Rising Threat of OXA-48 and other Carbapenemase Encoding Genes among Carbapenem Resistant Enterobacteriaceae in India

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Abstract

Members of Enterobacteriaceae family are responsible for both community and hospital acquired infections. Because of development of antimicrobial resistance carbapenem has remained as last resort of drug for treatment of infections caused by these bacteria. Mechanism for development of this resistance in carbapenem resistant Enterobacteriaceae (CRE) may due to production of carbapenemases, efflux mechanism or loss of outer membrane porins. The most common carbapenemase enzymes are Class A – KPC, Class B – NDM, VIM and IMP and Class D oxacillinase (OXA-48 like enzymes). In India, most prevalent carbapenemase encoding gene is NDM-1 but there is rising threat of OXA-48 prevalence. Unlike the phenotypic methods, the genotypic methods are useful to discriminate the type of carbapenemase enzyme, specifically for OXA-48 like enzymes. Total 170 CRE isolates were subjected for multiplex PCR study for their molecular characterization. Of the 170 CRE isolates, 68.2 % (n=116) were positive for NDM-1 gene while 44.1 % (n=75) of the isolates showed presence of OXA-48 gene. VIM (2.3%), KPC (1.7 %) were responsible for carbapenemase production while none of the isolates showed presence of IMP gene. NDM-1 and OXA-48 coexisted in 21.2 % (n=36) of the total isolates. OXA-48 causes weak hydrolysis of carbapenem because of which it is under reported with routine diagnostic methods. Early detection of OXA-48 and other carbapenemase encoding genes, helps for contact precautions and effective therapy which prevents further escalation and horizontal spread of CRE.

Keywords: OXA-48, NDM-1, Carbapenem Resistant Enterobacteriaceae, Carbapenemase

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INTRODUCTION

Most of microbiome of human intestine consists of Enterobacteriaceae family. The members from this family are responsible for both community and hospital acquired infections which includes wound infections, pneumonia, septicemia, meningitis, peritonitis, pylonephritis, cystitis and various catheter related infections. Because of development of antimicrobial resistance carbapenem has remained as last resort of drug for treatment of infections caused by these bacteria. But recently a major threat has emerged out as a result of carbapenem resistance leading to therapeutic failure. This has lead to serious healthcare concern especially in association with nosocomial outbreaks. Carbapenem resistance may occur due to production of carbapenemases, efflux mechanism or loss of outer membrane porins.

Carbapenemase enzymes confers resistance to most β-lactam antimicrobials including carbapenem. Also these isolates are coded with other antimicrobial resistant genes resulting in therapeutic failure. The most common carbapenemase enzymes belongs to three classes: Class A – KPC, Class B – NDM, VIM and IMP and Class D oxacillinase (OXA-48 like enzymes).

In India, most prevalent carbapenemase encoding gene is NDM-1 which is been proved in many studies. But of late the OXA-48 is seen to be closely following the NDM-1 prevalence, though it is more prevalent in Turkey, Morocco, Tunisia, Russia, Germany and other countries compared to India.

Various phenotypic methods are used by many clinical microbiology laboratories for detection of carbapenem resistance. Major disadvantage of these methods is failure to discriminate the exact type of carbapenemase produced which is very well identified and confirmed by genotypic methods. With development of number of newer resistance mechanisms by bacteria, identifying the most encountered resistance is important in a clinical setting. This will guide for infection control, contact precautions and understanding the prevalent carbapenem resistance mechanism in the geographical area. In the present study we aimed to identify the common blaNDM-1, blaKPC, blaOXA-48, blaVIM, and blaimp genes which are responsible for worldwide carbapenem resistance in clinical Enterobacteriaceae isolates by multiplex PCR study. Being one of the largest 1125 bedded hospital in this region, the study will provide prevalence of carbapenem resistance pattern from this place.

MATERIALS AND METHODS

Type of study
Observational cross-sectional study.

Study population
A total 170 carbapenem resistant Enterobacteriaceae isolates obtained from clinical specimens received for culture and sensitivity in the department of microbiology from June 2016 to May 2018 were included in the study.

Study setting
Department of Microbiology, KIMS Karad, Department of Molecular Biology and Genetics, KIMSDU, Karad.

