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EFFECT OF CAPSAICIN ON *TOL C*, *MDF A* AND *NOR E* GENES IN TETRACYCLINE RESISTANT *ESCHERICHIA COLI*

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ABSTRACT

The inhibition of efflux pumps plays a role in combating the antimicrobial resistance (AMR). Antimicrobial resistance in *Escherichia coli* (*E.coli*) is caused by the *AcrAB-TolC* efflux system, which is a form of efflux pump. *Mdf A* is a putative membrane protein of 410 amino acids that belongs to the major facilitator superfamily of transport proteins. In this study capsaicin, a phyto-alkaloid was used as modulator of efflux pump in combination with tetracycline in tetracycline resistant *E.coli*. Antimicrobial sensitivity was carried out using standard disc diffusion method with appropriate controls. Tube dilution method was carried out for MIC and the turbidity was measured at 600nm. mRNA expression for the respective genes (*Tol C, Mdf A, Nor E*) were carried out by Real time PCR. There was no zone of inhibition for capsaicin and tetracycline when they were used alone, while there was significant increase in the zone of inhibition for tetracycline when it was combined with capsaicin. Turbidity of the culture decreased when tetracycline is used in combination with capsaicin. Use of phytochemicals like capsaicin, which is a known inhibitor of efflux pump, could interfere with efflux pumping of antibiotics in resistant bacteria. mRNA expression for *Tol C* is decreased, whereas for *Mdf A* and *Nor E,* it is increased when tetracycline is used in combination with capsaicin indicating the blocking of efflux pumps.

Keywords: Antimicrobial resistance, *Tol C*, *Mdf A*, *Nor E*, *Escherichia coli*

INTRODUCTION

Antimicrobial resistance (AMR) is a growing challenge to the successful prevention and treatment of infections caused by bacteria, parasites, viruses, and fungi. Major surgery and cancer chemotherapy will be jeopardized without successful antibiotics (Shaheen *et al*., 2016). This results in fruitless therapy and leading to the endurance of infection. The resistance mechanisms evolved naturally over time by alteration in genetic makeup. Nevertheless,

overuse and misuse of antimicrobial agent hastens the resistance process (Davies and Davies, 2010). The MDR pumps of pathogenic bacteria known so far, belong to five families of transporters namely; major facilitator super-family (MFS), adenosine triphosphate (ATP)-binding cassette (ABC) super-family, small multi-drug resistance (SMR) family, resistance nodulation- cell division (RND) super-family and multidrug and toxic compound extrusion (MATE) family (Carlet *et al*., 2012). RND family's *Acr B* transporter and periplasmic accessory protein *Acr A* make-up *Acr AB*'s efflux system. The *Acr AB* genes are organised into an operon. *Tol C*, an outer membrane protein encoded by a gene elsewhere on the chromosome, is likely to work in tandem with *AcrAB* (Fralick, 1996).

MDR translocators, in bacteria, are an ideal model device for researching the MDR effect and its clinical implications. *Mdf A* encodes a 410-amino-acid putative membrane protein (*Mdf A*) that belongs to the main facilitator superfamily of transport proteins (Edgar and Bibi, 1997). Efflux pumps, unlike most other resistance determinants, are often intrinsic. Overexpression of these efflux pumps occurs as a result of mutations in regulatory proteins or promoters, resulting in drug resistance (Webber and Piddock, 2003). It is predicted that using efflux pump inhibitors (EPIs) like capsaicin with antibacterial agents would increase their sensitivity and efficacy. Plants from the *Capsicum* genus produce *capsaicinoids,* which are alkaloids. Capsaicin is thought to be made in the inter- locular septum of chilli peppers, and it is dependent on the AT3 gene, which is found at the pun1 locus (Tiwari *et al*., 2013). Hence the current study was aimed to analyse efflux pump inhibitory activity of Capsaicin in *E. coli*

MATERIALS AND METHODS

The bovine mastitis sample containing *E. coli* bacterial isolates were acquired from the Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala and Department of Veterinary Microbiology, Shivamogga. Capsaicin and tetracycline from Sigma Aldrich, Banglore were used. The isolate was assessed by disc diffusion susceptibility testing. The housekeeping 30S ribosomal subunit protein gene (*rpsL*) was used to calculate relative expression.

