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Genotypic Distribution and Antimicrobial Susceptibilities of Carbapenemase-Producing *Enterobacteriaceae* Isolated in Tertiary Care Hospital in South India

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Abstract

Antimicrobial resistance has emerged as a major danger to contemporary medicine around the world. Carbapenems are the highest class of B-lactam drugs which are considered as the most effective and safest antibiotics available. Increasing spread of carbapenemases has been noted across the world which restricts treatment options. The aim of this study was to determine the incidence of carbapenem resistant genotypic pathways in a tertiary care hospital. 130 clinical strains of *Enterobacteriaceae* were subjected to Kirby-Bauer disk diffusion tests and genotypic methods (PCR) for the identification of the genes NDM, VIM, and OXA-48. Carbapenem resistance was detected in 30% of the isolates by phenotypic methods. These 37 isolates on being subject to PCR showed OXA-48 followed by VIM and NDM as the most frequently isolated genotypes. All isolates had multiple genes encoding carbapenem resistance. Carbapenemases resistance is on the rise and is associated with multi drug resistance pattern. To minimize spread and initiate early appropriate therapy, early detection of carbapenem resistance is essential. Molecular methods remain gold standard for detection.

Keywords: Carbapenemases, *Enterobacteriaceae*, Genotypic, OXA-48, NDM, VIM

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INTRODUCTION

Antimicrobial resistance (AMR) has emerged as a major danger to contemporary medicine around the world.[1] As per the WHO Global Antimicrobial Resistance and Use Surveillance System report 2021, high rates of resistance have been reported for frequently used antimicrobials which are used to treat common bacterial infections such as UTIs.[2] This may result in ineffective empiric treatment and impede efforts to prescribe narrow spectrum antibiotics.[2] Low and middle-income countries have greater reported rates of AMR indicators than high-income countries, which is concerning.[2]

β-Lactam antibiotics have been the cornerstone of antibacterial therapy in recent times. Carbapenemases, enzymes that hydrolyzes all β-lactam antibiotics, including carbapenems, have been increasingly noted in *Enterobacteriaceae*.[2,3] The emergence of carbapenem- resistant *Enterobacteriaceae* (CRE) therefore threatens the success of modern medicine in treating infections and enabling safe surgeries. Hospital acquired infections with CRE have very limited safe treatment options. The increasing presence of these CRE in community acquired infections is of most crucial concern.

Exhibiting multiple mechanisms of resistance is a distinctive feature of Carbapenem resistant pathogens. Based on this criterion they are categorized as Carbapenemase producing Carbapenem resistant *Enterobacteriaceae* (CP-CRE) and Non Carbapenemase producing CRE (Porin deficiency AmpC, ESBL, and Carbapenem efflux mechanisms act as virulence factors in these pathogens).[4] Carbapenem resistance is regulated by multiple plasmid encoded genes that are positioned in various mobile genetic elements. The most frequently encountered genes in India are bla_{NDM}, bla_{OXA-48}, $blaI_{MP}$ and bla_{VIM}.[3,5,6]

With the introduction of novel successful therapeutic alternatives for CRE infections, the options for treatment could be targeted and personalized considering the individual antimicrobial susceptibility profiles, molecular resistance phenotypes, and the severity of the disease. Novel β-lactam/β-lactamase inhibitors such as aztreonam/avibactam (ATM/AVI, active against KPC, MBL, AmpC and OXA producers), ceftazidime/avibactam (CAZ/AVI, active against KPC and OXA-48 producers),meropenem/vaborbactam (active against KPC producers) and imipenem/relebactam (active against KPC and AmpC producers) are specifically targeted against a particular molecular resistance phenotype. If the presence of the specific resistance mechanism is detected early, these patients can be prioritized for therapy with novel CP-CRE active antibiotic agents, thereby minimizing treatment failure and drug resistance.[4]

This study was undertaken to establish the prevalence of bla_{NDM}, bla_{OXA-48}, and bla_{VIM} in clinical isolates collected from Microbiology laboratory in a tertiary care hospital in India.

