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DETECTION OF CARBAPENEMASE PRODUCTION IN *KLEBSIELLA PNEUMONIAE* IN A TERTIARY CARE CENTRE

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ABSTRACT

The study was done as a laboratory based study to detect the carbapenemase production in *Klebsiella pneumoniae* in a tertiary care hospital. A total of 150 clinically significant consecutive isolates of *Klebsiella pneumoniae* isolated between October 2012 and March 2013 were included in the study. Isolates from outpatients and from urine samples were excluded. The isolates were from blood (50), respiratory secretions (21) and exudates (79). Antibiotic Susceptibility testing to various drugs was performed by disc diffusion method. Carbapenemase production was screened by Modified Hodge Test (MHT) and *Klebsiella pneumoniae* carbapenemase(KPC) & Metalobetalactamase(MBL) production by inhibitor potentiated disc diffusion tests with Boronic acid and Ethylene Diamine TetraAcetic acid(EDTA) respectively. The isolates were subjected to Polymerase Chain Reaction (PCR) for the detection of bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{OXA-48} $bla_{OXA-181}$ & bla_{KPC} .Out of 150 isolates, 58(39%) exhibited carbapenem resistance. MHT was positive in 49(32.6%) isolates. Among the 49 MHT positive isolates 30 carried MBL genes. Among the 42 MBL screening was positive isolates 37 carried MBL genes. Overall, bla_{NDM-1}, bla_{VIM} & bla_{IMP} genes were detected in 34, 1 & 2 isolates respectively. MIC_{50} to imipenem was 0.125µg/ml. Bla_{KPC} , bla_{OXA-48} , $bla_{OXA-181}$ were not detected. Bla_{NDM-1} is the main mediator of carbapenem resistance in *Klebsiella pneumoniae* followed by bla_{VIM} & bla_{IMP}. Carbapenemase production (63.7%) is the most important reason for carbapenem resistance in carbapenem resistant *Klebsiella pneumoniae*. PCR remains the gold standard for the detection of carbapenemase production.

KEYWORDS: ***Klebsiella pneumoniae, carbapenem resistance, Modified Hodge test, Inhibitor potentiated disc diffusion***

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INTRODUCTION

Klebsiella pneumoniae, a normal inhabitant of gastrointestinal tract, skin, nasopharynx, genitourinary tract and eyes is an important pathogen causing a multitude of infections in the healthcare settings[1,2]. It is the causative agent of bacteremia, urinary tract infections, pneumonia, intra-abdominal and wound infections [3]. Multidrug resistance along with carbapenem resistance in *Klebsiella pneumoniae* is one of the major health problem presently [3,4,5,12,13]. Carbapenems are the only agents active against broad spectrum beta lactamase producing strains. Inappropriate and increased use of carbapenems has contributed to the development of its resistance in *Klebsiella pneumoniae*[6]. It has emerged as a real public health threat and has resulted in treatment failures with increased morbidity and mortality[7,8,9,10,11,12,13]. The various carbapenemase enzymes being reported in *Klebsiella* include class A carbapenemases (KPC), Class B metallo beta lactamases (NDM,VIM,IMP), Class D oxacillinase (OXA-48 like)[1,3,4,14]. The occurrence of non-beta-lactam resistance determinants and horizontal transmission of carbapenemase genes mediated by mobile genetic elements gives rise to multidrug and pan drug resistant isolates[15,16]. Additionally, there is a global explosion of CTX-M type extended-spectrum β lactamases and to a lesser extent, acquired AmpC enzymes in clinical isolates of *Klebsiella sp.* thereby resulting in burden of carbapenem resistance[15,16]. The mortality rates associated with MDR *Klebsiella pneumoniae* ranges from 50% to 70% in various studies across the globe[17,18]. The identification of carbapenemase producing strain is often not straight forward by the standard microbiological laboratory methods. The present study was undertaken to characterise carbapenemase production in *Klebsiella pneumoniae* by phenotypic and genotypic methods.

