The Effect of the Tannin Purified from Seeds of Rhus coriaria L. on the Efflux Pump (RamA) Gene Salmonella Typhimurium

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ABSTRACT

The aim of this study is to measure the gene expression of efflux pump gene (*RamA*) was conducted by using real-time quantitative PCR assay of *salmonella typhimurium* bacteria treated with minimal inhibitory concentration (MIC) of tannin from. The results of PCR reaction showed the presence of *RamA* gene in *Salmonella typhimuriu*m bacteria and this confirmed the role of this gene in the identification of *S.typhimurium* species and its intrinsic in this species. The gene expression of *RamA* gene was conducted by using real-time quantitative PCR assay. It was found that the value of gene expression fold to *RamA* gene was recorded (0.00007) in the *S. typhimurium*, in contrast with tannin untreated samples that which was (1.0). It was obvious there was a direct proportion between MICs values and folds of gene expression, therefore the increasing of tannin concentration in the growth medium leads to increasing of gene expression. *16S rRNA* gene expression results, which was used as reference gene, demonstrated that this gene was well suited as housekeeping gene because of the minimal variations of expression of this gene whether in tannin treated and untreated samples.

Key words: gene (*Ram*A), *S. typhimurium*, gene expression, Tannin,

INTRODUCTION

Salmonellae, Klebsiella pneumoniae, and Enterobacter aerogenes contain ramA, an additional member of the AraC/XylS family of transcriptional activators, which is absent from E. coli and Shigella spp. Overexpression of ramA confers multidrug resistance through induction of acrAB and tolC. Regulation of ramA is provided locally by ramR (STM0580), whose function can be ablated by internal point mutations and insertions within the helix-turn-helix motif and/or deletions within the DNA-binding region between ramR and ramA. All such events confer multidrug resistance (MDR), presumably through the prevention of RamR binding to an operator sequence near ramA, The role of RamA as a transcriptional regulator has been associated with MDR in Salmonella and other Enterobacteriaceae (Molitor *et al*., 2018).

The role is defined RamA using total genome transcription analysis and phenotypic array analysis as a global regulator . The transcriptomic experiments revealed that in response to the RamA inactivation there were considerable changes in gene expression

These included changes in genes related to MDR, genes co-regulated by other regulators such as marA, and soxS, as well as genes involved in the metabolic pathways. Such changes were also reflected in phenotype microarray analysis. The inactivation of ramA caused changes in the response of Salmonella to at least 100 compounds (Sun *et al*., 2014).

The increased expression of acrB was associated with the activation of RamA. decreased expression of acrA, acrB, tolC, and micF, and increased expression of ompF were observed in LTLramA::aph. Our data clearly showed that acrAB-tolC, ompF and micF were regulated by ramA at the transcriptional level. No change in the expression of marA, soxS, and rob indicated lack of the involvement of these regulators in the development of MDR (Zhang *et al*., 2018).

It is likely that RamA activates the MDR cascade independently of MarA. Consistent with results from a study by Ricci et al. phenotypic microarray data demonstrated that the inactivation of ramA increased Salmonella's susceptibility to an array of antimicrobials, confirming an important role of RamA in conferring MDR. Among those compounds, many are known or recently identified substrates of AcrAB efflux (Bailey et al.,2008 ;Ricci and Piddock, 2009).

Bailey et al. 2008 recently reported that inactivation of ramA led to altered expression of 223 genes in S. Typhimurium SL1344. Additionally, showed an overlapping regulation between soxS and ramA in Salmonella , encoding oxidoreductase that shuttles electrons from pyruvate to reduce nitrogenase. In line with data from other studies, it is likely ramA also plays a role as a transcriptional regulator of antioxidant defense in Salmonella .

Previous studies showed decreased expression of rob in S. Enteritidis was likely due to down-regulation by soxS and marA. found a putative transcriptional regulator (LysR family) was down-regulated due to the inactivation of RamA . The inactivation of ramA affected the response of Salmonella to many different chemicals. Of these compounds, acriflavine, blactams, chloramphenicols, fusidic acid, macrolides, novobiocin, puromycin, sulfonamides, tetracyclines and trimethoprim were known substrates of AcrAB-TolC system.

The data generated by the transcriptomic and phenotypic arrays suggested that ramA be a global transcriptional regulator that controls a set of genes with diverse physiological functions. It is possible that RamA plays a role in virulence regulation as well. Further studies on cross talk between global regulators including marA, soxRS and ramA, and the genes under their control in Salmonella may provide important details related to the mechanisms that govern how Salmonella enhance their fitness in new and challenging environmental landscapes.

MATERIALS AND METHODS

Extraction of genomic DNA

DNA was extracted from *Salmonella typhimurium* bacteria using a commercial purification system (Genomic DNA Mini Kit (Geneaid, Thailand); this kit was designed for the isolation

of DNA from Gram positive and Gram negative bacteria. DNA was extracted by this kit using bacterial protocol (for gram negative bacteria).