MATERIALS AND METHODS

A prospective, cross-sectional study was undertaken in the Department of Microbiology, Central lab, SRM Medical College and Research Centre, Kattankulathur, Tamil Nadu. Prior to undertaking the investigation, the institutional ethics committee provided approval for the project. A total of 130 *Enterobacteriaceae* spp isolated from 200 consecutive clinical specimens received at the laboratory during a period between April 2021 and May 2021 were included in the study. The clinical specimens consisted of various samples such as urine, pus and wound swab, blood culture bottles and tracheal aspirates. Non *Enterobacteriaceae* spp isolated in culture were excluded from the study. The general patient demographics such as age and ward were also collected from the laboratory database for analyzing the distribution of the CRE infections. No specific patient identifiers were included in this study.

Antimicrobial Susceptibility Test

The isolates were confirmed as *Enterobacteriaceae* spp by standard biochemical identification tests. According to the Clinical and Laboratory Standards Institute standards (2020), antimicrobial susceptibility was tested using the standard disc diffusion method on Mueller Hinton agar plate. The tested antibiotics were: Penicillins (Ampicillin and Amoxicillin),β-

lactam & β lactam inhibitor combination (Piperacillin Tazobactum and Ceftazidime-Clavulanic acid) Cephalosporins (Cefazolin, Cefoxitin, Cefuroxime, Ceftriaxone, Cefotaxime, Ceftazidine and Cefipime), aminoglycosides (Gentamycin, Amikacin) Tetracycline, Carbapenems (Meropenem, Imipenem and Ertapenem) Fluoroquinolones(Ciprofloxacin and Ofloxacin), Cotrimoxazole, Chloramphenicol and Nitrofurantoin. As per the CLSI guidelines, minimum inhibitory concentrations (MICs) for Colistin were calculated using the broth micro dilution method. The resistance to different classes of drugs was analyzed to classify the isolates as single class resistant or multi drug resistance (resistance to three or more classes of antibiotics).

Genotypic Identification of Carbapenemase Encoding Genes

To isolate genomic DNA, a pure culture of bacterial isolates was cultured overnight on nutrient agar culture plates. PCR amplification was used to detect carbapenemase-encoding genes in all carbapenem-resistant isolates.

Cell Lysis and DNA Extraction

Pure isolated colonies of CRE were transferred from cultures plates to 1.5ml centrifuge tube containing 1 ml of sterilized double distilled water using sterilized 4mm loop wire. These isolates were then subjected to boiling at 95°C for 15minutes in a water bath followed by centrifugation at 15000rpm for 10 minutes and were stored in deep freezer at -72°C for later use. Further Truescreen magnetic bead based extraction kit developed by TranScience Innovative Technologies Pvt. Ltd. was utilized for the extraction of plasmid DNA. The Proteinase K solution lyses the cell and expels out the DNA, proteins and other cell debris into the solution. Addition of Truescreen solution containing magnetic bead embedded with nucleic acid results in binding of the plasmid DNA present in the isolate to these beads. This solution is subjected to centrifugation to settle down all the contents of the tube at the bottom of the tube and when this solution is placed on the magnetic stand, the beads get attached to the walls of the tube due to magnetic attraction and are separated from rest of the cell debris. The supernatants are removed without disturbing the beads. After 2 series of washing steps the plasmid DNA is eluted out as supernatant.

Primer Designing

The forward and reverse primers were selected from Standard Operating Procedures Bacteriology, Antimicrobial Resistance Surveillance and Research Network 2nd Edition, published by Indian Council of Medical Research (ICMR), New Delhi, India in 2019. The primers were designed and developed at Eurofins Genomics, Bangalore, India. Complimentary and specific primers were designed for the detection of bla_{VIM}, bla_{NDM}, and bla_{OXA-48} genes.[6] (Table 1)

