Detection of Carbapenem Resistance Encoding Genes Among Gram Negative Bacteria from Urinary Tract Infection in Patients with Type 2 Diabetes Mellitus

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The emergence and spread of carbapenem-resistant *Enterobacteriaceae* (CRE) in urinary tract infection among diabetic patients have become an increasing concern for management and treatment of the patients. The aim of this study was to investigate the genotypic features of CRE strains isolated from urinary tract infection among type 2 diabetes mellitus patients. A total of 1560 diabetic patients were screened for suspected urinary tract infection. 277 Gram negative bacteria were identified by Phoenix 100 system (Becton-Dickinson, USA). These isolates were screened for their ability to produce carbapenemases by a disc diffusion test. A total of 45 CRE isolates were recovered from these Gram negative bacteria. Carbapenamase producing isolates were screened for bla_{SPM}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and bla_{GIM} genes The PCR products were sequenced in an ABI 3500 DNA sequencer (Applied Biosystems, USA). bla_{IMP-1}, bla_{IMP-8}, bla_{NDM-1}, bla_{NDM-2}, and bla_{NDM-4} were the predominant genes seen among *E.coli, Klebsiella pneumoniae, Citrobacter freundii, Acinetobacter baumannii* and *Proteus mirabilis*. Colistin and Amikacin were the drug of choice and Colistin had the MIC value of $\leq$ 1mg/l and for Amikacin 62% of isolates had MIC value of $\leq$ 4mg/l. This rising trend of carbapenem resistance among Gram negative bacteria stresses the increasing importance of continuous surveillance system and stewardship of antibiotics as strategies in the overall management of diabetic patients with urinary tract infection.

Keywords: Carbapenem resistance encoding genes, Gram negative bacilli,
Urinary tract infection, Type 2 diabetes mellitus.

Urinary tract infections (UTIs) are among the most common types of infectious disease, with approximately 150–250 million cases globally per year.[1-3] About 40–50% of women and 5% of men will develop a UTI at least once during their lifetime.[2,4] Owing to their high prevalence, UTIs are a major contributor to global antibiotic use and resistance.[5-6]

Urinary tract infections in diabetic patients have become a serious problem with the decisive effects on mortality rates and treatment outcome. Members of the Enterobacteriaceae are among the major causative agents of Urinary tract infection. Carbapenemase-producing Enterobacteriaceae have already been detected all over the globe, with a marked endemicity according to enzyme type. In India the prevalence of Carbapenemase-producing Enterobacteriaceae is found to be 12.3 to 22%[6,7].

Carbapenem resistance can be ascribed to several enzymes encoded by resistance genes

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including the production of various carbapenemases: K.pneumoniae carbapenemase (KPC; Ambler class A), Verona integron–encoded metallo-β-lactamase VIM), imipenemase (IMP), New Delhi metallo-β–actamase (NDM) (all Ambler class B), and oxacilinase-48 (OXA-48; Ambler Class D).[3,5,8]

The study was carried out to screen and characterize carbapenem resistance encoding genes among Gram negative bacteria from Urinary Tract Infection in patients with type 2 diabetes mellitus

MATERIALS AND METHODS

From July 2011 to June 2015, a total of 1560 diabetic patients were screened for suspected urinary tract infection. 277 Gram negative bacteria were identified by conventional microbiological techniques and confirmed by Phoenix 100 system (Becton-Dickinson, USA)[4,9] These isolates were screened for their ability to produce carbapenemases by a disc diffusion test, in which 10mg imipenem discs were used (Hi-Media, India)[7]. A total of 45 CRE isolates were recovered from these Gram negative bacteria. The MICs of eleven antibiotics, including imipenem, meropenem, ceftazidime, cefotaxime, cefuroxime, aztreonam, piperacillin-tazobactam, ciprofloxacin, amikacin, gentamicin and colistin were determined using the agar dilution method, and the results were analyzed according to the CLSI criteria of 2014[5,7]. Quality control for the MICs was performed using the reference strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.

DNA extraction and screening of carbapenem-resistance genetic markers

Bacterial genomic DNA was extracted from 1 ml of overnight cultures in Tryptic Soya Broth (Hi-Media, India) using the DNA Purification Kit (Kiagen, Germany) following the manufacturer's instructions. The DNA extracts were quantified using NanoDrop (Thermo Fisher Scientific, Wilmington, USA) and stored in a freezer at 20°C, to be used as templates in PCRs. The following genes were screened by PCR: bla_{SPM-1}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and bla_{GIM}[10-15]. The primers used in the study are depicted in the table-1. All the PCR experiments were performed in duplicate.

