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Effect of capsaicin in inhibiting efflux pump *AcrAB-Tol* C of tetracycline resistant *Escherichia coli*

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Abstract
Bovine mastitis has become extremely complex and costliest disease among the livestock diseases in India. The key factors for therapeutic failure in mastitis are the acquisition of antimicrobial resistance among bacteria. Multidrug efflux systems make a major contribution to the increased tolerance of bacteria to multiple antibiotics that strip antimicrobial agents from microbial cells and make them resistant to antimicrobials. Therefore, blocking the efflux pumps is one of the methods to circumvent antimicrobial resistance, thereby supplying bactericidal/ static concentrations inside the cytoplasm. Among various class of efflux pump *AcrAB-TolC* efflux system are responsible for antimicrobial resistance in *Escherichia coli (E.coli)*. 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 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. Turbidity of the culture decreases when tetracycline is used in combination with capsaicin. mRNA expression for *AcrAB-TolC* is decreased when tetracycline is used in combination with capsaicin indicating the blocking of efflux pumps. Use of phytochemicals like capsaicin, which is a known inhibitor of efflux pump, could interfere with efflux pumping of antibiotics in resistant bacteria. Thus combination of antibacterial agent with capsaicin can be a promising approach to combat antibiotic resistant bovine mastitis due to *E.coli.*

Keywords: Bovine mastitis, *AcrAB-TolC*, *Escherichia coli*

1. Introduction
Milk and milk products contribute a major source of protein for most of the population in low and middle income nations. India is one of the highest producer of milk owing to huge population of cattle and buffalo (Heringstad *et al*., 2000) [5]. Mastitis remains one of the most common economic problems of dairy industry worldwide (Seegers *et al*., 2003) [9]. Antibiotics are used both in dry cow therapy and in the therapeutic management of clinical or sub-clinical mastitis caused by bacterial pathogenic agents. Nevertheless, the irrational use of antibiotics will contribute to the development of resistance, which will compromise the cure sequentially abolishing the cure rates.

Antibiotic resistance is a common problem employed in the treatment of coliform mastitis in veterinary field practice. Among many bacterial pathogens, particularly among Gram-negative bacteria like *Escherichia coli* (*E.coli)*, multidrug resistance (MDR) has been an emerging issue. Enhanced efflux is an essential mechanism of MDR in gram-negative bacteria, thus decreasing drug access to their intracellular targets. Several reports indicated that, for almost all antibiotics, active efflux can be a mechanism of resistance. The majority of the efflux systems in bacteria are non-drug-specific proteins that without drug modification or degradation, can recognize and pump out a wide variety of chemically and structurally unrelated compounds from bacteria in an energy-dependent way (Carlet *et al*., 2012) [3]. Additionally, it is notable that the efflux mediated resistance frequency is always higher than the resistance frequency dependent on targ*et al*terations. The expression of efflux pumps has made many bacteria immune to multidrug in this way. This has gained importance in the era of frequent multidrug resistance (Bengtsson *et al*., 2009) [2].

The efflux mechanism of *AcrAB* consists of the *Acr B* transporter of the RND family and the *Acr A* periplasmic accessory protein. The *Acr AB* genes form an operon. The *Tol C* outer membrane protein, which is encoded on the chromosome by a gene located elsewhere is likely to function together with *AcrAB* (Fralick, 1996) [4].

Many of these transporters form multicomponent pumps in gram-negative bacteria that span both inner and outer membranes and are powered by a primary or secondary transporter portion energetically. The secondary transporter *Acr B* located in the inner membrane, and the periplasmic *Acr A* that bridges these two integral proteins of the membrane compose this pump assembly. A broad variety of compounds with little chemical similarity can be transported by the *AcrAB-TolC* efflux pump, thereby conferring resistance to a wide range of antibiotics. Many Gram-negative bacteria, including animal and plant pathogens, have homologous complexes (Parsek and Greenberg, 2000) [7].

