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Relative Expression of TLR9 Gene in Natural Sub-clinical and Clinical Cases of Bovine Mastitis caused by *Escherichia coli*

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A B S T R A C T

Mastitis is an inflammatory condition of the mammary tissue resulting in several physiological and metabolic changes, trauma, and more frequently it is caused by contagious or environmental pathogenic microorganisms, including Gram-positive and Gram-negative bacteria. *Escherichia coli* are most common infectious agents responsible for sub-clinical and clinical mastitis in dairy forms. The first line of defense against mammary bacterial infection was initiated by innate resistance of mammary gland. Toll-like receptors (TLRs) are a key family of innate immune proteins, serving as the principal recognition site of pathogens and signalling functions, enabling the host to eliminate pathogens. Hence, this study assessed the expression of an important *TLR9* gene in spontaneous bovine sub-clinical and clinical mastitis caused by *E. coli*. The total RNA was isolated from milk somatic cells converted as cDNA using oligo (dT) primers. Relative quantitation of mRNA of *TLR9* was analysed by RT-qPCR system. The mRNA expression of *TLR9* gene in sub-clinical mastitis was higher (3.22 fold) than clinical case (1.09 fold) when compared to normal bovine case. Relative difference in the expression of *TLR9* gene in sub-clinical and clinical mastitis was found to be significant ($P \leq 0.05$). During sub-clinical stage of infection the expression of *TLR9* gene was at high level, therefore most of the sub-clinical mastitis subsided by itself without precipitating into clinical mastitis. *TLR9* mediated signalling cascades will be fundamental to understand the host immune response against bovine mastitis.

Introduction

Mastitis is one of the major endemic diseases of dairy cattle and its prevalence is increasing in parallel with the development of new high milk producing breeds. *Escherichia coli* is a gram-negative most common infectious agents responsible for subclinical and clinical mastitis in dairy forms (Bradley *et al*., 2007). Innate resistance of mammary gland is one of the first line of host defense against mammary bacterial infection. Toll-like receptors (TLRs) are a key family of innate immune proteins, serving as the principal recognition site of pathogens and signalling functions, enabling the host to eliminate pathogens (Hallman *et al*., 2001). Currently, 13 TLRs have been identified in mammals (*TLR1* to *TLR13*) of which 10 TLRs are reported in cattle (Tabeta *et al*., 2004; Roach *et al*., 2005). Of these,

TLR9 is the main pathogen recognition receptors (PRR), for bacteria and virus. *TLR9* has been demonstrated as a receptor for bacterial DNA comprising unmethylated CpG dinucleotides (Hemmi *et al*., 2000). Specifically, *TLR9* links the innate and adaptive immune system by favouring a Th1 immune response and increasing the production of autoantibody. Bacterial DNA differs from mammalian DNA by its 20-fold-greater frequency of unmethylated CpG sequences (Wagner, 1999). These CpG sequences activate a signaling cascade via transcription factors NF--κB and AP-1 and stimulate the proliferation of B cells and the secretion of proinflammatory cytokines IL-6, IL-12, and tumour necrosis factor alpha are required to eliminate an invading pathogen (Zhao *et al*., 1997; Krieg, 2002). Hence in this study, we investigate the expression of *TLR9* 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 University farm and Veterinary Dispensaries and Teaching Veterinary Clinical Complex of the College of Veterinary and Animal Sciences at Thrissur Kerala. The collected milk samples subjected for California mastitis test (CMT) and somatic cell count (SCC) for detection of sub-clinical, clinical, and normal animals. Further the milk samples subjected for microbiological and biochemical examination for identification of *E. coli* (Quinn *et al*., 2002)

RNA isolation and cDNA synthesis

From the somatic cells, total RNA was isolated using TRIzol reagent of SIGMA (As per the manufacturer's protocol). The quality and concentration of extracted RNA was determined by agarose gel electrophoresis (1.5%) and nanodrop spectrophotometer. Residual DNA was removed from extracted total RNA by treatment with DNase1. For synthesis of cDNA, one microgram of total RNA was taken by using Revert Aid first strand cDNA synthesis kit.

Primers design and synthesis

Primers for RT-qPCR of *TLR9* 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).

RT-qPCR

Real-time PCR was carried out in an Illumina Eco® Q- RT PCR system. The reaction solution was prepared on ice, and consisted of: 10 microlitre of 2X SYBR Green PCR master mix, 10 pmole (1microlitre) of each gene-specific primers, 2 microlitre of cDNA template and 7 microlitre of nuclease free water to final volume of 20 microlitre. The reaction mixtures were incubated in 48 well plate at 95ºC for 10 min followed by 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. All reactions were performed in triplicates. After 40 amplification cycles, all the samples were submitted to dissociation curve analysis to verify the absence of non-specific products and primer dimers. 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 normalized to the housekeeping gene actin and the results are presented as $\Delta\Delta C_q$ values as described (Zheng *et al*., 2007). 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

Mastitis is an inflammatory condition of the mammary gland and that is usually caused by various bacterial infections. Among the bacterial infection, *Escherichia coli* is a gram-negative most common infectious agents responsible for subclinical and clinical mastitis in dairy forms (Bradley *et al*., 2007; Mitra *et al*., 2013). Innate resistance of mammary gland is one of the first line of host defense against mammary bacterial infection. Assessing the expression of important *TLR9* will be vital for improving our understanding of the early events controlling immune response.

