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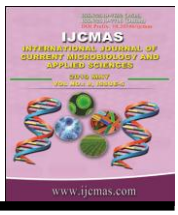

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Genotying of Carbapenem Resistant *Klebsiella pneumoniae* from Clinical Isolates

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A B S T R A C T

K. pneumoniae is responsible for a wide variety of hospital and community-acquired infections, affecting patients with normal immune systems as well as those with pre-existing conditions. The Carbapenems are β-lactam antibiotics that are used in the treatment of infections caused by Extended Spectrum beta-Lactamases (ESBL) producing gram negative bacteria (GNB). Carbapenem antibiotics are considered the drugs of choice for the treatment of ESBL -producing Enterobacteriaceae and other multidrug resistant bacteria. However, resistance to carbapenems is being increasingly detected and is mainly related to the action of carbapenemase-type enzymes. The emergence of bacterial strains that produce carbapenemases further limits the therapeutic options available to clinicians. Genotying of carbapenem resistant *Klebsiella pneumoniae* from clinical isolates. This was a hospital based prospective study, undertaken in the department of Microbiology. All clinical isolates like Urine, Pus, ET secretions, BAL, Blood and other Body fluids received in the laboratory were subjected to routine processing as per standard procedures. Identification &Antimicrobial susceptibility testing was performed by Vitek2 system. Modified Hodge test was carried out as confirmatory test. Genotypically, PCR was carried out for the detection of blaKPC, blaNDM-1 and blaOXA-48 gene. A total of 1,539 *Klebsiella pneumoniae* were isolated from various clinical samples over a period of 2 years from March 2015- April 2017. Among 1,539 *Klebsiella pneumoniae* isolates, 252(16.37%) isolates were carbapenem resistant by Vitek 2.190(75.39%) of the Carbapenem resistant *Klebsiella pneumoniae* were positive by Modified Hodge test. By PCR analysis *blaKPC* gene was found to be positive in 86(34.12%) of CRKP isolates. Followed by, blaNDM-1 gene showed 90(35.71%) positivity and 126(50%) isolates were positive for blaOXA-48 gene respectively. Carbapenem resistant is being progressively detected. This resistance is primarily related to the action of carbapenemase-type enzymes. The acceleratory frequency of carbapenemase-producing bacteria indicates the urgency of having tools convenient to monitor the appearance and the spread of each family of carbapenemase gene types.PCR is a compelling method for detection of carbapenemase genes. This overcomes the limitations of the phenotypic tests. Hence molecular characterization should be considered.

Introduction

K. pneumoniae is responsible for a wide variety of hospital and community-acquired infections, affecting patients with normal immune systems as well as those with pre-existing conditions *K. pneumoniae* is the most common cause of nosocomial respiratory tract and premature intensive care infections, and the most frequent cause of Gram-negative bacteraemia and urinary tract infections. 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.[1] The ability of *K. pneumoniae* to colonize the hospital environment, including carpeting, sinks, flowers, and various surfaces, as well as the skin of patients and hospital staff, has been identified as a major factor in the spread of hospital-acquired infections.[2] The Carbapenems are β-lactam antibiotics that are used in the treatment of infections caused by Extended Spectrum beta-Lactamases (ESBL) producing gram negative bacteria (GNB). Carbapenem antibiotics [Ertapenem, Imipenem, Meropenem, Doripenem, Razupenem (Faropenem)] are considered the drugs of choice for the treatment of Extended Spectrum Beta - lactamase (ESBL)-producing Enterobacteriaceae and other multidrug resistant bacteria.[3] However, resistance to carbapenems is being increasingly detected and is mainly related to the action of carbapenemas e-type enzymes. The emergence of bacterial strains that produce carbapenemases further limits the therapeutic options available to clinicians. Classes A to C have been well documented as both chromosomally encoded and plasmid-mediated enzymes.[4] The class D β-lactamases have been much more elusive and, for the most part, were identified only as plasmid-encoded β-lactamases in Gram-negative bacteria.

