International Journal of Health Sciences and Research
www.ijhsr.org ISSN: 2249-9571

Original Research Article

Molecular Detection of Carbapenem Resistant *Klebsiella pneumoniae* in Intensive Care Units

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ABSTRACT

Carbapenem Resistant *Klebsiella pneumoniae* carrying *bla*KPC, *bla*NDM-1 and *bla*OXA-48 genes are difficult to treat. This study was conducted to study the genotypic features of carbapenem resistance in isolates of *Klebsiella pneumoniae* isolated from Intensive care units from a tertiary care Hospital. *Klebsiella pneumoniae* (n=89) isolates resistant to at least one carbapenem were collected from 377 patients for 24 months. Carbapenem resistance was determined via VITEK-2 system and confirmed my Modified Hodge test. Genes were analyzed using Polymerase chain reaction (PCR). The rate of resistance by Vitek-2 system for carbapenems was 23.6% and Modified Hodge test against ertapenem was 83% for 89 CRKP isolates. There were 39%, 29% and 46% of isolates positive for *bla*KPC, *bla*NDM-1 and *bla*OXA-48 genes respectively. The presence of all three genes showed 8.9% positivity. The molecular classification of carbapenemases will be useful for the betterment of patients with concurrent carbapenem resistant Enterobacteriaceae (CRE) infections. This could prevent outbreaks and complications in Intensive care units.

Keywords: *Klebsiella pneumoniae*, Carbapenem resistance, *bla*KPC, *bla*NDM-1 and *bla*OXA-48 gene.

INTRODUCTION

Klebsiella pneumoniae is a gram negative bacteria belonging to Enterobacteriaceae family. Carbapenem resistance in *Klebsiella pneumoniae* is mainly associated with K. pneumoniae carbapenemase. [1] It is a carbapenem hydrolyzing beta-lactamase encoded by transmissible plasmids, which facilitate spread of the enzyme among bacterial species. [2] During the last decade, infections due to carbapenem resistant *Klebsiella pneumoniae* (CRKP) have been reported throughout the world. Outbreaks of CRKP have been reported in several countries. [3] Rapid and global dissemination of CRKP is of great concern in health care facilities. It can cause diverse infections including primary bacteremia, urinary tract infections, pneumonia, intra-abdominal infections, and wound infections.

Crude mortality rate of CRKP infections ranges from 30% to 44%. CRKP strains have higher mortality rate compared to strains susceptible to carbapenem. [4] Prompt initiation of appropriate antibiotic therapy for CRKP infections is crucial for patient survival. [5] However, appropriate antibiotics like colistin are not administered routinely to these patients until the cultures yield CRKP isolate. Knowing that a patient is colonized by CRKP may be beneficial in deciding to start empirical CRKP active treatment in suspicion of a Gram negative infection.

Drug resistant isolates remain an important hospital-acquired bacterial pathogen, add significantly to hospital stays,

and are especially problematic in high-impact medical areas such as intensive care units. This antimicrobial resistance is thought to be attributable mainly to multidrug efflux pumps. [6] Risk factors for acquisition of CRKP colonization have been mainly assessed in studies involving patients and were identified as antibiotic exposure, especially carbapenem, intensive care unit stay, prolonged hospitalization, poor functional status, invasive devices. [4] CRKP colonization of the host is a major predisposing factor for developing sub-sequent CRKP infection. [4]

Currently, the bacteria receiving the most attention is New Delhi metallo-beta-lactamase-1 (NDM-1) producing superbug that confers resistance to most antibiotics including carbapenems. This carbapenemase is class B carbapenemase (metallo β-lactamases), which require zinc at their active site. *Klebsiella pneumoniae* Carbapenemases (KPC), identified in 2001. [7] The spread of antibiotic resistance genes such as NDM-1 and KPC is facilitated by horizontal gene transfer (HGT) between bacteria. [8] In 2001, a *Klebsiella pneumoniae* isolate was obtained from a patient in Istanbul, Turkey, which was found to be multidrug resistant, including resistance to the carbapenems. In this isolate, a new OXA-type beta lactamase was identified and named OXA-48. [9] These enzyme and its variants are now widespread in *K. pneumoniae*. PCR is a sensitive and gold standard method for the detection of carbapenemase resistant *Klebsiella pneumoniae* isolates. Therefore, the present study was planned to detect *bla*KPC gene, *bla*NDM-1 gene and *bla*OXA-48 gene in Carbapenem Resistant *Klebsiella pneumoniae* isolates from Intensive Care Units.

