Endemicity of OXA-48 and NDM-1 Carbapenemase Producing *Klebsiella pneumoniae* and *Escherichia coli* from a Tertiary Hospital in Varanasi, India

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SA and JF contributed to conception and designing of study. Author JF managed the literature searches. Author JF and TB wrote the protocol, managed data collection, analysis and drafting of manuscript. All authors read and approved the final manuscript.

Article Information

Original Research Article

ABSTRACT

Background: Increase in global dissemination of NDM-1 and OXA-48 particularly among *Klebsiella pneumoniae* and *Escherichia coli* pose serious threat to antimicrobial therapy.
Aim: This study investigated the prevalence of OXA-48 like and NDM-1 among clinical isolates of *K. pneumoniae* and *E. coli*. *E. coli* and *K. pneumoniae* were isolated from clinical samples collected from patients' referred to microbiology laboratory for diagnosis.
Study Design: Investigative.
Method: Minimum inhibitory concentration break point for carbapenem and third generation cephalosporin on multidrug resistant isolates of *K. pneumoniae* and *E. coli* were determined by agar dilution method and carbapenem resistant isolates identified. DNA templates from the carbapenem resistant isolates were extracted by boiling and centrifugation method, and the extracted DNA template were later subjected to a multiplex PCR-based detection of the bla_{NDM-1} and bla_{OXA-48}

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genes. Amplicon positive for NDM-1 and OXA-48 were sequenced and a blast search was performed on the sequenced data on NCBI data base. A total of 293 *E. coli* and 236 *K. pneumoniae* were isolated, and 391 of these isolates were multidrug resistant. 159 isolates; comprising of 64 *E. coli* and 75 *K. pneumoniae,* of the multidrug resistant isolates were identified as carbapenem resistant enterobacteriaceae. Fifty; 50/159(31.4%) isolates were positive for NDM-1 and 44/159(27.7%) for OXA-48, while 17/159(10.7%) co-harboured NDM-1 and OXA-48 like genes. Sequence data for NDM-1 and OXA-48 revealed 99% sequence identity to sequences containing NDM-1 and OXA-181 respectively.

Conclusion: OXA-48-like carbapenemases are the most difficult to identify in routine diagnosis hence the need to formulate a robust routine diagnostic and infection control policy to curb the spread of pathogens habouring these carbapenem resistance genes.

1. INTRODUCTION

Over years, the world has witnessed continuous change in the epidemiology of multidrug resistant enterobacteriaceae. This is evidenced by the successive evolution of antibiotic resistance mechanisms particularly those involving β-lactamases such as the extended spectrum β-lactamases (ESBL) and its variants, and more recently the carbapenemases [1,2,3,4]. Genes that encode β-lactamases predominantly ESBL are frequently located on plasmids and often conjugated with other antibiotic resistance determinants. Consequently, ESBL producing enterobacteriaceae are frequently multidrug resistant [1]. The carbapenem; a class of antibiotic developed to resist the hydrolytic activity of ESBL, has been used as last line effective therapy against multidrug resistant enterobacteriaceae (MDRE), however the emergence of carbapenemases; a group of enzyme that hydrolyzes all β-lactam antibiotics including the carbapenems, constitute threat to continuous use of this class of antibiotics in medical practice. Although several types of carbapenemases and their variants have been identified, NDM-1 and OXA-48 are the frequently reported among enterobacteriaceae, particularly in *K. pneumoniae* and *E. coli* [2].

OXA-48 represents the main enzyme of class D carbapenemases. First identified in *K. pneumoniae* isolate from Turkey in 2001, subsequently, OXA-48 producing strains became major sources of nosocomial outbreaks in many parts of the world, notably in Mediterranean countries [2]. It is one of the most common carbapenemase types circulating in Spain, France and the Indian subcontinent, with the Middle East and North Africa considered as reservoirs. A point mutant analog of OXA-48;

OXA-181, with similar carbapenemase activity, has been identified in enterobacterial strains from India and from patients with a link to the Indian subcontinent [3]. Other types of OXA carbapenemase recently reported include, OXA-54, OXA-162, OXA-163, OXA-199, OXA-247, OXA-204 and OXA-234 [4,5].

