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ARTICLE

# In-vitro susceptibility testing methods for the combination of ceftazidime-avibactam with aztreonam in metallobeta-lactamase producing organisms: Role of combination drugs in antibiotic resistance era

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## Abstract

Resistance in Gram-negative organisms has become one of the leading threats in recent years. Of the different mechanisms described in the literature, resistance due to beta-lactamases genes have been overcomed by the use of a beta-lactamase inhibitor in combination with a beta-lactam antibiotic. When this combination is insufficient to counter metallo-beta-lactamases, a third antibiotic, has been added to restore susceptibility. One such recent combination is ceftazidime-avibactam with aztreonam. In this study, 60 isolates of multidrug-resistant organisms producing metallo-beta-lactamases were included to perform in-vitro antibiotic susceptibility testing against ceftazidime-avibactam and aztreonam alone and in combination using three different methods. Individual testing revealed 100% (60/60) resistance to both ceftazidime-avibactam and aztreonam in all the isolates. The disk diffusion method showed an inhibition zone size of 21 mm in all the isolates, with 16 isolates showing an increase in inhibition zone size of >16 mm. In the E-test fixed ratio method, MICs of ceftazidime-avibactam and aztreonam when used alone ranged from 8/4 μg $l^{-1}$ to ≥256/4 μg $l^{-1}$ and 16 μg $l^{-1}$ to 256 μg $l^{-1}$, respectively, but in combination, these MICs were reduced to 0.016/4 μg $l^{-1}$ to 2/4 μg $l^{-1}$ with FIC < 0.5 in all the isolates. Similar results were obtained with the E-test agar dilution method with more than a 16-fold reduction in MIC in all the isolates when avibactam concentration was fixed at 4 μg $l^{-1}$. All three methods showed a 100% correlation with each other. The current study depicted the usefulness of combining ceftazidime-avibactam with aztreonam against organisms producing metallo-beta-lactamases and that disk diffusion methods can be used as a method for performing in-vitro antibiotic susceptibility testing of this combination.

## Introduction

In the current era of increasing antimicrobial resistance, the problem of multidrug-resistant organisms is emerging at a very fast rate. For Gram-positive organisms like methicillin-resistant *Staphylococcus aureus*, vancomycin still remains the drug of choice as the overall incidence of VRSA is small [1]. Multi-drug resistant Gram-negative organisms are often treated with carbapenems and their increased use has resulted in the rapid emergence of resistance to these drugs. The reason for this sudden rise in resistance is multifactorial viz., continuous exposure to antibiotics including a steep rise in carbapenem use, use of lower doses than the approved dose of antibiotics, contamination of surgical equipment, and long-term stay in tertiary care hospitals [2]. Very few options such as colistin/polymyxin B, tigecycline,

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and fosfomycin are available, and resistance is being reported against these drugs also [3–6].

The success of treatment with beta-lactams against Gram-negative organisms has been greatly reduced because of the high prevalence of ESBLs, AmpC beta-lactamases, and carbapenemases such as the metallo-beta-lactamases (MBLs), and OXA-48 producing pathogens. Studies have shown that the carbapenem-resistance rates and presence of different types of carbapenemases genes vary in different geographical regions and therefore knowledge about the presence of the types of carbapenemases in a defined geographical region is of utmost importance in deciding the choice of empirical antibiotics [7]. The co-production of metallo-beta-lactamases along with ESBLs and serine carbapenemase enzymes is not uncommon in most of these organisms, which ultimately affects the choice of antibiotic and the patient outcome. In India, multiple studies have reported high rates of $bla_{\text{NDM-1}}$ in Gram-negative organisms [8–10]. The currently available first-generation beta-lactamase inhibitors such as clavulanic acid, tazobactam, or sulbactam have a narrow spectrum and do not inhibit the carbapenemases. To circumvent the overuse of carbapenems, newer beta-lactamase inhibitors were developed in combination with older beta-lactams. Avibactam is one such beta-lactam inhibitor (diazabicyclooctane or DBO) having in-vitro activity against the carbapenemases belonging to class A and class D beta-lactamases. Hence avibactam was used in combination with ceftazidime to act against these resistant isolates. But this combination was found to have no activity against metallo-beta-lactamases like $bla_{\text{NDM}}$, $bla_{\text{VIM,}}$ and $bla_{\text{IMP}}$ [11, 12].

NDM is mainly inhibited by aztreonam, a monobactam beta-lactam, but the presence of other beta lactamases like ESBLs that are also found in the same organism can hydrolyze and inhibit the activity of aztreonam against NDM beta-lactamases. This situation is very common in patients being referred to tertiary care facilities. Therefore, combining ceftazidime-avibactam (CAZ-AVI) and aztreonam (ATM) in these organisms will result in inhibition of ESBLs and other beta lactamases by the former drug and the intact ATM will act against the metallo-beta-lactamases. Only a few studies and case reports have described this combination to be a better option in the successful treatment of multi-drug-resistant organisms. Even though the phase 3 clinical trial of this drug combination showing its efficacy is still pending, Food and Drug Administration (FDA) has approved aztreonam for the treatment of MBL-producing organisms and is supported by the Infectious Diseases Society of America (IDSA) [13]. Still, there is scanty data regarding the use of this drug in the Indian context and currently, no method is approved by CLSI or any other organization for the in-vitro susceptibility testing of this combination. Hence the current study aimed to perform in-vitro susceptibility testing of ceftazidime-avibactam along with aztreonam by comparing three different methods of susceptibility testing, disk diffusion, MIC determination by the use of E-strips, and E-strips agar synergy to find a suitable method of testing which can be easily performed in hospital settings in resource-limited countries.

