

AcrAB-TolC Efflux Pump Mediated Resistance to Carbapenems among Clinical Isolates of *Enterobacteriaceae*

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Abstract

AcrAB-TolC is a resistance nodulation division type of efflux pump present in *Enterobacteriaceae*. It non-specifically effluxes antibiotics out of the bacterial cell, thus conferring drug resistance. Increase in the expression of the AcrAB-TolC efflux pump increases resistance to antibiotics. We aimed to study the expression levels of *acrA* and *acrB* that encodes AcrAB-TolC efflux pump, to understand efflux pump mediated resistance to carbapenem among clinical isolates of *Enterobacteriaceae* obtained from various clinical samples. Additionally, co-production of carbapenemase was also detected in the isolates demonstrating efflux pump mediated resistance to carbapenems. A total of 127 carbapenem resistant clinical isolates of *Enterobacteriaceae*, isolated from a tertiary care hospital were included in the study. An efflux pump inhibition (EPI) assay with reserpine, an efflux pump inhibitor, was performed to screen for isolates exhibiting efflux pump activity. Real Time Reverse Transcriptase qPCR was performed to detect the mRNA over expression levels of *acrA* and *acrB* that encodes AcrAB-TolC efflux pump. The control strains *K. pneumoniae* BAA2146 and *E. coli* AcrB were used. EPI assay with carbapenem showed that 56 /127(44%) isolates were screen positive indicating efflux pump activity. A total of 12 isolates showed 10¹ to 10⁷ increase in the expression of both *acrA* and *acrB* when compared with the controls indicating a strong efflux pump activity, in addition to producing carbapenemase. The study highlights the role of efflux pump AcrAB-TolC in conferring resistance to carbapenem among clinical isolates of *Enterobacteriaceae*.

Keywords: AcrAB-TolC, Reserpine, Carbapenem Resistance, Clinical Isolates of *Enterobacteriaceae*, Real Time RT qPCR

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INTRODUCTION

Enterobacteriaceae are a large family of Gram negative bacilli that often cause a variety of clinical infections – urinary tract infections, wound infections, sepsis, respiratory infections, etc.¹ Infections caused by *Enterobacteriaceae* often require treatment with antibiotics. The efficient antimicrobial agents against this group of bacteria are cephalosporins, carbapenems, aminoglycosides and fluoroquinolones.^{2,3} Antibiotic resistance (including that related to *Enterobacteriaceae*) to the commonly used antimicrobials is a growing problem in both developed and developing countries and especially in ICUs.

Resistance to carbapenems is often mediated by enzymes called as carbapenemase and also by non-enzyme mediated methods like over expression of efflux pumps and loss of porin proteins. Efflux pump mediated resistance to carbapenems is one of the few other studied mechanisms of resistance among clinical isolates of *Enterobacteriaceae*. AcrAB-TolC is a multidrug Resistance Nodulation Division (RND) type of efflux pump, which is present among *Enterobacteriaceae*. This is made up of three components AcrA – the periplasmic adaptor protein that connects AcrB and TolC, AcrB – the active pump that pushes the substrate from within the bacterial cell and TolC – the outer membrane channel that efflux out the substrate.^{4,5} Over expression of the AcrA and AcrB, contributes to drug resistance by effective effluxing out of carbapenems, cephalosporins, fluoroquinolones and aminoglycosides. The AcrAB-TolC efflux pump plays an important role in producing multidrug resistance in Gram-negative bacteria since it is not substrate specific.⁶

Genes for efflux pumps are present on plasmids as well as chromosome. Mutations in the local repressor genes or activation of a regulon regulated by a global transcriptional regulator such as MarA or SoxS of *E. coli*, results in over expression of efflux pumps.⁷⁻¹⁰ Since efflux pumps have a broad substrate range, over expression of a pump will confer resistance to more than one class of antibiotics, as well as to some dyes and detergents.

