Original Article

DOI: 10.7860/JCDR/2022/55467.16258

Microbiology Section

Carba M Test for Rapid Detection and Simultaneous Differentiation of Carbapenemases among Clinical Isolates of Gram Negative Bacteria

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ABSTRACT

Introduction: Carbapenemase production is the most common mechanism of carbapenem resistance. The carbapenemases belongs to class A, class B and class D of Ambler's molecular classification. Various tests have been designed for screening and confirmation of these enzymes. The acidimetric based Carba NP (Nordmann Poirel) test is simple and detects carbapenemases within two hours. The test could not differentiate between the serine carbapenemases from Metallo β-Lactamase (MBL). Differentiation of these two classes of enzymes will help in choosing the newer carbapenemase inhibitors like avibactam which selectively act on serine carbapenemases.

Aim: To design a test that can simultaneously confirm and differentiate Amblers class A and D from class B carbapenemase enzymes among clinical strains of the Gram-Negative Bacteria (GNB).

Materials and Methods: An experimental cross-sectional study was conducted between January-December 2018 on 195 strains of carbapenem resistant and 40 strains of carbapenem sensitive GNB. The carbapenemase genes were detected among all the bacteria by multiplex Polymerase Chain Reaction (PCR). The Carba M test was designed and evaluated for the detection and differentiation of class A and D from class B carbapenemases among the study isolates.

Results: The Carba M test had 100% sensitivity and specificity for identification of New Delhi Metallo-β-lactamase (NDM) and Verona Integron-encoded Metallo-β-lactamase (VIM) enzymes. The strains which co-produced two MBL enzymes were detected with 100% sensitivity. The test had 42.85% sensitivity for the detection of Oxacillinase (OXA)-48-like enzymes.

Conclusion: The Carba M test is useful in detection and simultaneous differentiation of carbapenemase content of the GNB and will help to choose appropriate carbapenemase inhibitors judiciously.

Keywords: Carbapenem resistance, Metallo-β-lactamases, Phenotypic detection, Serine carbapenemases

INTRODUCTION

Carbapenems are antibiotics that are used as a last resort to treat infections caused by Multidrug-Resistant (MDR) GNB [1]. Infections caused by carbapenem-resistant organisms are associated with increased morbidity and mortality [2]. Carbapenem resistance can be due to carbapenemases, efflux pump and hyperproduction of Extended Spectrum beta-Lactamase (ESBL) and/or Ampicillinase C (AmpC) with porin loss [3,4]. The carbapenemases are versatile enzymes that belongs to Ambler's molecular class A, B and D [3]. Serine is found in the catalytic region of class A (*Klebsiella pneumoniae* carbapenemase; KPC) and class D (Oxa-48-like) enzymes. While class B enzymes such as NDM, VIM, Imipenemase (IMP) have one or two zinc ions in their active site, they are referred to as MBL [3]. Except for monobactams, the MBL enzymes hydrolyse all β-lactam antibiotics efficiently. ESBL and AmpC β-lactamases do not hydrolyse carbapenem antibiotics [3,5].

The phenotypic tests based on substrate and inhibitor profile have been proposed for routine detection of carbapenemases in laboratory but their performance is variable [3,4]. Molecular based method like PCR is confirmatory but it is limited in terms of its availability and the number of targets that can be detected. The Clinical Laboratory Standards Institute (CLSI) recommended using the acidimetric-based Carba NP test for carbapenemase confirmation [6]. The Carba NP test is highly sensitive and specific for KPC and MBLs, but has a very poor sensitivity for OXA-48-like enzymes [3,4]. A major limitation of the Carba NP test is its inability to differentiate class A/D from class B enzymes. CLSI has recently introduced the modified Carbapenem Inactivation Method (mCIM) and its variant eCIM to identify carbapenemase production among Enterobacterales and *Pseudomonas aeruginosa* [7]. When used in conjunction with the eCIM, the mCIM can distinguish between class A and D and class B enzymes. While the procedure is very sensitive and specific for detecting these enzymes, the test is cumbersome, labour intensive and will take at least 18-24 hours to interpret the results [8]. There is a need for rapid test capable of simultaneously detecting and classifying carbapenemases among the clinical isolates of GNB.