The expected amplicons were visualized in 1.5% agarose gel stained with ethidium bromide. The 100 bp DNA ladder was used as molecular weight standard (Life Technologies, USA). Positive controls for PCR reactions were carried out by sequencing randomly selected amplicons comprising 10% of the total reactions. The PCR products were sequenced in an ABI 3500 DNA sequencer (Applied Biosystems, USA)[16].

RESULTS

Out of 1560 diabetic patients were screened for suspected urinary tract infection. 277 Gram negative bacteria were isolated; of which 45 were carbapenem resistant. The MIC value for different antibiotics of all 45 carbapenem resistant Gram negative bacteria is depicted in table 2. All the isolates were resistant to aztreonam, ceftazidime, ciprofloxacin, cefotaxime, cefuroxime, imipenem, meropenem, piperacillin-tazobactam and gentamicin. All isolates were susceptible to colistin and 62% to amikacin (Table-2). Molecular detection of carbapenem resistance encoding genes among Gram negative bacteria revealed the presence of

Table 1. Primers used and expected amplicons

Gene	Primer sequence	Amplicon (bp)
NDM	5' – GCAGTCGCTTCCAACGGTTTGATCGT – 3'	468
	5' – CTCAGTGTCGGCATCACCGAGATTGC – 3'	
IMP	5' – CTTGATGAAGGCGTTTATGTTCATAC – 3'	584
	5' – AAGAGTGATGCGTCTCCAGCTTCACT – 3'	
VIM	5' – ATGGTCTCATTGTCCGTGATGGTGATG – 3'	377
	5' – GTATAGCACGTTCGCTGACGGGACGTA– 3'	
GIM	5' – TGGCTAGCATCTCAACTCATTCTCATG – 3'	422
	5' – GATTCAGCAAGATCAATGGTGTGATC – 3'	
SPM	5' – ATGTCTTAGTAGCGAAAATGCTTGATG – 3'	509
	5' – CTTCACATTGGCATCTCCCAGATAAC – 3'	

Table 2. Minimum Inhibitory Concentration of Carbapenem Resistant Gram Negative
Bacteria isolated from Urinary tract Infection in patients with type 2 diabetes mellitus

