

Original Research Article

Quantitation of TLR-2 mRNA Expression in Bovine Mastitis caused by *E. Coli*

ABSTRACT

Aims: This study aim was to assay the expression of the *TLR2* gene in naturally infected cows with sub-clinical and clinical mastitis caused by *E. coli*.

Methodology: cDNA from *E. coli* used for *TLR2* expression analysis were obtained from (x) quarters of healthy animal, (x) animals with subclinical mastitis, (x) animals with clinical mastitis using commercial kit and oligo (dT) primers. Quantitative reverse transcription PCR (RT-qPCR) were standardized for the *TLR2* and β -actin gene, genes expression level was analyzed by Illumina Eco® Q- RT PCR system..

Results: The mRNA expression of *TLR2* gene in animals sub-clinical mastitis was significant ($P \leq 0.01$) higher (3.63 fold) than clinical case (1.89 fold) when compared to healthy bovine.

Conclusion: The expression of *TLR2* gene during early stage of infection was at high level, therefore most of the sub-clinical mastitis subsided by itself without precipitating into clinical mastitis. It is suggested that *TLR2* may consider as candidate gene for mastitis screening in cattle population.

Keywords: *TLR2*, expression, mastitis and *E. coli*

1. INTRODUCTION

In dairy industry, mastitis is one of the most important disease conditions and it is caused various groups of bacteria, among which a gram negative bacteria *E. coli* plays a considerable role in bovine mastitis (reference). These infection lead to severe milk loss and are a major cost to dairy farmers. For this reason, there is a need to understand the pathogenesis of mastitis. *E. coli* has the ability to grow in mammary secretions and to liberate lipopolysaccharide (LPS) is crucial in the pathogenesis of mastitis (reference). The innate immune response to pathogen in the mammary gland is crucial for effective clearance of the pathogen [1]. It is the predominant defense strategy during the early stages of an infection (reference). There is difference in host response towards experimentally induced infection of gram positive and gram negative bacteria [2]. Toll-Like Receptors are vital to immune function through the sensing of pathogenic agents through pattern recognition receptors [3]. In mammals, 13 TLRs are reported that recognize some specific ligands from pathogens to trigger immunological responses [4]. Of these, *TLR2* is the main pathogen recognition receptors (PRR), recognizes lipoteichoic acid (LTA) from Gram-negative (reference). The activation of TLRs may commonly lead to early activation of the transcription factors and the release of several proinflammatory mediators [5, 6]. Assessing the expression of an important *TLR2* will be vital for improving our understanding the pathogenesis of mastitis

caused by *E.coli*. Hence, the objective of this study was to investigate the mRNA expression of *TLR2* gene in naturally infected *E. coli* cases of sub-clinical and clinical bovine mastitis.

2. MATERIAL AND METHODS

2.1 Milk Sample collection and identification of bacteria

Milk samples (how many?) were collected from University farm and Veterinary Dispensaries at Thrissur Kerala. By using California mastitis test (CMT) and somatic cell count (SCC), the milk samples were categorized into subclinical, clinical and healthy. During sampling, the samples were collected aseptically and put into sterile centrifuge tube and kept in an ice box containing ice packs and taken immediately to laboratory for bacteriological analysis (reference, National Mastitis Council?). The milk samples were streaked on McConkey's agar plates for identification of coliforms. The positive cultures were further subjected to biochemical tests for identification of *E. coli* [7].

2.2 RNA isolation and cDNA synthesis

Total RNA was obtained from milk somatic cells using TRIzol reagent of SIGMA (as per the manufacturer's protocol). The quality of extracted RNA was determined by agarose gel electrophoresis (1.5%) and ethidium bromide staining. Residual DNA was removed by DNase1 treatment. The concentration of RNA was assessed by using nanodrop spectrophotometer. Total RNA was subjected to cDNA synthesis using oligo dT primers (Thermo Scientific, K1622).

2.3 Primers design and synthesis

Primers for RT-qPCR of *TLR2* and β -actin were designed from published bovine mRNA sequences available from GenBank. Designing and checking of primers were done with Primer3 software (Table 1).