Even though most of the bacteria carry genes that encode MDR efflux pumps on

their chromosomes, some of these efflux pump encoding genes have been mobilized onto plasmids that may have the potential to transfer between bacteria. This situation is disquieting, since it would result in dissemination of multidrug resistance, more so because efflux pumps have a broad substrate profile. A recent study reported an IncH1 plasmid that was isolated from a *Citrobacter freundii* strain that contained the RND MDR efflux pump and also carried the gene for the beta lactamase NDM.¹¹

In order to understand enterobacterial resistance to carbapenems, it is imperative to comprehend different mechanisms of drug resistance. *Enterobacteriaceae* that over expresses efflux pumps have been isolated from patients earlier.¹² Understanding the mechanism of over expression may help in designing novel therapeutics to prevent the production of efflux-pump proteins. A study from Turkey reported efflux pumps as the causative mechanism for multidrug resistant *K. pneumoniae* isolates.¹³ Tsai et al.¹⁴ from Taiwan showed that the over expression of AcrAB-TolC by *ramR* deletion in *K. pneumoniae* resulted in increased antibiotic resistance and reduced virulence at the same time. There are limited studies that have explored both beta lactamase as well as efflux pump mediated resistance, among clinical isolates of *Enterobacteriaceae* cultured from subjects in different tertiary care hospitals. Here we have demonstrated AcrAB-TolC efflux pump mediated resistance to carbapenems among clinical isolates of *Enterobacteriaceae*.^{15,16}

MATERIALS AND METHODS

Study Type: Cross Sectional

Inclusion Criteria

Clinical isolates of *Enterobacteriaceae* obtained from various clinical samples (urine, blood, sputum, body fluids) that were resistant to carbapenem were included in the study.

Study Isolates

A total of 127 carbapenem resistant clinical isolates of *Enterobacteriaceae* from clinical samples – urine, blood, sputum, body fluids, obtained from patients attending a tertiary care hospital were included in the study. The

bacterial isolates were identified by standard microbiological procedures.

Phenotypic Screening of Efflux Pump Mediated Resistance to Carbapenems¹⁷

Reserpine, a non-specific efflux pump inhibitor was used to block the activity of efflux pumps involved in conferring antibiotic resistance. An efflux pump inhibition assay (EPI assay) was performed by determining MIC (agar dilution method) of imipenem in the presence and in the absence of reserpine at a fixed concentration of 25µg/mL. A 2 or more fold decrease in the MIC of imipenem in the presence of Reserpine when compared to the MIC of imipenem alone was considered to indicate the activity of efflux pump in conferring drug resistance.

Real Time Reverse Transcriptase qPCR to Confirm the Over Expression of Efflux Pump AcrAB-TolC

Real Time Reverse Transcriptase qPCR (Real Time RTqPCR) (Step One Plus™ Real Time

PCR system, Applied Biosystems™) was performed to study the mRNA expression levels of *acrA* and *acrB* of the AcrAB-TolC efflux pump, that is known to confer resistance to antimicrobial drugs. Representative isolates which were screen positive for efflux pump activity against imipenem by EPI assay were grown overnight in LB broth (Hi Media). Total RNA was extracted using FavorPrep tissue total RNA mini kit and RNA was quantified for purity and concentration using Nanodrop at 260/280 and 260/230 nm. RNA was converted to cDNA by RT-PCR using Thermo scientific Verso cDNA synthesis kit. RT-PCR was performed in a conventional thermal cycler. Real Time RT qPCR was performed with SYBR Premix Ex Taq from Takara Clontech in Step One Plus™ Applied Biosystem™ using Comparative $\Delta\Delta CT$ program. The test was done in triplicates. *gapA* was used as the housekeeping gene for normalizing the transcription levels of target genes. The mean of the normalized expression (transcription) values were calculated for *acrA* and *acrB* for each

