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In-vitro Evaluation of Synergy Testing of Ceftazidime-Avibactam-Aztreonam among Carbapenem-Resistant Gram-Negative Organisms

Sridevi Dinakaran, Sheela Devi Chandrakesan and K. Sandhya Bhat*

Department of Microbiology, Pondicherry Institute of Medical Sciences, India

**Corresponding author*

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A B S T R A C T

The prevalence of severe infections due to Carbapenemase –resistant Enterobacterales (CRE) and *Pseudomonas aeruginosa* (CR-PA) has increased worldwide. The treatment has become challenging and limited among the metallo-β-lactamases (MBLs) producing organisms due to the rise in polymyxin resistance. Due to the paucity of novel drugs, the ceftazidime-avibactam-aztreonam serves as a wonder drug combination for these infections. In 2024, Clinical and Laboratory Standards Institute (CLSI) approved broth disk elution method for synergy testing. This study was performed to evaluate the ceftazidime-avibactam-aztreonam synergy testing against the MBL-producing clinical isolates. We used a total of 34 archived multidrug-resistant organisms (MDROs) from the Microbiology department over a six-month period, from July to December 2023. All these isolates were subjected to modified carbapenem inactivation method (m CIM) and EDTA carbapenem inactivation method (e CIM) for phenotypic detection of carbapenemases. The ceftazidime-avibactam-aztreonam synergy testing was performed by two methods: Disk diffusion method and disk elution method among MBL-producing isolates. The study revealed that among the 34 MDR clinical isolates, 21 (62%) were MBL producers, followed by 5 (15%) Serine beta lactamase producers, 3 (9%) Indeterminate and 5(15%) were negative. MBL producers were subjected to two methods of synergy testing. Among 21 MBL-producing isolates, synergy was observed in 13 (38.2%) and no synergy was seen in eight (24%) isolates by both methods. There were no discrepant results. Detection of synergism for ceftazidime-avibactam-aztreonam combination can be determined by the disk diffusion method in a low-resource setting and thereby aid in clinical decision-making.

Introduction

Antibiotic resistance poses the greatest threat to human health. Globally, the World Health Organization (WHO) developed a priority list for the research and development of newer antibiotics. Carbapenem-resistant Enterobacterales (CRE) and carbapenem-resistant *Pseudomonas aeruginosa* (CR-PA) are deemed in the critical category of the priority list due to their rapid global spread and association with severe, hard-to-treat

infections (Tacconelli *et al.,* 2018). The treatment has become challenging and limited, especially among the metallo-β-lactamases (MBLs) producing organisms. Due to the paucity of novel drugs, different combinations were tried to address this problem. Ceftazidime-avibactam-aztreonam (CAZ-AVI -ATM) serves as a wonder drug combination for these infections.

Ceftazidime-avibactam (CAZ-AVI) effectively combats ESBLs (Extended-spectrum β-lactamases), KPCs (*Klebsiella pneumoniae* carbapenemases), OXA-48 (oxacillinase-48), and class C cephalosporinases but is ineffective against MBLs like NDM (New Delhi metallo-beta-lactamase), VIM (Verona integron-encoded metallo-beta-lactamase), and IMP (imipenemase).

On the other hand, aztreonam (ATM) is often susceptible to ESBLs, KPCs, and other enzymes, but it resists MBLs (Marshall *et al.,* 2017). Various techniques, including strip stack, strip cross, disk stacking, and broth disk elution, were employed to assess the synergistic effects of combining CAZ-AVI-ATM (Khan *et al.,* 2021).

As of 2024, the Clinical and Laboratory Standards Institute (CLSI) has endorsed the broth disk elution method (BDE) as the standard approach for evaluating the synergistic effects of this combination (CLSI, 2024). Due to logistical constraints, the disk elution method may be challenging in resource-limited settings. Therefore, we evaluated the suitability of the disk diffusion method, a readily available laboratory technique, as an alternative for synergy testing, comparing its performance to the reference method (Sreenivasan *et al.,* 2022).

Materials and Methods

This retrospective study analyzed 34 multidrug-resistant (MDR) Gram-negative bacterial isolates, randomly selected from clinical samples (blood, aspirated fluid, ear swab, tissue and wound swab) collected at Pondicherry Institute of Medical Sciences Department of Microbiology between July and December 2023.

