Original Article

Molecular Characterization of Carbapenemases among Clinical Strains of *Enterobacteriaceae* at a Tertiary Care Hospital in Rural Uttar Pradesh

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Abstract

Introduction: Emergence of Carbapenemase producing *Enterobacteriaceae* is one of the most important Global Health care concerns. In recent years Carbapenemase producing *Enterobacteriaceae* have been implicated in numerous outbreaks. The data regarding carbapenemase genes associated with carbapenemases production among clinical strains of *Enterobacteriaceae* in rural Uttar Pradesh is very limited. Thus, in the present study we aimed to perform Phenotypic and genotypic characterization of carbapenemases among clinical strains of *Enterobacteriaceae* at a tertiary care centre in rural Uttar Pradesh

Material & Methods: A total of 695 clinical strains of *Enterobacteriaceae* comprising of 482 *Escherichia coli* and 213 *Klebsiella pneumoniae* were subjected to antibiotic susceptibility testing for carbapenem by Kirby Bauer disc diffusion method. Phenotypic detection of carbapenemase activity was performed by Modified Hodge test and Molecular detection of *bla*$_{NDM}$, *bla*$_{KPC}$ & *bla*$_{VIM}$ genes was performed by Real Time Multiplex PCR.

Result: A total of 204 (29.35%) *Enterobacteriaceae* strains showed carbapenem resistance by Kirby Bauer disc diffusion method, 68.14% (139/204) among them were *Escherichia coli* whereas 31.86% (65/204) were *Klebsiella pneumoniae.* Modified Hodge test for carbapenemase activity was positive in 163/204 (79.90%) strains. The most prevalent gene associated with carbapenemases was found to be *bla*$_{NDM}$ (54.90%, 112/204) followed by *bla*$_{KPC}$ (19.11%, 39/204) and *bla*$_{VIM}$ (5.88%,12/204).

Conclusion: New Delhi Metallo-β-lactamase was found to be the most prevalent carbapenemases among clinical strains of *Enterobacteriaceae* in present study. The findings of present study highlight the urgent need of antimicrobial surveillance to monitor antimicrobial patterns in rural community in Uttar Pradesh.

Keywords: Carbapenem, MHT, PCR, NDM, KPC, VIM

INTRODUCTION:

Emergence of Carbapenemase producing *Enterobacteriaceae* (CPE) is one of the major Global Health care concerns. In recent years CPE have been implicated in numerous outbreaks. [1,2] Among Enterobacteriaceae *Klebsiella pneumoniae* (K.pneumoniae) and *Escherichia coli* (E.coli) have been associated with notable drug resistance and are the very common reason of majority of nosocomial and community acquired infections. [3] Carbapenems are broad-spectrum antibiotics and were mostly preferred as the last resort in multidrug‑resistant *Enterobacteriaceae* infections. [4] Carbapenems are β-lactams which differ from penicillin by substitute the carbon atom with sulfur atom and adding the double bond to the penicillin nucleus five membered ring. Carbapenems act by binding to penicillin binding proteins (PBP), by preventing the binding of peptidoglycan strands & further synthesis of the cell wall of the bacteria. [5] The Carbapenem resistance may occur via 3 major mechanisms production of carbapenemases (β-lactamases enzyme), increased efflux pump action, and the combined effect of β-lactamases with permeability of bacterial cell membrane due to alteration or porin mutation. Carbapenemases production is the commonest mechanism of resistance to Carbapenems among *Enterobacteriaceae*. [6] The classes of carbapenemases produced by an *Enterobacteriaceae* isolate mainly depends on the carbapenemase gene harbored by the organism. The encoding genes of these carbapenemases are located either on the mobile genetic elements (MGEs) or on the chromosomes. Carbapenemases class A (*Klebsiella pneumoniae* carbapenemases (*KPC)* types), Class B Metallo β-carbapenemases (MBL types) i.e., *IMP*, *VIM* and *NDM* types, & Class D oxacillinases (*OXA*-types) are keeping importance in *Enterobacteriaceae* epidemiologically. [7] Carbapenemases like *NDM* (New Delhi Metallo β-carbapenemases) & KPC producing *Enterobacteriaceae* are showing resistance to most of the *β-lactams*, aminoglycosides and fluoroquinolones. Generally, *OXA* type carbapenemases shows less activity towards carbapenems but can induce a high resistance when associated with Extended-spectrum β-lactamases (ESBLs). [8]