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, also called metallo β-lactamases as they require zinc at their active site. This enzyme is coded by a gene called *bla*NDM-1. The NDM-1 enzyme was named after New Delhi, the capital city of India, as it was first described by Yong *et al.*, in December 2009 when a Swedish national who fell ill with an antibiotic-resistant bacterial infection that he acquired in India.[5] The infection was identified as a carbapenem-resistant *Klebsiella pneumoniae* strain bearing the novel gene bla_{NDM-1}.5Thereby organisms bearing *bla*NDM-1 gene have been identified from different parts of the world and it was considered that most of the cases were arisen from Indian subcontinent.[6]

Klebsiella pneumoniae Carbapenamases (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]. Among globally disseminated pathogens, HGT facilitates combination of the most effective antibiotic resistance genes from diverse geographies into multidrug resistance plasmids that spread between strains. Both HGT and clonal expansion have enabled KPC and NDM-1 to rapidly spread to distant locations after their emergence[9].

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[10]. This enzyme and its variants are now widespread in *K. pneumoniae* and other *Enterobacteriaceae* and have now been reported in *A. baumannii*as well[11], and they represent one of the most concerning developments in carbapenem resistance in the last decade.

The best therapeutic approach to *Klebsiella pneumoniae Carbapenemase* producing organisms has yet to be defined; however, common treatments based on in-vitro susceptibility testing are the polymyxins, tigecycline, and less frequently

aminoglycoside antibiotics are been used[26]. Hence, this study was undertaken to assess Genotying of carbapenem resistant *Klebsiella pneumoniae* from clinical isolates.

Materials and Methods

This was a hospital based prospective study, undertaken in the department of Microbiology, JSS Hospital. All clinical isolates of *K. pneumoniae* from patients attending out-patientand in-patient department at JSS hospital was included in the study. Fecal *K. pneumoniae* was excluded from this study. Inclusion criteria were phenotypically confirmed ESBL *K. pneumoniae* isolates for molecular detection of *bla*KPC, *bla*NDM-1 and *bla*OXA-48 gene. The samples collected were processed as per standard methods. The study protocol was approved by the ethics committee of the institute (Ref: JSS/MC/IEC/02/655/2015-16)

Urine, Pus, ET secretions, BAL, Blood and other Body fluids were received to the laboratory and were subjected to routine processing as per standard[2]procedure by Vitek2 system.

Modified Hodge test

0.5 McFarland dilution of the *E.coli* ATCC 25922 was prepared in 5 ml of broth or saline. 0.5ml of the 0.5 McFarland was added to 4.5 ml of Mueller Hinton Broth (MHB) or 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 disc [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 disc to the edge of the plate. Up to four organisms can be tested on the same plate with one drug. 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 cloverleaf-like indentation at the intersection of the test organism and the *E. coli* 25922, within the zone of inhibition of the 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.

*bla*KPC, *bla*NDM-1 and *bla*OXA-48Gene Detection

DNA was extracted from overnight broth culture of *K. pneumoniae,* using HiPur A Bacterial Genomic DNA Purification Kit MB505 as per the manufacture's protocol. The *bla*KPC, *bla*NDM-1 and *bla*OXA-48 gene was identified by PCR using primers.

PCR tube consisted of 29 µl of Master Mix + 1 µL of DNA. The conditions included an initial denaturation step of 5 min at 95°C, followed by 30 cycles of Holding Temperature: 95°C, Annealing Temperature: 58°C and Extension for 45 sec at 72°C, Final extension step of 5 min at 72°C. Isolates were screened for acquired *bla*KPC, blaNDM-1 and *bla*OXA-48 gene by PCR using primers and conditions described previously.

Post PCR Analysis by Gel Electrophoresis was carried out using 1% Agarose (DNA grade, low melting, Himedia). The results were recorded on gel documentation system.

Sequencing

The PCR amplified product were purified using HiPurA PCR purification kit from HIMEDIA Labs Pvt. Ltd. The sequence

chromatograms were analysed by BLAST and compared with known alleles to identify the replicons and identification of blaKPC, blaNDM-1 and blaOXA-48 alleles were done by using National Centre of Biotechnology Information database (www.ncbi.nlm.nih. gov/) (Table 1).

Results and Discussion

A total of 1,539 *Klebsiella pneumoniae* was isolated from various clinical samples (Bile, Blood, Urine, Sputum, drain, CSF, ET, CT, PF, Pus) over a period of 2 years.

