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Imipenem Induced Transcriptional Modulation of Multidrug Efflux Pumps and Porin Genes in Carbapenem Resistant *Escherichia coli* Isolates of Animal Origin

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ABSTRACT

Background: The emergence of a multidrug resistance phenotype is most likely outcome of co-existing multiple mechanisms. The underlying molecular mechanism of carbapenem resistance is poorly understood in bacterial isolates of animal origin. The present study investigates the resistance mechanism for reduced susceptibility to carbapenem in *E. coli*.

Methods: The study includes 12 multidrug resistant *E.coli* isolates showing decreased susceptibility to imipenem. Carbapenemase production was evaluated by the mCarba-NP test. The presence of the carbapenem resistance gene was confirmed by multiplex PCR. Efflux pump activity was accessed by broth microdilution method with or without selective efflux pump inhibitor. We analyzed differential expression of the outer membrane porin genes (*ompF*, *ompC*) and efflux pump genes (*acrA*, *acrB*) by quantitative real-time PCR analysis.

Result: We observed increased expression of *acrA* and *acrB* genes in all the *E. coli* isolates under imipenem stress. A positive correlation ($p<0.001$) was evident between imipenem MIC and *acrA* and *acrB* over-expression in these isolates. A significant decrease in *ompF* and *ompC* expression was observed in these isolates under imipenem stress. The change in MIC was not correlated with the corresponding OmpC and OmpF gene expression. The results suggest the importance of efflux pump and porins proteins in the emergence of multidrug resistance besides carbapenemase production.

Key words: Carbapenem resistance, Efflux pump, *Escherichia coli*, Gene expression, Porins.

INTRODUCTION

Escherichia coli are commensal gut microbes in humans and animals and are known to harbor transmissible antimicrobial resistance genes (ARG) conferring them with multidrug resistance (MDR) capabilities. The fecal shedding of these MDR *E. coli* and resulting environmental contamination pose an increased risk of infection to humans. Carbapenem resistance *E. coli* strains in particular, are a major public health concern. Carbapenem is a critically important antimicrobial for human medicine that has higher resistance potential (WHO 2019). The regulatory restrictions of carbapenem use in veterinary medicine have kept the occurrence of carbapenem-resistant *E. coli* (CREC) low in animals. However, growing instances of CREC recoveries from animal sources have been reported globally of late (Stolle *et al.,* 2013; Braun *et al.,* 2016; Melo *et al.,* 2017) and more recently in India (Pruthvishree *et al.,* 2017, Nirupama *et al.,* 2018; Ghatak *et al.,* 2013, Sankar *et al.,* 2021).

A higher rate of recovery of carbapenem resistance bacterial strain from animal sources has increased the interest in unpinning the underlying molecular mechanism of development of resistance. The efflux pumps hyperactivity, loss of outer membrane porin transport channels and, carbapenemase production are known to play important role in developing carbapenem resistance (Bhardwaj *et al.,* 2015). The progressive accumulation of several different mechanisms may result in the evolution of CRE phenotype in a bacterial subpopulation (Choi and Lee, 2019). The activity of AcrAB-TolC efflux pump along with porins (OmpF and OmpC) has been investigated for their role in carbapenem resistance in human strains of pathogenic *E. coli* (Chetri *et al.,* 2019; Pal *et al.,* 2019).

The emergence of carbapenem resistance *E. coli* isolates in animals in absence of direct carbapenem selection pressure create an interesting backdrop to investigate how these molecular mechanism of resistance unfolds. Here we investigated the interplay of efflux pumps, outer membrane porins and carbapenemase production and

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their possible effect on minimum inhibitory concentration in fecal origin carbapenem-resistant *E. coli* isolates from bovine.

MATERIALS AND METHODS

Bacterial strain identification and characterization

The study includes 12 MDR *E. coli* isolates recovered from bovine fecal samples in and around the Mathura region during May 2020 to June 2021 (Table 2). The antimicrobial susceptibility profile of *ampicillin, amoxicillin-clavulanic acid, ceftazidime, cefotaxime, ceftriaxone, cefpodoxime, cefoxitin, ciprofloxacin, gentamicin* and *ertapenem* was investigated using the Kirby-Bauer disc diffusion method. The results were interpreted following CLSI guidelines. Carbapenemase production was assayed by the mCARBA-NP biochemical test as described by Rudresh *et al.,* (2017). *Escherichia coli* ATCC-25922, *Klebsiella pneumoniae* ATCC BAA-1705 (bla KPC positive) and *Klebsiella pneumoniae* ATCC BAA-1706 (HiMedia, India) were used as the experimental control with known broad-spectrum antimicrobial agent susceptibility. All laboratory work was carried out in the Department of Veterinary Microbiology, College of Veterinary Sciences and AH, DUVASU, Mathura, India.

