Indian J Med Res 149, April 2019, pp 528-538
DOI: 10.4103/ijmr.IJMR_716_17

Quick Response Code:

Contribution of *acrB* upregulation & OmpC/Ompk36 loss over the presence of bla_{NDM} towards carbapenem resistance development among pathogenic *Escherichia coli & Klebsiella* spp.

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Received May 3, 2017

***Background & objectives*: The global spread of carbapenem-resistant *Enterobacteriaceae* (CRE) is an emerging clinical problem. Hence, in this study, the plausible role of extended-spectrum beta-lactamases (ESBLs)/carbapenemases, OmpC/Ompk36, *acrB* and their combinations was explored among CRE.**

***Methods*: The minimum inhibitory concentration (MIC) of meropenem, enzyme-phenotypes (ESBLs/IR and metallo-beta-lactamase (MBL)/non-MBL carbapenemase), genotypes ($bla_{TEM,}$ bla_{SHV} and bla_{CTX-M}; bla_{NDM} and $bla_{VIM;}$ bla_{KPC} and bla_{OXA-48}-like variants), *acrB* and outer membrane protein (OMP) expressions were analyzed with a total of 101 non-duplicate clinical isolates, obtained from various samples of patients visiting two tertiary care units of Eastern India during May 2013 - October 2016. This included *Escherichia coli* (n=36) and *Klebsiella pneumoniae* (n=65), categorized into two groups, namely Group I (resistant to all carbapenems; n=93; *E. coli*=34 and *Klebsiella* spp.=59) and Group II (non-resistant to all the carbapenems; n=8; *E. coli*=2 and *Klebsiella* spp.=6).**

***Results*: Though 88.17 per cent of Group I isolates exhibited ESBL property, the presence of carbapenemase activity (70.96%) and that of bla_{NDM} gene (42/66: 63.63%) indicated their contributions towards the emergence of CRE. Further, porin loss and/or efflux pump activation among ESBL/carbapenemase-producing isolates heightened the MIC of meropenem from 64 to 256 mg/l (range exhibited by only ESBL/carbapenemase-producing isolates) to >256 mg/l.**

***Interpretation & conclusions*: These findings implied the major contribution of porin loss and/or efflux pump activation over the presence of ESBLs/carbapenemases in imparting carbapenem resistance in pathogenic bacteria.**

Key words *acrB*, bla_{NDM} - carbapenem-resistant *Enterobacteriaceae* - epidemiology - extended-spectrum beta-lactamase - OmpC/Ompk36

The global emergence of antibiotic resistance, which results in tremendous morbidity and mortality worldwide, has been considered to be one of the greatest threats in international public health[1,2]. Among all the commercially available antibiotics, resistance towards several carbapenems, considered to be the 'drugs of last resort', is supposed to be clinically most important because it delimits the treatment options against bacterial infections[2,3]. Although fewer medicines such as aztreonam, tigecycline, colistin and fosfomycin

retain *in vitro* activity against carbapenem-resistant *Enterobacteriaceae* (CRE), their usages are restricted by either their side effect profile or uncertainty of *in vivo* efficacy[3]. From 2008 onwards, resistance towards several widely used carbapenems, namely imipenem, meropenem and ertapenem, had increased significantly in India[4]. While, only 11-22 per cent of enterobacterial isolates were found to be resistant towards at least one of these three carbapenems during 2007-2008, such resistance has been found to rise to at least 37.9 per cent in recent years[4,5].

Combined expression of extended-spectrum beta-lactamase (ESBL)-type enzymes with either loss/reduced expression of major outer membrane proteins (OMPs) (OmpF/Ompk35 of MW 36kDa and OmpC/Ompk36 of MW 38kDa in *Escherichia coli*/ *Klebsiella* spp., respectively) or overexpression of efflux pumps (AcrAB-TolC) as well as expression of metallo (NDM; VIM)/non-metallo-beta-lactamase (KPC; OXA)-type carbapenemases alone was known to be responsible for carbapenem resistance development among pathogenic *Enterobacteriaceae*[4,6,7]. However, the nature of contribution of these mechanisms towards carbapenem resistance development, whether additive, augmentative or diminutive, has not yet been well documented. Although the association of expression of porins and efflux pumps with ESBL is well known for carbapenem resistance development, but their relation with carbapenemases has not been studied before. Besides, reports on contribution of all these mechanisms for such resistance development among isolates of Indian origin are limited. Thus, the present cross-sectional study was undertaken to address all the aforesaid lacunae for better understanding the increasing emergence of CRE in India.

Material & Methods

Enterobacterial samples were obtained from urine, blood, sputum, body fluid, wound and pus of different non-duplicate and unrelated patients visiting the outpatient department of Seth Sukhlal Karnani Memorial Hospital-Institute of Post Graduate Medical Education and Research (SSKM-IPGMER) and Calcutta School of Tropical Medicine (CSTM, Kolkata, India), from May 2013 to October 2016. Samples were subjected to Gram staining and streaked on nutrient agar, blood agar and MacConkey's agar media and incubated overnight at 37ºC. Identification of *E. coli* and *Klebsiella* spp. isolates was done by growing isolates on organism-specific HiCrome™ selective agar (HiMedia Laboratories Pvt. Ltd., India) as well as by standard biochemical methods[8]. The study was approved by the Institutional Ethical Committee of CSTM, Kolkata (Ref. No. CREC-STM/260 dated 9/1/2013).

