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Original Article

Microbiology Section

Closing the Gap Between Phenotypic and Genotypic Detection of Carbapenem Resistant Enterobacteriaceae by New Modified Carbapenem Inactivation Method

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ABSTRACT

Introduction: Carbapenems have become one of the last resort of antimicrobials. But in last few years, Carbapenem Resistant *Enterobacteriaceae* (CRE) have been reported worldwide. Various phenotypic tests have been proposed for detection of carbapenemase activity including the newer modified Carbapenem Inactivation Method (mCIM) as advised by Clinical Laboratory Standard Institute (CLSI) 2017 guidelines.

Aim: Detection of CRE from clinical specimens with new mCIM method and its comparative evaluation with phenotypic and genotypic methods.

Materials and Methods: Study was conducted between January 2017 and December 2017 at KIMS, Karad. Total 66 CRE, isolated from 1634 clinical specimens and identified by VITEK 2 (Biomerieux, France) were included in the study. Phenotype screening was done by mCIM (CLSI 2017) method and was compared with Modified Hodge test (MHT) and Combined Disc Test (CDT) methods. *Klebsiella pneumoniae* ATCC BAA-1705 and *Klebsiella pneumoniae* ATCC BAA-1706 were used as positive and negative controls respectively. Molecular confirmation of these isolates for carbapenemase producing genes bla_{NDM-1}, bla_{OXA-48}, bla_{KPC} was done by multiplex Polymerase Chain Reaction (PCR) study.

Results: *Klebsiella pneumoniae* (n=35) outnumbered the other bacterial species among 66 CRE included in the study. mCIM was positive for 65 (98.48%) out of 66 isolates while MHT and CDT was positive for 50 (75.75%) and 59 (89.39%) of CRE isolates respectively. All the CRE isolates showed presence of at least one carbapenemase producing gene.

Conclusion: The mCIM method is simple, less subjective, cost effective, reproducible and most sensitive method and plays important role in detection and prevention of spread of CRE, thereby, reducing morbidity and mortality, especially where there is lack of automation and molecular diagnostic facility.

Keywords: Carbapenemase, Combined disc test, mCIM, Modified Hodge test

INTRODUCTION

Antimicrobial resistance has become one of the major health related concern all over world. Right from invention of β-lactam antibiotic penicillin to date, bacteria are inactivating the antimicrobials with newer mechanisms and production of various enzymes capable of inactivating almost all groups of antimicrobials [1]. This is more evident in β-lactam antimicrobial groups, making bacteria resistant to penicillin, all generations of cephalosporins, monobactams and β-lactam- β-lactamase inhibitor combinations. Mechanisms for this resistance consist of productions of β lactamases, extended spectrum β-lactamase, AmpC and other enzymes. This is more evident since last three decades or so [1]. Predominantly, this mechanism was seen in gram negative bacilli. With treatment options compromised with these groups of antimicrobials, carbapenem emerged out as a last resort of drug against many of these resistant bugs [2].

Carbapenem antimicrobials drugs include imipenem, meropenem, ertapenem and doripenem, which are useful in multi drug resistant bacterial infections. But since last few years, this last frontier of antimicrobials has also been overcome by production of carbapenemase enzymes [3]. Most of the gram negative bacilli producing these carbapenem hydrolysing enzymes belong to *Enterobacteriaceae* family. Among this Carbapenem Resistant *Enterobacteriaceae* (CRE) family. *E.coli* and *Klebsiella pneumoniae* are the leading species producing these enzymes [3]. These carbapenemase enzymes belong to Class A, B and Class D of Ambler classification system. [4,5] These enzymes are encoded by genes which are mostly present on plasmid while few are chromosomal mediated [6]. Besides carbapenemase enzyme production, over expression of efflux pump and outer membrane porin loss are the other mechanism of development of carbapenem resistance [7].