Modified carbapenem inactivation method (m CIM) and EDTA-carbapenem inactivation method (e CIM)

This method employed meropenem (30µg) disks (HiMedia Laboratories Private Limited, Mumbai, Maharashtra, India) to differentiate serine beta-lactamases (SBLs) and MBLs. For each isolate to be tested, 1µL loopful of bacteria for Enterobacterales isolate or 10µL of bacteria for *P. aeruginosa* from an overnight blood agar plate was resuspended in two tubes containing 2 mL of TSB. One tube was devoid of EDTA (mCIM), while the other was supplemented with 0.5M EDTA (eCIM). A meropenem (MEM) disk was submerged in each tube, and the tubes were incubated at 35 ±2°C for 4 h ± 15 min. The disks were then removed from the tubes and placed on Mueller-Hinton agar (MHA) plates, upon which a carbapenem-susceptible *Escherichia coli* (*E. coli*) ATCC 25922 had been freshly applied. The plates were incubated at 35 ±2°C for 16 to 20 h before the zone sizes were recorded. The results for mCIM and eCIM were interpreted based on Clinical and Laboratory Standards Institute (CLSI) 2024 guidelines (Tables 1and 2).

eCIM was interpreted when mCIM was positive.

An increase of ≥5mm in the zone diameter compared to the zone diameter of the mCIM was considered indicative of metallo-β-Lactamase (MBL) production.

An increase in zone diameter compared to the zone diameter of the mCIM but ≤4mm was considered indicative of no metallo-β-lactamase (MBL) production, suggesting the presence of other β-lactamases, such as serine β-lactamase (SBL).

The accuracy of this test was confirmed using *Klebsiella pneumoniae* (*K. pneumoniae*) ATCC BAA-1705 and an in-house NDM strain as reference controls.

Rapid detection of Metallo-β-lactamases

To identify MBL-producing strains, a rapid diagnostic kit TRURAPID O.K.N.V.I RESIST 5 test (3B BlackBio Dx limited, India), based on membrane technology and colloidal gold nanoparticles was used. This kit detects KPC, NDM, OXA-48-like, VIM, and IMP genes. The test was performed according to the manufacturer's instructions, with *E. coli* ATCC 25922 serving as a negative control and in-house KPC and NDM strains as positive controls for validation.

Disk diffusion method (Sreenivasan *et al.,* 2022)

The MBL-producing strains underwent synergy testing with the CAZ-AVI-ATM combination using the disk diffusion method. Ceftazidime-avibactam (30/20 µg) and aztreonam (30 µg) disks (HiMedia Laboratories Private

Limited, Mumbai, Maharashtra, India) were employed. The test involved placing the ceftazidime-avibactam disks into inoculated Mueller-Hinton agar plates, incubating them for 1 hour, and then replacing them with aztreonam disks. After overnight incubation, the zones of inhibition were measured. Isolates with inhibition zones ≥21 mm zone size were considered susceptible, aligning with CLSI guidelines for ceftazidime-avibactam (Khan *et al.,* 2021).

Broth disk elution (BDE) method

The BDE method employed ceftazidime-avibactam (30/20 µg) and aztreonam (30 µg) disks (HiMedia Laboratories Private Limited, Mumbai, Maharashtra, India) to evaluate synergy. The process involved adding 5 ml of cation adjusted Mueller Hinton Broth (CAMHB) to four sterile tubes, followed by the addition of ATM in one tube, CAZ-AVI in one tube, both disks CAZ-AVI-ATM in one tube and no disks in one tube, which acted as growth control. The tube was incubated at room temperature for 30 min to allow the antimicrobials to elute from the disk. A 0.5 McFarland standard inoculum was prepared by suspending fresh colonies from an overnight sheep's blood agar plate in normal saline. Then, 25 µl of this suspension was added to the tube with the eluted disks and vortexed to reach a final inoculum of around 7.5×10^5 CFU/ml and incubated at 35±2°C. After 16 to 20 hours of incubation, the results were visually assessed in comparison to the growth control tube. A strain was considered synergy-positive if it showed susceptibility to the CAZ-AVI-ATM combination and showed no growth in the tube. This test was validated by *E. coli* ATCC 25922 and *K. pneumoniae* ATCC BAA-1705 as per 2024 CLSI guidelines.