Antimicrobial susceptibility assay for the categorization of isolates: Antimicrobial susceptibility of the isolates was determined by Kirby-Bauer standard disc diffusion method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI), 2013[9], for the following antimicrobial agents (µg/disc): ceftazidime (30), cefotaxime (30), cefpodoxime (10), imipenem (10), meropenem (10) and ertapenem (10) (HiMedia Laboratories Pvt. Ltd., Mumbai). Isolates resistant and non-resistant to all carbapenems were categorized as Group I (resistant to all carbapenems) and Group II (non-resistant to all the carbapenems), respectively. The minimum inhibitory concentration (MIC) value (mg/l) of meropenem (AstraZeneca, UK) against Groups I and II isolates was determined using microdilution method and interpreted according to the guidelines of CLSI[9].

Phenotypic characterization of Group I and Group II isolates: Screening of ESBLs was done according to the guidelines of CLSI[9]. Isolates exhibiting an increase of >5 mm in inhibition zone of the combined ceftazidime/cefotaxime (30 µg)-clavulanic acid (10 µg) disc, compared to ceftazidime/cefotaxime (30 µg) alone, were categorized as ESBL positive. Carbapenemase activity was assayed among these isolates following the protocol described by Bernabeu *et al*[10]. Briefly, the isolates were sonicated and the supernatant was used to determine meropenem-hydrolyzing activity spectrophotometrically. Percentage activity was calculated by the following formula:

$$\% \text{ activity} = \frac{\text{Absorbance of cleaved meropenem in experimental well} \times 100}{\text{Absorbance of uncleaved meropenem in control well}}$$

Detection of metallo-β-lactamases (MBLs) among isolates with carbapenemase activity was evaluated by double-disc synergy test (DDST)[4], where imipenem (10 µg) and imipenem-ethylenediaminetetraacetic acid (10/750 µg) combination disc (HiMedia Laboratories Pvt. Ltd., Mumbai) were placed 40-50 mm apart over the lawn of bacteria, spread aseptically over Mueller-Hinton agar and incubated overnight at 37°C[4]. Isolates exhibiting an increase of >5 mm in the inhibition zone of the combination disc, compared to imipenem alone, were categorized as MBL positive.

Genomic DNA isolation, PCR detection of ESBLs and carbapenemases: Plasmid and chromosomal DNA was extracted from Groups I and II isolates by alkaline lysis and Hancock's method, respectively[11,12]. PCR amplification was carried out for detection of the following ESBL, MBL and non-MBL genes - ESBL: $bla_{TEM,}$ bla_{SHV} and bla_{CTX-M}; MBL: bla_{NDM} and bla_{VIM} and non-MBL: bla_{KPC} and bla_{OXA-48}-like variants using DNA and gene-specific primers, designed through Primer3 server, web version 4.0.0 (*http://bioinfo.ut.ee/primer3/*) (Table I). Based on PCR data, Group I isolates were further genotypically categorized into four subgroups, namely ESBL only (Ia), carbapenemase only (Ib), both ESBL and carbapenemase-producing (Ic) and none present (Id).

Analysis of expression of acrB efflux pump gene: Group I and II isolates were grown in Müller-Hinton Broth at 37°C with continuous orbital shaking and harvested at mid-log phase by centrifugation 12,300 × *g* 10 min. Bacterial total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and was reverse transcribed to cDNA by first-strand cDNA synthesis kit (Thermo Fisher Scientific Inc., MA, USA) following manufacturer's standard protocols. Expression of *acrB* was normalized against that of housekeeping gene, *RNA polymerase* B (*rpoB*), used as internal control[13]. The level of *acrB* expression among isolates of different subgroups was determined by calculating $2^{-\Delta\Delta CT}$ and compared to that of Group II isolates, using DataAssist™ software v2.1 (Thermo Fisher Scientific Inc., USA).

Analysis of bacterial outer membrane protein (OMP) expression: Group I and group II isolates were grown in Müller-Hinton Broth at 37ºC with continuous orbital shaking and harvested at mid-log phase by centrifugation at 12,300 × *g* 10 min. Bacterial OMPs were isolated and purified following the protocols described earlier[14]. OMPs were quantified in Lowry's method[15]. About 100 µg of OMPs/well were separated by 15 per cent sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining using 0.25 per