Out of 1,539 *Klebsiella pneumoniae* isolates, 252(16.37%) isolates were carbapenem resistant *Klebsiella pneumoniae* (CRKP) (resistant to Imipenem/ Ertapenem/ Meropenem antibiotics) by Vitek 2.

Modified Hodge Test (MHT) Result

Out of 252 carbapenem resistant *Klebsiella pneumoniae* (CRKP) isolates, 190(75.39%) were positive with clover-leaf indentation by Modified Hodge Test (MHT) and 62(24.60%) were negative respectively. Chi square= 65.016; P value= 0.000 (very highly significant).

Genotyping result

The molecular detection of *bla*KPC gene, *bla*NDM-1 gene and *bla*OXA-48 genes was carried out for 252 CRKP isolates. 86(34.12%) isolates were positive for *bla*KPC gene, followed by 90(35.71%) were positive for *bla*NDM-1 gene and 126(50.0%) were positive for *bla*OXA-48 gene by 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 CRKP isolates is shown in Table 2. Among 252 CRKP isolates 15(5.95%) isolates showed the presence of 3 genes (blaKPC+ blaNDM-1+ blaOXA-48). Combination of KPC gene + NDM-1 gene showed 28(11.11%) positive, followed by KPC gene + OXA-48 gene showed 38(15.07%) positive and NDM-1 gene + OXA-48 gene showed 43(17.06%) positive with P value= 0.000 (Very Highly significant) each respectively.

In the present study, an attempt was made to detect the Carbapenem Resistance in *Klebsiella pneumoniae* from various clinical samples by genotyping in a tertiary care hospital.

In our study, a total of 254(16.50%) *Klebsiella pneumoniae* isolates were Carbapenem resistant from Vitek 2 result. Vijaya Doddaiah *et al.*,[12] (2014) showed 16.03% and Shalley Dahiya *et al.*,[13] (2015) showed the prevalence of 14.75%for CRKP.

Modified Hodge Test (MHT) is carried out to identify the existence of carbapenemase enzyme for isolates showing resistance to one/more carbapenems as advised by CDC.[14]. In the present study, 252 CRKP were tested for KPC and MBL production by Modified Hodge Test (MHT). Out of 252 carbapenem resistant *Klebsiella pneumoniae* (CRKP) isolates, 190(75.39%) were positive with clover-leaf indentation for Modified Hodge Test (MHT) and 62(24.60%) were negative respectively.

Our results are similar with many other studies like, 75% positive result showed by McGettigan *et al.*,[15] (2009) followed 69% by Amjad *et al.*,[16] Cury *et al.*, (MHT 71% positive) [17](2012) and little higher rate was seen in Galani *et al.*, (2008) with 98% [18],and study by Shawn Vasoo *et al.*,[19] (2013) also showed 97.7% positive result respectively.

In the current study, out of 252 CRKP isolates, 86(34.12%) isolates were positive for *bla*KPC gene by polymerase chain reaction.

Similar to our study, Priyadarshini Shanmugam *et al.,*[20] *in* (2013) detected *blaKPC* gene, which showed 47.82% and another study by Efthymia Protonotariou *et al.,*[21](2018) showed 45% positive.

Discordant results were observed in the study by Ana Carolina Ramos *et al.,*[22] (2018) detected 9.09% KPC gene, where as a study by Saritha Naya *et al.,*[23] (2014) showed 16.6% which was comparably low to our study. Sotgiu *et al.,*[24] (2018) observed 61% positive for blaKPC gene followed by Aditya Raghunathan *et al.,*[25] (2011) conducted a study that observed 96% showing higher positive rates.

By PCR analysis, 90(35.71%) isolates were positive for blaNDM-1 gene in our study. Identical to our study, Patrice Nordmann *et al.,*[26] (2012) investigated and reported that 37.5% of Carbapenem resistant *Klebsiella pneumoniae* were NDM-1 gene producers in 2012. Amin Abdelfattah Aqel *et al.,*[27] (2017) showed 50%. In discordance with our study, Karthikeyan Kumaraswamy *et al.,*[28] (2010) investigated and reported that 9.9% of *K. pneumoniae* were NDM-1 producers from Chennai and in the same study 13% isolates showed positive for NDM-1gene from Haryana. A study conducted by Nagaraj *et al.,*[29] in 2012 showed that 75% isolates were positive for NDM-1gene. Another study by Arijit Bora *et al.,*[30] (2014) showed 71.79% isolates were positive for NDM-1 gene.