NDM was initially identified in *E. coli* and *K. pneumoniae* in a patient returning to Sweden from India in 2008, subsequently, it was reported across various countries [2,6]. Most outbreaks indicated a link with the Indian subcontinent, Balkan countries and the Middle East [2,7,8,]. Eight variants of NDM (NDM-1 to NDM-8) have been described, among which NDM-1 has acquired worldwide dissemination surpassing level reported for other carbapenemases [4].

Although the NDM-1 has been widely studied in India, there is paucity of data addressing the prevalence and distribution of OXA-48 like carbapenemases [2,9,10]. This study highlights high prevalence of NDM-1 and OXA-48 carbapenemases, and hypothesizes a possible spread of OXA-181 among clinical isolates of *K. pneumoniae* and *E. coli* from a tertiary care hospital.

2. MATERIALS AND METHODS

2.1 Bacterial Isolates

Non duplicate isolates of *E. coli* and *K. pneumoniae* were recovered from various clinical specimens such as urine, blood, sputum, endotracheal tube, pus aspirates, intravascular catheter tip, ascitic fluid and wound swab received in microbiology laboratory for routine analyses, from patients attending the various outpatient and inpatient departments of the

University hospital. Isolation was done by plating on cysteine lactose electrolyte deficient (CLED) agar or blood and MacConkey agar as per nature of the specimen. Standard bacteriological methods such as; colour and morphology of colonies on media, gram's reaction, sulfide-indole-motility test, simmon's citrate reaction, urease test, triple sugar iron test (TSI) and sugar fermentation of colonies were followed for identification of enterobacteriaceae isolates [11].

2.2 Identification of Carbapenem Resistant *K. pneumoniae* and *E. coli* Isolates

The MIC break point of ertapenem (MSD-Chibret, Franch), meropenem (Zuventus, India), imipenem (Ranbaxy, India), doripenem (Aqua vice laboratory, India), ceftazidime (GSK, Italy), ceftriaxone (Alkem, India) and cefotaxime (Akorn, India) on previously characterized 391(n= 528) clinical isolates of multidrug resistant *K. pneumoniae* and *E. coli* [12] was determined by agar dilution method [13]. Carbapenem resistant *K. pneumoniae* and *E. coli* was defined as non susceptibility to one or more of the carbapenem and resistance to all the third generation cephalosporin [13,14].

2.3 DNA Isolation

DNA templates from carbapenem resistant *K. pneumoniae* and *E. coli* isolates were prepared by boiling centrifugation method [15]. Briefly, five colonies of overnight culture of bacterium were transferred to 400 µl of sterile distilled water in eppendorf tube. The mixture was vortexed and incubated at 90°C for 15 minutes. After incubation, the mixture was allowed to cool and then centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and used as DNA template for PCR assay.

2.4 Amplification of *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$ Genes by Multiplex PCR

Carbapenem resistant *K. pneumoniae* and *E. coli* were screened for presence of *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$ carbapenemase genes by targeting 621bp and 438bp fragments for *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$ respectively, using primers specific for these genes (Table 1). DNA amplification for the carbapenemase genes fragments for each isolates was done in a multiplex PCR reaction mixture of 25 µl containing 5µl of DNA, 1µl of 10 pmol of each primer (8 forward and reverse primers) [Table 1], 2 µl of 10 mM dNTPs, 1 U *Taq* DNA, polymerase (Genei, Bangalore, India), and 3 µl of 10x *Taq* buffer (Genei, Bangalore, India). Amplification reaction condition in thermal cycler (BioRad USA) includes, initial denaturation at 94°C for 10 min followed by 36 cycles of 94°C for 30 sec, 53.5°C for 40sec and 72°C for 50 sec, and a final extension at 72°C for 5 min. DNA fragment of *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$ gene amplified by PCR were identified by agarose gel electrophoresis; briefly 5 µl of PCR amplicon mixed with 2 µl DNA loading dye was electrophoresed on 2% agarose gel treated with Tris Acetate EDTA (TAE) buffer and ethidium bromide (Genei, Bangalore, India), at constant 70 volts for 90 min. Molecular marker of 100bp DNA ladder, (Genei, Bangalore, India) was electrophoresed concurrently with the test samples. The gel was visualized under ultraviolet illumination in a gel documentation system [16].

