

Assessing The In Vitro Activity Of Ceftazidime - Avibactam And Aztreonam Among Carbapenemase Producing Enterobacterales: Defining The Zone Of Hope By Disc Diffusion Method

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KEYWORDS

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ABSTRACT

Background: Antimicrobial resistance is one among the top global public health and development threats creating a great challenge in therapy. Infections caused by carbapenem resistant *Enterobacterales* (CRE) are extremely difficult to treat and are associated with high mortality. The Indian Council of Medical Research has reported an increasing carbapenem resistance among *Enterobacterales*. The clinical use of Polymyxin and Tigecycline are limited and can be avoided with the availability of beta-lactam and beta-lactamase inhibitor - Ceftazidime-avibactam (CAZ-AVI) approved in India. But this combination is found to have no activity against metallo-beta-lactamases. Therefore, combining Ceftazidime-avibactam (CAZ-AVI) and Aztreonam (ATM) for these resistant organisms becomes essential. **Methods:** A total of 137 carbapenem resistant *enterobacterales* isolates were included in the study. Phenotypic detection of carbapenemases (MBL- metallo beta lactemases and serine carbapenemase) was identified using modified carbapenem inactivation method (mCIM) and EDTA-carbapenem inactivation method (eCIM). The synergy between in vitro activity of Ceftazidime-avibactam and Aztreonam among Carbapenemase producing *Enterobacterales* is identified by Disc diffusion method.

Results: Among the 1220 *enterobacterales* species, 137 (11.23%) isolates were resistant to carbapenems. Out of 137 isolates 76 isolates were metallo betalactamase (MBL) producers and 61 isolates were serine carbapenemase producers. The 76 MBL producing isolates were processed further for the Ceftazidime avibactam and Aztreonam synergy. Out of the total MBL isolates the drug synergy was identified in 68 (89.47%) isolates.

Conclusion: This simple in vitro antimicrobial susceptibility testing of Ceftazidime-avibactam with Aztreonam, gives us a hope on this combination therapy for potential MBL-CRE infections in the developing countries like India, where MBL producing infections predominate. Phenotypic detection of carbapenemases may be used as reliable alternative to traditional molecular diagnostic methods.

Introduction:

Antimicrobial resistance is one of the major global concerns. It threatens the effective prevention and treatment of an ever increasing range of infections caused by bacteria, parasites, viruses and fungi [1].It is a natural process that happens over time through genetic changes but its spread is accelerated by human activity, mainly by misuse and overuse of antimicrobials to treat, prevent or control infections in humans, animals and plants. Rapidly progressing antibiotic resistance is a great challenge in therapy.It is estimated that bacterial antimicrobial resistance was directly responsible for 1.27 million global deaths in 2019 [2].The world faces an antibiotics pipeline access crisis, as we are in urgent need of additional new antibiotics.In addition to death and disability antimicrobial resistance has significant effect over economy of a country [3].Carbapenem resistant bacteria have become a major concern nowadays. The emergence of carbapenem resistance in *Enterobacterales* is a growing public health problem worldwide because of their high prevalence, wide range of clinical infections, multidrug resistance and rapid dissemination of plasmid-mediated resistance genes from *Enterobacterales* to other organisms. They are associated with resistance genes to β -lactam agents,

aminoglycosides, quinolones producing extremely drug-resistant bacteria [4, 5].

Carbapenem resistance in *Enterobacterales* is mediated by three major mechanisms: i) Carbapenemase production, ii) Overexpression of the efflux pump, and iii) Membrane porin mutation. The carbapenemase enzyme production is the major resistance mechanism. Three groups of Carbapenemases are accountable for carbapenem resistance: 1. *Klebsiella pneumoniae* carbapenemases -KPC (Ambler class A), 2. MBL - Metallo- β -lactamases, (Ambler class B) including NDM, VIM, IMP etc, 3. OXA (Ambler class D) such as OXA-48. Since all these enzymes are plasmid-mediated, they facilitate the horizontal transfer of the genes and global spread of the strains. The prompt and precise identification of CRE is essential not only for suitable and appropriate antibiotic therapy but also for stringent execution of infection control practices [6-8]. The clinical use of Polymyxins and Tigecycline for treatment of multi drug resistant bacteria are limited and can be avoided with the availability of beta-lactam and beta-lactamase inhibitor- Ceftazidime-avibactam (CAZ-AVI) which is approved in India [9].