In recent years there are several reports of Carbapenem resistance *Enterobacteriaceae* having resistance mechanism as the acquisition of carbapenemase genes. [9,10] Accurate & early detection of carbapenem resistance *Enterobacteriaceae* is necessary for effective appropriate antimicrobial therapy. The most used phenotypic tests for Carbapenem resistant *Enterobacteriaceae* is Kirby Bauer disc diffusion (KB Test) method and Modified Hodge test (MHT). MHT is CLSI recommended growth-based phenotypic method for detection of carbapenemase. The MHT is based on the ability of

carbapenemase producers to decrease the concentration of carbapenem antibiotics, which allow the carbapenem-susceptible *E. coli* ATCC 25922 strain to grow producing a cloverleaf appearance. [11,12] Although molecular detection of carbapenemase genes using RT-PCR remains the standard procedure; it has limited significance in resource constrains settings. [10] The data regarding genes associated with carbapenemase production among carbapenem resistance clinical strains of *Enterobacteriaceae* in rural Uttar Pradesh is very limited. Thus, in this present study we aimed to perform phenotypic & genotypic characterization of carbapenemases among carbapenem resistant clinical strains of *Enterobacteriaceae* at a Tertiary Care Hospital in Rural Uttar Pradesh

MATERIAL & METHODS:

Study Design and Settings: Study Design and Settings: A Cross sectional study was performed at a tertiary care hospital in rural Uttar Pradesh for a period of 3 year from January 2018 to March 2021 after obtaining approval from Institutional Research & Ethical Committee [SU/2017/187(4)].

Bacterial strains and Antibiotic Susceptibility Testing for Carbapenem Resistance: A total of 695 clinical strains of *Enterobacteriaceae* isolated over a period of 3 years were used for the present study. Four hundred eighty-two (69.35%) among them were *Escherichia coli* whereas 213 (30.65%) were *Klebsiella pneumoniae.* All clinical strains of *Escherichia coli & Klebsiella pneumoniae* were processed for antibiotic susceptibility testing for carbapenem resistance using Imipenem (10ug), Meropenem (10ug) & Ertapenem (10ug) by K B disc diffusion method following CLSI guidelines 2019. [13]

Phenotypic detection: Phenotypic testing for carbapenemase activity was performed by MHT following CLSI guidelines. [14] 0.5 McFarland's suspension of ATCC *E. coli* 25922 (indicator organism) was diluted 1:10 in sterile saline. With the help of sterile swab stick, this 1:10 dilution of indicator strain (*E. coli* ATCC 25922) was lawn cultured on Mueller Hinton Agar plate. Plate was air dried for 5-6 minutes and a disc of Imipenem 10 µg was placed on the centre of the agar plate. With the help of sterile loop 4-5 colonies of the test organism were picked and inoculated in a straight line towards the periphery, from the edge of the disc. Positive control (*K. pneumoniae* ATCC 1705) and negative control (*K. pneumoniae* ATCC 1706) were also streaked on the same plate. The plates were incubated at 37^0C for 18-24 hours. The inoculated plates were checked for an enhanced growth around the test organism, at the intersection of the streak for a zone of inhibition. Carbapenemase producing isolate was detected by the MHT when the test isolate produces the enzyme and allowed the growth of the carbapenem susceptible *E. coli* ATCC 25922 strain towards the disk. The presence of an enhanced growth indicated carbapenemase production, and the absence of an enhanced growth meant that the test isolate did not produce carbapenemase.

Molecular detection of *bla*$_{NDM}$, *bla*$_{KPC}$ & *bla*$_{VIM}$ genes by Real Time Multiplex PCR: Deoxyribonucleic acid (DNA) was extracted from all isolates by column base DNA Extraction Kit following Kit Protocol. [15] For PCR 6.0 µl of Master Mix (Genetix Biotech Asia Pvt. Ltd) & 3.0 µl of each Primer Probe Mix [16] (Table:1) and 5.0 µl of isolated DNA templates were mixed in PCR tubes and amplification was performed in CFX96 Machine (BioRad) following Initial denaturation step was done for 3 min at 95^0C followed by Denaturation step for 10 sec at 95^0C & Annealing/Extension for 30 sec at 60^0C. The denaturation and annealing/extension step was repeated for 45 cycles and Acquisition on FAM, HEX & CY5 was taken at annealing/extension step of each cycle. After finishing the complete cycle graph was read considering positive Quantification cycle (Cq) value for FAM, HEX and CY5 between 10-30 and accordingly results were interpreted. If sigmoid curve appeared between the Quantification cycle (Cq) range of 10-30 was considered positive for presence of above gene in the sample. Isolates which did not showed sigmoid curve were considered negative. Control Strains [17] for KPC and NDM Negative Control (*K. pneumoniae* ATCC strain BAA-1706), KPC Positive Control (*K. pneumoniae* ATCC strain BAA-1705), NDM Positive Control (*K. pneumoniae* ATCC strain BAA-2146) & for No Template control (NTC) DNase & RNase free water were used for each experiment.

