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Occurrence of *bla*$_{NDM-1}$ & absence of *bla*$_{KPC}$ genes encoding carbapenem resistance in uropathogens from a tertiary care centre from north India

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***Background & objectives*: Carbapenem resistance mediated by carbapenemases is increasingly being reported worldwide. This study was conducted to know the occurrence of important carbapenem resistance encoding genes in Gram-negative bacilli (GNB) causing complicated urinary tract infection (CUTI), and to look at the genetic diversity of these isolates.**

***Methods*: The study was carried out on 166 consecutive carbapenem resistant uropathogens (CRU) isolated from cases with CUTI during 2008 and 2012. Carbapenemase production was characterized phenotypically and polymerase chain reaction was used to detect *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{KPC}$, and *bla*$_{NDM-1}$. BOX-PCR was done on 80 randomly selected isolates for molecular typing.**

***Results*: The *bla*$_{VIM}$ gene was present in 34 (43.6%), *bla*$_{IMP}$ in five (6.4%) and none of the isolates from 2008 had *bla*$_{NDM-1}$ or *bla*$_{KPC}$ genes. Among the isolates from 2012, *bla*$_{NDM-1}$ gene was present in 47 (53.4%), *bla*$_{VIM}$ in 19 (24.4%), *bla*$_{IMP}$ in one (1.1%) and none had *bla*$_{KPC}$. There were nine isolates during the two years which had multiple genes encoding carbapenemases; while 66 did not have any of the genes tested. Of the 80 isolates subjected to BOX-PCR, 58 could be used for analysis and showed, presence of multiple clusters of carbapenem resistant isolates and absence of a single dominant clone.**

***Interpretation & conclusions*: The *bla*$_{NDM-1}$ gene was absent in our isolates obtained during 2008 but was present amongst *Enterobacteriaceae* isolated in 2012. The *bla*$_{KPC}$ gene was also not found. Nine isolates obtained during the two years had multiple genes encoding carbapenemases confirming the previous reports of emergence of GNB containing genes encoding multiple carbapenemases. Typing using BOX-PCR indicated that this emergence was not because of clonal expansion of a single strain, and multiple strains were circulating at a single point of time.**

Key words *bla*$_{NDM-1}$ - *bla*$_{KPC}$ - carbapenem resistance - complicated urinary tract infection - uropathogens

The carbapenems are β-lactam antibiotics that are used in the treatment of infections caused by extended spectrum beta-lactamases (ESBL) producing Gram-negative bacteria (GNB) and several serious bacterial infections like meningitis, nosocomial pneumonia, nosocomial sinusitis and sepsis of unknown origin. Resistance against carbapenems is mediated mainly by metallo-β-lactamases. A decade ago the genes encoding metallo-β-lactamases (MBL) were mainly present in the non-fermenting GNB like *Pseudomonas aeruginosa* and *Acinetobacter* species[1]. However, the latter data suggest that these have disseminated at an alarming rates to the members of family *Enterobacteriaceae* as has been seen with epidemics of bla_{KPC} clones in USA, and Europe and the worldwide epidemic with bla_{NDM-1} producing Gram-negative bacteria[2]. The New Delhi metallo β-lactamase (NDM-1) producing *Escherichia coli* and *Klebsiella pneumoniae* and other resistant GNBs, such as *Acinetobacter* species have been isolated more frequently from cases of urinary tract infection (UTI)[3]. This study was designed to observe the presence of the genes encoding important carbapenemases in uropathogens causing complicated urinary tract infection (CUTI) in a tertiary care centre in north India, and their genetic relatedness.

Material & Methods

The study was performed on carbapenem resistant uropathogens (CRU) isolated from patients with complicated urinary tract infection attending the outpatient department or admitted at the Nehru hospital of the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, during 2008 and 2012. Baseline prevalence of carbapenem resistant uropathogens was obtained from laboratory records maintained during 2008 and 2012. Complicated UTI was defined as infection developing in a patient with anatomically, physiologically or functionally compromised urinary tract[4].

