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Research article

EVALUATION OF MODIFIED HODGE TEST AS AN INDICATOR OF KLEBSIELLA PNEUMONIAE CARBAPENEMASE (KPC) PRODUCTION BY USING bla $_{KPC}$ GENE PCR

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ABSTRACT

Introduction: Carbapenems belong to the Beta Lactam group of antimicrobial agents. They are often used as "last-line agents" or "antibiotics of last resort" in critically ill patients. Carbapenem resistance in *Enterobacteriaceae* may be due to various reasons but *Klebsiella pneumoniae* Carbapenemase (KPC) enzyme production is the commonest. Phenotypic as well as genotypic methods can be used to detect Carbapenemases. Among the phenotypic tests, Modified Hodge Test (MHT) is relatively easy to perform. **Aims and Objectives:** This study aimed to determine the prevalence of carbapenem resistance among Enterobacteriaceae isolates and calculate the sensitivity of MHT as an indicator of KPC production. **Materials and Methods:** All Enterobacteriaceae isolates from clinical samples were included in this study and were screened for Carbapenem resistance. 45 randomly chosen Carbapenem Resistant Enterobacteriaceae isolates were subjected to MHT and bla$_{KPC}$ gene detection by PCR. **Results:** 2035 Enterobacteriaceae isolates were tested and 5.2% were found to be resistant to Imipenem, 22.9 % were resistant to Meropenem and 4.42 % were resistant to both Imipenem and Meropenem. The sensitivity of MHT was calculated to be 90% and specificity was calculated to be 60%

Keywords: Carbapenemase, Modified Hodge Test, Enterobacteriaceae, bla $_{KPC}$ gene

INTRODUCTION

Bacteria belonging to the *Enterobacteriaceae* family are normally present as harmless human gut flora. These bacteria are the leading cause of a wide range of opportunistic infections.[1] Carbapenems belongs to the Beta Lactam group of antimicrobial agents, which kill bacteria by inhibiting the bacterial cell wall synthesis.[2] They possess the broadest spectrum of activity and greatest potency against Gram-positive and Gram-negative bacteria. [3] As a result, they are often used as "last-line agents" or "antibiotics of last resort" when patients with infections become gravely ill or are suspected of harboring resistant bacteria.[3] Carbapenem resistance among *Enterobacteriaceae* members is of great concern as these bacteria are easily transmissible among patients, leading to hospital acquired infections (HAI), but can also spread into the community, resulting in community acquired cases.[1]

Carbapenem resistance in *Enterobacteriaceae* may be due to various reasons that include hyper production of the Amp C beta lactamase, loss of porins, production of metallo beta lactamases (MBL) and production of *K pneumonia* carbapenemases.[1]

Carbapenemases increasingly have been reported worldwide in *Enterobacteriaceae* in the past 10 years.[4, 5] A large variety of carbapenemases has been identified in *Enterobacteriaceae* belonging to 3 classes of β-lactamases: the Ambler class A, B, and D β-lactamases. [3] In addition, rare chromosome encoded cephalosporinases (Ambler class C) produced by *Enterobacteriaceae* may possess slight extended

activity toward carbapenems.[4] Their identification is of primary importance since carbapenemase producers are resistant not only to most Beta-lactams but also to other main classes of antibiotics. [5]

Carbapenemases can be detected by phenotypic as well as genotypic methods.[6] Modified Hodge Test (MHT) is a phenotypic method which is relatively simple and easy to perform in a laboratory. [6] This cloverleaf technique, or Modified Hodge test, has been extensively used as a phenotypic technique for detecting carbapenemase activity. [5]

Limitations of the MHT in terms of clinical performance are its lack of specificity and the delay in obtaining the results upto 24 or 48 hours after isolation of a bacterial colony.[5] The molecular detection of bla_{KPC} is the gold standard for diagnosis, but the majority of laboratories in our country does not have the resources necessary to perform this test. [7]

The aim of this study was to determine the prevalence of Carbapenem resistance among Enterobacteriaceae and detect the bla $_{KPC}$ gene prevalence among randomly chosen Carbapenem resistant Enterobacteriaceae clinical isolates in our 750 bed hospital. The study also aimed to evaluate the performance of the Modified Hodge test and calculate the sensitivity of MHT as an indicator of Klebsiella Pneumoniae Carbapenemase (KPC) production.

