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ANALYSIS OF POTENTIAL BIOMARKERS FOR BOVINE MASTITIS USING REAL-TIME PCR

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ABSTRACT

Bovine mastitis is a significant disease that causes an estimated loss of $2 billion per year in the United States. The current diagnostic methods for mastitis are based on bacteriological analysis and somatic cell count (SCC) of milk samples. These techniques have low sensitivity and require a significant amount of time for a diagnosis, and are therefore insufficient. A test using real-time PCR and cytokine profiles of milk could provide a faster, easier, and more accurate diagnosis. This study looked at three possible mastitis biomarkers, TNF-α, IL-8, and IL-10, in conjunction with 28S as the control. The real-time PCR reaction was standardized for the four target proteins using a serial dilution of mRNA from milk. Quarter milk samples were collected from six Penn State Dairy Herd 2nd lactation cows 45-60 days in milk for a total of 23 samples (one cow had three quarters). The samples were subjected to bacteriological analysis using PSU-QUAD plates and SCC to categorize them as healthy or mastitic. The quarter samples were then analyzed using uniplex RT-PCR. The relative expression of each target protein in the healthy and mastitic quarters was compared using comparative Ct analyses and a statistical t-test. The expression of TNF-α among the previous studies is similar to that observed in our study, where TNF-α expression was similar in both mastitic and healthy quarters. Therefore, it can be inferred that TNF-α is not a suitable marker for mastitis. Although expression of IL-10 was elevated in mastitic quarters, the level of expression (Ct value) was not sufficient to allow its use as a mastitis biomarker. The relative expression of IL-8 was consistently higher in the mastitic quarters (high SCC) than the healthy quarters (low SCC). The relationship between high SCC and IL-8 expression indicates that IL-8 could be a promising biomarker for mastitis.

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CHAPTER 1: INTRODUCTION

Mastitis is inflammation of the mammary gland commonly caused by infectious microorganisms or udder trauma. The inflammatory response of the immune system is activated to eliminate the pathogens in the mammary gland, causing an increased number of cells of the immune system to be present. These somatic cells include lymphocytes, epithelial cells, polymorphonuclear leukocytes, and macrophages. Therefore, the somatic cell count (SCC), the number of somatic cells in milk, is a useful indicator of the inflammatory response to an intramammary infection. Somatic cell counts help detect mastitis (Herlekar, 2007). Uninfected mammary glands generally have SCC of about 150,000 cells/ml. SCC of above 200,000 cells/ml is indicative of mastitic infection.

In a healthy mammary gland, macrophages are the main cell type of the immune system, while in early inflammation neutrophils are the major cell population. After a bacterial invasion, neutrophil recruitment is caused by inflammatory mediators that are produced in the infected mammary gland by cells. These cells are possibly macrophages activated by bacteria phagocytosis or responding to bacterial toxins or metabolites. Several cytokines are known to be significant in eliciting the acute phase response and allow the accumulation of leukocytes at the infection site. In addition to their role in early non-specific defenses, macrophages are also important in the specific immune system as antigen processing and presenting cells for the T cells (Riollet et al., 2000).

Mastitis in cattle is a noteworthy disease for several reasons. It reduces milk production and milk quality, preventing it from being sold to the public. The economic losses from mastitis are significant; the total economic loss in the United States is estimated at $2 billion each year.

This number includes indirect and direct costs, such as reduced production, increased replacement costs, discarded milk, medication costs, veterinary fees, and labor costs (Herlekar, 2007).

The current diagnostic tests for mastitis are inadequate because they have low sensitivity and take too much time. Leutenegger et al. (2002) described a real-time polymerase chain reaction method to develop cytokine profiles in milk cells of cattle. Techniques such as these could be used to analyze changes in cytokine levels during mastitis compared to healthy levels, potentially leading to a diagnostic test superior to those currently being employed.

Hypothesis

The purpose of the study is to identify bovine mRNA targets that can be used as biomarkers to diagnose mastitis. An RT-PCR assay targeting tumor necrosis factor α (TNF-α), interleukin 8 (IL-8), and interleukin 10 (IL-10), will be standardized with 28S as the control protein. Healthy and mastitic samples will then be analyzed by RT-PCR for each of the four proteins and the relative expression of each protein in healthy and mastitic quarters will be compared. We predict that IL-8 will have a higher relative expression in the mastitic quarters than 28S and that TNF-α and IL-10 will be expressed at relatively similar levels in both healthy and mastitic quarters.

CHAPTER 2: REVIEW OF LITERATURE

Mastitis is an inflammation of the mammary gland caused by udder trauma or microorganisms. As the inflammatory response in the mammary gland acts to eliminate pathogens, anatomical, cellular, and soluble factors act in concert to reduce vulnerability to infection. The teat sphincter muscles of the teat canal keep the opening of the teat closed and prevent pathogens from gaining entry to the interior of the udder. The immune cells, or somatic cells, control the innate and acquired immune responses that are activated upon invasion by pathogens (Herlekar, 2007).

The innate and acquired immune responses work together to defend the mammary gland from pathogens. In early stages of infection the innate immune system is the main response, and macrophages, neutrophils, natural killer cells, and cytokines are the mediators. The chemo-attractants released by macrophages and epithelial cells recruit polymorphonuclear leukocytes, mainly neutrophils, to the site of the infection. The production and release of tumor necrosis factor, interferons, and interleukins initiate the inflammatory response (Herlekar, 2007).

The acquired immune response follows the activation of the innate immune response, allowing an antigen specific response to the pathogen. The acquired immune system provides immunological memory, so that the immune system can respond more quickly upon subsequent infections with the same pathogen. Macrophages, lymphoid cells, and immunoglubulins mediate the recognition of pathogens for elimination (Herlekar, 2007).

The protective mechanisms of the immune system can also cause tissue damage. Reactive oxygen molecules and other chemical mediators can be detrimental to the mammary secretory cells, causing reduced milk yield. Epithelial cells are also important for the production of chemo-

attractants, particularly interleukin-8. Mastitis results in alterations in milk composition as ions, proteins, and enzymes leak from blood to milk. This is due to changes such as increased permeability and the presence of phagocytic cells in the mammary gland tissue (Herlekar, 2007).

The somatic cell count (SCC) is a useful indicator of the inflammatory response to an intramammary infection and is often used to detect mastitis. The somatic cell composition in a mammary gland is a reflection of the infective stage, as the major cell types present changes as the infection progresses. Healthy and infected quarters can also be distinguished with SCC. Uninfected mammary glands generally have an SCC of about150,000 cells/ml. SCC of above 200,000 cells/ml indicates mastitis. Generally during infection there is a temporary increase in SCC followed by a return to normal levels following the clearing of the infection. In chronic cases the infection is not completely cleared and the SCC is elevated long-term. Low SCC is not a guarantee that mastitis is not present. Bacteriological culture analysis of milk can also be used to diagnose mastitis (Herlekar, 2007).