Objective: Molecular detection of Carbapenem Resistant *Klebsiella pneumoniae* in Intensive Care Units.

MATERIALS AND METHODS

This was a hospital based prospective study, undertaken in the department of Microbiology. Clinical isolates of *Klebsiella pneumoniae* from Intensive Care Units at JSS hospital were collected from January 2015 to December 2016. Only *Klebsiella pneumoniae* subspecies pneumoniae were included and Fecal *K. pneumoniae* were excluded from this study. The study protocol was approved by the Institutional ethical committee.

Blood, Urine, CSF, Sputum, Pus, ET, PF, CT samples were received in the microbiology laboratory were subjected to routine processing as per standard operating procedures. Phenotypic Identification of *K. pneumoniae* and Antimicrobial susceptibility testing (AST) was performed as per the CLSI guidelines [8] by Vitek2 automated system to detect Carbapenem resistant *K. pneumoniae* (CRKP) as primary test.

Further, confirmatory test was carried by Modified Hodge test.0.5 McFarland dilution of the *E.coli* ATCC 25922 was prepared in 5ml of nutrient broth. 0.5ml of the 0.5 McFarland was added to 4.5ml of saline to get 1:10 dilution. 1:10 dilution of *E.coli* ATCC 25922 was streaked as a lawn culture on to a Mueller Hinton agar plate and allowed to dry for 3–5 minutes. 10µg Ertapenem susceptibility disk (CT1761B-ETP 10mcg, [B. No.-178667] Oxoid, UK.) was placed at the center of the test area. Test organism was streaked in a straight line from the edge of the disk to the edge of the plate. Inoculated plates were incubated at 35°C±2°C in ambient air for 16-24 hours. After 16-24 hours of incubation, the plates were examined for a clover leaf-type indentation at intersection of test organism &*E. coli* 25922, within zone of inhibition of carbapenem susceptibility disc. MHT Positive test: Clover leaf-like indentation of *E.coli* 25922 growing along test organism growth streak within disk diffusion zone. MHT Negative test: No growth of *E.coli* 25922 along test

organism growth streak within disc diffusion zone.

***Detection of bla*KPC, *bla*NDM-1 and *bla*OXA-48 gene:**

DNA was extracted from overnight broth culture of *Klebsiella pneumoniae,* using HiPurA Bacterial Genomic DNA Purification Kit (MB505) as per the manufacture's protocol. The *bla*KPC, *bla*NDM-1 and blaOXA-48 gene were identified by PCR. The primers are namely, *bla*KPC gene: KPC _F: *F*- 5'-GTATCGCCGTCTAGTTCTGC-3', KPC_R: *R*- 5'-GGTCGTGTTTCCCTTTAGCCA-3' with amplicon size635bp.*bla*NDM-1 gene: NDM-1 _F: F- 5'-GGTTTGGCGATCTGGTTTTC-3', NDM-1_R: R-5'-CGG AAT GGC TCA TCA CGA TC-3' with amplicon size 621bp.*bla*OXA-48 gene: OXA-48_F: F- 5'-TTGGTGGCATCGATTATCGG-3', OXA-48_R: R- 5'-GAGCACTTCTTTTGTGATGGC-3' with amplicon size 744bp.