Mastermix Preparation

Commercially available SYBR™ Green PCR Master Mix (1X5ml vial) was used for this assay which includes 2X mixture of SYBR Green 1 Dye, AmpliTaq Gold™ DNA polymerase, dNTPs with dUTP, Passive Reference 1 (ROX), and optimized buffer components. 12.5µL of SYBR™ Green PCR Master Mix was used for each sample. To this 12.5µl mix, 0.3 µM (0.3µL) each of forward and reverse primer was added (for each of the three genes under study), to which 3µl of template DNA was added. Nuclease free molecular grade water was added to make up the final volume at 25µl. This mix was stored at -20°C for later use.[7]

Real Time Quantitative PCR Assay

In this study, ABI PRISM 7900HT Fast 384-Well System was used for performing Absolute quantification based real time PCR. Assay design was performed using the SDS Automation Control software installed in the computer which acts as a user interface software. One step PCR conditions

Table 1. The primers used in this study

Target gene	Primer pairs	Amplicon (Base pairs)
bla_{VIM}	F - GATGGTGTTTGGTCGCATA R - CGAATGCGCAGCACCAG	390
bla_{NDM}	F - CCGTATGAGTGATTGCGGCG R - GCCCAATATTATGCACCCGG	779
bla_{OXA-48}	F – GCTTGATCGCCCTCGATT R - GATTTGCTCCGTGGCCGAAA	570

were selected for the assay Assay set-up was performed according to system guide published by the manufacturer for Absolute quantification curve assay.[8] Cycle conditions were as followed: 15 min hold at 95°Cand 30 cycles of amplification consisting of 30 sec at 94°C, 90 sec at 59°C and 90 sec at 72°C, with 10 min at 72°C for the final extension6. Amplification confirmed the presence of bla_{NDM}, bla_{OXA-48} and bla_{VIM} genes.

RESULTS

Population and Pathogen Distribution

In the present study, 130 clinical isolates of *Enterobacteriaceae* were isolated from patients admitted in a tertiary care hospital. 52% (67 patients) of these patients belong to female population and 48% (63 patients) belong to male population. Among the patients whose sample were used for this study, 34% belong to the age group of more than 61, 32% belong to the age group 41-60, 25% belong to age group of 18-40 and comparatively only 14% who belong to the age group of less than 18 years of age. Among the 130 clinical samples which showed the presence of *Enterobacteriaceae* family as pathogen, 96 (74%) were isolated from urine samples, 18 were pus and wound swabs(18%), 4 were blood sample (3%) and 12 belong to miscellaneous samples (9%). Among the 130 *Enterobacteriaceae* isolates collected 72(55%) were found to *Escherichia coli*, 44 (34%) were found to *Klebsiella pneumonia*, 11 (8%) were found to *Citrobacter* spp., 2 were *Proteus mirabilis* and 1 was *Morganella morganii.*

In this study, the Carbapenem resistant isolates from the patient's samples were collected from different units of the hospital. CRE was most frequently isolated from General Medicine ward (12 isolates) followed by Major Intensive Care Unit (MICU) (6 isolates) and urology and pulmonary ward respectively (5 isolates from each ward). Rests of the isolates were found in patients admitted in Causality, COVID-ICU and Respiratory ICU. Further looking at the sample wise distribution 82% (32 isolates) were obtained from urine samples followed by 12% from pus and wound swab (5 isolates) and one isolate each from blood culture bottles and tracheal aspirates.

Antimicrobial Susceptibility

The analysis of the antimicrobial susceptibility of the *Enterobacteriaceae* isolates revealed interesting findings (Figure 1). The highest resistance was observed for Penicillins (Ampicillin 86.2% and Amoxicillin 46.9%) followed by First, Second, Third and Fourth generation Cephalosporins [Cefazolin (63.8%), Cefuroxime (68.5%) Cefotaxime (60%) and Cefipime (48%)]. Ceftazidime (30.8%) was found to be the most effective Cephalosporin antibiotics. Among the β-lactam & β lactam inhibitor combinations, Piperacillin-Tazobactum (29.2%) was superior to Ceftazidine-Clavulanic acid (56.2%) for therapy. Carbapenem class of antibiotics was also found to be highly sensitive to these isolates (Meropenem 26.2%, Imipenem 26.9% and Ertapenem 28.5%) compared to other β lactam class of antibiotics. Among the aminoglycosides, Amikacin (29.2%)