Lactating cows were screened for sub-clinical and clinical mastitis Based on this CMT, SCC and biochemical test, 8 samples revealed the presence of *E. coli* in sub-clinical case of mastitis. Similarly, from clinical mastitis cases 5 samples showed the presence of *E. coli*. From each group of sub-clinical and clinical mastitis, three animals selected for RT-qPCR expression study. In addition milk samples from three apparently healthy crossbred cows were also selected as control for expression studies.

Table.1 Primer sequence for TLR9 and β-actin genes used in RT-qPCR

Gene Name		Sequence (5'→3')	Expected product size
TLR9	F	AAGGCTTGAGGAACCTGGTC	119 bp
	R	GTTATTGTCCCGGAGACGCA	
β-actin	F	CCACACCTTCTACAACGAGC	105 bp
	R	ATCTGGGTCATCTTCTCACG	

Table.2 ANOVA for TLR9 gene expression in *E. coli* caused mastitis

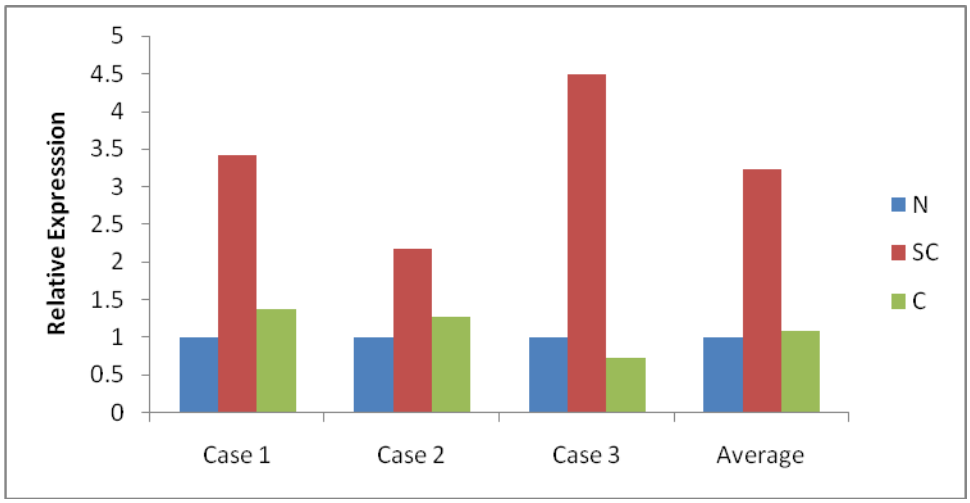
Source of Variation	df	MSS	F value
Between Groups	2	5.30**	10.97
Within Groups	6	0.48	

Table.3 Expression of TLR9 gene in *E. coli* caused sub-clinical and clinical mastitis

Sample	Cq Mean ± SE		ΔCq	ΔCq Mean	ΔΔCq	RQ
	TLR9	*β-actin*				
Normal	29.24 ± 0.62	15.94 ± 0.03	13.30	**13.30 ± 0.03**		
Sub-Clinical						
Case 1	27.21 ± 0.08	15.68 ± 0.25	11.53		-1.77	3.42
Case 2	27.53 ± 0.27	15.36 ± 0.24	12.18		-1.13	2.18
Case 3	27.19 ± 0.10	16.05 ± 0.11	11.14		-2.17	4.49
				11.61 ± 0.30	**-1.69**	**3.22** a^{*}
Clinical						
Case 1	28.88 ± 0.05	16.04 ± 0.07	12.84		-0.46	1.38
Case 2	29.27 ± 0.18	16.31 ± 0.02	12.96		-0.34	1.27
Case 3	30.20 ± 0.42	16.46 ± 0.01	13.74		0.44	0.74
				13.18 ± 0.28	**-0.12**	**1.09** $b^{ns}c^{*}$

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

Fig.1 Frequency distribution of TLR9 gene expression in *E. coli* caused mastitis



Analysis of variance for *TLR9* gene revealed significant difference ($P \leq 0.01$) for expression level between the groups (Table 2). The mean values of C_q, ΔC_q, $\Delta\Delta$Cq along with standard error and relative quantification of *TLR9* expression in *E. coli* caused mastitis are given in table 3. The relative expression of *TLR9* gene was ranged between 2.18 and 4.49 fold for sub-clinical mastitis and it was ranged from 0.74 to 1.38 fold, in clinical mastitis when compared with healthy crossbred cows (Figure 1). *E. coli* infected mammary gland shows mRNA levels of *TLR9* was higher in sub-clinical mastitis (3.22 fold) followed by clinical mastitis (1.09 fold) when compared to normal animal. Relative expression of *TLR9* gene was significantly ($P \leq 0.05$) higher in the sub-clinical mastitis, and also exhibits significant difference ($P \leq 0.05$) between sub-clinical and clinical mastitis (Figure 1).