The reaction mixture was prepared for 25µl by adding 0.5µl of Std. Taq Polymerase(2U/ µl), 0.5µl of dNTP's mix(10mM), 2.5µl of 10x Taq Buffer, 0.5µl of both forward and reverse primer (10mM) (blaKPC, blaNDM-1 and blaOXA-48), 18.5µl of Nuclease Free Water and 2.0µl of DNA Template. For negative control instead of DNA template 2.0µl of Nuclease free water (sample)was added. The thermal conditions includes, an initial denaturation step at 94°C for 5 min, followed by 30 cycles, annealing temperature for *bla*KPC gene, *bla*NDM-1 gene and *bla*OXA-48 was 55.4°C, 57°C and 55.4°C respectively and followed by a final extension at 72°C for 5minutes.

PCR products were analyzed by Agarose Gel `Electrophoresis (1% gel). The gel was documented using gel documentation unit (SYNGENE). The presence of *bla*KPC gene, *bla*NDM-1 gene and *bla*OXA-48 gene was further confirmed by DNA sequencing. The Nucleotide sequence was analysed by Basic Local Alignment search tool available at the National Centre of Biotechnology Information (NCBI)-Primer-BLAST search.

Statistical Analysis: Inferential statistical test like Chi-square test was applied to find out association between the various tests. Cross table to find out Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) was calculated. The difference, association and correlation are expressed statistically significant at p-value less than 0.005.

RESULTS

Between January 2015- December 2016, 377 patients were admitted to the Intensive care units of our Institution (NICU, SICU, ICCU, RICU, MICU, PICU). Throughout the study period, 89(23.6%) isolates were colonized with carbapenem resistant *Klebsiella pneumoniae* (CRKP) by Vitek 2 method. Out of those 89 patients carrying CRKP, 31 patients admitted to NICU (35%), followed by SICU-25(28%), ICCU-12(13%), RICU-9(10%), MICU-7(8%) and PICU-5(6%) respectively with P value= 0.000 (very Highly significant) (Graph 1).

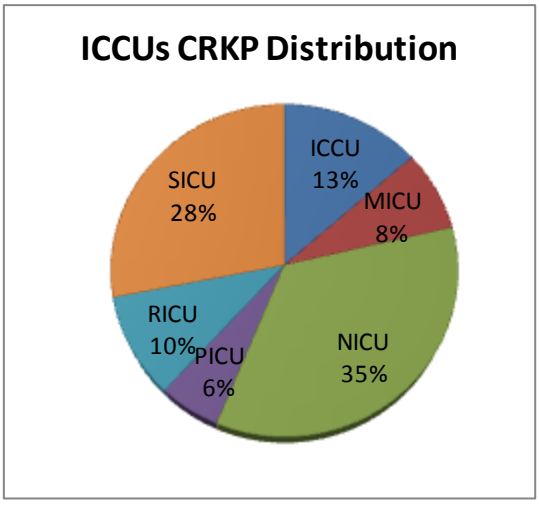
Patients' ages ranged from 1day to 80+years showing the maximum no. of CRKP isolates were from the age group 0-12 months -28(31.46%), followed by 61-70 years - 12(13.48%), 21-30years - 9(10.11%), 41-50 years- 8(8.98%) respectively with P value= 0.000(very Highly Significant). 63(70.83%) were male patients and 26(29.21%) were from female patients having CRKP colonization

The highest no. of CRKP isolates were from Pus-82(32.53%), followed by Urine- 64(25.39%), ET- 45(17.85%), Sputum-26(10.31%) and Blood-25(9.92%). The distribution of isolates collected from blood, cerebrospinal fluid (CSF), catheter tube (CT), drain, endotracheal tube (ET), pus, sputum, urine samples in various ICU's were summarized in the Table 1 with P value= 0.003(Highly significant).

The Modified Hodge test, confirmatory test carried out for 89 ICUs carbapenem resistant *Klebsiella pneumoniae* (CRKP) isolates, in which 74(83.14%) were positive with clover-leaf indentation and 15(16.85%) were negative without clover-leaf indentation with P value= 0.000(very Highly significant) (Table 2). The molecular detection of *bla*KPC gene, *bla*NDM-1 gene and *bla*OXA-48 genes was carried out for 89 ICUs CRKP isolates. 29(32.58%) isolates were positive and 60(67.41%) were negative for *bla*KPC gene, followed by 35(39.32%) were positive and 54(60.67%) were negative for *bla*NDM-1 gene and 46(51.68%) were positive and 43(48.31%) were negative for *bla*OXA-48 geneby Polymerase chain reaction respectively with P value= 0.000(Very Highly significant).

