International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2017; 5(4): 154-156

Received: 08-05-2017
Accepted: 10-06-2017

R Lakshmi
Post-Doctoral Fellow, Department of Veterinary Biochemistry, Rajiv Gandhi Institute of Veterinary Education & Research, Puducherry

KK Jayavardhanan
Professor and Head, Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences, Thrissur, Kerala

J Thanislass
Associate Professor and Head, Department of Veterinary Biochemistry, Rajiv Gandhi Institute of Veterinary Education & Research, Puducherry

Correspondence
R Lakshmi
Post-Doctoral Fellow, Department of Veterinary Biochemistry, Rajiv Gandhi Institute of Veterinary Education & Research, Puducherry

Toll like receptor-4 gene expression assay in mastitis caused by *Stapylococcus aureus* in crossbred cattle

R Lakshmi, KK Jayavardhanan and J Thanislass

Abstract
Mastitis is inflammation of mammary gland characterized by infiltration of leukocytes in udder tissue in response to various bacterial invasions. *Staphylococcus aureus* is the etiological agent more commonly associated to the disease and is normally related to both subclinical and chronic infections leading to severe economic loss to dairy farms. *TLR4* is involved in the initiation of the immune response to different pathogens, including Gram-negative and Gram-positive bacteria, fungi, and viruses. This study was carried out to assess the expression of *TLR4* gene in spontaneous bovine sub-clinical and clinical mastitis caused by *S. aureus*. RNA isolated from milk somatic cells was converted as cDNA using oligo (dT) primers. mRNA expression of *TLR4* was analysed by RT-qPCR system. The mRNA expression of *TLR4* gene in sub-clinical mastitis was higher (2.22 fold) than clinical case (1.62 fold) when compared to normal bovine case. The expression of *TLR4* gene at high level during sub-clinical stage of infection, may be subsided itself without precipitating into clinical mastitis. This study sheds light into the innate immune system represents the first line of defence in the host response to infection and is poised to immediately recognize and respond to the earliest stages of infection.

Keywords: expression assay, mastitis caused, *Stapylococcus aureus*, crossbred cattle

1. Introduction
Mastitis is inflammation of mammary gland characterized by infiltration of leukocytes in udder tissue in response to various bacterial invasions. The contagious pathogens are important in causing the subclinical and clinical form of mastitis. Although mastitis can be caused by 137 different microorganisms [1], *Staphylococcus aureus* is the etiological agent more commonly associated to the disease and is normally related to both subclinical and chronic infections leading to severe economic loss to dairy farms [2, 3]. The cell wall of gram positive bacteria comprises a thick layer of PGN (peptidoglycan) within which lipoteichoic acids and lipoproteins are embedded, can incite immune responses of the host system. Innate immune mechanism forms the bottom line of defence in neutralizing constant threat of micro-organism invasion. Activation of innate immunity is essential to initiate subsequent adaptive immune responses. Early recruitment of neutrophils at the site of inflammation will reduce the severity of infection. Toll-like receptors (*TLR*) are the best-described innate receptors and provide critical host defence during bacterial infection. Currently, in mammals 13 TLRs have been identified of which 10 TLRs are reported in cattle [4]. Each TLRs recognize the distinctive molecular signatures of microbes and activate the innate immune system as well as adaptive system [5]. *TLR4* was the first described mammalian *TLR* [6]. *TLR4* is involved in the initiation of the immune response to different pathogens, including Gram-negative and Gram-positive bacteria, fungi, and viruses. Thus, this study was planned to assess the comparative expression levels of TLR4 in sub-clinical and clinical mastitis caused by *S. aureus* infection. The results presented help to facilitate deeper insight into the specific regulatory mechanisms of bovine mastitis.

Materials and Methods
Ethical Approval
The study was approved by the research committee framed by the University. Ethical approval was not required, as no life animals were used in this study. However, adequate measures were taken to minimize pain or discomfort in accordance with the International Animal Ethics Committee.

Milk Sample collection and identification of bacteria

Eighty Milk samples were collected from University farm and Veterinary Dispensaries at Mannuthy, Kerala. Collected milk samples were categorized into subclinical, clinical and normal based on California mastitis test (CMT) and somatic cell count (SCC). The *S. aureus* infected milk samples were identified by culture and gram's staining for identification of *Staphyllococcus* and then further subjected to biochemical tests for identification of species specific *S. aureus* [7]. From each group three samples were selected for further study.