2.5 Sequencing of Amplicon Positive for *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$

Two PCR amplicons that indicated positive gel picture for OXA-48 band and NDM-1 band respectively were randomly selected and submitted to Xcelris Laboratory Ltd. India for sequencing with specific primers earlier used in amplification of the fragments during PCR (Table 1). Subsequently, a blast search was performed on NCBI data base to determine the sequence identity.

Table 1. Primer sequence for amplification of *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$genes

Primer pairs	Sequence(5^l - 3^l)	Product size (bp)	Reference	Primer source
OXA – 48 – F	GCG TGG TTA AGG ATG AAC AC	438	[16]	Hysel India
OXA – 48 – R	CAT CAA GTT CAA CCC AAC CG			
NDM-1 – F	GGT TTG GCG ATC TGG TTT TC	621	[16]	Hysel India
NDM-1– R	CGG AAT GGC TCA TCA CGA TC			

3. RESULTS

A total of 292 *E. coli* and 236 *K. pneumoniae* were recovered from patients' samples and 391 were identified as MDR isolates. Using CLSI and CDC screening criteria for carbapenem resistant enterobacteriaceae (CRE), 64 *E. coli* and 75 *K. pneumoniae* of the MDR isolates were resistant to carbapenem (Table 2).

Multiplex PCR amplification for bla_{NDM-1} and bla_{OXA-48} among the carbapenem resistant isolates, revealed that; 27 isolates of *E. coli* and 40 isolates of *K. pneumoniae* were positive for bla_{NDM-1}, 32 isolates of *E. coli* and 29 isolates of *K. pneumoniae* were positive for bla_{OXA-48}, while 10 *E. coli* and 7 *K. pneumoniae* were positive for both bla_{NDM-1} and bla_{OXA-48} (Table 2 and Fig. 1).

Two randomly selected PCR amplicons that indicated positive gel picture for OXA-48 band and NDM-1 band respectively were submitted for sequencing with the specific primers used in amplification of the respective fragments during PCR. Subsequent blast search performed on NCBI data base revealed that the amplified fragment sequenced with OXA-48 primer was 99% identical to the OXA-181 sequence in *M. morganii* [Fig. 2]. Similarly a blast search performed on the sequence result of amplicon sequenced with NDM-1 primer revealed 99% similarity to a range of sequences containing the NDM-1 sequence on NCBI data base [Fig. 3].

Table 2. Distribution of NDM-1 and OXA-48 genotype among carbapenem resistant isolates

Bacterial	MDR*	CR**	bla_{NDM-1}†	bla_{OXA-48}†	$bla_{NDM-1}bla_{OXA-48}$†
E. coli	218	64(29.4)	17(26.6)	22(34.4)	10(15.6)
K. pneumoniae	173	75(43.4)	33(44)	22(29.3)	07(9.3)
Total	391	159(40.7)	50(31.4)	44(27.7)	17(10.7)

Multidrug resistance
***Carbapenem resistance (proportion in % calculated with MDR as n)**
†*Proportion in % calculated with MDR as n*
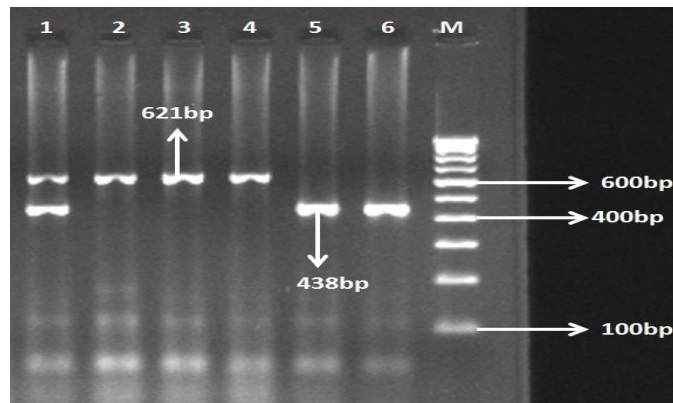
Fig. 1. Multiplex PCR amplification of bla_{NDM-1} and bla_{OXA-48} M - 100bp DNA ladder; bla_{NDM-1}-621bp; bla_{OXA-48} – 438bp

Fig. 2. Sequences producing significant alignment for amplified fragment sequenced with OXA-48 primers

Sequences producing significant alignments:

Select: All None   Selected:0

Description	Max score	Total score	Query cover	E value	Ident	Accession
Acinetobacter baumannii genome assembly Acinetobacter baumannii CHI-32, plasmid : pNDM-32	619	619	43%	2e-173	99%	LN833432.1
Enterobacter cloacae isolate GN574 plasmid pNDM-Ec1GN574, complete sequence	619	619	43%	2e-173	99%	KJ812998.1
Providencia stuartii isolate GN576 plasmid pNDM-PstGN576, complete sequence	619	619	43%	2e-173	99%	KJ802405.1
Escherichia coli isolate GN568 plasmid pNDM-EcoGN568, complete sequence	619	619	43%	2e-173	99%	KJ802404.1
Pseudomonas aeruginosa strain PSA-1.2 New Delhi metallo-beta-lactamase NDM-1 (blaNDM-1) gene, partial cds	619	619	43%	2e-173	99%	KP172295.1
Acinetobacter genomosp. NB14 strain JVAP01 plasmid pNDM-JVAP01, complete sequence	619	619	43%	2e-173	99%	KM923969.1
Acinetobacter baumannii ISAba125, blaNDM-1, bleMBL, trpF, dsbD, groL, insE, ISAba125 genes for transposase, NDM-1 metallo-beta-lactamase, bleomycin re	619	619	43%	2e-173	99%	LC032101.1

Fig. 3. Sequences producing significant alignment for amplified fragment sequenced with NDM-1 primers

4. DISCUSSION

Recent data obtained from communities and hospitals across the globe illustrated increasing prevalence of carbapenemase producing enterobacteriaceae, especially among *E. coli* and *K. pneumoniae*, and MBLs of the NDM type and OXA-48-like oxacillinases are the most frequently reported [17]. In this study, *K. pneumoniae* expressed higher rate of resistance to carbapenem than *E. coli*, and a corresponding high prevalence of NDM-1 and OXA-48 was equally observed among these isolates.

Although the multidrug resistance rate was higher in *E. coli* than *K. pneumoniae* from the data obtained in the previous study [12], susceptibility profile of *K. pneumoniae* (43.4%) and *E. coli* (29.4%) in this study, revealed higher carbapenem resistance rate among *K. pneumoniae* than *E. coli*. This finding validated a recent works showing higher resistance rate to carbapenems among *K. pneumoniae* than *E. coli* [17]. This trend has also been demonstrated across Asia, where data from each country/region showed consistent rise in resistance to carbapenems among *K. pneumoniae* [18]. Report from mainland China showed that resistance to imipenem among *E. coli* and *K. pneumoniae* in 2004 - 2005 was 0.0% and 0.7%, but increased to 0.5% and 2.7% in 2010. Similarly, a report from Korea indicated that *E. coli* and *K. pneumoniae* were completely sensitive to imipenem in 2000, while in 2009, resistance rates rose to 0.1% and 0.5% respectively. A comparative study conducted in Taiwan to evaluate resistance profile of enterobacteriaceae to carbapenem, showed resistance rate of 56.3%, 31.3% and 15.6% to imipenem, meropenem and doripenem respectively in 2010 and a rate of 72.1%, 58.1%

and 51.2% to imipenem, meropenem and doripenem respectively in 2012 [19].

The *bla*$_{NDM-1}$ has emerged globally as the most prevalent type of carbapenemases followed by *bla*$_{OXA-48}$, and their incidences have surpassed other types of carbapenemase genes [20]. A retrospective analyses on stored cultures of enterobacteriaceae isolated from New Delhi, Pune and Mumbai since 2006 identified the gene encoding *bla*$_{NDM-1}$ and OXA-181; a variant of OXA-48 carbapenemases [3]. Although a coordinated multicenter and several other single center studies on the prevalence and distribution of the NDM carbapenemases have been conducted in India, not many reports are available on OXA-48 carbapenemases. In this study, the distribution rate for NDM-1 and OXA-48 among the carbapenem resistant isolates was 31.4% and 27.7% respectively, while 10.7% co-harboured NDM-1/OXA-48 (Table1).This result does not corroborate exactly with a study conducted by Sharma et al. [17] who reported equal distribution of NDM (32%) and OXA-48 like (32%) genes in isolates of *E. coli* and *K. pneumoniae* collected from blood stream infection between 2013-2015. Similarly, a study on meropenem resistant enterobacteriaceae conducted by Srinivasan et al. [21], reported that NDM-1 was most prevalent (62%) than OXA-48 (1.4%). High prevalence of *bla*$_{NDM-1}$ carbapenemase gene carriage among CRE ranging from 31.2% - 91.6% have been reported in India [20,22,23,24]. While a study on clinical isolates of enterobacteriaceae from South India, showed *bla*$_{NDM-1}$ encoding gene as most prevalent (57.65%) [25], a report from North India documented a relatively lower prevalence of 5.18% in *E. coli* [26]. In this study, the observed NDM-1 positivity rates in *E. coli* (26.6%) and *K. pneumoniae* (44%), OXA-48 rates in *E. coli* (43.4%) and *K. pneumoniae* (29.3%) denotes a

