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Phenotypic and genotypic characterization of carbapenem resistance mechanisms in *Klebsiella pneumoniae* from blood culture specimens: A study from North India

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Abstract:

BACKGROUND: Emergence of carbapenem resistance among *Enterobacteriaceae* in different geographical regions is of great concern as these bacteria are easily transmissible among patients. Carbapenem-resistance in *Enterobacteriaceae* is due to production of carbapenemases of various classes and hyper production of the ESBLs (Extended spectrum beta lactamases) and Amp C beta lactamases with reduced cell wall permeability mechanisms. Phenotypic detection and differentiation is important for proper infection control and appropriate patient management. This study was done to know the prescence of various beta lactamases and carbapenemases with other mechanisms of resistance in *Klebsiella pneumoniae* isolates.

MATERIALS AND METHODS: 50 non-duplicate carbapenem resistant isolates of *Klebsiella pneumoniae* from blood culture specimens were included and various mechanisms of resistance were studied based on phenotypic and genotypic methods.

RESULTS: Out of 50 isolates, 39 (78%) of *K.pneumoniae* isolates were Extended Spectrum Beta Lactamase (ESBL) producers based on CLSI guidelines. All 50 showed positive Modified Hodge Test (MHT) and 32 showed Metallo Beta Lactamase (MBL) by Combined Disc Test (CDT). Four isolates showed AmpC production with porin loss . None of the isolates showed Class A KPC production by CDT . In our study all the 10 isolates evaluated by genotypic technique produced CTX-M group 1 enzyme by multiplex PCR. Seven out of 10 strains which showed positive MBL results were positive for NDM .

CONCLUSIONS: Carbapenems are often considered last resort antibiotics in the treatment of infections due to multidrug-resistant organisms. It is therefore mandatory to maintain the clinical efficacy of carbapenems by early detection of various enzymes . For routine clinical laboratories both phenotypic and genotypic tests need to be followed to detect various mechanisms of carbapenem resistance and this is of epidemiological relevance also.

Key words:

Amp C, carbepenem, *Enterobacteriaceae*

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Introduction

The acquired resistance to various beta-lactams in *Klebsiella pneumoniae* is a growing problem worldwide. This resistance is primarily due to the production of diverse beta lactamases. Carbapenems are the most favored antibiotics commonly used to treat infections which are caused by multidrug-resistant *Enterobacteriaceae*.[1] According to a recent WHO report, resistance

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to the last resort choice of treatment, i.e., carbapenem antibiotics for life-threatening infections caused by *K. pneumoniae*, has spread to all regions of the world.[2]

In India also, carbapenem-resistant *Enterobacteriaceae* (CRE) are now being increasingly reported having resistance mechanism as the acquisition of carbapenemase genes.[3,4]

Among the various mechanisms which have been described for carbapenem-resistance, predominant is the production of different classes of carbapenemases such as A, B, or D and others are the production of extended spectrum–β lactamases (ESBLs)/AmpC-β lactamases with a porin mutation or drug efflux.[5] This emergence of carbapenem resistance in *K. pneumoniae* due to carbapenemases is of great concern as this is plasmid mediated and these bacteria can easily disseminate not only among indoor patients leading to hospital associated infections but may also spread into the community.[1]

Further, *K. pneumoniae* isolates that coproduce both metallo beta lactamases (MBL) and *K. pneumoniae* carbapenemases (KPC) or other combinations have also been documented, and recently, they have become widespread in several hospitals.[6,3] Such coexistence of carbapenem-hydrolysing enzymes in bacteria may further compromise the therapeutic alternatives not only due to the carbapenemases mediated resistance to every β-lactam, but also due to the linkage with non-β-lactam resistance determinants.[7]

Appropriate detection of carbapenem-resistant *Enterobacteriaceae* in *K. pneumoniae* (CRE-KP) is vital for patient care to institute correct therapeutic options, and also epidemiological data are important to know which genes are circulating in our environment to control their spread. Although for detection, molecular techniques such as polymerase chain reaction (PCR) are the gold standard to assess the prevalence of different classes of carbapenemases but that is feasible only in research laboratories so for routine clinical laboratories detection is based mainly on phenotypic methodology.[6]

In this setting, the aim of the present study was to study the different classes of beta lactamases and carbapenemases in carbapenem-resistant isolates of *K. pneumoniae* isolated from blood culture specimens using phenotypic and genotypic methods.