MATERIALS AND METHODS

The isolates in this study were collected in a period of one year from July 2012 to June 2013. The study was conducted at Chettinad Hospital and Research Institute, Chennai. The institutional ethical committee approval was obtained prior to commencement of this study. All Enterobacteriaceae isolates from clinical samples like pus, wound swab, sputum, endotracheal aspirate, blood and urine were included in this study. The samples were collected after obtaining informed consent from the patients. For evaluation of Modified Hodge Test by PCR, 45 isolates which were resistant to both or either Imipenem or Meropenem were randomly selected. MHT and bla $_{KPC}$ PCR were performed on these 45 isolates

These isolates were screened for Carbapenem resistance in addition to the routine antibiotic susceptibility testing, which was performed by the Kirby Bauer method, as per the CLSI guidelines 2012. [8] Antimicrobial discs used were Ampicillin (10µg), Gentamicin (10µg), Amikacin (30µg), Cefazolin (30 µg), Cefuroxime (30µg) Ceftazidime (30µg), Cefotaxime (30µg), Ceftriaxone (30µg), Cefepime (30µg), Ciprofloxacin (5µg), Cotrimoxazole (23.75/1.25µg), Piperacillin/ tazobactam (100/10µg), Imipenem (10µg), Meropenem (10 µg), Polymyxin B (300 units) and Colistin (10µg).

Modified Hodge Test

Phenotypic detection of Carbapenemase production was done by using the Modified Hodge test. This test was performed as per the CLSI guidelines 2012. [8] A 0.5 Mac Farland's suspension of ATCC *Escherichia coli* 25922 was diluted 1:10 in sterile saline. This was inoculated on a Mueller Hinton agar plate, as for the routine disc diffusion testing. The plate was dried for 5 minutes and a disc of Imipenem 10 µg was placed in the centre of the agar plate. 3-5 colonies of the test organism were picked and inoculated in a straight line, from the edge of the disc, up to a distance of at least 20mm. The plates were incubated at 37^0C overnight and they were examined next day. They were checked for an enhanced growth around the test organism, at the intersection of the streak and for a zone of inhibition. The presence of an enhanced growth indicated Carbapenemase production, and the absence of an enhanced growth meant that the test isolate did not produce carbapenemase. [8]

bla $_{KPC}$ gene detection

The isolates which gave positive results for the modified Hodge test were submitted to molecular detection of the bla $_{KPC}$ gene by PCR. The PCR Kits were procured from Helini Biomolecules, Chennai. Isolates with negative test results were also randomly chosen for PCR. The Polymerase Chain Reaction was set up in a PCR vial, after adding the master mix, the forward and reverse primers and the extracted DNA from the isolates. The primers used for PCR amplification and the reaction conditions were Forward Primer: 5'-GCT CAG GCG CAA CTG TAA G-3' Reverse Primer: 5'-AGC ACA GCG GCA GCA AGA AAG-3'. The PCR vial was placed in PCR machine (Corpect Research 96 wells, Australia) and it was subjected to initial denaturation at 94ºC for 3 min, followed by 30 cycles of denaturation at 94ºC for 1 minute, annealing at 60ºC for 1minute and extension at 72ºC for 1minute. A final extension procedure was carried out at 72º C for 5 min. Next the PCR products were subjected to electrophoresis on 2% agarose gel stained with ethidium bromide and visualized with UV

light The antibiogram for the Carbapenem Resistant Enterobacteriaceae was analyzed

RESULTS

2035 non repetitive isolates of Enterobacteriaceae were obtained from clinical samples such as pus, urine, blood, sputum and endotracheal aspirates, over a period of 1 year. Of these, 1178 (57.88%) were *Escherichia coli*, 444 (21.81) were Klebsiella species, 178 (8.7%) Proteus species, 154 (7.56%) Citrobacter species and 81 (3.9%) were Enterobacter isolates (Figure1). Figure 2 shows the number of carbapenem resistant isolates among the individual genera. The antibiogram of the Enterobacteriaceae isolates is tabulated in Table 1 and the antibiotic resistance pattern of the CRE are tabulated in Table 2.