MATERIALS & METHODS

2.1 Bacterial strains

The study was conducted in an 1850-bedded university teaching hospital from October 2012 to March 2013. A total number of 150 clinically significant, non-duplicate, consecutive isolates of *Klebsiella pneumoniae* resistant to one of the cephalosporins subclass III were included. The isolates were obtained from hospitalised patients and were recovered from clinical specimens such as blood(50), respiratory secretions(21) and exudative specimens(79). Isolates from outpatients, those considered as colonisers, repetitive samples from the same patients and urine isolates were excluded.

2.2. Methodology

All the isolates were identified as per the standard biochemical bacterial identification methods[19] and stocked in 0.2% semisolid agar until analyzed. Patient's diagnosis, specimen types and demographic data were recorded. Susceptibility testing was performed by disc diffusion and results interpreted according to CLSI 2013 guidelines[20.] MIC was determined for imipenem. Carbapenemase production was screened by the Modified Hodge Test(MHT) KPC & MBL production was screened by inhibitor potentiated disc diffusion tests using Boronic acid and EDTA respectively. The isolates were subjected to Polymerase Chain Reaction (PCR) for detection of $bla_{\text{NDM-1}}$, bla_{VIM}, bla_{IMP}, $bla_{\text{OXA-48}}$, $bla_{\text{OXA-181}}$ & bla_{KPC} genes.

2.2.1. Antimicrobial susceptibility testing

Susceptibility to various classes of antibiotics was determined by the Kirby Bauer disc diffusion method on Muller Hinton agar plates in accordance with CLSI guidelines 2013[20.]The antibiotics tested were Amikacin(30µg), Cefoxitin(30µg), Cefotaxime(30µg), Ceftazidime(30µg), Cefepime(30µg), Ciprofloxacin(5µg), Aztreonam(30µg), Imipenem(10µg), Ertapenem(10µg), Meropenem(10µg)(BD), Polymyxin-B(300 units) procured from Hi-Media Laboratories, India. Disc diffusion susceptibility testing was also performed for tigecycline using 15µg disks(BBLTM BD,USA). The interpretation of zone diameters of tigecycline was done using the United States and Food and Administration's breakpoint criteria listed for *Enterobacteriaceae* (susceptible ≥19mm,

intermediate 15-18mm, resistance≤14mm) guidelines(US-FDA)[20].

2.2.2. Determination of Minimum inhibitory concentration (MIC)

MIC to imipenem was determined by agar dilution method with the range tested being 0.03-128µg/ml in accordance with CLSI guidelines 2013[20].

2.2.3. Phenotypic tests

2.2.3.1. Modified Hodge's Test[21, 22] : The phenotypic detection of carbapenemases production was done by the modified Hodge test & the test performed as per the CLSI guidelines 2013[20.] ATCC *Escherichia coli* 25922 was cultured overnight and suspended in 1:10 dilution in sterile saline to achieve a 0.5 McFarland standard turbidity and was lawn cultured onto a Muller-Hinton agar plate using a sterile cotton swab. After drying the plate for 5 min, a disk containing imipenem (10µg) was placed at the center of the plate, and an overnight cultured test strain of 3-5 colonies were picked and streaked in a straight line heavily from the edge of disc in centre to the periphery of the plate (distance of about 20mm) in four different directions and incubated overnight at 37ºc and examined next day. The presence of enhanced growth around the test organism streak at the intersection of the streak and the zone of inhibition(clover leaf shaped inhibition) after overnight incubation was interpreted as a positive result and positive for carbapenemase production and its absence meant the test isolate did not produce carbapenemase.

2.2.3.2. Detection of MBL screening and KPC screening[23] :

Tests were performed by inhibitor potentiated disc diffusion methods. Three meropenem discs of 10µg concentration was placed at 10mm distance on a MHA lawn cultured with 0.5McFarland adjusted test organism to one of the meropenem disc 5µL of 0.5 M EDTA(Sigma chemicals) solution of pH 8 and to the another 20µl of Boronic acid was added and one was left without adding any inhibitors and the plates were kept overnight in the incubator at 37ºc and examined next day for zone enhancement . An increase ≥5mm in zone diameter around disks containing β lactamase inhibitors compared with meropenem disk alone was considered to be positive. If there is the zone extension around meropenem disk with EDTA then the test organism is considered as MBL producer and similarly zone enhancement with boronic acid impregnated meropenem disk then that test organism is considered as KPC producer respectively.

