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Research Paper

Effect of concentration gradient carbapenem exposure on expression of bla_{NDM-1} and *acrA* in carbapenem resistant *Escherichia coli*

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ABSTRACT

Escherichia coli, one of the major pathogens, frequently exhibits carbapenem resistance. It would be of interest to investigate which of the mechanisms responds when a strain that carries both bla_{NDM-1} and over expressed AcrAB-TolC efflux pump system is exposed against carbapenem under differential concentration gradient stress. Four different sets of strains were used in the study; (i) Strain that have bla_{NDM-1} and over expressed *AcrA*B-TolC system (ii) Strain that harbour bla_{NDM-1} and express *AcrA*B-TolC at basal level (iii) the strain that is devoid of bla_{NDM-1} but having over expressed *AcrA*B-TolC systems and (iv) *E. coli* AG100A (Δ*AcrAB*) and *E. coli* HUE 1 (Δ*AcrAB-TolC*) where $bla_{NDM-1}$was cloned. The Quantitative Real time PCR showed bla_{NDM-1} was over expressed under meropenem and imipenem stress irrespective of concentration gradient. In case of ertapenem, at lower concentration *AcrA* were over expressed whereas, at higher concentration bla_{NDM-1} showed elevated expression. A consistent elevated expression of *AcrA* and *AcrB* was observed against all carbapenems in the strains devoid of bla_{NDM-1} where as in case of the strain with basal level expression of *AcrA*, no significant over expression could be observed for bla_{NDM-1}. In case of clones in group IV, expression of bla_{NDM-1} was elevated in the presence of carbapenem stress.

1. Introduction

Carbapenem resistant Enterobacteriaceae (CRE) is a significant clinical concern. They are resistant to all beta-lactam antibiotics and often harbour multiple resistance determinants thereby restricting therapeutic options (Lutgring and Limbago, 2016). People most at risk for getting CRE are those who have a prolonged hospital stay especially at ICU, receiving multiple antibiotics and undergone a surgery with a medical device placed in the body (https://healthywa.wa.gov.au/Articles/A_E/Carbapenem-resistant-Enterobacteriaceae-CRE, 2019). Resistance against carbapenems are essentially mediated by acquisition of carbapenemases like New Delhi metallo beta-lactamases (NDM), OXA-48, KPC and other types which hydrolyse carbapenem and other beta-lactam antibiotics. Additionally, they also exhibit intrinsic resistance through efflux pump mechanisms. *AcrA*B-TolC is one of the active efflux pump systems which are responsible for flushing out toxic materials, chemical agents and antimicrobials. Over-expression of *AcrA*B-TolC often changes a bacterial phenotype in to a resistant one (Keeney et al., 2008). However, it is not known how both the mechanisms in *E. coli* within a patient respond when carbapenem therapy is initiated and whether both the mechanism supplements each other or expressed specifically against a particular concentration of antibiotic. Thus, it would be of interest to investigate how these mechanisms transcriptionally respond when the strain harbouring bla_{NDM} with over expressed *AcrA*B-TolC efflux pump system is exposed against different concentration gradients of carbapenems. Hence, the present study was designed to investigate transcriptional expression of *AcrA*, *AcrB* and bla_{NDM-1} under concentration gradient carbapenem stress.

2. Methodology

2.1. Bacterial isolates

A total of 27 clinical isolates of carbapenem resistant *Escherichia coli* were obtained from the patients admitted to or attended the clinics of Silchar medical college and hospital, Assam, India from September 2016 to August 2017. The selected isolates were subjected to detect efflux pump activity by an inhibitor based method (Quale et al., 2003). *E. coli* AG100 (Δ*AcrAB*) (kindly donated by Prof. Hiroshi Nikaido, University of California, Berkeley USA) and *E. coli* HUE 1 (Δ*AcrAB-*

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TolC) (kindly donated by Prof. T. Sato, Sapporo Medical University, Japan) were used in the present study.