RNA isolation and cDNA synthesis

Total RNA was extracted from milk somatic cells by using TRIzol reagent of SIGMA (As per the manufacturer's protocol). The isolated RNA samples treated with enzyme DNase to remove DNA contamination. The quality or integrity of extracted RNA was assessed electrophoretically using 0.8 per cent agarose (Invitrogen) gel electrophoresis unit. Complementary DNA was synthesised from isolated RNA using RevertAid first strand cDNA synthesis kit by (Thermo Scientific, K1622) using oligo dT primers (Thermo Scientific, K1622).

Primers design and synthesis

Primers for RT-qPCR for *TLR4* 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).

Real Time PCR

The mRNA expression level of TLR4 were analysed by Illumina Eco® Q- RT PCR system by using gene specific primers. The β-actin was used as endogenous control gene because it showed a stable expression from all milk samples. A reaction solution was prepared on ice, a total volume 20 microlitre consists of 10 microlitre of 2X SYBR Green PCR master mix, 10 pmole (1microlitr) of each gene-specific primers, 2 microlitre of cDNA template and 7 microlitre of nuclease free water. All reactions were performed as triplicates. The dissociation curve analysis PCR products was carried out to determine the specificity of the amplicons. 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 $2^{-\Delta\Delta CT}$ method [8]. All statistical analyses were done using Statistical Product and Service Solution (SPSS) version 21.0 software

Results and Discussion

S. aureus is a common contagious pathogen recovered from cases of sub-clinical and clinical mastitis. Recurrent and persistent mammary infection by *S. aureus* 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 Based on biochemical test, 10 revealed the presence of *S. aureus* in sub-clinical case of mastitis. Similarly, from clinical mastitis cases 8 samples showed the presence of *S. aureus*. 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.

Analysis of variance for 4 gene revealed significant deference ($P \leq 0.01$) 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 *TLR*4 expression in *S. aureus* caused mastitis are given in Table 3. Relative expression of *TLR4* expression in sub-clinical mastitis ranged from 1.57 to 3.20, with a mean of 2.22 fold upregulation. In case of clinical mastitis, relative expression of TLR4 varied between 1.44 and 1.79 Relative expression of *TLR*4 was found to be significantly higher ($P \leq 0.01$) in sub-clinical mastitis cows when compared with healthy (normal) cows, and clinically-affected cows.

Toll-like receptors (*TLR*) are the best-described innate receptors, can be quickly activated, and comprise of functional molecule that provide critical host defence during bacterial infection. Different TLRs recognize the distinctive molecular signatures of microbes and activate the innate immune system as well as adaptive system [5]. *TLR4* is involved in the initiation of the immune response to different pathogens, including Gram-negative and Gram-positive bacteria, fungi, and viruses. In present study, the mRNA expression of *TLR4* in *S. aureus* caused mastitis was higher and significant ($P \leq 0.01$) in sub-clinically affected animals as compared with non-affected animals by 2.22 fold, indicating higher expression of *TLR4* in sub-clinical mastitis. This observation was in agreement with study by Goldammer *et al*. [9] who found increased levels of *TLR4* mRNA in mastitis infections caused by S. *aureus*. Moyes *et al.* [10] also reported up-regulation of *TLR4* during intramammary infection by *S. aureus*. These results disclose the vital role of *TLR*s in early innate immune response in udder against mastitis. The *TLR4* signalling pathway was activated by the interaction of *TLR4* with microbial compounds from gram positive and negative bacteria and proceeds through MyD88 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 During early stage of infection these *TLR*4 are expressed at high level to defend against pathogens, hence most of the sub-clinical mastitis may subsided by itself without precipitating into clinical mastitis.

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

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

Table 2: ANOVA for *TLR*4 gene expression in *S. aureus* caused mastitis

Source of Variation	df	MSS	F value
Between Groups	2	1.31**	5.53
Within Groups	6	0.24	