2.2.4. Molecular detection of genes [24, 25]

A single colony was inoculated into Luria bertani broth, incubated for 20hrs with shaking in between. 1.5ml of this was centrifuged for 5mins. The pellets were suspended in 500µl of distilled water and lysed by heating at 95ºc for 5 mins and centrifuged for 1 min. 5µl of this extract was used as a template for amplification. All the isolates were subjected to PCR using consensus primers targeting $bla_{NDM,}$, bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{OXA-48}[24,25,1,2,3]. For optimisation, strains previously confirmed by PCR and gene sequencing were used as positive controls and Escherichia coli ATCC 25922 as negative control.

Table 1
Primers used for detection of MBL genes, KPC and OXA genes

Primers	Primer sequence 5' - 3'	Product size	Annealing temp.
bla$_{NDM}$-F	GGG CAG TCG CTT CCA ACG GT	475	55 ^{o}c
bla$_{NDM}$-R	GTA GTG CTC AGT GTC GGC AT		
bla$_{VIM}$-F	TTT GGT CGC ATA TCG CAA CG	500	56 ^{o}c
6bla$_{VIM}$-R	CCA TTC AGC CAG ATC GGC AT		
bla$_{IMP}$-F	GTT TAT GTT CAT ACW TCG	432	45 ^{o}c
bla$_{IMP}$-R	GGT TTA AYA AAA CAA CCA C		
bla$_{OXA\ 48}$-F	GCT TGA TCG CCC TCG ATT	238	56 ^{o}c
bla$_{OXA\ 48}$-R	GAT TTG CTC CGT GGC CGA AA		
bla$_{KPC}$-F	ATG TCA CTG TAT CGC CGT CT	893	55 ^{o}c
bla$_{KPC}$-R	TTT TCA GAG CCT TAC TGC CC		
bla$_{OXA\ 181}$-F	ATG CGT GTA TTA TAG CCT TAT CG	888	56 ^{o}c
bla$_{OXA\ 181}$-R	AAC TAC AAG CGC ATC GAG CA		

PCR conditions
PCR conditions for bla$_{OXA}$ gene
Initial denaturation - 95°C for 5 mins
Denaturation - 94°C for 30 secs
Annealing - 56°C for 1 min
Extension - 72°C for 1 min (30 cycles)
Final extension - 72°C for 5 mins
PCR product of 238 bp, 888bp for *bla$_{OXA48,}$ bla$_{181}$* was visualised by agarose gel electrophoresis.

PCR conditions for MBL genes
Initial denaturation - 95°C for 5 mins
Denaturation - 94°C for 30 secs
Annealing - 56°C for 1 min for *bla$_{VIM,}$*
45°C *bla$_{IMP}$* and
55°C for *bla$_{NDM,}$*
Extension - 72°C for 1 min (30 cycles)
Final extension - 72°C for 5 mins
PCR product of 475 bp *bla$_{NDM}$* , 500 bp *bla$_{VIM}$* , 432 bp *bla$_{IMP}$* , 893 bp *bla$_{KPC}$* were visualised by agarose gel electrophoresis.

RESULTS

A total number of 150 *Klebsiella pneumoniae* isolates were included in the study.

Table2
Distribution of carbapenem susceptible and resistant isolates in various specimens is listed below

Specimen	Isolates(150) No. (%)	Carbapenem resistant(58) No. (%)	Carbapenem susceptible(92) No. (%)
Blood	50(33.4)	29(58)	21(42)
Exudative specimens	79(52.6)	21(26.6)	58(73.4)
Respiratory secretions	21(14)	8(38)	13(62)

Table 3
Distribution of carbapenem resistant strains in various wards

Type of specimen	Medical ICU	Surgical ICU	Neonatal ICU	Non ICU
Blood(29)	20	4	5	0
Respiratory secretions (8)	3	2	1	2
Exudative specimens(21)	8	7	3	3

3.1 Antimicrobial Susceptibility profile

Susceptibility to the various classes of antimicrobials were follows: amikacin(66.6%),cefotaxime(2%),ceftazidime(8.7%),cefepime(41.9%), ciprofloxacin(2%),, imipenem(61.4%), meropenem(59.3%),ertapenem(52%), aztreonam(24%) polymyxin-B(100%) and tigecycline(100%). The MIC to imipenem for bla_{NDM-1} producers ranged from 32 to 64µg/ml. The MIC to imipenem for $bla_{VIM,}$ bla_{IMP} producers was 64µg/ml. Of the total 150 isolates 58(38.6%) were resistant to imipenem by disc diffusion and MIC determination.