Evaluation of efflux pump activity

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used in combination with imipenem to analyze the inhibition of bacterial efflux pumps and MIC reduction. Briefly, 100 µl of the imipenem solution (1280 µg/ml) was serially diluted twofold in Muller Hinton Broth (MHB) in a 96 well microtitre plate. Standardized bacterial inoculums (1×10^5 cfu/ml) were added to each well of the microtitre plate. Growth and sterility controls were included in columns 11 and 12 of microtiter plates. The procedure was repeated in a duplicate plate containing 20 µl/well of CCCP (20 µg/ml). Plates were incubated at 37°C for 72 h. The MIC was determined for imipenem alone and in a combination of the CCCP. A >2 fold reduction in MIC was interpreted as inactivation of the efflux pump.

Extraction of bacterial DNA and PCR assay

E. coli isolates were inoculated on Trypticase Soy Agar plates and incubated overnight at 37°C. A single colony of each isolate was resuspended and boiled in 100 µL of nuclease-free water at 100°C for 10 min followed by immediate cooling on ice for 10 min. Samples were briefly centrifuged at 8000 rpm for 5 min. The supernatant was used for PCR analysis. The primers are listed in Table 1.

Extraction of bacterial total RNA

Total RNA extraction was performed using QIAamp RNA Mini Kit (Qiagen, Germany) with slight modification. Briefly, 1-2 colonies of each bacterial culture from overnight grown Trypticase soy agar plate was inoculated into 5 ml of Trypticase soya (TSB) containing a sub-inhibitory concentration of imipenem (1 µg/ml). 1.5 ml of mid-log phase culture was taken in microcentrifuge tubes and pelleted by centrifugation at 7000 rpm for 6 min. The bacterial pellet was resuspended in lysis buffer provided with the QIAamp RNA Mini Kit supplemented with 20 µl lysozyme and 10 µl proteinase-K. The lysate was mixed with ethanol and transferred to the 2 ml QIAamp spin columns followed by the standard procedure of washing and elution as per the manufacturer. The quantity and purity of purified RNA samples were analyzed in Bio-Photometer plus™ (Eppendorf, Germany).

Table 1: Details of primers used.

PCR name	Primer name	Sequence (5'-3')	Primer Concentration (Pmol)	Amplicon Size (bp)	References
OXA- 48-like	OXA-48-for	GCTTGATCGCCCTCGAT	15	281	(Dallenne *et al.,* 2010)
	OXA-48-rev	GATTTGCTCCGTGGCCGAAA	15		
Multiplex I IMP,	MultiIMP-F	TTGACACTCCATTTACDGa	15	139	
VIM and KPC	MultiIMP-	GATYGAGAATTAAGCCACYCTa	15		
	MultiVIM-F	GATGGTGTTTGGTCGCATA	25	390	
	MultiVIM-R	GATGGTGTTTGGTCGCATA	25		
	MultiKPC-F	CATTCAAGGGCTTTCTTGCTGC	25	538	
	MultiKPC-R	ACGACGGCATAGTCATTTGC	25		
E. coli ompF	*E. coli ompF-Fwd*	AAGTAGTAGGTTGCGCCCAC	10	118	(Chetri *et al.,* 2019)
	E. coli ompF-Rev	AGTTCGATTTCGGTCTGCGT	10		
E. coli ompC	*E. coli ompC-Fwd*	ATTCTGGCAGTACGTCGGTC	10	125	
	E. coli ompC-Rev	AAACAACTCCTGGACCCGTG	10		
AcrA	*acrA-Fwd*	CTCTCAGGCAGCTTAGCCCTAA	10	107	(Swick *et al.,* 2011)
	acrA-Rev	TGCAGAGGTTCAGTTTTGACTGTT	10		
AcrB	*acrB-Fwd*	AGCTTCCTGATGGTTGTCGG	10	107	
	acrB-Rev	ACGGCTGATGGCATCTTTCA	10		
rpsL	rpsL-*Fwd*	GCAAAAACGTGGCGTATGTACTC	10	104	
	rpsL-*Rev*	TTCGAAACCGTTAGTCAGACGAA	10		