2.2. Assessment of efflux pump activity using inhibitor based method

The phenotypic detection of efflux pump mediated carbapenem was performed using an efflux pump inhibitor CCCP (carbonyl cyanide *m*-chlorophenylhydrazone, 12.5 mM, Himedia, Mumbai) in combination with meropenem (10 mg, Himedia, Mumbai) and meropenem alone (Quale et al., 2003). CCCP alone was used. *E. coli* AG100 and *E. coli* HUE1 (wild type) was used as positive control while *E. coli* AG100A (Δ*AcrAB*), *E. coli* HUE1(Δ*AcrAB*-TolC) and *E. coli* ATCC 25922 was used as negative control in the present study.

2.3. Antimicrobial susceptibility testing and minimum inhibitory concentration determination

Antibiotic susceptibility testing of the selected isolates was performed by Kirby-Bauer disc diffusion method on Mueller Hinton agar (Himedia, Mumbai, India) and was interpreted as per CLSI guidelines (CLSI, 2017). Antibiotics tested were amikacin (30 µg), ampicillin (10 µg), aztreonam (30 µg), carbenicillin (100 µg), cefepime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), cotrimoxazole (25 µg), gentamicin (10 µg) and piperacillin/tazobactam (10 µg). The minimum inhibitory concentration of the test isolates was determined by agar dilution method against imipenem, meropenem and ertapenem at a concentration ranging from 0.25 µg/ml to 512 µg/ml. *E. coli* ATCC 25922 was used as control.

2.4. Detection of Carbapenemase genes

Investigations regarding the presence of carbapenemases (bla_{VIM}, bla_{NDM}, bla_{IMP}, bla_{OXA-48}, $bla_{OXA-23,\ -24/40,\ -58}$) in the selected isolates were performed by polymerase chain reaction (PCR) assay using specific primers and the reaction condition as previously described (Yong et al., 2009; Yum et al., 2002; Shibl et al., 2013; Mendes et al., 2009).

2.5. Transfer of plasmid carrying bla_{NDM-1} within the knockout mutants

A plasmid carrying bla_{NDM-1} was purified and transferred in the mutants *E. coli* AG100A (Δ*AcrA*B) and *E. coli* HUE 1 (Δ*AcrA*B-TolC) by heat shock method and transformants were selected on the media containing 1 µg/ml of imipenem. Both the recipient strains were lacking *AcrA*B-TolC efflux pump system. Hence, they were selected for interplay study.

2.6. Sequencing of AcrR and RamA

The local regulator acrR and global regulator RamA was amplified using primers acrR(F): 5′TATCGTCGTGCTATGGTACATAC3′ and acrR (R): 5′CACGACGCAGTGAACCAGAATAGC3′, RamA(F):5′CGGCCCTAA ACACAACCCC3′ and RamA(R): 5′TATAAAACCCTTCGCATAAAGG3′. the amplified products were sequenced by sanger's method to detect any mutation present in the gene.

2.7. Study of interplay of AcrAB-TolC and bla_{NDM-1}

Four different sets of strains were used in the study; (i) strains that have bla_{NDM-1} and over expressed *AcrA*B-TolC system, (ii) strains that harbour$bla_{NDM-1}$and express *AcrA*B-TolC at basal level, (iii) strains that is devoid of bla_{NDM-1} but having over expressed *AcrA*B-TolC systems, and (iv) *E. coli* AG100A (Δ*AcrA*B) and *E. coli* HUE 1 (Δ*AcrA*B-TolC) where $bla_{NDM-1}$was cloned. *E. coli* ATCC 25922 was used as control Strains were grown into LB broth containing 1 µg/ml,2 µg/ml and 4 µg/ml of imipenem, meropenem and ertapenem for 16 h at 200 rpm shaking to the late log phase. A total RNA was collected using QIAGEN Rneasy Mini Kit (QIAGEN, Germany) as per manufacturer's instructions. The RNA concentration was verified by Pico drop (Pico 200, Cambridge, UK). Ribosomal protein encoding gene, *rpsL* was used as reference strain to normalize the transcription levels of the target genes. The total RNA was reverse transcribed into cDNA using QuantiTect® reverse transcription kit (Qiagen, Germany). Quantitative Real-time PCR was performed using Power SYBR Green Master Mix (Applied Biosystems, Warrington, UK) following the manufacturer's instructions in Step One Plus Real Time PCR (Applied Biosystems, USA) using primers *AcrA*(F): 5′CTCTCAGGCAGCTTAGCCCTAA3′, *AcrA*(R): 5′TGCAG AGGTTCAGTTTTGACTGTT3′ (Swick et al., 2011),*AcrB*(F): 5′AGCTTC CTGATGGTTGTCGG3′, *AcrB*(R): 5′ACGGCTGATGGCATCTTTCA3′, NDM(F): 5′GGGCAGTCGCTTCCAACGGT3′, NDM(R):5′GTAGTGCTCAG TGTCGGCAT3′) (Yong et al., 2009) for amplification of *AcrA* and bla_{NDM-1}.