3.2 Phenotypic tests

Table 4
Comparison of MBL screening and PCR in MHT positive isolates(n=49)

Test	No. of positive isolates(%)
Carbapenem resistant by disc diffusion method	49(100)
MBL positive by phenotypic screening	42(85.7)
PCR positive for MBL genes	30(61)

Table 5
Comparison of MHT and PCR in MBL screen positive isolates (n=42)

Test	No. of positive isolates(%)
Carbapenem resistant by disc diffusion method	42(100)
MHT positive by phenotypic screening	42(100)
PCR positive for MBL genes	37(88)

3.3 Results of PCR

58(39%) were resistant to carbapenem(imipenem) by disc diffusion test and MIC determination. All PCR positive 37(63.8%) isolates were carbapenem resistant.

Bla_{NDM-1}, bla_{VIM} & bla_{IMP} genes were detected in 34(91.9%), 1(2.7%)& 2(5.4%) of PCR positive isolates respectively. $Bla_{KPC,,}$ bla_{OXA} were not detected.

Table 6
Distribution of PCR positive carbapenem resistant isolates in various clinical specimens

Type of specimen	Blood(10)	Respiratory secretions(7)	Exudative specimens(20)
NDM(n=34)	9	7	18
VIM(n=1)	1	0	0
IMP(n=2)	0	0	2

Table 7
Resistance pattern of PCR positive isolates to various classes of antimicrobials

Antibiotics	No. of resistant isolates(%)
Amikacin	34(91.8)
Ciprofloxacin	35(95)
Aztreonam	35(94.5)
Colistin	0(0)

DISCUSSION

Carbapenems are a group of β-lactams and includes imipenem, meropenem, doripenem, ertapenem, panipenem and biapenem. Their antibacterial activity covers a wide group of organisms. They are mostly used for the treatment of serious infections with ESBL and/or AmpC-positive *Enterobacteriaceae*.. The increased use of carbapenems in the treatment of infection has contributed to the emergence of carbapenem hydrolyzing β-lactamases [26]. Their dissemination has been declared as "global sentinel event". Presently, there is a paucity of information on the prevalance of carbapenemases in *Klebsiella pneumoniae* . A substantially higher proportion of resistance to carbapenem i.e 38.6% (58) was observed in the study isolates. A similar high proportion of resistance has been cited in a few other studies [23, 27, 28]. The study isolates were predominantly from male patients (68%) . Similar predominant male sex distribution has been observed by other investigators[28, 29,30] . Majority of the isolates were recovered from patients aged 50 and above. Such age distribution has been observed by other authors also.[23, 28, 30] A substantially high proportion of isolates were from blood (33%) followed by pus(25%) ,wound swab(17%), aspirated fluids(10%), tracheal aspirate(4%) and the rest from BAL(Broncho alveolar Lavage) and sputum(11%). Higher isolation rates of *Klebsiella pneumoniae* from blood [23,31,32] is a frequent occurrence in hospitalised patients. In a study from China 38.6% isolates have been recovered from blood samples[33] The isolates were recovered from both ICU(55%) and non-ICU(45%) patients in this study. The predominant occurrence of these resistant strains in ICU is due to the variety of contributory factors such as invasive procedures, exposure to variety of different antimicrobials. Most authors have reported highest isolation of *Klebsiella* from critical wards(ICU)[23,28,29,30] . Co resistance to various antimicrobials among the carbapenem resistant isolates varied widely. The highest resistance rate was observed to quinolones, aminoglycosides, monobactams. This indicates that carbapenemase production is frequently accompanied by high level resistance to various other antibiotics leading to the evolution of extremely drug resistant isolates. Susceptibility to tigecycline and colistin was retained by all the isolates. Similar high percentage of susceptibility was reported in various other studies - 100%