In our study, occurrence of blaOXA-48 gene was found to be 126 (50%) of CRKP *K.pneumoniae*. Identical to our study, Sara Lomonaco *et al.,*[31] (2018) observed 50% blaOXA-48 gene type, followed by a study by Carole AyoubMoubareck *et al.,*[32] *(2018)* reported 53.3% positivity for blaOXA-48 gene. According to another study by Hamid Solgi *et al.,*[33](2018) reported blaOXA-48 gene encoding in majority of CRKP isolates as 100%. RymOuertani *et al.,*[34] (2018) and Joseph D. Lutgring *et al.,*[35] (2018) also reported 92.85% and 90% positivity in their respective studies which was 40% higher when compared to our study. In discordance with our study, MarcArgente *et al.,*[36](2018) reported 1.9%, followed by Marianne Lund [37](2018)- 4.70%, Sandra Pulss *et al.,*[38] (2018) 0.64%, Martin Kaase *et al.,*[39](2016)- 5.8%, these results were 50% lower when compared to the present study.

In the current study, 15(5.95%) isolates showed the presence of 3 genes (blaKPC + blaNDM-1 + blaOXA-48). Combination of (blaKPC gene + blaNDM-1 gene) showed 28 (11.11%) positive (P value 0.4), followed by (blaKPC gene + blaOXA-48 gene) showed 38(15.07%) positive (P value 0.1) and (blaNDM-1 gene + blaOXA-48 gene) showed 43(17.06%) positive (P value =0.5) respectively.

Similar to our study Sara Lomonaco *et al.,*[31] (2018) observed 20% positives in combination of (blaNDM-1 gene + blaOXA-48 gene). Followed by Carole Ayoub Moubareck *et al.,*[32] (2018) identified both NDM-1 gene and blaOXA-48 gene with 22.1% positivity.

Amin Abdelfattah Aqel *et al.,*[40] (2018) identified 8.6% positive rate for both blaNDM-1 and baOXA-48 gene type which slightly lower to our study. Another study by Hamid Solgi *et al.,*[33] (2018) observed 87% positives in combination of blaNDM-1 gene and blaOXA-48 gene showing higher positive result when compared to our study. The study conducted by Marianne Lund *et al.,*[41] (2018) and Fiona Senchyna *et al.,*[42](2017) to detect blaKPC, blaNDM-1 and blaOXA-48genes showed correlating results.

Major part of the gut flora is subsidized by family Enterobacteriaceae where bla_{NDM} producing *K.pneumoniae* are also efficient to colonize.

Table.1 Showing PCR primers used for KPC, NDM-1 and OXA-48 gene detection

Gene	Primer	Oligonucleotide Sequence 5-3	Amplicon Size (bp)
16SrDNA	16SrDNA_F 16SrDNA_R	F-5'-CCAGCAGCCGCGGTAATACG-3' R-3'-ATCGGTTACCTTGTTACGACTTC-5'	996bp
Class A: blaKPC gene	KPC _F KPC_R	*F*- 5'-GTATCGCCGTCTAGTTCTGC-3' *R*- 5'-GGTCGTGTTTCCCTTTAGCCA-3'	635bp
Class B: blaNDM-1 gene	NDM-1 _F NDM-1_R	F- 5'-GGTTTGGCGATCTGGTTTTC-3' R-5'-CGG AAT GGC TCA TCA CGA TC-3'	621bp
Class D: blaOXA-48 gene	OXA-48_F OXA-48_R	F- 5'-TTGGTGGCATCGATTATCGG-3' R- 5'-GAGCACTTCTTTTGTGATGGC-3'	744bp
KPC-*Klebsiella pneumoniae* Carbapenemase, NDM-1 - New Delhi metallo-β-lactamase,OXA-48 - Carbapenem-hydrolyzing oxacillinase			

