Expression of TLR2 gene in bovine mastitis associated with *Staphylococcus aureus*

R Lakshmi, KK Jayavardhanan and J Thanislass

Abstract

The present study evaluated the expression of TLR2 in milk somatic cells bovine mammary glands infected with *Staphylococcus aureus*. The research work was carried out in college of Veterinary and Animal Science, Mannuthy, Kerala, during April 2015 to December 2015. Milk samples were screened for subclinical and clinical mastitis caused by *S. aureus*. For the expression assay, total RNA from milk somatic cells was isolated and converted as cDNA using oligo (dT) primers. Relative expression of mRNA of TLR2 gene was analysed using Illumina Eco® RT-qPCR system. Analysis of variance revealed significant difference (P ≤ 0.01) for expression of TLR2 gene in subclinical and clinical mastitis caused by *S. aureus*. The relative expression of TLR2 gene varied from 7.04 to 8.87 fold increase in subclinical mastitis cases caused by *S. aureus* with a mean of 7.89 fold, as compared to normal. In case of clinical mastitis, relative expression varied between 1.67 to 3.58 with a mean of 2.28 fold, when compared with healthy (normal) cows. The relative expression of TLR2 was highly significant (P ≤ 0.01) in sub-clinical affected cattle compared with clinically affected cattle. The expression of TLR2 gene at high level in subclinical mastitis might assist to subside itself without precipitating into clinical mastitis. Therefore, TLR2 might consider as candidate gene to screen for mastitis resistance in cattle population, and *S. aureus*.

Keywords: TLR2, expression, mastitis

1. Introduction

Mastitis, a potentially fatal mammary gland infection is one of the most prevalent production diseases in dairy herds world-wide. This infection of the mammary gland is caused by both Gram-positive and negative bacteria [1], *Staphylococcus aureus*, a Gram-positive coccus, has been the most predominant contagious pathogen of bovine mastitis with a characteristic pathogenicity and poses serious problems to the dairy industry as well as drawing public concerns. Mastitis caused by *S. aureus* predominantly leads to chronic infection that can pursue for the life of the cattle [2, 3]. The innate immune response is the chief line of defence during the initial stage of infection and is induced rapidly at the site of infection [4]. 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 [5]. TLR2 is uniquely capable of recognizing peptidoglycan and lipotechoic acid from *S. aureus* and other gram positive bacteria to initiate host immunity [6]. Therefore, the present study was intended to assess the relative expression levels of TLR2 gene, in subclinical and clinical mastitis caused by *S. aureus* infection.

2. Materials and Methods

2.1 Animal care

All experimental procedures were performed according to the guidelines of the Institutional Animal Ethics Committee of Kerala Veterinary and Animal Sciences University.

2.2 Sample collection and identification causative organism

The research work was carried out in college of Veterinary and Animal Science, Mannuthy, Kerala, during April 2015 to December 2015. Raw milk samples from eighty animals were collected from Veterinary University farm and dispensaries at Mannuthy, Kerala. Milk samples were categorized into three groups subclinical, clinical and normal (apparently healthy) based on California mastitis test (CMT) and somatic cell count (SCC). The milk samples were streaked on Muller Hinton agar plates for identification of *Staphylococcus* genus.
Further *S. aureus* species specific colonies were identified from the positive *Staphylococcus* culture using biochemical test [7].

### 2.3 RNA isolation and cDNA synthesis

Total RNA extraction was carried out from milk somatic cells by using TRIzol reagent of SIGMA [8]. The RNA samples were quantified by NanoDrop spectrophotometer (Thermo Scientific, Waltham, USA) and integrity of extracted RNA was assessed electrophoretically using 0.8 per cent agarose gel in a horizontal submarine electrophoresis unit. Reverse transcription was performed to synthesize cDNA from total RNA by using Revert Aid first strand cDNA synthesis kit (Thermo Scientific, K1622). Primers for RT-qPCR of TLR2 and β-actin were designed from published bovine mRNA sequences available from GenBank using Primer3 software (Table 1).

### 2.4 RT-qPCR

Expression level of mRNA of TLR2 were analysed by real-time quantitative PCR (RT-qPCR) by using specific primers designed for TLR2 gene. Three samples from subclinical and clinical and a normal sample with three technical replicates were subjected for the expression assay of TLR2 along with β-actin gene. RT-qPCR assay was carried out in 20 microlitre reaction volume consists of 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. The reactions were performed using cycling values of 95°C for 5 min followed by 40 cycles of 95°C for 30 s, 58°C for 25 s, 72°C for 30 s. Melt curve analysis of PCR products was performed to determine the specificity of the amplicons. The expression of gene level was analysed by using the 2^ΔΔCq method [9]. The relative expression of TLR2 mRNA was normalized using β-actin gene.

### Statistical analysis

Analysis of variance (ANOVA) was performed to test the significance among the groups under study. Tukey’s HSD (Honestly Significant Difference) was applied to test the significance between the groups.

### 3. Results

Crossbred lactating cows were screened for subclinical and clinical mastitis using CMT and SCC. *S. aureus* positive samples (10 subclinical and 8 clinical) were identified using biochemical and microbiological examination. Based on the screening results, three positive samples were selected from each group of subclinical and clinical mastitis caused by *S. aureus* for the expression assay.