Ceftazidime-avibactam has emerged as a promising option, even as monotherapy, for the treatment of severe infections with carbapenemase producing Enterobacterales and better clinical outcome has been well established. Studies have also demonstrated the superiority of Ceftazidime-avibactam over Colistin based regimens in improving the survival, and reducing the risk of acute kidney injury. But this combination is found to have no activity against metallo-beta-lactamases [10]. Aztreonam is highly stable to MBLs, however it gets hydrolyzed by co-expressing ESBLs and AmpCs. To overcome this issue, Aztreonam & Ceftazidime-avibactam could be a potential therapy for treating MBL-CRE infections in the developing countries like India, where MBLs predominate. Therefore, combining Ceftazidime-avibactam (CAZ-AVI) and Aztreonam (ATM) for these resistant organisms becomes essential [11]. As a result, genotypic profiling is emerging as an important clinical tool to make therapeutic decisions for these difficult-to-treat infections. Unfortunately, one barrier to global implementation of molecular characterization in the clinical setting is the financial burden of molecular diagnostic systems. Few studies have described the in-vitro antimicrobial susceptibility testing of Ceftazidime-avibactam in combination with Aztreonam by disk diffusion method, E- test agar synergy and E-test fixed ratio method showing 100% concordance with each other in their susceptibility testing [12].

The simple in vitro method described in literature for antimicrobial susceptibility testing of Ceftazidime-avibactam in combination with Aztreonam and phenotypic characterization of carbapenemases may be used as reliable alternative to traditional molecular diagnostic methods for prediction of optimal anti microbial combinations for treatment of CRE [13]. This study proves that routine drug susceptibility testing by disk diffusion method should be made routinely available in a resource limited setting rather than the costly genotypic and E-test MIC methods that are performed for Carbapenemase identification. The increased prevalence of metallo-beta-lactamases was addressed so that the overuse of Carbapenems can be prevented, encouraging the alternate use of the newer beta-lactamase inhibitors in combination with older beta-lactams.

Objectives:

1. To determine the prevalence of Carbapenem resistant *Enterobacterales* in clinical isolates of a Tertiary care hospital.
2. To differentiate the carbapenemases into serine β -lactamases and metallo- β -lactamases by phenotypic methods.
3. To assess the feasibility of in-vitro susceptibility testing of Ceftazidime-avibactam along with Aztreonam by disk diffusion method.

Methodology:

Ethical Approval: Ethical clearance was obtained from the Institutional Ethical Committee of our college prior to the commencement of the study.

Study Design: This was a prospective cross-sectional study conducted in the Department of Microbiology at a newly established medical college in South India.

Sample Collection and Isolation: A total of 1220 *Enterobacterales* isolates were obtained from various clinical samples, including pus, urine, blood, body fluids, endotracheal aspirates, and sputum.

Screening for Carbapenem Resistance: Clinical isolates of *Enterobacterales* were identified as carbapenem-resistant through a screening test using Meropenem (10 μ g) and Ertapenem (10 μ g) .