Kirby- Bauer/ Disc Diffusion Procedure

The bacterial isolate of *E.coli* was selected and transferred to the broth. The turbidity of 0.5 McFarland was used for standardisation of antimicrobial susceptibility testing. The 0.5 McFarland

was prepared by mixing 0.05 mL of 1.175 % barium chloride dehydrate with 9.95 mL of 1 % sulphuric acid. Mueller Hinton agar was used for the culturing of colonies. It was followed by placing the filter paper discs impregnated with antimicrobial agents. After that, the plates were inverted and incubated for 16-18 h at 37 °C to determine the susceptibility and the zone of inhibition was measured. Treatment groups are listed in Table.1. Enrofloxacine, a fluoroqunolone is used as one treatment because *E.coli* was found susceptible to Enrofloxacin.

Broth dilution method

Bacterial inoculate adjusted to 0.5 McFarland standard was suspended in the Mueller Hinton broth with two fold dilution of antibiotic (tetracycline- 30 µg). It was then incubated for 16-18 h at 37 °C, along with control. The tube without turbidity was used as MIC (minimum inhibitory concentration) determinant. Each sample was analyzed for the optical density using UV -VIS spectrophotometer at a wavelength of 600 nm.

Gene expression studies

Amplification of genes was done using gradient PCR Master Mix (Emerald Amp GT PCR Master Mix, Takara, Japan) in the PCR reaction. The contents of the PCR mix is given in table 3. These were spun briefly and placed in a thermal cycler. The details of gradient PCR conditions used for amplification are given in Table 4. The results were expressed in fold change as compared to untreated control. The details of real- time PCR reaction mix and thermal cycling conditions are given in tables 5 and 6 respectively. Real-time quantitative polymerase chain reaction (rt-qPCR) was employed for studying the gene expression of *Tol C*, *Mdf A* and *Nor E*. To measure relative expression, the housekeeping 30S ribosomal subunit protein gene (*rpsL*) would be used (Pérez *et al*., 2007). It was done by the method described by Nisha *et al*. (2020). The reaction includes total RNA isolation, DNase treatment of RNA, quality of RNA samples, concentration and purity of total RNA, complementary DNA synthesis and selection of primers, synthesis and dilution of the primers followed by standardization of PCR and amplification of genes.

Gene specific-primers were designed using online Primer 3 primer design software (Primer 3, http://bioinfo.ut.ee/primer3/) and specificity was checked using Primer 3 and BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Melt curve analysis

A melt curve analysis was done after the reaction for checking specificity of the amplification. It had denaturation at

Table 1.

Group	TREATMENT
Treatment I	*E.coli* treated with Enrofloxacin
Treatment II	*E,coli* treated with tetracycline and 125ppm capsaicin
Treatment III	*E.coli* treated with tetracycline and 250 ppm capsaicin
Treatment IV	*E.coli* treated with tetracycline and 500 ppm capsaicin
Treatment V	*E.coli* treated with tetracycline alone
Treatment VI	*E.coli* treated with capsaicin alone

Table 2**.** Description of primers used

Genes	Sequence
tolC-F	AAGCCGAAAAACGCAACCT
tolC-R	CAGAGTCGGTAAGTGACCATC
rpsL-F	GCAAAAACGTGGCGTATGTACTC
rpsL-R	TTCGAAACCGTTAGTCAGACGAA
mdfA-F	CATTGGCAGCGATCTCCTTT
mdfA-R	TTATAGTCACGACCGACTTCTTTCA
norE-F	CTGGCGGCAGCGGTAA
norE-R	TGCCATACAGACACCCACCATA