Materials and Methods

This prospective study was carried out in the Department of Microbiology of a tertiary care hospital in North India on the *K. pneumoniae* isolated from blood samples collected from various patients admitted in wards/intensive care units (ICUs) and outpatient department (OPD) from January 2013 to June 2014. A total of 50 nonduplicate carbapenem-resistant clinical isolates of *K. pneumoniae* were included in the study; 35 from ICU (70%), 10 from medical wards (20%), and 5 from OPD (10%). The bacterial isolates were identified to species level according to standard microbiological procedures.[8]

Carbapenem susceptibility

All these strains were tested for susceptibility to imipenem/meropenem/ertapenem (Hi-media) by disc diffusion method according to Clinical and Laboratory Standard Institute (CLSI)-2015 criteria.[9] Those strains which showed reduced susceptibility based on disc diffusion susceptibility to ertapenem/meropenem/imipenem were confirmed for carbapenem resistance by E-test (BioMex rieux India Ltd., bioMex rieux, Marcy l'Etoile, France).Resistance of *K. pneumoniae* strains to carbapenem was reported if MIC to ertapenem was ≥2 μg/ml and/or MIC to imipenem/meropenem was ≥4 μg/ml. All *K. pneumoniae* isolates were also tested for susceptibility to amikacin, cefepime, ciprofloxacin, cefpodoxime, amoxicillin + clavulanic acid, piperacillin + tazobactam by disc diffusion method whereas colistin and tigecycline susceptibility were determined by E-test.[9]

ESBL production was detected using agar disc diffusion method CLSI 2015.[9]

Modified Hodge Test (MHT)-All CRE-KP isolates were tested by MHT.[9] For quality control, *K. pneumoniae* ATCC BAA 1705 and BAA 1706 were taken as positive and negative controls.

For determination of the different classes of carbapenemase enzyme, we used carbapenemase inhibition tests (the combined disc method) using meropenem disc (10 μg) with various inhibitors.

Combined disc tests (CDTs) methods for the detection of different classes of carbapenemases was used as described.[10] Stock solution of β-lactamase inhibitors was prepared by dissolving the powdered form of 100 mg/mL of dipicolinic acid (DPA), 60 mg/mL aminophenylboronic acid (APBA), and 75 mg/ml of cloxacillin. All the powders were obtained from Sigma, St. Louis, MO, USA. 10 μL of inhibitors were added to meropenem disks, all the disk were left at room temperature for 30 min to dry.

For Class A: KPC-type enzyme production was suspected when there was 4 mm increase of inhibition zone diameter around the meropenem/amino phenylboronic acid disk than meropenem disk (10 μg) alone. Positive

control for KPC– *K. pneumonia* ATCCBAA-1705 was used.

Class B-MBL detection method is based on the synergy between MBL inhibitor DPA and meropenem. This was achieved when there was 5 mm increase of inhibition zone diameter around the meropenem/DPA disc (10 µ g/1000 µg), than meropenem disc (10 µg).

Class D: There is no specific inhibitor used for class D carbapenemases. It was detected by genotypic method only.

AmpC detection: Class C (AmpC): For AmpC production, screening method by cefoxitin disc diffusion was used, and this was confirmed by 5 mm increase of inhibition zone diameter around the meropenem/cloxacillin (10 µg/750 µg) than meropenem disc alone.[11,12]

Further out of 50 isolates, 10 randomly selected strains which were both MBL and ESBL positive by phenotypic method were processed for multiplex PCR for the detection of ESBL, AmpCenzyme, and other classes of carbapenemases. Multiplex PCR was used for detection of most commonly found genes $bla_{\text{CTX-M}_{\text{gp1}}}$ for ESBL, $bla_{\text{CMY-2}}$, $bla_{\text{DHA-1}}$ for AmpC, bla_{KPC} for Class A carbapenemase, bla_{NDM}, bla_{VIM}, bla_{IMP} for Class B carbapenemases, and $bla_{\text{OXA-48}}$ for Class D carbapenemases and the various primers used for PCR amplification were as described.[3,2,13-15] The molecular work was carried out in ARM Laboratory, Erode 638002, Tamil Nadu.