Phenotypic Detection of Carbapenemases: All *Enterobacterales* isolates that showed resistance to Meropenem (10 μ g) by the disc diffusion method were further processed for carbapenemase identification. This was done using the phenotypic methods Modified Carbapenem Inactivation Method (mCIM) and EDTA

Carbapenem Inactivation Method (eCIM), as per CLSI guidelines [14].

mCIM and eCIM testing: A 1- μ l loop full of bacteria was inoculated into a 2-ml test tube of Tryptone soya broth. Another 1- μ l loop full of bacteria was inoculated into 2-ml test tube of Tryptone soya broth supplemented with EDTA (In house preparation of EDTA was done: 186.1 gm of disodium EDTA is first dissolved in NaOH at pH 8 and then distilled water is added to make it into one litre). A Meropenem 10 μ g disc was placed in each tube, and the tubes were incubated at 35 °C for 4 h $\pm$ 15 min. Subsequently, both the discs were removed and applied to Muller Hinton agar plates that is freshly inoculated with a 0.5 McFarland suspension of a carbapenem-susceptible ATCC strain of *E. coli* 25922. The plates were incubated at 35°C for 16 to 20 hours and the mCIM and eCIM results were interpreted. The mCIM is considered as negative (no metallo beta lactamases) if the zone size is $\geq$ 19 mm, as positive if the zone size is between 6 to 15 mm, or as intermediate (defined as positive) if pinpoint colonies are present within a 16- to 18-mm zone. An isolate is positive for metallo-beta lactamase production when the eCIM zone size increases by $\geq$ 5mm compared to the zone size observed for the mCIM and is considered negative for a metallo-beta lactamase if the increase in zone size is $<$ 4mm [15].

CAZ-AVI and AZT disc synergy testing: Individual testing of Ceftazidime 30 μ g, Aztreonam 30 μ g, and Ceftazidime-avibactam (30 μ g /20 μ g) by Kirby Bauer disc diffusion will be done for the MBL producing *Enterobacterales*. For combination testing the ceftazidime-avibactam antibiotic discs initially applied onto Muller Hinton agar plates inoculated with the test organism and incubated at 35 $\pm$ 2 °C for a period of one hour will be removed and replaced with Aztreonam discs on the same site. After disc inoculation, plates will be re-incubated overnight and then observed for a zone of inhibition on the next day as per standard CLSI recommendations for disk diffusion testing [14]. Zone size of $\geq$ 21mm is considered as synergy test positive [16].

Results:

A total of 1220 *Enterobacterales* species were isolated from various samples like blood, pus, sputum, urine and endotracheal aspirate over a period of eleven months. Among the 1220, 137 carbapenem-resistant isolates were found, with their distribution across diverse clinical samples indicated in Table I. *Klebsiella pneumoniae* was the most common pathogen, accounting for 44% (60/137) of all isolates, with the most isolates detected in pus (29) and urine (14). *Klebsiella oxytoca* accounted for 16% (22/137) of the total isolates, with 14 samples from pus and 4 from sputum. *Escherichia coli* accounted for 36.5% (50/137) of the isolates, with a large proportion (40) originating from urine samples. *Citrobacter koseri* and *Enterobacter* species were less common, accounting for 0.7% (1/137) of all isolates from pus and urine, respectively. *Proteus* species accounted for 2.1% (3/137) of all pus samples.

Table I: Distribution of Carbapenem Resistant Isolates (n=137):

Name of the Isolate	Pus	Urine	Blood	ET aspirate	Sputum	Total	Percentage
<i>Klebsiella pneumoniae</i>	29	14	10	3	4	60	44%
<i>Klebsiella oxytoca</i>	14	4	-	-	4	22	16%
<i>Escherichia coli</i>	10	40	-	-	-	50	36.5%
<i>Citrobacter koseri</i>	1	-	-	-	-	1	0.7%
<i>Enterobacter</i> species	-	1	-	-	-	1	0.7%
<i>Proteus</i> species	3	-	-	-	-	3	2.1%
Total	57	59	10	3	8	137	100%

Figure 1 shows the antimicrobial sensitivity pattern of carbapenem-resistant *Klebsiella* isolates. The majority of *Klebsiella pneumoniae* isolates showed substantial resistance to routinely used medicines, particularly extended-spectrum cephalosporins like Ceftriaxone and Cefotaxime. Resistance to additional antibiotics, such as aminoglycosides (e.g., Amikacin) and fluoroquinolones (e.g., Ciprofloxacin), was also seen, indicating that these multidrug-resistant bacteria had limited treatment choices. The findings highlight the significance of continued surveillance and judicious antibiotic management in managing carbapenem-resistant *Klebsiella* infections.