Mastitis diagnosis is based off of the SCC of milk, bacteriological culture, and compositional changes in milk. Bacteriological culture is the most reliable technique, though sub-clinical infections are often negative. One study found that 25% of clinical and sub-clinical mastitis cases were culture negative, and a significant portion of clinical cases have no known etiology (Herlekar, 2007).

Cytokines play a central role in the immunoregulation of the bovine mammary gland. Three major, well studied cytokines are TNF-α, a pro-inflammatory factor representative of the acute-phase response, IL-8, a chemoattractive factor representative of neutrophil recruitment, and IL-10, a regulatory and anti-inflammatory factor representative of immune regulation. A

study to determine the normal transcriptional levels and changes in levels with lactation stage found that early and late lactation have lower levels of TNF-α and IL-8 than mid-lactation. IL-10, however, had higher levels in early and late lactation than in mid-lactation. The levels of cytokines in the three lactation groups were not statistically significant, but there were significant differences in the total profile of the three cytokines over the lactation periods. This implies a connection between the expression levels of the cytokines, which is more pronounced in late lactation, possibly due to the imminent shift to the dry period (Britti et al., 2005).

Leutenegger et al. described a method to quantitate cytokines and growth factors contributing to immunity against bacterial infections in the bovine mammary gland, the real-time TaqMan® PCR system. Limitations of conventional quantitative RT-PCR protocols include the need for parallel reactions for a single sample, post-amplification steps, and a narrowed dynamic range. This leads to a low efficiency of sample throughput, inaccuracy, and high costs. TaqMan® PCR is based on cleavage of fluorescent dye-labeled probes by the 5'—3' exonuclease activity of the *Taq* DNA polymerase during PCR and measurement of fluorescence intensity. Bovine specific TaqMan® probes encompass an intron, allowing complementary and genomic DNA to be differentiated. In addition to junctional specific probes and primers, DNase treatment prevents amplification of residual genomic DNA. A key factor in the accuracy of the method is determining the C_T value within the exponential phase of amplification rather than endpoint determination, as well as the use of a third oligonucleotide, the TaqMan® probe, which adds specificity. Elimination of post-amplification steps also increases reliability and reproducibility (Leutenegger et al., 2000).

The universally expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control in the Leutenegger study, permitting variations in cell numbers in the

starting material to be corrected for. GAPDH allowed the data to be normalized, eliminating sources of error in the efficiencies of RNA extraction and real-time PCR, as well accounting for as differences in the number of cells in the starting material (Leutenegger et al., 2000).

IL-2, IL-6, IL-8, IL-12 p40, TNF-α, IFN-γ, and granulocyte-macrophage colony stimulating factor (GM-CSF) were analyzed in the Leutenegger study in healthy cows using the TaqMan® PCR system. Transcription of TNF-α, IFN-γ, and GM-CSF mRNA was high in all samples, IL-8 had lower transcription levels present in all samples, IL-12 p40 mRNA was found in six out of seven samples, IL-6 mRNA was in two out of seven, and IL-2 mRNA was not detected in any samples. Conventional PCR has found IL-12 p40 mRNA is not present in healthy cows, but the TaqMan® PCR system demonstrated its presence in most of the healthy cow samples (Leutenegger et al., 2000).

IL-8 is important for chemotactic signaling, which promotes recruitment of neutrophils and to some degree T-lymphocytes to the site of infection. IL-8 also activates neutrophils. Previous studies have found IL-8 mediated chemotaxis to be absent from non-mastitic milk; this could be due to no IL-8 expression or to regulation of IL-8 (Leutenegger et al., 2000).

The real time TaqMan® RT-PCR method was used in a study to evaluate cytokines in both healthy and mastitic cows. The distinction between healthy and mastitic was based on SCC. The samples were also subjected to bacteriological tests. Three major cytokines were studied: IL-8, IL-10, and TNF-α. It was found that samples with high SCC also had higher levels of IL-8, but IL-8 levels were not affected by the presence of bacteria. TNF-α levels were higher in samples with positive bacteriology, but were not linked to SCC levels. This indicates that bacterial infection, independent of chemotactic effects, stimulates TNF-α transcription. IL-10 seemed to

show a correlation with SCC, but the lack of data on its function and origin requires further study in order to explain this outcome (Peli et al., 2004).

CHAPTER 3: MATERIALS AND METHODS

3.1 Milk Samples

A total of six Penn State Dairy Herd 2nd lactation cows 45-60 days in milk were selected for the study. Milk samples were collected from all four quarters of six cows. Sterile tubes were labeled with the date, the cow's identification number, and the quarter (Table 1). Before sample collection, the teat ends were cleaned with an iodine pre-dip, allowing the pre-dip to be on the teats for twenty to thirty seconds before drying with a clean towel. After drying, the teat ends were scrubbed with cotton or gauze moistened with 70% alcohol. Before sampling the teat was completely dry. To prevent contamination, the closest teats were sampled first. The first few milk streams were discarded. Making sure that the teat end did not touch the collection tube, 50 ml of milk were collected from each quarter. The samples were stored on ice until they reached the lab, upon which time they were refrigerated.

3.2 Somatic Cell Count

The milk samples were each thoroughly mixed by inverting the tube several times. Then the somatic cell count (SCC) was taken of each sample using a DeLaval Cell Counter (DCC) (DeLaval International AB, Thumba, Sweden). To take the cell count, a very small amount of milk was aspirated through the cartridge and inserted in the DCC. Every sample was repeated three times and the average was taken as the final count of each sample.

3.3 Bacteriologic Analysis of Milk Samples

Using a sterile loop, 10 µl of milk were spread onto each quadrant of a PSU-QUAD and incubated for 48 hours at 37°C. The PSU-QUAD consists of four types of media. These include

MacConkey's Agar (MAC), which is selective for gram negative bacteria and can differentiate between lactose fermenters and non-lactose fermenters; Baird-Parker Agar (BPA), which is selective for *Staphylococcus* species and can differentiate *Staphylococcus aureus* from coagulase-negative *Staphylococci*; Edward's Modified Medium with Colistin Sulfate and Oxolinic Acid (EMCO), which is selective for *Streptococcus* species and differentiates environmental steps from contagious ones (esculin positive or negative, respectively); and Blood Agar, which is a non-selective media used to look for any other types of bacteria the other media do not select for. The positive or negative culture results were recorded. If there was anything growing in moderate or large quantities on any of the plates, the milk was categorized as mastitic. If there was no growth or only one or two colonies on any of the four plates, then the milk was categorized as healthy.