## Materials and methods

A total of 60 multidrug-resistant isolates belonging to the family *Enterobacterales* (55) and *Pseudomonas aeruginosa* (5) were collected in a tertiary care centre, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh in North India over a period of 6 months and included in the study. These isolates were collected from different sources such as blood, pus, respiratory specimens like sputum, bronchoalveolar lavage fluid, and other body fluids. The Kirby-Bauer disk diffusion method along with MIC determination using the Vitek 2 system revealed resistance to beta-lactams, cephalosporins, and carbapenems like imipenem and meropenem.

### PCR for carbapenemase gene detection

Conventional PCR was performed on all the DNA samples for detecting carbapenemase genes ($bla_{\text{KPC}}$, $bla_{\text{VIM}}$, $bla_{\text{NDM}}$, $bla_{\text{IMP,}}$ $bla_{\text{OXA-48}}$). Further subtypes of these genes were not identified. DNA bands were viewed under the Azure 200 gel imaging system (CA, USA). The carbapenemase genes were detected using the following primers (Table 1).

### Phenotypic tests for the expression of carbapenemase genes: Modified carbapenem inactivation method (mCIM) and EDTA-CIM (eCIM)

For the detection of carbapenemase genes in *Enterobacterales* - a modified carbapenem inactivation method (mCIM) was performed and to differentiate the metallo-beta-lactamases from the serine beta-lactamases, mCIM was used in conjunction with EDTA carbapenem inactivation method (eCIM) according to the recent CLSI guidelines [14]. Briefly, one loopful of the test isolate was emulsified in 2 ml of tryptone soy broth in two different tubes, vortexed, and labeled as eCIM and mCIM. 200 µl of 0.5 M EDTA was added to the first tube labeled as eCIM. Then,10 µg of meropenem disk was added to both the tubes and incubated at 35 °C ± 2 °C for 4 h. In the meantime, 0.5 McFarland suspension of *E. coli* ATCC 25922 was prepared and inoculated onto the MHA plate. Later, both the meropenem disks were removed, excess fluid drained, and placed on the MHA plate which had been inoculated with ATCC *E. coli* 25922. The plates were then

**Table 1** Primers for carbapenemase gene detection

| PCR name | Beta-lactamase targeted | Primers | Product size (bp) |
|---|---|---|---|
| Simplex | NDM-1 | NDM-1 F: GGTTTGGCGATCTGGTTTTC<br>NDM-1 R: CGGAATGGCTCATCACGATC | 264 |
| Simplex | OXA-48 | OXA-48 F: TATATTGCATTAAGCAAGGG<br>OXA-48 R: CACACAAATACGCGCTAACC | 302 |
| Multiplex IV metallo beta lactamases and carbapenamases | IMP, VIM and KPC | IMP F:TTGACACTCCATTTACDG<br>IMP R:GATYGAGAATTAAGCCACYCT | 139 |
| | | VIM F:GATGGTGTTTGGTCGCATA<br>VIM R:CGAATGCGCAGCACCAG | 390 |
| | | KPC F:CATTCAAGGGCTTTCTTGCTGC<br>KPC R:ACGACGGCATAGTCATTTGC | 538 |

inverted and incubated again at 35 °C ± 2 °C in ambient air for 18–24 h.

An inhibition zone size of 6–15 mm in mCIM was considered as indicative of the production of carbapenemase in the test isolate thereby proving that the meropenem disk had been hydrolyzed by the carbapenemase produced by the test isolate. An inhibition zone diameter of ≥19 mm was interpreted as susceptible and negative for carbapenemase production and between 16–18 mm as indeterminate. eCIM was interpreted only if the mCIM was positive. The presence of metallo-beta-lactamase is confirmed when there is an increase in zone size of ≥5 mm in eCIM when compared to mCIM. A ≤4 mm increase in zone diameter showed the absence of metallo-beta-lactamases in the test isolate.

Susceptibility to ceftazidime-avibactam and aztreonam was tested individually by both disk diffusion and E-strips. Following individual drug testing, these drugs were also tested together to determine their activity in combination. These were performed on isolates for which the molecular testing revealed the presence of at least one of the metallo-beta-lactamases like $bla_{NDM}$, $bla_{VIM,}$ or $bla_{IMP}$ and on isolates whose phenotypic tests confirmed the presence of carbapenemase production. We compared the three different methods of combined testing to look for a better and more affordable method that could be adapted to routine diagnostic testing.

## Combination testing

### Disk diffusion method

Ceftazidime-avibactam disks (30 µg /20 µg) (BD Diagnostic Systems Sparks, MD, USA) provided by Pfizer, New York, USA were used. In this method, ceftazidime-avibactam antibiotic disks were initially applied onto MHA plates inoculated with the test organism and incubated at 35 ± 2 °C for a period of one hour. Later, these disks were removed and replaced with aztreonam disks on the same site. After disk inoculation, plates were re-incubated overnight and then observed for a zone of inhibition on the next day as per standard CLSI recommendations for disk diffusion testing.