Quantitative real-time PCR

cDNA synthesis was carried out using Revertaid® first-strand cDNA synthesis kit following the manufacturer's instructions on a Veriti 96-Well Thermal Cycler (Applied Biosystem, USA). The resulting cDNA product was diluted 5-fold and used in the qRT–PCR. qRT-PCR was performed using 2× SYBR Green master mixes (Thermo Fischer Scientific, USA). The 20 µl reaction mixture consisted of a 10 µl SYBR Green master mix, 10 pmol final concentrations of forward and reverse primers and 1 µl of (1:5 dilution) of cDNA. The real-time PCR was performed on QuantStudio™ 3 Real-Time PCR System (Applied Biosystem, USA). All qRT-PCR included an initial denaturation step of 95°C for 2 min followed by 40 cycles of amplification with denaturation at 95°C for 15 sec, annealing at 60°C for 15 sec and extension at 72°C for 1 min each. The primers are listed in Table 1. The relative expression of the target gene was quantified using the $2^{-\Delta\Delta CT}$ method after normalization using the *rspL* gene. Pearson's correlation analysis was performed to analyze the correlation between the level of the log2 fold change in target gene mRNA expression and log2 MIC of imipenem for corresponding isolate using Graph Pad Prism 5 (USA). All tests were done at a 5% level of significance.

RESULTS AND DISCUSSION

A total of 12 multidrug resistances *E. coli* strains showing reduced susceptibility to ertapenem were investigated. All the isolates tested were hyper resistant to tested antibiotics (Table 2). The isolates were 100% resistant to *ampicillin, amoxicillin-clavulanic acid, cefotaxime, ceftriaxone, cefpodoxime, ceftazidime, cefoxitin, ertapenem*, 83.3% resistant to *ciprofloxacin* and 16.6 % resistant to *gentamicin*, herein described as MDR. Carbapenem resistance is one of the most important contributing factors for the evolution of MDR in Gram-negative bacteria. In a contemporary study, gentamicin was found to be active against most of the CRE strains, as highlighted in this study (Gonzalez-Padilla *et al.,* 2015). PCR assay was carried out for the detection of carbapenemase genes KPC, NDM, OXA-48, IMP and VIM (Table 2). Five out of 12 (41.6%) and 1/12 (8.33%) isolates carried IMP and VIM genes respectively, while the OXA-48 gene was detected in 3/12 (25.00%). Seven out of 12 isolates harboured one or more carbapenemase genes. The KPC and NDM genes were not amplified in any of the isolates.

The broth microdilution method was used to determine MICs of imipenem for all the isolates. The imipenem demonstrated antibacterial activity against the test