Table: 1 Primer used for *bla*$_{NDM}$, *bla*$_{KPC}$ & *bla*$_{VIM}$ genes [17]

Gene	Primer Sequence	Amplicon size
bla$_{NDM}$	F-GGTTTGGCGATCTGGTTTTC	621
	R-CGGAATGGCTCATCACGAT	
NDM Probe-FAM	FAM-TG GAT CAA GCA GGA GAT	
bla$_{KPC}$	F-CGTCTAGTTCTGCTGTCTTG	798
	R-CTTGTCATCCTTAGGCG	
KPC Probe-HEX	HEX-TG ATA ACG CCG CCG CCA ATT TGT	
bla$_{VIM}$	F-GATGGTGTTTGGTCGCATA	390
	R-CGAATGCGCAGCACCAG	
VIM Probe-Cy5	Cy-5-CA CGA GCT GAC GAC AGC CAT GCA	

RESULT

A total of 204 (29.35%) *Enterobacteriaceae* strains showed carbapenem resistance by Kirby Bauer disc diffusion method, 68.14% (139/204) among them were *Escherichia coli* whereas 31.86% (65/204) were *Klebsiella pneumoniae.* One hundred

eighty (88.23%) *Enterobacteriaceae* strains showed resistance to all three carbapenem drugs Imipenem, Meropenem & Ertapenem. Thirteen (6.37%) *Enterobacteriaceae* strains showed resistance to Imipenem and Meropenem both, seven (3.43%) showed resistance to imipenem alone, three (1.47%) strains showed resistance to imipenem and ertapenem both and one (0.49%) strain showed resistance to meropenem alone. A detailed description of antibiotic susceptibility patterns for carbapenem resistance among *Enterobacteriaceae* strains is shown in Table 2.

Table 2: A Detailed Description of Antibiotic Susceptibility Patterns for Carbapenem Resistance

Drugs	***Escherichia coli* (139)**	***Klebsiella pneumoniae* (65)**
Imipenem alone	07	00
Meropenem alone	01	00
Imipenem and Meropenem	09	04
Imipenem and Ertapenem	03	00
Imipenem, Meropenem and Ertapenem (Resistant to all)	119	61
Total	139	65

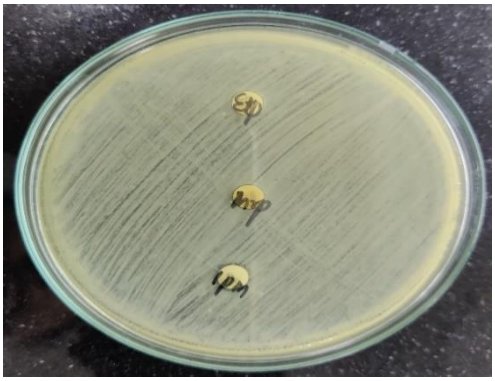

Figure 1: Result of Antibiotic Susceptibility Test

Modified Hodge test for carbapenemase activity was found to be positive in 163/204 (79.90%) strains. Molecular detection of bla_{NDM}, bla_{KPC} & bla_{VIM} genes by Real Time Multiplex PCR revealed that 61.92% (141/204) harboured one or more than one gene. The most prevalent carbapenemase gene was found to be bla_{NDM} (54.90%, 112/204) followed by bla_{KPC} (19.11%, 39/204) and bla_{VIM} (5.88%,12/204). Interestingly 22 (10.78%) strains showed the presence of both bla_{NDM} & bla_{KPC} genes. A detailed description of distribution of bla_{NDM}, bla_{KPC} & bla_{VIM} genes among *Enterobacteriaceae* strains is shown in Table 3.

Table 3: A detailed description of distribution of bla_{NDM}, bla_{KPC} & bla_{VIM} genes among *Enterobacteriaceae* strains

Organism	**NDM**	**KPC**	**VIM**	**NDM & KPC**	**Total**
Escherichia coli	72	06	11	09	98
Klebsiella pneumoniae	18	11	01	13	43

NDM – New Delhi Metallo β Lactamase, KPC – *Klebsiella pneumoniae* Carbapenemase, VIM – Verona Integron encoded Metallo β Lactamase

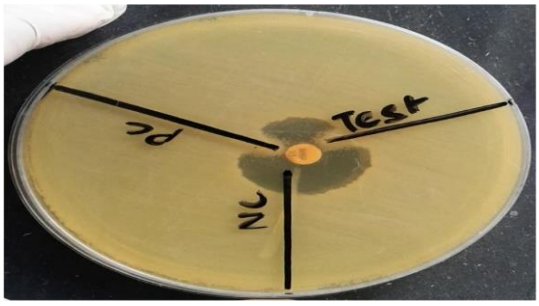

Figure 02: Result of Modified Hodge Test

Further on comparing the MHT and PCR results we observed that One hundread twenty five (61.27%) *Enterobacteriaceae* strains were positive for carbapenemases by both the methods, Thirty eight (18.63%) strains were positive by MHT alone, sixteen (7.84%) strains were positive by PCR alone, whereas twenty five (12.25%) strains were negative for carbapenemases by both the methods. There was a significant correlation between both methods (*Chi square* statistics 21.77, *P*=0.00001). A detailed comparison of MHT and PCR results for Carbapenemases among *Enterobacteriaceae* strains is given in Table 4.