RESULTS AND DISCUSSION

The Ct value of *16sRNA* , the housekeeping gene used in the present study is shown in Table (1) .The range of Ct value for *16sRNA* in the M. Tm. group was 14.05-14.30 with a mean ± SD (14.25 ± 0.62) . In the control group it ranged from 14.42-14.80 with a mean ± SD (14.61 ± 0.76) . The house keeping gene used in the present study was *16S rRNA* .The purpose of using this gene in molecular studies is that its expression remains constant in the cells or tissues under investigation and different conditions (Reboucas *et al*., 2013).

Table (1): Comparison between different groups in Ct value of *16sRNA* (Mean ± SD)

Group	Mean ± SD of Ct value	Range
Group M. Tm.	14.25 ± 0.62	14.05-14.30
Group C. Tm.	14.61 ± 0.76	14.42-14.80
T-test	1.064 NS	---

NS: Non-Significant .

The inherent assumption in the use of housekeeping genes in molecular studies is that their expression remains constant in the cells or tissue under investigation. *16s rRNA* has the advantage, that its evolution/mutation rate is slow. Therefore, there are regions which are highly conserved

Since the discovery of polymerase chain reaction (PCR) and DNA sequencing, comparison of the gene sequences of bacterial species showed that the 16S ribosomal RNA (rRNA) gene is highly conserved within a species and among species of the same genus. Therefore, *16S rRNA* gene sequencing has been used as the new gold standard for identification of clinical isolates with ambiguous biochemical profiles. The variation of total change in expression of *16sRNA* was studied in different study groups utilizing the 2^{-Ct} value and the ratio of 2^{-Ct} of the different study groups to that of control group. To further improve this and although there was a significant difference in the mean Ct value between groups in the present study, the variation of total change in expression of *16sRNA* was studied in different study groups utilizing the 2-Ct value and the ratio of 2-Ct of the different study groups to that of control group,(Table 2).

Table (2): Comparison of *16sRNA* Fold expression between study groups.

Group	Means Ct of *16sRNA*	2^{-Ct}	experimental group/Control group	Fold of gene expression

Group M. Tm.	14.51	4.28 E-5	4.28 E5/4.62 E5	0.93 ± 0.04
Group C. Tm.	14. 25	5.13 E-5	5.13 E5/4.62 E5	1.10 ± 0.07
T-test				0.379 NS

NS: Non-Significant.

The 2^{-Ct} value in M. Tm. Group was (4.28 E-5) for control gruop was (5.13 E-5) .The computed ratio for gene fold expression was (0.93 and 1.10 respectively). These small variations in gene fold expression between the study groups renders *16sRNA* gene a useful control gene . These small variations in gene fold expression between the study groups renders *16sRNA* gene a useful control gene. Plots of each run were recorded including the amplification plots and dissociation curves. Figures (1) (2) show the amplification plots and dissociation curves for *16sRNA* gene .

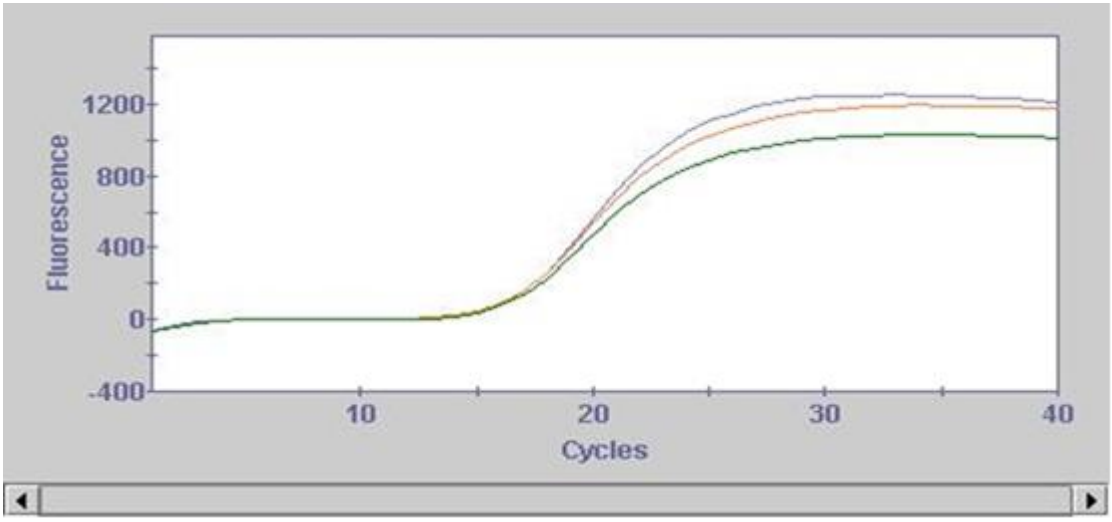

Figure (1) : *16sRNA* amplification plots by qPCR Samples included all study groups .Ct values ranged from 14.25-14.61. The photograph was taken directly from cepheid (smart cycler) qPCR machine .

