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Relative Expression of Toll-like Receptors, Cytokines and Acute Phase Protein by Real-Time PCR in Milk Somatic Cells of Subclinical Mastitis Affected Cattle

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ABSTRACT

Background: Subclinical mastitis is one of the most important diseases of economic importance affecting dairy animals worldwide. The present study was planned to evaluate the level of expression of toll-like receptors, cytokines and acute phase protein in milk somatic cells during subclinical mastitis of cattle.

Methods: The milk samples of subclinical mastitis-positive and healthy cattle were collected. After that, extraction of total RNA was done from milk somatic cells followed by cDNA synthesis by the process of reverse transcription. Following that qPCR was carried out and relative transcript levels were determined.

Result: In the present study, a relative up-regulated expression was found in TLR-2, IL-1β, IL-10 and Hp and down-regulation was found in TLR-4, TNF-α, IFN-γ and IL-6 in the milk of cattle with subclinical mastitis as compared to healthy ones. Monitoring of cytokines entangled in the modulation of immune responses during the infection is useful in deciding cytokine markers that could be employed as a forecasting tool in the early diagnosis of subclinical mastitis.

Key words: Acute phase protein, Cytokine, Subclinical mastitis, Toll-like receptors.

INTRODUCTION

Mastitis is one of the most important diseases of economic importance affecting dairy animals worldwide (Gulbe *et al*., 2020). It is an inflammatory response in the mammary gland, which is predominantly a result of the infectious challenge and is the most frequent disease of dairy animals (Fonseca *et al*., 2015). Subclinical mastitis (SCM) is subtle and more difficult to detect than clinical mastitis. SCM usually remains unnoticed because the milk and udder appear normal (Tanamati *et al*., 2019). In addition, SCM constitutes a reservoir of microorganisms that spreads the infection of other animals within a herd (Bhatt *et al*., 2012). So, the subclinical form of the disease is important because it is 15 to 40 times more prevalent than its clinical form (Singh *et al*., 2015a) and therefore usually persists longer in the herd, causing production losses (Kumar *et al.,* 2014).

Early diagnosis of SCM is extremely important to check its development in clinical cases. Changes in the expression patterns of toll-like receptors, cytokines and acute phase proteins of the mammary glands in healthy and diseased animals can help in detecting early infection. Studies indicated variations in toll-like receptors, cytokines and acute phase proteins expression in mastitis cases were associated with disease activity (Bhatt *et al.,* 2012). But, little information is available on toll-like receptors (TLR-2 and TLR-4) and IL-10 expression in natural SCM in cattle. Therefore, the present study was undertaken for the quantification of relative transcription levels of toll-like receptors, cytokines and acute phase protein in milk somatic cells of cattle suffering from subclinical mastitis.

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MATERIALS AND METHODS

Place of study

The study was conducted in the Department of Veterinary Microbiology, College of Veterinary and Animal Science, Navania, Udaipur from 2019-2021.

Source of milk samples

The milk samples were collected aseptically from the lactating cattle in and around the Udaipur district of

Rajasthan. Screening of the SCM was conducted by the California mastitis test (CMT) and somatic cell count (SCC).

Bacteriological examination

The milk samples found positive (based on CMT and SCC) were subjected to bacteriological examination as per standard procedures by Markey *et al*. (2013).

Extraction of total RNA from milk and cDNA synthesis

Total RNA was extracted from the milk sample (5×10^6 somatic cells) using the Trizol reagent (Genetix Biotech Asia Pvt. Ltd., New Delhi, India) as per the manufacturer's protocol. Total RNA was quantified by biospectrometer (Eppendorf, Hamburg, Germany) and A260/A280 was measured for RNA quality. RNA of different milk samples was used as a template for the synthesis of first-strand cDNA using reverse transcriptase as per the standard procedures by Singh *et al*. (2016). Synthesized cDNA was stored at -80°C until further use.

Real-time PCR assay

First of all the optimization of Real-Time PCR was done as per the standard procedures by Singh *et al*. (2016). The real-time PCR assay was carried out using a real-time PCR machine (Biorad Pvt. Ltd., California, U.S.A.) in triplicate for each sample. In the present study, toll-like receptors (TLR-2, TLR-4), cytokines (TNF-α, IL-1β, IFN-γ, IL-6 and IL-10) and acute phase protein *i.e*. Haptoglobin (Hp) were chosen as the target and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) acted as endogenous control. The specific oligonucleotide sequence of these genes has been presented in Table 1. Reactions were performed in a 20 µl volume reaction mixture comprised of various components (Table 2). Non-template control (NTC) comprises all other components except the template cDNA.

The thermal cycling conditions were performed as an initial step of denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 sec (cyclic denaturation), annealing (annealing temperature as described in Table 1 for each gene) for 30 sec. and extension at 72°C for 30 sec. For each sample, a dissociation curve was generated after the completion of amplification and analyzed to determine the specificity of the qPCR assay. Quantitative RT-PCR data were analyzed with the comparative Cq method ($^{\Delta\Delta}$Cq) (Livak and Schmittgen, 2001).

