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Characterization of TLR expression in *Staphylococcus aureus* induced mastitis in mice model by probe based real time PCR

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ABSTRACT

Staphylococcus aureus infection of the bovine mammary gland can result in a spectrum of clinical outcomes ranging from acute to chronic and subclinical. A molecular understanding of the principles causing this is particularly important to eventually develop innovative strategies for prevention and treatment in mastitis. The present study characterized the temporal (2, 4, 8, 12, 24 and 48h) expression of 5 important toll like receptors (TLR 2, TLR 4, TLR 9, TLR 11 and TLR 12) induced by *S. aureus* intramammary inoculation (IMI) in established mice model employing probe based real time PCR (RT-PCR). *S. aureus* challenge provoked transcriptional expression of each of the 5 TLRs in a temporal manner with maximum expression at 8 h after IMI. *S. aureus* maximally altered the mRNA concentration of TLR 9 and TLR 12 (> 5 fold) over the PBS control. We found TLR2 to be weakly (1- to 2-fold) regulated by the *S. aureus* challenge in this study. This study emphasized the contribution of other TLRs besides the most commonly studied TLR 2 and TLR 4 in activating immune response by switching on several receptors mediated signalling cascades. This study sheds light into the diverse pathogen recognition receptor (PRR) mediated mechanisms involved in host pathogen interaction.

Key words: Bovine mastitis, Intramammary infection, *Staphylococcus aureus,* Toll like receptors

Mastitis is a highly prevalent and costly disease of dairy cows that is commonly caused by intramammary bacterial infection. *Staphylococcus aureus* is one of the most common pathogen to induce bovine mastitis characterized by a moderate and delayed SCC increase (Bannerman *et al*. 2004) and infection can ultimately result in subclinical, chronic and sometimes lifelong infection (Wellnitz *et al*. 2012). For Gram positive bacteria like *S. aureus*, the cell wall component lipotechoic acid (LTA) has been shown to activate immune cells (Lee *et al.* 2006). Innate immunity is a critical first line defence against pathogens, sensed by the fundamental PRRs (Quinn and Neil 2011). TLRs are vital to immune function through the sensing of pathogenic agents and initiation of an appropriate immune response (Akira *et al*. 2006). Variations in TLR abundance are associated with altered immune responsiveness.

Currently, 13 TLRs have been identified in mammals (Tabeta *et al*. 2004). A study confirmed the presence of TLRs 1–10 in bovine tissues (Menzies and Ingham 2006). Until now, much work has been conducted with Peptidoglycan and LTA to study the level of TLR 2 and TLR 4 response, however very little is known about the other less studied receptors such as TLR 9 (Fournier and Philpott 2005). Assessing the expression of important TLRs will be vital for improving our understanding of the early events controlling immune response.

Hence, in the present study we used established mice model to investigate the relative mRNA transcript level of the 5 important TLRs (TLR 2, TLR 4, TLR 9, TLR 11 and TLR 12) expressions in *S. aureus* induced intramammary infection in a temporal manner (2, 4, 8, 12, 24, 48h) over PBS injected control tissue. RNA was extracted from infected tissue and the expression of the selected TLR genes was quantified by probe based RT- PCR at different time points during experimentally induced mastitis.

MATERIALS AND METHODS

Experimental challenge with S. aureus: Based on our previous study, the most predominant clone found endemic in the province causing subclinical mastitis (Mitra *et al*. 2013) was selected for animal experiments. We took one representative field isolate of *S. aureus* (HF16Y) from this predominant group, to challenge in the established mice model. This *S. aureus* was grown in brain heart infusion (BHI) broth overnight and centrifuged at 3000× g for 5 min

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Table 1. Sequence of primers used in quantitative RT- PCR

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Probe Cat No (Universal probe library)	AMP size (bp)
TLR 2	CTGCACTGGTGTCTGGAGTC	GGGCACCTACGAGCAAGAT	#2, cat.no. 04684982001	101
TLR 4	GGACTCTGATCATGGCACTG	CTGATCCATGCATTGGTAGGT	#2, cat.no. 04684982001	101
TLR 9	CTCGGAACAACCTGGTGACT	ACTGGAGGCGTGAGAGATTG	#106, cat.no. 04692250001	60
TLR 11	ATGGGGCTTTATCCCTTTTG	AGATGTTATTGCCACTCAACCA	#1, cat.no. 04684974001	60
TLR 12	TTTCAAGCACTGGCCTAACC	GAAGCCTAGGCATGGCAGT	#31, cat.no. 04687647001	60
GAPDH	AGCTTGTCATCAACGGGAAG	TTTGATGTTAGTGGGGTCTCG	#9, cat.no. 04685075001	62

