

# Phenotypic Detection of Carbapenemase Production in Gram Negative Bacilli from Clinical Isolates in a Tertiary Care Hospital in Telangana

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## Abstract

Antimicrobial resistance (AMR) in Gram negative bacteria (GNB) has become a critical health concern across the globe. Unveiling of  $\beta$ -lactamase, extended spectrum  $\beta$ -lactamase (ESBL) and AmpC  $\beta$ -lactamase producing bacteria has led to the development of multidrug-resistant organisms (MDRO's). Carbapenems are considered to be very effective in morbid infections caused by MDRO's. Now, the upsurge of carbapenem resistance among GNB is an issue of concern as these infections are very difficult to treat. Rapid and reliable methods to detect these CPO's in all Microbiology laboratories is essential to streamline the antimicrobial therapy. Accordingly, this study is conducted to determine the enormity of CPO's among clinical isolates by various phenotypic tests along with differentiation of serine  $\beta$ -lactamases from metallo- $\beta$ -lactamases. This is a Prospective Cross-sectional study meticulously planned & conducted for a period of one year. Among the 76 suspected Carbapenemase Producing Organisms (CPO's), 42% were *Klebsiella spp.* followed by *Escherichia coli* (25%), *Pseudomonas aeruginosa* (24%), *Citrobacter spp.* (5%) and *Proteus spp.* (4%). Out of the total isolates 82% of the isolates were confirmed as CPO's by Carba NP test, whereas 93% by mCIM test. 53% of the total isolates tested were Serine- $\beta$ -lactamase producers and 41% were Metallo- $\beta$ -lactamase producers. In conclusion, Carba NP test and mCIM in conjunction with eCIM test could be considered as reliable phenotypic diagnostic methods for carbapenemase detection to guide the clinicians for initiating antibiotic therapy.

**Keywords:** mCIM, eCIM, Carba NP, Serine- $\beta$ -lactamase Producers, Metallo- $\beta$ -lactamase Producers, Carbapenemase Producing Organisms

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## INTRODUCTION

Antimicrobial resistance among Gram negative bacteria (GNB) has become a major global health concern. Previously, antimicrobial agents such as penicillin's, 1<sup>st</sup> & 2<sup>nd</sup> generation cephalosporins have been used effectively as first line drugs for the effective management of infections with GNB. However, organisms had acquired resistance to these 1<sup>st</sup> line antibiotics, thereby enforcing the utilization of second-line drugs like the 3<sup>rd</sup> & 4<sup>th</sup> generation cephalosporins for treatment of life-threatening infections. Evolution of  $\beta$ -lactamase, extended spectrum  $\beta$ -lactamase (ESBL) and AmpC  $\beta$ -lactamase producing bacteria has led to the development of multidrug-resistant organisms (MDRO's).

Carbapenems (Imipenem, Meropenem, Ertapenem) are considered to be very effective in morbid infections caused by these MDRO's. So, they are considered as antibiotics of last resort by clinicians for critically ill patients due to their broad-spectrum activity. Now, the emergence of carbapenem resistance among GNB is becoming a significant community health issue due to their complexity for treatment. Also, Carbapenemase-Producing Organisms (CPO's) can cause outbreaks as Carbapenemase genes are transferable to the susceptible bacteria.<sup>1-4</sup> Infections due to CPO's are associated with disquieting rates of mortality.<sup>5</sup> It is essential to distinguish between CPO's from non-producers to prioritize the usage of antimicrobials which might hamper the emergence of resistance to untouched newly developed antimicrobials.

Expeditious methods to detect these CPO's in all Microbiology laboratories including those in resource-limited settings, is essential not only to streamline the antimicrobial therapy but also to minimize their spread in health-care facilities and the community, for epidemiologic and infection prevention & control purposes.

It is also important to differentiate between Carbapenemase classes because newly available  $\beta$ -lactam (BL) and  $\beta$ -lactamase inhibitor (BLI) combinations like ceftazidime-avibactam as well as others under research & development are mostly active against serine carbapenemases, but not against metallo- $\beta$ -lactamases (MBLs).