Bacterial isolates: A total of 166 non-duplicate consecutive carbapenem resistant uropathogens were collected over a period of two years (between 1st May to 31st August in 2008 and 2012) from patients with complicated UTI. All the isolates were identified using standard conventional biochemical tests. Antibiotic susceptibility was performed using the Kirby Bauer disc diffusion method[5] and results were interpreted as per the Clinical and Laboratory Standards Institute (CLSI) guidelines[6]. The antibiotic discs were obtained from Hi-media, Mumbai, India. Only those isolates that had a reduced susceptibility to meropenem (zone size ≤21 mm) and were found to produce carbapenemases and metallo-beta-lactamases by using both modified Hodge test and double disc synergy test were included in this study[7]. Briefly, the indicator organism, *E. coli* ATCC 25922, at a turbidity of 0.5 McFarland standard, was used to swab inoculate the surface of a Mueller-Hinton agar (Hi-Media, Mumbai, India) plate, and the test strain was heavily streaked from the plate centre to the periphery. After the plate was allowed to stand for 15 min at room temperature, a 10 µg imipenem disc (Hi-Media) was placed at the centre, and the plate was incubated overnight. The presence of a distorted inhibition zone was interpreted as a positive result for carbapenem hydrolysis screening. The detection of MBL production was also performed by the combined-disc test by using two imipenem discs (10 µg), one containing 10 µl of 0.5 M EDTA (SRL Laboratories, India), which were placed 25 mm apart on a Mueller-Hinton agar plate[7].

Molecular detection of carbapenemase genes: Polymerase chain reaction (PCR) was performed for bla_{IMP} (detects all imipenems except IMP-9, IMP-16, IMP-18, IMP-22 and IMP-25), bla_{VIM}, bla_{KPC} and bla_{NDM-1} to identify the presence of the resistance genes, using the primers described in Table I[8,9]. Total DNA (2 µl) was subjected to PCR in a 25 µl reaction mixture containing 1x PCR buffer, 0.4mM dNTP(Bangalore Genei, India), 0.6 µM each of forward and reverse primers (Sigma Aldrich, India), and 1 U of *Taq* polymerase (Bangalore Genei, India). Amplification was carried out as follows: initial denaturation at 94°C for 10 min; 30 cycles of 94°C for 40 sec, 55°C for 40 sec and 72°C for one min; and a final elongation step at 72°C for seven min. The annealing temperature was 55°C for bla_{IMP} and 58°C bla_{VIM}, and bla_{NDM-1} genes. A 100 bp DNA ladder was used as a size marker. Amplicons were visualized after running at 90V for one hour on a 2 per cent agarose gel containing ethidium bromide in a gel documentation system (Alpha Innotech, AlphaImager 3400).

Molecular typing of the carbapenem resistant uropathogens: Of the 166 isolates, 80 were chosen randomly for molecular typing using BOX PCR using the primer BOX-A1R primer (5`-CTACGGCAAGGCGACGCTGACG-3`) as described elsewhere[10]. Briefly, 500 ng of DNA was added to 25 µl reaction mixture containing 2.5 µl of PCR buffer with 1.5 mM $MgCl_2$, 0.8 µl of dNTPs, 2.25 µl of primer, 0.5 µl of *Taq* polymerase and PCR grade water. Amplification was carried out as follows: initial denaturation for two min at 94°C, 30 cycles of 94°C for 30 sec, 55°C for one min and 72°C for eight min;

Table I. Primer sets used for carrying the PCR reaction

Primer	Forward 5' to 3'	Reverse 5' to 3'	Product size (bp)
VIM[8]	GATGGTGTTTGGTCGCATA	CGAATGCGCAGCACCAG	390
IMP[8]	TTGACACTCCATTTACDG	GATYGAGAATTAAGCCACYCT	139
KPC[8]	CATTCAAGGGCTTTCTTGCTGC	ACGACGGCATAGTCATTTGC	538
NDM[9]	CACCTCATGTTTGAATTCGCC	CTCTGTCACATCGAAATCGC	561
BOXA1R[10]	CTACGGCAAGGCGACGCTGACG		