RESULTS AND DISCUSSION

As per International Dairy Federation criteria, quarters having SCC of more than 5 lakhs and bacteriologically positive, were identified as subclinically infected. Milk samples of 4 healthy and 4 SCM cattle were considered for this study. *Staphylococcus* was the microbe found associated with SCM in all these samples. From infected and healthy milk somatic cells, total RNA extracted was in the range of 300-400 ng/µl and A260/280 ratio of >1.75-1.8 indicated that the purity of RNA was good. The amplification plots and dissociation curves for the genes of interest showed a single peak indicative of primers specificity. Relative transcript levels of these genes in SCM are presented (Table 3).

TLR-2

In the present study, TLR-2 gene expression level was found to be upregulated ($p<0.01$) in diseased quarters compared to healthy quarters. These observations were in corroboration with the earlier report of a significant up

Table 1: Details of oligonucleotide sequences used in real-time PCR.

Gene	Primer sequence (5′-3′)	Amplicon size (bp)	Annealing temperature (°C)	Reference
TLR-2	F: 5′-CAT TCC TGG CAA GTG GAT TAT C-3′ R: 5′-GGA ATG GCC TTC TTG TCA ATG G-3′	198	54	Beecher *et al*. (2012)
TLR-4	F: 5′-TCT CTA CAA AAT CCC CGA CAA CAT-3′ R: 5′-AGA AAA GGC TCC CCA GGC TAA ACT-3′	238	57	Beecher *et al*. (2012)
TNF-α	F: 5′-TCTTCTCAAGCCTCAAGTAACAAGT-3′ R: 5′-CCATGAGGGCATTGGCATAC-3′	103	55	Leutenegger *et al*. (2000)
IL-1β	F: 5′-TGG GTA TCA AGG ACA AGA ATC-3′ R: 5′-CCA GTT AGG GTA CAG GAC AGA C-3′	182	54	Beecher *et al*. (2012)
IFN-γ	F: 5′-CAGAAAGCGGAAGAGAAGTCAGA-3′ R: 5′-CAGGCAGGAGGACCATTACG-3′	106	55	Shin *et al*. (2005)
IL-6	F: 5′-TCA TTA AGC GCA TGG TCG ACA AA-3′ R: 5′-TCA GCT TAT TTT CTG CCA GTG TCT-3′	105	54	Lee *et al*. (2006)
IL-10	F: 5′-CGC TGT CAT CGC TTT CTG-3′ R: 5′-AAC TCA CTC ATG GCT TTG TAG-3′	110	55	Beecher *et al*. (2012)
Hp	F: 5′-GTCTCCCAGCATAACCTCATCTC-3′ R: 5′-AACCACCTTCTCCACCTCTACAA-3′	174	57	Hiss *et al*. (2004)
GAPDH	F: 5′-GGCGTGAACCACGAGAAGTATAA-3′ R: 5′-CCCTCCACGATGCCAAAGT-3′	120	54	Bhupal Gramsci (2007)

regulation in the expression of the TLR-2 in SCM caused by *S. aureus*. The presence of *S. aureus* organism would have triggered the TLR-2 expression in milk somatic cells of SCM-affected cows (Karthikeyan *et al*., 2016).

TLR-4

In the current study, TLR-4 gene expression level was found to be down regulated (p>0.05) in diseased quarters. In our study, there was down-regulation of TLR-4 which was the prime TLR reported to be activated by gram-negative bacteria (Kayagaki *et al*., 2013). However, some studies reported an increased mammary abundance of TLR-4 in experimental induced mastitis with gram-positive bacteria (Beecher *et al*., 2012; Fonseca *et al*., 2015), natural infection with gram-positive bacteria (Tanamati *et al.,* 2019), induced infection with both gram-positive and gram-negative bacteria (Lee *et al*., 2006). The up regulation and down regulation of genes are under the control of various regulatory mechanisms in the host immune response. Any type of negative feedback will bring down the TLR expression and positive feedback will up-regulate the TLR level (Mitra *et al*., 2014).

TNF-α

In the present study, TNF-α gene expression level was found to be down regulated (p>0.05) in diseased quarters. Similar to the present study observations, Alluwaimi *et al*., (2003) observed down regulation of TNF-α at 32 h post-infection of *S. aureus* in the bovine mammary gland. The present study results are also in conformation with the finding of Bruno *et al*. (2010) who found a low levels of TNF-α associated with subclinical streptococcal-infected bovine mammary glands at dry-off. Low conc. of TNF-α mRNA transcript in diseased quarters could be due to timings as this TNF-α cytokine can be released in a pulse-like mode during inflammatory reactions to pathogens (Sordillo and Peel, 1992). Alluwaimi *et al*. (2003) were also of the opinion that differences in expression might be due to cyclic rise and decline in the number of viable pathogens. In the present study, there was a high level of IL-10 was found and it was able to inhibit the production of TNF-α by macrophages (Fiorentino *et al*., 1991). In contrast, multiple studies (Beecher *et al*., 2012; Fonseca *et al*., 2015; Singh *et al*., 2016) reported higher mRNA expression of TNF-α in animals with mastitis.