Table I. Primers used in the study

Primer	Forward and Reverse Primer Sequences	Amplicon size (bp)
Primer sets used in PCR		
bla_{TEM} type	5'-ATGAGTATTCAACATTTTCGTC-3'	860
bla_{SHV} type	5'-TTACCAATGCTTAATCAGTGAG-3	863
bla_{CTX_M} type	5'-GCGTTATWTTCGCCTGTG-3'	820
	5'-GCTTTTAKYGTTGCCAGT-3'	
	5'-GYCAGTTCACGCTGATGG-3'	
	5'-CGCCGACGCTAATACATC-3'	
bla_{NDM} type	5'-AAGCTGAGCACCGCATTA- 3'	758
	5'-CGGGCCGTATGAGTGATT-3'	
bla_{VIM} type	5'-GTCTATTTGACCGCGTCT-3'	778
	5'-CTCAACGACTGAGCGATT-3'	
bla_{OXA} type	5'-TGCGTGTATTAGCCTTATCG-3'	773
	5'- GAGCACTTCTTTTGTGATGG-3'	
bla_{KPC} type	5'-CTGTATCGCCGTCTAGTTC-3'	824
	5'-GCTGTRCTTGTCATCCTT-3'	
Primer sets used in qRT-PCR		
E. coli AcrB	5'-GAGAAATCATCCAGCAGCT- 3'	156
	5'-CTGTGAACCGAACAACTGA- 3'	
E. coli rpoB	5'-GAAGGCACCGTAAAAGACA- 3'	174
	5'-ACCCGAAGAGTGGGTTTTA- 3'	
Klebsiella AcrB	5'-GTTAATGACGCCGACAAC- 3'	125
	5'-TACGCTGACCTTGCAATC- 3'	
Klebsiella rpoB	5'-GTTGACTACATGGACGTATCC- 3'	175
	5'-AACAGCACGTTCCATACC- 3'	

cent Coomassie brilliant blue. Five microliters of PageRuler pre-stained protein ladder (10-170 kDa range) (Thermo Fisher Scientific Inc., USA) was also subjected to SDS-PAGE for determining the molecular weight of OMPs. De-stained gels were photographed through Gel documentation system (UVI-tech Ltd., Cambridge, UK), and OMP bands were subjected to densitometric scanning through Image Processing and Analysis in Java (ImageJ, NCBI, USA). OMP expression of Group I isolates was compared with that of Group II isolates and was expressed in terms of percentage reduction in expression. Inter-subgroup comparison was also done with the expression data normalized against Group II isolates.

Statistical analysis: MIC values were represented in range and the distribution pattern was depicted by bar diagrams. The mode values of MIC with frequencies were calculated for both *E. coli* and *Klebsiella* spp. of Groups I and II isolates. Carbapenemase activity, level of expression of *acrB* and OMPs among Group I isolates were represented in percentage activity, fold changes and percentage reductions normalized against the values of Group II isolates, respectively. First, Shapiro-Wilk normality test was performed to evaluate the distribution of data[16]. Data showing $P>0.05$ were considered to follow normal distribution. One-way ANOVA followed by Tukey's honestly significant difference (HSD) *post hoc* analysis test was performed to compare MIC values, percentage carbapenemase activity and expression level of *acrB* and OMP among various phenotypic-genotypic subgroups of Group I isolates[17]. The statistical analyses were performed through SPSS software v17.0 (IBM Inc., NY, USA). Overall contribution of all the strategies, namely expression of beta-lactamases (B: ESBL; C: carbapenemases), efflux pump's upregulation (E) and porin loss (P) and their combinations in carbapenem resistance development was represented through proportionate Venn diagram by eulerAPE v3.0.0[18].

Results

From May 2013 to October 2016, a total of 101 pathogenic *E. coli* and *Klebsiella* spp. were isolated from urine (71/101: 70.29%), wound pus (14/101: 13.86%), sputum (8/101: 7.92%), body fluids (5/101: 4.95%) and blood (3/101: 2.97%) of different non-duplicate unrelated patients (female: male: 1.24:1). Standard biochemical tests and overnight culture of the collected bacteria on organism-specific selective media identified 35.64 per cent (36/101) and 64.36 per cent (65/101) bacteria to be *E. coli* and *Klebsiella* spp., respectively. All the data generated from the above study settings followed normal distribution pattern as analyzed by Shapiro-Wilk normality test.

Antimicrobial susceptibility assay and selection of Group I and Group II isolates: Antibiograms of *E. coli* (n=36) and *Klebsiella* spp. (n=65) identified 93 (Group I: n=93; *E. coli*=34 and *Klebsiella* spp.=59) and eight (Group II: n=8; *E. coli*=2 and *Klebsiella* spp.=6) isolates as resistant and non-resistant towards all carbapenems, respectively, which were considered for further analyses. All the 93 Group I isolates demonstrated MIC values of meropenem in the range of 64 to >1024 mg/l, with central tendency (mode) of >1024 mg/l and having frequency of 0.706 and 0.644 for *E. coli* and *Klebsiella* spp., respectively. On the other hand, carbapenem non-resistant Group II isolates revealed MIC of meropenem in the range of 1-4 mg/l with mode value of 2 mg/l, having frequency of 1.0 and 0.667 for *E. coli* and *Klebsiella* spp., respectively.

Phenotypic screening of ESBLs and MBLs: Phenotypic screening identified 25.81 per cent (n=24; *E. coli*=7 and *Klebsiella* spp.=17) of Group I isolates to exhibit only ESBL property; 8.60 per cent (n=8; *E. coli*=4 and *Klebsiella* spp.=4) to demonstrate only carbapenemase activity and 62.36 per cent (n=58; *E. coli*=22 and *Klebsiella* spp.=36) to exhibit both ESBL and carbapenemase activities (Table II). However, 3.22 per cent (n=3; *E. coli*=1 and *Klebsiella* spp.=2) exhibited neither ESBL nor carbapenemase activity. Among the carbapenemase-producing isolates (n=66), 93.94 per cent of isolates (n=62; *E. coli*=25 and *Klebsiella* spp.=37) demonstrated to have MBL activity by DDST.