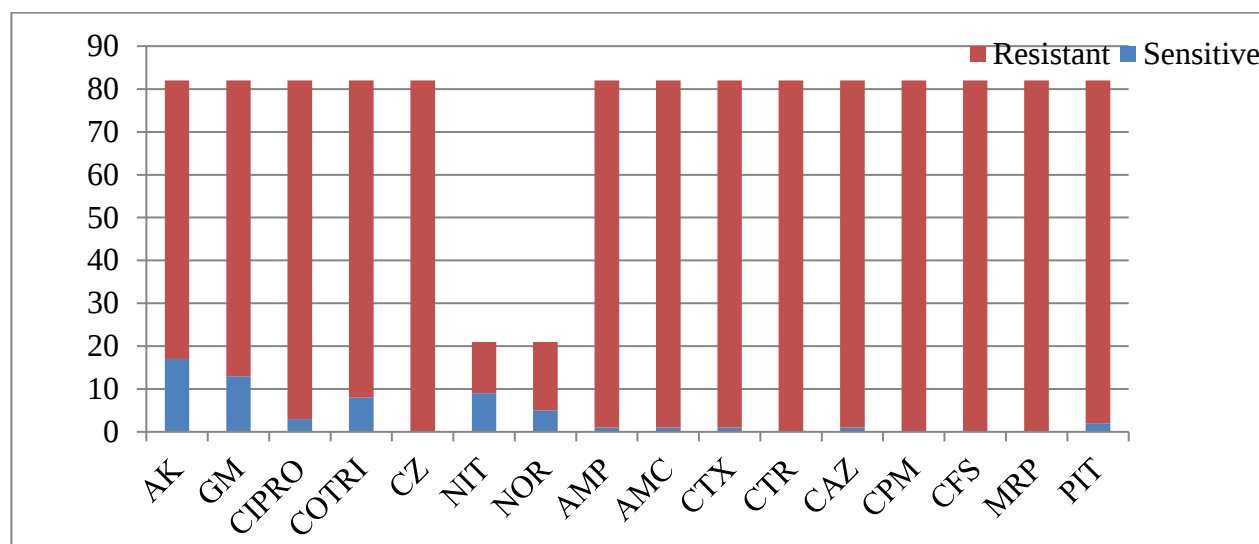


Figure: 1 Antimicrobial Sensitivity Pattern of Carbapenem Resistant Klebsiella Isolates.

Figure 2 shows the antimicrobial sensitivity patterns of carbapenem-resistant *Escherichia coli* isolates. *Escherichia coli* isolates, like *Klebsiella pneumoniae*, were highly resistant to popular antibiotics, including extended-spectrum cephalosporins like Ceftriaxone and Cefotaxime, as well as aminoglycosides like Gentamicin and Amikacin. Resistance to fluoroquinolones like Ciprofloxacin was also common. The discovered resistance patterns highlight the increasing difficulty of treating carbapenem-resistant *Escherichia coli* infections and underline the necessity for alternate therapeutic alternatives and strategic antibiotic use in clinical settings.