In the past few years many phenotypic and genotypic assays were developed for their detection. The advantage of phenotypic assay compared to genotypic assay is that they are less expensive and also they will detect the Carbapenemase activity but not specific genes which will help in detection of emergence of a new or a previously uncommon Carbapenemases.<sup>6</sup>

In this context, this study is intended to determine the enormity of CPO's among clinical isolates by various phenotypic tests along with differentiation of serine  $\beta$ -lactamases from metallo- $\beta$ -lactamases.

## MATERIALS AND METHODS

It is a prospective cross-sectional study meticulously planned & conducted in the Department of Microbiology in a tertiary care teaching hospital in Telangana, South India from February 2022 to 2023 for a period of 1 year after approval from Institutional Ethical Committee.

Varied clinical samples which include pus, urine, blood, body fluids, sputum from wards, intensive care units and OPD's were processed for identification of organisms as per standard operating procedures. Gram positive organisms were excluded from the study. All the GNB were screened for carbapenem resistance by Kirby Bauer disc diffusion method (KBDD) following CLSI M100 - Ed 32, 2022 guidelines using Ertapenem 10  $\mu$ g disk.<sup>7</sup> Isolates with zone diameter of  $\leq 18$  mm were considered as strains with suspected carbapenemase production.

These carbapenem resistant isolates were further evaluated for carbapenemase production by Carba NP and modified carbapenem Inactivation method (mCIM) in conjunction with EDTA modified carbapenem Inactivation method (eCIM). Carba NP and mCIM tests were used for detecting carbapenemase among Enterobacterales and *Pseudomonas aeruginosa* and eCIM is used along with mCIM to differentiate serine  $\beta$ -lactamases from metallo- $\beta$ -lactamases in Enterobacterales.

### Carba NP test

Carba NP test is a calorimetric microtube assay to detect Imipenem hydrolysis by CPO's.

The pH indicator present in the medium depicts a colour change as the medium gets acidified due to hydrolysis. 1µl loopful of bacterial growth from Muller Hinton Agar plate (MHA) is taken and emulsified in microcentrifuge tube containing 100µl of 20mM Tris Hcl lysis buffer and vortexed for 5 seconds. It is then emulsified using 100µl solution containing phenol red indicator along with 0.1mmol/litre ZnSO<sub>4</sub>, which is preadjusted to pH 7.8. Then 3mg/0.5ml of imipenem powder is taken and mixed in reaction tube and control tube contains indicator solution but will not have antibiotic. Tubes are then vortexed for about 10 seconds. Before reaction, both the tubes will be red to orange in colour. The tubes are then incubated at 37°C and observed for 2 hours for colour change from red orange to light orange/dark yellow/yellow [Figure 1]. Change in colour indicates that the organism is a carbapenemase producer.<sup>7</sup>

#### Modified Carbapenem Inactivation method (mCIM) in conjunction with EDTA Modified Carbapenem Inactivation method (eCIM)

Emulsify, 1µL loopful of test isolate for Enterobacterales and 10µL loopful for *Pseudomonas aeruginosa* from culture on Blood agar plate in 2ml Tryptic soy broth (TSB). Two test tubes containing 2mL of TSB with test isolate needs to be taken. One tube is without EDTA for mCIM and the other tube is with EDTA for eCIM. A meropenem (MRP) disk is immersed in each tube and incubated for 4 hours. Then the disks are then picked out of the test tubes and placed on MHA plates with a fresh lawn culture of carbapenem-susceptible *E. coli* ATCC 25922 and incubated overnight. Zone dimensions are to be recorded for interpretation of test results [Figure 2,3].<sup>7</sup>

#### Quality Control (QC)

Data quality is validated using standardized data collection tools. For laboratory investigations all the three phases of quality assurance as per standard guidelines of the lab were strictly followed. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 700603, *Klebsiella pneumoniae* ATCC BAA-1705 were used as QC strains.

#### RESULTS

Among the 76 suspected carbapenemase producing organisms (CPO's), 42% were *Klebsiella* spp. followed by *Escherichia coli* (25%), *Pseudomonas aeruginosa* (24%), *Citrobacter* spp. (5%) and *Proteus* spp.(4%) (Table 1 and 2).

7% of *Klebsiella* spp., 11% of *Pseudomonas aeruginosa* and 6% of *Escherichia coli* gave indeterminate results by mCIM in conjunction with eCIM test (Table 3).