IMP, imipenem hydrolysing enzyme; VIM, verona integron encoded metallo-β-lactamase; KPC, *Klebsiella pneumoniae* carbapenemase; NDM-1, New Delhi metallo-β-lactamase-1; BOXA1R, primer for repetitive element palindromic PCR

and a final elongation step at 72°C for eight min. Further steps till visualisation of the bands were the same as for PCR. The gel images were imported into Bionumerics 7.1 (Applied Maths NV, Belgium). *E. aerogenes, Morganella morganii, Proteus mirabilis* and *Providencia stuartii* were excluded from BOX PCR as the total number of individual isolates was < 10. Only those isolates with ≥ 7 bands (between 100 and 1500 bp) were included for analysis. Dendrogram was constructed using the band based UPGMA protocol[11]. The experiment was repeated twice.

Statistical analysis: The chi square test was applied to compare different proportions of samples and/or cases of complicated UTI during 2008 and 2012.

Results

High carbapenem resistance was observed amongst *Enterobacteriaceae* and non-fermenting GNBs during 2008 (9.3 and 61.6%) and 2012 (12.3 and 43.8%) (Table II). A total of 166 carbapenem resistant uropathogens which were isolated between May to August 2008 and 2012 at our centre, were analysed. Majority (57, 73.1%) of the isolates from 2008 were non-fermenting GNB and the rest (21, 26.9%) belonged to the family *Enterobacteriaceae*. Among the 88 isolates from 2012, 51 (58%) were members of family *Enterobacteriaceae* and 37 (42%) were non-fermenting GNB. In 2008 isolates the bla_{VIM} gene was present in 34 isolates (43.6%), bla_{IMP} gene was present in five (6.4%) and none had bla_{NDM-1} or bla_{KPC} gene. There were two isolates from 2008 which had both bla_{VIM} and bla_{IMP} genes. Among the isolates obtained during 2012, bla_{NDM-1} gene was present in 47 (53.4% of total and 84.3% of *Enterobacteriaceae*), bla_{VIM} in 19 (24.4%), and bla_{IMP} in one isolate. Six isolates from 2012 had bla_{VIM} and bla_{NDM-1} genes, while a single isolate with bla_{IMP} gene also had bla_{NDM-1} gene. Forty one (52.6%) and 25 isolates (28.4%) from 2008 and 2012 did not have any of the genes tested (Table III).

Table II. Details used to calculate baseline carbapenem resistance in isolates from cases of urinary tract infection (UTI)

Year	2008	2012
Total number of urine samples tested	10933	44278
Number (%) of samples positive for significant bacteriuria	6560 (20)	9299 (21)*
Number of samples with significant bacteriuria received from patients with complicated UTI (CUTI)	5248	7532
Enterobacteriaceae isolated from above samples	4093	5800
Number (%) of *Enterobacteriaceae* isolated from cases of CUTI tested for carbapenemases	867 (21)	1149 (19.8)
Carbapenem resistant *Enterobacteriaceae* from CUTI (%)	81 (9.3)	141 (12.3)*
Number (%) of non-fermenters isolated from CUTI	1115 (21.2)	1581 (21)
Number (%) of non-fermenters isolated from cases of CUTI tested for carbapenemase	297 (26.6)	288 (18.2)**
Carbapenem resistant non-fermenters isolated from cases of CUTI (%)	183 (61.6)	126 (43.8)**