3.3 Processing of Milk Samples for RNA Extraction

The milk samples were spun at maximum speed for ten minutes. The fat layer was removed with a swab and the supernatant was decanted into a new 50 ml tube. The pellet was resuspended in 15-20 ml of PBS, transferring to a new 50 ml tube to avoid carrying over milk fat. The resuspended pellet was spun again at maximum speed for ten minutes. The pellet was washed with PBS until the supernatant was clear after spinning, which was about two to three times. When the supernatant was clear, it was resuspended in 50 ml of PBS. The tube was pelleted and the supernatant was decanted.

3.4 RNA Extraction

RNA extraction was carried out on the pellet. The pellet was resuspended in 1-2 ml of Trizol, and then was let sit for five minutes at room temperature. After adding 1 ml of

chloroform and mixing vigorously, it was spun at maximum speed for ten minutes. The aqueous layer was transferred to a fresh tube and one volume of 70% ethanol was added and mixed well. This was transferred to spin column for Ambion RNA kit, spun for 30 seconds at maximum speed, and the flow through was discarded. This was continued until the entire ethanol aqueous layer had passed through the column. The mRNA was extracted using the Qiagen RNeasy kit, then frozen.

3.5 Primers and Probes

The primers and probes (Table 2) used in the study were selected using the PSU Probe Designer Software. The sequences for IL-8, IL-10, TNF-α, and 28S were taken from the published sequence in NCBI database. 28S is the internal control that is used in the real-time PCR.

3.6 RT-PCR Assay

In order to standardize the reaction, the mRNA samples were made into 1:10 serial dilutions. Then RT-PCR reactions were run separately for each target in triplicate a standard curve was created, and reaction efficiencies were compared. The targets were IL-8, IL-10, TNF-α, and 28S (internal control). After standardization, the samples were run in triplicate and analyzed using uniplex reactions for each target protein and the control. The mean Ct data was analyzed using the comparative ΔΔCt method.

A uniplex assay was run with the conditions seen in Table 3 for IL-8, IL-10, TNF-α, and 28S. They consisted of 20.8 µl of 10 µM forward primer, 20.8 µl of 10 µM reverse primer, 10.4 µl of 10 µM probe, 32.5 µl of RT-PCR enzyme mix, 130 µl of 5x reaction mix, 9.75 µl of Rox,

and 360.75 µl of water, for a total volume of 585 µl. In each well, 22.5 µl of mix and 2 µl of sample were placed.

The RT-PCR was run as seen in Table 4, with a reverse transcriptase step of 50°C and 30 minutes, initial denaturation of 95°C and 15 minutes, 40 cycles of denaturation at 94°C and 15 seconds and annealing at 63°C and 60 seconds, and a final extension stage at 72°C for 10 minutes. The above conditions were determined by trial and error to be the most efficient. The control was 28S, which is of use because it is universally expressed.

3.7 The Comparative Ct Method

The Ct values of TNF-α, IL-8 and IL-10 were examined relative to the control gene 28S. This was done to normalize for the differences in the efficiency of the RT step in the RNA extractions. The ΔCt for each experimental sample was subtracted from the ΔCt of the control (28S). This difference was called the ΔΔCt value. The amount of target in the sample was normalized to the control and the quantity of the target was calculated by $2^{-\Delta\Delta Ct}$. This resulted in the determination of the target amount and was expressed as an n-fold difference relative to the control (Leutenegger et al, 2000).

3.8 Statistical Analyses

The Ct values were used for conducting the statistical analyses. The Ct values for milk samples from healthy and mastitis quarters were transferred to Microsoft Excel and grouped as either healthy or mastitic. To estimate if there was a difference in the Ct values between healthy and mastitis quarters, Ct values were compared using one-way ANOVA. A *P*-value of < 0.05 was considered a significant association between the response and a category of the count. All statistical analyses were performed using JMP software version 4.0 (SAS Inst., Inc., Cary, NC).

Table 1. Source and number of milk samples used for the study

Sample #	Cow #	Quarter
1	1312	LF
2		LR
3		RF
4		RR
5	1645	LF
6		LR
7		RF
8		RR
9	1652	LF
10		LR
11		RF
12		RR
13	1448	LR
14		RF
15		RR
16	1712	LF
17		LR
18		RF
19		RR
20	1699	LF
21		LR
22		RF
23		RR

23 quarter milk samples were taken from 6 2nd lactation cows 45-60 days in milk from the Penn State Dairy Herd.

Table 2. Primers and Probes used for RT-PCR assays*

Gene	Function	Primer	Sequence	Probe Sequence	Gene Accession Number
28S	Internal Control	Forward	GATTTTGGCGGCAAGTTTCATTG	TTTCTCTCCATCCACTTCCG	AF154866
		Reverse	CCGGACTCTGGTTCCTTTCTG		
TNF-a	Pro-inflammatory factor	Forward	TCTTCTCAAGCCTCAAGTAACAAGT	AGCCCACGTTGTAGCCGACATCAACTCC	Z14137
		Reverse	CCATGAGGGCATTGGCATAC		
IL-8	Chemo-attractive factor	Forward	CACTGTGAAAAATTCAGAAATCATTGTTA	AATGGAAACGAGGTCTGCTTAAACCCCAAG	S74436
		Reverse	CTTCACCAAATACCTGCACAACCTTC		
IL-10	Anti-inflammatory factor	Forward	CAAGGAGCACGTGAACTCACT	CTGCGGCGCTGTCAT	NM_174088
		Reverse	TCTTGTTTTCGCAGGGCAGAA		

*Primers and Probes were designed using the PSU Probe Designer Software
IL-8, IL-10, TNF-α, and 28S sequences were taken from published sequences in the NCBI database.

Table 3. Uniplex RT-PCR assay conditions

Reagent	Concentration	Volume (µl)/reaction	Master mix (µl)
forward primer	10µM	0.8	20.8
reverse primer	10µM	0.8	20.8
Probe	10µM	0.4	10.4
RT-PCR enzyme mix		1.25	32.5
5x rxn mix	5x	5	130
Rox		0.37	9.75
water	-	13.87	360.75
total master mix volume		22.5	585

A uniplex assay was run with these conditions, which were determined through trial and error, for IL-8, IL-10, TNF-α, and 28S.