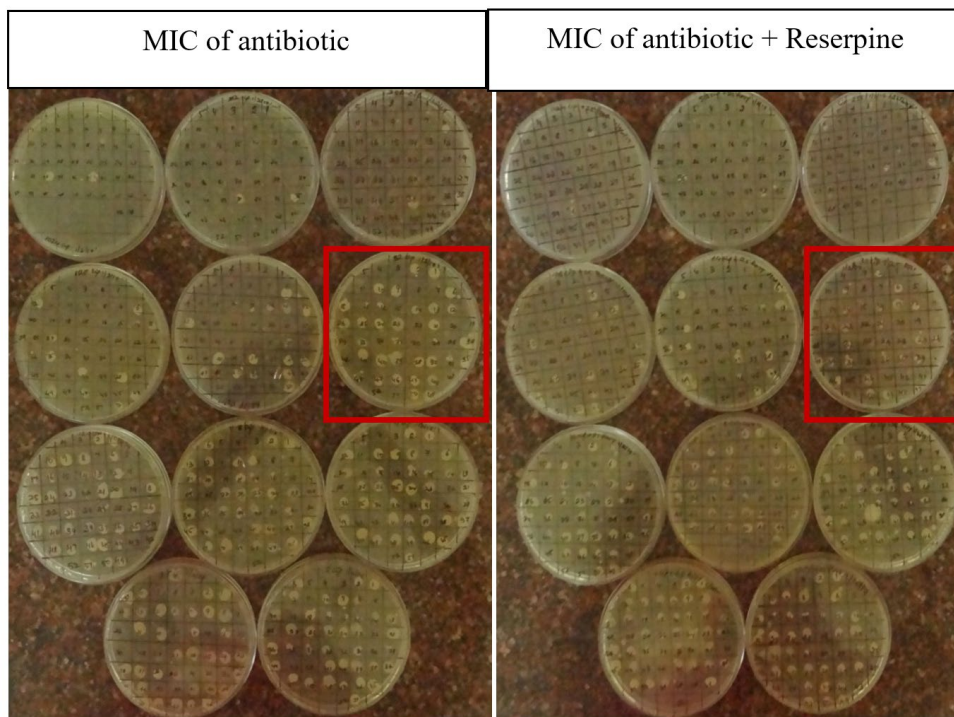


Figure 1. Representative picture of Efflux pump inhibition assay by MIC by agar dilution against Imipenem and Imipenem + Reserpine.

The highlighted boxes indicate the reduced MIC of antibiotic with reserpine when compared to MIC of antibiotic among the tested isolates.

strain manually as well as by using the Step One Plus™ software. *Klebsiella pneumoniae* BAA2146 was used as a positive control for studying and comparing the mRNA expression levels of both *acrA* and *acrB* among the screen positive isolates. *E.coli* AcrB, a strain that over expresses *acrB*, was used as a positive control for studying and comparing the mRNA expression levels of *acrB* among screen positive isolates.

The primers used for Real Time RT qPCR of *acrA* were forward primer-5'CTTAGCCCTAACAGGATGTG3', reverse primer-5'TTGAAATTACGCTTCAGGAT3'; of *acrB* were: forward primer-5'CGTACACAGAAAGTGCTCAA3', reverse primer-5'CGCTTCAACTTTGTTTTCTT3'. Real Time RT qPCR cycling conditions for *acrA* and *acrB* were as follows: initial denaturation at 95°C for 15 minutes, cycle denaturation at 94°C for 1 minute, annealing at 52°C for 1 minute, extension at 72°C

for 1 minute, melt curve stage (continuous) 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds.

Ethics

This study was approved by the Institute Ethics Committee of Dr. ALM PG IBMS, University of Madras, Taramani, Chennai. IHEC Approval No. UM/IHEC/01-2014-II, Dated 18-02-2015.

RESULTS

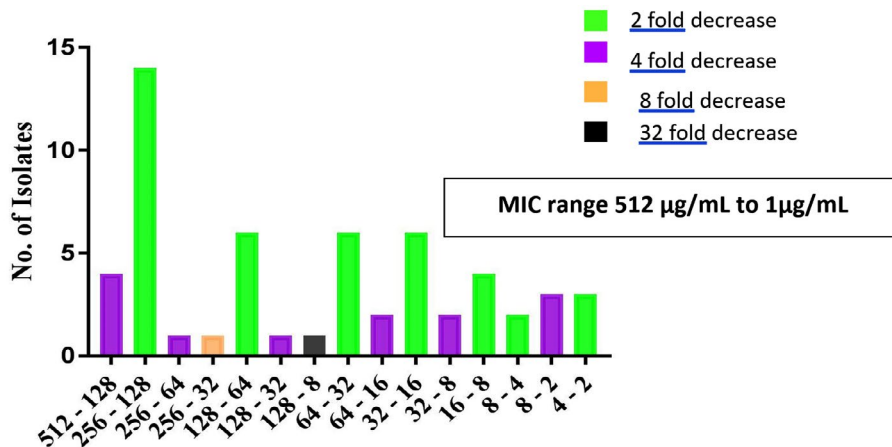
Phenotypic screening of efflux pump mediated resistance to carbapenems detected by efflux pump inhibition assay:

A total of 127 carbapenem resistant clinical isolates of *Enterobacteriaceae* were phenotypically screened for efflux pump mediated resistance to carbapenem. MIC of imipenem, a carbapenem, was determined for all the 127 isolates by agar dilution method. Concentration of imipenem ranged from 512 µg/mL to 1 µg/mL. MIC of imipenem with reserpine was determined. Isolates showing ≥ 2 fold decrease in MIC of imipenem with reserpine when compared to MIC of imipenem alone was considered to indicate the activity of efflux pumps in conferring resistance to imipenem (Figure 1).

Out of 127 carbapenem resistant clinical isolates of *Enterobacteriaceae*, 56 (44%) isolates showed ≥ 2 fold decrease in MIC of imipenem with

Table. Isolates that were screen positive for efflux pump activity detected by efflux pump inhibition assay with imipenem and reserpine

Fold decrease in MIC of imipenem + Reserpine Vs MIC of imipenem	No. of isolates (n=56)
2 fold decrease	41 (73%)
4 fold decrease	13 (23%)
8 fold decrease	1 (2%)
16 fold decrease	1 (2%)
Total No. of isolates	56



Fold decrease in MIC of imipenem with reserpine Vs MIC of imipenem

Figure 2. Isolates showing varying fold decrease in MIC of imipenem with reserpine when compared to MIC of imipenem alone.

reserpine when compared to MIC of imipenem alone.

Out of 56 isolates, that showed a decrease in MIC of imipenem with reserpine, 41 isolates (73%) showed a 2 fold decrease in MIC. One isolate showed a 16 fold decrease in MIC, from 128 µg/mL as MIC of imipenem to 8 µg/mL as MIC of imipenem with reserpine. (Table) (Figure 2).

Real Time RT qPCR to Study the mRNA Over Expression of *acrA* and *acrB* among Clinical Isolates that were Screen Positive for Efflux Pump Mediated Resistance to Carbapenem

Out of 56 isolates that were screen positive for efflux pump mediated resistance to imipenem, 12 representative isolates were selected for Real Time RTqPCR to study and compare the mRNA expression levels of *acrA* and *acrB* with known positive controls.

The mRNA expression levels of *acrA* ranged from 10¹ to 10⁷. The mRNA expression levels of *acrB* ranged from 10¹ to 10⁹. The mRNA expression levels of test isolates were either comparable to the control or highly over expressed when compared to control (Figure 3, Figure 4).

DISCUSSION

Carbapenem resistance among *Enterobacteriaceae* is mediated mainly by acquired carbapenemases.^{18,19} Resistance to carbapenems is also mediated by a combination of non-carbapenemase beta lactamases or ESBLs and reduced drug influx via loss of outer-membrane porins.²⁰ This study demonstrates, efflux pump mediated resistance to carbapenem group of antibiotics observed in clinical isolates of *Enterobacteriaceae* and also the potential of reserpine to be used along with carbapenem to treat infections caused by strains of carbapenem resistant *Enterobacteriaceae* showing efflux pump mediated antibiotic resistance.

A study by Adler et al.²¹ showed that mutations in *acrB* and *acrR* activates AcrAB-TolC mediated efflux thereby conferring high level carbapenem resistance in *E.coli*. Another study showed that over expression of efflux pumps expels carbapenems leading to carbapenem resistance associated with multidrug resistance.²² A study from Taiwan demonstrated that over expression of *acrB* was observed in 44% of

