Detection of *Klebsiella pneumoniae* Carbapenemase (KPC) Producing Enterobacteriaceae Isolates from Various Clinical Samples in a Rural Health Setup

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**Abstract**

Carbapenem resistance is increasing and emerging as a public health threat. The Indian subcontinent serves as a reservoir for all 3 types of carbapenemases: KPC, OXA-181 and NDM. The present study was done to determine the antibiotic resistance pattern of Gram negative bacilli (GNB) belonging to family Enterobacteriaceae and molecular detection of bla<sub>KPC</sub> gene among the Carbapenem resistant Enterobacteriaceae (CRE). Antibiotic sensitivity pattern of 301 gram negative isolates, members of Enterobacteriaceae (*E. coli*, *K. pneumoniae*, *K. oxytoca*, Proteus species, *Citrobacter* species, *Enterobacter* species and *Serratia* species) was determined and those resistant to carbapenem (ertapenem) were further processed in the study. Modified Hodge Test (MHT) was performed to phenotypically confirm the presence of carbapenemases (bla<sub>spc</sub>) and Polymerase Chain Reaction (PCR) was done to identify bla<sub>KPC</sub> gene. Of the total 301 isolates, 45 were resistant to ertapenem. Out of these 45 isolates 32 were MHT positive and three of these 32 MHT positive strains harboured bla<sub>KPC</sub> gene.

**Keywords:** bla<sub>KPC</sub>, Enterobacteriaceae, Gram negative bacilli, Carbapenem resistance, Modified hodge test

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(Received: December 14, 2019; accepted: March 29, 2020)

**Citation:** Arveen Sokhi, Priti Agarwal, Megha Maheshwari and Anita Chakravarti, Detection of *Klebsiella pneumoniae* Carbapenemase (KPC) Producing Enterobacteriaceae Isolates from Various Clinical Samples in a Rural Health Setup, *J. Pure Appl. Microbiol.*, 2020; 14(1):397-401. https://doi.org/10.22207/JPAM.14.1.41

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INTRODUCTION

The members of family Enterobacteriaceae are Gram negative bacilli (GNB) which are a part of human normal flora. They are also a source of community and hospital acquired infections. Carbapenems belong to a group of beta lactam antibiotics which have a broad spectrum activity. They are being used for treatment of severe life threatening infections caused by Multidrug resistant (MDR) GNB. These agents have become ineffective due to misuse and development of resistance against them.

Resistance has been developing against the antibiotics soon after they have been put to use. Alexander Fleming the discoverer of penicillin, while accepting his Noble Prize in 1945 warned about the possibility of drug resistance. Resistance to carbapenems was first demonstrated in the North Carolina strain of Klebsiella pneumoniae that produced an enzyme Klebsiella pneumoniae carbapenemase (KPC). The gene coding for resistance was found on plasmid. It was then when carbapenem resistance arrived.

KPCs are beta lactam enzymes produced by GNB (K. pneumoniae, E. coli, Enterobacter species) belonging to Enterobacteriaceae family harboring bla

\[ \text{KPC} \] gene. These enzymes hydrolyze and inactivate a wide range of beta lactam antibiotics such as penicillins, cephalosporins and carbapenems. There are different variants of \( \text{bla}_{\text{KPC}} \) gene (KPC-2 to KPC-15) which are seen in non fermenting bacteria also.

In order to contain the spread of bacteria harboring \( \text{bla}_{\text{KPC}} \) gene effective control measures and judicious antibiotic usage in hospitalized patients must be implemented.

The study focused on KPC producers because they are an important resistance mechanism for wide range of GNB which is not only limited to K. pneumoniae. KPC producing isolates can be misidentified by routine tests and should be suspected by ertapenem resistance. The study was done to identify \( \text{bla}_{\text{KPC}} \) and not other carbapenemases as no other study has been done previously to detect \( \text{bla}_{\text{KPC}} \) gene in a hospital situated in budhera village in Haryana.

MATERIALS AND METHODS

The study was conducted in the microbiology laboratory of SGT Medical College and Hospital for a period of 8 months (March-October 2018) on the patient samples received from various clinical departments.

The institutional ethical committee approval was taken prior to commencement of this study.

The GNB isolated from various clinical samples like blood, sputum, pus, urine, vaginal swabs, urinary catheter tip were included for the study. All the samples were inoculated on MacConkey and blood agar except urine samples for which cystine lactose electrolyte deficient (CLED) agar was used. The organisms were identified by Gram staining and biochemical tests (Indole test, Methyl red, Voges Proskauer, Citrate utilization test, Urea hydrolysis test and Triple Sugar Iron agar test) were done. Antibiotic susceptibility testing was done by Kirby bauer disc diffusion method as per CLSI guidelines 2018. Antimicrobial discs used were ampicillin (10µg), ampicillinsul bactum(10/10µg), ceftazidime (30µg), cefuroxime (30µg), cefotaxime(10µg), cefepime(5µg), levofloxacin (5µg), ciprofloxacin (5µg), tobramycin (10µg), cotrimoxazole (1.25/23.75µg), gentamicin (10µg), ertapenem(10µg), meropenem(10µg), imipenem (10µg), aztreonam (30µg). Resistance to ertapenem served as a screening test for \( \text{bla}_{\text{KPC}} \) gene detection. Out of 301 isolates, 45 isolates resistant to ertapenem were further processed for Modified Hodge Test and detection of \( \text{bla}_{\text{KPC}} \) gene.