2.8. Statistical analysis

The differences in relative expression of efflux pump gene *acrA*, *AcrB* andbla_{NDM-1} was compared with that of the wild type strain (under normal condition) between samples were determined with the help of one-way ANOVA followed by Tukey-Kramer (Tukey's W) multiple comparison test. Differences were considered statistically significant at both 5% and 1% level when $p < .05$. SPSS version 17.0 was used for statistical analysis (https://spss-64bits.en.softonic.com/download).

3. Result

Out of twenty-seven carbapenem resistant *E. coli,* fifteen were phenotypically predicted of efflux pump activity when tested against meropenem with and without an efflux pump inhibitor. Among them nine were found to harbourbla_{NDM-1} and three were showing both efflux pump activity and presence of bla_{NDM-1}. No other carbapenemase gene could be found with our target primers. The antibiogram profiling of the test isolates revealed highest susceptibility percentage towards gentamicin 74.07% (20/27) followed by cefepime 66.6% (18/27) and amikacin 55.5% (15/27). The MIC result showed that the isolates were above the break point for atleast one of the carbapenems (Supplementary Table 1). However, most of the isolates were resistant to ertapenem followed by imipenem and meropenem (Table.1). The bla_{NDM-1} was transferred within *E. coli* AG100A and *E. coli* HUE 1 and was selected in the media containing 1 µg/ml of ertapenem. These transformants were further analysed for expression of *NDM* gene. Sequencing analysis could not detect any mutation in *acrR* and *ram A*.

In group I it was observed that in presence imipenem transcriptional expression of bla_{NDM-1} was significantly increased irrespective of concentration gradient stress and similar trend was observed for meropenem as well (Fig. 1). However, in case of ertapenem higher expression of bla_{NDM-1} was noticed at 4 µg/ml. For *acrA* and *AcrB* change in expression was minimal when compared with that of without stress. Expression pattern was similar for both *acrA* and *acrB*.

In contrast, for group II and III no significant change in expression pattern for either of the target genes (bla_{NDM} for group II, *acrA* and *AcrB*) could be observed against concentration gradient exposure of three different carbapenem antibiotics (Fig. 2 and Fig. 3). In case of clones under group IV, expression of bla_{NDM-1} was elevated in the presence of carbapenem stress (Fig. 4). Statistical analysis results revealed that the values were found to be statistically significant i.e., $p < .01$.

4. Discussion

The threat of carbapenem resistant enterobacteriaceae (CRE) is emerging and multiple mechanisms are involved in their non-susceptibility towards carbapenem. This situation complicates therapeutic scenario in critical care conditions in hospital setting as carbapenem resistant organisms are most frequently found to be resistant to other

Table 1
Minimum inhibitory concentration Range of carbapenems tested.

Antibiotics	MIC range examined (μg/ml)														No. (%) of isolates above the breakpoint (N = 27)
	< 0.25	0.25	0.5	1	2	4	8	16	32	64	128	256	512	> 512	
Meropenem	3	2	2	3	1	4	1	4	2	1	1	1	1	1	62.9%(n = 17)
Ertapenem	1	–	1	4	1	3	2	3	3	2	3	1	2	1	92.5%(n = 25)
Imipenem	1	2	2	2	1	2	4	3	2	2	3	1	1	1	74.07% (n = 20)

N = Total number of isolates, n = Number of isolates above the break point.