Toll-like receptors (TLRs) are a key family of innate immune proteins, serving as the principal recognition site of pathogens and signalling functions, enabling the host to eliminate pathogens. *TLR9* is principal receptor for bacteria and virus. In our study *TLR9* was expressed with 3.22 fold upregulation in sub-clinical case of mastitis caused by *E. coli* infection. This observation was similar to a study by Zhu *et al.,* (2008), which demonstrated that murine mammary gland infected with *E. coli* showed *TLR9* expression significantly higher in infected group. Similarly, goat mammary gland infected with *E. coli* also showed increased expression of *TLR9* in infected group than control (Zhu *et al.*, 2007).

The *TLR9* signalling pathway was activated by the interaction of *TLR9* with CpG-DNA and proceeds through MyD88, TRAF6, IRAK, and consequently involves the activation of MAPK and NF-κB factors followed by production of pro inflammmatory cytokines, IL-6, IL-12, and tumour necrosis factor alpha are required to eliminate an invading pathogen.

In conclusion, the study majorly underpins the significant *TLR9* expression in sub-clinical mastitis eliciting prompt host immune response in *E. coli* intramammary infection. Sub-clinical mastitis is an early stage of infection, and hence the expression of *TLR9* is relatively higher at this stage compared to clinical stage. *TLR9* mediated signalling cascades will be fundamental to understand the host immune response before developing effective strategies to combat mastitis.

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References

Bradley, A.J., K.A. Leach, J.E. Breen, L.E. Green and Green, M.J. 2007. Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales. *Vet. Rec.*, 160: 253-7.

Hallman, M., M. Ramet and Ezekowitz, R.A. 2001. Toll-like receptors as sensors of pathogens. *Pediatr. Res.*, 50: 315-321.

Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda and Akira, S. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature*, 408: 740-745.

Krieg, A.M. 2002 CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.*, 20: 709-760.

Mitra, S.D., D. Velu, M. Bhuvan, N. Krithiga, A. Banerjee, R. Shome, H. Rahman, S.K. Ghosh and Shome, B.R. 2013. *Staphylococcus aureus* spa type t267, clonal ancestor of bovine subclinical mastitis in India. *J. Appl. Microbiol.*, 114: 1604-15.

Quinn, P.M., B. Carter, Markey and Carter, G. 2002. Clinical veterinary microbiology mos by International Ltd, Spain pp. 96-344.

Roach, J.C., G. Glusman, L. Rowen, A. Kaur, M.K. Purcell, K.D. Smith, L.E. Hood and Aderem, A. 2005. The evolution of vertebrate Toll-like receptors. *Proc. Natl. Acad. Sci. U.S.A*, 102: 9577-9582.

Tabeta, K., P. Georgel, E. Janssen, X. Du, K. Hoebe, K. Crozat, S. Mudd, L. Shamel, S. Sovath, J. Goode, L. Alexopoulou, R.A. Flavell and Beutler, B. 2004. Toll-like receptors 9 and 3 as

essential components of innate immune defense against mouse cytomegalovirus infection. *Proc. Natl. Acad. Sci. USA.*, 101: 3516-21.

Wagner, H. 1999. Bacterial CpG DNA activates immune cells to signal infectious danger. *Adv. Immunol.*, 73: 329-368.

Zhao, Q.J., R. Temsamani, Zhou and Agrawal, S. 1997. Pattern and kinetics of cytokine production following administration of phosphorothioate oligonucleotides in mice. *Antisense Nucleic Acid Drug Dev.*, 7: 495–502.

Zheng, Y., D.M. Danilenko, P. Valdez, I. Kasman, J. Eastham-Anderson, J. Wu, and Ouyang, W. 2007. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature*, 445: 648-651.

Zhu, Y.M., J.F. Miao., Y.S. Zhang, Z., Li, S.X. Zou and Deng, Y.E. 2007. CpG ODN enhances mammary gland defense during mastitis induced by *Escherichia coli* infection in goats. *Vet. Immunol. Immunop.*, 120: 168-176.

Zhu, Y., H. Fan., J. Miao and Zou, S. 2008. Protective effect of CpG-DNA against mastitis induced by *Escherichia coli* infection in a rat model. *Vet. J.*, 175: 369-378.

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