higher positivity rate of NDM-1 in *K. pneumoniae* than *E. coli* and the reverse for OXA-48.

Among the class D β-lactamases, OXA-48-like carbapenemases have increasingly been identified in enterobacteriaceae with a wide geographical distribution. Growing number of reports showed global dissemination of OXA-48-like carbapenemases, and in some European countries, OXA-48-like has become the predominant carbapenemase [27]. Findings from this study indicated high prevalence of OXA-48 in *E. coli* and *K. pneumoniae*. Although there is paucity of reports on the actual prevalence and distribution of OXA-48 carbapenemases in India, it might be endemic as the NDM-1 carbapenemase. The OXA-48 like and NDM carbapenemases were reported in India from stored cultured as far back as 2006, however, it's the 2011 NDM-1 epidemic associated with India that attracted research interest. Consequently, report from several single center and multicenter studies on NDM-1 became available, but with little or no corresponding studies on OXA-48 oxacillinases, until recently when few single center studies started emerging. Growing number of reports have shown global dissemination of OXA-48-like carbapenemases, and in some European countries, OXA-48-like has become the predominant carbapenemase [27]. Although a study from south India reported prevalence of 2(1.8%) for bla_{OXA-48} and $bla_{OXA-181}$ carbapenemase gene in *K. pneumoniae* and *C. freundii* [28], several recent reports from studies carried out in India indicated high prevalence of OXA-48 like carbapenemases among enterobacteriaceae [29,30]. We therefore posit that the OXA-48 like carbapenemase could be as endemic as the NDM-1 and spreading among bacteria unsuspectingly [30].

OXA-181 was first identified in isolates of enterobacteriaceae of India origin in SMART surveillance program and since then it has been reported in some parts of the world [2,3,10,]. In this study, a blast search performed on the sequence data of amplicons that corresponded to the band size of NDM-1 and OXA-48 on gel revealed 99% alignment to sequence carrying $bla_{OXA-181}$; a point mutant variants of bla_{OXA-48} and bla_{NDM-1} gene on NCBI data base respectively [Figs. 2, 3]. Several similar studies in India also reported OXA-181 variants of OXA-48 [3,21,30]. Although only one OXA-48 amplicon was sequenced, the finding of this study, is in tandem with other reports on the variant of OXA-48 in India [25,30], suggesting that OXA-181 could be

the prominent OXA-48 variant in circulation. Further studies on OXA-48 like carbapenemase is important considering the fact that bla_{OXA-48} like genes, unlike other carbapenemases, weakly hydrolase carbapenem and spare broad spectrum cephalosporin; a situation that could lead to non-detection of isolates carrying this gene in routine diagnosis. In addition, the OXA-48 like carbapenemase has been implicated in salient spread of carbapenemase producing isolates and outbreaks in hospitalized patients [17].

5. CONCLUSION

Although the emergence and rapid dissemination of NDM-1 among *K. pneumoniae* and *E. coli* has been widely studied, there is dearth of information on OXA-48 like carbapenemases. The prevalence of OXA-181 in recent reports from India denotes a changing epidemiological trends indicating possible endemicity of this variant of OXA-48 like carbapenemase gene. OXA-48 are the most difficult carbapenemase to identify routinely, hence there is need to formulate robust antimicrobial diagnostic and infection control policy as described by Center for Disease Control and Prevention (CDC) [31] and European Centre for Disease Prevention and Control (ECDC) [32], and implementing it to prevent the spread of isolates resistant to carbapenem.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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