Tetracycline resistance is one of the examples of efflux mediated resistance where tetracyclines are extruded by the Tet efflux pumps (belonging to the MFS family) using proton exchange as the energy source. More than 20 distinct Tet genes have currently been identified, most of which are harbored in MGEs. Most of these genes are preferentially found in Gram- negative organisms, with one of the few exceptions predominating in gram-positive organisms being Tet (K) and Tet (L) (Schulz *et al*., 2011) [8]. Inhibition of such efflux pump of bacteria (*E. coli*) enhances the therapeutic index of antibacterial drugs and will be helpful to overcome antibacterial resistance (Alekshun and Levy, 2007) [1].

The use of efflux pump inhibitors (EPI) like Capsaicin with these antibacterial agents is anticipated to enhance their sensitivity and efficacy. An active component of chili peppers is capsaicin (8- methyl-*N*-vanillyl-6-nonenamide) (Oyagbemi *et al.,* 2010). Plants exclusively of the Capsicum genus produce capsaicinoids, which are alkaloids. Capsaicin is believed to be synthesized in the interlocular septum of chili peppers and depends on the gene *AT3*, which resides at the *pun1* locus. Biosynthesis of the capsaicinoids occurs in the glands of the pepper fruit where capsaicin synthase condenses vanillylamine from the phenyl propanoid pathway with an acyl-CoA moiety produced by the branched-chain fatty acid pathway (Tiwari *et al*., 2013) [10].

2. Materials and Methods

The bacterial isolates of *E. coli* from bovine mastitis were procured from the department of Veterinary Public health, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala and department of Veterinary Microbiology, Shivamogga. Capsaicin purchased from Sigma Aldrich and tetracycline purchased from chemind chemicals was used. The bacteria will be sub-cultured and susceptibility towards antibacterial agents and capsaicin will be assessed by disc diffusion susceptibility testing (Jorgensen and Turnidge, 2015) [6].

2.1 Disc Diffusion/ Kirby- Bauer Procedure (Hudzicki, 2009)

E. coli was selected and it had been transferred to a broth (sterile saline). The turbidity of 0.5 McFarland (Standard equivalent to 10^7 CFU of bacteria /ml of any sample) was ensured for the standardization of antimicrobial susceptibility testing.

Muller Hinton agar was used for the inoculation of organism from 0.5 McFarland suspension in three different directions. Then, the filter paper discs were placed on the agar, impregnated with antimicrobial agents. The plates were allowed for incubation for 16-18 hours at temperature 37 ° C, in the inverted position. The zone of inhibition was measured for the determination of susceptibility.

2.2 Broth dilution method

Muller Hinton broth was inoculated with bacterial suspension (0.5 McFarland standard) and two-fold dilution of antibiotic (Tetracycline). It was incubated for 16-18 hours at 37 ° C along with the control (Muller Hinton broth without antibiotics and inoculum). The turbidity was noticed on the next day. The tube without turbidity was used as a determinant of MIC (minimum inhibitory concentration). All the tubes showing no turbidity are sub-cultured on solid agar culture plate (without any antibiotic).The tube corresponding to the plate showing no growth of bacteria is considered as tube representing the MBC (minimum bactericidal concentration) of the drug. The optical density of each sample was analyzed by using a UV -VIS spectrophotometer at a wavelength of 600 nm.

2.3 Gene expression studies

Real-time quantitative polymerase chain reaction (RT-qPCR) was employed for studying the gene expression of *Acr A, Acr B, Tol C.*