Table 4. RT-PCR Thermocycler Conditions

Step	Temperature	Time	Cycles
Reverse transcriptase step	50C	30 min	1
initial denaturation	95C	15 min	1
denaturation	94C	15 sec	40
annealing	63C	60 sec	
final extension	72C	10 min	1

RT-PCR was run on the mRNA samples using these conditions, which were determined through trial and error.

CHAPTER 4: RESULTS

4.1 Standardization of real time PCR assay

The standard curve for each target gene was developed as described in the materials and methods section. The threshold of detection for 28S (control) ranged from 10-10^{-5} RNA dilution with Ct values ranging from 27 to 40, while for TNF-α, the detection threshold ranged from 10^{-1} to 10^{-5} RNA with Ct values from 27-36. The assay could detect IL-8 RNA ranging from 10-10^{-5} RNA with Ct values from 21-37, while for IL-10, the detection threshold ranged from 10-10^{-5} RNA with Ct values from 23-39. Amplification efficiency (R^2 value) for IL-8, 1L-10, TNF-α and 28S were 0.998, 0.998, 0.997 and 0.989 respectively (Table 5). The serial dilution amplification plots for the four targets are shown in Figures 1-4 with the fluorescence plotted against the number of cycles for the different dilutions. Efficiency plots, where ΔCt was plotted against $\log_{10}$ RNA copies, were created for TNF-α, IL-8, and IL-10 (Figures 5-7). These plots showed that the reference gene (28S) and the target gene (TNF-α, IL-8, or IL-10) were amplified at the same rate so that the comparative Ct analysis could be used for subsequent studies related to biomarkers.

4.2 Somatic cell count and bacteriologic analysis of milk samples

Quarter milk samples (n=23) from 6 cows were examined for SSC and mastitis pathogens. Ten out of 23 quarters were identified to have sub-clinical mastitis (SCC > 200,000). Somatic cell count of quarter milk samples from cows with < 200,000 cells/ml ranged from 4,000-156,000 cells/ml, with an average of 60.15 cells/ml. The SSC of quarter milk samples from cows > 200,000 cells/ml ranged from 238,000–5,065,000 cells/ml with an average of 1,369,800 cells/ml (Table 6).

Of the 23 samples, mastitis pathogens were isolated from 16 of 23 (69.6%) quarter milk samples. Coagulase negative staphylococci (CNS) were isolated from 15 of 23 (65%) samples. Based on SCC values, it was observed that CNS was isolated from 9 healthy quarters and 6 mastitic quarters. Non-lactose fermenters (NLF) were found in two (8%) quarters LF and LR of cow 1652, one of which was healthy and the other of which mastitic. Esculin positive colonies (Esc+) were isolated from 7 of 23 (46.7%) quarters, 5 of which were from healthy quarters while 2 were from mastitic quarters. Coliforms, *Staphylococcus aureus*, and *Streptococcus agalactiae* were not detected in the quarter milk samples (Table 6).

4.3 Expression of TNF-α, IL-8 and IL-10 genes in quarter milk samples

Results of the analysis of expression of target genes in quarter milk samples are shown in Table 7. The mean Ct values for TNF-α, IL-8 and IL-10 gene expression in healthy quarters were at 32.62, 22.17 and 30.86 cycles respectively. While the Ct values for TNF-α, IL-8 and IL-10 gene expression in mastitic quarters were at 29.07, 18.05, and 26.69 cycles respectively (Table 7).

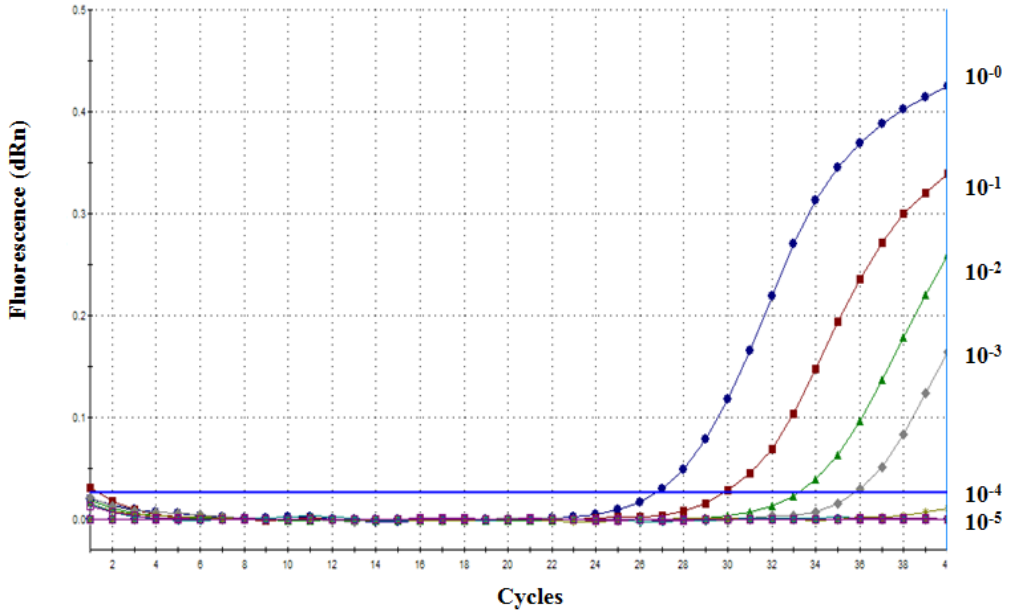
The mean Ct value for TNF-α expression in healthy quarters was 32.6 cycles with a confidence interval (CI) of 30.0-35.2 cycles. While the mean Ct value for TNF-α in mastitic quarters was 29.1 cycles with a CI of 24.3-33.9 cycles. There was no significant difference in the TNF-α Ct values between mastitic and healthy quarters. The relative expression of TNF-α in mastitic quarters was similar to that observed in healthy quarters (Tables 7 and 8).

The mean Ct value for IL-8 expression in healthy quarters was 22.7 cycles with a CI of 21.4-23.9 cycles. While the mean Ct value for IL-8 in mastitic samples was 17.1 cycles with a CI of 15.1-18.9 cycles. There was significant difference in the Ct values for Il-8 between mastitic

and healthy quarters. The relative expression of IL-8 in mastitic quarters was 2.3 fold higher than that observed in healthy quarters (Tables 7 and 8).