Recently, class A and D carbapenemase inhibitors like avibactam, relabactam and vaborbactam have been introduced into clinical practice. These inhibitors do not act on class B enzymes [9]. Thus, differentiating carbapenemase classes will aid clinicians in selecting the most appropriate carbapenemase inhibitor for critically ill patients. Therefore, study assessed the Carba NP test and its simplified modification Carba M test for rapid confirmation and differentiation of class B from class A and D carbapenemases.

MATERIALS AND METHODS

The present cross-sectional study was carried out at Department of Microbiology, ESIC MC PGIMSR and MH, Rajajinagar, Bengaluru, Karnataka, India, between January-December 2018. Ethics approval was obtained from Institutional Ethical Committee (IEC) vide reference No. 532/L/11/12/Ethics/ESICMC&PGIMSR/Estt.Vol.IVDtd: 04/12/2019. This work was conducted as part of a 'Study on mechanisms of carbapenem resistance among gram negative

bacteria' funded by Rajiv Gandhi University of Health Sciences Advanced Research Wing.

Inclusion criteria: The isolates which possessed one or more carbapenemase genes and randomly selected 40 carbapenem sensitive isolates were included in the study.

Exclusion criteria: All other carbapenem sensitive isolates were excluded from the study.

Study Procedure

The study comprised carbapenem (n=195) resistant and carbapenem sensitive (n=40) GNB isolated from a variety of clinical samples including exudates, blood, sputum, fluid, urine, and other samples submitted to the Department of Microbiology during the study period. The isolates were identified to species level on the basis of colony morphology, grams stain and a battery of biochemical tests like oxidase, indole, citrate, triple sugar iron agar, urea hydrolysis, sugar fermentation, decarboxylation of lysine, arginine and ornithine, Oxidation-Fermentation Test, cetrimide agar etc., [10].

The antibiotic susceptibility testing was done according to Kirby-Baur disc diffusion method and the results were interpreted in accordance with CLSI guidelines [6]. The imipenem Minimum Inhibitory Concentration (MIC) was determined using an agar dilution technique in accordance with CLSI guidelines [11]. Isolates of Enterobacteriaceae which had imipenem MIC of ≥4 µg/mL and isolates of *Pseudomonas aeruginosa* or *Acinetobacter* spp which had imipenem MIC of ≥8 µg/mL were interpreted as carbapenem resistant [11]. These isolates were subjected for multiplex PCR using bla_{NDM}, bla_{MP}, bla_{VIM}, $bla_{OXA\text{-}48\ like}$ and bla_{KPC} primers as described previously [12].