Table 3. Contents of PCR mix

Components	Volume(µl)	Final concentration of cell culture lysate cDNA
Template(cDNA)	1.0	250 ng
Forward primer (10 pM/µL)	0.5	5 pM
Reverse primer (10 pM/µl)	0.5	5 pM
Master mix	12.5	-
Nuclease free water	5.5	-
Total volume	20	-

Table 4. Conditions for gradient PCR

S.No.	Steps	Temperature		Time
1	Initial denaturation	95 °C		4 min
2	Denaturation	95 °C		25 sec
3	Annealing	*Mdf A*	60.6 °C	30 sec
		Tol C, Nor E	58.3 °C	
4	Extension	72 °C		1 min
5	Step 2 to 4 repetition	35 cycles		
6	Final extension	72 °C		10 min
7	Hold	4 °C		10 min

Table 5. Optimized concentrations of RT-qPCR mix (20µL)

Components	Volume (µL)	Final concentration
Template(cDNA)	1	250 ng
Maxima SYBR Green qPCR Master Mix(2X)	10	1X
Forward primer (10 *pM*/µL)	0.5	5 *pM*
Reverse primer (10 *pM*/µL)	0.5	5 *pM*
Nuclease free water	8	-
Total volume	20	-

Table 6. RT-qPCR conditions

Steps		Temperature		Time
Initial denaturation		95 °C		4 min
40 cycles of	Denaturation	95 °C		35 sec
	Annealing	*Mdf A*	60.6 °C	40 sec
		Tol C, Nor E	58.3 °C	
	Extension	72 °C		35 sec

Table.7. Relative expression for *tolC*, *mdfa, NorE* gene in *E.Coli* treated with tetracycine and capsacin

Treatment	fold of expression (*tol C*)	fold of expression (*mdfa*)	fold of expression (*Nor E*)
Tetracyclin + capsaicin 125 mg/L	0.45±0.38 ^{a}	1.2265±0.19^{b}	1.13±0.39^{b}
Tetracyclin + capsaicin 250 mg/L	0.56±0.27^{a}	1.02±0.29^{b}	1.21±0.05^{b}
Tetracyclin + capsaicin 500mg/L	0.159±0.08^{b}	1.13 ±0.098^{b}	1.28±0.025^{b}
F -value	7.189*	3.072	3.309
P- value	0.026	0.2	0.1

n=3 , r = 6 replicates maintained each treatment, values expressed as Mean ± SE, for each mean with different superscript indicate significant difference (P<0.05)

95 °C for 15 sec, annealing at 60 °C for 15 sec followed by 95 °C for 15 sec. Data acquisition was performed during the final denaturation step.

Relative quantification of genes

The relative change in expression of *Tol C, Mdf A, Nor E* genes was analyzed by comparative CT (Cycle threshold) method

and was expressed as 'n' fold change up/ down regulation of the transcripted gene in concerning untreated control group. The data was analysed by One way ANOVA with Dunnets multiple comparison test.

RESULTS AND DISCUSSION

Antimicrobial susceptibility testing

The zone of inhibition was in resistance range when tetracycline and capsaicin were used alone but showed increase in the zone of inhibition when capsaicin and tetracycline were used in combination (in the range of 18-23). The zone of inhibition is positively correlated to concentration of capsaicin in tetracycline capsaicin combination and showed maximum activity at capsaicin at 500mg/L level. This is in accordance with the findings of Raja *et al*. (2015).

Broth dilution method

Antimicrobial activity of tetracycline and capsaicin was determined spectrometrically by measuring (optical density) OD at 600 nm. When tetracycline was used alone below MIC level, the turbidity for the culture was more after incubation for 24 hours. On combination with capsaicin, turbidity value decreased and the turbidity decreased as the capsaicin concentration increased. OD value for the culture was 0.815 when tetracycline was used alone whereas OD value decreased to 0.04 at 500 mg/ L of capsaicin. At 250 mg/L OD value increased to 0.238 and at 125 mg/L OD value increases to 0.47(Figure 2). Similar values were reported by Raja *et al*. (2015) after using piperine and *Catharanthus roseus* Ofloxacin resistant *E.coli*

