Detection of carbapenem resistant enterobacteriaecae and the comparison between phenotypic methods and multiplex PCR

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Abstract
Introduction: Carbapenem resistant *Enterobacteriacea* (CRE) has created a remarkable health distress. Definitive detection of Carbapenemase producing CRE is the first step in combating this problem.
Objective: To detect and compare CP- CRE both by phenotypic and molecular methods.
Materials and Methods: A total of 52 carbapenem resistant clinical isolates were screened for the presence of carbapenemase genes by routine phenotypic methods like modified hodge test and combined disc test as well by multiplex PCR.
Results: Out of the total 52 meropenem resistant isolates, 35 were modified hodge test positive and 33 were combined disc test positive. 42 isolates were found harbouring one or more than one gene. blaKPC alone was present in 38 isolates, while as blaKPC with blaNDM were present in 1 isolate and blaKPC with blaIMP was seen in 1 isolate. blaNDM alone in 2 isoates, blaIMP and blaVIM alone in none of the isolates.
Conclusion: Accurate detection of carbapenemase producing genes by molecular methods overcomes the problem related to CRE. Though there is no signal method that is ideal for all situations.

Keywords: Modified hodge test, Combined disc test, Multiplex PCR, Carbapenem resistant *Enterobacteriaecae.*

Introduction
Enterobacteriaceae, are the most common causes of community-acquired and as well as nosocomial infections. These bacteria acquire resistance which in turn complicates the treatment. They can acquire genes that encodes for multiple antibiotic resistance mechanisms, including ESBLs, Amp Cs, and carbapenemases. Carbapenemases are clinically important because they destroy carbapenems as they are the antibiotic of last resort.[1] CRE are often resistant to β lactam drugs and can carry the resistance to other antimicrobial classes, thus limiting treatment options. Inappropriate treatment of severe infections caused by CP-CRE and lag in the detection is associated with increased mortality. So, they should be screened routinely for susceptibility to atleast one carbapenem. Several phenotypic methods are available for detection of carbapenemases like Modified Hodge test, combined disc test and Inhibitor based E test. The Modified Hodge test which is recommended by CLSI, is cheap and, simple to perform. However, its subjective, and it cannot distinguish among the different carbapenemase classes. Combined disc s tests have been extensively used because of low cost.

Recently, various molecular methods like multiplex PCR have been shown to be sensitive and scrupulous method for identification of genes. Both sensitivity and specificity of multiplex PCR assay is 100% and can determine class of β-lactamase present.[2]

Materials and Methods
Study Design
Prospective study was done for a period of one and a half year in the department of microbiology SKIMS institute soura.

Aims and Objectives
Comparing phenotypic methods like Modified Hodge test and Combined Disc test with multiplex PCR (VIM, IMP, KPC and NDM 1) for the detection of carbapenemases in CRE.

Isolates of *Enterobacteriaceae* obtained from blood of patients of all age groups admitted at SKIMS or attending the OPD were included in this study. Isolates other than *Enterobacteriaecae* were not included. Blood was processed for the recovery of bacterial pathogens as per standard microbiological techniques.[3] Further identification and susceptibility testing were done on the vitek 2 compact. So vitek 2 was the screening test. All clinically significant CRE isolates were included in this study.

Confirmation of MBL Production
All screen test positive isolates were subjected to combined disc test (CDT) using imipenem, meropenem and ceftazidime disc along with EDTA. The CDT was performed as described by Yong D et al.[4]

Confirmation of KPC
Carbapenemase (KPC) production was confirmed by Modified hodge test. The test isolate producing the carbapenemase enzyme will allow the growth of a carbapenem susceptible strain (*E. coli* ATCC 25922) towards a carbapenem disk.