*P<0.05, **<0.001 compared with the respective value in 2008

Of the 80 isolates randomly selected for typing, 25 were *P. aeruginosa*, 20 were *E. coli*, 20 were *K. pneumoniae*, and 15 belonged to *A. baumannii* complex. Only 58 (21 *P. aeruginosa,* 13 *E. coli*, 17 *K. pneumoniae,* and 7 *A. baumannii* complex) from the above had > 10 bands (between 100-1500 bp) and were analysed. The dendrogram is shown in the Figure. A cluster of possibly related isolates was defined as isolates having more than 70 per cent similarity, while those having similarity of >90 per cent were considered as closely related. Accordingly, it was found that there was no clustering among the *Acinetobacter* spp. Six possibly related clusters (PA1 to PA 6) were seen among the *P. aeruginosa* isolates; PA 4, PA 5 and PA 6 contained isolates only from 2008, while PA1, PA2 and PA3 had isolates from 2008 and 2012. Three and four possibly related clusters each were found among *K. pneumoniae* and *E. coli* isolates. It was seen that the isolates did not cluster according to the year of isolation among *K. pneumoniae*. While, the same was true for *E. coli,* except in cluster EC 3 which contained isolates only from 2012. Two closely related isolates were found in each of the following clusters: PA6, PA4, PA2, PA3, EC3, and KP2. Similarly, four closely related isolates were found in cluster PA1.

Discussion

In view of the increasing reports of carbapenem resistant pathogens from India and lack of data regarding the scenario in carbapenem resistant uropathogens

Table III. Beta lactamase gene profile of different carbapenem resistant *Enterobacteriaceae* (CRE) isolates obtained during 2008 and 2012

Pseudomonas aeruginosa (33)				
	2008			
Organism (number of isolates)	VIM	IMP	NDM	KPC
Non-fermenters (n=57)				
Pseudomonas aeruginosa (33)	15	2	0	0
Acinetobacter baumannii complex (24)	15	1	0	0
Percentage of total non-fermenters positive by PCR	52.6	5.2	0	0
Enterobacteriaceae(n=21)				
Klebsiella pneumoniae (15)	0	2	0	0
Escherichia coli (4)	4	0	0	0
Enterobacter aerogenes (2)	0	0	0	0
Percentage of *Enterobacteriaceae* positive by PCR	19	9.5	0	0
	2012			
Non-fermenters (n=37)				
P. aeruginosa (16)	0	0	4	0
Acinetobacter baumannii complex (21)	11	0	0	0
Percentage of non-fermenters positive by PCR	29.7	0	10.8	0
Enterobacteriaceae (n=51)				
K. pneumoniae (21)	2	1	17	0
E. coli (20)	3	0	16	0
Enterobacter aerogenes (7)	1	0	7	0
Morganella morganii (1)	1	0	1	0
Proteus mirabilis (1)	0	0	1	0
Providencia stuartii (1)	1	0	1	0
Percentage of *Enterobacteriaceae* positive by PCR	15.7	2.0	84.3	0

IMP, imipenem hydrolysing enzyme; VIM, verona integron encoded metallo-β-lactamase; KPC, *Klebsiella pneumoniae* carbapenemase; NDM-1, New Delhi metallo-β-lactamase-1

