Sokhi et al., *J. Pure Appl. Microbiol.,* **14**(**1**), 397-401 | March 2020
Article 5995 | https://doi.org/10.22207/JPAM.14.1.41

Print ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

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_{KPC} 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_{KPC}) and Polymerase Chain Reaction (PCR) was done to identify bla_{KPC} 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_{KPC} gene.

Keywords: $bla_{KPC,}$ 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

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[1].
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[2].

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 Carolinan 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[3].

KPCs are beta lactam enzymes produced by GNB (*K. pneumoniae*, *E. coli*, *Enterobacter* species) belonging to Enterobacteriaceae family harboring bla_{KPC} gene. These enzymes hydrolyze and inactivate a wide range of beta lactam antibiotics such as penicilllins, cephalosporins and carbapenems. There are different variants of bla_{KPC} gene (KPC-2 to KPC-15) which are seen in non fermenting bacteria also[4].

In order to contain the spread of bacteria harboring bla_{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 bla_{KPC} and not other carbapenemases as no other study has been done previously to detect bla_{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 cysteine 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[5]. Antimicrobial discs used were ampicillin (10µg), ampicillinsul bactum(10/10µg), ceftazidime (30µg), cefuroxime (30µg), cefotaxime(10µg), cefepime(5µg), levofloxacin (5µg), ciprofloxacin (5µg), tobramicin (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 bla_{KPC} gene detection[5].Out of 301 isolates, 45 isolates resistant to ertapenem were further processed for Modified Hodge Test and detection of bla_{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$_{KPC}$ gene detection

DNA Extraction, PCR and Electrophoresis were performed according to Shanmugam et al[6] with minor modifications. The following primers were used to amplify the bla$_{KPC}$ 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 25µl reaction mix. 12.5µl of HiChrom PCR Master Mix was added. To it 0.75µl of Forward Primer, 0.75µl of Reverse Primer, 2.5µl of Template DNA and 8.5µl 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, 23(51.1%) were from urine, 16(35.5%) from pus, 3(6.6%) from sputum and 3(6.6%) from blood. Of the forty five isolates twenty nine were E*. coli*, twelve were *K. pneumoniae*, three *K. oxytoca* and one *Proteus mirabilis*.

Of the 45 CRE, 32 (71.1%) were resistant to meropenem, 36 (80%) were resistant to imipenem. The resistance pattern of these isolates is given in table 1. All carbapenem (ertapenem) resistant isolates were subjected to Modified 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$_{KPC}$ gene was carried out for MHT positive isolates. Three (9.3%) of the 32 isolates were found to harbor bla$_{KPC}$ 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$_{KPC}$ 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$_{KPC}$ negative.

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

Antibiotics	*Klebsiella* (n^1=15)	*E. coli* (n^1=29)	*Proteus* (n^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%)
Tobramicin	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%)

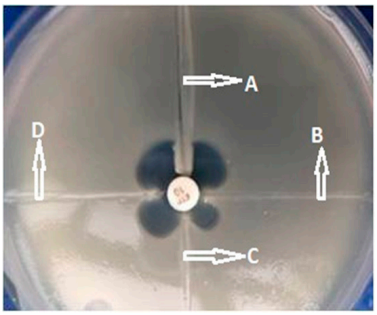

Fig. 1. Modified Hodge Test (A is Negative control, B is Positive control, C and D are two test isolates showing positive MHT)

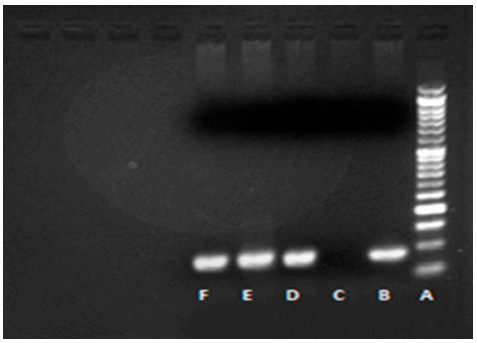

Fig. 2. Gel Electrophoresis for bla_{KPC} gene. Lanes: A- 50bp DNA ladder, B-Positive control, C-Negative control, D,E,F are test isolates showing bla_{KPC} gene at 90bp.

DISCUSSION

There are reports of increased prevalence of carbapenems 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[7]. 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[8].

Developed countries like 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[9].

However, various studies conducted in India before 2006 failed to show any evidence of resistance for *E. coli* and *K. pneumoniae* to carbapenems[10], Indian subcontinent serves as a reservoir for all 3 types of carbapenemases: KPC, OXA-181 and NDM[8].

Two different studies from Western Rajasthan reported 31.7% and 37% CRE respectively[11, 12]. 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[15] 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%[14].

MHT was positive in 32 (71.1%) isolates, whereas, only 3(9.3%) showed the presence of bla_{KPC} gene by conventional PCR. However, this non specificity of MHT was discussed by Tsakris et al[15].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)[7] .

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[16]. It reported six (5.8%) out of 103 isolates as bla_{KPC} 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_{KPC} gene.

The present study reported 9.3% KPC isolates which is more than the findings of a study conducted at Safdarjung Hospital, New Delhi [17] that reported 2.1% KPC producers. However, absence of bla_{KPC} was reported in a study conducted in North India by Mohan et al [18] 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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