Combined Disc Test (CDT)
For the test, imipenem (IMP) 10 µg, meropenem (MRP) 10µg and ceftazidime (CAZ) 30 µg discs was used. In addition to this 0.5mM EDTA solution was used. The EDTA impregnated discs were stored at -20°C in airtight vials after being dried in an incubator, till further use.[2] Increase in the zone size of 5-10 mm between inhibition

zone diameter of IMP, MRP, CAZ-EDTA disk and that of IPM, MRP, CAZ only.

Modified Hodge Test

5 ml of broth or saline was prepared by using 0.5 mcFarland of *E.coli* ATCC25922. After preparing 1: 10 dilution a lawn culture was made on a Mueller Hinton agar plate. 10µg meropenem susceptibility disk was placed in the centre. Test organism was streaked in a straight line from the edge of the disk to the edge of the plate. Plates were incubated overnight at 35^0C.

MHT Positive test will have a clover leaf-like indentation of the *E. coli* 25922 growing along the test organism growth streak.

MHT Negative test will have no growth of the *E. coli* 25922 along the test organism growth streak.

Quality Control

Positive and Negative Control: an in house known carbapenemase producing *Klebsiella* strain and carbapenemase negative *Klebsiella* strain.

Polymerase Chain Reaction

DNA was extracted by using bacterial lysates from overnight broths prepared by removing of 200µl of broth culture, centrifugation (1200x g; 2), re-suspension in 200µl of molecular grade water. Boiling at 95♌C for 20 min, and discarding the cellular debris by centrifugation. (12000 xg; 2 min at 4♌C). Extracted DNA was used for PCR. The resulting PCR products were analyzed in a 1% agarose gel with ethidium bromide staining and UV light. The design of the primers used for the detection of *bla*NDM-1, *bla*VIM, *bla*IMP and *bla*KPC genes are given below:

Primers	Sequence	Base pair
NDM 1 FP	GCATAAGTCGCAATCCCCG	237
NDM 1 RP	CTTCCTATCTCGACATGCCG	
VIM FP	GTTTGGTCGCATATCGCAAC	382
VIM RP	AATGCGCAGCACCAGGATAG	
IMP FP	GAAGGCGTTTATGTTCATAC	587
IMP RP	GTAAGTTTCAAGAGTGATGC	
KPC FP	TCGAACAGGACTTTGGCG	201
KPC RP	GGAACCAGCGCATTTTTGC	

Observation

The blood samples received over a period of one year and three months from in-patients and out-patients were processed for isolation and identification of bacterial pathogens according to the standard microbiological techniques.

A total of 120 non duplicate *Enterobacteriaceae* were isolated from patients admitted or attending the OPD. Out of these 52 were CRE. 40(76.9%) were *Klebsiella peumoniae*, 12(21.4%) were *Escherichia coli*, and 29(39.4%) were recovered from IPD, 23(62.1%) from ICU.

Out of the total 52 meropenem resistant isolates, 35(67.3%) were Modified hodge test positive. (Fig. 1). Combined disc test was also done on these 52 meropenem resistant isolates, out of them 33(63.4%) were combined disc test positive. (Fig. 2). Multiplex Polymerase chain reaction was done for *bla*KPC gene, *bla*NDM gene, *bla*IMP gene and *bla*VIM gene detection in 52 meropenem resistant isolates, 42(80.7%) isolates were found harbouring one or more than one gene. *bla*KPC alone was present in 38(73.0%) isolates, while as *bla*KPC with *bla*NDM were present in 1(1.9%) isolate and *bla*KPC with *bla*IMP was seen in 1(1.9%) isolate. *bla*NDM alone in 2(3.8%) isoates, *bla*IMP and *bla*VIM alone in none (0%) of the isolates. However in 10(19.2%) isolates, none of the gene was detected. (Fig. 3,4). Furthermore out of 35 MHT positive isolates, only 21 were CDT positive. However out of 33 CDT positive isolates 21 were MHT positive. (Table 1). Out of 42 PCR positive isolates MHT were positive in 31 isolates and out of 10 PCR negative isolates 4 were MHT positive. (Table 2). Again out of 42 PCR positive isolates 28 were positive by CDT as well and 14 were negative. Out of 10 PCR negative isolates 5 were CDT positive and 5 were CDT negative. (Table 3).