Isolates	MIC for the antimicrobial agents in mg/L and interpretation										
	CXM	CTX	CAZ	ATM	MEM	CIP	AN	COL	PIP	IMP	GEN
E.coli-5	32 R	32 R	32 R	8 R	16 R	8 R	2 S	1S	32 R	16 R	8 R
E.coli-8	32 R	32 R	32 R	8 R	16 R	8 R	2 S	1S	32 R	16 R	8 R
E.coli-9	32 R	32 R	32 R	8 R	16 R	8 R	2 S	$\geq$1S	32 R	16 R	8 R
E.coli-13	32 R	32 R	32 R	8 R	16 R	8 R	2 S	1S	32 R	16 R	8 R
E.coli-19	32 R	32 R	32 R	8 R	16 R	8 R	2 S	$\geq$1S	32 R	16 R	8 R
E.coli-23	32 R	32 R	32 R	8 R	16 R	8 R	2 S	1S	32 R	16 R	8 R
E.coli-29	32 R	32 R	32 R	8 R	16 R	8 R	2 S	1S	32 R	16 R	8 R
E.coli-37	32 R	32 R	32 R	8 R	16 R	8 R	2 S	1S	32 R	16 R	8 R
E.coli-48	32 R	32 R	32 R	8 R	16 R	8 R	2 S	1S	32 R	16 R	8 R
E.coli-55	32 R	32 R	32 R	8 R	16 R	8 R	2 S	1S	32 R	16 R	8 R
E.coli-62	32 R	32 R	32 R	8 R	16 R	8 R	2 S	1S	32 R	8 R	16 R
E.coli-84	32 R	32 R	32 R	8 R	16 R	8 R	2 S	1S	32 R	8 R	16 R
E.coli-86	32 R	32 R	32 R	8 R	16 R	8 R	2 S	1S	32 R	8 R	16 R
E.coli-87	32 R	32 R	32 R	8 R	16 R	8 R	2 S	1S	32 R	32 R	16 R
E.coli-88	32 R	32 R	32 R	8 R	16 R	8 R	2 S	1S	32 R	32 R	16 R
E.coli-89	32 R	32 R	32 R	8 R	16 R	8 R	2 S	$\geq$1S	32 R	8 R	16 R
K.pneumoniae-6	16 R	16 R	16 R	16 R	8 R	2 R	32 R	0.5 S	64 S	32 R	16 R
K. pneumoniae-14	16 R	16 R	16 R	16 R	8 R	2 R	32 R	0.5 S	64 S	32 R	32 R
K.pneumoniae-15	16 R	16 R	16 R	16 R	8 R	2 R	32 R	0.5 S	64 S	16 R	32 R
K.pneumoniae-19	16 R	16 R	16 R	16 R	8 R	2 R	32 R	0.5 S	64 S	16 R	32 R
K. pneumoniae-24	16 R	16 R	16 R	16 R	8 R	2 R	32 R	0.5 S	64 S	16 R	32 R
K. pneumoniae-25	16 R	32 R	16 R	16 R	8 R	2 R	4 R	0.5 S	64 S	16 R	32 R
K.pneumoniae-37	16 R	16 R	16 R	16 R	8 R	2 R	32 R	0.5 S	64 S	16 R	32 R
K.pneumoniae-47	16 R	16 R	16 R	16 R	8 R	2 R	32 R	0.5 S	64 S	16 R	32 R
K. pneumoniae-52	16 R	16 R	16 R	16 R	8 R	2 R	32 R	0.5 S	64 S	16 R	32 R
K. pneumoniae-53	16 R	32 R	16 R	16 R	8 R	2 R	4 R	0.5 S	64 S	16 R	32 R
C. freundii-12	16 R	16 R	16 R	16 R	8 R	2 R	32 R	0.5 S	64 S	16 R	32 R
C. freundii-17	16 R	16 R	16 R	16 R	4 R	2 R	32 R	0.5 S	64 S	16 R	32 R
C. freundii-19	16 R	16 R	16 R	16 R	4 R	2 R	32 R	0.5 S	64 S	32 R	32 R
C. freundii-25	16 R	32 R	16 R	16 R	8 R	2 R	32 R	$\geq$0.5 S	64 S	32 R	32 R
C. freundii-29	16 R	16 R	16 R	16 R	8R	2 R	32 R	0.5 S	64 S	32 R	32 R
C. freundii-30	16 R	16 R	16 R	16 R	4 R	2 R	32 R	0.5 S	64 S	32 R	$\geq$16 R
A. baumanii-3	16 R	16 R	16 R	16 R	8R	2 R	32 R	0.5 S	64 S	16 R	32 R
A. baumanii-4	16 R	16 R	16 R	16 R	8R	2 R	32 R	0.5 S	64 S	32 R	32 R
A. baumanii-14	16 R	16 R	16 R	16 R	8R	2 R	32 R	0.5 S	64 S	32 R	32 R
A. baumanii-20	16 R	16 R	16 R	16 R	8R	2 R	32 R	0.5 S	64 S	32 R	32 R
A. baumanii-28	16 R	16 R	16 R	16 R	8R	2 R	32 R	0.5 S	64 S	32 R	32 R
A. baumanii-29	16 R	16 R	16 R	16 R	8R	2 R	32 R	0.5 S	64 S	32 R	32 R
P. mirabilis-29	16 R	16 R	16 R	16 R	8R	2 R	32 R	0.5 S	64 S	32 R	8 R
P. mirabilis-33	16 R	16 R	16 R	16 R	8R	2 R	32 R	0.5 S	64 S	32 R	32 R
E. cloacae-11	16 R	64 R	16 R	16 R	32R	4 R	2 R	0.5 S	64 S	16 R	16 R
E. cloacae-11	16 R	64 R	16 R	16 R	32R	4R	2 R	0.5 S	64 S	16 R	32 R

I, intermediate; R, resistant; S, susceptible; AN, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; COL, colistin; CTX, cefotaxime; CXM, cefuroxime; MEM, meropenem; TZP, piperacillin-tazobactam, GEN, Gentamicin. Interpretation according to CLSI Guidelines-2014

