Original Article

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Microbiology Section

bla$_{KPC}$ gene Detection in Clinical Isolates of Carbapenem Resistant *Enterobacteriaceae* in a Tertiary Care Hospital

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ABSTRACT

Introduction: Carbapenem resistance among *Enterobacteriaceae*, especially in *Klebsiella pneumoniae* and *Escherichia coli*, is an emerging problem worldwide. A common mechanism of carbapenem resistance is the production of class-A, *Klebsiella pneumoniae* carbapenemase (KPC).

Aims and Objectives: The present study focused on determining the antibiotic resistance pattern and prevalence of bla $_{KPC}$ gene coding for KPC in carbapenem resistant *Enterobacteriaceae*.

Methodology: Forty six carbapenem resistant isolates belonging to the family *Enterobacteriaceae* were tested for antibiotic sensitivity pattern. Modified Hodge Test (MHT) and PCR for bla $_{KPC}$ gene detection were performed on these isolates. Of these, 22 were *Klebsiella pneumoniae*, 21 were *Escherichia coli*, 2 were *Citrobacter* species and 1 was *Proteus mirabilis*

Results: Forty three (93.4%) out of the 46 isolates were resistant to Meropenem, 34 (73.9%) were resistant to Imipenem and 30 (65.2%) were resistant to both Imipenem and Meropenem. Modified Hodge Test was positive in 38 (82.6%) out of 46 isolates and bla$_{KPC}$ gene was detected in 31 (67.4%) isolates. bla $_{KPC}$ gene was detected in 28 out of the 38 MHT positive isolates.

Keywords: Enterbacteriaceae, Carbapenem resistance, Modified Hodge Test, bla $_{KPC}$ gene

INTRODUCTION

Enterobacteriaceae are a family of Gram negative bacilli which form part of normal human intestinal flora. Carbapenems belong to the beta-lactam group of antimicrobial agents, which kill bacteria by inhibiting the bacterial cell wall synthesis [1]. They possess the broadest spectrum of activity and greatest potency against Gram-positive and Gram-negative bacteria. 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 [2]. Unfortunately, the recent emergence of multidrug-resistant (MDR) pathogens seriously threatens this class of lifesaving drugs.

Carbapenem resistance among *Enterobacteriaceae*, in particular among *Klebsiella pneumoniae* and *Escherichia coli*, is an emerging problem worldwide [3]. Several resistance mechanisms have been reported to circumvent the efficacy of carbapenems, and carbapenemases are the most prominent enzymes that neutralize carbapenems [3]. Carbapenemases have a breadth of spectrum unrivaled by other beta lactamases [4].

Carbapenem resistance in *Enterobacteriaceae* can be mediated by metalloblactamases, class A carbapenemases, or on rare occasions by OXA-type carbapenemases [5]. Other resistance mechanisms are attributed to altered affinity of PBPs for carbapenems, increased efflux of the b-lactam antibiotics, decreased permeability of the outer membrane,or to a combination of reduced permeability and high-level production of a b-lactamase, typically a class C β-lactamase [5].

A common mechanism of carbapenem resistance is the class-A, *Klebsiella pneumoniae* carbapenemase (KPC). In 2001, the first KPC-producing *K pneumoniae* isolate was reported in North Carolina. The enzyme (KPC-1) is an Ambler class A beta-lactamase [6]. KPCs have been reported in *K pneumoniae* and in *Klebsiella oxytoca, Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, Citrobacter freundii, Enterobacter* spp, *Serratia* spp, and *Salmonella* spp. The *bla*$_{KPC}$ genes that encode KPCs are present on transferable plasmids and are flanked by transposable elements, thus allowing for the gene to move from plasmid to the bacterial chromosome and back [7]. The KPC enzyme confers resistance to all beta lactam agents including penicillins, cephalosporins, monobactams, and carbapenems [8]. In order to control the spread of *bla*KPC-containing bacteria in hospitalized patients, effective infection control measures and controlled antibiotic usage must be complemented by the utilization of rapid and sensitive *bla* KPC diagnostic assay [1].

The present study focused on determining the antibiotic resistance pattern and prevalence of bla KPC gene in Carbapenem Resistant *Enterobacteriaceae*.