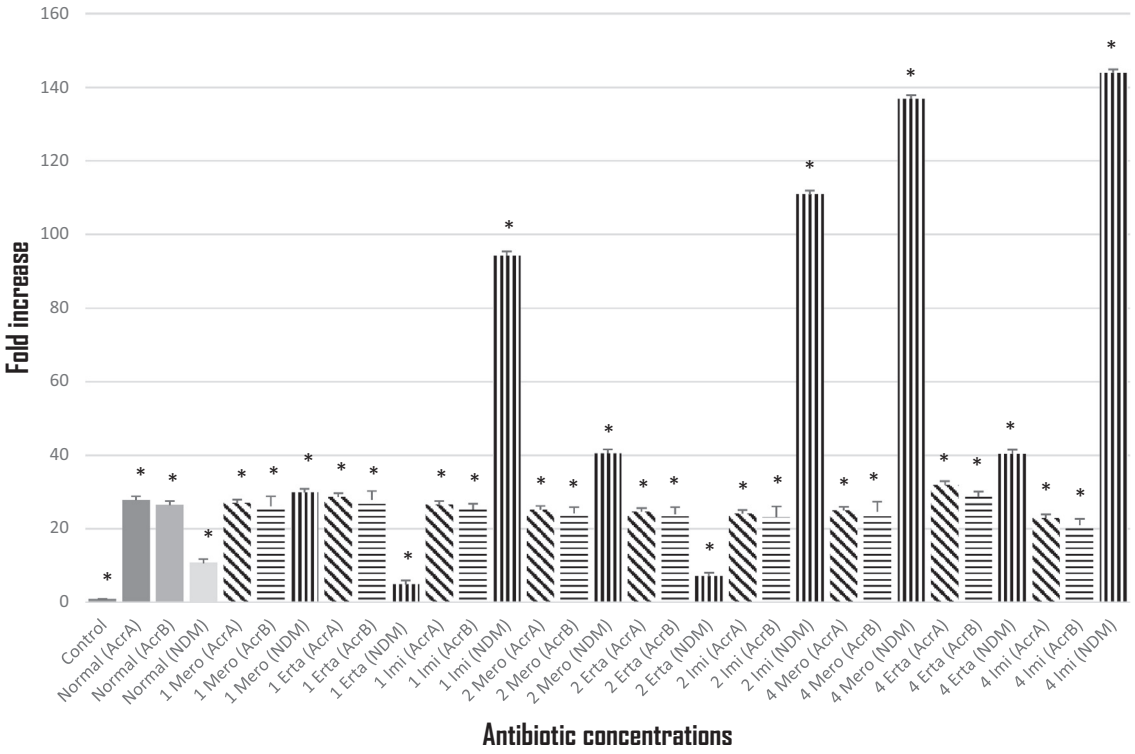

Fig. 1. Expression of *acrA* and *acrB* and bla_{NDM} gene (Group I) relative to *E. coli* ATCC 25922 (Without stress) under concentration gradient carbapenem stress. Control- *E. coli* ATCC 25922, Normal- wild type strain (without stress), * represents P < .01.