Table 3. Distribution of Carbapenem Resistant genes among Gram Negative Bacteria
isolated from Urinary tract Infection in patients with type 2 diabetes mellitus

Isolates	bla$_{IMP}$		bla$_{VIM}$			bla$_{NDM}$		bla$_{SPM-1}$
	IMP-1	IMP-8	VIM-1	VIM-24	NDM-1	NDM-2	NDM-4	
E.coli	3	1	-	1	4	1	2	-
Klebsiella pneumoniae	-	1	-	1	3	1	-	2
Citrobacter freundii	1	-	1	1	2	2	1	-
Acinetobacter baumanii	1	-	1	-	2	3	-	-
Proteus mirabilis	-	-	-	-	1	-	-	-
Enterobacter cloacae	-	-	-	-	-	-	-	-

bla$_{SPM}$, bla$_{NDM}$, bla$_{IMP}$, bla$_{VIM}$, and bla$_{GIM}$ genes.

On nucleotide sequence of *E.coli* for bla$_{SPM}$, bla$_{NDM}$, bla$_{IMP}$, bla$_{VIM}$, and bla$_{GIM}$ genes revealed that out of 16 carbapenem resistant *E.coli*, 4 isolates showed the presence of bla$_{NDM-1}$, 3 isolates the presence of bla$_{IMP-1}$, two isolates for bla$_{NDM-4}$, and one isolate each for bla$_{VIM-24}$, bla$_{NDM-2}$, and bla$_{IMP-8}$. Four isolates didn't show the presence of any genes screened (Table-3).

Nucleotide sequence of *Klebsiella pneumoniae* for bla$_{SPM}$, bla$_{NDM}$, bla$_{IMP}$, bla$_{VIM}$, and bla$_{GIM}$ genes revealed that out of 10 carbapenem resistant *Klebsiella pneumoniae*, three isolates showed the presence of bla$_{NDM-1}$, two isolates for bla$_{SPM-1}$, one isolate each for bla$_{VIM-24}$, bla$_{IMP-8}$ and bla$_{NDM-2}$. Two isolates didn't show the presence of any genes screened (Table-3).

Nucleotide sequence of *Citrobacter freundii* for bla$_{SPM}$, bla$_{NDM}$, bla$_{IMP}$, bla$_{VIM}$, and bla$_{GIM}$ genes revealed that out of 6 carbapenem resistant *Citrobacter freundii*, 2 isolates showed the presence of bla$_{NDM-1}$, two isolates showed the presence of bla$_{NDM-2}$, one isolate the presence of bla$_{IMP-1}$ and bla$_{NDM-4}$, and one isolate for bla$_{VIM-24}$ (Table-3).

Similarly on Nucleotide sequence of *Acinetobacter baumannii* for bla$_{SPM}$, bla$_{NDM}$, bla$_{IMP}$, bla$_{VIM}$, and bla$_{GIM}$ genes revealed that out of 6 carbapenem resistant *Acinetobacter baumannii*, 3 isolates showed the presence of bla$_{NDM-2}$, two isolates showed the presence of bla$_{NDM-1}$, one isolate the presence of bla$_{IMP-1}$ and bla$_{VIM-1}$ (Table-3).

Among 2 *Proteus mirabilis* one isolate showed the presence of bla$_{NDM-2}$ and Enterobacter cloacae did not show the presence of the any carbapenem genes evaluated (Table-3).

DISCUSSION

The prevalence of bacterial resistance to antibiotics continues to increase. Regrettably, infections caused by resistant organisms result in a tremendous morbidity and mortality worldwide. The mathematician William Foster Lloyd in 1833 published his lecture entitled "Checks to Population".[8] describing how farmers using common grazing areas for their sheep could diminish this shared resource to the subsequent disadvantage of all, while still acting in rational self-interest.[8] The economist Garret Hardin later encapsulated this concept as "the tragedy of the commons".[9] A similar impasse could be said to have arisen from our use of antibiotics. In seeking to maximize benefit for individual patients, clinicians now risk reduced utility of this precious shared resource for the many.