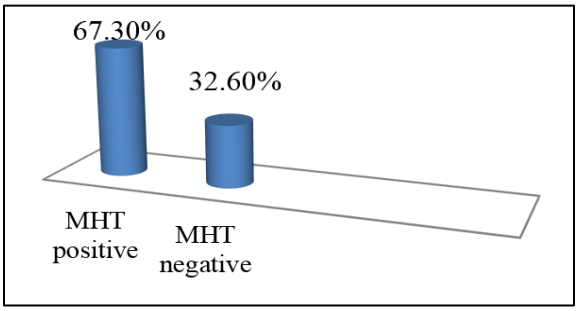

Fig. 1: Overall distribution of MHT positive and negative *Enterobacteriaceae* isolates

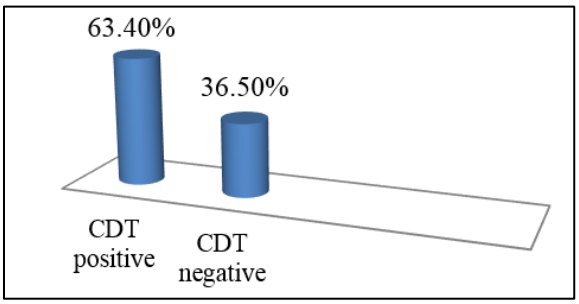

Fig. 2: Overall distribution of CDT positive and negative *Enterobacteriaceae* isolates.

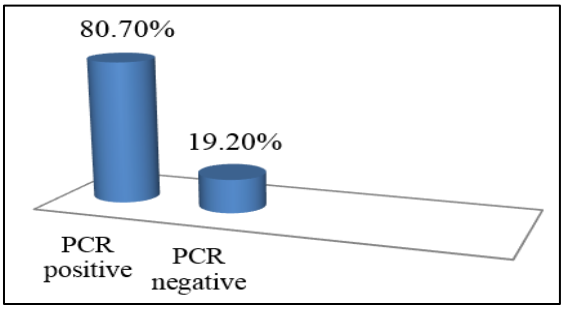

Fig. 3: Overall distribution of PCR positive and negative isolates

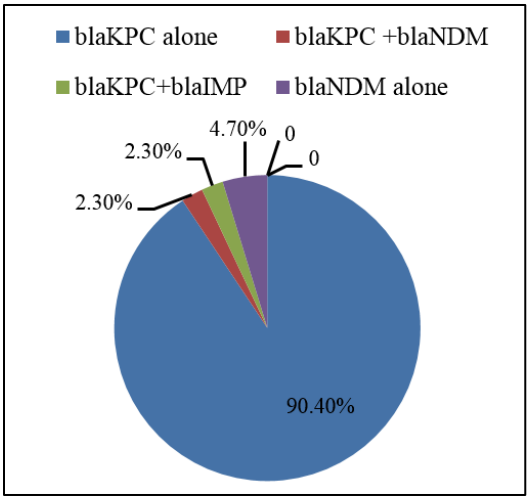

Fig. 4: Distribution of gene in PCR positive *Enterobacteriaceae* isolates.

Table 1: Comparison between Modified Hodge test and Combined Disc test.

MHT		CDT		
		Positive	**Negative**	**Total**
	Positive	21	14	35
	Negative	12	5	17
	Total	33	19	52

Table 2: Comparison between PCR and MHT among CRE

MHT		PCR		
		Positive	**Negative**	**Total**
	Positive	31	4	35
	Negative	11	6	17
	Total	42	10	52

Table 3: Comparison between PCR and CDT

CDT		PCR		
		Positive	**Negative**	**Total**
	Positive	28	5	33
	Negative	14	5	19
	Total	42	10	52