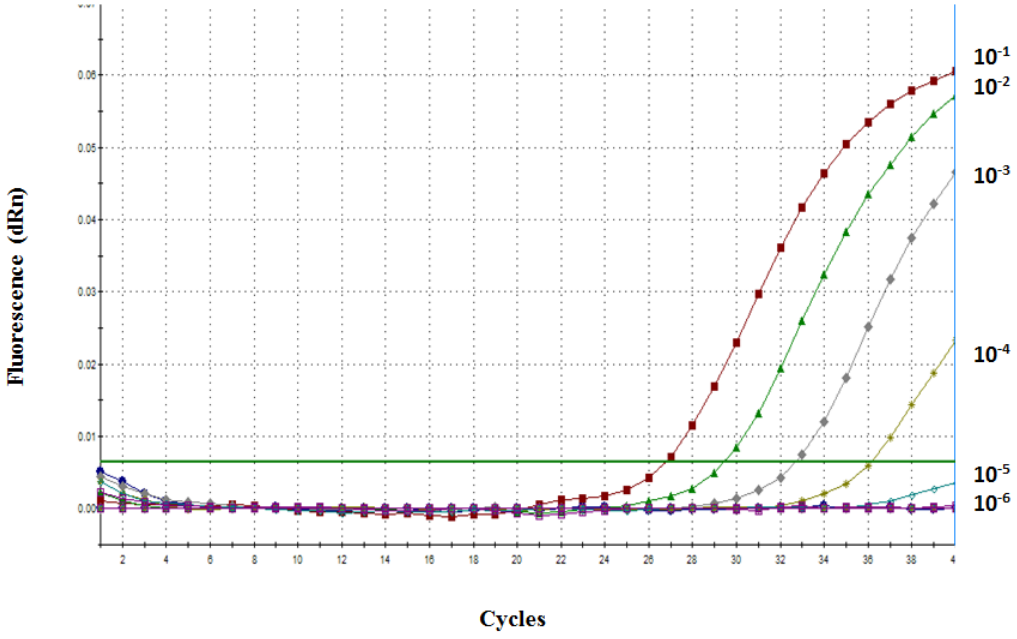
The mean Ct value for IL-10 expression in healthy quarters was 30.8 cycles with a CI of 29.4-32.1 cycles. The mean Ct value for IL-10 in mastitic samples was 26.6 cycles with a CI of 126.1-27.1 cycles. Therefore, there was a significant difference in the Ct values for Il-10 between mastitic and healthy quarters. The relative expression of IL-10 in mastitic quarters was 1.19 fold higher than that observed in healthy quarters (Tables 7 and 8).

Figure 1. 28S Serial Dilution Amplification Plot



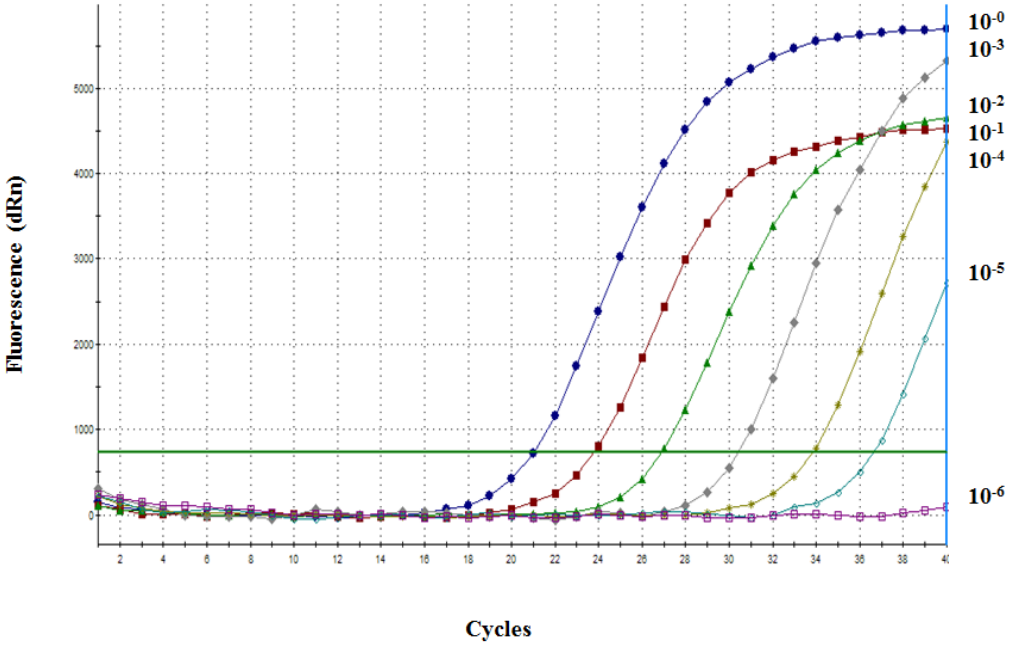
Fluorescence vs. the number of cycles plotted for the 1:10 serial dilution of mRNA samples for the target protein 28S.

Figure 2. TNF-α Serial Dilution Amplification Plot



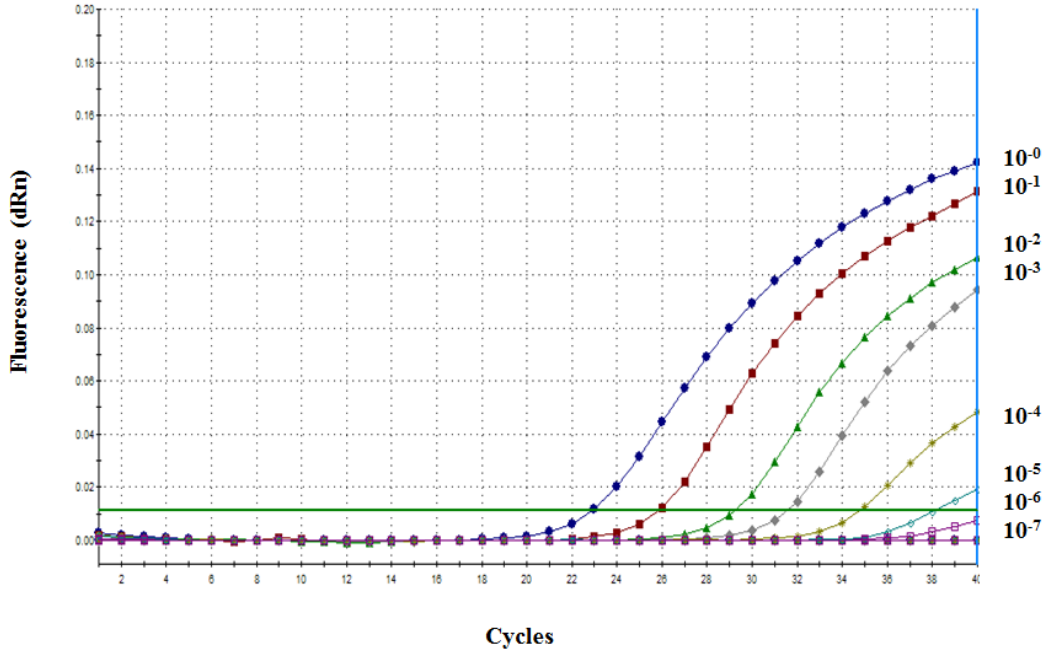
Fluorescence vs. the number of cycles plotted for the 1:10 serial dilution of mRNA samples for the target protein TNF-α.