PCR detection of ESBL and carbapenemase genes: PCR screening revealed 64.52 per cent (n=60; *E. coli*: 20/34 and *Klebsiella* spp.: 40/59) of Group I isolates to harbour at least one ESBL or carbapenemase gene. Among the ESBL and MBL genes, bla_{TEM} (20.40%, n=19; *E. coli*: 6/34 and *Klebsiella* spp.: 13/59) and bla_{NDM} (45.20%, n=42; *E. coli*: 18/26 and *Klebsiella* spp.: 24/40) were found to be the most prevalent ones among Group I isolates (Table II). These genes were mostly found among ESBL only and both ESBL- and carbapenemase-producing phenotypes. Distribution of beta-lactamase genes among different phenotypic groups is elaborated in Table II. Overall co-existence of multiple carbapenemase and/or ESBL genes was found among 24.73 per cent of group I isolates (n=23; *E. coli*: 10/34 and *Klebsiella* spp.: 13/59), of which

Table II. Distribution of various beta-lactamase genes among carbapenem-resistant (Group I) isolates

Organism (n)	Phenotype	Genotype						
		ESBL genes (%)			Non-MBL genes (%)		MBL genes (%)	
		bla_{TEM}	bla_{SHV}	bla_{CTX_M}	bla_{OXA-48}-like	bla_{KPC}	bla_{NDM}	bla_{VIM}
Escherichia coli (n=7)	ESBL only	1 (14.3)	-	-	-	-	-	-
Klebsiella spp. (n=17)		8 (47.1)	2 (11.8)	5 (29.4)	-	-	-	-
E. coli (n=4)	Carbapenemase only	-	-	-	1 (25.0)	-	1 (25.0)	-
Klebsiella spp. (n=4)		1 (25.0)	-	-	-	-	1 (25.0)	
E. coli (n=22)	Both ESBL and carbapenemase producing	5 (22.7)	-	2 (9.1)	3 (13.6)	4 (18.2)	17 (77.3)	3 (13.6)
Klebsiella spp. (n=36)		3 (8.3)	2 (5.6)	2 (5.6)	7 (19.4)	3 (8.3)	23 (63.9)	3 (8.3)
E. coli (n=1)	No beta-lactamase activity	-	-	-	-	-	-	-
Klebsiella spp. (n=2)		1 (50.0)	-	-	-	-	-	-
Total (n=93)		19 (20.4)	4 (4.3)	9 (9.7)	11 (11.8)	7 (7.5)	42 (45.2)	6 (6.4)

Percentage of individual beta-lactamase genes was calculated using the numbers in parentheses mentioned besides the name of organisms.
ESBL, extended spectrum beta lactamase; MBL, metallo beta lactamase

69.56 per cent (16/23) were found to harbour MBL gene(s) along with ESBL/non-MBL gene(s).

Expression analysis of acrB efflux pump gene: Expression analysis of *acrB* demonstrated that while 76.47 per cent (26/34) of Group I *E. coli* exhibited 34.2+5.11-fold higher expression of this gene than that of Group II isolates, 66.1 per cent (39/59) of Group I *Klebsiella* spp. demonstrated 10.65+3.05-fold higher expression of *acrB* compared to the respective Group II isolates. Overexpression level of *acrB* was found to be significantly associated with ESBL and carbapenemase production among both *E. coli* and *Klebsiella* spp. (one-way ANOVA: $P<0.05$; *post hoc* Tukey's HSD test: $P<0.05$ for both ESBL- and carbapenemase-producing isolates) (Table III). Isolates producing both ESBL and carbapenemase exhibited the highest level of *acrB* expression, while isolates having no such activity demonstrated minimal *acrB* overexpression when compared to the respective Group II isolates.

Expression analysis of bacterial OMPs: Comparative analysis of OMP expression between Groups I and II isolates revealed 61.76 per cent (21/34) of Group I *E. coli* to have 45.36 per cent of reduced OmpC expression, whereas 69.49 per cent (41/59) of Group I *Klebsiella* spp. exhibited approximately 30.1 per cent of diminished Ompk36 expression (Fig. 1). Complete loss of OmpC/Ompk36 was documented in 17.65 per cent (6/34) of *E. coli* and 30.51 per cent (18/59) of *Klebsiella* spp., respectively. Loss of OmpC/Ompk36 was found to be significantly associated with both ESBL and carbapenemase production among Group I *E. coli* and *Klebsiella* spp. isolates (one-way ANOVA: $P<0.05$; *post hoc* Tukey's HSD test: $P<0.05$ for both ESBL- and carbapenemase-producing isolates) (Table III).

Genotypic categorization of Group I isolates and their association with MIC, carbapenemase activity and acrB and OMP expression: Among the Group I *E. coli*, one, 13, six and 14 isolates were categorized under Ia (ESBL gene carrying), Ib (carbapenemase gene carrying), Ic (both ESBL and carbapenemase carrying) and Id (none present) subgroups, respectively. Similarly, nine, 27, four and 19 Group I *Klebsiella* spp. isolates were classified under these subgroups.