53% of the total isolates tested were Serine-β-lactamase producers and 41% were Metallo-β-lactamase producers whereas 6% of the isolates gave indeterminate test results (Table 4).

**Table 1.** Frequency distribution of suspected Carbapenemase producing Organisms

No.	Isolate (n= 76)	Number	Percentage
1.	<i>Klebsiella</i> spp.	32	42%
2.	<i>E. coli</i>	19	25%
3.	<i>P. aeruginosa</i>	18	24%
4.	<i>Citrobacter</i> spp.	04	05%
5.	<i>Proteus</i> spp.	03	04%
	Total (n)	76	--

**Table 2.** Phenotypic detection of CPO's by Carba NP and mCIM test

No.	Isolate (n= 76)	Carba NP test Positive		mCIM test positive	
		Number	Percentage	Number	Percentage
1.	<i>Klebsiella</i> spp.	26	81%	30	93%
2.	<i>Escherichia coli</i>	17	89%	18	94%
3.	<i>Citrobacter</i> spp.	04	100%	04	100%
4.	<i>Proteus</i> spp.	02	66%	03	100%
5.	<i>P. aeruginosa</i>	12	66%	16	88%
	Total	62	82%	71	93%

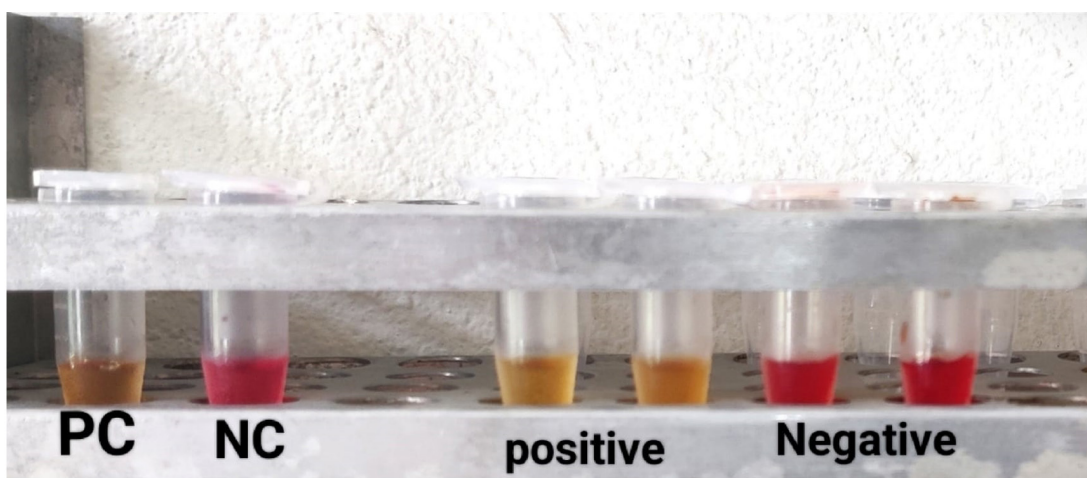
## DISCUSSION

Infections due to CPO's entail challenges in their detection in resource limited settings, thus emphasizing the need of optimization of diagnostics which are cost-effective and easy to use on a daily basis. Though genotypic tests are very precise in the identification of genes coding for resistance, it also has a disadvantage that if a new or uncommon enzyme coding gene which is not included in the testing panel cannot be detected and the mere presence of gene will not always confer resistance as the organism may not express the gene phenotypically. Genotypic tests are also very expensive. On the other side, Phenotypic tests detect the expression and probably will not miss the carbapenemase production and is also cost

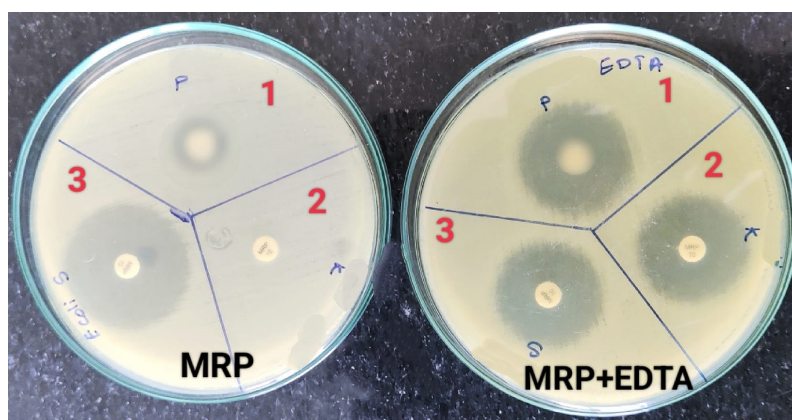
effective. Diagnostic perfection comes for a test which is cost-effective with a lower turnaround time and is highly sensitive and specific.

