

## The Bovine Mammary Glands Cytokines at the Periparturient Period

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### Abstract

Bovine mammary glands are susceptible to intramammary infection at the periparturient period. Cytokines are one of the sensitive means in examining the immune responses of mammary glands and they could serve as a suitable tool for the udder health control or in evaluating of mastitis treatment or vaccine efficiency at this period. The gene expression of cytokines, IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFN- $\gamma$  and TNF- $\alpha$  were examined in milk cells from cattle two weeks before their parturition and cattle at their mid-lactation with RT-PCR. All cytokines were detected in milk cells from periparturient period except IL-12, whereas in milk cells from mid-lactation, cytokines IL-2, IL-4, and IL-12 cDNA failed to be detected. The results indicated the versatility of this approach in providing flexible tool to reveal the status of the mammary glands at this period.

**Key Words:** Cytokines, bovine, mastitis, periparturient, IL-12, RT-PCR

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## Introduction

Bovine mammary glands are highly susceptible to intramammary infection (IMI) at the periparturient period (PP) (Burton et al, 2001; Cai et al., 1994; Sordillo et al., 1991; Park et al., 1992; Kimura et al., 1999; Lee and Kehrli; 1998, Nonnecke et al., 2003). The cells population reveal dramatic changes at the PP. Park et al. (1992) showed that T- lymphocytes decreased from 62% at late-lactation to 16% during PP. The CD4<sup>+</sup>/CD8<sup>+</sup> cell ratio reached its lowest level at the late stages of the PP (Asai et al., 1998; Park et al., 1992). Contrary to the T-lymphocytes percentage at the PP, B-lymphocytes increased from 7% at late lactation to 25% whereas, level of macrophages peaked to 69%.

Numerous studies delineated the sever impairment of neutrophils function at the PP (Cai et al., 1994; Kimura et al., 1999; Lee and Kehrli, 1998). Neutrophils of normal bovine mammary glands at PP expressed apparent reduction in ingestion capacity, antibody dependent cell mediated cytotoxicity and random migration (Cai et al., 1994). The capacity of neutrophils to express adhesion molecules like L-selectin and  $\beta$ 2- integrin diminished markedly (Kimura et al., 1999; Lee and Kehrli, 1998).

The level of cytokines and their gene expression indicated considerable increase at the PP. A marked increase in the mRNA expression of interleukin-2 (IL-2) and interleukin-4 (IL-4) was recorded (Asai et al., 1998). The level of IL-2 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were shown to increase as parturition approaches (Sordillo et al., 1991). However, interferon- $\gamma$  (IFN- $\gamma$ ) was barely detectable in the PP (Sordillo et al., 1991, Burton et al., 2001). Cytokines interleukin-1 $\alpha$  (IL-1 $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10) and interleukin-12 (IL-12) were not addressed at the PP. Interleukin-1 and IL-6 are major proinflammatory cytokines that play critical role in coliform mastitis (Shuster et al., 1997). Interleukin-8 is potent neutrophils-chemoattractant factor (Barber and Yang, 1998). Interleukin-4 and IL-10 in the bovine mammary glands were scarcely addressed. Interleukin-12 is potent cytokine that enhances the pro Th1 cytokines production and result in considerable mobilization of innate and humoral immunity (Trinchieri, 1995). Transcriptional activity of IL-12 at the late lactation was shown a significant increase in comparison to its level at mid-lactation (Alluwaimi and Cullor, 2002).

In recent years cytokines were employed as adjuvant or as innovative therapeutical means in treatment and/or diagnosis of mastitis (Alluwaimi, 2004).

This study is examining the possibility of using the reverse-transcriptase polymerase chain reaction (RT-PCR) to evaluate the udder health at the PP. This approach could also be useful in disclosing the efficiency of vaccines or other hygienic measures that are employed during the PP to amoderate the susceptibility of mammary glands to IMI. Further aim of this study is to explore the expression of other cytokines at the PP.

**Materials and Methods**

*Milk samples.*

The composite milk samples were collected from three Holstein cattle (Alreif dairy farm, Al-Ahsaa) at their last 2 weeks of gestation period. Composite milk samples that were used as control were collected from the same farm and from cattle at their mid-lactation.