### E-test fixed ratio method

Ceftazidime-avibactam E-test strips were purchased from (bioMérieux, Marcy-I 'Etoile, France), and aztreonam E -test strips from HiMedia were used for the MIC determination and E-test fixed ratio method. Here, E-test strips containing ceftazidime-avibactam and aztreonam were added sequentially to the bacterial lawn on Muller Hinton agar plates; the first E-test strip (strip CAZ-AVI) was incubated for a period of one hour which was then removed, cleaned with alcohol, and saved as an MIC reading scale. The second E-test strip i.e., aztreonam E strip was positioned exactly over the imprint of the first E-test strip, strip A. The plates were re-incubated again for 18 h at 35 ± 2 °C. Respective MIC strips/scales were used to read MICs by placing them in each gradient's position and the FIC (Fractional inhibitory concentration) index was calculated. *E. coli* ATCC 25922 was used as a quality control strain in all these experiments. The FIC was calculated and interpreted as described below

$$\text{FIC of agent A} = \text{MIC of agent A in combination}/\text{MIC of agent A alone}$$
$$\text{FIC of agent B} = \text{MIC of agent B in combination}/\text{MIC of agent B alone}$$
$$\text{Cumulative FIC} = \text{FIC OF agent A} + \text{FIC of agent B}$$

Synergy' is interpreted when the FIC index is ≤0.5, additivity when the index is >0.5–1, indifference or no interaction corresponds to the FIC index > 1–4.0, and antagonism when the FIC index is >4.0 [15, 16].

### E-test agar synergy method

Avibactam (AVI) was incorporated into MHA plates to a final concentration of 4 µg $ml^{-1}$. 0.5 McFarland standard of

the test organisms were inoculated onto the plates, and an ATM E-test strip (bioMérieux, Marcy-l'Etoile, France) was applied over it, and the plates were incubated for 18 h at 35 ± 2 °C.

Synergy was defined as a ≥3-fold dilution decrease in MIC, additivity 2-fold dilution decrease in MIC, and indifference 1-fold dilution change in MIC value [17, 18].

## Results

A total of 60 isolates including *Klebsiella pneumoniae* (38), *Escherichia coli* (11), *Enterobacter cloacae* (3), *Citrobacter freundii* (3), and *Pseudomonas aeruginosa* (5) from routine samples were collected for a period of 6 months. These isolates were found to be resistant to most antibiotics like cefotaxime, ceftriaxone, cefepime, ciprofloxacin, imipenem, meropenem, cefoperazone-sulbactam, and piperacillin-tazobactam in the routine antimicrobial susceptibility testing methods which included either Kirby-Bauer disk diffusion or by MICs (Vitek 2 platform). Among these isolates, minocycline showed the highest susceptibility of 80.3% followed by colistin (60%) and doxycycline (48.8%). After routine antimicrobial susceptibility testing, these isolates were subjected to molecular testing for the detection of metallo-beta-lactamases like $bla_{NDM}$, $bla_{VIM,}$ and $bla_{IMP}$. Testing indicated the presence of $bla_{NDM}$ alone in 25 isolates, $bla_{NDM}$ and $bla_{IMP}$ in 12 isolates, $bla_{NDM}$ and $bla_{VIM}$ in 4 isolates, and the presence of all of the three enzymes in 19 isolates. (Figure 1) PCR testing was followed by phenotypic methods like mCIM and eCIM and all the isolates were positive for both these methods further confirming the presence of carbapenemases genes in these resistant isolates.

### Disk diffusion method

Individual testing of ceftazidime, aztreonam, and even ceftazidime-avibactam by Kirby Bauer disk diffusion showed 100% resistance in all the 60 isolates. This was followed by testing of ceftazidime-avibactam in combination with aztreonam by replacing the ceftazidime-avibactam disk after one hour of incubation with aztreonam disk and overnight incubation at 35 ± °C. Susceptibility was reported in all the isolates with an inhibition zone diameter of ≥21 mm. This is in agreement with the susceptible criteria of ≥21 mm zone size recommended for ceftazidime-avibactam according to CLSI guidelines. (Fig. 2) Combining ceftazidime-avibactam and aztreonam resulted in an increase in zone size of 4–9 mm in 17 isolates, 10–15 mm zone size increase in 23 isolates, and 16 isolates showing a zone size increase of more than 16 mm. Four isolates did not show a significant increase in the inhibition zone size as they already had a higher zone of inhibition to ceftazidime-avibactam (Tables 2, 3).

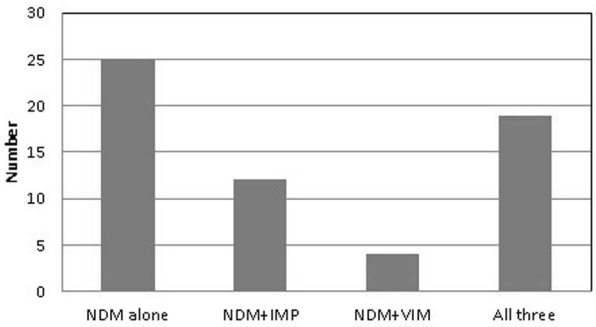


**Fig. 1** Isolates carrying MBL resistance genes

### Synergy by E-strip fixed ratio method

MIC determination by the E-test strip method for ceftazidime-avibactam and aztreonam by individually testing revealed MIC range between ≥8/4 μg $ml^{-1}$ to ≥256/4 μg $ml^{-1}$

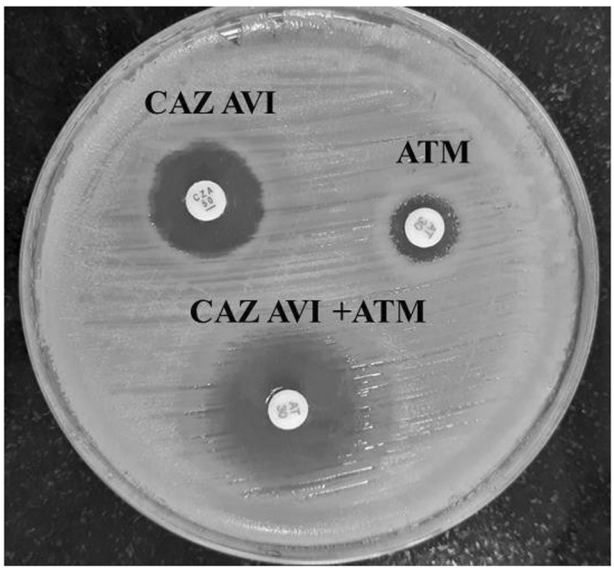


**Fig. 2** Susceptibility testing by disk diffusion showing ceftazidime-avibactam, aztreonam alone and in combination (CAZ-AVI + ATM)

