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Expression of *TLR4* Gene in Spontaneous Bovine Sub-Clinical and Clinical Mastitis Caused by *Escherichia coli*

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ABSTRACT

Escherichia coli infection of the bovine mammary gland can result in a spectrum of sub-clinical and clinical infection to a severe systemic mastitis disease. E. coli appear to mostly circumvent the host immune response through the signalling of Toll-Like Receptors (TLRs). Understanding the expression of TLR genes in bovine is particularly important to develop strategies for the prevention of mastitis. This study characterized the expression of an important TLR4 gene in spontaneous bovine sub-clinical and clinical mastitis caused by E. coli. The mRNA expression of TLR4 gene in sub-clinical mastitis was higher (6.55 fold) than clinical case (2.88) when compared to normal bovine case. Relative difference in the expression of TLR4 gene in sub-clinical and clinical mastitis was found to be significant ($P \leq 0.01$). E. coli elicits a strong and earlier response in sub-clinical mastitis, mainly through TLR4, and hence the expression of TLR4 is relatively higher at this stage compared to clinical stage. During early stage of infection the expression of TLR4 gene was at high level, therefore most of the sub-clinical mastitis subsided by itself without precipitating into clinical mastitis. This study sheds light into the innate immune system represents the first line of defense in the host response to infection and is poised to immediately recognize and respond to the earliest stages of infection.

Key words: *TLR4, expression, mastitis and E. coli*

INTRODUCTION

Mastitis is an inflammatory disease condition in dairy cows lead to severe milk losses is potentially fatal, and are a major concern for the dairy industry. Mastitis caused by both gram-negative and gram-positive bacteria. *E. coli* is a gram-negative bacteria line plays a considerable role in bovine mastitis. Innate immunity is a critical first line defense against pathogens[1], and the initial recognition of microbes by cells of the immune system is largely based on pattern recognition receptors (PRR) including Toll-like receptors (TLRs)[2,3].

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TLRs are vital to immune function through the sensing of pathogenic agents and initiation of an appropriate immune response[4]. So far 13 TLRs have been identified in mammals of which 10 TLRs are known to occur in cattle, and the expression of TLR transcripts varies among different mammalian species. Of these, *TLR4* is the main PRR, for lipopolysaccharides (LPS) from gram-negative bacteria. Signalling through TLRs may commonly lead to early activation of the transcription and nuclear-kappa B factors and subsequent expression and the release of several proinflammatory mediators. Assessing the expression of an important *TLR4* will be vital for improving our understanding of the early events controlling immune disease response. Hence, this study aims to investigate the mRNA expression of *TLR4* gene in natural infected *E. coli* cases of sub-clinical and clinical mastitis.

MATERIALS AND METHODS

Sample collection and identification

Milk samples were collected from sub-clinical, clinical, and normal animals from University farm and Veterinary Dispensaries and Teaching Veterinary Clinical Complex of the College of Veterinary and Animal Sciences at Thrissur Kerala. Mastitis was established on the basis of California mastitis test (CMT) and somatic cell count (SCC). The milk samples were streaked on "Mueller Hinton Agar" and "Mac Conkey's Agar" plates and incubated at 37ºC for 48 hours. A minimum of five colonies of the same type in the culture was considered positive for the causative agent. The positive cultures were further subjected to biochemical tests for identification of *E. coli*.

RNA isolation and cDNA synthesis

Total RNA of milk somatic cells were isolated using TRI reagent of SIGMA (As per the manufacturer's protocol). RNA aliquots were treated with deoxyribonuclease I (DNaseI) to eliminate genomic DNA, which can influence the interpretation of the real-time RT-PCR assay data. The quality of the RNA was assessed by agarose gel electrophoresis (1.5%) and ethidium bromide staining. The concentration of RNA was assessed by using nanodrop spectrophotometer. One microgram of total RNA was subjected to cDNA synthesis using Revert Aid first strand cDNA synthesis kit (Thermo Scientific, K1622).

Primers design and synthesis

Primers for RT-qPCR of *TLR4* and *β-actin* were designed from published bovine mRNA sequences available from GenBank. Designing of primers were done with Primer3 software. The primers selected were custom synthesized from Sigma Aldrich and were diluted to a concentration of 10 p*M*/µl (Table 1).