Although MIC value distribution of meropenem was found to be almost consistent among the various subgroups, carbapenemase activity was found to be significantly high among Ib, Ic and Id subgroups of both organisms when compared with that of Ia isolates (one-way ANOVA: $P<0.05$; *post hoc* Tukey's HSD test: $P<0.01$ and $P<0.05$ for Ib and Ic, respectively) (Table IV). Moreover, single and multiple gene harbouring isolates among *Klebsiella* spp. of subgroup Ib revealed marked difference in their meropenem-hydrolyzing proficiency ($P<0.01$).

Inter subgroup comparison revealed significant overexpression of *acrB* among beta-lactamase-

Table III. Changes in the expression levels of *acrB* and OmpC/Ompk36 among various phenotypic classes of carbapenem-resistant Group I isolates

Phenotypes	Organism (n)	Fold increase in *acrB* expression (mean±SE) Normalized against Group II isolates	Per cent reduction in Omp-C/-k36 expression (mean±SE) normalized against Group II isolates
ESBL only	*Escherichia coli* (7)	24.56±9.48	54.46±6.25
	Klebsiella spp. (17)	18.60±7.30	27.40±1.52
	Overall (24)	20.34±7.89*	35.29±3.65*
Carbapenemase only	*E. coli* (4)	35.45±1.76	47.34±2.37
	Klebsiella spp. (4)	11.92±0.84	0.682±0.26
	Overall (8)	23.68±1.56*	24.01±1.65*
Both ESBL and carbapenemase producing	*E. coli* (22)	38.45±3.79	57.34±4.59
	Klebsiella spp. (36)	18.78±3.28	37.03±1.86
	Overall (58)	26.24±3.35*,†	44.73±2.73*,†
No beta-lactamase activity	*E. coli* (1)	0.26	35.44
	Klebsiella spp. (2)	3.81±0.79	16.49±0.68
	Overall (3)	2.63±0.65	22.81±0.53

One-way ANOVA: *$P<0.05$; †$P<0.05$ through Tukey's HSD *post-hoc* test. Three, one and two *E. coli* isolates from ESBL only, carbapenemase only and both ESBL- and carbapenemase-producing phenotypic classes exhibited complete loss of porin respectively. Similarly, among *Klebsiella* spp., five, one, three and one isolates from ESBL only, carbapenemase only, both ESBL- and carbapenemase-producing and no beta-lactamase activity-showing phenotypic classes showed complete loss of porin, respectively. HSD, honestly significant difference; SE, standard error

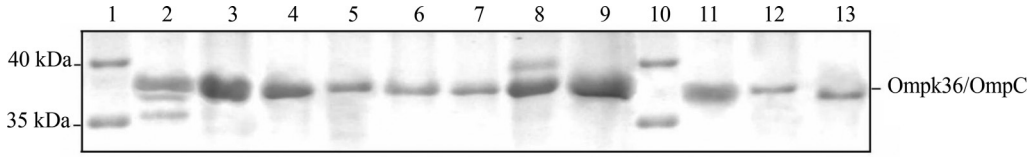

Fig. 1. Representative photograph of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of extracted outer membrane proteins (OMPs) exhibiting reduced expression of Ompk36/OmpC among Group I compared to Group II isolates. Lanes 1 and 10: Pre-stained protein marker, lanes 2-4: Ompk36 of carbapenem non-resistant (Group II) *Klebsiella* spp., lanes 5-7: Ompk36 of carbapenem-resistant (Group I) *Klebsiella* spp., lanes 8-9: OmpC of carbapenem non-resistant (Group II) *E. coli* and lanes 11-13: OmpC of carbapenem-resistant (Group I) *E. coli*.

producing *E. coli* of subgroups Ia, Ib and Ic with respect to Id (no beta-lactamase gene present) ($P<0.05$) (Table IV). However, this trend was not followed among *Klebsiella* spp., where *acrB* gene was found to be slightly underexpressed among these subgroups.

Comparable expression of OmpC was documented among all the subgroups of *E. coli*, whereas significant loss/reduced expression of Ompk36 was found among Id *Klebsiella* spp. (no beta-lactamase gene present) when compared to beta-lactamase-producing *Klebsiella* spp. of subgroups Ia, Ib and Ic ($P<0.01$) (Table IV).

Role of different strategies in carbapenem resistance emergence: Although the presence of only ESBL/carbapenemase genes (B/C), efflux pump upregulation (E), porin loss (P) or their combinations, namely B/C-E, B/C-P, PE and B/C-PE exhibited differential contribution in carbapenem resistance emergence among *E. coli* and *Klebsiella* spp., PE (10/34: 29.41%), B/C-PE (11/34: 32.35%) of *E. coli* and B/C-E (18/59: 30.51%), B/C-P (13/59: 22.03%) and PE (13/59: 22.03%) of *Klebsiella* spp. were found to be the most predominant strategies (Fig. 2A). Most of these isolates following these predominant strategic combinations exhibited very high level of meropenem resistance (MIC >1024 mg/l), whereas isolates with only B/C strategy demonstrated low level of meropenem resistance (MIC: 64-256 mg/l) indicating the major role of P, E and their combined strategies to increase meropenem resistance (one-way ANOVA: $P<0.01$; *post hoc* Tukey's HSD test: $P<0.01$ for all the six strategies including P, E and their combinations with respect to B/C alone) (Fig. 2B).