susceptible to tigecycline and 91% to polymyxin B [28,30,31] . Previous study by Shanthi et al, reported 97.4% and 93.1% susceptibility to polymyxins and tigecycline respectively. However emergence of colistin resistance is a great concern. All the PCR positive isolates were carbapenem resistant and among the 21 (36.2%) of the AST screen positive carbapenemase resistant isolates none of the genes targeted were detected, the possible mechanisms in these could be other non targeted genes or CTX-M or AmpC prodcution[28]. In the present study 61.2%(30) PCR postive isolates showed true positive results by MHT and 88%(37) by MBL screening .This is in accordance with study by by Arjit bora et al. Hence combined disc method can be used as a screening test for effective carbapenemase production in the laboratory. Overall MBL genes were encountered in 37 *Klebsiella pneumoniae* isolates. 63.8% are carbapenemase producers in carbapenem resistant strains and the remaining(36.2%) are non-carbapenemase producers. In the carbapenemase producers bla_{NDM-1} was the predominant gene encountered among the MBLs followed by $bla_{IMP,}$ $bla_{VIM.}$. Bla_{NDM-1} was the most prevalent being detected in 34(59%) of carbapenem resistant isolates . Various other studies have also noted that NDM-1 is the most common mediator of carbapenem resistance in *Klebsiella pneumoniae* in India[28,30]. Elsewhere in Japan ,Singapore,Taiwan,Lebanan IMP and VIM among MBL genes has been identified as common mediator of carbapenem resistance in *Klebsiella* (IMP) [36,37,38] whereas KPC is predominant carbapenemase producer in various studies from USA ,UK and other countries like In the present study 63.7% of carbapenem resistant *Klebsiella pneumoniae* produced carbapenemase . Hence there is high prevalence and hence all needed measures should be implemented for the control of spread of these carbapenemase producers. On comparing the MHT results with PCR,among the 58 carbapenem resistant isolates, clear true positive was observed in 30 isolates and the 7 showed false negative results and true negative results were observed in 21 isolates.In this study, we have observed the occurrence of false positive with MHT[n=19] may be due to other mechanisms other than carbapenemases such as efflux pump upregulation or porin mutation in association with hyperproduction of AmpC beta-lactamases and cephalosporin.It is the due reason for false positive results with MHT in other studies[27,40,41] . The false negative MHT has been observed in the isolates producing metallo beta lactamases especially NDM [27,39]. False positive with MBL screen [n=5] may be due to EDTA acting on cell membrane & increasing membrane permeability or other MBL genes production not targeted in PCR. Limitations of the MHT in terms of clinical performance are its lack of specificity and the delay in obtaining the results upto 24 to 48hrs after isolation of bacterial colony. The molecular detection is the gold standard for diagnosis, but the majority of laboratories in deveoloping countries do not have the resources necessary to perform this test. Hence MBL screening by combined disc diffusion can be used on routine basis for screening.

CONCLUSION

There is a high prevalence of carbapenem resistance in *Klebsiella pneumoniae* . There is a high degree of co resistance of other antimicrobials leading to MDR isolate. NDM-1 is the main mediator of carbapenem resistance in carbapenemase producing *Klebsiella pneumoniae* followed by IMP and VIM in an increasing rate and with increased prevalence of NDM about 92% . The KPC and OXA genes were not encountered in the study isolates. . PCR remains the gold standard for the detection of carbapenemase production . A Coordinated effort is urgently needed to prevent the spread of carbapenemase resistant strains of Klebsiella pneumoniae. Judicious use of antibiotic in the hospital and vigilance surveillance will curtail the emergence and further spread of carbapenemase producing *Klebsiella pneumoniae* . MHT or MBL screening tests can be effectively used on a routine basis to detect the carbapenemase producers . Continuous surveillance, source control and early intervention will curtail dissemination. Clinical laboratories must be aware of false positive and negative results in MHT . Finally, the natural reservoir of the *bla*NDM gene

needs to be identified. Control of this reservoir, might help to stop the current dissemination of these emerging resistance determinants (Patrice Nordmann et al., 2009).

ETHICAL APPROVAL

Institutional ethical commitee approval was obtained prior to the commencement of study.

Conflict of interest

Conflict of interest declared none.

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