*Cytokines*

The bovine cytokines, interleukin-1 $\alpha$  (IL-1 $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFN- $\gamma$  and TNF- $\alpha$  were studied with RT-PCR. The cytokines foreword and reverse primers were reported by Riollet et al. (2001) (Table-1).

*RNA extraction*

Milk samples were first centrifuged at 2000rpm for 15min and the pellets were washed once with RNase free phosphate buffer saline (Sigma). Total RNA (tRNA) was then extracted from approximately 5x10<sup>6</sup> total milk cells using Qiagen total RNA extraction kit (Qiagen Ltd, Valencia, CA, USA). The procedures were carried out according to the manufacturer's directions. The tRNA was eluted using 15-30  $\mu$ l RNase free water heated for 90 °C.

*RT-PCR*

Approximately 1 $\mu$ l of tRNA was reverse transcribed to Complementary DNA (cDNA) using 20  $\mu$ l reverse transcription reaction. The mixture containing final concentration of 5 mM MgCl<sub>2</sub>, 1X of a 10 X PCR buffer, 2.5 $\mu$ M random hexamers, 1 mM of each of dGTP, dATP, dTTP and dCTP, 1U/ $\mu$ l RNase inhibitor and 2.5 U/ $\mu$ l reverse transcriptase (Gene Amp<sup>®</sup> RNA PCR kits, Applied Biosystems, Branchburg, NJ, USA). The mixture was incubated at 42°C for 15 minutes heated to 99°C for 5

minutes and maintained at 5°C for 5 minutes using GeneAmp PCR thermocycler system 2400 (Applied BioSystems, USA). 1  
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*PCR amplification* 3

The RT product was brought up to 100 µl by adding 80 µl PCR mixture containing a final concentration of 2mM MgCl<sub>2</sub>, 1X of a 10X PCR buffer, and 2.5U/100 µl of *Thermus aquaticus* DNA polymerase (Ampli Taq® DNA polymerase) and approximately 40-45 pM of forward and reverse primers (Proligo, USA). 4  
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PCR mixture of 100 µl was amplified as stated in table-2 using GeneAmp PCR thermocycler system 2400 (Applied BioSystems, USA). 8  
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*Agarose gel electrophoresis* 10  
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The RT-PCR products were run on 1% agarose gels [1g agarose (Sigma chemical Co, Spain) in 1x tris-acetate EDTA (TAE) buffer (PH8) (40 mM Tris-acetate, 1 mM EDTA)]. The gel was fixed in the horizontal gel electrophoresis apparatus with the addition of 1 L running buffer (1 X TAE buffer) containing 25 µl of 0.5µg/ml ethidium bromide. The samples and the 100 bp ladder marker (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) were Loaded with 30% glycerol, and run at 90 V for 90 min. The gel was then visualized with ultra-violet illuminator (Fisher Scientific, USA) and photographed by the C-5060 digital camera (Olympus, Japan). 12  
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**Results** 20

Gel electrophoresis of the cytokines RT-PCR is shown in fig.1, 2 and 3. All cytokines were expressed in normal milk except IL-2, IL-4 and IL-12 (Fig.1, 2). However, gel electrophoresis of cDNA from milk cells at the PP revealed the expression of the whole studied cytokines except IL-12 (Fig.-3). The bands of IL-2 and IL-4 were too weak to be visualized in fig. 3. 21  
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**Discussion** 26

Mammary glands are highly susceptible to IMI at the PP (Burton et al, 2001; Nonnecke et al., 2003). Several studies investigated the cytokines activity at the PP (Sordillo et al., 1991; Asai et al., 1998; Burton et al., 2001). However, the majority of these studies limited their scope to IL-2, IFN-γ and TNF-α. In this study the gene expression of ten cytokines, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFN-γ and TNF-α was explored using RT-PCR. 27  
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The gel electrophoresis of RT-PCR of cytokines cDNA indicated the significant cytokines expression at the PP. Expression of IL-2, IL-4, and TNF-α at the PP was 33  
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reported previously (Sordillo et al., 1991; 1995; Asai et al., 1998). Despite the evident vulnerability of mammary glands to IMI at the PP, reports indicated the elevated expression of IL-2, IL-4 and TNF- $\alpha$  at this period (Asai et al., 1998, Sordillo et al., 1995).