I. Total RNA isolation

This was done by using the RNeasy Mini Kit (QUAGEN) as per the manufacturer's instruction with slight modifications. The bacterial cells were harvested with cooling centrifugation at 10,000 rpm for 3 minutes at 4 ^{0}C. The pellet was suspended with 4 µL of water in a centrifuge tube along with 2 µL of lysozyme. The suspension was freeze-thawed till the formation of turbidity. After 250 µL of RLT buffer was added to the centrifuge tube and it was centrifuged for 3 minutes at maximum speed. The Lysate is added with 350 µL of 70 % ethanol with proper mixing by pipetting. The reaction mixture (700 µL) was transferred to a pink colored RNA binding column to a 2 ml collection tube, which was centrifuged with a speed of 10,000 rpm for three minutes at 4 ^{0}C. The filtrate was discarded and 700 µL RW1 buffer was added to the spin column and centrifuged for a period of 15 seconds at 4 ^{0}C with discarding the flow-through. Next to this, 500 µL RPE buffer was added to the RNeasy spin column, allowed to centrifuge at 10,000 rpm for 15 seconds at 4 ^{0}C followed by removal of flow-through. Again, 500 µL RPE buffer was mixed with RNeasy spin column and subjected to centrifugation for 2 minutes with the rotation speed of 10,000 rpm at 4 ^{0}C. The spin column was transferred to a fresh 1.5 ml collection tube. The second tube wash was done by the addition of 50 µL of nuclease free water. Again centrifuged at 10,000 rpm for one minute.

II. DNase treatment of RNA

DNase treatment was done using a DNase kit (Sigma-Aldrich, USA). Forty µL of isolated RNA was added to five µL of 10 X reaction buffer and five µL of DNase I in an RNase free PCR tube. Mixed gently and incubated for 15 min at room temperature.

After DNase treatment, the DNase I was inactivated by adding five µL of stop solution (50 *mM* EDTA) and heating for 10 min having a temperature point at 70 ^{0}C, to denature both the DNase I and the RNA.

III. Quality of RNA samples

The quality and integrity of total RNA were analyzed by agarose gel electrophoresis. An appropriate sized clean gel casting tray was selected and set with suitable acrylic comb and sealing. Ultrapure agarose (1.8 percent) in 25 mL of 1X

TAE (Tris-acetate EDTA buffer) was dissolved by heating and allowed for cooling at 50 ^{0}C. 0.1µL of ethidium bromide (final concentration of 0.5µg/mL) was added into molten agarose, blended completely by gentle mixing, and then dropped into the tray to polymerize.

After solidification, poured a little buffer over the gel and removed the sealing and comb, then the gel with tray was immersed in a 1X TAE buffer in an electrophoresis tank with wells towards the negatively charged electrode.

Two µL of each RNA sample was mixed with five µL of 2X RNA gel loading dye and loaded into the wells. Horizontal electrophoresis was done at 5.3 V/cm until the end of the gel was reached by the tracking dye (bromophenol blue). It was visualized after the electrophoresis and recorded in a gel documentation system (BioRad, Gel Doc 2000™, USA).

IV. Concentration and purity of total RNA

The purity and concentration of total RNA isolated were estimated by spectrophotometric method.

The concentration of total RNA per µL of the sample was analyzed by placing one µL of the sample after calibrating with the blank solution. The purity of total RNA was assessed using the absorbance of the RNA samples taken at 260 and 280 nm in a spectrophotometer. The optical density ratio at A_{260} / A_{280} ratio was calculated and the samples showing a A_{260} / A_{280} ratio between 1.8- 2.0 were used for further study.

V. Complementary DNA synthesis

Complementary DNA (cDNA) synthesis was carried out from total RNA using Revert Aid first strand of cDNA synthesis kit (Thermo-scientific, USA) as per protocol from the manufacturer. Followed by thawing, all the components were mixed and briefly centrifuged and kept in ice. Total RNA (100 ng), one µL oligo $(dT)_{18}$ primer and 10 µL of nuclease free water were added into each of the sterile, nuclease-free 0.2 mL PCR tube, mixed gently, centrifuged briefly at 1000 rpm for one min and incubated at 65 ^{0}C for 5 minutes. After incubation, the tubes were snap chilled on ice.