IL-1β

IL-1 has been designated as an important mediator of neutrophil appointment at the place of inflammation and regulating the functions of infiltrating neutrophils, monocytes and the cytokines released by these cells (Fonseca *et al.,* 2009). In our study, up regulation of IL-1β was recorded. Our result is consistent with the finding of Beecher *et al*., (2012) who also reported higher mRNA expression of IL-1β in cattle with induced mastitis.

IFN-γ

In the present study, the IFN-γ gene expression level was found to be down regulated (p>0.05) in diseased quarters. This down regulation might be due to the *Staphylococcus* spp. infection. These results were in accordance with previous studies (Alluwaimi *et al*., 2003; Lee *et al*., 2006) which also reported down regulation of mRNA of IFN-γ after staphylococcal mastitis. *S. aureus* could deploy mechanisms to suppress IFN-γ expression in somatic cells to survive intracellularly (Lee *et al*., 2006). In the present study, there was an increase in the level of IL-10 found and IL-10 was able to suppress the secretion of IFN-γ by Th1 lymphocytes (Mosmann and Moore, 1991). In contrast, results of up regulation were observed by different researchers (Bhupal, 2007; Fonseca *et al*., 2009; Bhatt *et al*., 2012) in experimentally induced mastitis, natural mastitis and SCM.

IL-6

In the present study, the relative level of the IL-6 gene was down regulated (p>0.05). The findings in our study are in accordance with the previous study (Bruno *et al*., 2010). In the current study, *Staphylococcus* spp. infections were predominant and *Staphylococcus* caused a chronic type of mastitis. Perhaps this explains why expression of IL-6 was downregulated in our milk samples of SCM. Although IL-6 mRNA transcription has been detected in chronic *S. aureus* infected quarters, its transcriptional activity changed only slightly (Alluwaimi *et al*., 2003). The present study results contrast with the findings of many studies (Bhatt *et al*., 2012; Bochniarz *et al*., 2017) that reported IL-6 concentration to be significantly higher in subclinical mastitic milk.

IL-10

In the present study, the IL-10 gene expression level was found to be up regulated (p>0.05). The present study results

Table 2: Ingredients for standardized real-time PCR assay.

Ingredients	Volume (µl)	Non-template control (NTC)
2X GeneSureTM SYBR Green qPCR master mix (Genetix biotech Asia Pvt. Ltd., New Delhi, India)	10 µl	10 µl
50 pM forward primer	0.4 µl	0.4 µl
50 pM reverse primer	0.4 µl	0.4 µl
cDNA	2 µl	-
N.F.W.	7.2 µl	9.2 µl
Total	20 µl	20 µl

Table 3: Relative transcript levels of toll-like receptors, cytokines and acute phase protein in subclinical mastitis.

Gene	Relative transcript level [× Fold±SD]
TLR-2	3.929237±1.3584
TLR-4	0.211907±0.056899
TNF-α	0.117092±0.045502
IL-1β	6.45993±4.641494
IFN-γ	0.006771±0.006523
IL-6	0.012288±0.016768
IL-10	1.428391±0.875931
Hp	1.037812±0.233193

are consistent with the finding of other studies (Fonseca *et al*., 2009; Bochniarz *et al*., 2017) reported higher expression of the IL-10 gene in animals with mastitis. In contrast, a study (Beecher *et al*., 2012) observed no change in the transcript abundance of IL-10.

Hp

In the present study, the Hp gene expression level was upregulated. It is consistent with the previous studies (Kumar *et al*., 2014; Singh *et al*., 2015b) which also showed increased concentrations of Hp in the milk of animals suffering from mastitis and SCM.

Mastitis is a multi-etiological and multifactorial disease that is influenced by many genes. Divergent results were observed in the fold of expression level of target genes which can be explained by the fact that immune response differed according to the stage of SCM, bacterial strain and host with the observation of wide individual variation. Further studies including a larger number of genes and animals in different steps of infection are necessary to better understand the immune response mechanism and to develop a more systematic scheme for the control and eradication of SCM.

CONCLUSION

In the present study, a relative upregulated expression was found in TLR-2, IL-1β, IL-10, Hp and downregulation was found in TLR-4, TNF-α, IFN-γ and IL-6 in the milk somatic cells of subclinical mastitis affected cattle.

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Conflict of interest

All authors declare that they have no conflict of interest.

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