The gold standard for detection of carbapenemase production is by molecular methods. But because of its cost effectiveness, and time consumption, phenotypic tests have been developed [8,9]. Various phenotypic tests to detect CRE include methods like MHT, CDT and Double disc synergy test [8,9]. These tests are growth dependent, turnaround time is 18-24 hours, and interpretations are also subjective [9,10]. Methods like the MHT are useful for detection of carbapenemases but has disadvantage of its low sensitivity and low specificity for NDM-1 [11].

A new phenotypic test modified Carbapenem Inactivation Method (mCIM) has been proposed in CLSI guidelines [12]. The test is easiest method especially in a limited laboratory setup [10]. This study therefore was conducted with aim to access efficacy of mCIM and its comparison with other phenotypic and molecular method.

MATERIALS AND METHODS

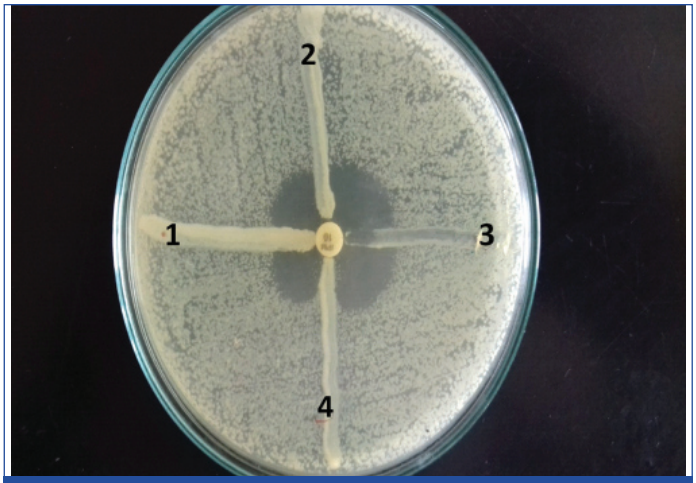
The laboratory-based prospective observational study was carried out at Department of Microbiology and Department of Molecular Biology and Genetics, KIMS, Karad.

After getting Institutional Ethical Committee Clearance (ECR/307/Inst/MH/2013/RR-16) and patient's informed written consent, non-repetitive specimens received in microbiology laboratory over a period of one year from January 2017 to December 2017 were included in the study. Total 1634 specimens received from indoor patients admitted at Krishna Hospital and Medical Research

Centre were processed for culture and sensitivity. Specimens of urine, endotracheal secretions, pus, sputum, cerebrospinal fluid, blood, and body fluids like pleural fluid, ascitic fluid, and catheter tips were included. Specimens from outdoor patients were excluded from the study. Processing of the specimens was done as per standard methods on Blood agar, Chocolate agar, and MacConkey's agar [13]. Blood specimens were processed using BacT/ALERT 3D system (Biomerieux, France) and after getting alert from the automation system, further subculture was done using standard methodology [13]. Bacterial colonies were identified by VITEK 2 compact (Biomerieux, France) automation system and antimicrobial susceptibility testing was done with the same system to detect Minimum Inhibitory Concentration (MIC) [14]. For this, gram negative antimicrobial panel was used which included imipenem, meropenem and ertapenem besides other antimicrobials. Interpretation of test was done as per CLSI-2017 guidelines. Out of the 499 clinical isolates grown on culture media from processed specimens, 242 belonged to *Enterobacteriaceae* family. Any *Enterobacteriaceae* isolate resistant to at least one carbapenem antibiotic is considered as CRE [12]. Total 66 CRE isolates were isolated and included for further study. Carbapenem sensitive *Enterobacteriaceae* were excluded from the study. Detection of carbapenemase production for these 66 isolates was done by phenotypic methods as follows.