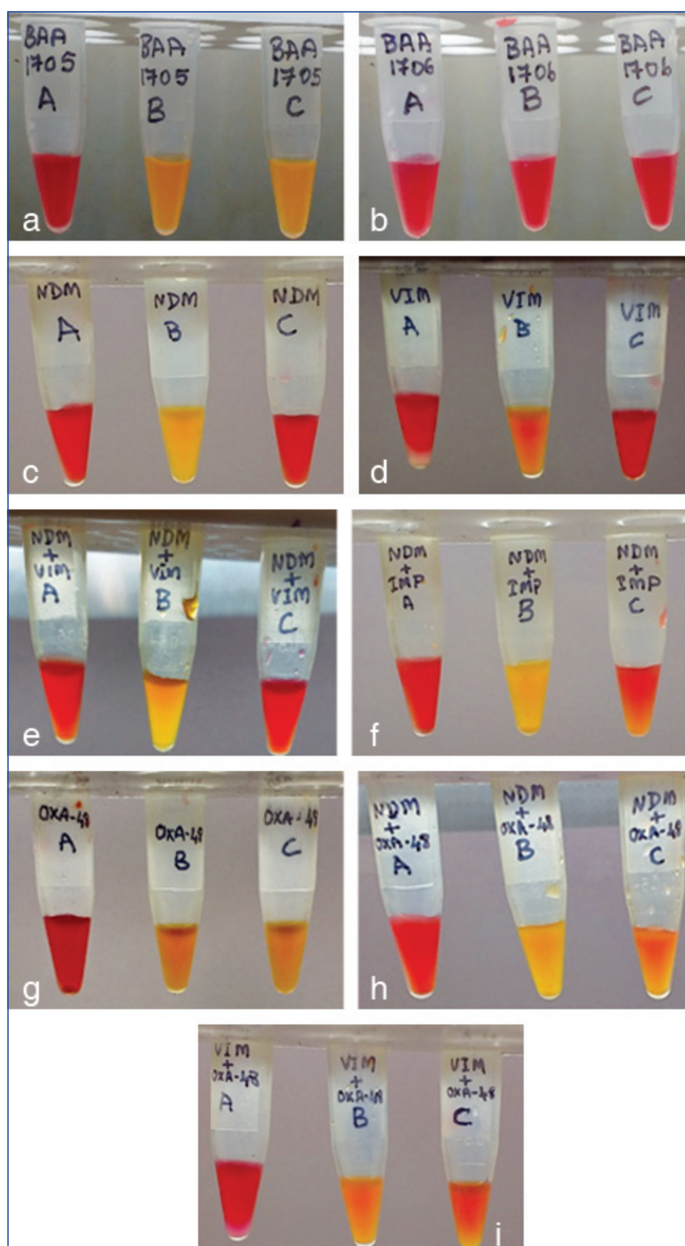
The *Klebsiella pneumoniae* ATCC 1705, *Klebsiella pneumoniae* ATCC 1706, *Escherichia coli* ATCC 25923 and *Klebsiella pneumoniae* ATCC 700603 were used as controls.

Modified carba NP test (mCNP): The test was performed on the test and control strains grown on Brain-Heart Infusion (BHI) agar or Sheep Blood Agar (SBA) according to the modified Carba NP test protocol as described previously [12].

Rapid MBL confirmatory test (Carba M test): This improved method of modified Carba NP [12] test detects carbapenemase production and simultaneously distinguish class B enzymes from class A and D enzymes. The test had three reagents which were named 'A', 'B' and 'C'. The reagent 'A' was prepared by adding 16.6 mL of molecular grade water, 180 µL of 10 mM $ZnSO_4$ solution and 2 mL of 0.5% phenol red solution. The pH was adjusted to 7.0±0.1. The reagent 'B' was prepared by adding 12 mg/mL of injectable imipenem-cilastatin powder (equivalent to 6 mg/mL of imipenem pure powder) to reagent A. The reagent 'C' was prepared by adding 15.6 mL of molecular grade water, 2 mL of 0.5% phenol red solution, 1 mL of 0.05 M EDTA solution and 12 mg/mL of injectable imipenem-cilastatin powder (equivalent to 6 mg/mL of imipenem pure powder), pH was adjusted to 7.0±0.1. Briefly, a loop full of bacterial colony from BHI agar or SBA plate was resuspended in 300 µL of normal saline and vortexed for a minute. The 100 µL inoculum was alliquoted to each of the three Eppendorf tubes labelled 'A', 'B' and 'C'. To tubes labelled 'A', 'B' and 'C' 100 µL of corresponding reagent 'A', 'B' and 'C' was added. All three tubes were incubated for a maximum of two hours at 37°C. Each tube's colour change was visually read and interpreted in accordance with [Table/Fig-1,2]. The isolates were tested thrice and interpreted by three different observers.

Tube "a": Solution A	Tube "b": Solution B	Tube "c": Solution C	Interpretation
Red or red-orange	Red or red-orange	Red or red-orange	Negative, no carbapenemase detected
Red or red-orange	Light orange, dark yellow or yellow	Red or red-orange	Positive, Class B carbapenemase
Red or red-orange	Light orange, dark yellow or yellow	Light orange, dark yellow or yellow	Positive, Class A/D carbapenemases
Red or red-orange	Dark yellow	Light orange or yellow	Positive, co-existence of Class A/D/B carbapeneases
Red or red-orange	Orange	Orange	Invalid

[Table/Fig-1]: Interpretations of Carba M test.