Analysis of variance for TLR2 gene revealed significant deference (P ≤ 0.01) for expression level between the groups (Table 2). The mean values of Cq, ΔCq, ΔΔCq along with standard error and relative quantification of TLR2 expression in *S. aureus* caused mastitis are given in Table 3. Relative expression of TLR2 gene varied from 7.04 to 8.87 fold increase in subclinical mastitis cases caused by *S. aureus* with a mean of 7.89 fold, as compared to normal (apparently healthy). In case of clinical mastitis, relative expression of TLR2 varied between 1.67 to 3.58 with a mean of 2.28 fold, when compared with normal cows (Fig. 1).

### 4. Discussion

Mastitis is the persistent inflammatory condition of udder tissue and fatal mammary gland infection in dairy cattle. The first line of defense against mammary bacterial infection was initiated by innate resistance of mammary gland. Stimulation of immune cells by LTA, the cell wall components of Gram-positive bacteria appears to be TLR2 [2, 5, 10]. Hence, assessing the relative expression pattern of TLR2 in somatic cells of milk during subclinical and clinical mastitis caused by *S. aureus* may help to monitor their involvement in mammary innate resistance and subsequent use of this information will be validated as markers.

In present study, the mRNA expression of TLR2 is highly significant (P ≤ 0.01) in subclinical affected cattle as compared with clinically affected cattle. These observations were in agreement with Moyes et al. [11] who reported that TLR2 mRNA was up-regulated by *S. aureus* induced mastitis. Mitra et al. [12] also observed an up regulation of TLR2 expression in *S. aureus* infected bovine mammary gland. Similar results were also observed by Goldammer et al. [13] in cattle udders with natural infections caused by *S. aureus*. The expression level of TLR2 gene in subclinical and clinical mastitis was compared. We found that the expression of TLR2 was found to be relatively high in sub-clinical mastitis than clinical mastitis. This finding is similar to that of Swanson et al. [14], who reported TLR2 mRNA to be up-regulated by *S. aureus* induced mastitis. Since TLR2 is a principle receptor for *S. aureus*, and consequently expression at high level would help to defend against pathogens during early stage of infection, hence most of the subclinical mastitis may subsided by itself without precipitating into clinical mastitis. These results disclose the vital role of TLR2 in early innate immune response in udder against mastitis, as it plays essential role in innate immunity by ligand recognition and signal transduction and involved in the recognition of bacterial lipoproteins, and zymosan [15,16].

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Sequence (5’→3’)</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>F ACGGAGTGGTGCAAGTATGA</td>
<td>114bp</td>
</tr>
<tr>
<td>β-actin</td>
<td>R CTTGGGAATGGCCCTTCTTG</td>
<td>105 bp</td>
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<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>df</th>
<th>MSS</th>
<th>F value</th>
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<tr>
<td>Between Groups</td>
<td>2</td>
<td>40.16**</td>
<td>63.62</td>
</tr>
<tr>
<td>Within Groups</td>
<td>6</td>
<td>0.063</td>
<td></td>
</tr>
</tbody>
</table>

### Table 1: Primer sequence for TLR2 and β-actin genes used in RT-qPCR

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cq Mean ± SE</th>
<th>ΔCq</th>
<th>ΔCq Mean</th>
<th>ΔΔCq</th>
<th>RQ</th>
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<tr>
<td><em>TLR2</em></td>
<td>22.27 ± 0.02</td>
<td>15.94 ± 0.03</td>
<td>6.33</td>
<td>6.33 ± 0.03</td>
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<tr>
<td><em>β-actin</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Subclinical</td>
<td>19.84 ± 0.31</td>
<td>16.48 ± 0.29</td>
<td>3.36</td>
<td>-2.98</td>
<td>7.86</td>
</tr>
<tr>
<td>Case 1</td>
<td>19.84 ± 0.31</td>
<td>16.48 ± 0.29</td>
<td>3.36</td>
<td>-2.98</td>
<td>7.86</td>
</tr>
<tr>
<td>Case 2</td>
<td>18.59 ± 0.34</td>
<td>15.40 ± 0.25</td>
<td>3.18</td>
<td>-3.15</td>
<td>8.87</td>
</tr>
</tbody>
</table>

### Table 3: Expression of TLR2 gene in *S. aureus* caused subclinical and clinical mastitis

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*Note: “1468”*
5. **Conclusion**

The present study results demonstrate that significant expression of TLR2 in subclinical mastitis, which is necessary to initiate a robust defensive action against \textit{S. aureus} infection of mammary gland. Subclinical mastitis is an early stage of infection and hence the expression of TLR2 was at high level during this stage compared to clinical stage. Therefore, it is suggested that TLR2 might consider as candidate gene to screen for mastitis resistance in cattle population.

6. **Acknowledgments**

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7. **References**


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**Fig 1:** Relative expression of TLR2 gene in \textit{S. aureus} caused mastitis

<table>
<thead>
<tr>
<th>Case 1</th>
<th>19.77 ± 0.05</th>
<th>16.25 ± 0.29</th>
<th>3.52</th>
<th>-2.82</th>
<th>7.04</th>
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<td>Case 2</td>
<td>21.72 ± 0.16</td>
<td>16.38 ± 0.25</td>
<td>5.34</td>
<td>-0.99</td>
<td>1.99</td>
</tr>
<tr>
<td>Case 3</td>
<td>20.96 ± 0.04</td>
<td>16.46 ± 0.27</td>
<td>4.49</td>
<td>-1.84</td>
<td>3.58</td>
</tr>
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Clinical

<table>
<thead>
<tr>
<th></th>
<th>3.35 ± 0.10</th>
<th>-2.98</th>
<th>7.89 a**</th>
</tr>
</thead>
</table>

5.14 ± 0.33 -1.19 2.28 b**c**

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