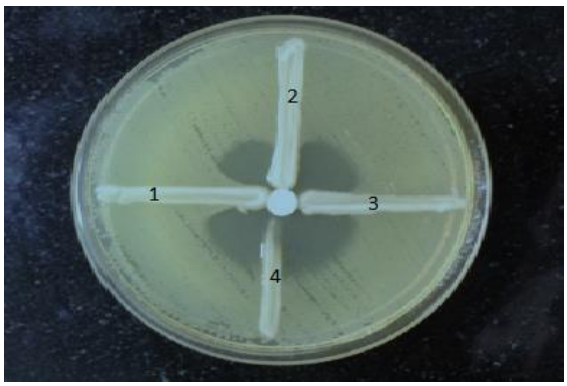

Fig. 5: Modified hodge test results for carbapenemase detection. **1:** Positive control, **2 and 3:** Rest strains showing positive result, **4:** Negative control.

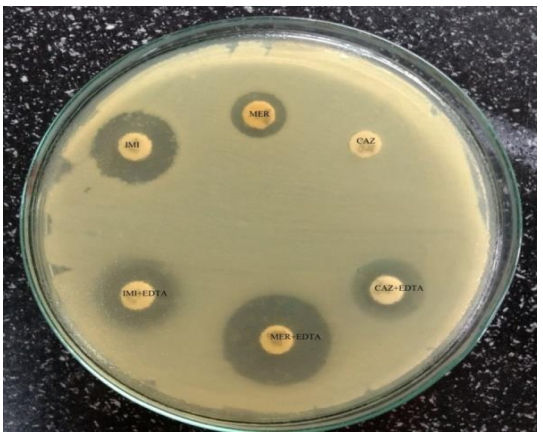

Fig. 6: Combined disc test (positive results) for detection of carbapenemase

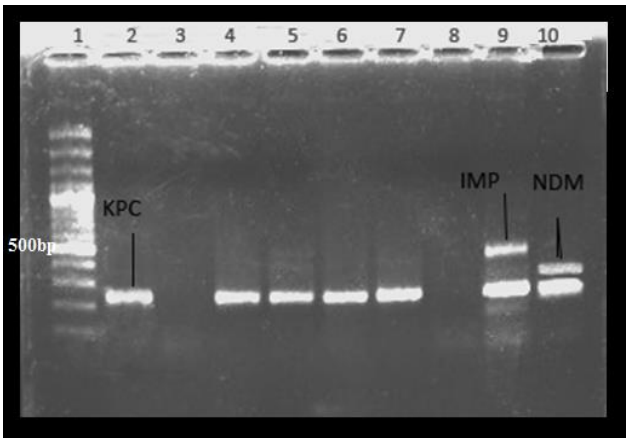

Fig. 7: Multiplex PCR results for *bla*KPC, *bla*NDM, *bla*IMP and *bla*VIM. **Lane 1:** 100bp ladder, **Lane 2:** *bla*KPC, **Lane 3 and 8:** negative results, **Lane 4-7:** *bla*KPC, **Lane 9:** *bla*KPC+IMP, **Lane 10:** blaKPC+NDM.

Discussion

Carbapenem-resistant *Enterobacteriaceae* (CRE) are worldwide a health problem. These multidrug-resistant organisms cause infections associated with high mortality and limited treatment options, and are increasingly recognized as an important cause of health care-associated infections.[5-9] Among *Enterobacteriaceae* members, *E. coli* and *K. pneumoniae* are the most important causative agents of hospital and community acquired infections.[10] In order to reduce and control the spread of carbapanem resistance, rapid identification is crucial. The screening for Carbapenamase producers in clinical specimens is based on phenotypic tests, whereas confirmation tests are mainly based on molecular assay. However, traditional phenotypic methods are time consuming, difficult to interpret, and the sensitivity/specificity vary between different species. To address this issue, a variety of molecular methods have been developed, PCR-based methods are rapid than conventional microbiological methods, but they require sophisticated instrumentation and technical expertise.