The following components [5X reaction buffer (4 µL), Ri block RNAase inhibitor (1 µL), 10 mM dNTP mix (2 µL), 1 µL Revert Aid M-Mul V RT (200 U/ µL)]. The total reaction volume was made to 20 µL using sterile nuclease-free water, mixed gently, and spun at 1000 rpm for about 2 minutes. Tubes were incubated at 42 ^{0}C for around 60 minutes. The reaction was terminated by heating the mixture to 70 ^{0}C for a period of 5 minutes. The cDNA product was stored at -80 ^{0}C before its use.

VI. Selection of primers

Gene specific-primers (Table 1) 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/).

VII. Synthesis and dilution of the primers

The primers, obtained from Sigma- Aldrich, USA in the lyophilized form, were centrifuged before to opening for preventing the loss of contents. They were reconstituted in sterile de-ionized double distilled water to a concentration of 100 *pM*/µL.

The solutions were incubated at room temperature for an hour and then stored at – 20 ^{0}C. Working solutions of each primer was made by diluting the stock solution to 10 *pM*/ µL.

Table 1: Description of primers used.

Genes	Sequence
AcrA-F	CTCTCAGGCAGCTTAGCCCTAA
AcrA-R	TGCAGAGGTTCAGTTTTGACTGTT
AcrB-F	GGTCGATTCCGTTCTCCGTTA
AcrB-R	CTACCTGGAAGTAAACGTCATTGGT
rpsL-F	GCAAAAACGTGGCGTATGTACTC
rpsL-R	TTCGAAACCGTTAGTCAGACGAA
tolC-F	AAGCCGAAAAACGCAACCT
tolC-R	CAGAGTCGGTAAGTGACCATC

VIII. Standardization of PCR conditions

The PCR conditions were optimized by setting different time-temperature combinations for the annealing process. A volume of 20 µL in a 200 µL PCR tube was carried out. The annealing temperature of the primers was determined by gradient PCR (Biorad, Thermal cycler, USA) with eight different temperatures ranging from 56^0 C to 68 ^{0}C. The temperature that gave the best result for amplification was taken for further studies.

IX. Amplification of genes using gradient PCR

Master Mix (Emerald Amp GT PCR Master Mix, Takara, Japan) was used in the PCR reaction. The contents of the PCR mix is given in table 2.

Table 2: Reaction mix for the gradient PCR reaction

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	-

These were spun briefly and placed in a thermal cycler. The details of gradient PCR conditions used for amplification were given in Table 3.

Table 3: Gradient PCR conditions for the amplification of genes

Sl. No.	Steps	Temperature		Time
1	Initial denaturation	95 ^{0}C		4 min
2	Denaturation	95 ^{0}C		25 sec
3	Annealing	*Acr A, Acr B*	60.6^0 C	30 sec
		Tol C	58.3^0 C	
4	Extension	72 ^{0}C		1 min
5	Step 2 to 4 repetition	35 cycles		
6	Final extension	72^0 C		10 min
7	Hold	4 ^{0}C		10 min

The PCR amplified products were checked in 1.8 percent agarose gel (1X TBE) electrophoresis. Five µL of the product was mixed with two µl of 2X gel loading buffer and loaded into wells carefully. As a molecular size standard, a100 bp DNA marker (5 µL) was also loaded into one of the wells. Electrophoresis was then carried out at 5.3 V/cm until bromophenol blue dye migrated more than four cm length of gel in 1X TBE buffer. The gel was visualized under UV transilluminator and documented in a documentation system (Biorad, GelDoc 2000^{TM} , USA).