Phenotypic Test for detection of Carbapenemases Modified Hodge Test (MHT)

Mueller hinton agar (MHA) plate was inoculated as a lawn culture with 0.5 Mc Farland E. coli ATCC 25922. The plate was dried for 3 to 10 minutes and ertapenem disc was applied on the centre of the plate. Using a 10-μL loop, 3 to 5 colonies of test and Quality Control organism which were grown overnight on a blood agar plate were inoculated in straight lines perpendicular to each other. After overnight incubation, the plate was looked for the presence of a “clover-leaf” indentation in the zone of inhibition. The isolates showing clover-leaf like indentation around the zone of inhibition of E. coli ATCC 25922 were considered as positive and absence of indentation was considered as negative. Quality control strains used were:

1. K. pneumoniae ATCC BAA- 1705 (MHT positive)
2. K. pneumoniae ATCC BAA-1706 (MHT negative).
bla\textsubscript{KPC} gene detection

DNA Extraction, PCR and Electrophoresis were performed according to Shanmugam et al\textsuperscript{6} with minor modifications. The following primers were used to amplify the bla\textsubscript{KPC} gene: Forward Primer: 5’-GCT CAG GCG CAA CTG TAA G-3’ and Reverse Primer: 5’-AGC ACA GCG GCA GCA AGA AAG-3’.

PCR Reaction

To make 25\mu{l} reaction mix. 12.5\mu{l} of HiChrom PCR Master Mix was added. To it 0.75\mu{l} of Forward Primer, 0.75\mu{l} of Reverse Primer, 2.5\mu{l} of Template DNA and 8.5\mu{l} of Molecular Biology Grade water was added.

The PCR cycles followed were:
1. Initial denaturation was done at 94°C for 3 minutes.
2. It was followed by 30 cycles of denaturation at 94°C for 1 minute.
3. Annealing was done at 60°C for 1 minute.
4. Extension at 72°C for 1 minute which was followed by final extension for 5 minutes at 72°C. Gel electrophoresis was performed on 2% agarose gel at 100volts for 15 minutes. The gel was observed under UV Transilluminator.

RESULTS

During the study period, 301 isolates of Enterobacteriaceae (E. coli, K. pneumoniae, K. oxytoca, Proteus spp, Serratia spp, Citrobacter spp., Enterobacter spp.) were tested by Kirby bauer disc diffusion method, of which 45(14.9%) were found to be Carbapenem Resistant Enterobacteriaceae (CRE). Citrobacter spp., Enterobacter spp. and Serratia spp. were susceptible to Ertapenem, so they were not processed further for Phenotypic and Genotypic tests. All (100%) of CRE were resistant to ertapenem according to CLSI 2018 guidelines. Of the 45 ertapenem resistant isolates, 32(71.1%) were resistant to meropenem and imipenem. Two of the bla\textsubscript{KPC} isolates were isolated from blood and one from pus sample from patients admitted in Surgery and ICU ward. Twenty nine (90.6%) of 32 MHT positive isolates were bla\textsubscript{KPC} negative.

Table 1. Co-resistance pattern of ertapenem resistant isolates.

| Antibiotics       | Klebsiella (n\textsuperscript{1}=15) | E. coli (n\textsuperscript{1}=29) | Proteus (n\textsuperscript{1}=1) |
|-------------------|--------------------------------------|----------------------------------|----------------------------------|
| Ampicillin        | 15 (100%)                            | 29 (100%)                        | 1 (100%)                         |
| Ampicillin sulbactum | 15(100%)                            | 29(100%)                         | 1 (100%)                         |
| Ceftazidime       | 15(100%)                            | 29(100%)                         | 1 (100%)                         |
| Cefuroxime        | 15 (100%)                           | 29 (100%)                        | 1 (100%)                         |
| Cefotaxime        | 15(100%)                            | 29(100%)                         | 1 (100%)                         |
| Levofloxacin      | 15(100%)                            | 28 (96.5%)                       | 1 (100%)                         |
| Cefepime          | 15(100%)                            | 29 (100%)                        | 1 (100%)                         |
| Ciprofloxacin     | 15(100%)                            | 29(100%)                         | 1 (100%)                         |
| Tobramycin        | 11(73.3%)                           | 21 (72.4%)                       | 0%                               |
| Gentamicin        | 11(80%)                             | 23 (79.3%)                       | 1 (100%)                         |
| Cotrimoxazole     | 14 (93.3%)                          | 27 (93.1%)                       | 0%                               |
| Aztreonam         | 11(73.3%)                           | 22 (75.8%)                       | 0%                               |
| Norfloxacin       | 4 (100%)                            | 28 (96.5%)                       | 1 (100%)                         |
| Nitrofurantoin    | 3 (75%)                             | 20 (70%)                         | 0%                               |
| Imipenem          | 14 (93.3%)                          | 21 (72.4%)                       | 1 (100%)                         |
| Meropenem         | 10(66.6%)                           | 22 (75.8%)                       | 0%                               |
| Ertapenem         | 15 (100%)                           | 29 (100%)                        | 1 (100%)                         |