Bacteriology Workup
Standard methodology was used for processing of samples. All the clinical specimens received during the period were cultured on blood agar, chocholate agar, and MacConkey agar. The growth in the culture media was identified by the Vitek® 2 system (BioMerieux, France). Antimicrobial susceptibility testing was done by Vitek® 2 system (BioMerieux, France). The growth in the culture media was identified by the Vitek® 2 system (BioMerieux, France). The growth in the culture media was identified by the Vitek® 2 system (BioMerieux, France).

Molecular Workup

Plasmid Extraction
Plasmid extraction was used for detection of carbapenem resistance genes. All 170 carbapenem resistant Enterobacteriaceae isolates were processed for plasmid extraction. (QIAGEN® Plasmid Mini Kits). The plasmid DNA were stored at -20°C for further use.

Polymerase Chain Reaction
Detection of carbapenemase encoded genes blaNDM-1, blaKPC, blaOXA-48, blaVIM, and blaimp was done with subset of primers. Primers used in the study were as shown in Table 1.
containing 2 U Taq polymerase (GeNei, India), 1X assay buffer consisting of 10 mM Tris-HCL (pH 9.0 at 25°C), 1.5 mM MgCl₂, 50 mM KCl and 0.01 % gelatin, each dNTP at a concentration of 200 µM and 10 pmols of each oligonucleotides primers per reaction.

Initially PCR was carried out to standardize the protocol for individual genes. For this PCR mixture used was as deionized distilled water -15µl, buffer -2 µl, dNTP-0.5 µl, forward primer -0.5 µl, reverse primer 0.5 µl, Taq DNA polymerase - 0.5 µl, Template DNA – 1 making total volume 20 µl.

Table 1. Primers used for carbapenemase encoded genes in the study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward / Reverse</th>
<th>Primer Sequences</th>
<th>Amplicon size(bp)</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDM-1 F</td>
<td>5’-GGGCAGTGCCTTCAACACGT-3’</td>
<td>475</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>5’-GTAGTGCTCAGTGTCGCGAT-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXA-48 F</td>
<td>5’-GCCTCTGCTTTAAAGAGAATACAC-3’</td>
<td>307</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>5’-CGCTCGTATAGTGTAACCTT-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KPC F</td>
<td>5’TGGTGTACCTAGTGTCGCGAT-3’</td>
<td>150</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>5’TGGTGTACCTAGTGTCGCGAT-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIM F</td>
<td>5’TGGTGTACCTAGTGTCGCGAT-3’</td>
<td>523</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>5’TGGTGTACCTAGTGTCGCGAT-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMP F</td>
<td>5’-GAAGGCGGTATGATGTCGCGAT-3’</td>
<td>587</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>5’-GAAGGCGGTATGATGTCGCGAT-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. PCR programs used for standardization of PCR

<table>
<thead>
<tr>
<th>Initial Denaturation</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C 10 minutes</td>
<td>95°C 30 seconds</td>
<td>55°C 30 seconds</td>
<td>72°C 30 seconds</td>
<td>4°C 10 minutes</td>
</tr>
<tr>
<td>Class A 1. KPC (150 bp)</td>
<td>30 cycles Class B 2. NDM-1 (475bp)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>95°C 10 minutes</td>
<td>95°C 30 seconds</td>
<td>55°C 30 seconds</td>
<td>72°C 30 seconds</td>
<td>4°C 10 minutes</td>
</tr>
<tr>
<td>Class B 2. NDM-1 (475bp)</td>
<td>30 cycles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>95°C 10 minutes</td>
<td>95°C 30 seconds</td>
<td>45°C 30 seconds</td>
<td>72°C 45 seconds</td>
<td>4°C 10 minutes</td>
</tr>
<tr>
<td>Class C 3. VIM (523 bp)</td>
<td>35 cycles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>95°C 5 minutes</td>
<td>95°C 30 seconds</td>
<td>55°C 1 minute</td>
<td>72°C 45 seconds</td>
<td>4°C 10 minutes</td>
</tr>
<tr>
<td>Class D 4. IMP (587 bp)</td>
<td>35 cycles Class E 5. OXA-48 (307 bp)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>95°C 10 minutes</td>
<td>95°C 30 seconds</td>
<td>55°C 30 seconds</td>
<td>72°C 30 seconds</td>
<td>4°C 10 minutes</td>
</tr>
</tbody>
</table>

bp – base pair
The PCR programs used for amplification were as shown in Table 2.