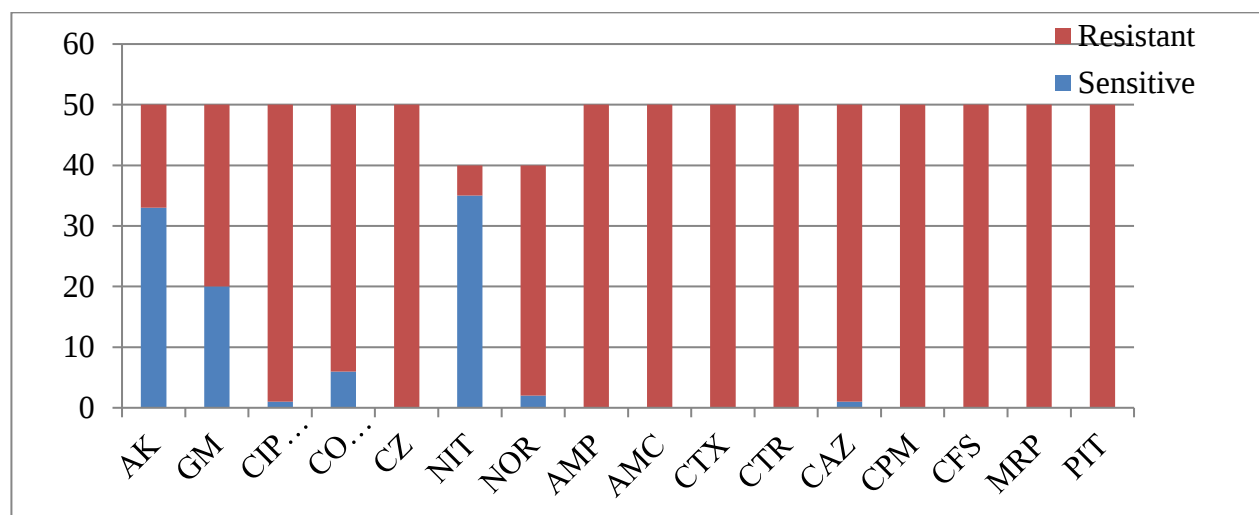


Figure: 2 Antimicrobial Sensitivity Pattern of Carbapenem Resistant Escherichia coli Isolates.

Figure 3 shows the distribution of metallo- β -lactamases (MBL) and serine carbapenemases in clinical samples. A considerable majority of carbapenem-resistant isolates produced MBL enzymes, with *Escherichia coli* and *Klebsiella pneumoniae* having the highest incidence. These MBL-producing isolates were primarily obtained from urine and pus samples. Serine carbapenemases, such as KPC and OXA kinds, were also found, albeit in lower numbers than MBLs. These serine carbapenemase-producing isolates were primarily detected in blood and sputum samples. The findings underscore the variability of carbapenemase mechanisms in clinical pathogens, with MBLs being more typically associated with urine and pus-based infections and serine carbapenemases being more prevalent in bloodstream and respiratory tract infections.