The distribution of *bla*NDM-1, *bla*KPC and *bla*OXA-48 genes among various ICUs is shown in Table 2. Among the total of 89 CRKP isolates from various ICUs, 8(8.9%) isolates showed the presence of all three genes. Positivity of both *bla*NDM-1+*bla*KPC genes showed 12(13.4%) isolates. Followed by *bla*KPC+*bla*OXA-48 gene combination was seen in 13(14.6%) isolates and the union of *bla*NDM-1+*bla*OXA-48 gene was seen in 19(21.3%) isolates.

Further, the results of Modified Hodge test were compared with *bla*KPC, *bla*NDM-1 and *bla*OXA-48 genes. The MHT with *bla*NDM-1 gene showed 27(30.33%) positive isolates and 07(7.86%) negative isolates (P = 0.383; NS). Modified Hodge test with *bla*KPC gene showed 22(24.71%) positive and 08(8.98%) negative isolates (P = 0.135; NS). Modified Hodge test with that of *bla*OXA-48 gene showed 41(46.06%) positive and 10(11.23%) negative isolates (P = 1.000; NS). Modified Hodge test v/s *bla*KPC, *bla*NDM-1 and *bla*OXA-48 genes showed 22(24.71%) true positives and 07(7.86%) true negatives respectively. The Sensitivity, Specificity, Positive predictive value and Negative predictive value for Modified Hodge test with different genes was calculated (Table:3). The sensitivity for MHT and *bla*KPC, *bla*NDM-1 and *bla*OXA-48 genes showed 75%,77% and 89%. The specificity was 13%, 12% and 23%. The PPV was 29%, 36% and 55% and NPV was 53%, 46% and 66%.



Graph 1: ICUs CRKP distribution

Table 1: Showing Isolates collected from various samples verses all ICU's

SAMPLES / ICU's	BLOOD	CSF	CT	DRAIN	ET	PUS	PF	SPUTUM	URINE	Total
NICU	15	-	01	-	07	-	-	-	08	31
SICU	01	01	-	01	14	05	01	02	-	25
ICCU	01	-	-	-	07	-	-	01	03	12
MICU	-	-	-	-	02	03	01	01	-	07
RICU	01	-	-	-	03	-	-	02	03	09
PICU	01	-	-	-	03	-	-	-	01	05
Total	19	01	01	01	36	08	02	06	15	89
Percentage	21.3%	1.1%	1.1%	1.1%	40.4%	2.2%	8.9%	6.7%	16.8%	100%

Table 2: Showing the distribution of Modified Hodge Test (MHT) Result, *bla*NDM-1, *bla*KPC and *bla*OXA-48genes among various ICUs:

ICUs	Modified Hodge Test		NDM-1 gene	KPC gene	OXA-48 gene	Presence 3 genes	NDM-1 + KPC	KPC + OXA-48	NDM-1+OXA-48
	Positive	Negative	Positive	Positive	Positive				
ICCU	08	04	05	04	06	01	02	01	03
MICU	07	00	01	05	03	01	01	01	01
NICU	28	03	19	08	18	4	6	5	11
PICU	03	02	01	02	00	00	00	00	00
RICU	06	03	03	04	06	02	02	03	02
SICU	22	03	06	06	13	00	01	03	02
Total	74	15	35	29	46	08	12	13	19
Percentage	83.14%	16.85%	39.3%	32.5%	51.6%	8.9%	13.4%	14.6%	21.3%

Table 3: Sensitivity, Specificity, Positive predictive value, Negative predictive value of different gene with Modified Hodge test:

Modified Hodge test	*bla*NDM-1gene Positive	*bla*KPC gene Positive	*bla*OXA-48gene Positive
Positive {N=74(83.14%)}	27(30.33%)	22(24.71%)	41(46.06%)
Sensitivity	77%	75%	89%
Specificity	12%	13%	23%
Positive predictive value (PPV)	36%	29%	55%
Negative predictive value (NPV)	46%	53%	66%

DISCUSSION

Carbapenem-resistant *K. pneumoniae* is a major problem in nosocomial infections with high mortality rates especially in immune compromised patients in the intensive care unit (Patel et al., 2008; Ulu et al., 2015). CRE were listed as one of the most urgent antibiotic resistance threats by the Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO) (Band et al., 2018). According to the CDC tracking program; KPC and OXA-48 type CRE have spread rapidly.

The potential of *Klebsiella pneumoniae Carbapenemase* (KPC) production among gram negative bacteria along with the development of resistance to carbapenem antibiotics have made difficult and important problem in the treatment of infection. Reduced susceptibility to carbapenems is indirect indicators, on which rapid identification of KPC can be followed. [10]

In our study, a total of 89(23.6%) *Klebsiella pneumoniae* isolates were Carbapenem resistant from Vitek 2 results. Similarly a study conducted by D. Moemenet al [10] showed 33.3% CRKP isolates. Previous Egyptian literature showed a prevalence of 44.3% of CRKP isolates, [11] others reported lower incidence at 13.9% in the Egyptian National Cancer Institute [12] and 14.2% in Al-Azhar University Hospital. [13] Similarly other studies showed varying rate from 20 to 40% in New York and Greece. [14] Higher result of 83% was shown in a study in USA. [15]

The high trend of CRKP in the current study could be attributed to the frequent use of carbapenems as an empiric therapy in ICUs at our institution.

In the current study, 31 patients admitted to NICU (35%), followed by SICU-25(28%), ICCU-12(13%), RICU-9(10%), MICU-7(8%) and PICU-5(6%). This was similar to the study by Hacer Akturk *et al* [16] showed 2.6% for NICU and 3.6% for PICU and in other study by Sebnem Bukavaz et al [17] 76% patients were in the intensive care unit were as our study showed comparatively less CRKP isolates(23.6%) in ICUs. Resistance rates to ertapenem, imipenem, and meropenem were 99, 97, and 96%, respectively. Based on VITEK-2 MIC values for all antibiotics, this was similar with the study by Sebnem Bukavaz et al. [17]

In the current study, non susceptibility to ertapenem, based on the updated CLSI criteria was used in the surveillance of CRKP isolates. The use of ertapenem has been suggested to screen for carbapenem resistance among Enterobacteriaceae. [18] Endimiani *et al.* (2009) reported that nearly 60% of CRKP

isolates are susceptible to IPM or MEM, and resistant to Ertapenem. [19] The isolates collected in this study were highly resistant to b-lactams, fluoroquinolones and variably to aminoglycosides. Therefore there is an urgent need to develop new antimicrobials for CRKP, together with strict infection control measures in particular hand-hygiene to control the cross-infection with K. pneumoniae. [10]

In the present study, 89(23.6%) CRKP were further tested for KPC and MBL production by Modified Hodge Test (MHT). Recently CLSI accepted MHT as specific and sensitive phenotypic method for carbapenemase detection. [20] Out of 89 carbapenem resistant *Klebsiella pneumoniae* (CRKP) isolates, 74(83.14%) were positive by Modified Hodge Test (MHT) and 15(16.85%) were negative respectively.

Our results are similar with many other studies like, 75% positive result showed by McGettigan SE *et* al., [21] followed 69% by Amjad A. *et al.,* [23] Cury et al.(MHT 71% positive) [20] and little higher rate was seen in Galani I *et al* [22] *and* Sebnem Bukavaz *et al* [17] each with 98% positive result respectively.