RT-qPCR

All RT-qPCR reaction was performed in triplicate. The amplification was carried out in 20 microlitre volume reaction containing 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. Negative controls (NTC) without cDNA were included for the real time PCR assay. Real-time PCR was carried out in an Illumina Eco® Q- RT PCR system. The thermal profile used for this was as follows: 95ºC 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. Dissociation (melt) curve analysis was done after each PCR. The protocol for melt curve analysis was 95^0C for 15 sec, 55^0 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 (Cq). The Cq value (the fractional cycle number at which the fluorescence exceeds a fixed threshold) was determined for each sample. To evaluate the relative mRNA expression, samples were normalised to the housekeeping gene (*β- actin*) and the results are presented as – $\Delta\Delta C_q$ values [5]. The *β-actin* was selected as housekeeping gene because it showed a stable expression from all milk samples.

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. All statistical analyses were done using Statistical Product and Service Solution (SPSS) version 21.0 software.

RESULTS AND DISCUSSION

E. coli is a common environmental pathogen recovered from cases of sub-clinical and clinical mastitis[6]. Recurrent and persistent mammary infection by *E. coli* is serious problem for the dairy animals. To eventually develop new strategies to prevent and treat sub-clinical and clinical mastitis, understanding the host pathogen interaction is necessary.

A total of 80 crossbred lactating cows were screened for sub-clinical and clinical mastitis using CMT. Based on this CMT, 28 cows were found positive for sub-clinical mastitis (35 per cent) and 19 were found positive for clinical mastitis (23.75 per cent). The rest were negative for CMT test and found to be healthy animals. Samples from all the three groups were subjected to SCC. Based on the result of CMT and SCC, 28 sub-clinical and 19 clinical samples were examined for identification of causative organism by microbial culture and biochemical test. Out of 28 sub-clinical samples, 8 revealed the presence of *E. coli*. Similarly, from clinical mastitis cases 5 samples showed the presence of *E. coli*. Based on the screening test, three animals from each group of sub-clinical and clinical mastitis caused by *E. coli* were selected for RT-qPCR assay. In addition milk samples from three apparently healthy crossbred cows were also selected as control for expression studies.

Analysis of variance revealed significant difference ($P \leq 0.01$) for expression level of *TLR4* genes between the groups (Table 2). The mean values of C_q, ΔC_q, $\Delta\Delta$Cq along with standard error and relative quantification of *TLR4* expression in *E. coli* caused mastitis are given in Table 3. The relative expression of *TLR4* gene was ranged between 5.63 and 7.26 fold for sub-clinical mastitis and it was ranged from 2.65 to 3.48 fold, when compared with healthy crossbred cows (Figure 4.21). *E. coli* infected mammary gland shows mRNA levels of *TLR4* was higher in sub-clinical mastitis (6.5 fold) followed by clinical mastitis (2.88) when compared to normal animal. Relative expression of *TLR4* gene was significantly ($P \leq 0.01$) higher in the sub-clinical mastitis, and also exhibits significant difference ($P \leq 0.05$) between sub-clinical and clinical mastitis (Figure 1).

Pathogen recognition receptors (PRR) are the first to recognize the invading pathogens to elicit an immune response. Stimulation of TLRs consequently induces a series of signalling cascades that ultimately result in the activation of NF-kB and other immune responsive genes[7]. The contribution of different TLRs to infection depends on the site of the infection and the pathogen. In the present study, *TLR4* was profoundly expressed with 6.5 fold upregulation in sub-clinical case of mastitis. *TLR4* is a pattern recognition receptor that is involved in immune signalling and plays a crucial role in cell survival through recognition of bacterial cell wall component LPS[8]. Specifically, *TLR4* as pattern recognition receptors has been implicated in a link between the innate and adaptive immune system by favouring a Th1 immune response and enhancing autoantibody production. Accumulated lines of evidence reveal that the innate immune system recognizes gram-negative bacteria mainly through *TLR4*[9]. Similarly this study also found significant upregulation of *TLR4* in infection caused by gram-negative bacteria, *E. coli*.

