ORIGINAL ARTICLE

Evaluation of broth disk elution method for detecting synergy between ceftazidime-avibactam and aztreonam against NDM-producing carbapenem-resistant Gram-negative bacteria

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Abstract

Background: The emergence of Gram-negative bacteria that produce carbapenemase has drastically restricted the available treatment options. The ceftazidime/avibactam-aztreonam combination is a good colistin-sparing strategy in such infections. *Material and Methods*: In total 135 carbapenem-resistant isolates of Enterobacterales and *Pseudomonas aeruginosa* were checked for NDM and OXA-48 genes by conventional multiplex Polymerase Chain Reaction (PCR). Broth disc elution method, a simple and cost-effective phenotypic test, was used to assess the synergistic effect of ceftazidime-avibactam combined aztreonam, against NDM-producing isolates. *Results: Klebsiella pneumoniae* was the most common carbapenem resistant organism (55%) isolated. NDM (37.0%) was the most prevalent carbapenemase gene detected followed by coproduction of NDM and OXA-48 (23.0%). A total of 80 isolates positive for NDM were subjected to synergy testing (ceftazidime + avibactam combined with aztreonam) by broth disc elution method of which 72 (90.0%) demonstrated a positive synergistic effect, while 8 (10.0%) were negative. *Conclusion:* Conventional multiplex PCR may serve as a useful screening tool for the detection of common carbapenemase genes. Broth disk elution method of synergy testing is a reliable method in resource limited settings. Thus, our study emphasizes the significance of using a feasible diagnostic test and monitoring the common genes that are responsible for carbapenem resistance.

Keywords: Carbapenemase, Ceftazidime-Avibactam, Enterobacterales, NDM, *Pseudomonas aeruginosa*

Introduction

Morbidity and death linked to bacterial infections are remarkably reduced by using antibiotics. Nonetheless it's over usage has led to increase in antibiotic resistance [1]. Carbapenems are potent antimicrobials for treatment of infections due to drug-resistant bacteria. Carbapenem resistance could be a result of the ability to resist antibiotic activity through a variety of mechanisms, including improper specific target or a variation in the cytoplasmatic membrane constitution or unable to pass the outer membrane [2]. In Enterobacterales, carbapenem resistance is mainly due to the carbapenemase [3]. The Metallo-Beta-Lactamases (MBL) have been found largely in *Pseudomonas aeruginosa*; with reports of global rise amongst Enterobaterales as well [4].

Presently, there are fewer Antimicrobial Susceptibility Testing (AST) techniques accessible for assessing the effectiveness of Aztreonam plus Ceftazidime/Avibactam (ATM + CZA) combination which is colistin sparing approach. A previous study showed that the ATM-CZA susceptibility testing in vitro can be done by using disk-based methods. Broth Disk Elution (BDE) and strip methods employing Minimum Inhibitory Concentration (MIC) test turned out to be the most suitable and less

complicated procedures. BDE method is especially useful in resource limited settings. As a result, BDE, a newer approach for MIC measurement has the convenience of disc diffusion and the dependability of broth elution method. The BDE technique performs exceptionally well, with 100 percent sensitivity and specificity [5]. Hence, the study aimed to explore for a simple lab synergy testing for MBL-producing Enterobacterales and *Pseudomonas aeruginosa* in this setting. There is a dearth of studies reporting the detection of synergy between ceftazidime-avibactam and aztreonam using BDE method [5].

Material and Methods

Study design and study setting: The project was initiated after receiving approval from the Institutional Ethics Committee of Kasturba Medical College, Mangalore (IEC/KMC/MLR06/2022/268). The microbiology lab at a tertiary care facility was the site of this prospective investigation in South India, spanning a period of 11 months (January 2023 to July 2023).

Sample size calculation: The formula used to determine the proportion of the sample: $n = Z^2pq/d^2$; Z = 1.96 is a standard normal value at 5.0% level of significance, p (prevalence) = 14.2% [6], q = 1-p, d (absolute precession) = 6.0%, n = 130 = minimum number of samples that are resistant to either imipenem or meropenem or resistant to both.

Inclusion criteria: Clinically significant, non-repetitive carbapenem resistant Enterobacterales and Pseudomonas aeruginosa strains [7] from exudate, blood, and urine samples.