Were as, 74(83.14%) isolates were found to produce carbapenemase enzyme by MHT and 15(16.85%) were found negative for MHT. The reason for negative result of bacterial strains which were carbapenem resistant but negative by MHT could be explained as the over production of ESBL or Amp C enzyme with porin loss. [18] The processing of MHT showed that it is easy, simple with minimum infrastructure and of less cost; reliable test and this can be implemented in routine microbiology laboratories for detection of carbapenemase producers.

The most prevalent gene among the 89(23.6%) CRKP isolates was blaOXA-48 gene at 51.6%. Similar studies like Sara Lomonaco *et al* [26] (2018) observed 50% blaOXA-48 gene type, followed by a study by Carole Ayoub Moubareck *et al* [27] *(2018)* reported 53.3% positivity for blaOXA-48 gene. Study conducted by D. Moemenet al [10] showed 10.9%.

The present study showed, 39.3% of positivity for bla NDM-1 gene, this was similar to the results of Patrice Nordmann *et* al [28] investigated and reported that 37.5% of Carbapenem resistant *Klebsiella pneumoniae* were NDM-1 gene producers in 2012. Followed by, a study conducted by NagarajS *et al* [29] in 2012 showed that 75% isolates were positive for NDM-1gene. Another study by Arijit Bora *et al* [30] showed 71.79% isolates were positive for NDM-1gene. Study conducted by D. Moemenet al [10] showed 4.3%.

The current study showed 32.5% positive rate for blaKPC gene. Similar to our study, study conducted by D. Moemen et al [10] showed 43.5% positive rate followed by Priyadarshini Shanmugam *et* al [31] in (2013)detected blaKPC gene, which showed 47.82% and another study by Efthymia Protonotariou *et al* [32] *(2018)* showed 45% positive. G. Sotgiu *et* al [33] observed 61% positive for blaKPC gene.

Comparing phenotypic test to PCR results, we noted that out of the 74(83.14%) MHT positive isolates, 27(30.33%) positive *bla*NDM-1, followed by 22(24.71%) for *bla*KPC gene and 41(46.06%) for *bla*OXA-48 gene. False-positive results (7 isolates) may be due to carbapenem hydrolysis by ESBLs, coupled with disrupted porin expression as reported by others. [24] Conversely, false negative results (10 isolates) may be explained by presence of metallo-beta-lactamase producing isolates with weak carbapenemase activity as reported by Miriagou *et al*. (2010). [25] And this may be because, the remaining MHT positive isolates which were negative for KPCs and MBLs presumably had the other types of carbapenemase enzymes like IMI-, SME-, GES-, NMCA-types, IMP and VIM-types, OXA-types. Not only do KPC-producing organisms hydrolyze carbapenems, but also, they are often resistant to multiple other antibiotics.

In our study, The Sensitivity and Specificity for Modified Hodge test with

different genes was calculated. The sensitivity for MHT and *bla*KPC, *bla*NDM-1 and *bla*OXA-48 genes showed 75%, 77% and 89%. The specificity was 13%, 12% and 23%. In other study by Girlich *et al.*, [34] showed 77.4% sensitivity and 38.9% specificity for MHT test in detecting KPC in overall test. This low sensitivity and specificity may be because, MHT detects other carbapenemase enzymes in addition to KPC as it is the only indicative enzymatic activity of carbapenemase and cannot differentiate class A carbapenemases from class B MBLs. And MHT cannot be used as a confirmatory test for recognition of the KPCs because of the difficult elucidation and false positive results. False-positive results are more common in isolates producing AmpC and CTX-M b-lactamase. [35]

PCR is an effective method for detection of carbapenemase genes which overcomes the limitations of the phenotypic tests. Infection control measures like Hand washing and isolation pre-cautions should be taken particularly in the intensive care unit. Education on hand washing of the intensive care unit staff and high level disinfection of the surfaces should be carried out. These preventive measures will help to decrease the rate of carbapenem resistant *K. pneumoniae* strains in ICUs.

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How to cite this article: Hegde MA, Tejashree A, Mehdi S. Molecular detection of carbapenem resistant *Klebsiella pneumoniae* in intensive care units. Int J Health Sci Res. 2018; 8(11):88-96.

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