Of the total of 2035 isolates, 106 (5.2%) were resistant to Imipenem and 468 (22.9 %) were resistant to Meropenem and 90 (4.42 %) were resistant to both Imipenem and Meropenem.

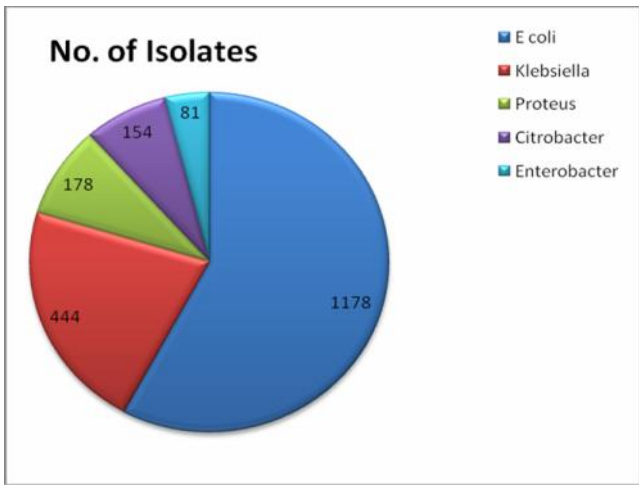

Fig 1: Total number of Enterobacteriaceae isolates

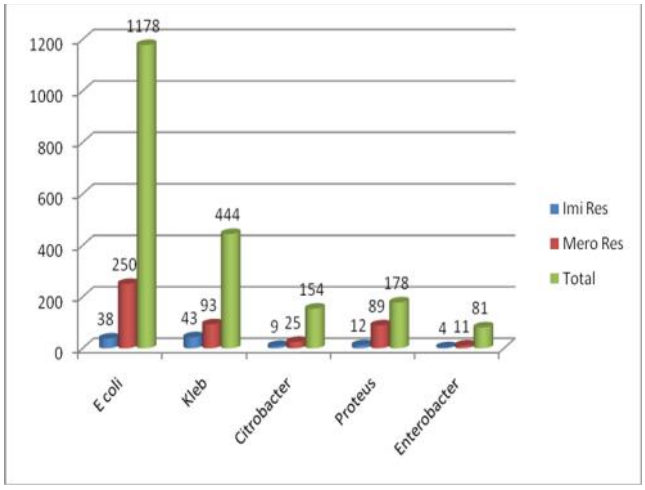

Fig 2: Carbapenem Resistance pattern of the Isolates

Table 1: Antibiotic Resistance pattern of the Enterobacteriaceae (% of Resistance)

ANTIBIOTIC	Ecoli	Klebsiella	Proteus	Citrobacter	Enterobacter
Amikacin	9.67 %	16.64 %	31.46 %	16.88 %	11.11 %
Ampicillin	85.7 %	100 %	71.51%	88.42 %	69.14 %
Cefazolin	71.3%	66.67 %	82.58 %	72.73 %	82.72 %
Cefuroxime	64.9 %	59.46 %	66.83 %	58.44 %	65.43 %
Cefotaxime	61.1 %	53.38 %	49.43 %	51.3 %	46.91 %
Cefepime	53.2 %	42.79 %	39.32 %	37.66 %	38.27 %
Ciprofloxacin	63.1 %	47.07 %	58.99 %	40.26 %	41.98 %
Cotirmox	55.2 %	50.9 %	68.02 %	46.75 %	45.68 %
Colistin	0 %	0 %	100 %	2.6 %	0 %
Genta	36.76 %	34.0 %1	46.07 %	34.42 %	32.1 %
Imipenem	3.23 %	9.68 %	6.82 %	5.84 %	4.94 %
Meropenem	21.3 %	20.95 %	50.15 %	16.23 %	13.58 %
Nitrofurantoin	11.7 %	31.15 %	87.87 %	33.78 %	43.48 %
Norfloxacin	59.54 %	31.15 %	49.2 %	36.49 %	30.43 %
Piperacillin Tazobactam	19.9 %	21.78 %	15.47 %	21.52 %	13.58 %
Polymyxin B	0 %	0 %	100 %	0 %	1.23 %
Tobramycin	39.47 %	16.17 %	48.14 %	29.49 %	34.78 %

Table 2: Antibiotic resistance pattern of the 45 Multi Drug Resistant Enterobacteriaceae