Figure 3. IL-8 Serial Dilution Amplification Plot



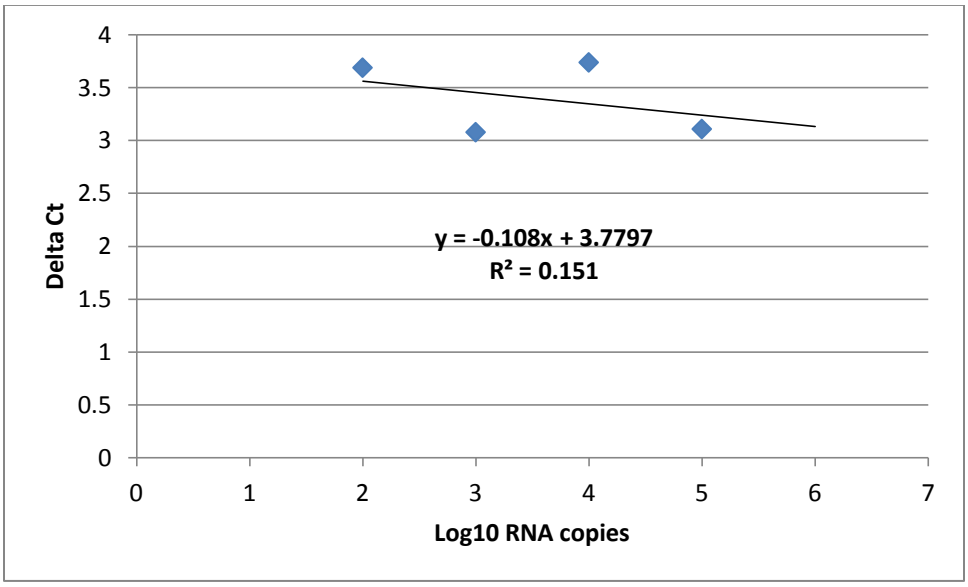
Fluorescence vs. the number of cycles plotted for the 1:10 serial dilution of mRNA samples for the target protein IL-8.

Figure 4. IL-10 Serial Dilution Amplification Plots



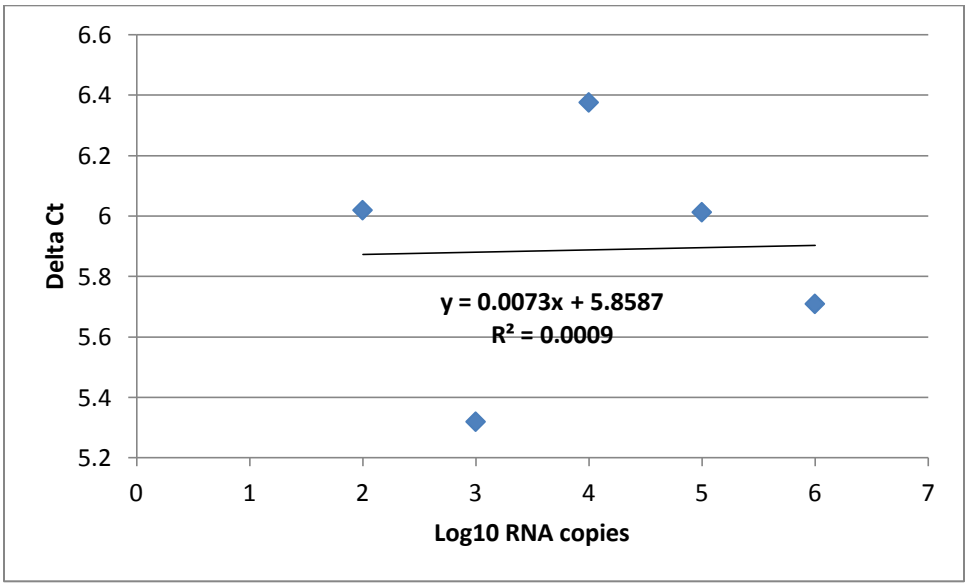
Fluorescence vs. the number of cycles plotted for the 1:10 serial dilution of mRNA samples for the target protein IL-10.

Figure 5. TNF-α Efficiency Plot



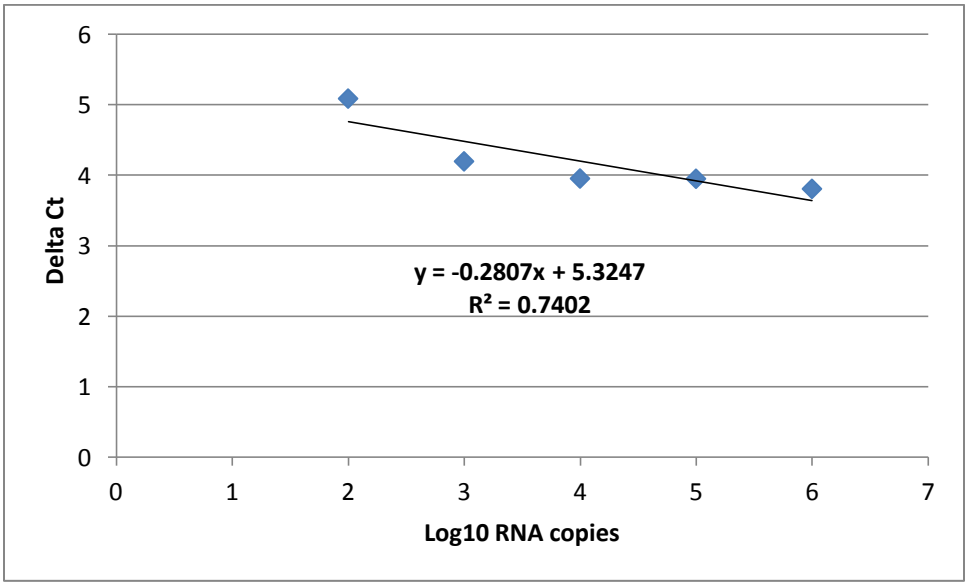
ΔCt plotted against $\log_{10}$ RNA copies using reference gene (28S) and target gene (TNF-α). These plots show similar amplification for both genes.

Figure 6. IL-8 Efficiency Plot



ΔCt plotted against $\log_{10}$ RNA copies using reference gene (28S) and target gene (IL-8). These plots show similar amplification for both genes.