CarbaNP & mCIM tests are two phenotypic detection methods with good diagnostic perfection as recommended by CLSI M100 guidelines.<sup>7</sup>

In the present study, a total of 76 isolates with suspected carbapenemase production were tested with Carba NP and mCIM test. Carba NP test is positive for 82% of the total isolates which correlated with a study by Patidar et al.,<sup>8</sup> where 88% of the isolates were positive in contrast to Sinha D. et al.<sup>9</sup> showing only 71% positivity. 93% of the total isolates were confirmed to be carbapenemase producers by mCIM test which is similar to studies by Tsai et al.<sup>10</sup> 100%, Li et al.<sup>11</sup> 97.5% , Sinha et al. 87% and Aboulela et al. 83%



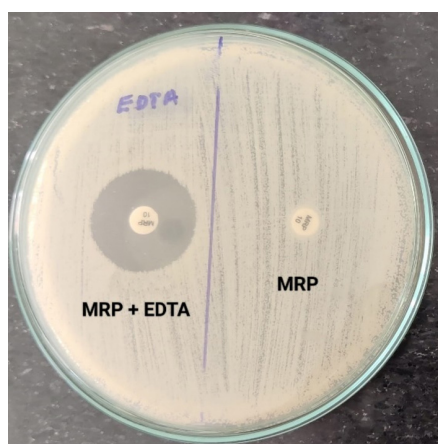
**Figure 1.** Carba NP test to differentiate between Carbapenemase Producers and non-producers



**Figure 2.** Isolate 1&2 in the figure shows Metallo-β-lactamase production Isolate 3 shows Serine-β-lactamase production with mCIM + eCIM test

**Table 3.** Frequency distribution of isolates tested with mCIM in conjunction with eCIM test

No. Isolate (n= 76)	mCIM	mCIM + eCIM	Indeterminate
1. <i>Klebsiella</i> spp.	30 (93%)	14 (43%)	02 (7%)
2. <i>Escherichia coli</i>	18 (94%)	07 (36%)	01 (6%)
3. <i>Citrobacter</i> spp.	04 (100%)	0	0
4. <i>Proteus</i> spp.	03 (100%)	01 (33%)	0
5. <i>P. aeruginosa</i>	16 (88%)	09 (50%)	02(11%)

**Figure 3.** Metallo- $\beta$ -lactamase production detected by mCIM + eCIM test**Table 4.** Differentiation of CPO's into different classes based on mCIM in conjunction with eCIM test

No. Organism n= 76	Serine- $\beta$ -lactamases	Metallo- $\beta$ -lactamases
1. <i>Klebsiella</i> spp.	16 (50%)	14 (43%)
2. <i>Escherichia coli</i>	11 (57%)	07 (36%)
3. <i>Citrobacter</i> spp.	04 (100%)	0
4. <i>Proteus</i> spp.	02 (66%)	01(33%)
5. <i>P. aeruginosa</i>	07 (39%)	09 (50%)
Total	40 (53%)	31(41%)

whereas Koul et al. reported only 53.5% Positivity.

In this study, mCIM detected carbapenemase production in 93% of *Klebsiella* spp which correlated with Li et al.<sup>11</sup> 96% whereas Tsai et al., Koul et al. and Alemayehu et al.<sup>12</sup> reported 65%, 48.48% and 30% respectively. Similarly 94% of *E. coli* were positive by mCIM test in correlation with Li et al. & Tsai et al. 100% and Alemayehu et al.<sup>12</sup> reported only 20% positivity. *Citrobacter* spp. and *Protues* spp. were 100% positive to mCIM test as with Li et al. & Tsai et al. 83% of *Pseudomonas aeruginosa* strains were positive as compared to 30% by Alemayehu et al.