Table 1. Primer sequence for *TLR2* and β -actin genes

Gene Name		Sequence (5'→3')	Expected size
TLR2	F	AGCGAGTGGTGCAAGTATGA	114 bp
	R	CTGGGGAATGGCCTTCTTGT	
β -actin	F	CCACACCTTCTACAACGAGC	105 bp
	R	ATCTGGGTCATCTTCTCACG	

2.4 RT-qPCR

Before real-time quantification, the PCR was optimized for *TLR2* and β -actin genes. Expression level of mRNA of *TLR2* was analysed by Illumina Eco® Q- RT PCR system. The β -actin was selected as housekeeping gene because it showed a stable expression from all milk samples. Real time PCR was carried out in triplicate for each sample in a total volume of 20 microlitre, 10 microlitre of 2X SYBR Green PCR mastermix, 10 pmole (1microlitr) of each gene-specific primers, 2 microlitre of cDNA template and 7 microlitre of nuclease free water. Real time PCR cyclic conditions were 95°C for 10 min then 40 cycles of 95 for 30 sec, 58 for 30 sec and 72 for 1 min with fluorescence recording at the end of each cycle. The final step was to obtain a dissociation curve analysis PCR products to determine the specificity of the amplicons. The protocol for melt curve analysis was 95°C for 15 sec, 55° C for 15 sec followed by 95° C for 15 sec. Data acquisition was performed during the final denaturation step. The result was

expressed at threshold cycle values (Ct). The relative expression of each sample was calculated using the $2^{-\Delta\Delta C_t}$ method [8].

2.5 Statistical analysis

Analysis of variance was performed to test the significance of among the groups under study. Tukey's HSD (Honestly Significant Difference) was applied to test the significance between two groups (with ones?please state). All statistical analyses were done using Statistical Product and Service Solution (SPSS) version 21.0 software.

3. RESULTS AND DISCUSSION

The successful development of an intramammary infection is dependent upon virulence factors of the bacteria and the nature of the host response to the pathogen [9]. Once a pathogen has entered to the teat cistern, the innate immune system represents the next line of defense in the host response to infection [10]. The innate immune response to intramammary infection with gram positive or gram negative bacteria differs in several respects. *E. coli* is frequent cause of sub-clinical and clinical mastitis [11]. Several authors proposed that specific *E. coli* strains are more adapted to the mammary gland [12,13,14]. Recurrent and persistent mammary infection by *E. coli* is a serious problem for the dairy animals. The innate immune response to pathogen in the mammary gland is crucial for effective clearance of the pathogen. Assessing the expression of important *TLR2* will be vital for improving our understanding the pathogenesis of mastitis.

Lactating crossbred cows (how many?) were screened for sub-clinical and clinical mastitis (clinical mastitis is detected though Tamis test and physical examination of the udder) by using CMT (reference) and SCC. Based on biochemical test, 8(eight) revealed the presence of *E. coli* in sub-clinical case of mastitis and 5 (five) samples showed the presence of *E. coli* in clinical mastitis. For RT-qPCR assay, milk from three animals from each group of sub-clinical and clinical mastitis

caused by *E. coli* were selected (randomly or with the closest SCC?) for expression study. Milk samples from three apparently healthy crossbred cows were also selected as control for expression studies.

Analysis of variance for *TLR2* gene revealed significant difference ($P \leq 0.01$) for expression level between the groups (Table 2). The mean values of C_q , ΔC_q , $\Delta\Delta C_q$ along with standard error and relative quantification of *TLR2* expression in *E. coli* mastitis are given in Table 3.

Table 2. ANOVA for *TLR2* expression in *E. coli* mastitis

Source of Variation	Df	MSS	F value
Between Groups	2	5.57**	24.72
Within Groups	6	0.23	

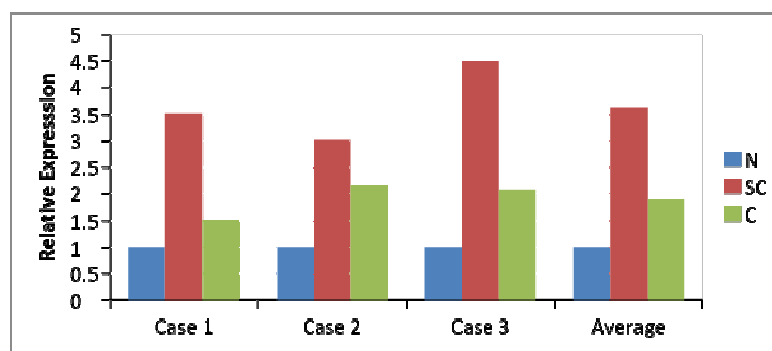
Table 3. Expression of *TLR2* gene in *E. coli* caused mastitis