MATERIAL AND METHODS

The study was conducted in Clinical Microbiology Lab at Chettinad Hospital and Research Institute, Chennai, for a period of 1 year. All *Enterobacteriaceae* isolates from clinical samples like pus, wound swab, sputum, endotracheal aspirate, blood and urine were screened for Carbapenem resistance. Antibiotic susceptibility testing was performed for these isolates by Kirby Bauer method, as per the CLSI guidelines 2012. Antimicrobial discs used were Ampicillin (20µg), Gentamicin (10µg), Amikacin (30µg), Cefazolin (30 µg), Cefuroxime (30µg) Ceftazidime (30µg), Cefotaxime (30µg), Ceftriaxone (30µg), Cefepime (30µg), Ciprofloxacin (5µg), Cotrimoxaole (1.25 µg/23.75 µg), Piperacillin/tazobactam (100/10µg), Imipenem (10µg), Meropenem (10 µg), Polymyxin B (300 units) and Colistin (10µg). The institutional ethical committee approval was obtained prior to commencement of this study.

46 isolates of *Enteobacteriaceae*, which were resistant to either Imipenem or Meropenem or both, were selected for further testing by Modified Hodge Test and *bla* $_{KPC}$ gene detection.

Modified Hodge Test

Carbapenemase production was detected by using the Modified Hodge test. This test was performed as per the CLSI guidelines

2012. 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, upto a distance of at least 20mm. The plates were incubated at 37°C 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 [9].

bla $_{KPC}$ gene Detection

DNA Extraction: The isolates to be tested were inoculated into sterile nutrient broth. After overnight incubation, the broth was transferred to sterile Eppendorf vials and centrifuged at 10000 RPM for 10 minutes. The supernatant was discarded while the pellet was used for the *bla* $_{KPC}$ gene detection

The pellet was suspended in 200µl of PBS and 50µl of Lysozyme was added and incubated at 37°C for 15min. Then 400µl of Lysis buffer and 40µl of proteinase K (10mg/ml) were added and gently mixed well. This was incubated in water bath at 70°C for 10 min. The whole lysate was transferred into Pure Fast spin column and centrifuged at 10000 rpm for 1min. The flow through was discarded and 500µl of Wash Buffer was added and centrifuged at 10000 rpm for 1 min. The wash procedure was repeated one more time. The flow through was discarded and the column was centrifuged for additional 2 minutes to remove any residual ethanol. DNA was eluted by adding 100µl of Elution Buffer and it was centrifuged for 1min. 1µl of extracted DNA was used for PCR amplification.

PCR Procedure

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. 25µl of Master Mix contained 10X Taq buffer, 2mM Mgcl2, 0.4mM dNTPs mix, and 2U *Proofreading* Taq DNA polymerase. The primers used were

Forward Primer: 5'-GCT CAG GCG CAA CTG TAA G-3'

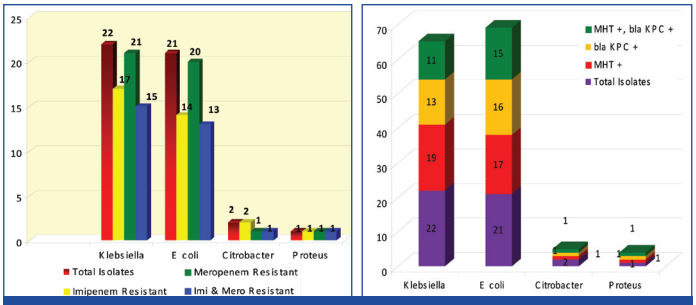
Reverse Primer: 5'-AGC ACA GCG GCA GCA AGA AAG-3'

The PCR vial was placed in PCR machine and it was subjected to initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1minute and extension at 72°C for 1minute. A final extension procedure was carried out at 72° C for 5 min. Gel electrophoresis was then carried out using 2% agarose gel. Gel was viewed in a UV Transilluminator and the bands pattern was observed.