at room temperature to pellet the cells. S. aureus pellet was washed with sterile cell culture tested PBS twice to remove all the media components and finally resuspended in PBS. Standard plate count (SPC) method was employed to adjust the bacterial load at a cell density of 5×10^3 cfu/ 50µl for intramammary inoculation. The bacterial challenge in mice model was done according to Modak *et al*. (2012) and Krishnamoorty *et al*. (2014). For each time point, a set of three Swiss albino mice were used for intramammary inoculation (IMI). For control sample PBS was inoculated in mice mammary gland. Mammary gland tissues were collected at different time points post infection (2, 4, 8, 12, 24 and 48 h). The samples were collected in RNAlater® and incubated at 4°C overnight. RNA stabilized tissues were flash frozen in liquid nitrogen after discarding the RNA-later and stored at –80°C.

RNA extraction and quantitative real-time PCR: Total RNA was extracted from mouse mammary tissues using RNAeasy mini kit as per manufacturer's instruction. RNA preparations were stored at - 80°C till further use. 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 spectrophotometer. The quantification of relative mRNA concentrations by quantitative real-time PCR (qRT-PCR) of TLR genes was performed using gene specific primers and probes from Universal Probe Library (Table 1) using Roche Lightcycler® 480 real-time PCR system. Total mRNA present in the samples was converted to cDNA. Quantitative RT- PCR was done using a kit. The 3 µg of total RNA was subjected to cDNA synthesis using cDNA synthesis kit under the following conditions: denaturation at 95°C for 10 sec and amplification via 40 cycles of 95°C for 5 sec and 60°C for 34 sec. To determine TLR mRNA levels, the TLR copy number was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using ΔΔCT inbuilt algorithim in the LC480 system.

RESULTS AND DISCUSSION

S. aureus is a common pathogen recovered from cases of subclinical mastitis (Gunther *et al*. 2011, Mitra *et al*. 2013). Recurrent and persistent mammary infection by *S. aureus* is serious problem for the dairy animals. To eventually develop innovative strategies to prevent and treat subclinical mastitis, understanding the host pathogen interaction is necessary. Pathogen recognition receptors (PRR) are the first to recognize the invading pathogens to elicit an immune response. In this study, *S. aureus* intramammary infection in mice model provoked transcriptional expression of each of the 5 TLRs viz., TLR2, TLR4, TLR9, TLR11 and TLR 12 in a temporal manner (Fig. 1, Table 2). The mRNA abundance of these target genes was calibrated with that of a reference gene (GAPDH) and expressed as fold over expression of the genes in *S. aureus* infected tissue over the PBS injected control tissue calculated and plotted as average of 3 biological replicates.

S. aureus challenge achieved the maximum impact on the mRNA levels of 5 TLRs at 8 h after IMI. Amongst the 5 TLRs, *S. aureus* maximally altered the mRNA concentration of TLR 9 and TLR 12 (> 5 fold) over the PBS control. TLR 4 and TLR 11 also showed up regulation (4- to 5-fold) at 8 h after IMI (Table 1). We found TLR 2 to be weakly (1-2 fold) regulated by the *S. aureus* challenge in this study.

Activation of TLRs subsequently induces a series of signalling cascades that ultimately result in the activation of NF-8B and other immune responsive genes (Modak *et al*. 2012). The contribution of different TLRs to infection depends on the site of the infection and the pathogen. In the present study, TLR 9 was profoundly expressed with 6 fold upregulation post infection (Fig 2 C). A TLR that is

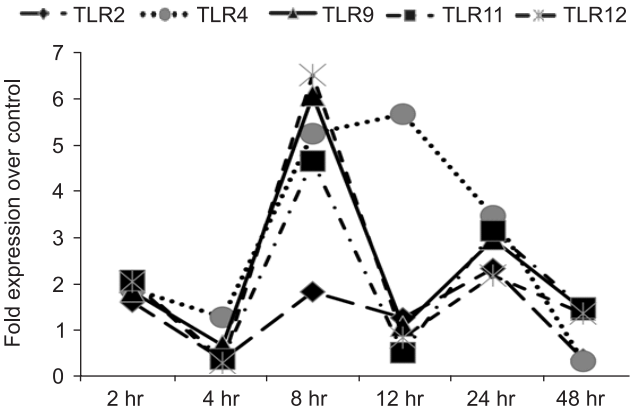

Fig. 1. Comprehensive representation of temporal expression of TLR genes in the *S. aureus* infected mice mammary tissue analyzed by real time quantitative RT-PCR. Fold over expression of the genes in *S. aureus* infected tissue over the PBS injected tissue was calculated and plotted as average of 3 biological replicates (Table 2).