Exclusion criteria: Carbapenem sensitive Enterobacterales and Pseudomonas aeruginosa strains from exudate, blood culture and urine and all isolates other than Enterobacterales and *Pseudomonas aeruginosa* were left out of the investigation. The estimated proportion was calculated based on the number of patients (n = 130). However, during the study period, some clinical samples exhibited polymicrobial growth, leading to the isolation of more than one organism from a single patient. Hence although 130 patients were included in the study a total of 135 Carbapenem Resistant Organisms (CRO) were obtained and subjected to analysis. Gram staining, colony morphology, and an automated technique (VITEK® 2) were used to identify every organism. Antibiotic susceptibility reports were taken from VITEK® 2 system. The OXA-48 and NDM were identified molecularly by conventional multiplex Polymerase Chain Reaction (PCR). Using the boiling approach, DNA was extracted, from a 24 hr old culture of the organism grown on 5.0 % sheep blood agar plates. The extracted DNA was kept at -20°C if it wasn't used right away. The primer sequence used were as follows:

Primer set NDM (984bp) [8]:

NDM: Forward sequence (5′ CACCTCATGTTT GAATTCGCC 3′)

NDM: Reverse sequence (5′ CTCTGTCACATC GAAATCGC 3′)

Primer set OXA - 48 (438bp) [9]:

OXA – 48: Forward sequence (5′ GCGTGGTTAA GGATGAACAC 3′)

OXA – 48: Reverse sequence (5′ CATCAAGTTCA ACCCAACCG 3′)

A total volume of the reaction mixture was 25 µl containing 12.5 µl of ready-to-use master mix, 0.625 µl of each primer (forward and reverse), 7.5 µl of nuclease free water and 2.5 µl of DNA template. The multiplex PCR was performed with the following conditions: 10 min in 94°C to initial denature, 30 cycles of 1 min in 94°C for denaturation, 30 secs in 55°C for annealing and 1min 30 secs in 72°C for

extension and 5mins in 72°C for complete extension. Gel electrophoresis was used for 45 minutes in 120 V to separate the DNA fragments and imaged using gel documentation system.

In vitro synergy test of CZA + ATM by BDE method was done on NDM positive isolates. Cation adjusted Muller Hinton Broth (MHB) measuring 2ml was taken in 5 sterile borosilicate tubes. One tube was kept uninoculated, as a sterile broth (clear) control, one for growth of test organism (turbid) as growth control and the rest of the 3 tubes were used for synergy testing, with 1 disk of ATM, 1 disk of CZA and 1ATM+1CZA discs added respectively. The tubes with antibiotic discs were kept for 30 mins at ambient temperature so the antimicrobials get eluted from the discs. Sheep blood agar plate colonies were suspended in normal saline to bring the turbidity equal to 0.5 McFarland. Twelve µl of this inoculum suspension was inoculated to the tubes except the sterile broth control tube and vortexed. For 16-20 hours inoculated tubes were incubated in 37°C. ATCC E. coli 25922 was utilized as strain control. The result was interpreted by visual observation for turbidity of the broth in comparison with clear control broth (Figure 1). Synergy was seen favorably when the test tube having both ATM and CZA discs was clear [5].

The study proformas and medical records were noted for demographic and other clinical data of patients. Statistical analysis was carried out with Minitab for windows. Chi-square test was employed to compare the groups. Statistical significance was defined as a p-value of less than 0.05.

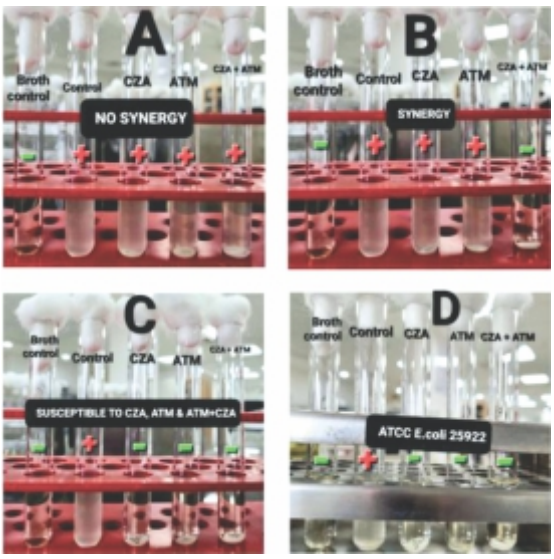

Figure 1: Broth disk elution synergy method

**A – No Synergy as the tubes with antibiotic discs are turbid, B – Synergy positive as the tube with both the discs is clear but tubes with individual discs are turbid, C – Susceptibly to all the tested antibiotic agents as the tubes containing individual discs and the tube with both the discs are clear, D – ATCC E. coli 25922 a negative control for synergy test.*