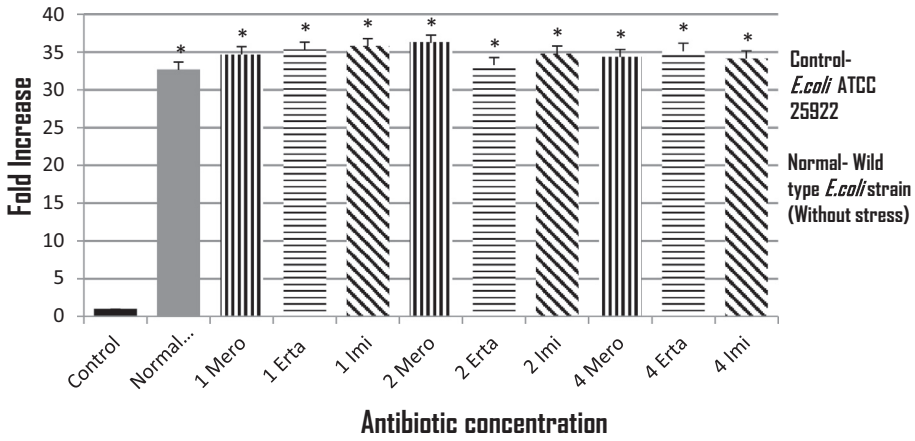

Fig. 2. Expression of the bla_{NDM} gene (Group II) expressing only NDM relative to *E. coli* ATCC 25922 (without stress) under concentration gradient carbapenem stress. * represents P < .01.

classes of antimicrobials. The epidemiology and occurrence rate of CRE varies in different geographical locations. In India *NDM* is the predominant carbapenem resistance determinant which is carried within members of enterobacteriaceae and non-fermenters (Yong et al., 2009) and different variants are reported from this country.

Resistance to the carbapenem is mostly a complex phenomenon which involves interplay between multiple intrinsic and acquired resistance mechanisms. These include down regulation or loss of porins,

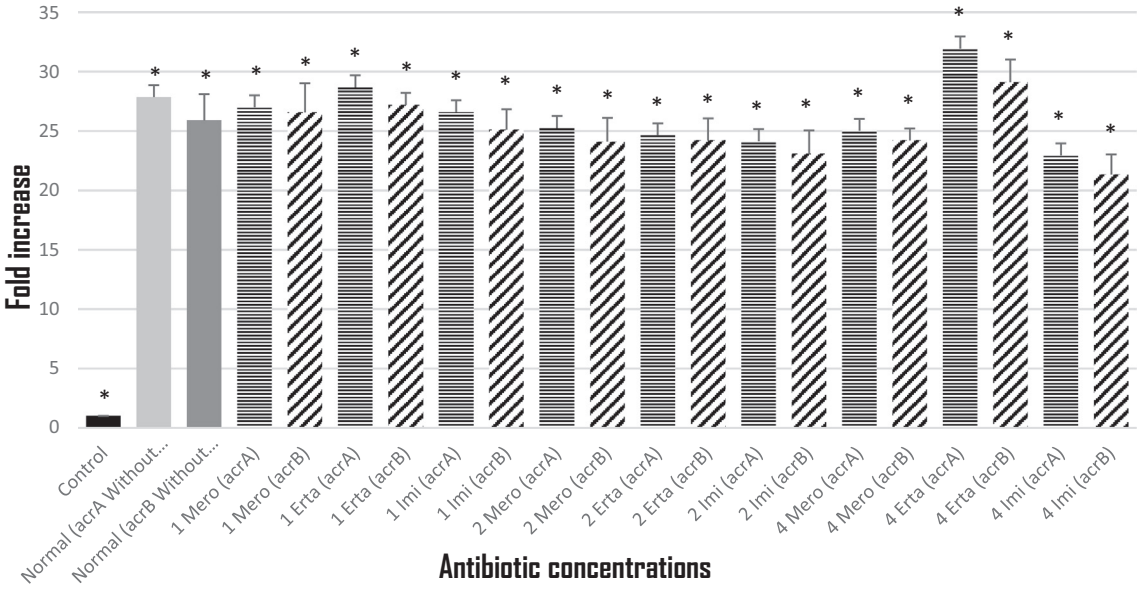

Fig. 3. Expression of *acrA* and *acrB* gene (Group III) expressing only efflux pump activity relative to *E. coli* ATCC 25922 (without stress) under concentration gradient carbapenem stress. * represents P < .01.