Table 2. Temporal relative expression of TLR in the *S. aureus* infected mice mammary tissue. Fold over expression of the genes in *S. aureus* infected tissue over the PBS injected tissue was calculated as average of three biological replicates

Time post infection	TLR2	TLR4	TLR9	TLR11	TLR12
2HRS A	1.306	1.436	0.9539	1.075	1.004
2HRS B	1.313	2.355	3.55	4.167	4.093
2HRS C	2.113	1.789	0.9033	0.9033	0.9526
MEAN	1.577333	1.86	1.8024	2.048433	2.016533
ST DEV	0.463914	0.463596	1.513677	1.83674	1.798457
SE	0.267849	0.267665	0.873948	1.060473	1.03837
4HRS A	0.2851	0.7812	0.7696	0.1096	0.1862
4HRS B	0.547	2.266	0.7667	0.758	0.505
4HRS C	0.161	0.6983	0.393	0.1646	0.083
MEAN	0.331033	1.2485	0.6431	0.344067	0.258067
ST DEV	0.197057	0.882155	0.216598	0.35953	0.219988
SE	0.113774	0.509327	0.125056	0.207581	0.127014
8HRS A	0.9675	8.636	9.254	3.914	5.849
8HRS B	1.964	1.597	5.761	6.346	9.482
8HRS C	2.46	5.437	3.113	3.633	4.152
MEAN	1.797167	5.223333	6.042667	4.631	6.494333
ST DEV	0.760108	3.524361	3.080174	1.491864	2.72297
SE	0.438861	2.03485	1.778391	0.861354	1.572154
12HRS A	1.299	2.576	1.367	0.826	1.081
12HRS B	1.048	4.985	0.6845	0.092	0.456
12HRS C	1.361	9.374	1.115	0.526	0.886
MEAN	1.236	5.645	1.0555	0.481333	0.807667
ST DEV	0.165738	3.446723	0.345118	0.369033	0.319779
SE	0.095692	1.990025	0.19926	0.213068	0.18463
24HRS A	1.87	1.802	1.809	1.665	1.335
24HRS B	1.711	1.469	5.559	6.665	4.004
24HRS C	2.303	4.439	1.424	1.034	1.105
MEAN	1.961333	2.57	2.930667	3.121333	2.148
ST DEV	0.306386	1.627143	1.865147	3.08508	1.611452
SE	1.13241	1.483834	1.692071	1.802156	1.240185
48HRS A	0.6664	0.8177	0.9269	1.013	0.8423
48HRS B	0.098	0.002	2.846	3.64	3.006
48HRS C	0.6056	1.505	1.056	0.8295	0.9276
MEAN	0.456667	0.7749	1.609633	1.8275	1.591967
ST DEV	0.312099	0.752414	1.072669	1.57235	1.225331
SE	0.180195	0.434419	0.619324	0.907823	0.707466

well positioned to respond to microbial invasion is TLR 9. Toll-like receptor 9 (TLR9) is a pattern-recognition receptor that is involved in immune signalling and plays a crucial role in cell survival through recognition of various bacterial components including unmethylated CpG-DNA (Tanaka *et al*. 2010). Specifically, TLR 9 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. Similarly, there are reports on TLR 11 playing an important role in mediating TNF-α induction and systemic inflammation in response to *Salmonella* infection (Shi *et al*. 2012). Accumulated reports revealed that innate immune system recognizes Gram positive bacteria mainly through TLR 2 (Gerold *et al*. 2007, Takeda *et al*. 2003) (Fig 2A). However, a recent study reported that mastitis, induced by gram positive or gram negative bacteria increases mammary abundance of both TLR 2 and TLR 4 (Lee *et al*. 2006). The present study did not show significant up-regulation of TLR 2 which is the prime TLR reported to be activated by gram positive bacteria. Several studies showed that the induction of TLR 2 is mainly controlled by NF-êB and Sp1 transcriptional factors. There are studies revealing the critical role of transcription factors in the regulation of TLR expression and function in cells of the innate immune system. Moreover, host innate responses against both extracellular and intracellular bacterial pathogens are more dramatically impaired in mice that lack the common adaptor molecule MyD88 than in mice that are deficient in a single TLR (e.g., TLR 4 or TLR 2). Collectively, these data indicated that multiple MyD88–dependent TLRs are required for the maintenance and/or full expression of protective innate antibacterial responses.