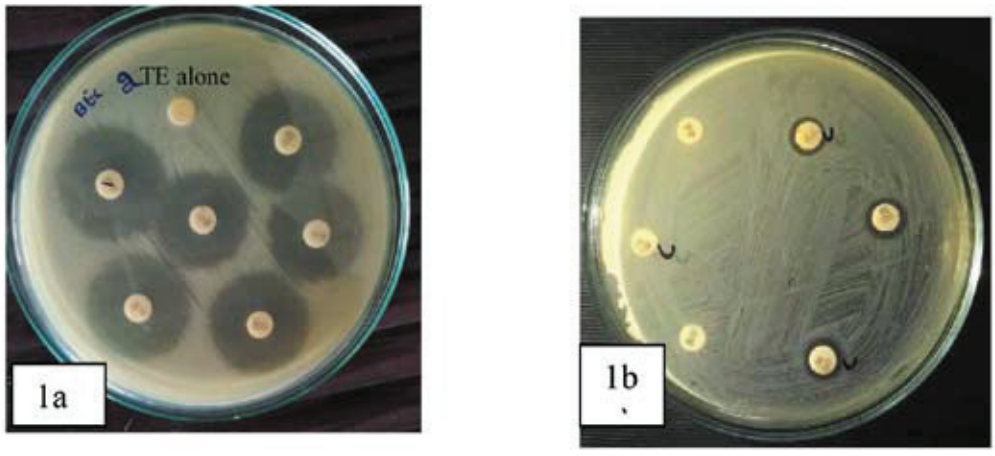

Fig 1: Antimicrobial disc diffusion assay of *E.coli* with tetracycline and 500mg/L capsaicin in combination (1a) antimicrobial disc diffusion assay of *E.coli* with capsaicin alone and tetracycline alone. Capsaicin is marked as C in picture (1b)

3. Gradient PCR

Real time analysis

The mRNA expression level for *Tol C* is decreased to 0.4, whereas for *Mdf A* and *Nor E* remain static around the range of 1(Table.7, Figure 4, 5, 6)

The mRNA expression level for *Tol C* was decreased on exposure to capsaicin, whereas it increased the mRNA expression for *Mdf A* and *Nor E* genes. The fold of expression for *Tol C* was reduced to 0.4, whereas for *Mdf A* and *Nor E,* not decreased. Capsaicin down-regulated the genes encoding for efflux pump mainly *Tol C* of AcrAB- tolC efflux pump of RND family. Down regulation of efflux

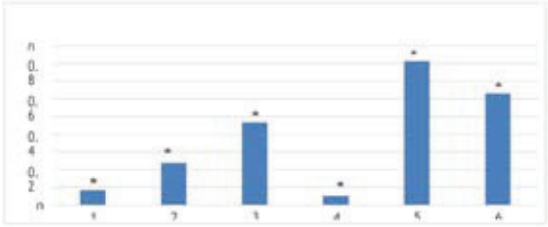

Fig 2. Results of OD values analyzed by spectrophotometer at 600 nm. 1.Enrofloxacin 2.Capsaicin with tetracycline at 125mg/L, 3.Capsaicin with tetra 250 mg/L, 4. Capsaicin with tetracycline 500 mg/L, 5. Tetracycline alone 6. Capasaicin alone (* values are significant at 5% level, ** values are significant at 1%)

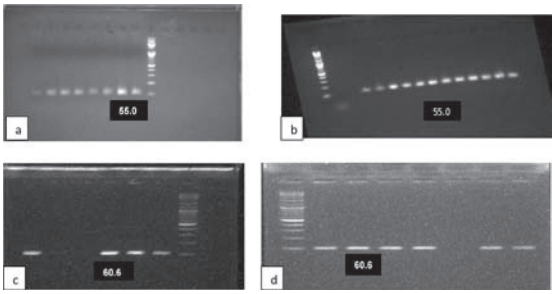

Fig 3. Results of gradient PCR for *rspL* (a), *Tol C* (b), *Nor E* (c), *Mdf A* (d)