Figure 7. IL-10 Efficiency Plot



ΔCt plotted against $\log_{10}$ RNA copies using reference gene (28S) and target gene (IL-10). These plots show similar amplification for both genes.

Table 5. Standardization of RT-PCR assays using serially diluted 10-fold RNA of 28S, TNF-α, IL-8 and IL-10 target genes

Target	Mean Ct values							Amplification Efficiency	R^2
	10-fold RNA serial dilution								
	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}		
28S	26.78	29.86	33.28	35.82	39.94	No Ct	No Ct	107.7	0.989
TNFα	-	26.75	29.55	32.74	36.25	38.79	No Ct	108.2	0.997
IL-8	21.07	23.85	26.91	30.5	33.92	36.7	No Ct	105.4	0.998
IL-10	22.97	25.91	29.33	31.62	34.85	38.13	No Ct	116.2	0.998

The data used to standardize the RT-PCR assay showing the Ct values for the target proteins and the 28S reference gene, the amplification efficiencies, and the R^2 values; this showed that the assay worked well.

Table 6. Somatic cell count and bacteriologic analyses of quarter milk samples

Sample[a]	Somatic Cells[b]		Bacteriology[c]		
Cow-Quarter	SCC X1000	SCC-based Quarter Status	MAC	BPA	EMCO
			NLF	CNS	Esc+
1652- RF	4	Healthy	-	-	-
1652- LF	12	Healthy	+	+	-
1652- RR	12	Healthy	-	+	-
1448- RR	25	Healthy	-	+	-
1645- LF	37	Healthy	-	+	+
1448- RF	44	Healthy	-	+	+
1712- LR	52	Healthy	-	+	+
1645- RF	54	Healthy	-	+	-
1712- RR	58	Healthy	-	-	-
1712- LF	71	Healthy	-	+	+
1312- LF	128	Healthy	-	-	-
1312- RR	129	Healthy	-	-	-
1699- RR	156	Healthy	-	-	+
1699 -LF	238	Mastitic	-	+	-
1699- RF	309	Mastitic	-	-	-
1645- LR	318	Mastitic	-	+	-
1712- RF	350	Mastitic	-	+	-
1652- LR	356	Mastitic	+	+	-
1312- LR	459	Mastitic	-	+	-
1699- LR	469	Mastitic	-	+	+
1448- LR	3,039	Mastitic	-	+	+
1312- RF	3,095	Mastitic	-	-	-
1645- RR	5,065	Mastitic	-	-	-

[a] RF – right front; RR- right rear; LF-left front; LR- left rear
[b] SCC: Somatic Cell Count
[c] MAC: MacConkey's Agar; NLF, non-lactose fermenter
[c] BPA: Baird Parker Agar; CNS, coagulase negative staphylococci
[c] EMCO: Edwards Modified Agar supplemented with Colistin and Oxolinic acid; Esc+, Esculin positive colonies

23 quarter milk samples were taken from 6 2nd lactation cows 45-60 days in milk from the Penn State Dairy Herd. Samples with SCC > 200,000 were categorized as mastitic, and samples with SCC< 200,000 were categorized as healthy. Bacteriologic analysis was done using PSU-QUAD plates at 37°C for 48 hours.

Table 7. Expression of target genes in healthy and mastitic quarters

Sample	Biomarkers [a]			
	Ct Values			
Healthy Cow- Quarters	**TNFa**	**IL-8**	**IL-10**	**28S**
1652- RF	30.62	25.05	34.35	33.94
1652- LF	31.00	25.33	33.50	34.07
1652- RR	31.75	25.25	35.13	35.79
1448- RR	39.41	19.62	29.21	32.64
1645- LF	26.79	22.46	30.23	33.74
1448- RF	37.78	21.73	30.34	36.41
1712- LR	34.13	24.17	31.67	33.10
1645- RF	- [a]	21.19	28.79	32.64
1712- RR	29.64	22.63	30.68	32.01
1712- LF	30.54	22.53	29.64	33.40
1312- LF	30.55	20.33	29.46	29.80
1312- RR	29.97	21.42	30.86	31.08
1699- RR	39.21	16.46	27.26	30.56
Mean Ct values of healthy quarters	32.62	22.17	30.86	33.01
(A) Δ Ct value (TNFα /IL-8/Il-10 Ct value- 28S Ct value)	0.40	10.85	2.16	
Mastitic Cow-Quarters	**TNFα**	**IL-8**	**IL-10**	**28S**
1699 –LF	27.87	16.10	26.82	29.43
1699- RF	- [a]	17.11	27.55	29.62
1645- LR	39.23	20.49	29.37	32.40
1712- RF	28.47	21.04	27.84	30.86
1652- LR	24.56	18.85	28.79	29.60
1312- LR	28.18	18.48	28.86	28.99
1699- LR	29.43	16.24	25.01	28.85
1448- LR	20.12	13.42	21.94	24.72
1312- RF	25.16	12.89	25.32	27.11
1645- RR	38.62	15.91	25.37	29.44
Mean Ct values for mastitic quarters	29.07	17.05	26.69	29.10
(B) Ct Value (TNFα /IL-8/Il-10 Ct value- 28S Ct value)	0.03	12.05	2.42	
ΔΔ Ct (A-B)	0.37	-1.20	-0.26	
Relative expression in mastitic quarters as compared to healthy quarters (2^- ΔΔ Ct)	0.78	2.30	1.19	

[a] Ct values for TNF-α for cow quarters 1645-RF and 1699-LF could not be determined

Mean Ct values for RT-PCR of 23 quarter milk samples taken from 6 2nd lactation cows 45-60 days in milk from the Penn State Dairy Herd. Samples with SCC > 200,000 were categorized as mastitic.

Table 8. Statistical analysis of C_T values of TNF-α, IL-8 and IL-10 gene expression

Sample	Statistic	TNF-α	IL-8	IL-10
Healthy quarters	Ct range	26.8-39.4	16.4-25.3	27.3-35.1
	Mean Ct (CI)	32.6 (30.0-35.2)	22.7 (21.4-23.9)	30.8 (29.4-32.1)
Mastitic quarters	Ct range	20.1-39.2	12.9-21.0	21.9-29.4
	Mean Ct (CI)	29.1 (24.3-33.9)	17.1 (15.1-18.9)	26.6 (26.1-27.1)
	P (< 0.05)	1.57 (0.06)	4.67 (0.00)	4.34 (0.00)

Analysis of Ct values obtained from RT-PCR on 23 quarter milk samples from 6 2nd lactation Penn State Dairy Herd cows 45-60 days in milk comparing healthy and mastitic quarters. SCC of > 200,000 was considered mastitic, and 28S was used as the internal control.