Table IV. Comparative account of MIC_{mrp}, per cent carbapenemase activity and changes in the expression levels of *acrB* and OmpC/Ompk36 among carbapenem-resistant isolates of various subgroups (Ia-Id)

Sub groups	Organism (n)	Number of genes present per isolate (n)	MICmrp (mg/l) (frequency) (%)	Per cent carbapenemase activity (mean±SE) Normalized against Group II isolates	Fold increase in acrB expression (mean±SE) Normalized against Group II isolates		Per cent reduction in Omp-C/-k36 expression (mean±SE) Normalized against Group II isolates	
					Individual subgroup data	Comparison between Ia+Ib+Ic and Id	Individual subgroup data	Comparison between Ia+Ib+Ic and Id
Ia	*Escherichia coli* (1)	Single (1)	512 (100.0)	0.91	43.38	M=41.14±10.13* O=10.78±3.50	47.05	M=45.79±4.40 O=13.98±3.73**
		Multiple (0)	-	-	-		-	
	Klebsiella spp. (9)	Single (5)	512 (60.0)	0.0±0.93	12.40±7.11		20.32±1.54 [1]	
		Multiple (4)	>1024 (75.0)	3.45±1.21	6.02±2.11		10.3±2.6	
		Overall (10)	>1024 (50.0)	1.47±0.85	12.97±4.39		18.98±4.39 [1]	
Ib	*E. coli* (13)	Single (9)	>1024 (88.9)	21.79±1.88	31.99±11.22		48.24±5.61	
		Multiple (4)	>1024 (50.0)	17.74±3.20	52.29±15.94		49.86±4.74s [1]	
	Klebsiella spp. (27)	Single (22)	>1024 (72.7)	7.48±1.98	13.93±3.78		18.53±5.30 [11]	
		Multiple (5)	>1024 (60.0)	23.93±0.24**	3.45±0.81*		1.18±0.93**	
		Overall (40)	>1024 (65.0)	13.78±1.86*,†	20.52±6.30		26.18±4.39[12]	
Ic	*Escherichia coli* (6)	Multiple (6)	>1024 (50.0)	16.69±0.38	47.07±6.30		39.21±3.09 [1]	
	Klebsiella spp. (4)	Multiple (4)	>1024 (50.0)	8.78±0.52	5.41±2.27		0.77±0.97	
		Overall (10)	>1024 (50.0)	13.53±0.44*,†	30.41±4.69		23.83±2.24 [1]	
Id	*Escherichia coli* (14)	NA	≥1024 (85.7)	9.41±0.33	23.31±5.01		37.11±1.99 [4]	
	Klebsiella spp. (19)	NA	≥1024 (68.4)	8.64±0.29	12.46±3.40	N=23.31±5.01 *P*=12.46±3.40	33.22±0.65 [11]	N=37.11±1.99 *P*=33.22±0.65
		Overall (33)	≥1024 (75.75)	8.97±0.31*	17.06±4.08		34.87±1.22 [15]	

*P**<0.05, ***<0.01 (one-way ANOVA); †*P*<0.05 (Tukey's HSD *post-hoc* test); Ia, Ib, Ic and Id represented ESBL only, carbapenemase only, both ESBL- and carbapenemase-producing and none present isolates, respectively. Number of isolates showing complete loss of OMPs was mentioned in square brackets. M=Average expression of acrB/OmpC among (Ia+Ib+Ic) *E. coli*; N=Average expression of acrB/OmpC among Id *E. coli*; O=Average expression of acrB/Ompk36 among (Ia+Ib+Ic) *Klebsiella* spp. and P=Average expression of acrB/Ompk36 among Id *Klebsiella* spp. One-way ANOVA and Tukey's HSD *post-hoc* analysis test was conducted: (*i*) to compare MIC values, carbapenemase activities and changes in the expression levels of *acrB*, OmpC/Ompk36 among various subgroups of Group I isolates (Ia-Id), (*ii*) to detect meropenem-hydrolyzing proficiency and changes in expression levels of *acrB*, OmpC/Ompk36 among single and multiple resistant gene harbouring *E. coli* and *Klebsiella* spp. isolates of each Ia/Ib subgroup, (*iii*) to identify the role of *acrB* upregulation and OMP loss in carbapenem resistance among beta-lactamase-producing and non-beta-lactamase-producing *E. coli* (M vs. N) and *Klebsiella* spp. (O vs. P) isolates. NA, not applicable; HSD, honest significant difference; SE, standard error; OMPs, outer membrane proteins