Table.2 Showing genotyping result

Test	Number	Percentage
KPC gene Positive	86	34.12%
NDM-1 gene Positive	90	35.71%
OXA-48 gene Positive	126	50.00%
Presence all 3 genes(KPC+NDM-1+OXA-48)	15	5.95%
KPC gene + NDM-1 gene	28	11.11%
KPC gene + OXA-48 gene	38	15.07%
NDM-1 gene + OXA-48 gene	43	17.06%

Image.1 Showing modified Hodge test result

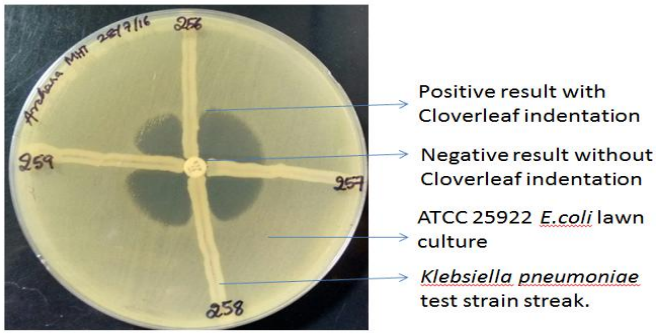
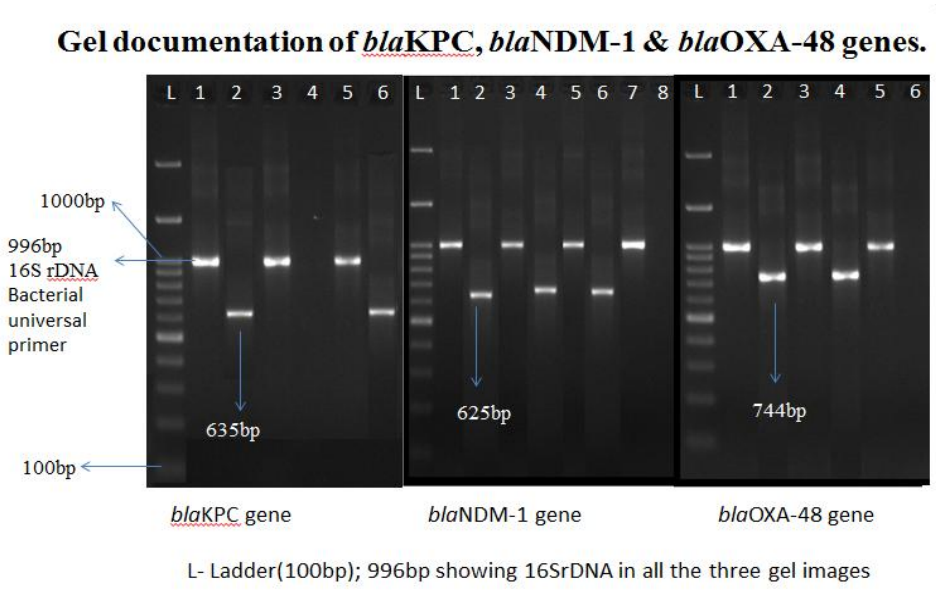


Image.2 Showing gel electrophoresis of KPC, NDM-1 and OXA-48 genes



L- Ladder(100bp); 996bp showing 16SrDNA in all the three gel images

Based on these findings, genotypic assay could be considered in the diagnostic as confirmatory method for carbapenemase production and/or as an identification tool for the most important different carbapenemase genes.PCR is the rapid way to detect which family of β-lactamase is present when the presence of a carbapenemase is suspected. This finding is supported by Anjana Shenoy *et al.,*[43] (2014); Hrabak *et al.,*[44](2014).

These in turn distribute as source for widening the infection especially in healthcare framework. In order to restraint the spread, disinfection procedures need to be implemented. Contact isolation of these infected patients is not routine. This may not be achievable in all healthcare settings, principally in the developing countries. Microbiological scrutiny at the time of admittance, contact isolation of potentially colonized patients, will go a rambling in hindering contamination of the environment and transmission to other patients. In addition, proper use of carbapenems will also hamper the selecting resistant bacteria in a habituated geographical area.

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