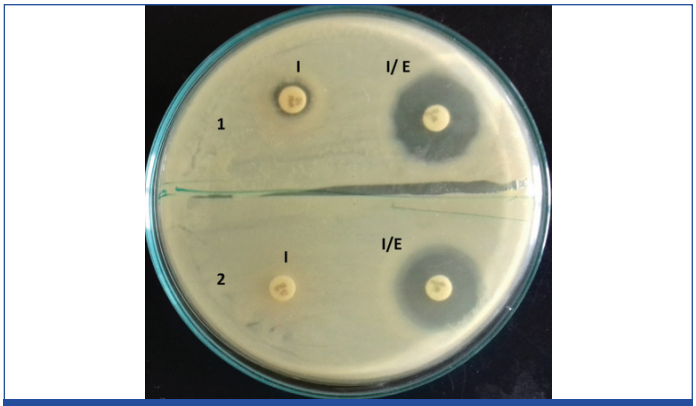
Modified Hodge Test (MHT): The test was performed according to the standard CLSI guidelines for the detection of carbapenemase in *Enterobacteriaceae* [12]. *E.coli* ATCC 25922 matching 0.5 McFarland turbidity was uniformly swabbed onto Muller Hinton Agar (MHA) and test isolate was streaked as a straight line from the edge of the meropenem disc (10 µg), to the edge of the plate. An indentation in the growth of the negative control towards the meropenem disc on either side of the test isolate was considered as positive for the production of the beta lactamase by the test isolate [Table/Fig-1]. Controls used in the test procedure were MHT Positive *Klebsiella pneumoniae* ATCC BAA-1705 and MHT Negative *Klebsiella pneumoniae* ATCC BAA-1706 [12].

Combined Disc Test (CDT): The combined disc test was performed as described by Pournaras S et al., with use of imipenem disc [15].



[Table/Fig-1]: Modified Hodge Test: 1) Positive isolate; 2) Negative isolate; 3) Negative Control; 4) Positive Control.

The test organisms were inoculated on Muller Hinton agar plates as per CLSI guidelines. After 10 minutes, two disc imipenem (10 µg) and imipenem/EDTA (10/750 µg) (Himedia) were placed on Muller Hinton agar plates. Plates were incubated at 37°C for overnight. The inhibition zones of imipenem and imipenem plus EDTA were compared after incubation. The test was considered positive for carbapenemase production when there was increase in zone of inhibition for imipenem plus EDTA of ≥5 mm than imipenem alone [Table/Fig-2] [15].

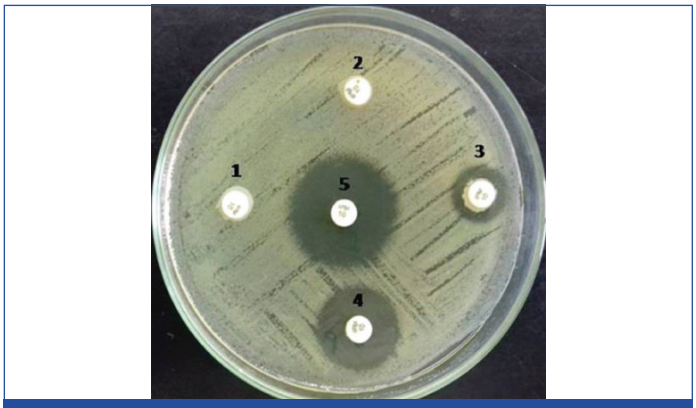


[Table/Fig-2]: Combined Disc Test. 1 and 2: CDT positive isolates.
I: Imipenem Disc; I/E: Imipenem+EDTA Disc

Modified Carbapenem Inactivation Method (mCIM): The mCIM method was carried for the all 66 isolates as per methodology led down by CLSI guidelines with use of imipenem disc [12]. A loopful of bacterial isolate from an overnight blood agar plate, was emulsified in 2 mL of Tryptone Soya Broth (TSB). A 10-µg imipenem disc was immersed in each broth using sterile forcep and was incubated at 37°C for four hours. Immediately following completion of the TSB-imipenem disc suspension incubation, a 0.5 McFarland suspension (using the direct colony suspension method) of *E. coli* ATCC 25922 in nutrient broth was made and inoculated on a Muller Hinton Agar (MHA) plate. *E. coli* ATCC 25922 act as susceptible strain for potent imipenem disc. Plates were allowed to dry for 3-10 minutes. Imipenem disc from TSB-imipenem disc suspension was dragged and pressed with help of sterile inoculating loop along the inside edge of the tube to expel excess liquid from the disk. Using the loop the disc was removed from the tube and then placed on the MHA plate previously inoculated with the imipenem-susceptible *E. coli* ATCC 25922 indicator strains [12]. MHA plates were incubated at 37°C in ambient air for 18-24 hours. To validate the results, *Klebsiella pneumoniae* ATCC BAA-1705, and *E. coli* ATCC 25922 were used as positive and negative controls.