Significant activation of 4 TLRs (TLR 4, 9, 11 and 12)

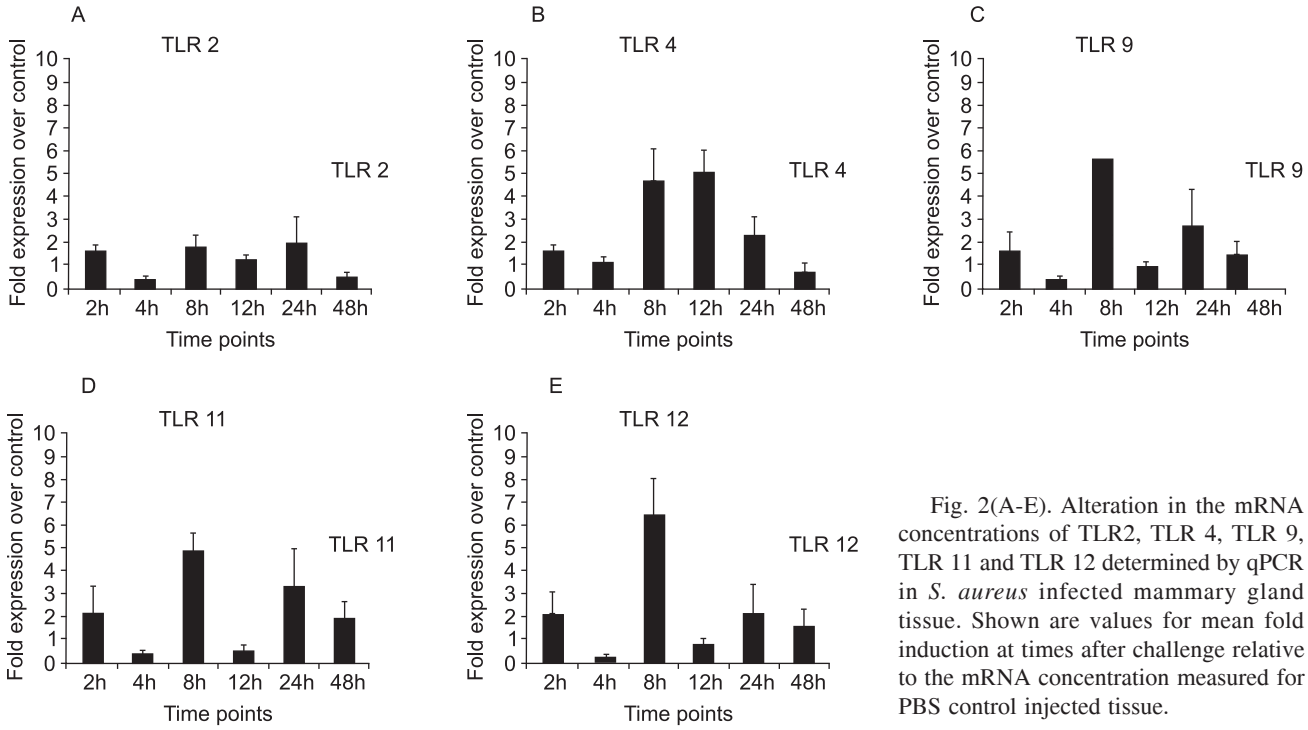

Fig. 2(A-E). Alteration in the mRNA concentrations of TLR2, TLR 4, TLR 9, TLR 11 and TLR 12 determined by qPCR in *S. aureus* infected mammary gland tissue. Shown are values for mean fold induction at times after challenge relative to the mRNA concentration measured for PBS control injected tissue.

(Fig. 2) indicated stronger response, which will be further able to activate 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 all toll-like receptors (TLRs) (except TLR3) (Gazzinelli and Denkers 2006, Takeuchi and Akira 2010), which is essential for the optimal production of IL-10, IL-12, TNF-α, and IFNγ, all of which are important mediators of host survival (Yone *et al.* 2012).

The upregulation and downregulation of genes are under the control of several regulatory mechanisms in host immune response. TLR-signalling pathways are also likely to stringently regulate tissue maintenance and homeostasis by elaborate modulator mechanisms. These include physical interactions, conformational changes, phosphorylation, ubiquitylation, and proteasome-mediated degradation involving various regulatory molecules. Among the many regulatory molecules, microRNAs (miRNAs) have received considerable attention as a newly identified family of regulators involved in fine-tuning the TLR expression (He *et al*. 2014). Any kind of negative feedback will bring down the TLR expression and positive feedback will up regulate the TLR level. Further investigations are necessary to understand and elucidate the role of key players regulating the immune response in *S. aureus* IMI.

In conclusion, the study majorly underpins the significance of various TLR in eliciting prompt host immune response in *S. aureus* IMI. It emphasizes the role of the less studied receptors like TLR 9, TLR 11 and TLR 12, which may also significantly contribute to the activation of NF-kB and production of cytokines in response to *S. aureus* infection. These results collectively indicated an important role for various TLRs in concert to tightly control the outcome of *S. aureus* intramammary infection. Further downstream investigation of the wide range of immune genes transcribed as a result of these TLR mediated signalling cascades will be fundamental to understand the immune response before developing effective strategies to combat mastitis.

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