X. Real-time quantitative reaction conditions

After validation of primer annealing temperature, the cDNA

of target genes *Acr A, Acr B, Tol C* along with reference gene rspL was studied for mRNA expression by real- time q- PCR (Applied Biosystems, USA). The expression of *Acr A, Acr B, Tol C* gene was studied (Maxima SYBR green qPCR master mix, Thermo Scientific, USA). Separate PCR reactions were set up for target gene and housekeeping gene/reference rspL. Furthermore, the reaction also included one non-template control (NTC) for each gene and one reverse transcription minus (RT minus) control for each sample, and negative control (with only nuclease-free water). Each sample was run in triplicate in a 20 µL volume. Besides, one non-template control (NTC) for each gene and Reverse Transcription minus (RT minus) control for each sample, and negative control (with only nuclease free-water) were also included in the reaction. The real-time qPCR reaction conditions were initial incubation at 95^0 C for four min followed by 40 cycles of amplification cycle with denaturation at 95^0 C for 35 sec. Fluorescence signals were measured in each cycle. For each sample, the curve was generated after completion of analysis and was analyzed in positive and negative controls to detect the specificity of PCR reaction. The results were expressed as threshold cycle values (C_T). Glyceraldehyde – 3- phosphate dehydrogenase was used as an internal control and the relative expression was analyzed using the formula,

Fold change=$2^{-\Delta\Delta C_T}$,

Where $\Delta\Delta C_T = \Delta C_T$ treatment- ΔC_T control.

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 4 and 5 respectively.

Table 4: 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 5: RT-qPCR conditions

Steps		Temperature		Time
Initial denaturation		95^0 C		4 min
40 cycles of	Denaturation	95^0 C		35 sec
	Annealing	*acrA,acrB*	60.6^0 C	40 sec
		TolC	58.3^0 C	
	Extension	72^0 C		35 sec

XI. Melt curve analysis

A melt curve analysis was performed after the reaction for checking specificity of the amplification. The program for melt curve analysis consisted of denaturation at 95^0 C for 15 sec, annealing at 60^0 C for 15 sec followed by 95^0 C for 15 sec. Data acquisition was performed during the final denaturation step.

XII. Relative quantification of genes

The relative change in expression of *Acr A, Acr B, Tol C* genes was analyzed by comparative C_T (Cycle threshold) method and was expressed as 'n' fold change up/ down regulation of the transcripted gene in concerning untreated control group.

Fold change=$2^{-\Delta\Delta C_T}$,

Where ΔΔCT = **(**CT of target gene - CT of GAPDH**)** treatment-(CT of target gene-CT, rspL) control.

3. Results and Discussion

3.1 Results

3.1.1 Antimicrobial susceptibility testing

Tetracycline on combination with capsaicin, shows the zone of inhibition higher than the individual tetracycline. The zone of inhibition is positively correlated to concentration of capsaicin in tetracycline-capsaicin combination.

Fig 1: Antimicrobial disc diffusion assay of *E.coli* for tetracycline, oxytetracycline in combination with capsaicin.

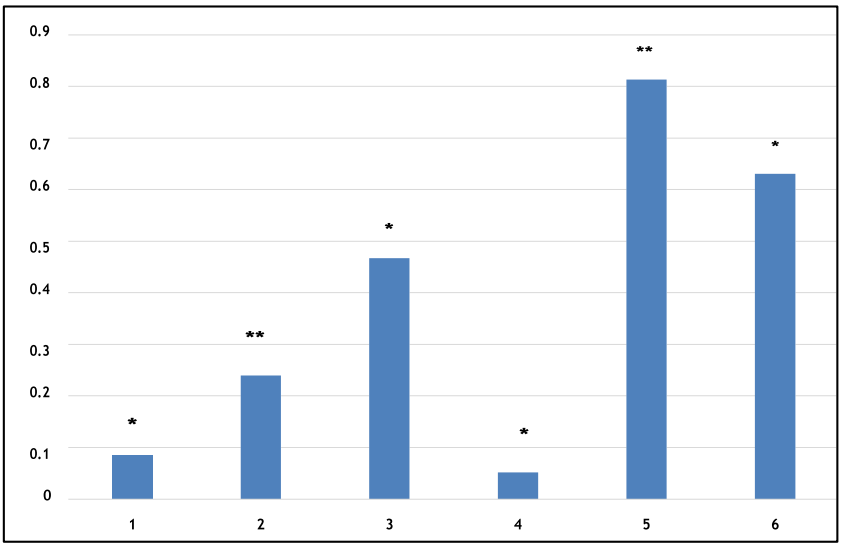

Fig 2: Results of OD values analyzed by spectrophotometer at 600 nm. 1.Enrofloxacin 2.Capsaicin with tetracycline at 125mg ml–1, 3.Capsaicin with tetra 250 mg ml–1, 4. Capsaicin with tetra mg ml–1, 5. Capsaicin alone (* values are significant at 5% level, ** values are significant at 1% level).

