and variable [18].

In our study, we collected a total of 100 *Pseudomonas aeruginosa* samples from inpatients across various clinical units. The findings revealed a higher infection rate in males,

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accounting for 56% of the cases, which is consistent with the findings of Ahmed et al., [19] who reported a similar male preponderance of 59.3%. This male predominance may be attributed to several factors, including outdoor activities, personal habits, work environments, and increased exposure to environmental organisms found in soil, water, and other regions.

Age-wise, the majority of isolates were from patients aged 41–60 years (39%), followed by those aged >60 years (35%). These findings align with Choudhary et al.,[20] who also reported a peak prevalence in the 41–60 age group. The higher incidence in older patients may reflect age-related immune compromise and the burden of comorbidities, which predispose them to opportunistic infections like *P. aeruginosa*.

Exudate samples were the most common source of *P. aeruginosa* isolates (58%), consistent with Choudhary et al.,[20] who reported 55.5% of isolates from similar specimens. The high prevalence in exudates can be attributed to the organism's ability to thrive in moisture-rich environments, form protective biofilms on damaged tissue, and utilize the nutrient-rich components of wound exudates [21]. Additionally, impaired local immunity and frequent exposure to hospital environments further facilitate colonization and infection by *P. aeruginosa* in such specimens.

Carbapenem resistance was detected in 21% of the isolates, all of which were resistant to both imipenem and meropenem. This resistance rate is higher than the 11.2% reported by Kresken et al., [22] indicating an alarming local trend, possibly driven by the overuse of broad-spectrum antibiotics, inadequate infection control practices, and the clonal spread of resistant strains in healthcare settings.

Among the 21 CRPA isolates, 67% (14/21) were confirmed to be MBL producers using the Imipenem-EDTA disc synergy test. These findings are in line with Hong DJ et al.,[23] while Radhika et al., [15] reported a much lower prevalence (15%). The rise of MBL-producing *P. aeruginosa* is largely linked to the excessive use of carbapenems and the plasmid-mediated spread of resistance genes in healthcare settings, underscoring the importance of stringent antimicrobial stewardship and regular surveillance of resistant strains [24].

MBL-producing strains were frequently isolated from the ICU (28.5%) and general surgery wards (14.2%), similar to Gupta et al.,[24] who found a 28% MBL rate in ICU samples—likely reflecting increased antibiotic use, invasive procedures, and critically ill patients in these settings, all of which contribute to the selection and spread of resistant pathogens.

At the molecular level, blaVIM was the most prevalent MBL gene, detected in 90% of MBL-positive *P. aeruginosa* isolates. This aligns with a Chinese study[25] that reported an 84.1% prevalence, suggesting regional dominance of blaVIM, possibly due to clonal expansion or local antibiotic selection pressures. In contrast, significantly lower rates were observed in studies by Wang et al.,[26] at 16.1% and Abaza et al.,[27] at 0%, indicating geographical variation in gene prevalence. Notably, no blaIMP genes were detected in our isolates, in stark contrast to the studies by Wang et al[26], which reported a 28.2% prevalence, and Shibata et al.[28], where blaIMP was predominant in 82.8% of isolates. The absence of blaIMP in our setting may reflect limited horizontal gene transfer of this particular gene or the presence of other, more competitive resistance mechanisms, such as blaVIM, which may have outcompeted blaIMP under local selective pressures.

Antibiotic susceptibility testing revealed that 33.3% of CRPA isolates were susceptible to CZA, while 66.6% were susceptible to aztreonam. Conversely, resistance to CZA was observed in 66.6% of the isolates, and 33.3% were resistant to aztreonam. Despite aztreonam's theoretical advantage against MBLs, our study found no significant inhibitory effect of aztreonam or its combination with CZA on either MBL or non-MBL-producing strains. This outcome contradicts studies by Davido et al.[29] and Benchetrit et al.,[30] which reported successful outcomes using this combination against multi-drug resistant organisms, including blaNDM-1, blaOXA-48, and AmpC-producing strains.

The reduced efficacy observed here may stem from the co-production of other β-lactamases such as ESBLs or AmpC, which can hydrolyze aztreonam. Additionally, diminished synergy may result from local resistance mechanisms, altered β-lactamase expression, or permeability barriers. These findings emphasize the importance of localized resistance surveillance and tailored susceptibility testing to guide effective use of combination therapies.

Limitations of the study

This single-center study had a limited sample size, which may affect the generalizability of the results. Synergy testing using the disk diffusion method may not be as reliable as MIC-based methods. Additionally, the absence of whole-genome sequencing and clinical outcome data limited deeper analysis of resistance mechanisms and therapeutic impact.

Conclusion

Our study found that both MBL- and non-MBL-producing *P. aeruginosa* isolates demonstrated in-vitro resistance to the ceftazidime–avibactam (CZA) and aztreonam (ATM) combination. Although this combination has shown promising activity in other studies, our findings align with reports of no inhibitory effect. While pharmacokinetic and pharmacodynamic studies support the clinical use of CZA–ATM, our results suggest limited efficacy against resistant *P. aeruginosa* strains. Further large-scale studies

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are essential to validate disk diffusion methods for synergy testing and to refine treatment strategies based on local resistance mechanisms.

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