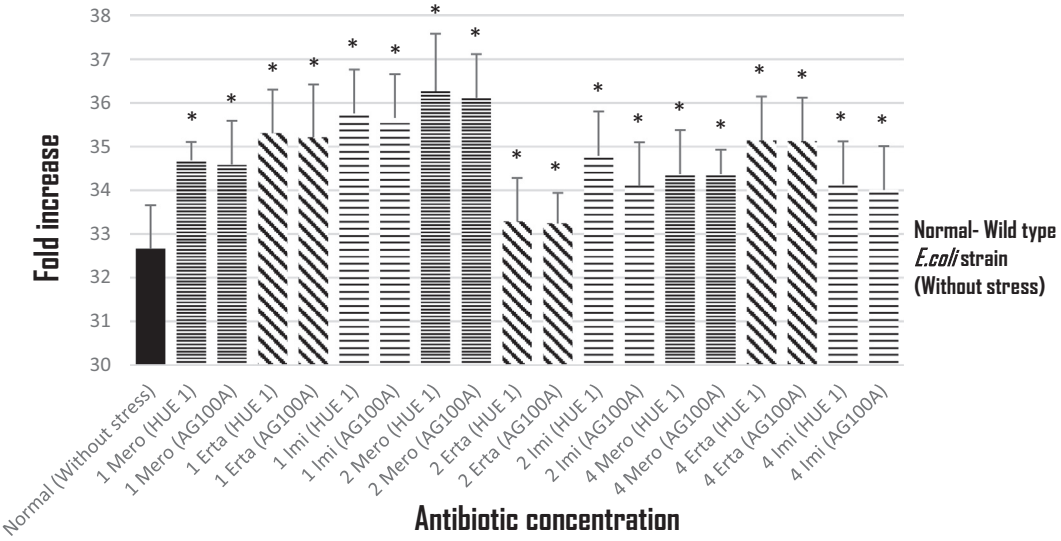

Fig. 4. Expression of the bla_{NDM} in *E. coli* mutant strains (Group IV) relative to *E. coli* wild type strain (without stress) under concentration gradient carbapenem stress. * represents P < .01.

over expression of efflux pump systems and plasmid mediated acquisition of carbapenemase genes (Choudhury et al., 2016). However, no such study has been conducted so far to analyse the interplay between two mechanisms in *E. coli.* A previous study has observed that efflux pump system plays an important role in carbapenem resistance compared to bla_{NDM-1} in *Pseudomonas aeruginosa* (Choudhury et al., 2016). In another study, simultaneous expression of single component and multi component efflux pump was attempted in *E. coli* and *Pseudomonas aeruginosa* but it did not increase antibiotic resistance (Lee et al., 2000). In similar work interplay between overexpression of *Acr*AB-TolC and mutation in *gyrA* and *parC* in quinolone resistance in *E. coli* was observed (Singh et al., 2012). Also, a previous study has highlighted greater role of *acrB* in beta-lactam and quinolone resistance (Padilla et al., 2010). However, our study showed same pattern of transcriptional expression for both *acrA* and *AcrB*, which is probably due to the presence of both the genes in same transcript. The present study showed that in the isolate with both over-expressed *Acr*AB-TolC and bla_{NDM-1} the relative level of mRNA is higher for both the genes while compared with other groups (II, III and IV). It was also noticed that even a smaller quantity of imipenem and meropenem was able to trigger the expression of *NDM* whereas; the role of ertapenem in induction was unclear. However, no previous study is on record to compare our finding. Unlike *Pseudomonas aeruginosa* where expression of efflux pump system MexAB-OprM was significant than that of bla_{NDM-1} (Choudhury et al., 2016), the current study found contrasting pattern in *E. coli.*

5. Conclusion

In Indian subcontinent carbapenem resistance is an emerging problem. Although acquisition of New Delhi metallo beta-lactamase is regarded as the prime reason for treatment failure, efflux pump mediated resistance too remains a matter of concern as together both the mechanisms pose a greater threat. Present study was able to highlight a higher mRNA transcript of both the resistance genes when coexist

within an isolate. This study warrants further investigation to identify the concentration of antibiotics, duration of exposure and other factors responsible induction of these intrinsic and acquired mechanisms that exert an isolate a multidrug resistant phenotype.

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Authors' contribution

SC performed the experimental work, data collection & analysis and prepared the manuscript. DB analysed the data. DDC and AC have designed the work plan. AB has conceived the plan and supervised the whole study.

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Conflict of interest statement

The authors declare that they have no competing interests.

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