RESULTS

Forty six isolates belonging to the family *Enterobacteriaceae* were tested. Of these, 22 were *Klebsiella pneumoniae,* 21 were *Escherichia coli*, 2 were *Citrobacter* species and 1 was *Proteus mirabilis* [Table/Fig-1]. These isolates were from pus, urine, blood, sputum and endotracheal aspirates. Of the 46 isolates, 43 (93.48%) were resistant to meropenem, 34 (73.91%) were resistant to imipenem and 30 (65.21%) were resistant to both imipenem and meropenem. Modified Hodge Test was positive in 38 (82.6 %) out of 46 isolates and *bla* $_{KPC}$ gene was detected in 31 (67.4%) isolates.

The results of Modified Hodge Test (MHT) and PCR for bla $_{KPC}$ gene detection are tabulated in [Table/Fig-2]. The antibiotic resistance pattern of the isolates is tabulated in [Table/Fig-3]. Among the 30 isolates which were resistant to both imipenem and meropenem, MHT was positive in 27 isolates (90%) and negative in 3 (10%) isolates. Twenty one (77.7%) of the 27 MHT positive isolates were found to be having the *bla* $_{KPC}$ gene while the remaining 6 isolates



[Table/Fig-1]: Identification of the isolates & their carbapenem sensitivity
[Table/Fig-2]: Results of MHT and bla KPC gene detection

Percentage of Resistance	*Klebsiella* (n =22)	*Escherichia* coli (n =21)	*Citrobacter* (n =2)	*Proteus* (n = 1)
Ampicillin	100%	100%	100%	100%
Cotrimoxazole	100%	100%	100%	100%
Gentamicin	73%	67%	100%	100%
Tobramycin	94%	73%	100%	100%
Amikacin	59%	33%	100%	100%
Nitrofurantoin (urine)	100%	20%	100%	100%
Ciprofloxacin	91%	91%	100%	100%
Cefazolin	100%	100%	100%	100%
Cefuroxime	100%	100%	100%	100%
Cefotaxime	100%	100%	100%	100%
Cefipime	96%	100%	100%	100%
Piperacillin Tazobactam	100%	100%	100%	100%
Imipenem	77%	67%	100%	100%
Meropenem	96%	95%	50%	100%
Polymixin B	0%	0%	0%	100%
Colistin	0%	5%	0%	100%

[Table/Fig-3]: Antibiotic resistance pattern of the Multi Drug Resistant isolates

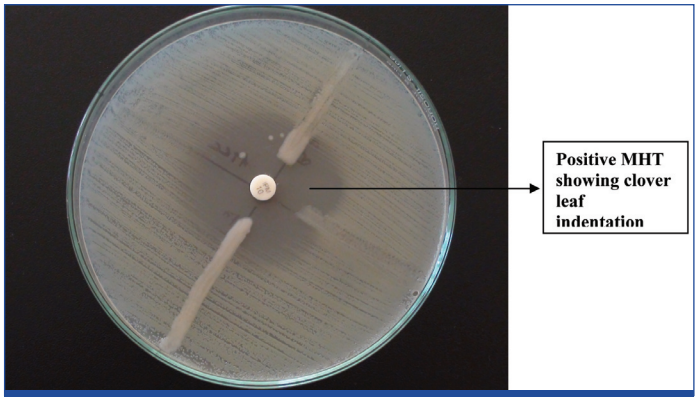
were negative for the gene. Of the 3 MHT negative isolates, one was positive for bla $_{KPC}$ gene. Three isolates were resistant to imipenem and sensitive to meropenem. Two of these isolates were MHT positive and one was MHT negative. bla $_{KPC}$ gene was detected in one of the MHT positive isolates. One isolate which was resistant to imipenem and Intermediate to meropenem, gave a positive result with the MHT and for the bla KPC gene detection.