**Table 2** Isolates showing an increase of inhibition zone size with a combination of drugs (Ceftazidime-avibactam + aztreonam)

| Zone inhibition size (mm) | No of isolates |
|---|---|
| <4 mm | 4 |
| 4-9 mm | 17 |
| 10-15 mm | 23 |
| >16 mm | 16 |

**Table 3** Disk diffusion Zone size for all the isolates

| Disk diffusion zone diameter | 6 mm | 7–12 mm | 13–16 mm | 17–20 mm | 21–24 mm | >24 mm |
|---|---|---|---|---|---|---|
| ALL(60) | | | | | | |
| CAZ AVI | 5 | 3 | 14 | 38 | | |
| ATM | 16 | 40 | 4 | | | |
| CAZ-AVI + ATM | 0 | 0 | 0 | 0 | 18 | 42 |
| *Klebsiella pneumoniae(38)* | | | | | | |
| CAZ AVI | 1 | 1 | 9 | 27 | | |
| ATM | 11 | 24 | 3 | | | |
| CAZ-AVI + ATM | | | | | 7 | 31 |
| *Escherichia coli(11)* | | | | | | |
| CAZ AVI | 2 | | 4 | 5 | | |
| ATM | 3 | 8 | | | | |
| CAZ-AVI + ATM | | | | | 6 | 5 |
| *Pseudomonas aeruginosa(5)* | | | | | | |
| CAZ AVI | 2 | 2 | 1 | | | |
| ATM | 1 | 3 | 1 | | | |
| CAZ-AVI + ATM | | | | | 3 | 2 |
| *Citrobacter freundii (3)* | | | | | | |
| CAZ AVI | | | 1 | 2 | | |
| ATM | 1 | 2 | | | | |
| CAZ-AVI + ATM | | | | | 1 | 2 |
| *Enterobacter cloacae(3)* | | | | | | |
| CAZ AVI | | | | 3 | | |
| ATM | | 3 | | | | |
| CAZ-AVI + ATM | | | | | 1 | 2 |

and 16 μg $ml^{-1}$ to 256 μg $ml^{-1}$ respectively. The combination of ceftazidime-avibactam and aztreonam by replacing the first strip with that of the second strip significantly reduced the MIC of both the agents ranging from 0.016/4 μg $ml^{-1}$ to 2/4 μg $ml^{-1}$ with FIC < 0.5 for all the isolates. All the 60 isolates showed synergy with FIC of <0.5 and none of the isolates had additive or antagonistic responses by this method (Fig. 3 & Table 4).

### Synergy by E- test agar synergy method

In the E-test agar synergy method, the use of aztreonam E-strips on Muller Hinton agar plate incorporated with avibactam reduced the MIC of aztreonam by a minimum of 16-fold in all the isolates, 64–256-fold in 19 isolates, 256–1000-fold decrease in 16 isolates and more than 1000-fold decrease has been noted in 18 isolates (Fig. 4).

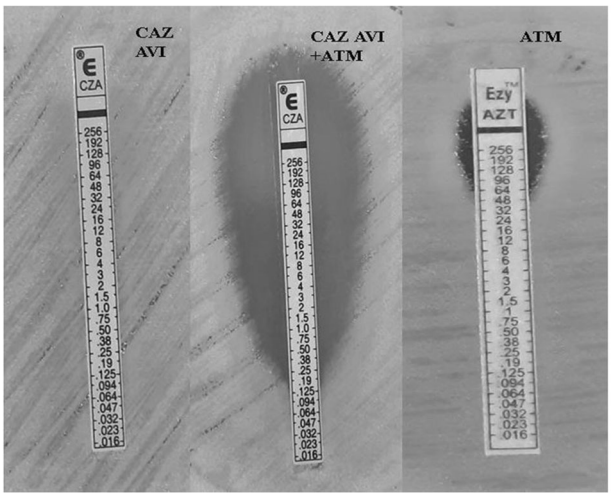


**Fig. 3** Susceptibility testing with ellipsometry showing the effect of the individual as well as a synergistic combination of CAZ-AVI and ATM

## Discussion

Multi-drug resistance in Gram-negative organisms has become a great concern in recent times [19]. In the COVID-19 pandemic, there are multiple concerns regarding the rise in anti-microbial resistance rates due to the increased use of antibiotics for empirical therapy [20, 21]. A study from China has shown that 95% of their COVID patients were started on antibiotics even though only 15% of them were actually found to have a secondary bacterial infection [22].