[Table/Fig-2]: The results of Carba M test for different carbapenemases.
a) *Klebsiella pneumoniae* ATCC BAA-1705 known KPC producing strain as Positive control organism; b) *Klebsiella pneumoniae* ATCC BAA-1706 as Negative control organism; c) NDM producing Escherichia coli; d) VIM producing *Pseudomonas aeruginosa*; e) NDM with VIM producing *Pseudomonas aeruginosa*; f) NDM with IMP producing *Klebsiella pneumoniae*; g) OXA-48-like producing *Acinetobacter* spp; h) NDM with OXA-48-like producing *Escherichia coli*; i) OXA-48-like with VIM producing *Pseudomonas aeruginosa*

STATISTICAL ANALYSIS

To compare the performance of the two carbapenamase detection methods, the PCR was used as a reference method. The simple frequencies were tabulated, and the tests' sensitivity and specificity were calculated. To determine statistical significance, the Chi-square test was used. A statistically significant p-value <0.05 was considered.

RESULTS

A total of 195 carbapenem resistant GNB were isolated during study period and were positive for one or more carbapenemase

genes by PCR [Table/Fig-3]. Additionally, 40 carbapenem-sensitive GNB were chosen at random to serve as carbapenemase negative controls. Majority of the isolates belonged to the family Enterobacteriaceae (n=157) followed by *Acinetobacter* spp (n=46) and *Pseudomonas aeruginosa* (n=32) [Table/Fig-3]. Among the 195 bacteria that produced carbapenemases, 134 had NDM enzyme, 35 isolates had OXA-48-like enzymes and two had VIM. Twenty four isolates had multiple carbapenemase genes: NDM+OXA-48-like (n=10), NDM+VIM (n=11), OXA-48-like+VIM (2), and NDM+IMP (n=1). None of the isolates had KPC enzymes, hence *Klebsiella pneumoniae* ATCC 1705 was used as KPC producing isolate. All the 40 carbapenem sensitive bacteria lacked carbapenemase genes.

Strain (N=235)	Carbapenemase gene	No. of isolates	MIC (µg/mL) of imipenem
Acinetobacter spp (n=46)	NDM	18	>32
	OXA-48-like	14	>4
	NDM+OXA-48-like	3	>32
	VIM+OXA-48-like	1	>32
	No carbapenemase	10	<1
Citrobacter freundii (n=4)	NDM	3	>32
	NDM+OXA-48-like	1	>32
Citrobacter koseri (n=5)	NDM	5	>32
Escherichia coli (n=80)	NDM	64	>32
	NDM+OXA-48-like	4	>32
	NDM+VIM	2	>32
	No carbapenemase	10	<1
Enterobacter cloacae (n=7)	NDM	6	>32
	OXA-48-like	1	>4
Klebsiella oxytoca (n=2)	NDM	2	>32
Klebsiella pneumoniae (n=57)	NDM	24	>32
	OXA-48-like	19	>4
	NDM+OXA-48-like	2	>32
	NDM+VIM	1	>32
	NDM+IMP	1	>32
	No carbapenemase	10	<1
Morganella morgani (n=2)	NDM	2	>32
Pseudomonas aeruginosa (n=32)	NDM	10	>32
	NDM+VIM	8	>32
	VIM	2	>32
	OXA-48-like	1	>8
	VIM+OXA-48-like	1	>32
	No carbapenemase	10	<1

[Table/Fig-3]: Isolates used in Carba M test validation.

The modified Carba NP test detected carbapenemases in 175 of 195 carbapenem resistant isolates [Table/Fig-4] with an overall sensitivity of 89.74% and a specificity of 100%. The Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of mCNP test was found to be 100% and 66.6%, respectively. The mCNP test confirmed carbapenemase production in all isolates that produced class B enzymes with 100% sensitivity and specificity. But the sensitivity decreased to 42.85% (15/35) when isolates carried class D (Oxa-48 like) enzymes. Ten isolates each of *K. penumoniae* and *Acinetobacter spp* which had Oxa-48 like enzymes were interpreted as negative using mCNP test.