Hodge Test. MHT was positive for 32(71.1%) of 45 ertapenem resistant isolates confirming the presence of significant carbapenems hydrolyzing activity (Fig. 1). PCR for detection of bla\textsubscript{KPC} gene was carried out for MHT positive isolates. Three (9.3%) of the 32 isolates were found to harbor bla\textsubscript{KPC} gene. Band formation was seen at 90 basepair as shown in Fig. 2. All the KPC positive isolates were K. pneumoniae and they showed resistance to both meropenem and imipenem.

Two of the bla\textsubscript{KPC} isolates were isolated from blood and one from pus sample from patients admitted in Surgery and ICU ward. Twenty nine (90.6%) of 32 MHT positive isolates were bla\textsubscript{KPC} negative.

Fig. 1. Modified Hodge Test (A is Negative control, B is Positive control, C and D are two test isolates showing positive MHT)
**DISCUSSION**

There are reports of increased prevalence of carbapenem-resistant GNB over few years. The rapid dissemination of carbapenemases like KPC is a major challenge for physicians and clinical laboratories. However, identifying the different bacterial mechanisms of resistance is crucial for infection control and epidemiological studies. KPC-producing isolates have been reported from USA, Italy, China, Greece, Argentina, Israel, Poland, Taiwan, and Columbia. There are reports of sporadic occurrence of KPC from South East Asia including India, Australia, and South Korea.

Developed countries like the United States reported 62.3% CRE in the year 2012-2013. Greece reported <1% CRE in 2001 which in 2008 rose to 30% in hospital wards and 60% in ICUs.

However, various studies conducted in India before 2006 failed to show any evidence of resistance for *E. coli* and *K. pneumoniae* to carbapenems, Indian subcontinent serves as a reservoir for all 3 types of carbapenemases: KPC, OXA-181, and NDM.

Two different studies from Western Rajasthan reported 31.7% and 37% CRE respectively. Whereas, the present study showed 14.9% CRE, this is lesser than the other two studies. However, it is in accordance to a study conducted in a Tertiary Care Hospital, North India which showed 14.7% CRE.

In the present study, CRE isolates exhibited increased prevalence of multidrug resistance to various antibiotics ranging from 66.6% to 100% which is comparable to a study from Northeast India with resistance rate ranging from 85.7% to 100%.

MHT was positive in 32 (71.1%) isolates, whereas, only 3 (9.3%) showed the presence of bla<sub>KPC</sub> gene by conventional PCR. However, this non-specificity of MHT was discussed by Tsakris et al. Their report states that the false positive results of MHT could be due to presence of CTX-M ESBL positive or AmpC–hyperproducing Enterobacteriaceae. This also suggests the involvement of other resistance mechanisms, such as production of carbapenemases (OXA, NDM, MBL) alone or in combination with porins loss, ESBL (TEM, SHV, CTX-M).

KPC-producing strains in the current study were 100% resistant to ertapenem, imipenem, and meropenem. The first report of KPC producer was from South India (Pondicherry) in 2010. It reported six (5.8%) out of 103 isolates as bla<sub>KPC</sub> positive, that were 100% resistant to carbapenem (imipenem, ertapenem, and meropenem).

However, this is the first study conducted in SGT medical college and hospital to detect the prevalence of bla<sub>KPC</sub> gene.

The present study reported 9.3% KPC isolates which is more than the findings of a study conducted at Safdarjung Hospital, New Delhi that reported 2.1% KPC producers. However, absence of bla<sub>KPC</sub> was reported in a study conducted in North India by Mohan et al. that is contrary to the present study. Although, there are a few studies from India that shows the prevalence of CRE in clinically relevant isolates but not many laboratories follow the standard protocol for CRE identification.

**CONCLUSION**

The emergence of carbapenem resistance globally and in India is a cause of concern because of the limited treatment options for carbapenem-resistant organisms. More studies should be undertaken in different regions of the country to avail the information on prevalence of KPC.

Early diagnosis of KPC can improve the patient outcome. Whereas, the diagnostic technique to be introduced in the laboratory workflow is a choice which should be done carefully, as per the resources available and personnel in each hospital.

**ACKNOWLEDGEMENTS**

None.
CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS’ CONTRIBUTIONS

AS conducted the isolation and identification of bacterial strains, performed the antibiotic sensitivity tests, phenotypic and genotypic tests, tabulation of data and drafting of the manuscript. PA and MM guided in the phenotypic and genotypic test and correcting the manuscript. AC guided in the final editing of the manuscript. All the authors read and approved the final manuscript.

FUNDING

None.

DATA AVAILABILITY

All datasets created or investigated during this study are involved in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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