*Klebsiella pneumoniae* ATCC BAA-2146 was used as control for NDM-1 gene and *Klebsiella pneumoniae* ATCC BAA-1705 was used for KPC. For VIM and OXA-48, in house control strains confirmed by gene sequencing were used as control during PCR study. For detection of *bla*IMP conventional PCR method was used. After standardization of PCR, all DNA plasmids were subjected for multiplex PCR study. (Qiagen Multiplex PCR Kit). This was used for detection of *bla*NDM, *bla*KPC, *bla*OXA-48, and *bla*VIM. For this reaction mixture was prepared as mastermix (Qiagen) -10 µl, primers NDM-1, KPC, OXA-48, VIM – forward and reverse- 0.5 µl each, RNAse free water- 3 µl, Q buffer - 2µl, template- 1 µl making 20 µl total reaction volume. PCR program used to carry multiplex PCR reaction is as shown in Table 3.

The amplification product of multiplex PCR were analyzed by electrophoresis in 2.0% agarose gel at 100 V for 1 h in 1X Tris acetate EDTA (TAE) buffer stained with 0.01 mg/ml ethidium bromide. For molecular weight marker 1 Kb DNA ladder was loaded in a gel along with the samples to confirm specific size of the corresponding gene. For staining of the gel ethidium bromide (10mg/ml) was used and UV transilluminator was used for visualization and results were photographed in gel documentation system (Bio- Rad Laboratories).

**DNA Sequencing**

DNA sequencing was done for OXA-48 and VIM gene as no control strains were available for the same. Two clinical isolates from the study encoded with likely OXA-48 and VIM gene with PCR amplification of 307 bp fragments and 523 bp fragments respectively were used for sequencing purpose. PCR products were first purified and were sent for DNA sequencing by direct cycle sequencing. DNA sequencing was carried out using ABI PRISM 310 Analyzer from commercial source by dyeoxy nucleotide chain termination method. DNA sequencing of the core region was

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**Table 3.** PCR program used for Multiplex PCR

<table>
<thead>
<tr>
<th>Initial Denaturation</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIM (523 bp), NDM-1 (475 bp), OXA-48 (307bp), KPC (150 bp)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>95°C 10 minutes</td>
<td>95°C 30 seconds</td>
<td>45°C 1 minute</td>
<td>72°C 1 minute</td>
<td>72°C 10 minutes</td>
</tr>
<tr>
<td>4°C ∞</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 1.** Distribution of Carbapenemase encoded genes in CRE isolates
performed using the sense and antisense primers separately.

The analysis of derived nucleotide sequences for identification of sequence similarity was performed using the NCBI/BLAST network service of National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/BLAST). The obtained DNA sequences from isolates were aligned with representative of the sequences for identification of sequence similarity from gene bank database (NCBI blast search) with the help of nucleotide alignment program. The DNA sequence of both OXA-48 and VIM genes were sent for publication in National Centre for Biotechnology Information (NCBI) database and were assigned with GenBank accession number MK183750.2 and MK183751.2 respectively.

**Statistical Analysis**

Data were filled in the MS Excel Software. Analyzed results were expressed as percentage and p values by Chi square test using GraphPad Instat Software. If the probability is less than 0.05, the association or difference is said to be significant.

**OBSERVATION AND RESULTS**

Of the total 170 CRE isolates, 158 (93%) of

<table>
<thead>
<tr>
<th>Patterns of Genotype</th>
<th>NDM-1</th>
<th>OXA-48</th>
<th>KPC</th>
<th>VIM</th>
<th>IMP</th>
<th>Number of CRE isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>74</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>38</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>170</td>
</tr>
</tbody>
</table>

Fig. 2. Multiplex gene specific PCR - Agarose gel image
Lane L: 100 bp DNA ladder, Lane +Ve: Positive control, Lane -Ve: Negative control and Lanes 1 to 8: Clinical isolates for Carbapenem Resistant *Enterobacteriaceae* family showing presence or absence of carbapenemase encoding genes. Lane 1- NDM-1 Positive, Lane 2-NDM-1 & OXA-48 Positive, Lane 3- NDM-1,OXA-48 & KPC Positive, Lane 4 –OXA-48 Positive, Lane 5- VIM positive, Lane 6 - VIM & NDM-1 positive, Lane 7 - KPC positive.
the isolates confirmed carbapenemase activity by presence of at least one or more carbapenemase encoded plasmid gene in the multiplex PCR study. Remaining 12 (7%) of the isolates were negative for presence of any one of the gene tested in the multiplex study.