Results

Fifty nonduplicate carbapenem resistant clinical isolates of *K. pneumoniae* were included in the study. All of the isolates were recovered from blood samples, ICU 70% (n = 35), medical wards 20% (n = 10), and OPD 10% (n = 5). All *K. pneumoniae* isolates were resistant to cefepime, ciprofloxacin, cefpodoxime, amoxicillin + clavulanic acid, piperacillin + tazobactam but 36/50 (72%) isolates were susceptible to amikacin by disc diffusion method. In addition, susceptibility to colistin and tigecycline which was determined by E test was 100%. Out of 50 isolates, 39 (78%) of *K. pneumoniae* isolates were ESBL producers based on CLSI guidelines. All 50 showed positive MHT, and 32 showed MBL production by CDT using DPA as inhibitor. Four isolates showed AmpC production with porin loss based on the algorithm followed for CDTs (APBA + Cloxacillin positivity). None of the isolates showed Class A KPC production (APBA + Cloxacillin negative) alone as well as KPC with MBL (APBA + DPA positivity) based on CDT [Table 1]. Multiplex-PCR was done on 10 randomly selected strains showing ESBL and MBL positivity, and the results are shown in Table 2.

Table 1: Number of positive isolates based on the algorithm for detection of various β-lactamases

β-lactamase	APBA	DPA	Cloxacillin	*n*
KPC	Positive	-	-	0
MBL	-	Positive	-	32
AmpC + porin loss	Positive	-	Positive	4
MBL + KPC	Positive	Positive		0

KPC = *Klebsiella pneumoniae* carbapenemase, MBL = Metallo β-lactamases, APBA = Amino phenyl boronic acid, DPA = Dipicolinic acid

Discussion

In this study, fifty *K. pneumoniae* isolates which were carbapenem resistant by disc diffusion method and also by E–test have been evaluated. All the isolates were resistant to most of the antibiotics tested except for amikacin (72% susceptible). Various studies show high level of resistance to many antibiotics in carbapenem resistant *K. pneumoniae* similar to the findings in our study.[16,17]

Out of 50 carbapenem resistant isolates, 39 (78%) showed ESBL production, 32 (64%) isolates showed MBL positivity and 4 (8%) showed AmpC positivity with porin loss based on inhibitors based phenotypic tests.

Thirty-two strains were both ESBL and MBL producers. In a previous study from our institution 19/26 (73%), MBL positivity was seen but none of the 26 strains showed KPC production in *Enterobacterial* strains.[18] In the present study, there is little decrease in the trend of (64% vs. 73%) MBL production. The studies from different parts of India have reported varying resistance mechanisms in *K. pneumoniae* including combination of various resistance genes.[6,2] In a recent study, both MBL and KPC type carbapenemases were seen among clinical isolates of *Escherichia coli* and *Klebsiella* spp. and supplementing MHT with a CDT is a reliable phenotypic test to identify both the class A and class B carbapenemase producers.[19] However, in our study despite of using CDT, we were not able to show KPC enzyme production based on the phenotypic test, and this was also confirmed in ten strains by PCR test, similar finding has been highlighted in a recent study from North India regarding absence of KPC enzyme.[20] We were not able to show any Class D enzyme also as till now there is no phenotypic method for Class D enzyme detection. However, recently a disc diffusion test and MIC based testing with Temocillin has been shown to be a good indicator of OXA-48, but this needs further evaluation.[21,22]

Out of 50, eighteen strains which were resistant to carbapenems (MIC) failed to show MBL or KPC production by phenotypic tests since another important cause of carbapenem resistance among *Enterobacteriaceae*

Table 2: Results of multiplex-polymerase chain reaction done on 10 randomly taken strains showing extended spectrum beta-lactamase and metallo beta lactamase positivity based on phenotypic tests

Strains	DDST and CDT for MBL phenotype	NDM	Other MBL genes (IMP/VIM)	KPC	OXA-48	CTX-M groups	Plasmid mediated AmpC genes (CMY-2 and DHA-1)
V1	+	+	-	-	-	CTX-M group 1	-
V2	+	+	-	-	-	CTX-M group 1	-
V3	+	+	-	-	-	CTX-M group 1	-
V4	+	+	-	-	-	CTX-M group 1	-
V5	+	+	-	-	-	CTX-M group 1	-
V6	+	+	-	-	-	CTX-M group 1	-
V7	+	+	-	-	-	CTX-M group 1	-
V8	+	-	-	-	-	CTX-M group 1	-
V9	+	-	-	-	-	CTX-M group 1	-
V10	+	-	-	-	-	CTX-M group 1	-