Results

The present study included samples of exudate, urine and blood from 130 patients. The mean age of patients was 61 years (60.8%), amongst which 88 (67.7%) were males and rest were female 42(32.3%). Up to 41 (32%) CRO were isolated from patients having urinary tract infection, 31 (24%) from respiratory tract infection, 30 (23%) from skin and soft tissue infection, 21 (16%) from blood stream infection and 7 (5%) from sterile body fluid infection. Among 135 CRO isolated, 74 (55%) were *Klebsiella pneumoniae*, 28 (21%) were *Pseudomonas aeruginosa*, 22 (16%) were *E. coli*, 7 (5%) were *Enterobacter spp*, 3 (2%) were *Proteus spp* and 1 (1%) was *Providencia rettgeri*.

Antibiotic resistance pattern of carbapenem non-susceptible Enterobacterales and *Pseudomonas aeruginosa* is as shown in Figure 2.

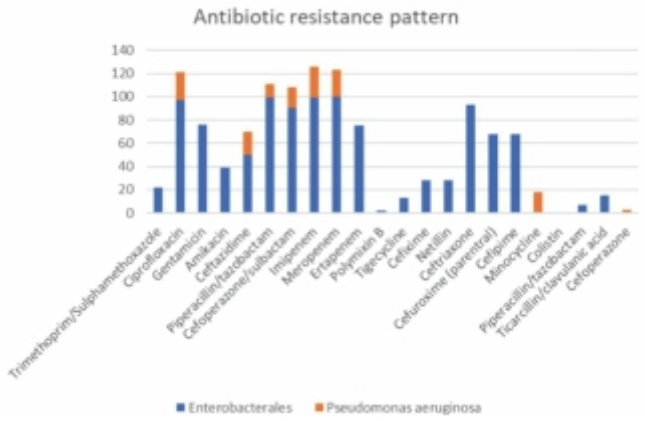

Figure 2: Antibiotic resistance pattern

The molecular detection of NDM and/ OXA-48 was done by PCR as shown in Figure 3. Among the 135 isolates, 50 (37.0%) were NDM, 31 (23.0%) were NDM and OXA-48 co-producers and 18 (13.3%) were OXA-48 producers. A total of 36 (26.7%) were negative for NDM and/OXA-48 production. Out of 50 NDM isolates, 11 (22.0%) were *E. coli*, 27 (54.0%) were *Klebsiella pneumoniae*, 7 (14.0%) were *Pseudomonas aeruginosa,* 4 (8.0%) were *Enterobacter spp*. and 1 (2.0%) was *Providencia retggeri.* Among the 31 isolates positive for both NDM and OXA-48, 2 (6.5%) were *E. coli*, 27 (87.1%) were *Klebsiella Pneumoniae*, 1 (3.2%) was *P. aeruginosa* and 1 (3.2%) was *Enterobacter aerogenes*. Amongst 18 OXA-48 producing isolates 2 (11.1%) were *E. coli* and 16 (88.9%) were *Klebsiella pneumoniae*.

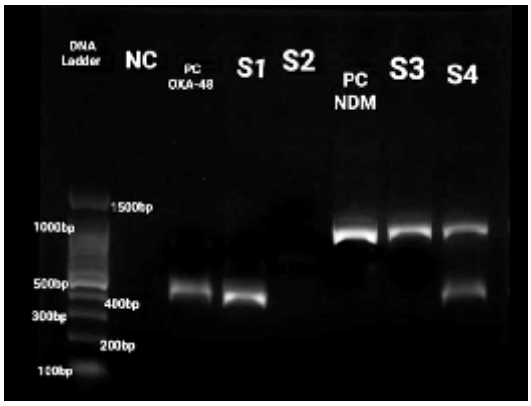

Figure 3: Detection of NDM and OXA-48 by multiplex PCR

**NC – negative control; PC OXA-48 - positive control for OXA-48; PC NDM positive control for NDM; S1, S2, S3, S4 – sample 1, 2, 3 and 4. S1-positive for OXA-48, S2-negative, S3-positive for NDM and S4-positive for NDM and OXA-48*

Statistical analysis of the type of carbapenemase detected revealed that association of *Klebsiella pneumoniae* with coproduction of NDM+OXA-48 and OXA-48 production was significant (p= 0.00 and 0.002 respectively). The infection of urinary tract was substantially associated with NDM (p = 0.05) production. Respiratory infections, and skin and soft tissue infections were significantly associated with NDM+OXA-48 coproduction (p = 0.004 and 0.023 respectively).