**Table 4** Antimicrobial susceptibility test showing MIC of ceftazidime-avibactam, aztreonam, and ceftazidime-avibactam in combination with aztreonam by the E- strip-fixed ratio method for all the tested isolates

| Isolate category | Antimicrobial agent | Number of isolates at each MIC (μg/mL) for each antimicrobial Agent[a] | | | | | | | | | | | | | MIC-50 | MIC-90 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | <0.032 | 0.064 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | >64 | | |
| ALL (60) | CAZ AVI | | | | | | | | | 3 | 4 | 1 | 2 | 50 | 256 | 256 |
| | ATM | | | | | | | | | | 7 | 9 | 12 | 32 | 256 | 256 |
| | CAZ-AVI + ATM | 5 | 2 | 18 | 15 | 7 | 6 | 7 | | | | | | | 0.19 | 1.5 |
| *Klebsiella pneumonia* (38) | CAZ AVI | | | | | | | | | 2 | 3 | 1 | | 32 | 256 | 256 |
| | ATM | | | | | | | | | | 4 | 7 | 8 | 19 | 64 | 256 |
| | CAZ-AVI + ATM | 3 | 1 | 13 | 13 | 4 | 1 | 3 | | | | | | | 0.19 | 0.38 |
| *Escherichia coli* (11) | CAZ AVI | | | | | | | | | 1 | | | | 10 | 256 | 256 |
| | ATM | | | | | | | | | | 2 | 1 | 3 | 5 | 64 | 256 |
| | CAZ-AVI + ATM | 2 | | 2 | | 2 | 3 | 2 | | | | | | | 0.5 | 1.5 |
| *Pseudomonas aeruginosa* (5) | CAZ AVI | | | | | | | | | | 1 | | | 4 | | |
| | ATM | | | | | | | | | | | 1 | | 4 | | |
| | CAZ-AVI + ATM | | 1 | 1 | | | 2 | 1 | | | | | | | | |
| *Citrobacter freundii* (3) | CAZ AVI | | | | | | | | | | | | 2 | 1 | | |
| | ATM | | | | | | | | | | 1 | | | 2 | | |
| | CAZ-AVI + ATM | | 1 | 2 | | | | | | | | | | | | |
| *Enterobacter cloacae* (3) | CAZ AVI | | | | | | | | | | | | | 3 | | |
| | ATM | | | | | | | | | | | | 1 | 2 | | |
| | CAZ-AVI + ATM | | | | 1 | 1 | | 1 | | | | | | | | |

[a]MIC-50 and MIC-90 were not calculated in isolates <10 in number

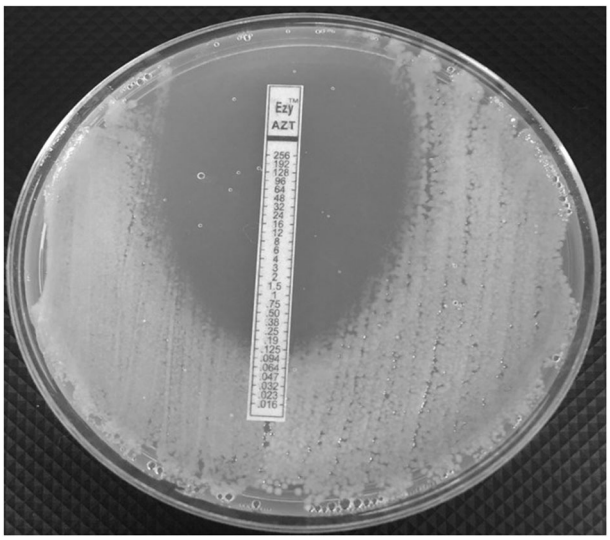


**Fig. 4** Synergy testing showing MIC determination of aztreonam E-strip in Muller Hinton agar incorporated with avibactam at a fixed concentration of 4 μg $l^{-1}$

Hence the proper choice of antibiotic becomes pivotal in the management of these multi-drug resistant organisms.

Metallo-beta-lactamases (MBLs) are the predominant carbapenemases in South East Asia, particularly in India, whereas serine carbapenemases dominate in other parts of the world [8, 23]. The newly developed fifth-generation cephalosporin cefiderocol has better in vitro activity against MBLs and preliminary clinical trials also showed promising results, but further clinical studies are needed for their routine clinical use [13, 24, 25]. Monobactams are the only drugs that show good activity against MBL and so a combination of ceftazidime-avibactam along with aztreonam can be used to overcome their activity. Even though other new beta-lactamases like relebactam or vaborbactam can be used in combination with aztreonam, they have been found to have a lesser activity against class A serine beta-lactamases which makes the avibactam-aztreonam combination a promising drug for the treatment of MBL producing organisms [26, 27].

An initial study of the same combination was done by Marshall et al. in 2017 where they had shown 21 MBL positive isolates to be individually resistant to CAZ-AVI but with the addition of ATM, 17 out of 21 isolates became susceptible to the disk diffusion method, and all the 21 isolates displayed a reduction in agar dilution MICs with this drug combination [28]. In addition to this, they also studied a thigh infection model in neutropenic mice and found almost a 4-$\log_{10}$-CFU reduction at 24 h with this drug combination. In another study by Karlowsky et al. 267 MBL positive *Enterobacterales* from 40 different countries were collected and ATM-AVI combination at a MIC of ≤8 μg $ml^{-1}$ was inhibitory to 99.9% of isolates showed no regional differences [29]. Many case reports have been

published showing the successful outcome of this combination of drugs in multi-drug resistant organisms. One such case report is by Davido et al. where *Klebsiella pneumoniae* isolate was found to be producing $bla_{OXA48}$ and $bla_{NDM-1}$ carbapenemases and another patient grew $bla_{NDM-1}$-producing, AmpC-hyper producing strain of *Pseudomonas aeruginosa*, susceptible only to amikacin and colistin by routine testing. Both were treated with a combination of CAZ-AVI/ATM for a period of ten days and were found to be free of infection even though the first patient died of heart attack [30]. Another case report that showed a better clinical outcome of CAZ-AVI with ATM was in two organ transplant patients who presented with septic shock with NDM-1-producing *K. pneumonia* [31].