In this study IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-10 and IFN- $\gamma$  were also detected at this period. However, IL-12 cDNA at PP and mid-lactation was not detected. Although, a significant elevation of gene expression pattern was recorded for IL-12 at late-lactation (Alluwaimi and Cullor, 2002), the failure in detection of IL-12 at the PP despite the elevation of monocytes and macrophages requires further analysis. IL-8 was detected, but its transcriptional activity at late-lactation was not increased (Alluwaimi et al., 2003).

Nevertheless, expressions of these cytokines at the PP most probably do not enhance the weakened immunity at this period due to the domination of several immunosuppressant factors, mainly glucocorticoid, at the mammary glands (Guidry et al., 1976).

Cytokines have been considered important criteria in development of techniques in monitoring the udder health. However, lack of user-friendly cytokines-based techniques considered a major factor in hampering the development of these techniques (Alluwaimi, 2004). RT-PCR could be one of the candidates of cytokines-based techniques in udder health control. In this study, RT-PCR of cytokines RNA reflected promising signs of differences at the PP from that of mid-lactation. Nevertheless, sensitivity and the reproducibility of RT-PCR in udder health control required further analysis.

**Conclusion:**

Variation in cytokines gene expression at PP could provide considerable avenue in evaluation of the mammary glands at this period. Expression of IL-2 and IL-4 in addition to other cytokines that are expressed in mid-lactation indicates that the mammary glands at PP are not entirely deprived from any protection. However, the cytokines level and their biological activity need to be elucidated. In conclusion, this technique could play useful approach as explicit, reasonably sensitive and straightforward in evaluating the udder health at the PP.

**Acknowledgment**

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Table -1. The foreword (F) and reverse (R) sequences of bovine cytokines primers

| Gene                 | Primers 5'→ 3' |                      | cDNA (bp) |
|----------------------|----------------|----------------------|-----------|
| IL-1 $\alpha$        | *F             | TGCAAGCTATGAGCCACTTC | 291       |
|                      | *R             | GCATTCCTGGTGGATGACTC |           |
| IL-1 $\beta$         | F              | TGGGAGATGGAAACATCCAG | 231       |
|                      | R              | TTTATTGACTGCACGGGTGC |           |
| IL-2                 | F              | CTACTTCAAGCTCTACGGGG | 248       |
|                      | R              | TTGATCTCTCTGGGGTTCAG |           |
| IL-4                 | F              | TGCCCCAAAGAACAACAATG | 200       |
|                      | R              | TTTAGCCTTTCCAAGAGGTC |           |
| IL-6                 | F              | TGAAAGCAGCAAGGAGACAC | 187       |
|                      | R              | TGACATTTTCCTGATTTCCC |           |
| IL-8                 | F              | ACTGGCTGTTGCTCTCTTGG | 260       |
|                      | R              | ACCTGCACAACCTTCTGCAC |           |
| IL-10                | F              | TGCACAGCTTACCTGTGACC | 177       |
|                      | R              | CGCAGGGTCTTCAGCTTCTC |           |
| IL-12 <sub>P40</sub> | F              | AGGTCGTGGTAGAAGCTGTG | 270       |
|                      | R              | CCTTGTGGCATGTGACTTTG |           |
| IFN- $\gamma$        | F              | AGCCCAGATGTAGCTAAGGG | 210       |
|                      | R              | CTCCAGTTTCTCAGAGCTGC |           |
| TNF- $\alpha$        | F              | AACAGCCCTCTGGTTCAAAC | 310       |
|                      | R              | TCTTGATGGCAGACAGGATG |           |

\* F= Foreword

\* R= Reverse

Table -2. PCR profile times and temperature for amplification of the mouse cytokine cDNA

| Initial step     | Each 35 Cycle   |                 | Final step     |
|------------------|-----------------|-----------------|----------------|
|                  | Melt            | Anneal-extend   |                |
| 105 sec.<br>95°C | 15 sec.<br>95°C | 30 sec.<br>60°C | 7 min.<br>72°C |

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Fig. 1: The gel electrophoresis of cytokines cDNA from normal milk cells. The bands are, **M**= marker, **1**= IL-1 $\alpha$ , **2**= IL-1 $\beta$ , **3**= IL-6, 4=IL-8

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Fig. 2: The gel electrophoresis of cytokines cDNA from normal milk cells. The bands are, **M**= marker, **1**= IL-10, **2**= IFN- $\gamma$ , **3**= TNF- $\alpha$ .