Results and Discussion

Amongst the thirty-four MDR isolates, the majority were from wound swabs (18, 53%), followed by tissue (7, 20%), Aspirated fluid (2, 6%), blood (6, 18%), and ear swabs (1, 3%).

The majority of MDR isolates used for testing were *K. pneumoniae* (18, 53%), followed by *E. coli* (9, 26%), *Klebsiella spp.* (3, 9%), *Pseudomonas aeruginosa* (2, 6%), *Providencia rettgeri* (1, 3%) and *Enterobacter spp* (1, 3%).

All 34 MDR isolates were subjected to the modified carbapenem inactivation method (m CIM) & EDTA-carbapenem inactivation method (e CIM) for detection of carbapenemase. It revealed the majority of the isolates were MBL-producing strains (21, 61%), followed by SBLs (5,14%), negative (5, 14%), and indeterminate (3,9%) for carbapenemase production (Fig. 1)

The 21 MBL-producing strains were further confirmed using a rapid test kit, which detected the presence of MBL genes. The results showed that the majority of strains (14, 67%) carried the NDM gene, while (7,33%) co-produced both OXA-48 and NDM genes. Notably, none of the isolates tested positive for KPC, IMP, or VIM genes (Fig.2).

The 21 MBLs were employed in the disk diffusion method for testing the synergy of the CAZ-AVI-ATM combination. Most isolates (13, 62%) exhibited a positive synergy effect, whereas 8, 38% did not show any synergy (Fig. 3).

The 21 MBL-producing isolates were retested for synergy using the BDE method, and the results were consistent with the initial findings (Fig. 4).

The majority of isolates (28,82%) in this study were obtained from exudate samples, which is consistent with the findings of Kalaivani *et al.,* (2024) who also reported a high proportion of exudate samples 933,41%). Likewise, the majority of isolates in this study were *K. pneumoniae* (18,53%) and *Escherichia coli* (9,26%), which is similar to the findings of Kalaivani *et al.,* (2024) who reported a predominance of *K. pneumoniae* (67,85%) and a smaller proportion of *Escherichia coli* (7,7%) (Kalaivani *et al.,* 2024).

The present study identified carbapenemase-positive isolates in 76% of cases, with 15% producing SBLs and 61% producing MBLs. Conversely, Deepashree *et al.,* reported a detection rate of 54% for SBLs and 45.5% for MBLs in blood culture isolates (Rajshekar *et al.,* 2024).

In the current study, synergism is seen in thirteen MBL-producing isolates by the disk diffusion method this is similar to the study conducted by Sreenivasan *et al.,* (2022) which reported synergism in sixty MBL producing isolates and proved this method can be incorporated in routine testing (Sreenivasan *et al.,* 2022). CLSI recognized BDE also yielded similar results in our study. Harish *et al.,* (2023) observed that synergy testing using the BDE method is more precise and reliable (Harris *et al.,* 2023).

Table.1 mCIM interpretation

mCIM Zone diameter	Interpretation
6-15mm or pinpoint colonies within 16-18mm	mCIM positive
≥19mm clear zone	mCIM negative
16-18mm zone or zone of ≥19mm pinpoint colonies within the zone	Inconclusive

Table.2 mCIM and eCIM combination test interpretation

mCIM result	eCIM result	Interpretation
Negative	Do not interpret	Carbapenemase not detected
Positive	Negative	Serine β-Lactamase detected
Positive	Positive	Metallo-β-Lactamase detected
Inconclusive	Do not interpret	Testing inconclusive for the presence of carbapenemase

Table.3 Broth disk elution method results and interpretation

CAZ-AVI	ATM	Synergy	Possible mechanism
Susceptible	Susceptible	+/-	No Carbapenemase production
Susceptible	Resistant	+/-	Hyper ESBL/Amp C producer
Resistant	Susceptible	+	MBL producer
Resistant	Resistant	+	MBL, ESBL/Amp C producer

Figure.1 Modified carbapenem inactivation method (m CIM) and EDTA-carbapenem inactivation method (e CIM); A) m CIM positive and e CIM positive, B) m CIM positive and e CIM negative.