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

Sample	Cq Mean ± SE		ΔCq	ΔCq Mean	ΔΔCq	RQ
	TLR4	*β-actin*				
Normal	21.68 ± 0.10	15.94 ± 0.03	5.75	**5.75 ± 0.03**		
Sub-clinical						
Case 1	20.55 ± 0.07	16.48 ± 0.29	4.07		-1.68	3.20
Case 2	20.50 ± 0.32	15.40 ± 0.25	5.09		-0.65	1.57
Case 3	20.87 ± 0.08	16.25 ± 0.29	4.62		-1.13	2.19
				4.59 ± 0.30	**-1.15**	**2.22** a^{**}
Clinical						
Case 1	21.41 ± 0.15	16.38 ± 0.25	5.03		-0.72	1.64
Case 2	21.37 ± 0.25	16.46 ± 0.27	4.91		-0.84	1.79
Case 3	21.58 ± 0.17	16.36 ± 0.10	5.22		-0.53	1.44
				5.05 ± 0.09	**-0.69**	**1.62** $b^{ns}c^{**}$

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

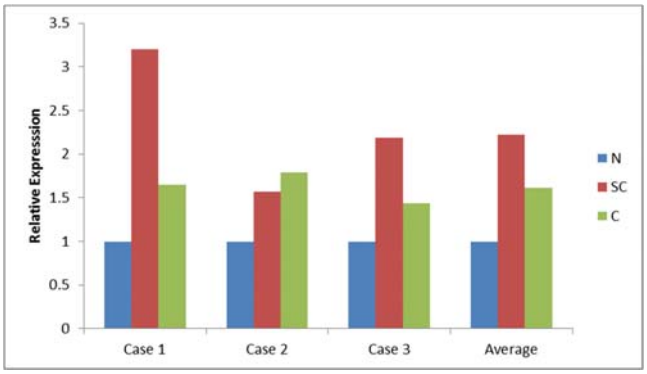

Fig 1: Relative expression of *TLR4* gene in mastitis caused by *S. aureus*

Conclusion

The study majorly underpins the significant *TLR4* expression in subclinical mastitis eliciting prompt host immune response in *S. aureus* intramammary infection. Sub-clinical mastitis is an early stage of infection, and hence the expression of *TLR*4 is relatively higher at this stage compared to clinical stage.

Acknowledgments

The research work mentioned in this article is a part of the Ph.D. research work of the first author under the guidance and supervision of the second author. The first author acknowledging the INSPIRE Fellowship program of Department of Science and Technology for providing fellowship for the Ph. D program. The authors are thankful to the Dean, College of Veterinary and Animal Science for providing facilities to conduct this experiment.

References

1. Ranjan RD, Swarup RC, Patra, Nandi D. Bovine protothecal mastitis: a review. Perspectives in Agriculture; Veterinary Sciences; Nut. Nat. Res. 2006; 17:1- 7.
2. Philpot WN, Nickerson SC. Winning the Fight Against Mastitis. Westfalia-Surge Inc.; Naperville, 2000.
3. Kubota M, Hayashi T, Iwasaki K, Ohtsuka H, Kohiruimaki M, Kawamura S *et al*. and Effective Method for Separation of *Staphylococcus aureus* from Somatic Cells in mastitis milk. J. Dairy. Sci. 2007; 90: 4100 4107
4. Tabeta K, Georgel P, Janssen E, Du X, Hoebe K, Crozat K, *et al*. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc. Natl. Acad. Sci. USA*. 2004; 101:3516-21.
5. Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S. Host innate immune receptors and beyond: making sense of microbial infections. *Cell Host Microbe.* 2008; 3:352-63.
6. Takeda K, Kaisho T, Akira S. Toll-like receptors. Ann. Rev. Immunol. 2003; 21:335-376.
7. Quinn PM, Carter B, Markey, Carter G. Clinical veterinary micro biology mos by international Ltd, Spain 2002, 96-344.
8. Kumar R, Tiwari AK, Chaturvedi U, Kumar GR, Sahoo AP, Rajmani RS, *et al*. Velogenic newcastle disease virus. Appl Biochem Biotechnol 2012; 167(7):2005-2022.
9. Goldammer T, Zerbe H, Molenaar A, Schuberth HJ, Brunner RM, Kata SR *et al*. Mastitis increases mammary mRNA abundance of *β* -Defensin 5, Toll-like-receptor 2 (TLR2) and TLR4 but not TLR9 in cattle. *Clin. Diag. Lab. Immunol*. 2004; 11:174-185.
10. Moyes KM, Drackley JK, Morin DE, Bionaz M, Rodriguez-Zas SL, Everts RE *et al*. Gene network and pathway analysis of bovine mammary tissue challenged with *Streptococcus uberis* reveals induction of cell proliferation and inhibition of PPAR gamma signaling as potential mechanism for the negative relationships between immune response and lipid metabolism. *BMC Genomics*. 2009; 10:542.