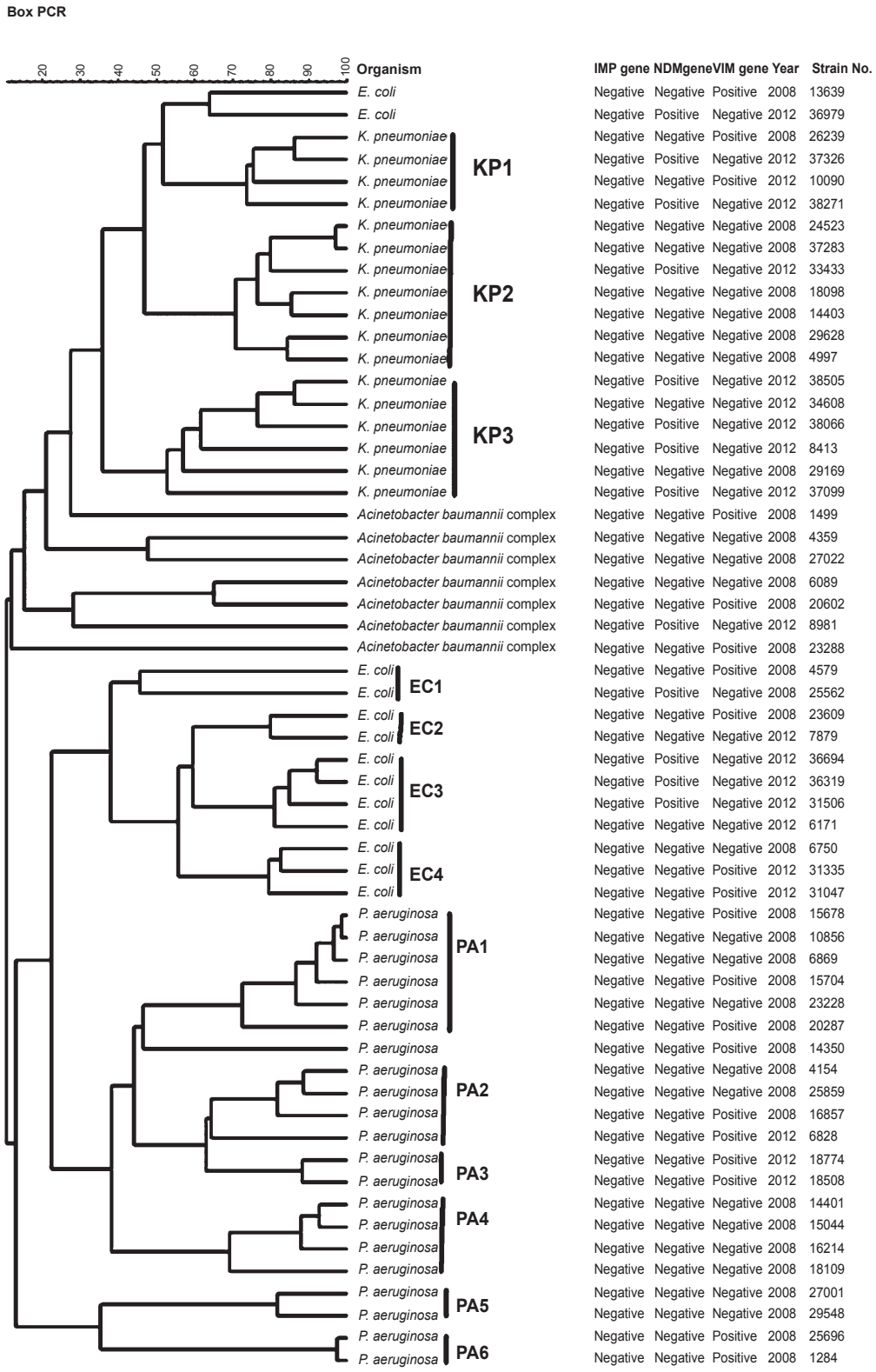

Figure . UPGMA (unweighted pair group method with arithmetic mean) tree fo BOX-PCR patterns from 58 carbapenem resistant uropathogens isolated during 2008 and 2012.

isolated from cases of complicated UTI, this study was done to know the occurrence of commonly occurring carbapenemase encoding genes in the above and to study the genetic relatedness between these isolates.

High carbapenem resistance was found among uropathogens at our centre. It was observed that carbapenemase resistance which was a problem in non-fermenters, became more common in *Enterobacteriaceae* during 2012, and majority of our isolates that fulfilled the criteria of carbapenem resistance in 2008 were non-fermenting GNB while members of the family *Enterobacteriaceae* (83.60%) formed the bulk of carbapenem resistant isolates during 2012. A number of methods like modified Hodge test, double disc synergy test, molecular detection of carbapenemase encoding genes are available for detection of carbapenem resistance in GNB. Since, there are no uniform guidelines for detection of carbapenemase production among GNB; we performed both modified Hodge test and double disc synergy test simultaneously[12]. Only those isolates positive by both the tests were included in the study.