Following incubation, zones of inhibition were measured as for the routine disc diffusion method [Table/Fig-3]. Interpretation of test was done as:

1. **Carbapenemase positive:** Zone 6-15 mm or presence of colonies within a 16-18 mm zone. If the test isolate produces carbapenemase, the imipenem in the disc will be hydrolysed and there will be no inhibition or limited growth inhibition of the imipenem-susceptible *E. coli* ATCC 25922.
2. **Carbapenemase negative:** Zone 19 mm or more. If the test isolate does not produce carbapenemase, the imipenem in the disc will not be hydrolysed and will inhibit growth of the imipenem-susceptible *E. coli* ATCC 25922.
3. **Indeterminate:** Zone 16-18 mm. The presence or absence of a carbapenemase cannot be confirmed.



[Table/Fig-3]: mCIM Test: 1) Positive Control; 2&3) Carbapenemase Positive; 4) Carbapenemase Negative; 5) Negative Control.

Molecular Detection of *bla*$_{NDM-1}$, *bla*$_{OXA-48}$, *bla*$_{KPC}$ by Multiplex PCR

Confirmation of CRE isolates was done by gold standard method that is multiplex PCR. Plasmid DNA was extracted from the screened positive 66 isolates by using the QI Aprep Miniprep Kit (QIAGEN) as per the manufacturer's instructions. Plasmid DNA concentrations were determined by the UV-Visible spectrophotometer (Shimadzu) by checking absorbance at 260/280 nm and the quality of plasmid DNA was checked on 1% agarose gel electrophoresis after staining with ethidium bromide. The PCR was first optimised to obtain all possible amplicons according to described protocols [3,16-18]. The extracted plasmid DNA of each isolates were subjected to multiplex PCR of the *bla*$_{NDM-1}$, *bla*$_{OXA-48}$, *bla*$_{KPC}$ genes by using the specific primer sets as shown in [Table/Fig-4]. For multiplex PCR, 20 µL PCR reaction mixture included 10 µL of master mixture (Qiagen), 0.5 µL of each primer making total 3 µL of all primers together, nucleic acid free water 4 µL, Q buffer 2 µL and 200 nanogram (ng) of purified DNA template of each sample. The PCR amplification programme was performed as per the following sequence; total of 30 cycles of multiplex PCR consisting initial denaturation step for 10 minutes at 95°C, denaturation step for 30 seconds at 95°C, annealing step for one minute at 55°C and extension step for one minute at 72°C and final extension of 10 minutes at 72°C with a thermal cycler (Master cycler gradient, Eppendorf). After multiplex PCR, the amplification products were analysed by 2.0% agarose gel electrophoresis at 100 V for one hour in 1X Tris Acetate EDTA (TAE) stained with 0.01 mg/mL ethidium bromide. A 1 Kb DNA ladder as a molecular weight marker was loaded in a gel along with the samples for confirmation of specific size of the corresponding gene fragment. The gels were stained with ethidium bromide (10 mg/mL) and visualised under UV transilluminator and photographed in gel documentation system (Bio-Rad Laboratories). Also, control strains were used as standards for presence or absence of corresponding genes. *Klebsiella pneumoniae* ATCC BAA-2146, *Klebsiella pneumoniae* ATCC BAA-1705 were used as positive control for *bla*$_{NDM-1}$, *bla*$_{KPC}$ respectively [3,17]. *Klebsiella pneumoniae* ATCC BAA-1706 was used as negative control. The isolate producing PCR product with 307 bp size of *bla*OXA-48 confirmed by DNA sequencing was used as positive control to compare with other results [Table/Fig-5].