In the current study, all the 60 isolates were subjected to molecular methods and phenotypic tests for the detection of metallo-beta-lactamases. The presence of at least one MBL was reported in 25 isolates and more than one MBL was detected in a total of 35 isolates. Initially, these isolates were resistant to ceftazidime-avibactam and aztreonam when they were tested alone by both the disk diffusion method and the E-test strip method. However, in combination testing, all the isolates were found to be susceptible to all the three different methods described in our study. Regarding in-vitro susceptibility testing of this combination in the literature, only a few studies have been reported. Zou et al. compared the in-vitro susceptibility of 120 carbapenem-resistant *Enterobacterales* from three different hospitals in South West China and found 96.7% of isolates that were initially resistant to aztreonam alone showed a 128-fold (range: ≤0.125–4 mg $l^{-1}$) reduction in MIC after the addition of avibactam [32]. Wenzler et al. have studied synergy testing of different drug combinations (CAZ + ATM, CAZ + CAZ-AVI, CAZ-AVI + ATM) against 7 different isolates containing serine and metallo-beta-lactamases by two different methods i.e., E-test MIC: MIC ratio method and agar-E-test method. CAZ-AVI with ATM was found to be the best combination in that study with the demonstration of synergy in five of seven tested isolates with a minimum of 128-fold reduction in MIC. In the agar-E test method also, synergy was demonstrated in 86% of their isolates proving that the agar-E test method could demonstrate greater numbers for the most synergistic combination, causing a reduction of at least 17,000-fold in MIC value (from > 256 mg $l^{-1}$ to <0.016 mg $l^{-1}$) [18]. Zhang et al. from China tested 161 MBL positive *Enterobacterales* for in-vitro susceptibility testing of AVI and ATM combination and found that 96.9% of isolates were inhibited with that combination. Their study also found that patients with pre-existing lung disease were associated with the worst disease outcome [33]. Even though these studies have used broth microdilution and E-strips for MIC determination until now, there is no accepted or gold standard method for in-vitro susceptibility testing of this drug combination. Recently, a study has been published where the authors have used broth microdilution panels for aztreonam –avibactam susceptibility testing and found that in all the isolates, this drug combination has reduced the MIC of aztreonam by ≥4-fold for 85% (51/60) of the tested isolates [34]. In our study, 18 isolates have shown a 1000-fold decrease in MIC value and 36 isolates have shown a decrease in MIC by 64–1000-fold with the remaining 6 isolates showing less than 64-fold reduction in MIC by the E-test agar synergy method. By the E-strip fixed ratio method, all the 60 isolates have shown FIC < 0.5 showing synergy.

Most of these studies have evaluated only a few samples by only one method and none of the studies have described disk diffusion as a part of their in vitro susceptibility testing. This is the first study to be reported in the literature that describes the in-vitro antimicrobial susceptibility testing of ceftazidime-avibactam in combination with aztreonam by disk diffusion method in comparison with E- test agar synergy and E-test fixed ratio method. All the three methods have shown 100% susceptibility to the drug combination and they also had a 100% concordance with each other in their susceptibility testing. Hence, the disk diffusion method can be readily used as an alternative method for susceptibility testing of ceftazidime-avibactam and aztreonam combination effectively in a resource-limited setting.

Apart from this in-vitro susceptibility testing and clinical studies, many clinical trials assessing the pharmacokinetic properties of this combination also showed promising results explaining the safety and pharmacodynamics of this drug combination [35, 36]. Even though CAZ-AVI with ATM has shown better activity against MBL-producing organisms, some studies have reported the resistance of these isolates to this drug combination. This resistance is because of PBP3 protein showing four amino-acid insertions, being either YRIN or YRIK or production of CMY-42, which ultimately reduces the susceptibility to the drug combination causing a significant rise in their MIC [37, 38]. However, in clinical isolates, this type of resistance is still rarely detected and the current study showed 100% sensitivity in MBL positive isolates making it a useful antibiotic combination. Further studies with a substantial number of isolates are needed to validate disk diffusion as a method for testing the synergy combination of ceftazidime-avibactam and aztreonam.

## Limitations of the study

One main drawback of the current study was the limited number of isolates i.e., only 60 samples were used for testing by all the three methods and all the samples were

collected from patients attending a single tertiary care centre. Therefore, the results of this study cannot be generalized for all the organisms and for all hospital settings as the rate of carbapenem resistance and types of beta-lactamases vary in different geographical areas. Another limitation is 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 other factors like pharmacodynamics and host immune response also play a role in the clinical outcome of the patient. The current study also did not compare with the standard method such as the checkerboard method.

## Compliance with ethical standards

**Conflict of interest** The authors declare no competing interests.

**Ethical clearance** The study has been approved by the Institution Ethics Committee, PGIMER, Chandigarh vide no INT/IEC/2021/SPL-863, Dated – 25/05/2021.

**Patient's consent** This study has been conducted from patient's samples which are collected for routine diagnostic purposes. No separate sample was collected for the study purpose. Hence patient consent was not taken for conducting this study.