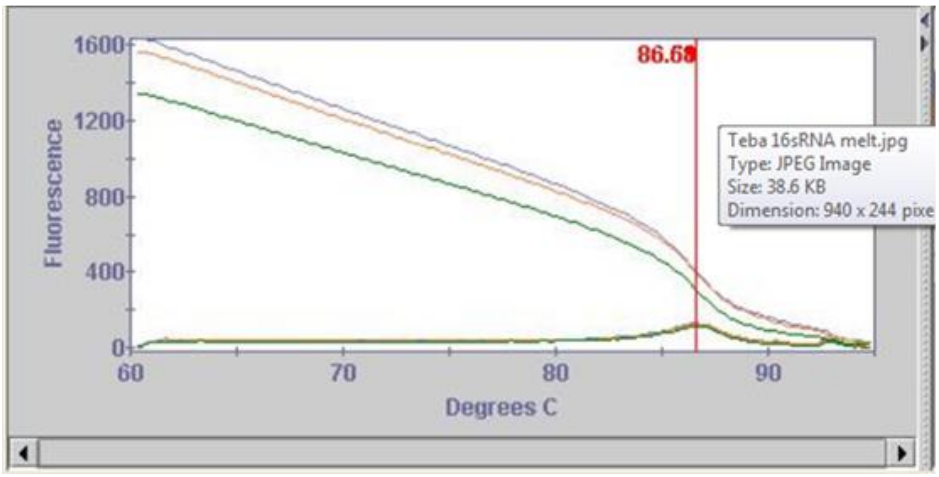

Figure (2) : *16sRNA* dissociation curves by qPCR Samples included all study groups. Melting temperature 86.68°C, No primer dimer could be seen. The photograph was taken directly from cepheid (smart cycler) qPCR machine .

Normalization of Ct (cycle threshold) Values

The main purpose of this step was to measure the gene expression of the *RamA* gene and compare the gene expression in the present of the tannin and in the absence of it in order to inhibition the role of this gene in the resistance of *S. typhimurium* bacteria to the antibiotics . In the present study, quantitative RT- PCR assay analyzed the mRNA expression of *RamA* gene by comparing the treated and untreated samples of bacterial growth with tannin by using the concentration below the dose of MIC for each sample. In the present study, quantitative RT -PCR assay analyzed the mRNA expression of *RamA* gene and compared its expression between apparently control group and M. Tm Group. The calculation of gene expression fold change was made using relative quantification (Livak and Schmittgen, 2008).

The Ct values of genes amplification were recorded from the software of quantitative RT PCR .This depends on normalization of Ct values calculating the ΔCt which is the difference between the mean Ct values of replica of *RamA* cDNA amplification of each single case and that of the *16sRNA*. Shows the detailed ΔCt values of each participant in the study.

Table (3) was show the means Ct of *RamA* for M.Tm. group and apparently control group was 26.88 and 12.80 , respectively. The means of ΔCt (normalization Ct values) for M.Tm. group was 12.37 while ΔCt for apparently control group was 1.45 . Results of means 2-ΔCt in M.Tm. group was 0.000188 and apparently control group was 2.73 .

Table (3): Fold of *RamA* expression Depending on $2^{-\Delta Ct}$ Method

Groups	Means Ct of *RamA*	Means Ct of 16sRNA	ΔCt (Means Ct of *RamA* - Means Ct of 16sRNA)	$2^{-\Delta Ct}$	experimental group/ Control group	Fold of gene expression
Group M. Tm.	26.88	14.51	12.37	0.000188	2.73 / 0.000188	0.00007
Group C. Tm.	12.80	14.25	1.45	2.73	2.73/ 2.73	1

Fold = $2^{-\Delta Ct}$

To calculate the gene expression folds in relation to the housekeeping genes the result of 2-ΔCt of each group was measured in relation to that of control group. The results are shown in Table (3). The fold of gene expression in M.Tm. group was lower than apparently control groups in 0.00007 times. These results indicate a decrease expression of *RamA* gene M.Tm. group compare with control group and there are few copies of mRNA in M.Tm. group according to their fold of gene expression values. In calculation of the relative expression of

RamA gene in all study groups the $2^{-\Delta Ct}$ results was applied. A calibrator was used and it was one of the samples of the control with low expression of *RamA*, the mean of $2^{-\Delta Ct}$ values of M.Tm. group was (0.000188) and for control gruop was (2.73) .