Out of these 120 isolates only 52(43.3%) were found to be meropenem resistant, which includes 40(76.9%)

Klebsiella pueumoniae isolates and 12(21.44%) *E. coli* isolates. None *Enterobacter cloacae* isolate was found resistant to meropenem. Most of the isolates 83(69.1%) were isolated from IPD. Of these 83 isolates recovered, 29(35%) were meropenem resistant. Contrary to this out of 37 isolates recovered from ICU, 23(62%) were meropenem resistant. Also according to Bhatt et al., most of the resistant isolates were obtained from acute wards (42.9%) and intensive care units (29.5%), followed by other wards (23.2%) and the outpatient department (4.4%).[11] In our study, out of 52 meropenem resistant isolates Modified Hodge test was positive in 35(67.3%) isolates. Combined disc test was positive in 33(67%) of the isolate. Similar results were seen in a study conducted by Rachana Solanki et al. where out of the 100 carbapenem resistant isolates, 70(70%) isolates were MHT positive, and 65(65%) isolates were CDT positive.[12]

In this study out of 52 meropenem resistant isolates, 42(80.7%) were PCR positive. The most prevalent gene being *bla*KPC which was detected in 38(90.4%) isolates, followed by *bla*NDM alone (4.7%). Furthermore, the KPC gene co-existed in the same isolate with at least two other carbapenamase genes in 2 isolates. *bla*KPC+IMP was detected in 1(2.3%) isolates and *bla*KPC+NDM in 1(2.3%) isolates. *bla*IMP alone and *bla*VIM alone was not detected. Our results are in accordance with the study conducted by Asifa et al. who in their study found that out of 55 meropenem resistant organism, 36(64.5%) were PCR positive harbouring *bla* KPC gene. Contrary to our results, a study conducted by Nicolas Kieffer et al. found that among 57 carbapenemase-producing isolates, 50 were found positive for the $bla_{\text{OXA-181}}$ gene and 7 were positive for the *bla*NDM-1 gene. None of the isolates co-produced two carbapenemases.[13] In our study while comparing MHT with CDT we found that out of 35 MHT positive isolates only 21 were positive by CDT. And out of 33 CDT positive isolates MHT was positive in 21 isolates. Both MHT and CDT were positive in 12 isolates. Our results were in discordance with the study conducted by Sathya Pandurangani, who found that out of 65 CDT positive 61 were detected by MHT. Both MBL and MHT screen were positive in 12 isolates.[14]

While comparing MHT with PCR, it was seen that 31 were positive both by MHT and PCR, 11 were PCR positive and MHT negative which and 4 were PCR negative and MHT positive, this variable susceptibility can be explained by presence of silenced gene, or it can be observed with strains producing ESBLs or AmpC with decreased porins. Currently the widely accepted method for MBL confirmation is the E-test. But its high cost has forced many laboratories to use alternative methods like CDT.[15] In our study, by comparing CDT with PCR we found that 28 isolates were positive both by PCR and CDT. While 14 were PCR positive and CDT negative. Also 5 isolates were PCR negative and CDT positive, giving us the sensitivity of 66.6% and specificity 50% which is discordant with the specificity of 100% seen by Rachana Solanki et al.[12] CDT have shown discordant results depending upon the methodology employed, β-lactam substrates, MBL inhibitors and the bacterial genus tested.[16]

Conclusion

We found that Modified hodge test and Combined disc test were both sensitive for detecting CP CRE, so can be used for screening all isolates of *Enterobacteriaceae* for carbapenemase production. It is cost effective method for detection of carbapenemases among *Enterobacteriaceae*. However the sensitivity of these phenotypic methods was slightly low as compared to molecular methods like PCR. Keeping in view the higher cost factor associated with the molecular testing and their limited role in diagnostic practice, we recommend the use of routine phenotypic methods for detection of carbapenemases in laboratory and molecular methods for epidemiological surveillance purpose.

Conflict of Interest: None.

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