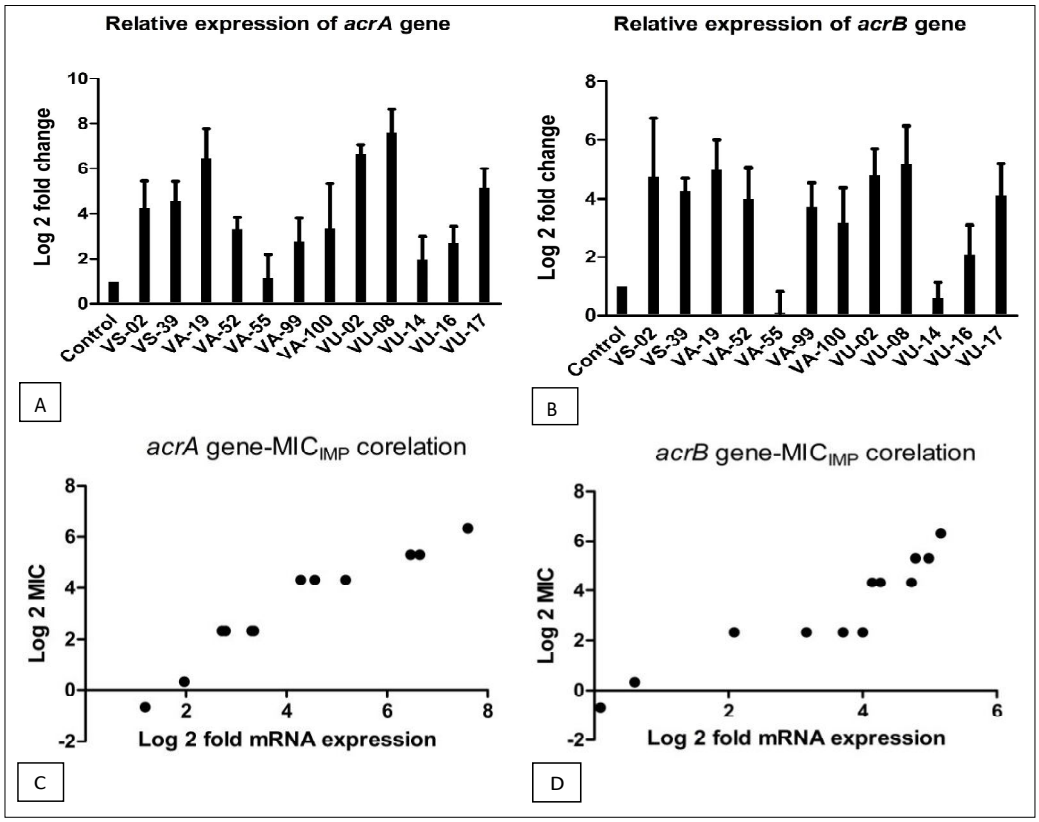

Fig 1: Expression of *acrA* and *acrB* gene in carbapenem non-susceptible *E. coli* isolates. Relative changes in the expression of *acrA* (A) and *acrB* (B) *genes* were quantified by real-time PCR by comparison with expression levels of the housekeeping gene *rspL* and expressed as the fold-change. The Log_2 MIC value of Imepenem was correlated with Log2 fold expression of *acrA* (C) and *acrB* (D) *mRNA*. Upregulation of both *acrA* and *acrB* genes was observed in all the isolate with positive correlation between MIC and upregulated gene expression.

carbapenem resistant *E. coli* strain (ImpR) with MIC values of 1.25 µg/mL to 80 µg/mL, whereas the MIC for the control strain (ImpS-*E. coli* ATCC 25922) was 0.625 µg/ml (Table 2). A two to eight-fold MIC reduction was seen in 9 out of 16 isolates when tested in combination with CCCP, indicating the inhibitory activity for the efflux pump. CCCP reduced efflux activity in carbapenem-resistant Gram-negative bacteria, therefore, reducing the MIC (Huang *et al.,* 2008).

We evaluated the imipenem-induced differential expression of *acrA* and *acrB* genes in *E. coli* isolates. We observed a 2-8 fold (log) increase in *acrA* and *acrB* transcripts in all the isolates post imipenem exposure (Fig 1a and 1b). More recent studies showed AcrA porin hyperactivity in ertapenem resistance *E. coli* (Rosenberg *et al.,* 2000; Chetri *et al.,* 2019). Bacterial efflux pumps flush out antibiotics from the cell resulting in multidrug resistance. AcrAB-TolC efflux pump is most frequently associated with multidrug resistance *E. coli* showing reduced susceptibility to β-lactam antibiotics (Li and Nikaido, 2016). We found that transcriptional expression of *acrA* and *acrB* genes