There have been several reports of GNB producing carbapenemases from both India and abroad. The most common among them being imipenem hydrolysing enzyme (IMP), Verona integron encoded metallo beta lactamase (VIM), *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi metallo beta-lactamase-1 (NDM-1) encoded by bla_{IMP}, bla_{VIM}, bla_{KPC} and bla_{NDM-1} genes, respectively[13-15]. The bla_{NDM-1} gene was first described in 2008 in a *Klebsiella* isolate obtained from a patient in Sweden who had been previously hospitalized in New Delhi[16]. Castenheira *et al*[17] reported the presence of bla_{NDM-1} (15 out of 39 CRE, 38.5%) and its dissemination in Indian CRE isolated between 2006-2007. Several reports have been published on the presence of this gene in non-fermenters both from India and abroad[18-20]. However, none of the CRUs isolated during 2008 in our study had bla_{NDM-1} gene, though, it was the commonest carbapenem encoding gene in carbapenem resistant *Enterobacteriaceae* (CRE) isolated during 2012. We also found four isolates of *P. Aeruginosa* obtained during 2012 having bla_{NDM-1} gene. Such a high occurrence of bla_{NDM-1} indicates an endemic occurrence and appearance and rapid spread of this gene after 2008 in northwest India.

KPC carbapenemase was first reported from United States of America during 2001. These were virtually resistant to all antibiotics and spread rapidly globally[21]. A prevalence of 34.8 per cent of bla_{KPC} has been reported by Lascols *et al*[22] in CRE isolated from intra-abdominal infection from India. However, in our study none of our isolates were positive for bla_{KPC}. Our finding was in agreement with that of Nagaraj *et al*[14] who also did not find bla_{KPC} in any of their CRE[10].

The bla_{IMP} gene first detected in the 1980s in Japan and subsequently reported worldwide[23], was the least common gene detected during 2008 and 2012 in our study. This finding was in agreement with that of Amudhan *et al*[15] who also found a low level of bla_{IMP} during 2010. However, it was contrary to the findings of Dwivedi *et al*[24] who reported an occurrence of bla_{IMP} in seven out of 12 carbapenem resistant *Enterobacteriaceae* isolated in 2005 and 2006. This could be due to differences in the local circulating strains and not due to differences in type of clinical samples since prior antibiotic therapy which is a risk factor for the development of carbapenemase production was present in both patients of ventilator associated pneumonia (VAP), complicated UTI and hospitalized patients[15,24,25].

Among the non-fermenters bla_{VIM} was the commonest gene detected both during 2008 and 2012. This finding was similar to that reported earlier[22,26]. Forty one (52.6%) and 25 (28.4%) isolates obtained during 2008 and 2012, respectively did not have any of the genes tested for by PCR. The carbapenem resistance was possibly mediated by other genes not tested in the present study (*e.g.* GES, OXA-48, PER and VEB) or other mechanisms like absence of OprD which diminishes the permeability of cell wall to carbapenems, or presence of efflux pumps and altered penicillin binding proteins[8,27,28].

Molecular typing of the isolates was done using BOX-PCR technique. It is a type of repetitive element palindromic-PCR, which has been previously used for typing of both Gram-positive and Gram-negative organisms[29-31]. In previous studies, it was found to be rapid and have similar discriminatory capability as pulse field gel electrophoresis (PFGE)[32,33]. We found that there were multiple clusters of possibly related isolates, though there was no evidence for a single dominant clone. Our study also demonstrated that multiple clusters of possibly related strains were circulating at a particular point of time and some which were present in 2008, were also obtained in 2012, showing the persistence of some closely related strains. In this study, an increased carbapenem resistance was found among the members of *Enterobacteriaceae* from

2008 to 2012, which was mainly due to carbapenemases encoded on plasmids. These carbapenemases have a potential to be transferred both intra- and intergenically. Hence, this calls for better infection control measures and continued surveillance for carbapenem resistance.

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