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Relative expression of proinflammatory cytokines by real time PCR in milk somatic cells of subclinical mastitis affected buffaloes

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ABSTRACT

The expression profile of proinflammatory cytokines including TNFα, IL1β, IL6 and IFNγ were investigated in milk somatic cells of subclinical mastitis affected buffaloes using real time PCR assay. Relative expression levels of target mRNAs were analysed by comparison with the expression of the housekeeping gene, β-actin within each animal. Highest relative transcript level of TNFα (44.93 ± 1.16) was observed followed by IL1β (19.92 ± 1.78), IL6 (4.72 ± 1.96) and IFNγ (2.55 ± 2.23) from milk somatic cells of subclinical mastitis with respect to healthy ones. Lowest upregulation of IFN-γ in the present study attributed to the isolation of only Gram positive bacteria (staphylococci and streptococci) from subclinical mastitis cases. Monitoring of cytokines involved in the regulation of immune responses during the infection is useful in determining cytokine markers that could be utilized as a forecasting tool in the early diagnosis of subclinical mastitis.

Key words: Milk somatic cells, Proinflammatory cytokines, Real time PCR, Subclinical mastitis

Mastitis, a global problem affecting dairy animals, is causing hindrance in development of dairy industry by incurring heavy financial losses (Charaya *et al*. 2015). Subclinical mastitis is more important than its clinical counterpart because it is 15 to 40 times more prevalent and difficult to diagnose, and therefore, usually persists for longer duration in the herd, causing production losses (Ali *et al*. 2015). Early diagnosis of subclinical mastitis is extremely important to check its development to clinical cases. Changes in expression pattern of proinflammatory cytokines of mammary gland in healthy and diseased animals can help in detecting early infection. Studies indicated variations in cytokine expression in mastitis cases were associated with disease activity (Lee *et al*. 2006, Wenz *et al*. 2010, Bhatt *et al*. 2012). But, little information is available on cytokine expression patterns in lactating buffaloes. Therefore, the present study was undertaken for quantification of relative transcription levels of proinflammatory cytokines in milk somatic cells of Murrah buffaloes suffering from subclinical mastitis.

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MATERIALS AND METHODS

Collection of milk samples: The milk samples were collected aseptically from the lactating Murrah buffaloes reared at an organised farm separately in sterile vials. Before sample collection, the udder was thoroughly washed and the teats were swabbed with 70% ethyl alcohol.

Bacteriological examination and somatic cell count: For bacteriological examination, milk samples were inoculated on blood agar and MacConkey's lactose agar plates and incubated at 37°C for 18–24 h. The somatic cell count was determined after staining the milk smears with modified Newman-Lampert stain.

Extraction of total RNA from milk: Milk sample (5×10^6 somatic cells) was diluted 1:1 with 1× PBS, pH 7.2 and 0.5 M EDTA was added to the final concentration of 100 µM EDTA and centrifuged at 1000g for 15 min at 4ºC. The fat layer and supernatant was discarded. The cell pellet was washed twice with 1× PBS with 100 µM final conc. of EDTA. Total RNA was extracted from cell pellet using Trizol method. The eluted RNA was treated with DNase I, RNase-free and stored at –20ºC

Quantitation of total RNA and cDNA synthesis: Total RNA was quantified by Nanodrop and A_{260}/A_{280} was measured for RNA quality. Total RNA (4 µl) of different milk samples was used as a template for the synthesis of first stand cDNA using Revertaid first strand cDNA synthesis kit at thermal conditions 25ºC for 10 min, 42ºC for 60 min, 70ºC for 5 min and finally at 4ºC.

Optimization of real time PCR: Specificity of all the primers was determined by conventional PCR. The optimal primer concentration for the primer pair was determined by performing real time PCR assay in duplicate at different primer concentrations over the range of 100 to 300 nM. The melt curve assisted in establishing primer specificity.

Real time PCR assay: Real-time PCR assay was carried out using Piko real 96 well PCR in triplicate for each sample using SYBR GREEN dye as the active chemistry and ROX as passive dye. TNFα, IL1β, IFNγ and IL6 genes were chosen as targets and β actin as an endogenous control. Reaction mixture comprised of the following components (Table 1). Samples from healthy buffaloes were taken as reference.

Table 1. Ingredients for standardised real time polymerase chain reaction assay

Ingredients	Volume	Non-template control
2 × Quantitect SYBR master mix	10 µl	10 µl
Forward primer (10 µM)	0.4 µl	0.4 µl
Reverse primer (10 µM)	0.4 µl	0.4 µl
cDNA	2 µl	-
Nuclease free water	7.2 µl	9.2 µl
Total	20 µl	20 µl

The reactions were performed as initial step at 95°C for 10 min, followed by 40 cycles at 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec in a 20 µl total reaction volume. For each sample, a dissociation curve was generated after completion of amplification and analysed to determine the specificity of qPCR assay. The specific primers used in the study are encapsulated in Table 2. The qPCR data were analysed with the comparative Cq method (ΔΔCq) (Livak and Schmittgen 2001).