Majority of patients had diabetes mellitus (39.0%) and hypertension (36.0%). Twenty-three patients were immunocompromised. The mean duration of hospital stays, and Intensive Care Unit (ICU) stay in individuals afflicted with microbial producing NDM and/ OXA-48 was as shown in Table 1.

Out of the 130 individuals infected with carbapenem resistant Enterobacterales and *Pseudomonas aeruginosa*, the overall mortality rate was 22.3% (n = 29). The mortality rate amongst the patients infected with NDM/OXA-48 producing organisms was as shown in Table 1.

A total of 80 NDM producers were subjected to synergy test among which 72 (90.0%) were positive and 8 (10.0%) were negative.

Table 1: Duration of hospital stay and mortality amongst patients infected in NDM and OXA-48 producing organism infected patients

Enzymes	Duration of hospital stay (days)		Duration of ICU stay (days)	
	Mean ± SD	Median	Mean ± SD	Median
NDM	13.30 ± 8.70	11	3.386 ± 5.027	2
NDM and OXA48	19.47 ± 13.44	16	6.71 ± 6.29	7
OXA48	17.04 ± 8.78	17	5.35 ± 6.91	3
Enzymes	**Mortality rate**			
	Yes (Dead)	**No (Alive)**	***p***	χ^2
NDM (n=50)	9	36	0.294	1.101
NDM and OXA48 (n=31)	11	17	0.05*	3.692
OXA48 (n=18)	5	12	0.683	0.167

SD – Standard deviation; ICU – intensive care unit

**Association of ndm+oxa-48 coproduction with mortality was statistically significant (p-value = 0.05)*

Discussion

Clinical isolates that produce carbapenemase are increasing worldwide causing serious threat to public health, particularly in developing nations like India [10, 11]. During this study period the commonest carbapenem resistant bug recovered was *Klebsiella pneumoniae* (56.9%) followed by *Pseudomonas aeruginosa* (21.5%). A study done in Mahabubnagar, India, showed that *Klebsiella species* (56.7%), *E. coli* (17.7%), and *Pseudomonas species* (10.9%) were predominant [12]. A study from Thailand in the year 2022 showed that *Klebsiella spp.* (76.0%), E. coli (17.0%), and other Enterobacterales (6.0%), were the most common species of carbapenemase producing Carbapenem-Resistant Enterobacterales (CRE) infections [13]. The multiplex PCR revealed NDM (38.5%) gene was more prevalent than OXA-48 (13.8%), the coproduction of NDM plus OXA-48 was 23.8%. The previous study in 2020 from the same set up, showed similar results with 17% of NDM, 7% of OXA-48 and 5% of NDM plus OXA-48 genes identified from a total of 101 isolates of Gram-negative bacilli [14]. Similarly, a study from North India revealed that 44 out of the 164 CRE isolates (26.8%) showed presence of both the NDM and OXA-48 genes. The NDM gene alone tested positive in 94 out of 164 (57.3%) CRE isolates, following OXA-48 in 62 out of 164 (37.8%) isolates [15]. However, the findings of our study is in contrast to the findings from Hungary, in which out of the total 18 carbapenemase genes from 50 isolates, NDM was less prevalent (n = 2) compared to OXA-48 (n = 6) [16].

A study from Turkey also revealed that, NDM (1.3%) was less prevalent than OXA-48 (71.9%) [17]. In our study, 36 (26.7%) out of 135 isolates were negative for NDM and OXA-48 production but were carbapenem resistant. The mechanism of resistance could be through synthesis of additional carbapenemase (such as KPC, IMP, VIM, and OXA-23/24) or by efflux pump/porin loss [18-22]. Statistically substantial correlation was discovered between *Klebsiella pneumoniae* and coproduction of NDM+OXA-48 and OXA-48 production (p-value 0.00 and 0.002 respectively). Similar relevance was found in a study done on carbapenem resistant *Klebsiella pneumoniae* where most isolates co-harbored NDM + OXA-48 (64.5%) followed by OXA-48-like (33.3%) [23].