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Fig-3: The gel electrophoresis of cytokines cDNA of milk cells at the periparturient period. The bands are, **M**=markers, **1**= IL-1 $\alpha$ , **2**= IL-1 $\beta$ , **3**= IL-6, 4=IL-8, 5=IL-10, 6= = IFN- $\gamma$ , 7=TNF- $\alpha$ . Note that IL-2 and IL-4 bands (between bands 2 and 3) were detected but they were so weak to be visualized in this figure.

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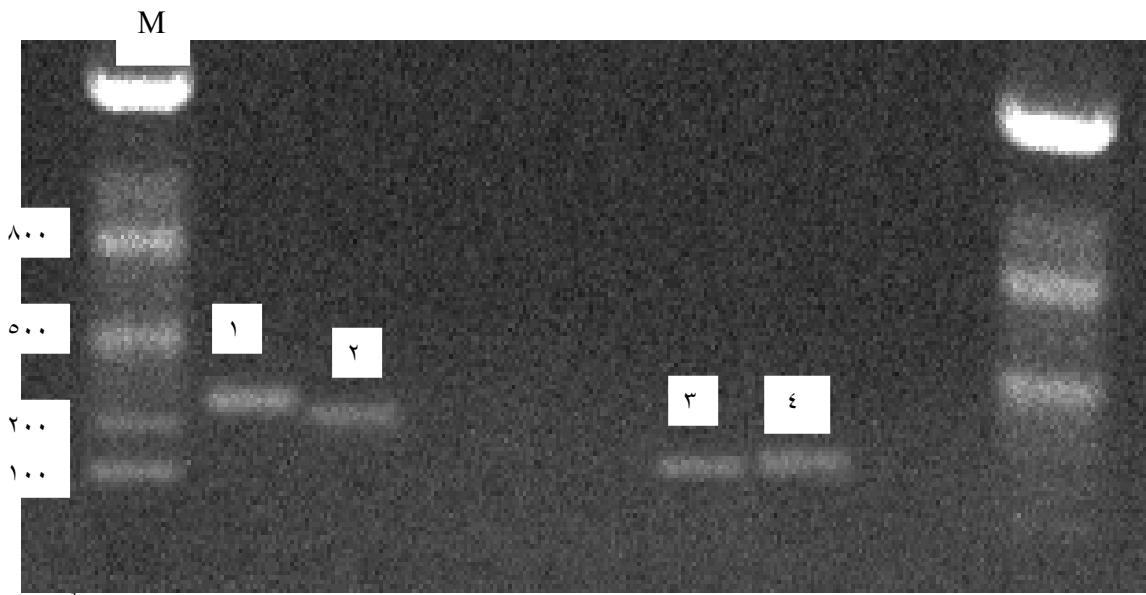
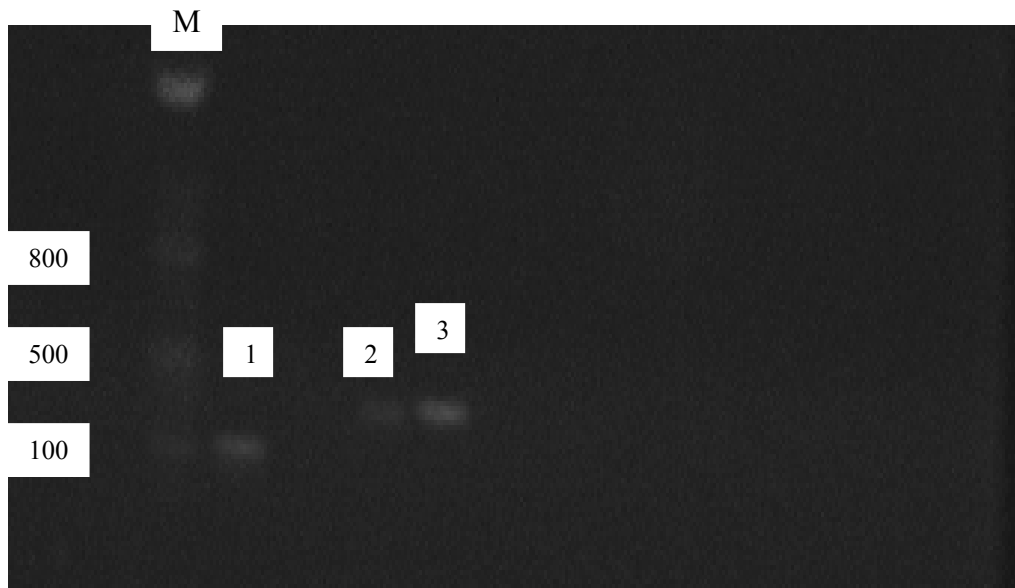