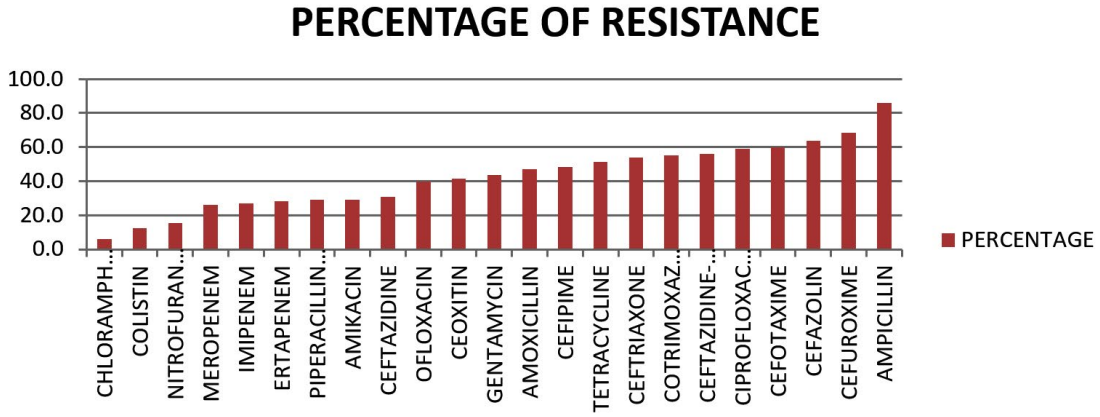

Figure 1. Graphical representation the antibiotic resistance (%) of the *Enterobacteriaceae* isolates

was found to be more effective than Gentamicin (43.8%). Nitrofurantoin (15.4%) is found to be the most effective antibiotic as 85% of the isolates were found to be sensitive to it. Chloramphenicol (6%) and colistin showed high sensitivity, though these antibiotics are not often used as therapy due to the known side effects.

Overall prevalence of CRE was found to be 30% (39 isolates). Resistance to at least three different antibiotic classes was regarded as multidrug resistance. (Table 2) The prevalence of multi drug resistance (MDR) *Enterobacteriaceae* were found to be 32% (42 isolates) in this study and 30% (39 isolates) of the isolates were found to be MDR and CRE. 55% of the isolates were found to be resistant to only one or two different class of antibiotics and remaining 13% of the isolates were susceptible to all antibiotics.

Real time Quantitative PCR was performed to detect the bla_{NDM}, bla_{VIM}, bla_{OXA-48} gene encoding Carbapenem resistance in the 39 CRE isolates. (Figure 2) It was observed that majority of isolates were found to have more than one Carbapenem resistance encoding gene (bla_{NDM}, bla_{VIM}, bla_{OXA-48})

Table 2. Distribution of multi drug resistance (MDR) pathogens in the collected isolates

	Type Of Pathogen			
	Single Antibiotic Class Resistant	Two Antibiotic Class Resistant	Multidrug Resistant	Multidrug Resistant & CRE
No. of Isolates	35	36	42	39
Percentage %	27	28	32	30

Table 3. Distribution of gene combinations observed among CRE isolates in the study

Gene Combinations	No. of Isolates
NDM And OXA - 48	4
VIM And OXA - 48	15
All Three	20
Total	39

within their plasmid DNA. (Table 3) All 39 isolates were found to have bla_{OXA-48} gene in their plasmid DNA. bla_{VIM} was the second most prevalent gene present in 35 isolates followed by bla_{NDM} present in 26 isolates. The majority of isolates have all the three genes present in them (51%). 38% of isolates had bla_{VIM} and bla_{OXA-48} genes embedded in the genome. bla_{NDM} and bla_{OXA-48} gene combination