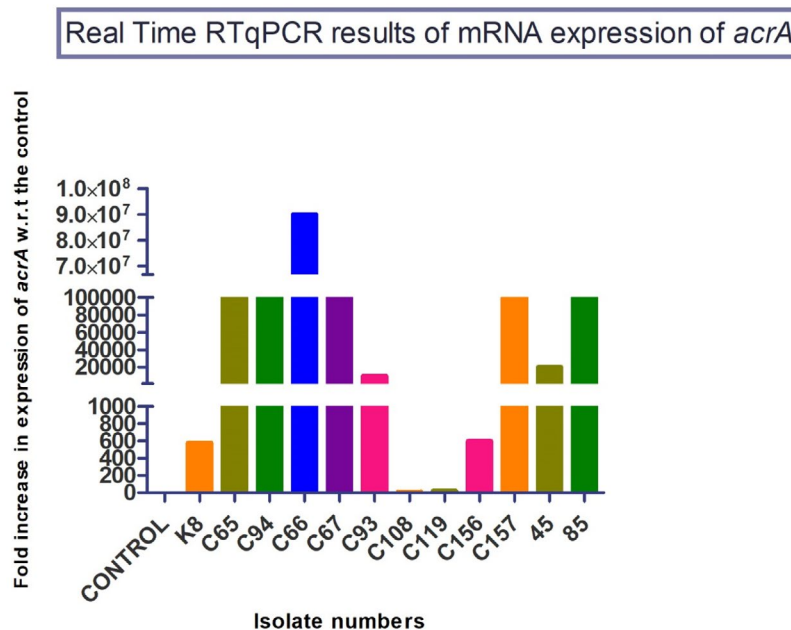


Figure 3. Real Time RTqPCR results of mRNA expression of *acrA*.

clinical isolates from UTI showing resistance to carbapenem and tigecycline.²³ A study from India reported that 35% clinical isolates of *E.coli* showed efflux pump mediated resistance to carbapenems by efflux pump inhibition assay using CCCP as an efflux pump inhibitor. The same study reported that 52% of the isolates selected for Real Time qRT PCR showed upregulation of *acrA*.²⁴

In the current study, 127 carbapenem resistant clinical isolates of *Enterobacteriaceae* were phenotypically screened for efflux pump mediated resistance to carbapenem by efflux pump inhibition assay using reserpine as an efflux pump inhibitor.

Of the 127 isolates, 56 (44%) isolates showed ≥ 2 fold decrease in MIC of imipenem with reserpine when compared to MIC of imipenem alone. This indicates role of an efflux pump inhibitor in mediating resistance to imipenem.

It was interesting to note that one isolate showed a 16 fold decrease in MIC from 128 $\mu\text{g}/\text{mL}$ MIC of imipenem to 8 $\mu\text{g}/\text{mL}$ MIC of imipenem

with reserpine, suggesting the potential use of an efflux pump inhibitor in tandem with an antibiotic to effectively treat infections instead of resorting to higher classes of antibiotics.

Of the 56 isolates that were screen positive for efflux pump mediated resistance to imipenem, 12 representative isolates were selected for Real Time RTqPCR to study the mRNA expression levels of *acrA* and *acrB*. All the 12 strains had increased expression levels for *acrA* that ranged from 10^1 to 10^7 and for *acrB* that ranged from 10^1 to 10^9 .

Out of 12 isolates, three showed the highest expression of *acrA* i.e., 10^7 . In addition to the over expression of *acrA*, these isolates also produced beta lactamases. (a) Isolate no. C65 showed an 8 fold decrease in MIC of imipenem with reserpine, and was also positive for *bla_{NDM}*. (b) Isolate no. C66 showed a 4 fold decrease in MIC of imipenem with reserpine, and was also positive for *bla_{OXA}*-48. (c) Isolate no. C67 showed a 4 fold decrease in MIC of imipenem with reserpine, and