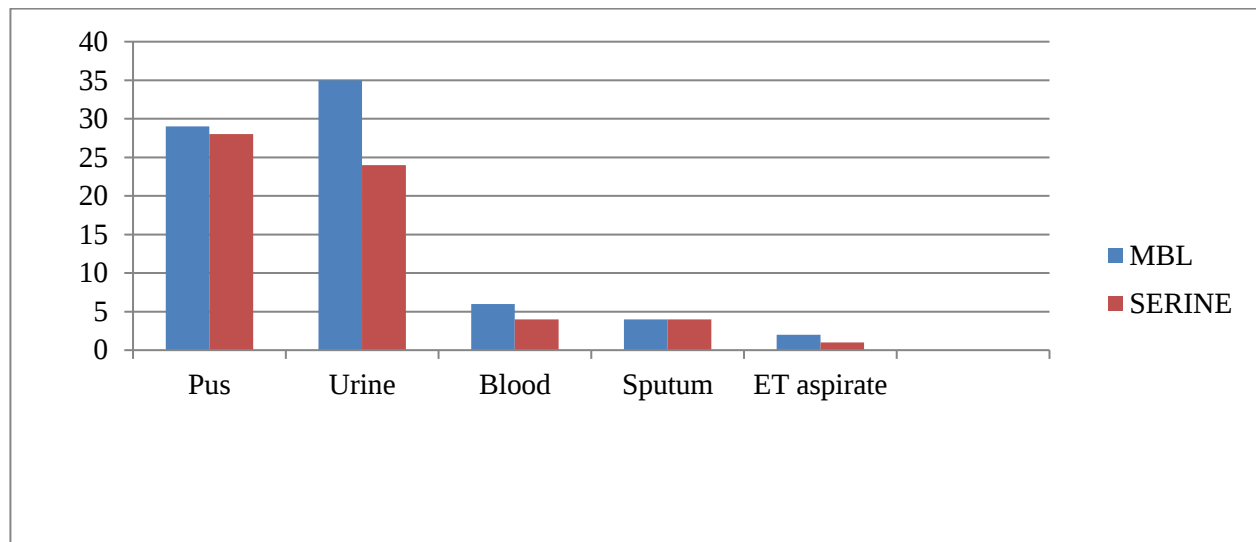


Figure: 3 Distribution of MBL and Serine carbapenemases among clinical samples:

Figure 4 depicts the distribution of Metallo- β -lactamases (MBL) and Serine carbapenemases among carbapenem-resistant isolates. Among the 137 carbapenem-resistant isolates, MBL producers were more common, accounting for a greater proportion of resistance mechanisms. The most frequent MBL producers were *Klebsiella pneumoniae* and *Escherichia coli*. MBL-producing isolates were primarily detected in pus and urine samples. Serine carbapenemase manufacturers, including KPC and OXA kinds, were less commonly discovered but were found in *Klebsiella pneumoniae* isolates, primarily from blood and sputum samples. The distribution of various carbapenemase types demonstrates the complexities of carbapenem resistance, with MBLs being the most common resistance mechanism among clinical isolates.

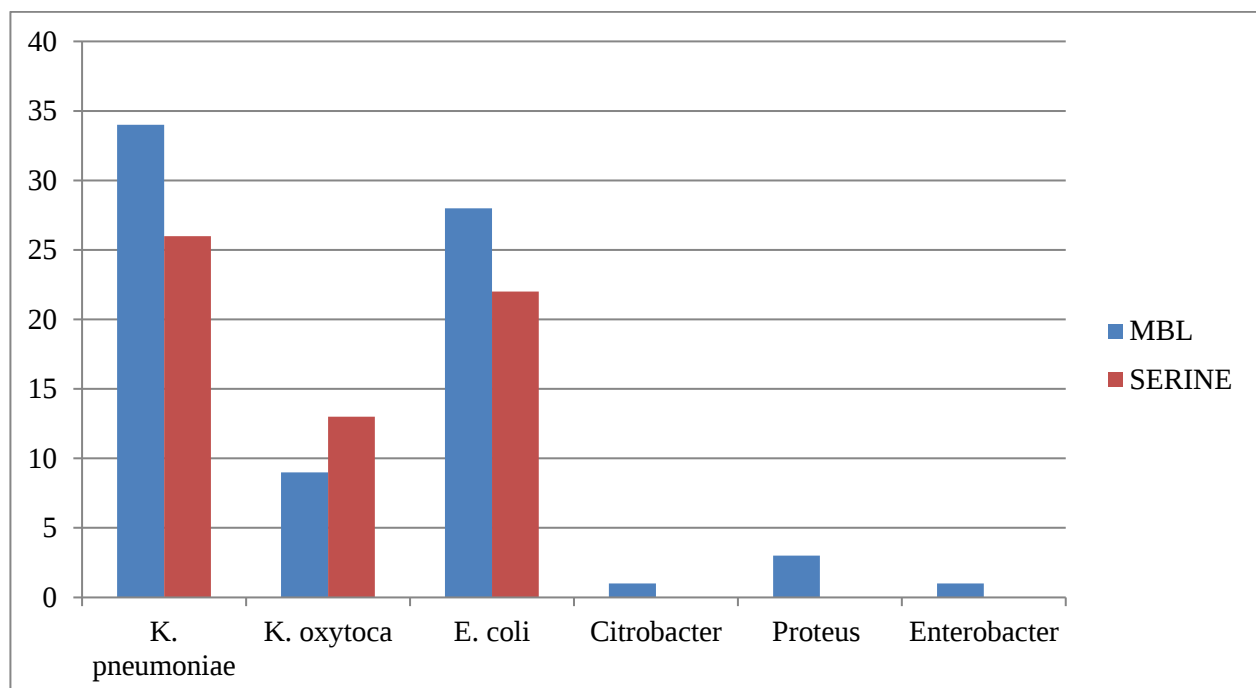


Figure: 4 Distribution of MBL and Serine carbapenemases among carbapenem resistant isolates:

Table 2 shows the zone diameter distribution for MBL (Metallo- β -lactamase) isolates evaluated with Aztreonam (AZT), Cefazolin (CZA), or both. The majority of *Klebsiella pneumoniae* (34 isolates) had variable zone sizes, with 30 isolates having a 6-12 mm zone for AZT and 28 isolates exhibiting a 6-12 mm zone for CZA. However, the combination of CZA and AZT produced >24 mm for 30 isolates, showing greater effectiveness. *Klebsiella oxytoca* (9 isolates) showed 6-12 mm zones for both AZT and CZA, however with the combination, all isolates showed >24 mm, showing increased susceptibility. In *Escherichia coli* (28 isolates), 25 isolates showed a 6-12 mm zone for AZT and 24 isolates showed a 6-12 mm zone for CZA, with

25 isolates displaying >24 mm with the combination, demonstrating high susceptibility to CZA + AZT. Overall, the combination of CZA and AZT was the most effective treatment particularly *Klebsiella pneumoniae* and *Escherichia coli*. The results of eCIM, mCIM, and the Synergy test revealed the presence of both MBL and serine carbapenemases in the *Enterobacterales* isolates, with a high prevalence of KPC-type carbapenemases and MBLs in the clinical samples. These findings highlight the importance of comprehensive diagnostic testing to guide optimal antibiotic therapy for carbapenem-resistant infections (Fig.5).

Table: 2 Distribution of Zone Diameter of the MBL Isolates:

Name of the Isolate	Name of the Disk	6 – 12 mm	12- 21 mm	<21 mm	21- 24 mm	>24 mm
<i>Klebsiella pneumoniae</i> (34)	AZT	30	4			
	CZA	28	6			
	CZA + AZT		1	3	30	
<i>Klebsiella oxytoca</i> (9)	AZT	9				
	CZA	9				
	CZA + AZT					9
<i>Escherichia coli</i> (28)	AZT	25	3			
	CZA	24	4			
	CZA + AZT		2	1	25	
<i>Citrobacter species</i> (1)	AZT	1				
	CZA	1				
	CZA + AZT		1			
<i>Proteus species</i> (3)	AZT	3				
	CZA	3				
	CZA + AZT		3			
<i>Enterobacter species</i> (1)	AZT	1				
	CZA	1				
	CZA + AZT		1			



Modified Carbapenem Inactivation Method

Test of synergy between CAZ-AVI and AZT

Figure: 5 eCIM, mCIM and Synergy test.

Discussion:

It is important to differentiate Carbapenemase classes because newly available β -lactam (BL) and β -lactamase inhibitor (BLI) combinations like ceftazidime-avibactam as well as others under research are mostly active against serine carbapenemases, but not against metallo- β -lactamases [17]. The monobactam Aztreonam (ATM) is resistant to hydrolysis by MBLs but susceptible to hydrolysis by serine class A, C and D extended spectrum

beta lactamases, whereas ceftazidime-avibactam (CAZ/AVI), a beta lactam with a beta-lactamase inhibitor, has broad activity against serine beta-lactamases but is hydrolysed by MBLs. A total of 1220 *Enterobacterales* were isolated in our microbiology laboratory for the past 11 months from various samples like pus, urine, blood, sputum, and endotracheal aspirate. Among the isolates about 137 had shown resistance to carbapenems (11.23%). It is very similar to the study conducted by Bhatt et al, where the prevalence of resistance to carbapenems was 11.96%. The prevalence of carbapenem resistance in various parts of India varies from 14-69%. This was mostly owing to infection control practices, hospital infrastructure, and the number of antibiotics used [18]. Among the carbapenem resistance organisms *Klebsiella* species accounts for 60% and *Escherichia coli* 36.5%, shows similarity to the study done by Sreeja K Vamsi et al. Most of the carbapenem resistant isolates were from pus samples followed by urine and other samples [19].