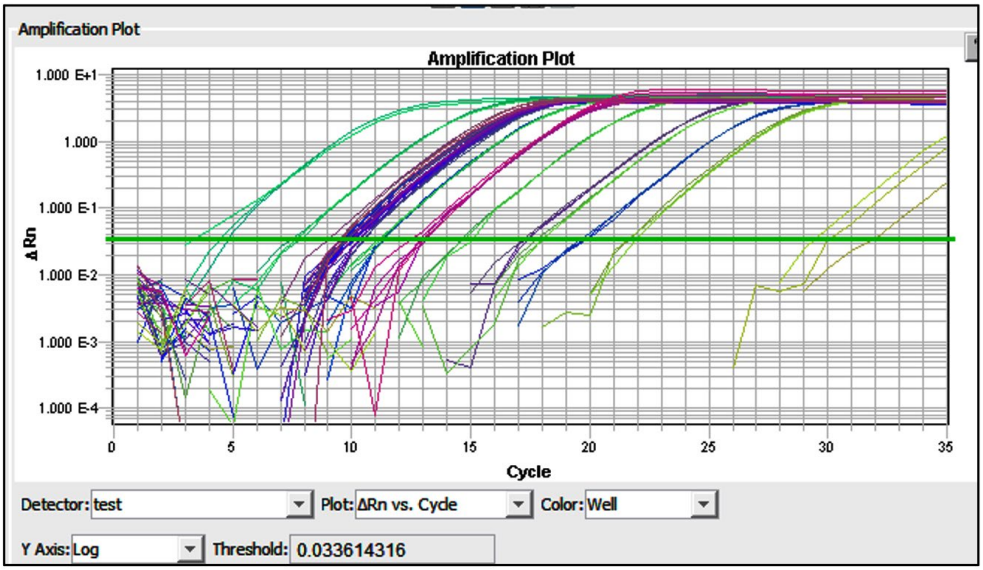

Figure 2. Isolates showing presence of bla_{NDM}, bla_{VIM}, bla_{OXA-48} in RT- PCR

was found to be least prevalent in the collected isolates. No isolate had a single genetic mechanism of resistance.

DISCUSSION

Carbapenem resistant *Enterobacteriaceae* are becoming more prevalent around the world, which is causing concern as *Enterobacteriaceae* are common commensals of the gastrointestinal tract. CRE can be spread within hospitals and communities with asymptomatic carriage, only to contribute to hard to cure hospital acquired infections as well as community acquired infection. CRE are enlisted as critical pathogens in the World Health Organization's list of Priority Pathogen 2018. Here we report the antimicrobial susceptibility profile and genotyping analysis of 130 clinical isolates of *Enterobacteriaceae* collected from patients admitted in a tertiary care hospital.

As per our findings, there was no significant gender bias (52% female vs 48% male) in the rate of CRE infections. However, maximum CRE infections occurred in older patients, according to our findings, with all the CRE isolated from patients >41 years. Similar trends in age distribution was observed by recent studies.[9,10] The rise in CRE prevalence in older patients can be observed due to high incidence of co-morbidities, previous exposure to antimicrobial therapy, weaker immune response and longer hospital stays in these individuals. Maximum isolates were obtained from urine samples (96), followed by pus and wound swabs (18), blood (4) and others (12). The most frequently isolated pathogen was *E.coli* (55%) followed by *Klebsiella pneumonia* (34%) and *Citrobacter* spp. (8%). This can be explained by the fact that urine samples yielded the most isolates, and E.coli is the most common uropathogen. It was found out via AST (KBDDM) that 30% of the 130 samples collected (39 isolates) were carbapenem resistant. Similar results were found in several other studies. [11,12] Some studies also showed lower prevalence of CRE 13 (18% CRE).This deviation maybe because of larger sample space used in the mentioned study (519) compared to this study (130) as well as the difference in geographical area as the study was conducted in Northern India whereas this study was performed in Southern India.

The predominant bacteria showing carbapenem resistance was *Klebsiella pneumonia* (29 isolates)which constituted 74% of the CRE isolates whereas the remaining was carbapenem resistant *E.coli* (10 isolates). This emphasized the fact that a higher proportion of *Klebsiella pneumonia* isolates are carbapenem resistant as compared to *E.coli.* Similar results were observed by Pawar et al.[11] (64% CR- *Klebsiella pneumonia* and 19% CR-E.coli) and Saeed et al.[9] (87% CR-*Klebsiella pneumonia* and 8% CR-*E.coli*).