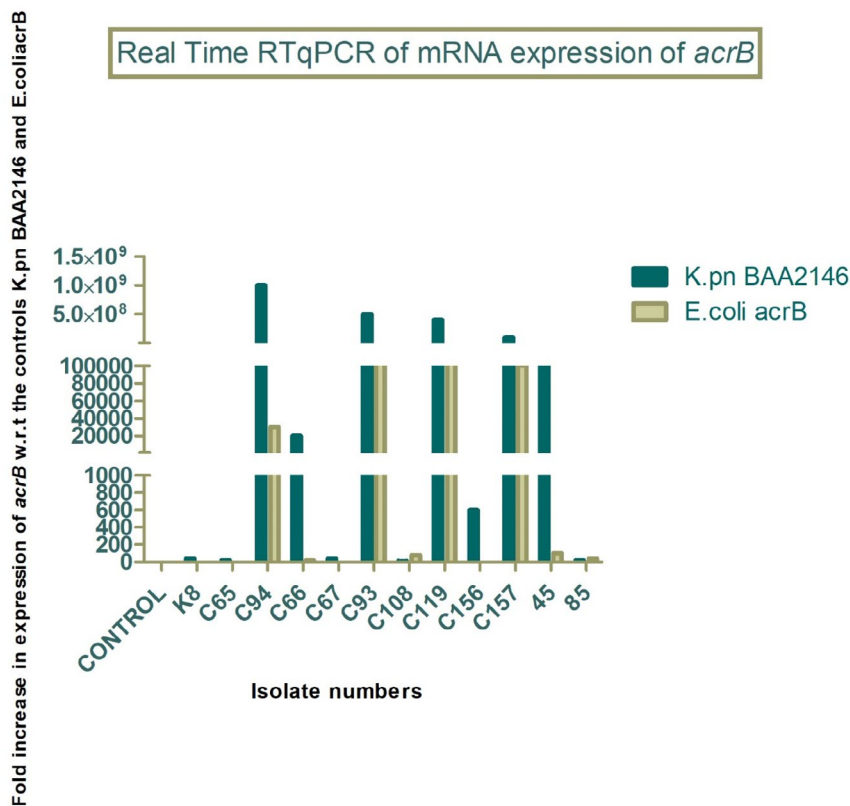


Figure 4. Real Time RTqPCR results of mRNA expression of *acrB*.

was also positive for *bla*_{NDM} and *bla*_{OXA}-48. All the three isolates were *Klebsiella pneumoniae* isolated from urine samples of three patients who were above 70 years old suffering from UTI.

Out of 12 isolates, four showed a very high expression of *acrB* i.e., 10⁸ - 10⁹. In addition to the over expression of *acrB*, these isolates also produced beta lactamases. (a) Isolate no. C94 from a drain sample of a 50 year old female patient showed a 16 fold decrease in MIC of imipenem with reserpine, and was also positive for *bla*_{NDM}. (b) Isolate no. C93 from a urine sample showed a 4 fold decrease in MIC of imipenem with reserpine, and was also positive for *bla*_{NDM}. (c) Isolate no. C119 from a urine sample showed a 4 fold decrease in MIC of imipenem with reserpine, and was also positive for *bla*_{OXA}-48 and *bla*_{KPC}. (d) Isolate no. C157 from a urine sample showed a 4 fold decrease in MIC of imipenem with reserpine, and was also positive for *bla*_{NDM}. All the four isolates (C94, C93, C119 and C157) were *E. coli*.

Over expression of the efflux pump AcrAB-TolC contributes to antibiotic resistance. If the genes that encode for over expression of the efflux pump are present on plasmids, along with genes that encode for enzyme mediated resistance, then it would result in rampant dissemination of multi-drug resistance that is mediated by multiple mechanisms. However, the presence of genes for AcrAB-TolC on plasmids is not explored in this study.

CONCLUSION

The present study demonstrates efflux pump AcrAB-TolC mediated resistance to carbapenems among clinical isolates of *Enterobacteriaceae*. Antibiotic resistance, mediated by over expression of *acrA* and *acrB* that encodes the efflux pump AcrAB-TolC in *Enterobacteriaceae* enhances the non-susceptibility of the bacteria to a range of antibiotics, because of the non-specificity of substrates that could be effluxed out of the efflux pump.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

PS conceptualized, carried out the work, and wrote the manuscript. PK supervised, provided critical feedback and reviewed and edited the manuscript. DM edited the manuscript and provided the strains included in the study. PB provided the strains included in the study. All authors read and approved the final manuscript for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by the Institute Ethics Committee of Dr. ALM PG IBMS, University of Madras, Taramani, Chennai, India with reference number UM/IHEC/01-2014-II.

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