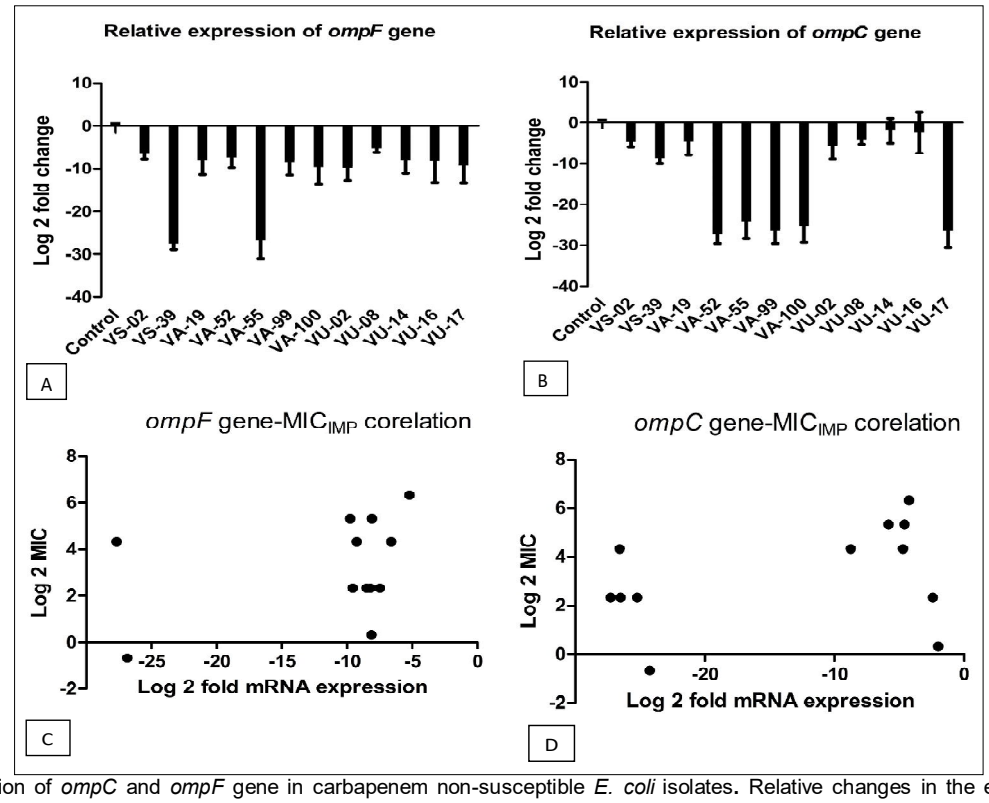

Fig 2: Expression of *ompC* and *ompF* gene in carbapenem non-susceptible *E. coli* isolates**.** Relative changes in the expression *ompF* (A) and *ompC* (B) *genes* were quantified by real-time PCR by comparison with expression levels of the housekeeping gene *rspL* and expressed as the fold-change. The Log_2 MIC value of Imepenem were correlated with Log_2 fold expression of *E. coli ompF* (C) and *ompC* (D) mRNA. Down regulation of *E. coli ompC* and *ompF* genes were observed in all the isolates however, no correlation observed between MIC and down regulated gene expression.

Table 2: Detail of bacterial isolates used and carbapenem genotypes identified.

Isolate no.	Antibiotic resistant profile	MIC-Imipenem(µg/ml)	blaVIM	bla IMP	blaKPC	blaOxa48
VS-02	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	20.0	-	+	-	-
VS-39	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	20.0	+	+	-	-
VA-19	AMC, CPD, CRO,CTX, CAZ, AM, CIP, GM, FOX, ETP	40.0	-	-	-	+
VA-52	AMC, CPD, CRO,CTX, CAZ, AM, CIP, GM, FOX, ETP	5.0	-	+	-	-
VA-55	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	0.625	-	-	-	+
VA-99	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	5.0	-	-	-	-
VA-100	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	5.0	-	+	-	-
VU-02	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	40	-	+	-	+
VU-08	AMC, CPD, CRO,CTX, CAZ, AM, FOX, ETP	80	-	-	-	-
VU-14	AMC, CPD, CRO,CTX, CAZ, AM, FOX, ETP	1.25	-	-	-	-
VU-16	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	5.0	-	-	-	-
VU-17	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	20	-	-	-	-

seemingly had a positive correlation with a corresponding increase in MIC to imipenem (P<0.001) in all the *E. coli* isolates (Fig 1c and 1d). The previous report suggests a positive correlation between reduced susceptibility to carbapenem and AcrA over-expression in *E. coli* (Chetri *et al.,* 2019). Previously, carbapenemase production was shown to be associated with *acrA* and *acrB* upregulation among CREs (Pal *et al.,* 2019). However, such association could not be established in our study, as some of the resistant isolates with no detectable carbapenemase genes also exhibited *acrB* and *acrB* upregulation.

MDR *E. coli* isolates showed diminished expression of *ompF* and *ompC* genes compared with *E. coli* ATCC 25922 (Fig 2a and 2b). A recent report highlights the potential linkage of OmpC porin to the development of carbapenem resistance *E. coli* (Larkin, *et al.,* 2020). Reduced expression of OmpF and OmpC has been demonstrated frequently in carbapenem-resistant strains of *E. coli* (Yoshida *et al.,* 2006). In *E. coli*, OmpC and OmpF are well-known porins associated with the uptake of antibiotics and the general influx mechanism of small molecular weight compounds (Cowan *et al.,* 1992). We assessed the effect of porin gene expression on MIC for imipenem. There was no significant effect on MIC for imipenem(p>0.05) with an associated decrease in *ompF* and *ompC* expression (Fig 2c and 2d). Netikul *et al.* (2015) observed no significant change in carbapenem MIC values with corresponding changes in OmpK35/36 (an OmpC/F analog of *E. coli*) expression in resistant *Klebsiella* isolates when compared to non-resistant isolates. The finding suggests that differential change in OmpF/OmpC expression is independent of the concentration gradient of the target molecule. Previous studies have shown that AcrB overexpression has a repressive effect on OmpF porin activity (Jaskulski *et al,* 2013; Philippe *et al.,* 2015). Decreased activity of OmpF and OmpC porins in carbapenem-resistant isolates observed in this study could be the result of concurrent AcrAB over-expression.

CONCLUSION

In summary, decreased or loss of expression of outer membrane porins coupled with increased activity of AcrAB-TolC efflux pump is asscociated with carbapenem resistance in *E. coli*. Increase activity of AcrAB-TolC efflux pump is directly correlated with increased level of resistance to imipenam. Hence AcrAB efflux pump can be potential target for counteracting growing challenge of carbapenem resistance in Gram negative bacteria.

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Conflict of interest: None.

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