Carbapenemase Gene Primers				
Gene	Forward/ Reverse	Primer Sequences	Amplicon size (bp)	Reference
NDM-1	Forward	5'-GGGCAGTCGCTTCCAACGGT-3'	475	3
	Reverse	5'-GTAGTGCTCAGTGTCGGCAT-3'		
KPC	Forward	5'-GCT CAG GCG CAA CTG TAA G-3'	150	17
	Reverse	5'-AGC ACA GCG GCA GCA AGA AAG-3'		
OXA-48	Forward	5'-GCGTGGTTAAGGATGAACAC-3'	307	18
	Reverse	5'-CGCTCCGATACGTGTAACTT-3'		

[Table/Fig-4]: Primer sequence used for carbapenemase encoded genes and their size.

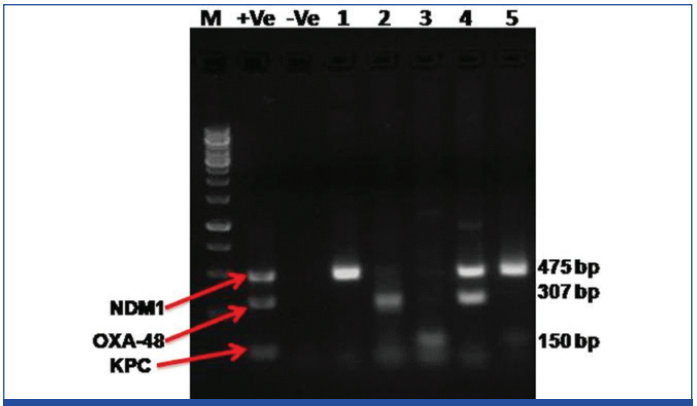
STATISTICAL ANALYSIS

All the data was analysed using software SPSS Statistics Version 20.

RESULTS

According to CLSI definition, 66 were CRE isolates, 37 (56%) of the isolates were from ICU, as compared to 29 (44%) from the wards. Leading isolate was *Klebsiella pneumoniae* (n=35) among different CRE species followed by *E.coli* (n=15) [Table/Fig-6]. Urine (n=27) was leading specimen which contributed for CRE isolates followed by pus (n=15) [Table/Fig-6]. Different types of specimens showed no significance with isolated bacterial species with Pearson Chi-square test value 35.3 and p-value is 0.501.

All the 66 isolates [Table/Fig-7] showed presence of at least one of the three carbapenemase encoded genes in the multiplex PCR study. Out of the 66 isolates, 54 showed presence of *bla*$_{NDM-1}$, while



[Table/Fig-5]: Representative agarose gel image showing multiplex gene specific PCR amplification of 475 bp fragment of NDM-1, 307 bp fragment of OXA-48 and 150 bp fragment of KPC genes. Lane M: 1 Kb DNA ladder, Positive control, Negative control, Lane 1and 5-NDM-1 Positive, Lane 2-OXA-48 Positive, Lane 3-KPC Positive, Lane-4 NDM-1 and OXA-48 positive isolates.

Bacterial species	Blood	Pus	Urine	Sputum	CSF	ETT secretion	Peritoneal fluid	Total
Citrobacter frieundii	0	1	0	0	0	0	0	1
Enterobacter cloacae	0	1	2	0	1	2	0	6
Escherichia coli	0	5	9	1	0	0	0	15
Klebsiella oxytoca	0	1	0	1	0	0	0	2
Klebsiella pneumoniae	5	7	9	4	2	7	1	35
Proteus mirabilis	0	0	3	0	0	0	0	3
Providencia rettgeri	0	0	4	0	0	0	0	4
Total	5	15	27	6	3	9	1	66

[Table/Fig-6]: Distribution of CRE isolates among different Clinical Specimens.