Sample	Cq Mean $\pm$ SE		Δ Cq	Δ Cq Mean	$\Delta\Delta$ Cq	RQ
	<i>TLR2</i>	β -actin				
Normal	22.27 $\pm$ 0.02	15.94 $\pm$ 0.03	6.33	6.33 $\pm$ 0.03		
Sub-Clinical						
Case 1	20.20 $\pm$ 0.04	15.68 $\pm$ 0.25	4.51		-1.82	3.52
Case 2	20.09 $\pm$ 0.06	15.36 $\pm$ 0.24	4.74		-1.60	3.02
Case 3	20.22 $\pm$ 0.03	16.05 $\pm$ 0.11	4.17		-2.17	4.49
				4.47 $\pm$ 0.17	-1.86	3.63 _a ^{**}
Clinical						
Case 1	21.77 $\pm$ 0.54	16.04 $\pm$ 0.07	5.73		-0.60	1.52
Case 2	21.54 $\pm$ 0.18	16.31 $\pm$ 0.02	5.23		-1.11	2.15
Case 3	21.75 $\pm$ 0.04	16.46 $\pm$ 0.01	5.29		-1.04	2.06
				5.41 $\pm$ 0.16	-0.92	1.89 _b ^{** c}

a = Normal vs Sub-clinical; b = Normal vs Clinical; c = Sub-clinical vs Clinical

The relative expression of *TLR2* gene was ranged between 3.02 and 4.49 fold for sub-clinical mastitis and it was ranged from 1.52 to 2.15 fold, when compared with healthy crossbred cows (Figure 1). *E. coli* infected mammary gland shows mRNA levels of *TLR2* was higher in sub-clinical mastitis (3.63 fold) followed by clinical mastitis (1.89 fold) when compared to normal animal. Relative expression of *TLR2* gene was significantly ($P \leq 0.01$) higher in the sub-clinical mastitis, and also exhibits significant difference ($P \leq 0.01$) between sub-clinical and clinical mastitis (Figure 1).

Fig. 1. Relative expression of *TLR2* gene in *E. coli* caused mastitis



N- Normal healthy; SC- Sub-clinical mastitis; C-Clinical mastitis

Toll like receptors are key component of innate immune system that recognize a variety of pathogen through pattern recognition receptor [15]. *TLR2* is principal receptor for peptidoglycan and lipotechoic acid from gram positive gram negative bacteria. In our study *TLR2* was expressed with 3.63 fold upregulation in sub-clinical case of mastitis caused by *E. coli* infection. *TLR2* mRNA expression were increased by mastitis infected by *E. coli* [16]. Similarly Ibeagha-Awemu *et al.* [17] also reported increased mRNA expression of *TLR2* in mammary glands infected with LPS. Similarly, *TLR2* and *TLR4* mRNA expression increased in infected mammary glands of cows with mastitis caused by either *S.aureus* or *E. coli* [18]. Stimulation of *TLR2* consequently induces a series of signalling cascades that ultimately result in the activation of NF- κ B. The ability of the *TLR2*-induced NF- κ B pathway to cross-talk with other signaling

molecules is a key element in shaping the overall pattern of host responses, such as production of cytokines and chemokines, antimicrobial killing mechanisms, maturation of antigen presenting cells, and recruitment of the adaptive immune response [19,20,21].

4. CONCLUSION

In our study the expression of *TLR2* gene was significantly higher in sub-clinical mastitis compared to clinical case of mastitis infected by *E. coli*. Sub-clinical mastitis is an early stage of infection, and hence the expression of *TLR2* is relatively higher at this stage compared to clinical stage. The result suggested that mastitis increases the expression of *TLR2* contribute to innate immune system in the cow udder. Therefore, the sub-clinical stage of mastitis may subsided by itself without precipitating into clinical mastitis. It is suggested that *TLR2* might be considered as a candidate gene for screening mastitis resistance in cattle population.

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