Twelve isolates were imipenem sensitive, but meropenem resistant. MHT was positive in 8 (66.6%) of these isolates. Of this only 5 (41.7%) were *bla* $_{KPC}$ positive, while 3 were negative for the gene. MHT was negative in the remaining 4, of which 2 each tested to be bla $_{KPC}$ gene positive and negative respectively. [Table/Fig-4] shows an isolate which is MHT positive and [Table/Fig-5] shows the positive and negative PCR results for the bla $_{KPC}$ gene

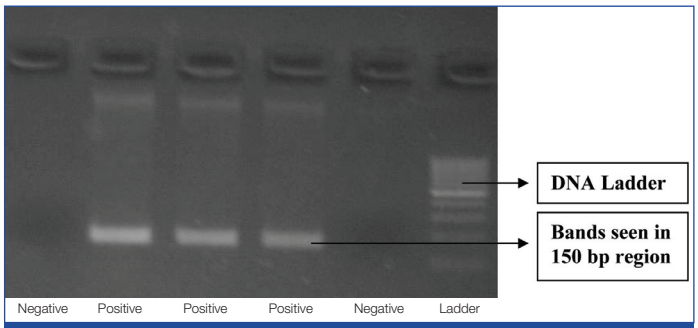
DISCUSSION

In this study carbapenem resistance was seen mainly among *Klebsiella* species, followed closely by *Escherichia coli* [Table/Fig-1]. The majority of the carbapenem resistant isolates were from urine (39.2%), followed by pus 34.7 %, respiratory 13% and blood 8.6%. This correlates with the findings of Nagaraj et al., [1] who have reported 42% isolates from urine, followed by 18% from wound infections.

All the carbapenem resistant isolates showed 100% resistance to ampicillin, cotrimoxazole, all 4 generations of cephalosporins and piperacillin tazobactam [Table/Fig-3]. The resistance to aminoglycoside antibiotics varied from 33% for amikacin to 94% to tobramycin [Table/Fig-3]. In the present study, *Klebsiella* showed a 77% resistance to imipenem and 96% resistance to meropenem, while *E coli* showed 67% resistance to imipenem and 95% resistance to meropenem. A study by Francis RO et al., [10]



[Table/Fig-4]: Modified Hodge Test



[Table/Fig-5]: PCR for bla $_{KPC}$ gene – Gel Electrophoresis

showed that among the samples that were confirmed positive for carbapenem resistance, the rate of resistance for each organism was 29% for *Klebsiella pneumoniae*, 2.8% for *Escherichia coli* and 3% for *Enterobacter* spp.

Modified Hodge Test [Table/Fig-2] was positive in 38 (82%) out of 46 isolates and *bla* $_{KPC}$ gene was detected in 31 (67.4%) isolates. PCR was positive in 28 (73.7%) out of 38 MHT positive isolates and negative in 5 (62.5%) out of the 8 MHT negative isolates. A study by Mosca A et al., in Italy showed that 84% of the carbapenem resistant strains evaluated by MHT showed the production of carbapenemase and that PCR for bla KPC gene showed a 100% positivity [11] Another study by Ragunathan A et al., [7] showed that PCR assay for *bla* $_{KPC}$ gene was positive in 82% of the MHT positive test isolates and was negative in 87% of the MHT negative isolates. The MHT results of the present study correlate with the findings of the other 2 studies. The present study showed *bla*$_{KPC}$ gene prevalence of 67.4 % among the carbapenem resistant *Enterobacteriaceae*. United States-wide surveillance study estimated the prevalence of *bla*$_{KPC}$ among various *Enterobacteriaceae* was 0.5%, whereas a study of Brooklyn hospitals reported 38% prevalence in *K. pneumoniae* [12]. Another study by Deshpande et al., reports a positivity rate for *bla* $_{KPC}$ gene as 44 out of 51 Carbapenem resistant isolates [13].

Of the 3 MHT negative isolates, one was positive for bla KPC gene. Cabral AB et al., [14] have also reported such isolates carrying silent genes. 8 (17.3%) isolates were resistant to carbapenems, but were MHT negative. They may have developed a different resistant mechanism other than carbapenemase production.

bla $_{KPC}$ gene was not detected in 10 MHT positive isolates. This indicates the presence of a carbapenemase other than KP Carbapenemase. Resistant to both imipenem and meropenem is a strong indicator of carbapenemase production rather than resistance to either one of the carbapenems, as this may imply a different resistance mechanism.

CONCLUSION

The prevalence of carbapenem resistance is increasing at a rapid pace. Carbapenem resistance by the KPC production is the most common mechanism as evidenced by MHT and PCR. Timely intervention in the form of rapid detection, good infection control practices and judicious use of antibiotics will ensure that the spread of drug resistance among bacteria is kept under control.

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