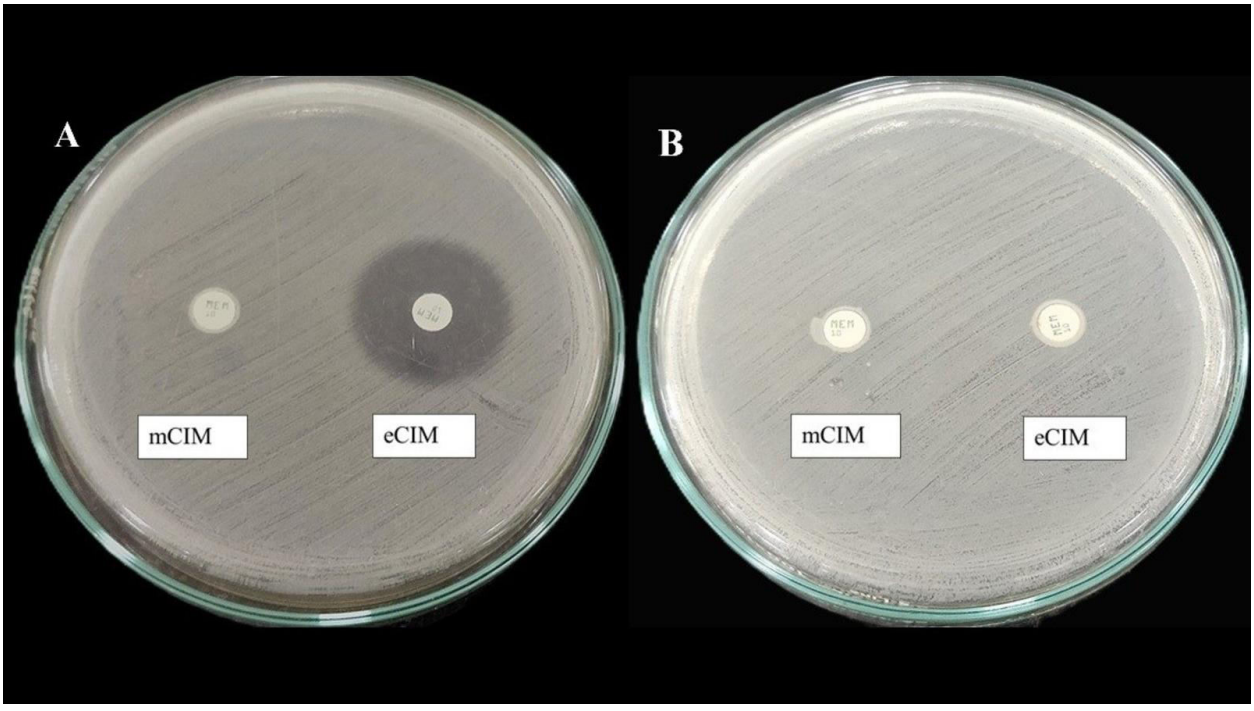

Figure.2 TRURAPID O.K.N.V.I RESIST 5 rapid test kit for detection of *OXA-48 like*, *KPC*, *NDM*, *VIM*, *IMP* carbapenemases; A) *OXA-48 +NDM* positive, B) *NDM* positive

Figure.3 Synergy testing demonstrated by Disk diffusion method; A)Synergy positive, B) Synergy negative.

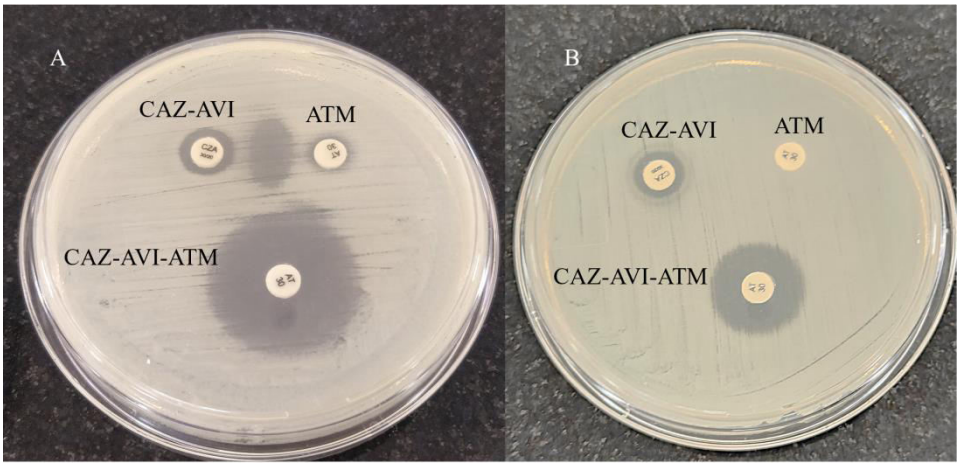

Figure.4 Synergy testing demonstrated by broth disk elution method; Left side showing synergy positive, right side showing synergy negative.