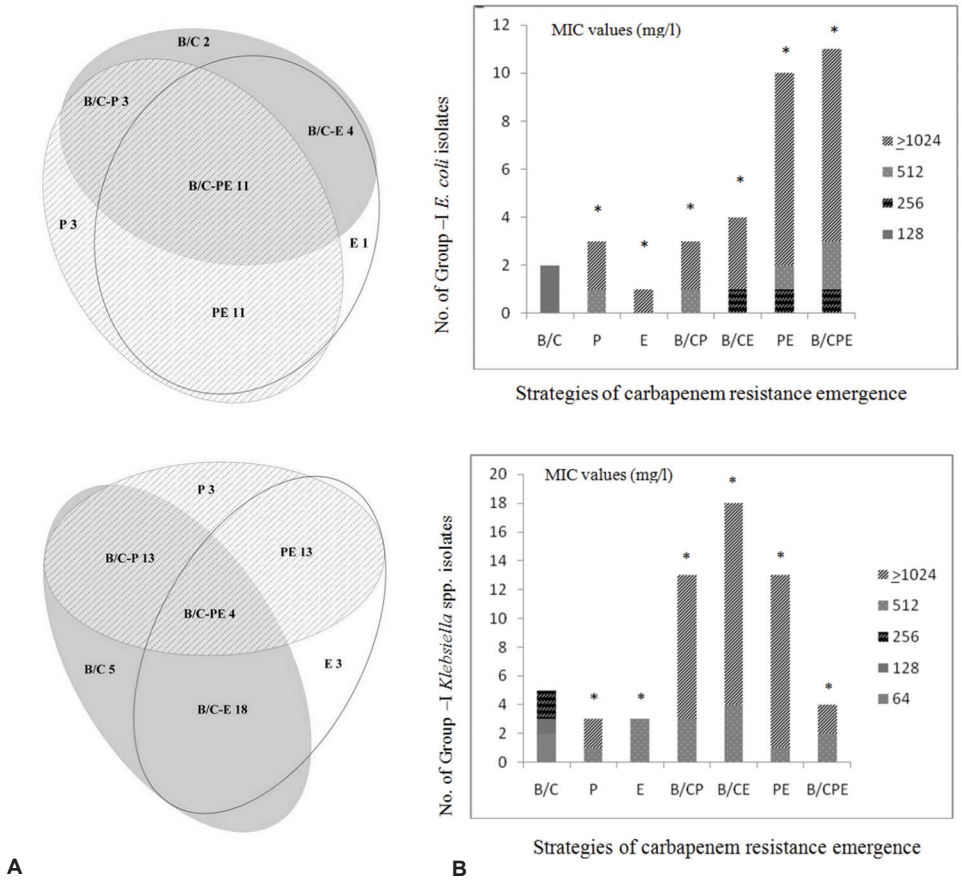

Fig. 2. (**A**) Venn diagram showing the prevalence of various strategies in carbapenem resistance emergence. Numerical after abbreviations for individual strategies indicate the number of isolates exhibiting that strategy. (**B**) Contribution of various strategies in carbapenem resistance emergence [as indicated by the minimum inhibitory concentration (MIC) value ranges]. *indicates significant changes in (MIC) values when compared to B/C strategy (P<0.01 for one-way ANOVA with *post hoc* Tukey' honestly significant difference test P<0.01). B/C, ESBL/Carbapenemases; P, porin loss; E, efflux pump's upregulation; CP, carbapenemase expressing isolates with porin loss; CE, carbapenemase expressing isolates with efflux pump's upregulation; PE, both porin loss and efflux pump's upregulation; CPE, carbapenemase expressing isolates with both porin loss and efflux pump's upregulation.

Further, *E. coli* of subgroups Ib/Ic following PE strategy always had higher percentage of OmpC reduction when compared to those following P strategy within the same subgroups - indicating the positive role of *acrB* in augmenting OmpC reduction (one-way ANOVA: P<0.01 for Ib and Ic) (Table V). Unlike this mechanism, *Klebsiella* spp. of subgroups Ia following PE strategy had higher level of *acrB* upregulation when compared to those following E strategy within the same subgroups - indicating the positive role of Ompk36 in augmenting *acrB* upregulation (one-way ANOVA: P<0.05). These two findings implicated mutual complementation of P and E among Ia, Ib and Ic isolates following PE strategy.

Discussion

In this study, majority of the CREs were isolated from urinary infections, treatment of which renders a serious challenge to clinicians. As most of the CREs isolated from urine exhibited resistance to aminoglycosides, fluoroquinolones and carbapenems - commonly used to treat urologic infections, there is an increased risk of receiving inappropriate empiric treatment, thereby resulting in higher rate of morbidity/morality associated with CRE infections[19,20]. Although treatment options are limited, old antibiotics - temocillin, fosfomycin, pivmecillinam, ceftolozane-tazobactum and combination therapy - were often recommended as available line of treatment against such infections[20].