The Carba M test also detected all the isolates producing carbapenemase, with the exception of the 20 OXA-48-like producing strains [Table/Fig-4] which were found to be carbapenemase negative. The overall sensitivity and specificity for detecting carbapenemases activity among the isolates was 89.74% and 100%, respectively. Additionally, the test could differentiate class B from class A and D enzymes. The isolates producing class B enzymes (NDM, VIM, NDM+VIM, NDM+IMP; n=148) were identified with 100% sensitivity and specifity. For class B enzymes, the Carba M test was in 100% agreement with PCR ($p<0.05$). Only 15 of the 35 isolates expressing class D (Oxa-48-like) enzymes were interpreted as class A and D enzymes, whereas the remaining isolates exhibited no colour change and were classified as negative or non carbapenemase producers. The test was 42.85% sensitive for detecting class D (OXA-48-like) enzymes.

The strains which co-produce NDM or VIM with OXA-48-like (class B with class A/D) enzymes (n=12) were interpreted as either class B or class A and D. Nine isolates were interpreted as class B enzymes and three isolates were interpreted as class A and D despite having class A and D and class B enzymes together. Hence test could not differentiate the MBL from non MBL carbapenemases, when they co-existed together in same isolate. There was not much interobserver variation and results were consistent when repeated.

DISCUSSION

The infection with Carbapenem Resistant Organism (CRO) is associated with poor outcome. The emergence and spread of carbepenem resistant organisms has become a problem of global concern [2]. Carbapenem-resistant Enterobacteriaceae have been reported all over the world, owing primarily to the acquisition of carbapenemase genes [3]. The carbapenemase

Carbapenemase	β-lactamase (n)	Carba M test			Modified Carba NP	
		Class B enzymes	Class A/D enzymes	Negative	Positive	Negative
Class B enzymes (MBLs) (n=148)	NDM (134)	134	0	0	134	0
	VIM (2)	2	0	0	2	0
	NDM+VIM (11)	11	0	0	11	0
	NDM+IMP (1)	1	0	0	1	0
Class D enzymes (n=35)	OXA-48-like (35)	0	15	20	15	20
Co-production of Class B and Class D enzymes (n=12)	NDM+OXA-48-like (10)	7	3	0	10	0
	VIM+OXA-48-like (2)	2	0	0	2	0
No carbapenemase (n=40)	None	0	0	40	0	40
Control strains	*K. pneumoniae* ATCC 1705 (KPC)	0	1	0	1	0
	K. pneumoniae ATCC 1706	0	0	1	0	1
	K. pneumoniae ATCC 700603	0	0	1	0	1
	E. coli ATCC 25923	0	0	1	0	1

[Table/Fig-4]: Results of Carba M test and modified Carba NP test.

genes reside on mobile genetic elements enabling easy spread among gram negative bacteria [4,13]. A rapid, reliable, accurate and cost-effective phenotypic test for detection of carbapenemase producing organisms is necessary to aid clinicians in chosing appropriate carbapenemase inhibitor drug and for better infection control practices.

The sensitivity and specificity of the mCNP test and the Carba M test for detecting carbapenemase content were 89.7% and 100%, respectively. The Carba M test gave superior results compared to mCNP test, as it detected and simultaneously differentiated class B from class A and D carbapenemases ($p < 0.05$). The addition of third tube with EDTA, without $ZnSO_4$ in the presence of imipenem will inhibit the class B enzymes and prevent the colour change in tube C. Whereas the class A and D enzymes which are unaffected by EDTA could still hydrolyse the imipenem and change the colour in tube C. This could easily differentiate class B (NDM, VIM, IMP) from those of class A and D (KPC, OXA-48 like) carbapenemases.