Significant activation of *TLR4* indicated stronger response, which will be further able to stimulate downstream signalling cascades to provide profound immune response to fight against the pathogen. Such TLR expression can result in activation of MyD88, at universal adaptor for many TLRs,[10,11] which is necessary for the optimal production of IL-10, IL-12, TNF and IFN all of which are important mediators of host survival[12].

Table 1: Primer sequence for *TLR4 and β-actin* genes used in RT-qPCR

Gene Name		Sequence (5'→3')	Expected product size
TLR4	F	GCCGTGGAGACAAACCTAGT	138 bp
	R	CTCCAGGTTGGGCAGGTTAG	
β-actin	F	CCACACCTTCTACAACGAGC	105 bp
	R	ATCTGGGTCATCTTCTCACG	

Table 2: ANOVA for *TLR4* gene expression in *E. coli* caused mastitis

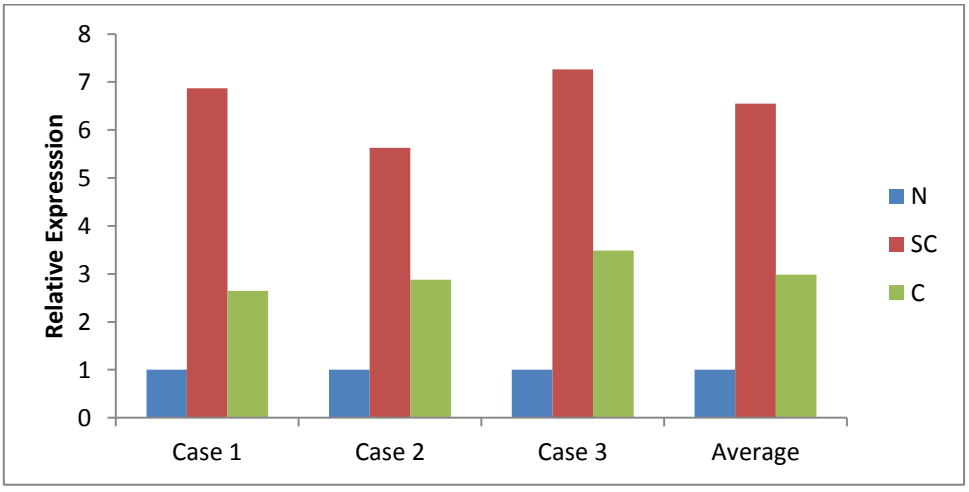
Source of Variation	df	MSS	F value
Between Groups	2	24.03**	79.25
Within Groups	6	0.30	

Table 3: Expression of *TLR4* gene in *E. coli* caused sub-clinical and clinical mastitis

Sample	Cq Mean ± SE		ΔCq	ΔCq Mean	ΔΔCq	RQ
	TLR4	*β-actin*				
Normal	21.68 ± 0.02	15.94 ± 0.03	5.75	**5.75 ± 0.03**		
Sub-Clinical						
Case 1	18.65 ± 0.04	15.68 ± 0.25	2.97		-2.78	6.87
Case 2	18.61 ± 0.06	15.36 ± 0.24	3.25		-2.49	5.63
Case 3	18.94 ± 0.03	16.05 ± 0.11	2.89		-2.86	7.26
				3.04 ± 0.11	**-2.71**	**6.55** a^{**}
Clinical						
Case 1	20.38 ± 0.54	16.04 ± 0.07	4.34		-1.40	2.65
Case 2	20.53 ± 0.18	16.31 ± 0.02	4.22		-1.53	2.88
Case 3	20.41 ± 0.04	16.46 ± 0.01	3.95		-1.80	3.48
				4.17 ± 0.12	**-1.58**	**2.98** $b^{**}c^{**}$

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

Fig. 1: Frequency distribution of *TLR4* gene expression in *E. coli* caused mastitis



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

CONCLUSION

Expression of *TLR4* 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 *TLR4* is relatively higher at this stage compared to clinical stage. Hence, most of the sub-clinical mastitis subsided by itself without precipitating into clinical mastitis. This study sheds light into the innate immune system represents the first line of defense in the host response to infection and is poised to immediately recognize and respond to the earliest stages of infection.

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