Percentage of Resistance	Klebsiella(n =22)	Ecoli (n =20)	Citrobacter (n =2	Proteus (n = 1)
Amikacin	59 %	33%	100 %	100 %
Ampicillin	100 %	100 %	100 %	100 %
Cefazolin	100 %	100 %	100 %	100 %
Cefuroxime	100 %	100 %	100 %	100 %
Cefotaxime	100 %	100 %	100 %	100 %
Cefipime	96%	100 %	100 %	100 %
Ciprofloxacin	91 %	91%	100 %	100 %
Cotrimoxazole	100 %	100 %	100 %	100 %
Colistin	0 %	5%	0 %	100 %
Gentamicin	73 %	67%	100 %	100 %
Imipenem	77 %	67%	100 %	100 %
Meropenem	96 %	95%	50 %	100 %
Nitrofurantoin (urine)	100%	20%	100 %	100 %
Piperacillin Tazobactam	100 %	100 %	100 %	100 %
Polymyxin B	0 %	0%	0 %	100 %
Tobramycin	94 %	73%	100 %	100 %

For evaluation of Modified Hodge Test by bla $_{KPC}$ detection using PCR, 45 isolates which were resistant to both or either Imipenem or Meropenem had been selected. Of these, 22 were *Klebsiella pneumoniae*, 20 were *Escherichia coli*, 2 were Citrobacter species and 1 was *Proteus mirabilis*). Of the 45 isolates, 43 (95.5 %) were resistant to Meropenem, 34 (75.5%) were resistant to Imipenem and 29 (64.4%) were resistant to both Imipenem and Meropenem. Modified Hodge Test was positive in 37 (82.2%) out of 45 isolates and bla $_{KPC}$ gene was detected in 30 (66.7 %) isolates (Table 3). Of the 30 bla$_{KPC}$ gene positive isolates, MHT was positive in 27 and negative in 3 isolates. Of the 15 bla$_{KPC}$ gene negative isolates, MHT was positive in 10 (66.6%) and negative in 5 (33.3%) isolates. Considering PCR for bla $_{KPC}$ gene as the gold standard for the detection of *Klebsiella pneumoniae* Carbapenemase, the sensitivity and specificity of MHT was calculated using the formula: Sensitivity = a/a+b and Specificity = d/ c+d. [Where 'a' is True Positive (27), 'b' is False Negative (3), 'c' is False Positive (10) and'd' is True Negative (15)]. In the present study, the sensitivity of MHT was calculated to be 90 % and the specificity was 60%. The positive predictive value was 72.97% and the Negative predictive value was 83.33%. Figure 3 shows isolates with positive MHT, displaying the characteristic clover leaf like indentation and Figure 4 shows the visualization of the PCR products by gel electrophoresis.

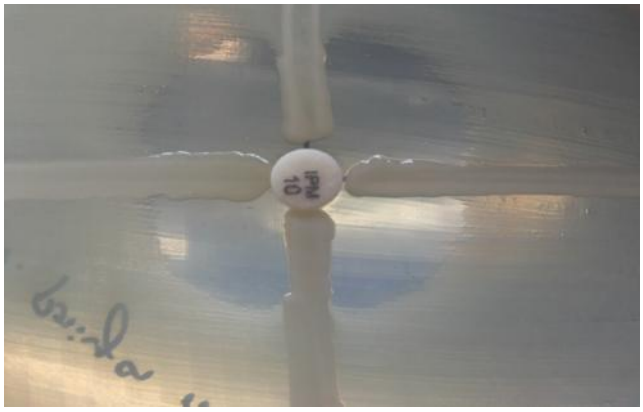

Fig 3: Positive Modified Hodge Test

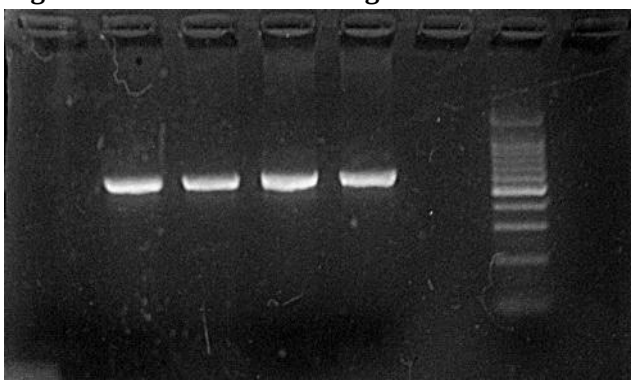

Fig4: bla $_{KPC}$ gene PCR : Gel Electrophoresis showing positive and negative isolates