The Carba M test had 100% agreement with PCR in identifying class B enzymes (NDM, VIM, NDM plus VIM and NDM plus IMP; n=148) and this agreement was statistically significant ($p < 0.05$). The test had 100% sensitivity, specificity, PPV and NPV against class B enzymes. These results were comparable to the findings of Dortet L et al., who also showed the test could differentiate class B from class A and D enzymes with good efficacy [14]. Both mCNP and Carba M tests failed to detect 20 OXA-48-like enzymes, which are know for their low level resistance and false negative results [4,14].

The test could not differentiate class A and D from class B enzymes, when they co-existed in the same isolate. For such isolates the tube “A” was red and tube “B” was yellow and results of tube “C” mainly depends up on the expression levels of Oxa-48-like eznymes. If OXA-48 like enzymes are highly expressed then imipenem was hydrolysed and colour changed in tube “C” to yellow and was interpreted as class A and D. If the expression of OXA-48 like enzyme was very less, then they couldn’t act on imipenem and was interpreted as class B [Table/Fig-2]. Similar findings were made by Dortet L et al., [14]. By looking at the intensity of colour difference between tube “B” and “C”, sometimes one could suspect the co-existance of class A/D and class B enzymes in an isolate.

Numerous phenotypic tests have been developed to detect and differentiate carbapenemase classes utilising carbapenemase inhibitors (EDTA, dipicolinic acid, and boronic acid derivatives) in combination with imipenem [8,15]. These tests were based on reduction in imipenem MIC or increase in zone of imipenem when added with specific inhibitors. These tests require an additional 24 hours for interpretation. Additionally, they lacked sensitivity and specificity when bacteria produced two carbapenemases or when various resistance mechanisms were present in the same isolate [8]. The Carba M test can be done directly on isolates grown from clinical specimens and this will save at least 24 hours compared to the previously described phenotypic methods. The Dortet L et al., designed the Carba NP II test where they used Bacterial Protein Extraction Reagent (B-PER) II bacterial protein extraction reagent and commercially available imipenem pure powder [14]. The Carba M test was performed using direct colony without the need of extraction reagent, and in present study injectable imipenem-cilastatin was used as substrate. This modification could result in a cost reduction from 0.7$ (Carba NP II) to 0.008$ (Carba M test). Hence, the test is relatively inexpensive, gives quick results, and requires chemicals that are readily found in routine microbiology laboratory.

Infection with carbapenemase producing isolates are left with limited treatment options [5]. Avibactam and relebactam are diazabicyclooctane (DBO) serine β-lactamase inhibitors that inhibit a wide range of β-lactamases [2,16]. These molecules when combined with imipenem or ceftazidime have shown promising results for treatment of infections with bacteria producing serine carbapenemases and ESBL and/or AmpC with porin loss types of carbapenem resistance [17,18]. The molecules didn’t potentiate the action of imipenem or ceftazidime for the isolates producing VIM, IMP and NDM type of metallo-β-lactamases [18]. Rapid detection and differentiation of the class A and D from class B carbapenemases will help the clinicians to decide the judicious use of the newer carbapenemase inhibitors. Hence the Carba M test can be a useful test as it identifies the carbapenemase production as well as it differentiate the class of carbapenemase enzymes.

Limitation(s)

The test was validated for only four different carbapenemases and common bacterial isolates obtained from clinical specimens. Further studies using different carbapenemase producing bacteria are required.

CONCLUSION(S)

The serine carbapenemase inhibitors like avibactam and relebactam can inhibit class A and D enzymes but have no action against class B enzymes. Using the Carba M test, simultaneous detection and differentiation of class of carbapenemase enzymes will help the clinicians to use the new molecules judiciously.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]

- Plagiarism X-checker: Feb 11, 2022
- Manual Googling: Feb 16, 2022
- iThenticate Software: Feb 22, 2022 (6%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:

- Financial or Other Competing Interests: RGUHS Advanced Research Grants Vide Code No. M107 for the year 2016-17
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? No
- For any images presented appropriate consent has been obtained from the subjects. No

Date of Submission: **Feb 05, 2022**
Date of Peer Review: **Feb 16, 2022**
Date of Acceptance: **Mar 02, 2022**
Date of Publishing: **Apr 01, 2022**