Based on the findings, ceftazidime-avibactam-aztreonam combination will be a promising treatment option for infections caused by metallo-beta-lactamase (MBL) producing Enterobacterales.

Concerning the co-production of *NDM* and *OXA-48,* which was about 33% in the present study, five *K. pneumoniae* isolates and two *Escherichia coli*, this is similar to the study by Filgona *et al.,* (2018) which showed the co-production of *NDM* and *OXA-48* is seen in 17 isolates (Filgona *et al.,* 2018).

NDM is a carbapenemase that confers resistance to a wide range of beta-lactam antibiotics, including carbapenems, OXA-48 on the other hand, is a carbapenem-hydrolyzing class D beta-lactamase that also contributes to carbapenem resistance. The combination of these two genes in *K. pneumoniae* has a synergistic effect, rendering the bacteria even more resistant and limiting the treatment options.

Interestingly, there are similar study that resonates with our study's findings in failure to detect the presence of *IMP*, *VIM*, and *KPC* genes in clinical isolates (Aqel *et al.,* 2017). The production of IMPs and VIMs has mostly been described in *Pseudomonas aeruginosa,* Mahesh *et al.,* (2017) reported a significant presence of these carbapenemase genes, with 42% of isolates harbouring *VIM* genes and 14% carrying *IMP* genes in *Pseudomonas aeruginosa* (Mahesh Reddy *et al.,* 2017).

Although aztreonam shows promise against metallo-beta-lactamase (MBL)-producing Enterobacterales, its effectiveness is limited by the presence of serine beta-lactamases (SBLs), such as AmpC and extended-spectrum beta-lactamases (ESBLs), which can inactivate aztreonam. Fortunately, the study found that these isolates were susceptible to the combination of ceftazidime-avibactam-aztreonam (CAZ-AVI-ATM).

This combination therapy may offer a potential treatment option for infections caused by these multidrug-resistant *K. pneumoniae* isolates (Benchetrit *et al.,* 2020). The rise of highly resistant bacteria highlights the urgent need for enhanced surveillance, strengthened infection prevention measures, and innovative solutions, including novel antibiotics and therapies, to effectively counter the growing threat of antibiotic resistance.

The study revealed that the disk elution and disk diffusion methods produced similar results notably, the disk diffusion method stands out as a cost-effective, widely applicable technique and is suitable for laboratories with limited access to advanced diagnostic tools.

On the other hand, RT-PCR (Real-time polymerase chain reaction) and conventional PCR are reliable diagnostic methods; however, due to their significant drawbacks, such as high operational costs and difficulties in standardization, they are not suitable for widespread use in resource-limited settings.

To overcome these limitations, the study recommends utilizing rapid diagnostic tests for identifying MBL genes, which can provide timely results at a reasonable cost.

The study's drawback is its small sample size and the failure to compare the results of the rapid card test with the reference method, RT-PCR. Further studies with a more extensive and diverse sample population are needed to understand the generalizability and reliability of the findings.

Author Contributions

Dr. Sridevi Dinakaran: Conceived the original idea, Conducted research work, analysed the data, and wrote the manuscript.; Dr. Sheela Devi Chandrakesan: Designed the model and the computational framework and critically reviewed the manuscript.; Dr Sandhya Bhat K: Conceived the original idea, supervised the conduct of research, analysed the data and critically reviewed the manuscript

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Declaration

Conflict of Interest Statement: Nil

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Statement of Image Integrity and Standards: NA

References

Aqel, A.A., Giakkoupi, P., Alzoubi, H., Masalha, I., Ellington, M.J. and Vatopoulos, A., 2017. Detection of OXA-48-like and NDM carbapenemases producing *Klebsiella pneumoniae* in Jordan: A pilot study. *Journal of Infection and Public Health*, *10*(2), pp.150-155. https://doi.org/10.1016/j.jiph.2016.02.002

Benchetrit, L., Mathy, V., Armand-Lefevre, L., Bouadma, L. and Timsit, J.F., 2020. 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. *International Journal of Antimicrobial Agents*, *55*(1), p.105842.https://doi.org/10.1016/j.ijantimicag.2019.10.023

CLSI. Performance Standards for Antimicrobial Susceptibility Testing. 34th ed. CLSI supplement M100. Clinical and Laboratory Standards Institute; 2024.