Multiplex PCR study showed presence or absence of different carbapenemase encoded genes. Of the 170 CRE isolates, 116 (68.2 %) had presence of NDM-1 gene. OXA-48 genes were seen in 75 (44.1 %) of the isolates. Only 7 (2.9 %) were positive for VIM gene (Fig. 1). All 170 isolates were negative for presence of IMP gene.

Total 74 CRE isolates showed presence of only NDM-1genes, while 36 isolates were having presence of NDM-1 and OXA-48 like genes (Table 4). Total 48 % of OXA- positive isolates (36/75) were having presence of NDM-1 gene which is extremely significant. \( \chi^2 = 22.09 \) and \( p < 0.0001 \). NDM-1 and VIM gene combination was present in 5 of the total 170 CRE isolates. One isolate had presence of NDM, OXA 48 and KPC genes. Twelve isolate were negative for any of the carbapenemase gene studied in the project. Fig. 2 is representative agarose gel image of amplification of VIM-523bp, NDM-1-475 bp, OXA-48-307 bp and KPC gene-150 bp.

**DISCUSSION**

Members of *Enterobacteriaceae* got main focus of attention especially around year 2000 because of sudden rise of Extended Spectrum β- Lactamase (ESBL) producing isolates. This resistance scenario got worsened with rapid spread of CTX-M enzyme producing *Enterobacteriaceae* isolates. But in last decade or so the focus has shifted to the rise of carbapenem resistance caused by carbapenemase producing *Enterobacteriaceae* isolates which inactivates almost all types of β-lactam antimicrobials.

Molecular methods are gold standard method for detection of carbapenemase production. Also it discriminates different types of carbapenemase production unlike phenotypic methods which fails to discriminate the types of carbapenemase. Of the 170 CRE isolates 158 isolates were encoded by at least one carbapenemase producing gene. According to Centre for Disease Control (CDC) definition, remaining 12 isolates were CRE. These 12 isolates might be having other mechanism for carbapenem resistance like target site mutation, OMP alteration and efflux pumps. Chromosomal mediated genes like *bla_NMC* are also having carbapenem resistant activity. In the present project, plasmid mediated genes were focused and because of low prevalence of other mechanisms, they were not studied. Multiplex PCR study is one of the reference method for simultaneous detection of genes encoded for carbapenemases of different classes.

NDM-1 has been predominant carbapenemase in Indian CRE isolates. In a SENTRY Antimicrobial Surveillance Program, 2006-2007 Castanheira M. et al. retrospectively found a prevalence rate of 38.5 % of NDM-1 among *Enterobacteriaceae* isolates collected from multiple Indian cities. In one of the pioneer study from India, Kumarasami K. found it as 31.2 % and 55.3 % in isolates from Chennai and Haryana respectively. A higher prevalence of 91.6 % was found by Deshpande P. et al. in clinical isolates from a tertiary care hospital in Mumbai but the study included only 24 isolates. In a study from Mumbai Kazi M. et al. have found prevalence of 75.2 % in 2012. In blood stream *Enterobacteriaceae* isolates Mohanti et al. found 65.6 % of the CRE isolates conferred with NDM-1 gene. Almost similar but slightly higher that is 68.2 % of the CRE isolates in present study were encoded with *bla_NDM-1* gene.

Next to NDM-1 gene, multiplex PCR study showed OXA-48 (44.1 %), VIM (2.3 %), KPC (1.7 %) responsible for carbapenemase production among 170 CRE isolates in the present study. Mohanti et al. found OXA-48 in 24.7 %, OXA-181 in 23.6 %; VIM in 6.4 %; and KPC in 2.1 % of the isolates in their study. In a study conducted by Khajuria A. et al. found 42 % (19/45) of *E. coli* isolates showed presence of OXA-48 gene.