3.2 Gradient PCR

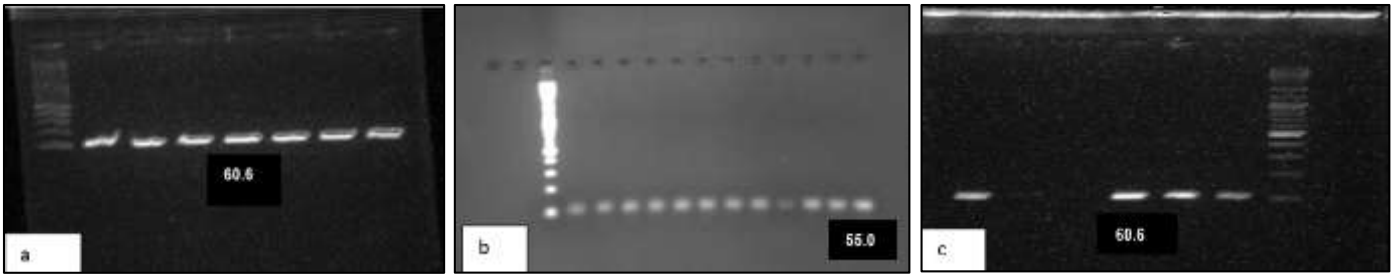

Fig 3. Results of gradient *PCR* for *AcrA(a), tolC(b), AcrB(c).*

3.3 Real-time analysis

The mRNA expression level for *Acr A, Acr B, Tol C* is decreased on exposure to capsaicin. The fold of expression for *AcrA* gene is reduced to 0.798, for *AcrB* gene is reduced to 0.9, for *Tolc* is reduced to 0.4.