Figures (3) and (4) show the amplification plots and dissociation curves for *RamA* .Each quantificative PCR reactions was run in triplicate for each sample, In each run , samples from control group. and M. Tm. group were run in addition to non-template and non- primer controls.

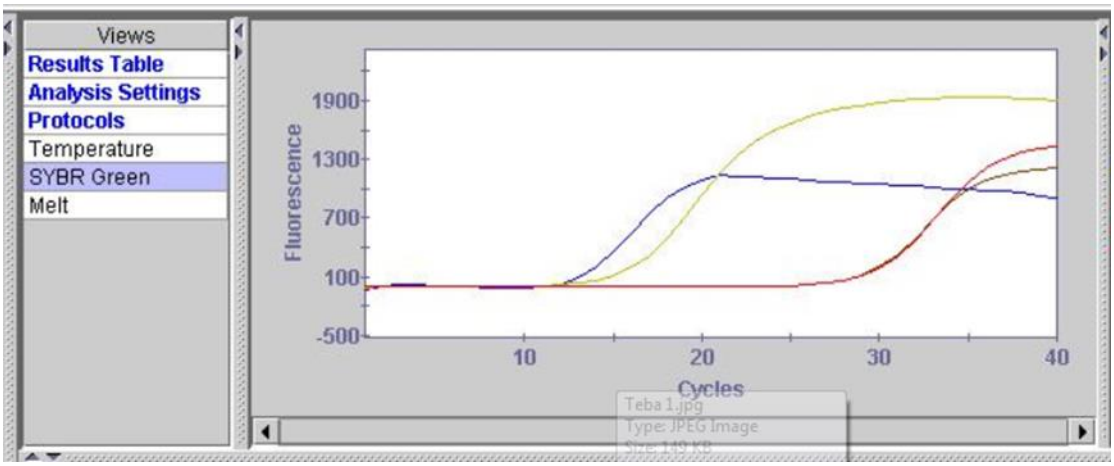

Figure (3): *RamA* amplification plots by qPCR. Samples included all study groups .Ct values ranged from 20-26. The photograph was taken directly from cepheid (smart cycler) qPCR machine .

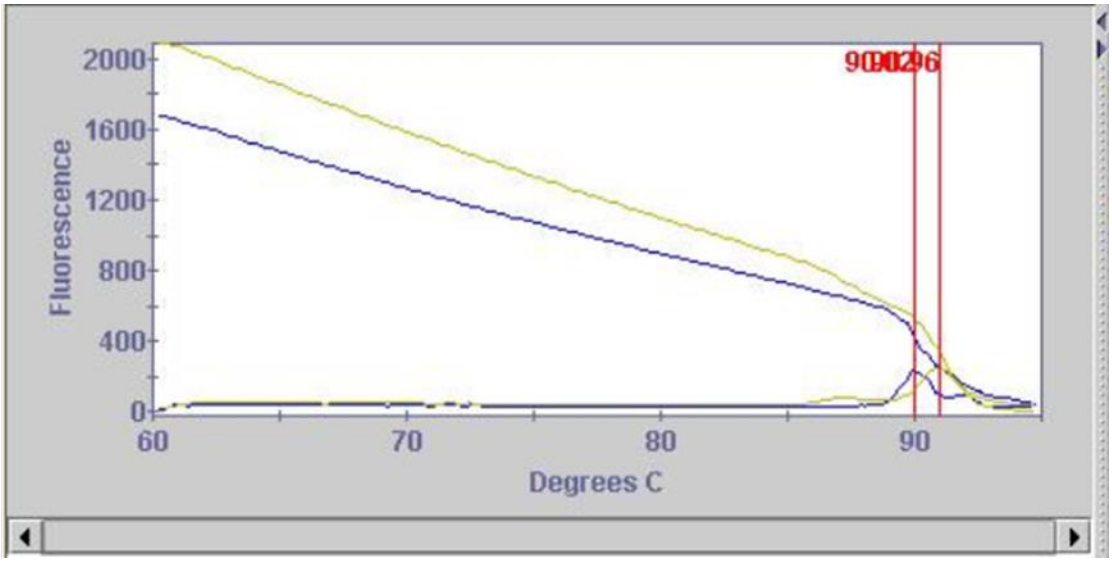

Figure (4): *RamA* dissociaition curves by qPCR Samples included all study groups. Melting temperature ranged from 90.02°C to 90.69°C, No primer dimer could be seen. The photograph was taken directly from cepheid (smart cycler) qPCR machine.

The study conclude that our study demonstrated the role of *RamA* gene for molecular detection *S.typhimurium* at the level of genus and species respectively. *16S rRNA* gene (190

bp) gave ideal results when used a housekeeping gene in the gene expression experiment with the minimal variation in different conditions.

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