Out of the 137 carbapenem resistant isolates 76 were MBL producers (55.5%) and 61 were Serine carbapenemase producers (44.5%). This indicates the equal distribution of both genes among the carbapenem resistant isolates. This is very much comparable to the study done by Sulakshana Sony Cheemala et al, in Andhra Pradesh during 2022 to 2023 reported 53% were serine and 41% were MBL producers, this again showed the MBL production has been constantly increasing among the carbapenem resistant isolates. In the same year Ravi Kumar Sharma et al in UP reported 62% of MBL among carbapenemase producers [20, 21]. Among the MBL producing *Enterobacterales*, *Klebsiella* species accounts for 56.5% (43/76), *Escherichia coli* 36.8% (28/76) and others 6.5% (5/76). All the 76 MBL producing *Enterobacterales* isolates were resistant to Aztreonam and Ceftazidime avibactam with a zone diameter of <21 mm when tested individually. But when tested for synergy using Ceftazidime avibactam and Aztreonam most of the MBL isolates exhibit synergy. In the 76 MBL producers, *Klebsiella pneumoniae* showed synergy in 33/34 isolates (97.05%), *Klebsiella oxytoca* has 100% synergy (9/9), *Escherichia coli* showed synergy in 26/28 isolates (92.85%) and other enterobacteriaceae isolates did not exhibit synergy. The overall synergy shown by enterobacteriaceae isolates was 89.47% (68/76). This is similar to the study done in 2017 by Marshall et al. where 17 out of 21 isolates became susceptible to synergy testing by disk diffusion method and by Rawan et al. in 2021 observed 98.75% synergy in *Klebsiella* species and 95% in *E. coli* isolates [22, 23]. This gives us an additional clue that metallo beta lactamases are more common among the *klebsiella* isolates when compared to other members of *Enterobacteriaceae* family and exhibit more synergy. Therefore the combination of CAZ/AVI and AZT is considered as effective therapeutic option for Metallobeta lactamase producing *Enterobacteriaceae* isolates. However, safety of this combination and in vivo efficacy have to be evaluated.

Conclusion:

Carbapenem resistance is a significant global public health issue, particularly among Gram-negative bacteria. Infections caused by these organisms provide considerable detection issues, particularly in resource-constrained settings, underlining the need for cost-effective and simple diagnostic techniques for everyday usage. Furthermore, resistance mechanisms to beta-lactam antibiotics evolve quickly, hence combination therapy with two or more antibiotics is frequently suggested. The combination of Aztreonam (ATM) and Ceftazidime/Avibactam (CAZ/AVI) has shown a synergistic impact against Metallo- β -lactamase (MBL)-producing bacteria in vitro, with supportive clinical trials establishing the in vivo efficacy of ATM-AVI. To test for susceptibility to the ATM-CZA combination, focus on carbapenem-resistant *Enterobacterales* (CRE) with metallo- β -lactamases. The IDSA (Infectious Diseases Society of America) and ESCMID both endorse this combination as an empiric therapeutic option. To effectively treat CRE infections, it is critical to design a method that can be repeated on a regular basis in microbiology laboratories. One alternative is to do synergy testing utilizing the disc diffusion method with Ceftazidime, Avibactam, and Aztreonam.

Limitations:

The absence of data regarding the clinical use of this drug combination since the in-vitro susceptibility cannot always predict the in-vivo activity of the drugs in the patient because pharmacodynamics of the drug and host immune response also play a role in the clinical outcome of the patient.

Funding:

None

Conflict of Interest:

The authors report no conflicts of interest in this work.

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