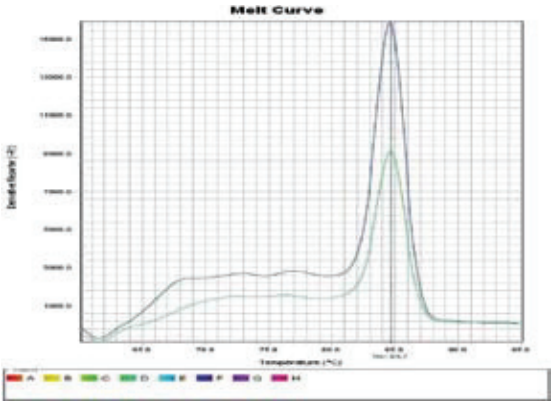

Fig 4. The melt curve of *Tol C* obtained in real time PCR

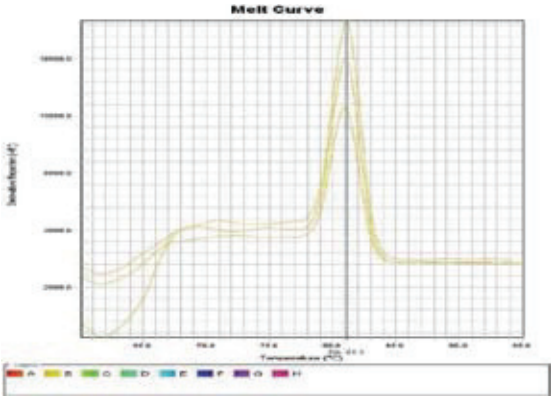

Fig 5. The melt curve of *Mdf A* obtained in real time PCR

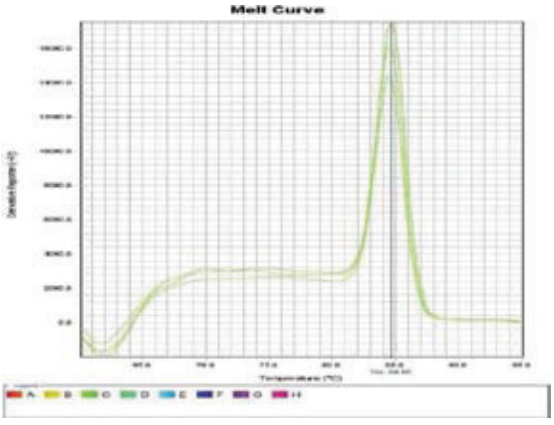

Fig 6. The melt curve of *Nor E* obtained in real time PCR

pump genes belonging to AcrAB- tolC associated with reduction in antimicrobial resistance (Perez *et al*., 2007). Hence the present research shows that antibacterial enhancing activity of Capsaicin is not mediated through inhibition of *Mdf A* and *Nor E* genes.

SUMMARY

Approaches to prevent the over-expression of efflux genes by targeting their transcription regulators using efflux pump inhibitors like capsaicin provide an alternative emerging strategy to overcome antimicrobial resistance. Capsaicin could be further studied for its inhibitory action on efflux pump to overcome antimicrobial resistance in both gram positive and gram negative bacteria. It could be used as lead compound for further drug development process with proper clinical models. Tetracycline is used extensively for the treatment of mastitis. Among various classes of efflux pump, AcrAB tol C efflux system, *MdfA* and *NorE* genes are responsible for antimicrobial resistance in *E. coli*. In this study Capsaicin, a phyto-alkaloid was used as modulator of efflux pump in combination with tetracycline in tetracycline resistant *Escherichia coli*. Antimicrobial sensitivity carried out by using standard disc diffusion method with appropriate controls. Tube dilution method is carried out and the turbidity is measured at 600nm. mRNA expression for the respective genes is carried out by Real time PCR. There was no zone of inhibition for capsaicin and tetracycline when they were used alone, while there was significant increase in the zone of inhibition for tetracycline when it was combined with capsaicin. Further research can be carried to evaluate the efficiency of capsaicin in combination with tetracycline or other antibiotics.

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