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## References


1. Santajit S, Indrawattana N, Mechanisms of antimicrobial resistance in ESKAPE Pathogens. *Biomed Res Int*. 2016;2016. https://doi.org/10.1155/2016/2475067.
2. Taggar G, Attiq Rheman M, Boerlin P, Diarra MS. Molecular epidemiology of carbapenemases in enterobacteriales from humans, animals, food, and the environment. Antibiotics. 2020;9:1–22. https://doi.org/10.3390/antibiotics9100693.
3. Kaye KS, Pogue JM, Tran TB, Nation RL, Li J. Agents of last resort: polymyxin resistance. Infect Dis Clin North Am. 2016;30:391–414. https://doi.org/10.1016/j.idc.2016.02.005.
4. Pormohammad A, Mehdinejadiani K, Gholizadeh P, Nasiri MJ, Mohtavinejad N, Dadashi M, et al. Global prevalence of colistin resistance in clinical isolates of Acinetobacter baumannii: A systematic review and meta-analysis. Micro Pathog. 2020;139:103887. https://doi.org/10.1016/j.micpath.2019.103887.
5. Prim N, Turbau M, Rivera A, Rodríguez-Navarro J, Coll P, Mirelis B. Prevalence of colistin resistance in clinical isolates of Enterobacteriaceae: A four-year cross-sectional study. J Infect. 2017;75:493–8. https://doi.org/10.1016/j.jinf.2017.09.008.
6. Petrosillo N, Taglietti F, Granata G. Treatment options for colistin resistant klebsiella pneumoniae: present and future. J Clin Med. 2019;8:934. https://doi.org/10.3390/jcm8070934.
7. Zhang X, Li F, Cui S, Mao L, Li X, Awan F, et al. Prevalence and distribution characteristics of blaKPC-2 and blaNDM-1 genes in Klebsiella pneumoniae. Infect Drug Resist. 2020;13:2901–10. https://doi.org/10.2147/IDR.S253631.
8. Kazi M, Drego L, Nikam C, Ajbani K, Soman R, Shetty A, et al. Molecular characterization of carbapenem-resistant Enterobacteriaceae at a tertiary care laboratory in Mumbai. Eur J Clin Microbiol Infect Dis. 2015;34:467–72. https://doi.org/10.1007/s10096-014-2249-x.
9. Deepjyoti P, Atul G, and Amitabha B. Microbial drug resistance. 2017. 815-21. https://doi.org/10.1089/mdr.2016.0124.
10. Fomda BA, Khan A, Zahoor D. Danish NDM-1 (New Delhi metallo beta lactamase-1) producing Gram-negative bacilli. Indian J Med Res. 2014;140:672–8.
11. Queenan AM, Bush K. Carbapenemases: The versatile β-lactamases. Clin Microbiol Rev. 2007;20:440–58. https://doi.org/10.1128/CMR.00001-07.
12. Evans BA and Amyes SGB, "OXA β-lactamases", *Clin Microbiol Rev*., 27, 2, 241–263, 2014. https://doi.org/10.1128/CMR.00117-13.
13. Tan X, Kim HS, Baugh K, Huang Y, Kadiyala N, Wences M, et al. Therapeutic options for metallo-β-lactamase-producing enterobacterales. *Infect Drug Resist*. 2021;14:125-42. https://doi.org/10.2147/IDR.S246174.
14. CLSI, *CLSI M100-ED29: 2019 Performance Standards for Antimicrobial Susceptibility Testing, 29th Edition*. 2019.
15. Pankey GA, Ashcraft DS. In vitro synergy of ciprofloxacin and gatifloxacin against ciprofloxacin-resistant Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2005;49:2959–64. https://doi.org/10.1128/AAC.49.7.2959-2964.2005.
16. Laishram S, Pragasam AK, Bakthavatchalam YD, Veeraraghavan B. An update on the technical, interpretative, and clinical relevance of antimicrobial synergy testing methodologies. Indian J Med Microbiol. 2017;35:445–68. https://doi.org/10.4103/ijmm.IJMM_17_189.
17. Sopirala MM, Mangino JE, Gebreyes WA, Biller B, Bannerman T, Balada-Llasat JM, et al. Synergy testing by etest, microdilution checkerboard, and time-kill methods for pan-drug-resistant Acinetobacter baumannii. Antimicrob Agents Chemother. 2010;54:4678–83. https://doi.org/10.1128/AAC.00497-10.
18. Wenzler E, Deraedt MF, Harrington AT, Danizger LH. Synergistic activity of ceftazidime-avibactam and aztreonam against serine and metallo-β-lactamase-producing gram-negative pathogens. Diagn Microbiol Infect Dis [Internet]. 2017;88:352–4. https://doi.org/10.1016/j.diagmicrobio.2017.05.009.
19. McLaughlin M, Advincula MR, Malczynski M, Qi C, Bolon M, Scheetz MH. Correlations of antibiotic use and carbapenem resistance in enterobacteriaceae. Antimicrob Agents Chemother. 2013;57:5131–3. https://doi.org/10.1128/AAC.00607-13.
20. Afshinnekoo E, Bhattacharya C, Burguete-García A, Castro-Nallar E, Deng Y, Desnues C, et al. COVID-19 drug practices risk antimicrobial resistance evolution. Lancet Microbe. 2021;2:e135–e136. https://doi.org/10.1016/S2666-5247(21)00039-2.
21. Lucien MAB, Canarie MF, Kilgore PE, Jean-Denis G, Fénélon N, Pierre M, et al. Antibiotics and antimicrobial resistance in the COVID-19 era: Perspective from resource-limited settings. Int J Infect Dis. 2021;104:250–4. https://doi.org/10.1016/j.ijid.2020.12.087.
22. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet 2020;395:1054–62. https://doi.org/10.1016/S0140-6736(20)30566-3.
23. van Duin D, Doi Y. The global epidemiology of carbapenemase-producing Enterobacteriaceae. Virulence 2017;8:460–9. https://doi.org/10.1080/21505594.2016.1222343.
24. Bassetti M, Echols R, Matsunaga Y, Ariyasu M, Doi Y, Ferrer R, et al. Efficacy and safety of cefiderocol or best available therapy for the treatment of serious infections caused by carbapenem-resistant Gram-negative bacteria (CREDIBLE-CR): a randomised, open-label, multicentre, pathogen-focused, descriptive, phase 3 trial. Lancet Infect Dis. 2021;21:226–40. https://doi.org/10.1016/S1473-3099(20)30796-920.