Distribution of NDM and OXA-48 in isolates from urinary tract infections, respiratory infections and skin and soft tissue infections were statistically significant (p = 0.05, 0.004 and 0.023 respectively). The outcome is comparable to the research conducted by Pudpong *et al*. (2022) where most of the patients infected with carbapenemase producing organisms were diagnosed with urinary tract infections (28.1%) [13]. A study done in a Spanish hospital showed that 42% of the cases with urinary tract infections were due to carbapenemase producing organisms [24]. NDM+OXA-48 (41.5%) coproduction in this study was significantly associated with higher rate of mortality (p = 0.05) and these patients had a longer hospital (18 days) and ICU stay (7 days). Study by Jean *et al.* (2022) showed varying case-fatality rates among patients with CRE (22.0 to 72.0%) and *Pseudomonas aeruginosa* (44.7 to 64.0%) infections [25]. This increased mortality among NDM and OXA-48 co-producers could serve as a critical alert for clinicians. Study by Priyendu *et al.* (2014) showed that median length of hospital stay in carbapenem-sensitive patients was lower (9 days) compared to

that in case of carbapenem-resistant patients (23.5 days) [26]. Another study from Thailand showed that the duration of ICU stays (10 days) was longer in the patients infected with NDM+OXA-48 co-producers [13]. In the present study, DM (n = 75, 57.8%) was the commonest co-morbid condition, followed by hypertension (n = 69, 53.0%) and immunocompromised state (n = 44, 33.8%). Literature quote other risk factors like mechanical ventilation, presence of multiple indwelling devices, surgical procedures in the event of sepsis or a localized infection which attribute to the risk of CRO acquisition [27].

Therapies to CRE that produce metallo-β-lactamase (like NDM) + OXA-48 in India include a 3-hour prolonged infusion of ceftazidime, avibactam, and aztreonam, or polymyxins plus other agents to which the organism has shown to be susceptible MIC, such as tigecycline, amino-glycosides, or intravenous fosfomycin. If the MIC is ≤ 16µg/ml, high-dose carbapenems are an approved treatment option, tigecycline (permitted for skin-soft tissue infections and intra-abdominal infections) or aminoglycosides. If susceptible *in vitro*, *Pseudomonas aeruginosa* resistant to carba-penems can be treated with antibiotics with β-lactam (ceftazidime/cefepime) or β-lactam-β-lactamase inhibitor combos (piperacillintazo-bactam/cefoperazone sulbactam). For infections for which there are no other options, amino-glycosides or polymyxins can be used [18].

In our study, a combination of CZA+ATM was administered to 10 patients who were NDM producers, with 8 surviving (80.0% survival rate). The effectiveness of ATM in combination with CZA aligns with studies conducted by Radha *et al.* (2023) (20.9%) and Swaminathan *et al.* (2022) (17.2%), both of which demonstrated a reduction in mortality [28, 29]. However, the observed clinical response in our study to CZA+ATM combination therapy is to be interpreted with caution as the data was available for small subset of patients (n = 10). Hence these observations are only exploratory and do not allow generalization or definitive conclusion regarding clinical effectiveness.

A simple and reliable phenotypic method, the BDE test, was adopted to evaluate the synergy between CZA and ATM. Furthermore, recent Clinical and Laboratory Standards Institute (CLSI) guidelines have recognized this in-vitro susceptibility testing method using BDE as a dependable approach for synergy testing. Our results revealed, synergy was positive amongst majority of NDM (88.0%, n = 44) and NDM plus OXA-48 (93.0%, n = 28) producing CRO. The results align with the research conducted by Taha *et al.* (2023) where 98.0% of the NDM producers were positive for synergy testing [30]. The results of this simple and cost-effective synergy test can assist clinicians in initiating the CZA + ATM combination therapy with greater confidence, minimizing concerns about treatment failure due to the rapid turnaround time of the BDE method. Aztreonam + ceftazidime-avibactam together are only useful in treating Enterobacterales that express MBL. Avibactam is not able to reverse ATM activity in *P. aeruginosa* and *Acinetobacter spp*. that express MBL, where efflux resistance mechanisms make ATM less efficient. However, our study had certain limitations. Convenience sampling and testing was employed which restricts the findings' potential to be applied generally. Presence of other carbapenemase genes (KPC, IMP or VIM) was not assessed in our study, because molecular characteri-zation of carbapenemase resistance was restricted to NDM and OXA-48.

Conclusion

In the current research, out of the total 135 CRO, majority were from urinary tract infection (32.0%) and the most common carbapenem resistant *Enterobacterale* isolated was *Klebsiella pneumoniae* (55.0%). NDM (37.0%) was most common following by NDM and OXA-48 (23.0%) coproduction. Conventional multiplex PCR may serve as useful screening tool for the detection of common carbapenemase genes. BDE method of synergy testing is a reliable method in resource limited settings. As a result, our research highlights the significance of monitoring common carbapenemase genes alongside the judicious use of feasible diagnostic methods.

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