In life threatening or invasive infections carbapenems are most used antimicrobials because of their bactericidal effect which is concentration independent. Also because of their broad spectrum activity which includes action against Gram-positive, Gram-negative bacteria including anaerobes, their use is widespread. The widespread use, selection pressure of antimicrobial surviving resistant strains, horizontal plasmid spread among *Enterobacteriaceae* isolates...
might be contributing factors for raised prevalence of NDM-1 and OXA-48 in the present study. Also being tertiary care centre many patients referred have already received antibiotics, β –lactams being preferred one.

There is variation among predominant carbapenemase encoding gene among CRE isolates around the globe. Pollet S. et al. found 78.3% of isolates were having blaKPC in a study from USA.38 Predominant gene reported were OXA-48 (86%)39, KPC-2 (54.9%)40 NDM (50 %)41 in studies from Turkey39, China40 and Oman41 respectively.

In the present study NDM-1 and OXA-48 coexisted in 21.2 % (36/170) of the isolates. Simultaneous presence of blaOXA-48 and blaNDM-1 among E. coli in the study by Khajuria A et al. was found to be (25/300) 8.3%35. Certainly there have been increasing reports of outbreaks and case reports seen across the globe42. This rapid spread of OXA-48 like gene is mediated by plasmid mediated transfer of genetic elements which is seen quite common among Enterobacteriaceae family43. OXA-48 like enzymes causes weak hydrolysis of carbapenems. As a result minimum inhibitory concentration of carbapenems remains on lower side44. Also routine phenotypic methods fail to detect presence of OXA-48 like enzymes. Therefore molecular detection of OXA-48 like genes is a must at present time. Early detection of the OXA-48 and other genes helps for active management and prevents further horizontal spread7.

None of the isolates showed presence of blaIMP in the study. Similar findings were observed by Mohanti et al.34 and Solankhi et al.20 among Indian studies. IMP type MBL gene is endemic in Japan and Taiwan though rest of world does show some sporadic cases45. Similarly VIM is more prevalent in Greece but outbreaks and single reports are from all over world46. blaNDM-1 carrying clinical isolates is characterized by presence of other resistance factors.29 Total 42 (24.2%) of the isolates positive for NDM-1 were carrying at least one other carbapenemase encoded gene. Mohanti et al. found multiple gene in 39.4 % of the isolates44. Such co-occurrence of multiple carbapenemase gene was detected in 5.9%, 10% and 13% from studies in Sultanate of Oman41, China40 and Turkey29 respectively. Higher percentage of multiple gene co-occurrence in NDM-1 strains specifically in combination with OXA-48 in the present study is a cause of concern.

CONCLUSION

Though NDM-1 is leading carbapenemase encoded gene in carbapenem resistant Enterobacteriaceae isolates, the spread of OXA-48 is also quite noticeable. As there is no well defined phenotypic method available for detection of OXA-48-like producer emphasis should be on its molecular detection. This will help in contact precautions and to prevent further escalation of carbapenem resistance. Further considering a raised prevalence of coexistence of OXA-48 and NDM-1 gene, a study on role of NDM-1 in transfer of plasmid mediated transfer of OXA-48 is warranted.

ACKNOWLEDGMENTS

We thank all the staff at the department of Microbiology, KIMS and at Department of Molecular Biology and Genetics, KIMSDU, Karad for their support.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS’ CONTRIBUTION

SKP and STM designed the research project. SKP performed the bacteriology workup. SKP performed molecular workup along with KDD and MNP. SKP analyzed the data along with SVK. SKP wrote the manuscript. STM supervised and reviewed the manuscript. STM, KDD and SKP approved the manuscript.

FUNDING

Krishna Institute of Medical Sciences, "Deemed to be University", Karad KIMSDU / DR / 81/2016.

ETHICS STATEMENT

Informal consent was taken before the study from each patient. Study approval was issued and maintained by the Institutional Ethical Committee of KIMSDU, Karad before starting the research project.
DATA AVAILABILITY
All datasets generated or analyzed in relation to aims and objective of above research project are included in the manuscript.

REFERENCES