OXA-48-class D, CTX-M-Class A, CMY-2, DHA-1, AmpC enzymes. DDST = Double disk synergy test, CDT = Combined disc test, MBL = Metallo β-lactamases, NDM = New Delhi metallo-beta-lactamase-1, VIM/IMP = Class B Enzyme, KPC = *Klebsiella pneumoniae* carbapenemease,+ = Found, - = not found

could be overproduction of ESBL or AmpC enzyme with porin changes, this could be the reason in these eighteen bacterial strains.[10] In our study, four strains were positive for APBA and cloxacillin CDT thus showing AmpC enzyme with porin loss, by phenotypic methods. In an earlier study done at our center, we were able to detect AmpC in 32% of *K. pneumoniae*.[15]

In this study, all the 50 isolates were MHT positive. Since MHT test can give false-positive results in isolates showing ESBL or AmpC enzyme with porin loss, it cannot be relied on completely, especially in areas with high prevalence of CTX-M type of ESBLs and should be supplemented with CDT tests for carbapenemase detection.[23] Further, in our study, 32 strains showed both ESBL and MBL, and additional 7 strains showed ESBL positivity (total ESBL = 39), so these could be responsible for carbapenem resistance with some permeability defect association. Six Seven out of 50 isolates could not fit into any ESBLs, AmpC, KPC, MBLs phenotypically assuming that these could have some other carbapenemase genes probably OXA enzymes which were not detectable by CDT.

In our study, all the 10 isolates evaluated by genotypic technique produced CTX-M group 1 ESBL enzyme by multiplex PCR. CTX-M carriage has been reported from South East Asia and other Mediterranean countries.[24] In India also, CTX-M has been shown to exist in members of *Enterobacteriaceae*.[25] Seven out of 10 strains which showed positive MBL results by phenotypic method were positive for NDM gene by PCR and showed absence of any other MBL gene, so this seems to be the most prevalent MBL type of enzyme in our setup. NDM-1 has been found not only in hospital but community settings also.[26] In 10 MBL positive strains, 3 did not show any other Class B enzyme (NDM/VIM/IPM), so there could be some rare Class B gene in these isolates. Thus, in this study, CTX-M type ESBLs and NDM type of MBLs were the predominant types of enzymes.

In spite of many phenotypic tests, isoelectric focusing and genotypic characterization based on multiplex PCR are considered gold standard as the results with the phenotypic tests can be ambiguous and unreliable. Attempts are being made to standardize some phenotypic methodology, as for most of the diagnostic laboratories it is difficult to do molecular techniques on a routine basis.

The limitation of our study is that we were able to perform multiplex PCR on 10 strains only out of 50 and also limited set of genes could be looked for because of financial constraints. However, the results of these 10 isolates are 100% in agreement with our phenotypic strategy of testing based on CDT method. Further limitation, we were not able to look for Class D enzymes phenotypically in our laboratory, and in this regard, further study is being planned to look for feasibility of using Temocillin for detection of Class D enzyme.

Carbapenems are one of the important last resort antibiotics in the treatment of infections due to multidrug-resistant bacteria. It is therefore essential to maintain the clinical efficacy of carbapenems by early detection of carbapenemases because these enzymes can easily be transmitted through transposon and/or integron, and there is a possibility of widespread dissemination among susceptible Gram-negative bacterial isolates in the hospital. Further, knowledge regarding the epidemiology of which carbapenemases are prevalent in a particular geographic area helps in planning treatment strategy as clinical isolates with a combination of mechanisms causing carbapenem resistance (e.g., impermeability plus ESBLs and/or hyperproduced AmpC) could still respond to carbapenem treatment, whereas carbapenemase-producing *Enterobacteriaceae* as such would rule out the use of β-lactams to treat patients, thus significantly limiting treatment options for life-threatening infections.[17] Care in detection is needed because high carbapenem MICs are not always evident, especially in OXA producers. So updating ourselves,

exploring new diagnostic options and implementing new testing strategies can help to detect these enzymes for early and proper patient management.

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Conflicts of interest
There are no conflicts of interest.

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