Table 3: Results of MHT and bla$_{KPC}$ gene PCR

	Total Isolates	**MHT (+ve)**	**Bla $_{KPC}$ gene(+ve)**	**MHT & bla $_{KPC}$ gene (+ve)**
Klebsiella	22	19	13	11
E coli	20	16	15	14
Citrobacter	2	1	1	1
Proteus	1	1	1	1
Total	45	37	30	27

DISCUSSION

In our study, 5.2% of the isolates were resistant to Imipenem, 22.9 % were resistant to Meropenem and 4.42 % were resistant to both Imipenem and Meropenem. The highest percentage of resistance to Carbapenems was seen in Klebsiella species, 9.68% to Imipenem and 20.9% to Meropenem, followed by *Escherichia coli*, Proteus, Citrobacter and Enterobacter (Table 1). A study by Ramana et al [1] showed that, among the different *Enterobacteriaceae* members tested, *Klebsiella spp*. showed the highest percentage of carbapenem resistance at 30%, whereas *Proteus spp*. and *Citrobacter* spp revealed comparatively low carbapenem resistance of 17% and 12%, respectively. The prevalence of carbapenem resistance in our study was less than that of Ramana et al. Another study by Parveen et al [9] showed that 45 (43.6%) of *K. pneumoniae* from clinical specimens, were resistant to meropenem by the disk diffusion test. Among isolates reported to the National Healthcare Safety Network in 2006–2007 carbapenem resistance was reported in up to 4.0% of Escherichia coli and 10.8% of K. pneumoniae isolates that were associated with certain device-related infections. [10]

In the present study, the sensitivity of Modified Hodge test was calculated to be 90%. A similar study by Anderson et al [11] which had also evaluated the modified Hodge test for detection of KPC-mediated resistance inferred that the test demonstrated 100% sensitivity and specificity for detection of KPC activity. Diana Doyle et al [12] in her study showed that MHT had a sensitivity of 98% for detecting KPC producers and 93% for OXA-48-like enzyme producers but was less than optimal for detecting MBLs. The sensitivity of MHT as inferred by our study was less than that of the sensitivity of the other 2 studies. This could necessitate changes in the MHT to make it more sensitive. A study was carried out by Pasteren et al [13] using an optimized MHT known as Pseudomonas aeruginosa MHT (PAE MHT) which demonstrated 100% sensitivity and 98% specificity for detection of KPC activity without any indeterminate result. Another study in Greece, showed that Modified Hodge test detected 98% KPC producers, keeping PCR as the gold standard [6] In contrast, another study by D. Girlich et al [5] showed that the overall sensitivity and specificity of the MHT was low (77.4% and 38.9%, respectively). In our study too, the specificity of MHT was low- only 60%. Hatipoglu et al [14] also state that the MHT has a low specificity. This could be because Modified Hodge Test detects other carbapenemase enzymes in addition to KPC.

Of the 3 MHT negative isolates, one was positive for bla $_{KPC}$ gene. Adriane BC et al [15] have also reported such isolates carrying silent genes. 8 (17.7%) isolates were resistant to carbapenems, but were MHT negative. They may have developed a different resistance mechanism other than carbapenemase production.

Prevalence of bla $_{KPC}$ gene was found to be 66.67% among the carbapenem resistant isolates. bla$_{KPC}$ gene was not detected in 10 MHT positive isolates. This could indicate the presence of a carbapenemase other than KP Carbapenemase. Resistance to both imipenem and meropenem is a strong indicator of carbapenemase production rather resistance to either one of the carbapenems, as this may imply a different resistance mechanism

CONCLUSION

Modified Hodge Test is a sensitive test for Klebsiella pneumoniae Carbapenemase production. It is recommended that all isolates showing intermediate sensitivity or resistance pattern to carbapenems be screened for the production of carbapenemases by Modified Hodge test, which will provide a cost-effective and rapid approach for the detection of carbapenemases in *Enterobacteriaceae.*

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