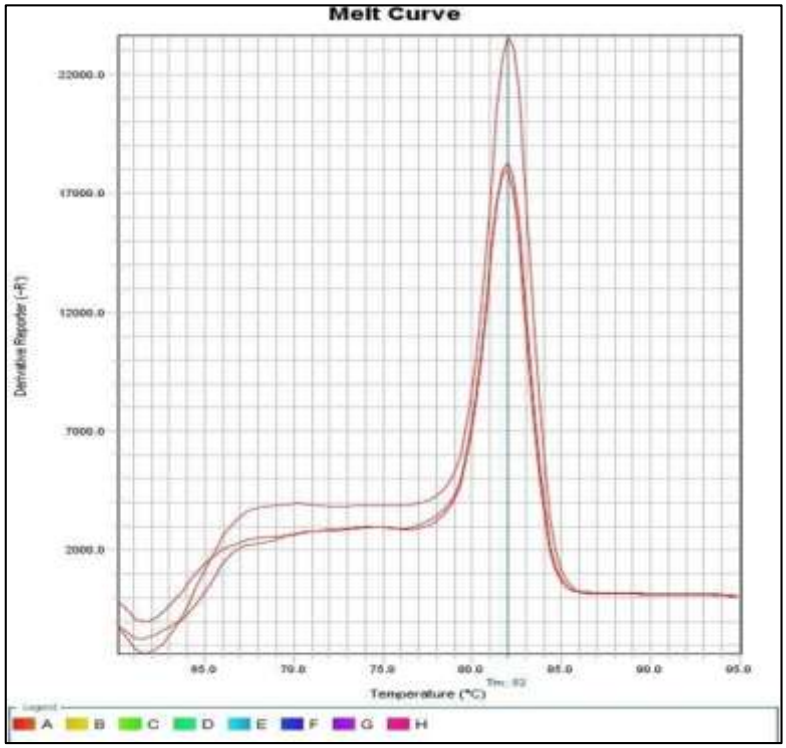

Fig 4: The melt curve of *Acr A* obtained in real time PCR

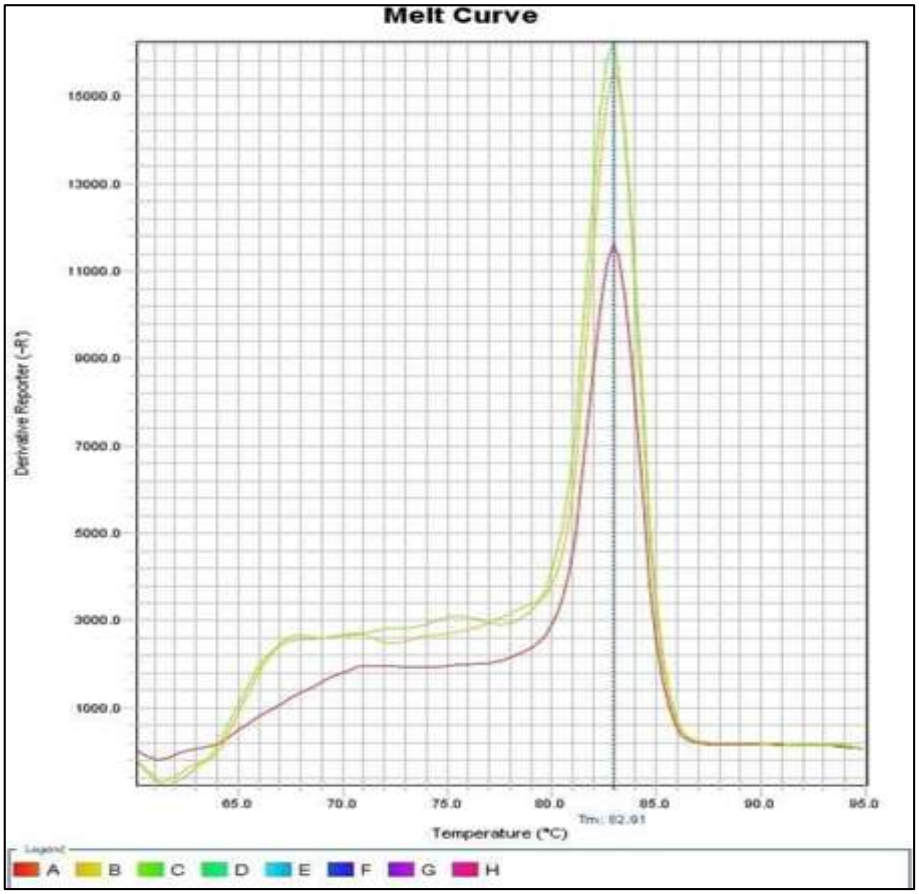

Fig 5: The melt curve of *Acr B* obtained in real time PCR

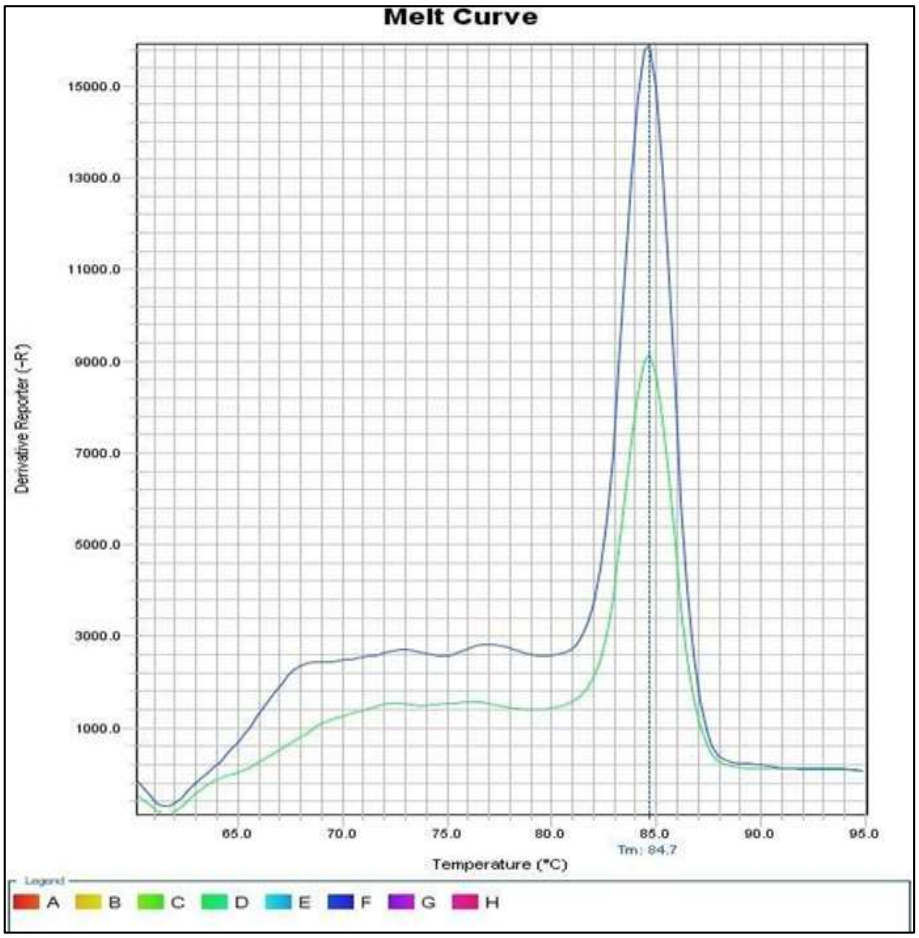

Fig 6: The melt curve of *Tol C* obtained in real time PCR

In the present study, capsaicin increases the antibacterial activity of tetracycline in resistant *E. coli* isolates from bovine mastitis. Combination of tetracycline and capsaicin shows antibacterial activity in resistant *E.coli* strains. Capasicin down-regulates the genes encoding for efflux pump mainly *Acr A, Acr B* and *Tol C* of AcrAB- tolC efflux pump of RNDfamily. AcrAB- TolC system is the efflux pump *E. coli* belongs to the RND family responsible for multiple drug resistance. Inhibition of the respective pump reverses the resistance mechanism. The antimicrobial activity of tetracycline and capsaicin is determined spectrometrically by measuring (optical density) OD at 600 nm. When tetracycline is used alone below MIC level the turbidity for the culture is more after incubation for 24 hours. In combination with capsaicin turbidity value decreases and the turbidity decreases as the capsaicin concentration increases. The combination of tetracycline and capsaicin increases the antibacterial activity of tetracycline in resistant *Escherichia coli* strains by blocking the efflux pump. The mRNA expression level for *Acr A, AcrB, Tol C* is decreased on exposure to capsaicin. The fold of expression for *Acr A* gene is reduced to 0.798, for *AcrB* gene is reduced to 0.9, for *Tol C* is reduced to 0.4. This study evidence that there is a significant role of efflux pumps in antibiotic resistance in bacteria. To maximize the efficacy of current and future antimicrobials, the effect of efflux pumps needs to be considered in the design of future antibiotics and the role of inhibitors assessed. This study also showed that EPI (efflux pump inhibitors like capsaicin can be used as adjuvant therapy in tetracycline-resistant E*scherichia coli* from bovine mastitis to overcome antimicrobial resistance. Also, capsaicin can be used to target the different efflux pumps in both gram-positive and gram-negative bacteria to overcome multidrug resistance.

4. Conclusion

Tetracycline is used extensively for the treatment of mastitis. To combat the resistance mediated by the efflux pump, capsaicin can be used concomitantly with tetracycline to overcome antibiotic resistance in treating mastitis. Further research can be carried to evaluate the efficiency of capsaicin in combination with tetracycline or other antibiotics.

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