Table 4: Comparison of MHT and PCR results for Carbapenemases among *Enterobacteriaceae* strains

Organism	**NDM**	**KPC**	**VIM**

	MHT	PCR	MHT	PCR	MHT	PCR
Escherichia coli	73	81	14	15	11	11
Klebsiella pneumoniae	26	31	22	24	01	01

DISCUSSION

The objective of this study was to perform Phenotypic & Molecular characterization of carbapenemases among carbapenem-resistant *Enterobacteriaceae* strains which were isolated from a tertiary care hospital in rural Uttar Pradesh. Among *Enterobacteriaceae, Klebsiella pneumoniae* (70%) and *E. coli* (55%) were associated with high carbapenem resistance by recent World Health Organization report. [18] ICMR-AMRSN also reported carbapenem resistance prevalence of 40-50% in *Klebsiella pneumoniae* and 15-25% in *E. coli* in general population in India. [19] Thus, we included *Klebsiella pneumoniae* and *E. coli* strains for Phenotypic and Genotypic characterization of carbapenemases in the present study. The overall prevalence of carbapenem resistance in present study was found to be 29.35% which is a matter of concern and highlights the urgent need of strategies to restrict spread of carbapenem resistance in this region.

Class B carbapenemases NDM, VIMs and IMPs are generally associated with carbapenem resistance in *Enterobacteriaceae*. [2] Among them NDM a plasmid mediated metallo-β-lactamase is one of the most clinically significant carbapenemases. It was first discovered in a *K. pneumoniae* strain from the Swedish patient's urine sample who was hospitalized in India and since then it has spread Worldwide. [20, 26] NDM-producing *Enterobacteriaceae* are widely spread in almost all part of India. In present study also the most prevalent gene was found to be bla_{NDM} (54.90%, 112/204) that encodes for New Delhi metallo-β-lactamase. Verona integron-encoded metallo-β-lactamase (VIM) are often associated with patients with immunodeficiency and drug resistance nosocomial infections. [21] Twelve (5.88%) strains in present study also harboured bla_{VIM}. *K. pneumoniae* carbapenemase (KPC) a Class A serine carbapenemases was first reported in a *K. pneumoniae* isolate in 1996 in the USA and since then it is spreading endemically throughout the world, causing a various type of severe infections. [22] KPC encoding bla_{KPC} gene was observed in 19.11% (39/204) *Enterobacteriaceae* strains in present study. Coexistence of KPC with other carbapenemase eg. IMP, VIM & NDM is also common. 22 (10.78%) *Enterobacteriaceae* strains in present study also showed the presence of both bla_{NDM} & bla_{KPC} genes. [6,23]

On comparing MHT and PCR results we observed that in 38 (18.63%) MHT positive strains none of the genes included in our study were amplified, which can be explained by presence of bla_{OXA} or any other genes responsible for carbapenemases production. Authors in past has reported poor performance of MHT for detection of carbapenemases for metallo-β-lactamases (NDMs, VIMs and IMPs) 13 PCR positive NDM strains in present study were also found to be negative by MHT. [24] Cabral AB et al have reported that in MHT negative isolates some of the isolates carrying silent bla KPC gene likewise we have also observed 3 such strains with silent bla KPC gene. [25]

Further 25 (12.26%) *Enterobacteriaceae* strains were negative by both PCR and MHT which suggests that the carbapenem resistance in those strains may be due to hyper production of ESBL or AmpC enzymes combined with alteration or upregulated efflux pump or porin loss. [1,2] Due to financial constrains we have performed only K B disc diffusion method, MHT and PCR for bla_{NDM}, bla_{KPC} & bla_{VIM} genes which remains one of the major limitations of this study. Including other phenotypic tests like E-test, MIC, Carba NP and including other genes for carbapenemases producers might have provided better insight of carbapenem resistance in present study.

CONCLUSION

New Delhi metallo-β-lactamase was found to be the most prevalent carbapenemases among clinical strains of *Enterobacteriaceae* in present study. The finding of present study highlights the urgent need of antimicrobial surveillance to monitor antimicrobial patterns in rural community in Uttar Pradesh.

LIMITATION OF THE STUDY: Genes responsible for carbapenem resistance other than NDM, VIM and KPC can be characterize and other organisms of family *Enterobacteriaceae* can also be studied for carbapenemase production.

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CONFLICT STATEMENT: Authors declare no conflict of interest.

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ETHICS STATEMENT: Not applicable

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