The prevalence of MDR *Enterobacteriaceae* were found to be 32% in this study and when correlated with the prevalence of CRE, it was found out that all the CRE isolated in this study were found to be MDR (30%). This data further authenticate the findings confirming the extensive spread of these Superbugs in the Hospital settings in India.[11,12] The alarming rise in the resistance against several antibiotics can be explained by the irrational prescription and inappropriate adherence to oral antibiotic treatment, with minimum resistance being noted for injectable antibiotics such as aminoglycosides. Carbapenemase genes are frequently found on mobile genetic components that can easily be transmitted between bacteria. These also carry resistance genes to other antibiotics resulting in CRE being associated with multidrug resistance as evidenced in this study.

qPCR gene amplification was performed on the phenotypically confirmed CRE isolates to detect the presence of three genes that encode for carbapenem resistance (bla_{NDM}, bla_{VIM}, bla_{OXA-48}).67% of the isolates had bla_{NDM} gene present in their genome, 90% of the isolates have bla_{VIM} in their genome and all isolates (100%) were found to constitute bla_{OXA-48} within their genome. It was revealed in this study that all the CRE isolates tested displayed at least 1 gene within their genome that encodes for Carbapenem resistance. 51% of the isolates had all the three genes present within their genome, 38% isolates had both bla_{VIM} and bla_{OXA-48} within their genome and only 11%of isolates had both bla_{NDM} and bla_{OXA-48} present in them. The reason behind the co-existence of multiple Carbapenemase gene

in a single isolate is that the these pathogen utilize multiple mechanisms to tackle the activity Carbapenem class of antibiotics and each gene govern specific mechanism of resistance which is yet to be explained.[13,14] The prevalence of these genes varies vastly according to the geographical areas where the respective studies are conducted. Govindaswamy et al.,[5] found higher prevalence for bla_{NDM} gene (61.7% prevalence)followed by bla_{VIM} (30.8% prevalence) and bla_{OXA-48} (5% prevalence). Pawar et al.[15] found also had a coinciding result which reveals bla_{NDM} as most prevalent Carbapenemase gene followed by bla_{OXA-48} and reflected that 21%of the CRE isolates had both bla_{NDM} gene and bla_{OXA-48} coexisting together in their genome. The results of the current study is comparable to the study Nachimuthu et al.[16] conducted in Tamil Nadu in 2016 (nearby geographical area) which concluded that bla_{NDM} is the most prevalent gene in this region and bla_{OXA-48} is an emerging Carbapenemase gene. There is a possibility that shift in prevalence of these genes have taken place within the span of 5 years (2016-2021). These findings cast a shadow on the utility of the newer antibiotics which specifically target certain genotypic mechanisms in India.[17]

There are certain limitations in this study which in future could be rectified to further addition of relevant data related to this study. Inclusion of additional isolates and detection of additional genetic mechanisms such of bla_{KPC} and bla_{IMP} could make this study more comprehensive. Inclusion of Patient consent form and portfolio could have helped in gathering information about the history of patient, duration of hospitalization, type of infection and empirical treatments given to them. All these information could have helped in better understanding the spread of CRE in hospital settings and to devise protocol for better screening of patients with CRE infection. Inclusion of novel Cephalosporin class antibiotics like Cefiderocol in the panel of antibiotics could generate more relevant AST results.

CONCLUSION

Carbapenemases resistance is on the rise and is associated with multi drug resistance patterns, which may render multiple antibiotic classes ineffectual. To minimize spread and initiate early appropriate therapy, early detection of carbapenemases is a must. Molecular methods are a gold standard for the detection of carbapenemases. Further high-quality epidemiology and resistance mechanism–centered research is critically needed to standardize effective, tailored and targeted therapy for CRE infections.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made an equal, substantial, direct and intellectual contribution to the work, and approved it for publication.

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None.

DATA AVAILABILITY

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

This study was approved by the Institutional Ethics Committee, SRM Medical College Hospital and Research Centre, India with reference number 2889/IEC/2021.

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