Carbapenemase production by Carba NP test in this study was confirmed in 100% of *Citrobacter* spp., 89% *Escherichia coli*, 81% *Klebsiella* spp. and 66% of *P.aeruginosa* & *Proteus* whereas a study by Pragasam et al.<sup>13</sup> detected 97% and 89% of *E.coli* & *Klebsiella* respectively.

Current study reveals, 53% of total isolates tested were Serine- $\beta$ -lactamase producers and 41% were Metallo- $\beta$ -lactamase producers

which is comparable to Aboulela et al.<sup>14</sup> & Koul et al.<sup>15</sup> who reported 52.8% MBL, 30.2% Serine carbapenemase producers & 58.3% MBL, 41.6% Serine carbapenemase producers respectively.

50% of *Pseudomonas aeruginosa*, 43% of *Klebsiella* spp., 36% of *E.coli*, 33% of *Proteus* spp. were MBL producers in the study. A study by Koul et al.<sup>15</sup> reported 75% *Klebsiella* spp., 25% of *E.coli* were MBL producers.

By analysing the findings of the present study mCIM test detected more number of carbapenem producing strains compared to Carba NP test. The strength of Carba NP test is that it can be considered as rapid biochemical test with a turnaround time of less than 2 hours for detection of CRO's. Nordmann et al.<sup>16</sup> had reported this test to be 100% sensitive for detection of carbapenemase production but subsequent studies revealed a lower sensitivity of <90%.<sup>17-19</sup> Limitations of Carba NP test is that it cannot differentiate between different classes of carbapenemases, low sensitivity for detection

of class D carbapenemases and false negative results were obtained with some mucoid strains of *Enterobacteriaceae*. Tamma et al. reported a lower detection rate of 40% OXA-48 like enzyme producing Enterobacterales.<sup>20</sup> Tijet et al. reported that false negative results were probably due to partial lysis of the cell wall in mucoid MBL producing Enterobacterales.<sup>21</sup>

Modified CIM has shown good results in this study which corroborated with foregoing studies who have revealed excellent precision to detect KPC, VIM, NDM, OXA-48 like carbapenemases.<sup>22,24-28</sup> It is preferable for resource limited laboratories as it is easy to do and inexpensive test. As eCIM is also performed along with mCIM for the detection of MBL's it will be helpful for differentiation between serine & metallo beta-lactamases.<sup>23,29,30</sup> The disadvantage with this method is a turnaround time of 18-24 hours and also when class B and class A/D carbapenemases are co-expressed, eCIM test cannot detect class B enzymes but the prevalence of isolates encoding both classes is very low.<sup>22</sup> Also, it has lower sensitivity for IMP type of MBL detection 79.6% but is 100% sensitive for NDM enzyme detection.<sup>23</sup>

## CONCLUSION

The Health implications caused by CPO's were increasing day by day as reported in several studies globally, accentuating the expeditious demand for optimization of diagnostics and therapeutics and also for establishment of definitive and effective infection prevention and control practices. Thus, understanding the mechanisms causing carbapenem resistance has gained significance. The study results showed that mCIM combined with eCIM test was superior to CarbaNP test. Newer classes of  $\beta$ -lactam &  $\beta$ -lactamase inhibitors, like ceftazidime-avibactam have a significant therapeutic effect on serine carbapenemase producers whereas concomitant therapy with ceftazidime-avibactam along with aztreonam and colistin were highly active on MBL producers. As mCIM with eCIM can differentiate between both, it could be utilised as simple, reliable, cost-effective phenotypic method for carbapenemase detection which will contribute in the formulation of better treatment plan to

curtail therapeutic failures. Into the bargain, it will help the resource limited laboratories to consider restricting the genotypic testing for carbapenemase production.

## Limitations of the study

With this study's results, one cannot extrapolate the carbapenemase resistance in this particular region, as a multitude of people suffering from various infections visit the tertiary care hospitals after taking many antibiotics over the counter.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

## FUNDING

None.

## DATA AVAILABILITY

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

## ETHICS STATEMENT

This study was approved by the Institutional Ethics Committee, Mamata Academy of Medical Sciences, Hyderabad, India, with review letter number IEC/MAMS/2023/019.

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