Deepashree Rajshekar, Sujatha S R , Krishna Karthik M V S, Sreejith Raveendran. 2024 Evaluation of phenotypic carbapenem inactivation methods among carbapenem resistant gram-negative bacteria isolated from blood culture specimens and their synergy testing. Indian Journal of Microbiology Research. 11(3): 175-179. https://doi.org/10.18231/j.ijmr.2024.032

Filgona, J., Banerjee, T. and Anupurba, S., 2018. Endemicity of OXA-48 and NDM-1 Carbapenemase producing *Klebsiella pneumoniae* and *Escherichia coli* from a tertiary hospital in Varanasi, India. *JAMB*, *12*, pp.1-8. DOI: 10.9734/JAMB/2018/43928

Harris, H., Tao, L., Jacobs, E.B., Bergman, Y., Adebayo, A., Tekle, T., Lewis, S., Dahlquist, A., Abbey, T.C., Wenzler, E. and Humphries, R., 2023. Multicenter evaluation of an MIC-based aztreonam and ceftazidime-avibactam broth disk elution test. *Journal of clinical microbiology*, *61*(5), pp.e01647-22. https://doi.org/10.1128/jcm.01647-22

Kalaivani, R., Kali, A., Surendran, R., Sujaritha, T. and Babu, C.G., 2024. Rapid characterization of carbapenem-resistant Enterobacterales by multiplex lateral flow assay and detection of ceftazidime-avibactam-aztreonam synergy. *Indian Journal of Medical Microbiology*, *47*, p.100530. https://doi.org/10.1016/j.ijmmb.2024.100530

Khan, A., Erickson, S.G., Pettaway, C., Arias, C.A., Miller, W.R. and Bhatti, M.M., 2021. Evaluation of susceptibility testing methods for aztreonam and ceftazidime-avibactam combination therapy on extensively drug-resistant gram-negative organisms. *Antimicrobial agents and chemotherapy*, *65*(11), pp.10-1128. https://doi.org/10.1128/AAC.00846-21

Marshall, S., Hujer, A.M., Rojas, L.J., Papp-Wallace, K.M., Humphries, R.M., Spellberg, B., Hujer, K.M., Marshall, E.K., Rudin, S.D., Perez, F. and Wilson, B.M., 2017. Can ceftazidime-avibactam and aztreonam overcome β-lactam resistance conferred by metallo-β-lactamases in Enterobacteriaceae?. *Antimicrobial agents and chemotherapy*, *61*(4), pp.10-1128. https://doi.org/10.1128/AAC.02243-16

Rajshekar D, Sujatha S R, Krishna Karthik M V S, Raveendran S, Evaluation of phenotypic carbapenem inactivation methods among carbapenem resistant gram-negative bacteria isolated from blood culture specimens and their synergy testing. *Indian J Microbiol Res* 2024;11(3):175-179. https://doi.org/10.18231/j.ijmr.2024.032

Reddy, R.M., Kumar, S. and Kumar, M., 2017. Characterization of VIM and IMP Metallo-β-lactamases (MBL) in Pseudomonas aeruginosa isolated in a tertiary care hospital. *Int J Curr Microbiol Appl Sci*, *6*(3), pp.1461-1467. DOI: 10.3947/ic.2015.47.2.81

Sreenivasan, P., Sharma, B., Kaur, S., Rana, S., Biswal, M., Ray, P. and Angrup, A., 2022. 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. *The Journal of Antibiotics*, *75*(8), pp.454-462. https://doi.org/10.1038/s41429-022-00537-3

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D.L., Pulcini, C., Kahlmeter, G., Kluytmans, J., Carmeli, Y. and Ouellette, M., 2018. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *The Lancet infectious diseases*, *18*(3), pp.318-327. https://doi.org/10.1016/S1473-3099(17)30753-3

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