Similar to a previous study by Dhara and Tripathi[21] in the current study phenotypic screening identified ESBL property among majority of the isolates. The presence of carbapenemase activity and bla_{NDM} indicated their contribution towards the emergence of

Table V. Differential expression pattern of *acrB* and outer membrane proteins C/outer membrane proteins (OMP) k36 among isolates of various subgroups exhibiting various carbapenem resistance strategies

Organisms	Strategies	Ia (ESBL expressing)		Ib (carbapenemase expressing)		Ic (both ESBL and carbapenemase expressing)		Id (none expressing)	
		Mean±SE							
		Fold increase in *acrB* expression	Per cent reduction in Omp-C/-k36 expression	Fold increase in *acrB* expression	Per cent reduction in Omp-C/-k36 expression	Fold increase in *acrB* expression	Per cent reduction in Omp-C/-k36 expression	Fold increase in *acrB* expression	Per cent reduction in Omp-C/-k36 expression
Escherichia coli	E	None	None	48.87±13.91	-	None	None	10.69±5.15	-
	P	None	None	-	11.34±0.9 (1)	-	11.86±2.09 (1)	-	42.00±1.72 (1)
	PE	43.38	47.05	36.34±12.27	54.55±5.03**	47.07±7.72	45.03±4.74**	26.11±6.52*	44.77±2.89 (3)
	B/C only	None	None	-	-	-	-	NA	NA
Klebsiella spp.	E	5.85±1.09	-	7.45±3.65	-	5.71±3.17	-	8.03±2.56	-
	P	-	18.67	-	22.06±0.92 (5)	None	None	-	33.29±1.62 (1)
	PE	15.76±1.87*	4.94 (1)	13.22±7.90	5.75 (6)	None	None	17.46±6.48	0.0 (10)
	B/C only	-	-	-	-	None	None	NA	NA

*P**<0.05, **<0.01 (one-way ANOVA) Number of isolates showing complete loss of OMPs was mentioned in brackets. None, no representative; NA, not applicable; ESBL, extended spectrum beta lactamase; E, efflux pump's upregulation; P, porin loss; PE, both porin loss and efflux pump's upregulation; B/C, ESBL/Carbapenemases

carbapenem resistance among these bacteria. However, percentage prevalence of carbapenemase/MBL/ bla_{NDM} gene was comparable among Group I E. *coli* and *Klebsiella* spp. isolates, and the co-existence of ESBL/MBL/non-MBL genes was identified among isolates of both the genera. Similar observation has earlier been reported[22-24]. Besides, rare co-existence of bla_{OXA-48}-like variants and bla_{NDM} genes was noted among carbapenem-resistant *E. coli* isolates, which was consistent with the earlier findings[25].

Although previous studies reported the additive role of ESBL production, decreased OMP expression and an active efflux pump system towards carbapenem resistance development, association of carbapenemases with OMP loss and *acrB* upregulation was not explored before[26-28]. In addition to exploring the well-known association between ESBL production and *acrB* upregulation/porin loss, a significant association of carbapenemase production with *acrB* upregulation was observed in the current study among CREs. Although similar significant association between ampC production and efflux pump upregulation was reported among *Pseudomonas aeruginosa* previously[29], there is no report of such association among CREs. Significant association between ESBL/carbapenemase production and OmpC/Ompk36 reduction was also found in the present study, which was consistent with the results of Wozniak *et al*[30]. The current study demonstrated the positive role of *acrB* in augmenting OmpC reduction among carbapenem-resistant *E. coli* and that of Ompk36 in facilitating *acrB* upregulation among carbapenem-resistant *Klebsiella spp*. The overall study indicated greater contribution of Ompk36 loss over *acrB* expression, particularly among *Klebsiella* spp. Similar association between the presence of IMP-4 MBL production and loss of Ompk36 was reported in carbapenem-resistant *Klebsiella oxytoca* isolate ZC101 of Chinese origin[31]. In the present study, isolates having no ESBL and carbapenemase activities demonstrated minimal *acrB* overexpression and OmpC/Ompk36 loss. Thus, ESBL or carbapenemase production only in combination with *acrB* overexpression and porin downregulation could have played a major role in imparting carbapenem resistance among these bacteria.

Though difference in carbapenemase activity was noted among inter subgroups (Ia-Id), but mode MIC value against meropenem remained almost consistent all throughout indicating the secondary role of carbapenemases and the contribution of other mechanisms in imparting carbapenem resistance among these subgroups. Though similar findings were reported earlier among pathogenic *Pseudomonas aeruginosa*[32], such observations were not reported for CRE. This was also evident on comparing the role of different strategies on MIC values of meropenem. In case of both *E. coli* and *Klebsiella* spp., presence of only ESBL/carbapenemases imparted MIC of meropenem within only 64-256 mg/l range, whereas the presence of ESBL/carbapenemases along with porin loss and/or efflux pump activation heightened the MIC value above 256 mg/l. All these findings indicated the contribution of efflux pump activation and porin loss over the presence of ESBLs/carbapenemases towards imparting carbapenem resistance among pathogenic enterobacterial isolates. Since the drugs targeting these mechanisms are limited in commercial markets, targeted mechanisms towards porin loss and efflux pump activation must be developed to address the carbapenem resistance issue of pathogenic bacteria.

***Acknowledgment*:** The authors acknowledge the suggestions of Dr Syamsundar Mandal, Statistical Officer, Department of Epidemiology and Biostatistics, Chittaranjan National Cancer Research Institute, Kolkata.

***Financial support & sponsorship*:** The first author (AP) acknowledges the financial support in the form of Junior Research Fellowship from the Indian Council of Medical Research, New Delhi [no. 3/1/3/WL/JRF-2011/HRD-129 (41937)].

***Conflicts of Interest*:** None.

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