Fig.1: Alluwaimi, A. M., The bovine mammary gland cytokines.....etc.

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Fig.2: Alluwaimi, A. M., The bovine mammary gland cytokines.....etc.

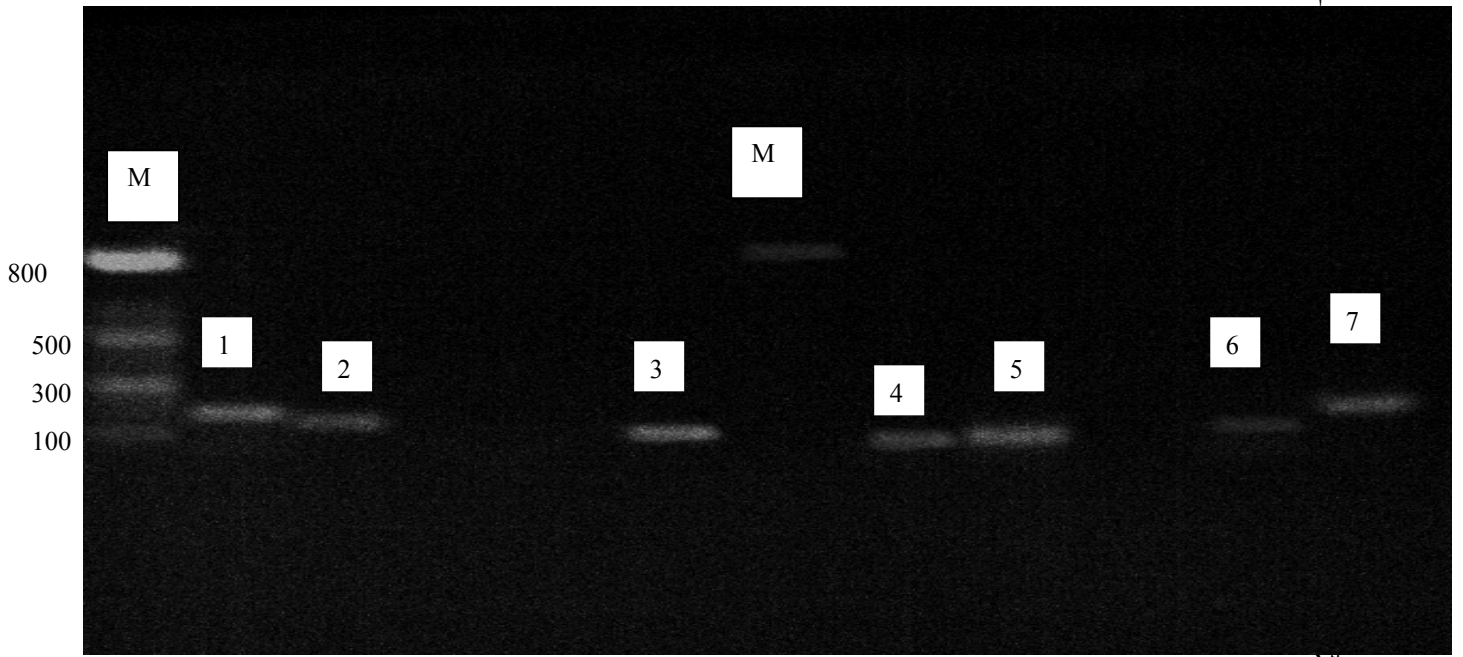


Fig.3: Alluwaimi, A. M., The bovine mammary gland cytokines.....etc.

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