CHAPTER 5: DISCUSSION

The National Mastitis Council recommends the use of Somatic Cell Count along with bacteriological culture analysis to determine the udder health status of a cow (Herlekar, 2007). Milk samples from a quarter or pooled milk sample from an udder with Somatic Cell counts of < 200,000 cells/ml is considered to be from a healthy udder, while SCC > 200,000 cells/ml is suggestive of subclinical mastitis. Bacteriologic culture analysis is definitive confirmation of the bacteriologic status of the udder which allows the determination on the type of bacteria associated with intramammary infection, and provides a rationale for mastitis prevention and control practices. The need for prognostic molecular biomarkers that aid veterinarians in determining the status of udder health is greatly needed to refine the findings of SCC and bacteriologic analysis and aid in making more effective treatment decisions.

Only three types of bacteria were present in the samples: coagulase negative staphylococci, non-lactose fermenters, and Esculin positive colonies. Coagulase negative staphylococci and Esculin positive colonies were found more often in quarters categorized as healthy (SCC < 200,000) than in mastitic quarters (SCC > 200,000). The non-lactose fermenters were only present in one cow, in both a healthy and a mastitic quarter. Hence, the data indicate that coagulase negative staphylococci, NLF, and Esc+ colonies were present in many of the quarters and therefore did not specifically indicate the healthy or mastitic status of the quarters.

Bacteriologic analysis using culture methods, which has the advantage of identification of bacterial species involved in mastitis, also has the limitations of being time consuming and that it cannot be used on-site (Viguier et al., 2009). All these factors have led to the development of new diagnostic methods, namely the use of biomarkers. There are many potential biomarkers, including

IL-12, TNF-α, IL-10, IL-8, INF-γ, and IL-1β (Leutenegger et al., 2000). The three biomarkers analyzed in this study, TNF-α, IL-10, and IL-8, were chosen because they are major cytokines that have been previously examined and are known to be a part of the immune response to intramammary infection (Peli et al., 2004). They also represent different components of the immune response; TNF-α is a pro-inflammatory factor representative of the acute-phase response, IL-8 is a chemoattractive factor representative of neutrophil recruitment, and IL-10 is a regulatory and anti-inflammatory factor representative of immune regulation (Britti et al., 2005). TNF-α is a pro-inflammatory cytokine that mediates the inflammatory response at local and systemic levels. It promotes neutrophil recruitment and induces fever and the acute phase response. Variable results have been obtained regarding TNF-α and mastitis with random increases or no change in its levels in the milk. Experimental infection of *S. aureus* did not show any detectable level of TNF-α (Bannerman et al., 2004a). Another study showed that TNF-α levels increased with *K. pneumoniae* challenge and peaked around 12-24 hours after infection (Bannerman et al., 2004b). In addition, intramammary infection with either *S. marcescens* or *S. uberis* induced increased levels, but the increase was transient and highly variable (Bannerman et al., 2004c). Furthermore, TNF-α levels were significantly higher with increased bacteriology, but were not correlated with SCC (Peli et al., 2004). Variations have also been observed in the level of TNF-α in response to different bacterial agents. For example, experimental infection with *E. coli* showed an increase in TNF-α, while no detectable level could be detected when challenged with *S. aureus*. The expression of TNF-α across these studies is similar to that observed in our study in which TNF-α expression was similar in both mastitic and healthy quarters. Therefore it can be inferred that TNF-α is not suitable marker for mastitis.

IL-8 is a chemotactic cytokine; one of its main functions is the recruitment and activation of neutrophils. Previous studies have found IL-8 mediated chemotaxis to be absent from healthy milk; this could be due to a lack of IL-8 expression or to regulation of IL-8 (Leutenegger et al., 2000). Previous studies have also shown a positive correlation between SCC and the level of IL-8 in the milk. It was found that IL-8 levels were higher in milk samples with higher SCC compared to samples with low SCC. This is in contrast with TNF-α, which has been shown to have higher levels with milk samples of positive bacteriology and not linked to SCC; this indicates that TNF-α is not necessary to stimulate IL-8 synthesis and that IL-8 expression is not induced by the presence of bacteria (Peli et al., 2004). Another study showed that initial increases in IL-8 were concurrent with increases in milk SCC following bacterial challenge (Bannerman et al., 2004b, c). This is comparable to the result of our study, as the relative expression of IL-8 was consistently higher in the mastitic quarters (high SCC) than the healthy quarters (low SCC). The relationship between high SCC and IL-8 expression indicates that IL-8 could be a promising biomarker for mastitis.

IL-10 is a regulatory and anti-inflammatory cytokine which downregulates pro-inflammatory cytokine production. Previous studies in relation to IL-10 and bacterial infections in milk quarters have shown variable results. The levels of IL-10 were found to increase with *E. coli* infection, and there was slight increase with *S. aureus* infection (Bannerman et al., 2004a). In another study, the level of IL-10 increased and stayed high with *K. pneumoniae* infection throughout the study period (Bannerman et al., 2004b). Bannerman et al. (2004b) also observed that gram negative bacteria are more potent inducers of IL-10 than gram positive bacteria. In a separate study, when challenged with *S. marcescens*, a gram negative bacteria, after an initial increase, the IL-10 levels returned to pre-challenge levels >18 hours later; when challenged by *S. uberis*, a gram positive species, elevated levels of IL-10 persisted until the end of the study and the peak level was higher (Bannerman et al.,

2004c). The level of IL-10 is inversely related to TNF-α as IL-10 inhibits production of TNF-α (Bannerman et al., 2004a). A similar pattern was observed in our study where we found low levels of IL-10 along with high levels of TNF-α.

Some studies have shown a correlation with IL-10 levels and SCC, though the researchers stated that due to the lack of data on the function and origin of IL-10 further study was needed to explain the outcome (Peli et al., 2004). In our study, the expression of IL-10 in mastitic quarters was 1.19 fold higher than that of healthy quarters. Based on the findings in our study it can be inferred that although IL-10 expression was elevated in mastitic quarters; however the level of expression (Ct value) was not sufficient to allow its use as a mastitis biomarker.

Overall, based on our indicator study, TNF-α and IL-10 are less likely to serve as biomarkers for mastitis. However, it was observed that expression of IL-8 was significantly higher in mastitis quarters as compared to healthy quarters. Based on this observation it can be inferred that IL-8 could be used as a potential biomarker to evaluate the health condition of cattle quarters. However, further study on a larger scale would be needed to further establish the validity of IL-8 as potential biomarker.

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