25. Wunderink RG, Matsunaga Y, Ariyasu M, Clevenbergh P, Echols R, Kaye KS, et al. Cefiderocol versus high-dose, extended-infusion meropenem for the treatment of Gram-negative nosocomial pneumonia (APEKS-NP): a randomised, double-blind, phase 3, non-inferiority trial. Lancet Infect Dis. 2021;21:213–25. https://doi.org/10.1016/S1473-3099(20)30731-3.
26. Haidar G, Clancy CJ, Chen L, Samanta P, Shields RK, Kreiswirth BN, et al. Identifying Spectra of Activity and Therapeutic Niches for Ceftazidime-Avibactam and Imipenem-Relebactam against Carbapenem-Resistant Enterobacteriaceae. *Antimicrob Agents Chemother*. 2017;61. https://doi.org/10.1128/aac.00642-17.
27. Lomovskaya O, "Vaborbactam: Spectrum of beta-lactamase inhibition and impact of resistance mechanisms on activity in enterobacteriaceae," *Antimicrob. Agents Chemother*., 61, 2017. https://doi.org/10.1128/AAC.01443-17.
28. Marshall S, Hujer AM, Rojas LJ, Papp-Wallace KM, Humphries RM, Spellberg B, et al. Can ceftazidime-avibactamand aztreonam overcome β-lactam resistance conferred by metallo-β-lactamases in *Enterobacteriaceae*? Antimicrob Agents Chemother. 2017;61:e02243–16. https://doi.org/10.1128/AAC.02243-16.
29. Karlowsky JA, Kazmierczak KM, De Jonge BLM, Hackel MA, Sahm DF, Bradford PA. In vitro activity of aztreonam-avibactam against enterobacteriaceae and pseudomonas aeruginosa isolated by clinical laboratories in 40 countries from 2012 to 2015. *Antimicrob Agents Chemother*., 2017;61:e00472–17. https://doi.org/10.1128/AAC.00472-17.
30. Davido B, Fellous L, Lawrence C, Maxime V, Rottman M, Dinh A. Ceftazidime-avibactam and aztreonam, an interesting strategy to overcome β-lactam resistance conferred by metallo-β-lactamases in *Enterobacteriaceae* and *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 2017;61:e01008–17. https://doi.org/10.1128/AAC.01008-17.
31. Benchetrit L, Mathy V, Armand-Lefevre L, Bouadma L, Timsit J-F. Successful treatment of septic shock due to NDM-1-producing Klebsiella pneumoniae using ceftazidime/avibactam combined with aztreonam in solid organ transplant recipients: report of two cases. *Int J Antimicrob Agents*. 2020;55. https://doi.org/10.1016/j.ijantimicag.2019.10.023.
32. Zou C, Wei J, Shan B, Chen X, Wang D, Niu S. In vitro activity of ceftazidime-avibactam and aztreonam-avibactam against carbapenem-resistant enterobacteriaceae isolates collected from three secondary hospitals in southwest China between 2018 and 2019. Infect Drug Resist. 2020;13:3563–8. https://doi.org/10.2147/IDR.S273989.
33. Zhang B, Zhu Z, Jia W, Qu F, Huang B, Shan B, et al. In vitro activity of aztreonam–avibactam against metallo-β-lactamase-producing Enterobacteriaceae—A multicenter study in China. Int J Infect Dis. 2020;97:11–18. https://doi.org/10.1016/j.ijid.2020.05.075.
34. Ransom E, Bhatnagar A, Patel JB, Machado MJ, Boyd S, Reese N et al. Validation of Aztreonam-Avibactam susceptibility testing using digitally dispensed custom panels. J Clin Microbiol. 2020;58:e01944–19. https://doi.org/10.1128/JCM.01944-19.
35. Chauzy A, Buyck J, de Jonge BLM, Marchand S, Grégoire N, Couet W. Pharmacodynamic modeling of β-lactam/β-lactamase inhibitor checkerboard data: illustration with aztreonam–avibactam. Clin Microbiol Infect. 2019;25:515.e1–515.e4. https://doi.org/10.1016/j.cmi.2018.11.025.
36. Cornely OA, Cisneros JM, Torre-Cisneros J, Rodríguez-Hernández MJ, Tallón-Aguilar L, Calbo E. COMBACTE-CARE consortium/REJUVENATE Study G.Pharmacokinetics and safety of aztreonam/avibactam for the treatment of complicated intra-abdominal infections in hospitalized adults: results from the REJUVENATE study. J Antimicrob Chemother. 2020;75:618–27. https://doi.org/10.1093/jac/dkz497.
37. Sadek M, Juhas M, Poirel L, Nordmann P, Genetic Features leading to reduced susceptibility to Aztreonam-Avibactam among Metallo-β-Lactamase-Producing Escherichia coli Isolates. *Antimicrob Agents Chemother*. 2020;64. https://doi.org/10.1128/AAC.01659-20.
38. Niu S, Wei J, Zou C, Chavda KD, Lv J, Zhang H, et al. In vitro selection of aztreonam/avibactam resistance in dual-carbapenemase-producing Klebsiella pneumoniae. J Antimicrob Chemother. 2020;75:559–65. https://doi.org/10.1093/jac/dkz468.