Bacterial resistance to our antibiotic arsenal is not a new phenomenon. Indeed, multiple resistance determinants have been found in bacteria isolated from environments that have been separated from human activity for millions of years.[10] However, the antimicrobial resistance crisis we currently face reflects the rapid expansion, diversification and extension of host range for a multitude of resistance determinants under selection pressure from the widespread use of antibiotics. The impact of multidrug resistance (MDR) extends into all aspects of medicine and threatens the significant progress which has been made in field of modern medicine. In past decades much emphasis has been applicably placed on MDR among Gram positive cocci and several new treatment options have become available for

it. However, the threat of MDR in Gram-negative organisms has not led to a similar increase in novel therapeutics. The prevalence of carbapenem resistance in Gram negative bacilli isolated from clinical samples continues to increase globally.[11,14]

Carbapenems was developed in the 1980s are derivatives of thyanamycin. Imipenem and meropenem were the first members of the class, had a broad spectrum of antimicrobial activity back then, nearly all Enterobacteriaceae were susceptible to carbapenems.[8,9,11,14] In the 1990s, Enterobacteriaceae started to develop resistance to cephalosporins, till then, the cephalosporins were the first-line antibiotics for these organisms. Enterobacteriaceae by acquiring extended-spectrum beta-lactamases, which inactivate those agents, became resistant to cephalosporins. Consequently, the use of cephalosporins had to be restricted, while carbapenems, which remained impervious to these enzymes, had to be used more.[9] In pivotal international studies in the treatment of infections caused by strains of K $pneumoniae$ that produced these inactivating enzymes, outcomes were better with carbapenems than with cephalosporins and fluoroquinolones.[10,11,17]

Currently, MBL has spread through most Gram negative bacteria, which are prevalent in community-acquired and health care-associated infections[12-14]. Since some MBL-producing Gram negative bacteria show low-level resistance or even sensitivity to carbapenems, the CLSI breakpoints of carbapenems among them have changed since June 2010. Imipenem breakpoints of 4mg/l (susceptible [S]), 8mg/l (intermediate [I]), and 16mg/l (resistant [R]) and meropenem breakpoints of 4mg/l (S), 8mg/l (I), and 16 mg/l (R) have moved to 1mg/l (S), 2mg/l (I), and 4mg/l (R) and 1mg/l (S), 2mg/l (I), and 4mg/l (R). In the present study all the isolates had breakpoint of $\geq$ 4mg/l for meropenem and $\geq$ 8mg/l for imipenem. The drug of choice for carbapenem resistance isolates was Colistin. 12 out of 45 isolates had breakpoint of 1mg/l and 33 isolates had 0.5mg/l. Amikacin was the next drug of choice for MDR Gram negative isolates. 62% of isolates were sensitive to Amikacin with the breakpoint of $\leq$ 4mg/l.

In China, currently available data tend to suggest that bla_{NDM-1} is only present at a relatively low frequency and spreading sporadically amongst Enterobacteriaceae[16,17]. In the present study we demonstrated high incidence of bla_{NDM-1}, bla_{NDM-2}, and bla_{NDM-4} among $E.coli$, $Klebsiella$ $pneumonia$, $Citrobacter$ $freundii$ and $Acinetobacter$ $baumannii$. No data available to compare this results from India.

This is the ûrst report of IMP-8 MBL among Enterobacteriaceae in India. IMP-8 is very uncommon in Europe, only once reported from Portugal in a $Pseudomonas$ $mendocina$ strain[18]. In contrast, IMP-8 is frequently encountered in Asia, especially in Taiwan, where IMP-8-producing Enterobacteriaceae are involved in serious infections[19,20]. Yan et al. reported on a case series of 37 patients with bloodstream infections caused by a large variety of IMP-8-producing Enterobacterial species including $Escherichia$ $coli$, $K.$ $pneumoniae$, $Enterobacter$ $cloacae$ and $C.$ $freundii$[21]. In India, workers have detected bla_{IMP} and bla_{VIM} genes in 59% of $Pseudomonas$ $aeruginosa$ isolates in Chennai[22] and 61.1% strains carried bla_{VIM} and 3% carried bla_{IMP} in Tamil Nadu[23].

CONCLUSION

We report for the first time Carbapenemase producing $E.coli$, $Klebsiella$ $pneumonia$, $Citrobacter$ $freundii$, $Acinetobacter$ $baumannii$, and $Proteus$ $mirabilis$ isolated from urinary tract infection among type 2 diabetes mellitus patient. Epidemiological control and adequate identification of carbapenemase production among these isolates will enable proper management of UTI among diabetic patients.

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