RESULTS AND DISCUSSION

As per International dairy federation criteria, quarters having somatic cell count more than 5 lakh and bacteriologically positive, were identified as subclinically infected. Milk samples of 10 healthy and 10 subclinical mastitis buffaloes were considered for this study. Staphylococci and streptococci were the microbes found associated with subclinical mastitis. From infected and healthy milk somatic cells, total RNA extracted was in the range of 300–400 ng/µl and $A_{260/280}$ 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 single peak indicative of primers specificity. Highest relative transcript level of TNFα was observed followed by IL1β, IL6 and IFNγ from milk somatic cells of subclinical mastitis with respect to healthy ones (Table 3). Only few reports are available on proinflammatory cytokine expression in buffalo milk.

Table 3. Relative transcript levels of bovine proinflammatory cytokines in subclinical mastitis

Cytokine	Relative transcript level [× Fold ±SD]
TNF α	44.93 ± 1.16
IL1β	19.92 ± 1.78
IFN γ	2.55 ± 2.23
IL 6	4.72 ± 1.96

TNFα is a highly proinflammatory cytokine. Multiple studies (Alluwaimi *et al*. 2003, Bannerman *et al*. 2004, Lee *et al*. 2006) of experimental intramammary infections with Gram-positive bacteria, *S. aureus* and *Streptococcus uberis*, described up-regulation of mammary gland leukocytes and a wide variety of associated cytokines and inflammatory mediators including TNFα. In contrast, Fonseca *et al*. (2009) showed no significant difference in the expression of TNF-α in two breeds with mastitis animals infected with two different types of microbes. Whereas Bhatt *et al*. (2012) reported higher level of TNFα expression in Gir and crossbred cattle as compared to Kankrej cattle as a consequence of *E. coli* infection. Although *S. aureus* and coliform mastitis are associated with high influx of neutrophils but their activation state and CD markers expression are different.

IL1 is indicated as an important mediator of neutrophil recruitment at the sites of inflammation. In the present study, up regulation of IL1β was recorded. Similar to our findings, Riollet *et al*. (2001) determined up regulation of IL1β from animals chronically infected with *S. aureus*. Wenz *et al*. (2010) reported greater concentration of IL1β in animals infected with Gram negative bacteria than animals infected

Table 2. Oligonucleotide primers used in real time polymerase chain reaction assay

Genes	Sequences	Product size (bp)	Reference
TNFα	F:TCTTCTCAAGCCTCAAGTAACAAGT		
	R: CCATGAGGGCATTGGCATAC	103	Leutenegger *et al*. (2000)
IL1β	F: AAATGAACCGAGAAGTGGTGTT		
	R: TTCCATATTCCTCTTGGGCTAGA	185	Strandberg *et al.* (2005)
IFNγ	F:CAGAAAGCGGAAGAGAAGTCAGA		
	R: CAGGCAGGAGGACCATTACG	106	Shin *et al*. (2005)
IL6	F: CTGCAATGAGAAAGGAGATA		
	R: GGTAGTCCAGGTATATCTGA	192	Mingala *et al.* (2009)
β actin	F: CGCACCACCGGCATCGTGAT		
	R: TCCAGGGCCACGTAGCAGAG	227	Mingala *et al.* (2009)

with Gram positive bacteria. The induction of IL1β is delayed after intramammary infection with Gram-positive or wall-less bacteria compared with infection with Gram-negative bacteria (Bannerman *et al*. 2004); however, the magnitude of the IL1β response was comparable.

In the present study, relative transcript level of IL6 was reported as 4.72 ± 1.96. However, qPCR failed to identify presence of mRNA of IL6 in some healthy milk samples. Similarly, Sakemi *et al*. (2011) reported significantly higher concentration of IL6 in mastitic milk. Contrary to this, Alluwaimi *et al*. (2003) reported only slight change and Bruno *et al*. (2010) reported lower IL6 transcriptional activity in infected quarters. Lee *et al*. (2006) and Bhatt *et al*. (2012) reported different patterns in IL6 profile among different organisms and among different breeds of animals. This difference might be related to immunosuppressive picture of mastitis.

IFNγ was lowest up regulated in our study and in some healthy animals; no signal for IFNγ was detected. Lowest upregulation of IFNγ might be due to the isolation of only Gram positive bacteria (staphylococci and streptococci) from subclinical mastitis. Our study supports the finding of Lee *et al*. (2006) who observed delayed and less pronounced expression in staphylococcal mastitis and observed a striking difference between the *E. coli* and *S. aureus* infections. *S. aureus* could deploy mechanisms to suppress IFN-γ expression in somatic cells to survive intracellularly. On the other hand, the expression was gradually upregulated in glands challenged with *E. coli*. In contrast, Alluwaimi *et al*. (2003) reported down regulation of mRNA of IFNγ after *S. aureus* intramammary challenge.

Mastitis is a multietiological and multifactorial disease which is influenced by numerous genes. Divergent results were observed in fold of expression level of target genes which can be explained by the fact that immune response differed according to bacterial strain, stage of subclinical mastitis, and host with the observation of wide individual variation. Monitoring cytokines involved in the regulation of immune responses during the infection could be useful in determining cytokine markers that could be utilized as a forecasting tool in the early diagnosis of mastitis.

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