31 isolates were carrying *bla*$_{OXA-48}$ like gene. Only three isolates were having presence of *bla*$_{KPC}$. MHT was positive only in 50 (76%) isolates. A total of 59 isolates (89%) were positive by CDT method. The mCIM showed positive results for 65 isolates [Table/Fig-7]. mCIM was the most sensitive (98.48%) of all phenotypic methods in the study. In MHT and CDT there was significant positive correlation with correlation coefficient (r)=0.264, p-value 0.032 (p<0.05). In CDT and mCIM there was highly significant positive correlation with correlation coefficient (r)=0.36, p-value 0.003 (p<0.01). mCIM positive results (98.48%) were almost matching the results of the gold standard PCR method (100%).

Result	MHT	CDT	mCIM	Multiplex PCR
Positive	50	59	65	66
Percentage (%)	75.75	89.39	98.48	100

[Table/Fig-7]: Results for phenotypic and genotypic test of 66 CRE isolates.

DISCUSSION

Prevalence of CRE has increased worldwide very rapidly in last decade or so [19]. This is more critical, especially in Indian set up because of predominance of *bla*$_{NDM-1}$ as carbapenemase encoded gene in the CRE isolates [20]. This is also evident from multiplex PCR test in present study, as 54 out of 66 clinical isolates were carrying *bla*$_{NDM-1}$. The *bla*$_{NDM-1}$ and other plasmid mediated genes *bla*$_{KPC}$, *bla*$_{OXA-48}$, *bla*$_{IMP}$ and *bla*$_{VIM}$ are responsible for horizontal transmission of carbapenem resistance among *Enterobacteriaceae* family [6]. Among CRE isolates *Klebsiella pneumoniae* and *E.coli* were two leading Carbapenemase Producing-Carbapeneme Resistant *Enterobacteriaceae* (CP-CRE) clinical isolates. Similar findings have been found in Indian and global studies [21,22]. This becomes more worrisome factor as these two organisms are also present more in number in intestinal flora making it possible for rapid dissemination of CP-CRE in hospitalised patient especially in ICU setup

[21]. Early detection of this CP-CRE is very useful to prevent further spread among hospitalised patient and health care providers [23].

Molecular methods though gold standard for detection of CP-CRE has major disadvantage of high cost, making it unavailable in a limited resource laboratory [9]. In such scenario, phenotypic test becomes important. MHT method in the study, showed sensitivity of 75.75% only. Almost similar findings (75%) were observed by Dutta S et al., [24]. The MHT method has limitations because of its subjective interpretation and lower sensitivity and specificity [25]. CDT test showed more sensitivity (89.39%) compared to MHT in the present study. This is justifiable in present study, as genotype study showed presence of bla_{NDM-1} in 54 out of 66 isolates which is a metallo β lactamase encoding gene. CDT mechanism is based on inhibition of metallo β lactamase [6,15]. The disadvantage of CDT is its failure to detect serine based carbapenemase making it less sensitive to detect all isolates of CP-CRE [6,15].

Contrary, mCIM is simple, inexpensive, less subjective, reproducible and most sensitive method [10,25,26]. In the present study, only one isolate which was positive by PCR study, was negative by mCIM method, making this new method most sensitive (98.48%) among the phenotypic test studied and closing gap between phenotypic and genotypic methods. The mCIM method was able to detect serine as well metallo β lactamase producing isolates, although it has the disadvantage of inability to discriminate the type of carbapenemase and is time consuming [26,27].

LIMITATION

One of the limitation in the study was, it did not assess CRE isolates which were PCR negative. As a result, there was limitation in calculating sensitivity and specificity of phenotypic methods in relation to gold standard method and applying statistics. In the present study, all the CRE isolates were molecular positive and there was limitation to assess false positivity of phenotypic methods. But considering positive results, study recommends mCIM method for early and easy detection of CRE.

CONCLUSION

In a limited setup laboratory, early phenotypic detection of CRE is of great importance as this will guide clinicians and help to control the spread of carbapenemase producing CRE (CP-CRE) infections by contact precautions of the patient.

In a microbiology laboratory where molecular methods are unavailable, mCIM method will play important role compared to MHT and CDT, almost matching efficacy as that of gold standard molecular method for easy and early detection of CRE, thereby reducing morbidity and mortality.

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