The Bovine Mammary Glands Cytokines at the Periparturient Period

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Abstract

Bovine mammary glands are susceptible to intrammamary 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 mastitis treatment or vaccine efficiency at this period. The gene expression of cytokines, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFN-γ and TNF-α 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.

Introduction:

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

Numerous studies delineated the sever impairment of neutrophils function at the PP (Cai *et al.*, 1994; Lee and Kehrli, 1998; Kimura *et al.*, 1999). 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 β2-intgrin diminished markedly (Lee and Kehrli, 1998; Kimura *et al.*, 1999).

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-α (TNF-α) were shown to increase as parturition approaches (Sordillo *et al.*, 1991). However, interferon-γ (IFN-γ) was barely detectable in the PP (Sordillo *et al.*, 1991, Burton *et al.*, 2001). Cytokines interleukin-1α (IL-1α), interleukin-1β (IL-1β) 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 colifrom mastitis (Shuster *et al.*, 1997). Interleukin-8 is potent neutrophils-chemoatractant 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 results in considerable mobilization of innate and humoral immunity (Trinchieri, 1995). Transcriptional activity of IL-12 at the late lactation has 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 theraputical means in treatment and/or diagnosis of mastitis (Alluwaimi, 2004). Cytokines could provide swift, reliable and highly susceptible means in bovine mastitis diagnosis. Cytokines represents the signals that dictate the scenario of immune responses of normal and mastitic udder. Hence, Subtle changes in cytokine network of mammary gland in health and disease could be considered promising candidate in monitoring the udder health through early detection of infection and monitoring the effectiveness of therapeutic strategies.

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 amolerate 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 the same number of cattle at their mid-lactation (3-4 months postpartum).

Cytokines

The bovine cytokines, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFN-γ and TNF-α were studied with RT-PCR. The cytokines foreword and reverse primers were reported by Riollet *et al.* (2001) (Table-1).

Table (1)

The foreword (F) and reverse (R) sequences of bovine cytokines primers

Gene	Primers 5′ → 3′		cDNA (bp)
IL-1α	*F	TGCAAGCTATGAGCCACTTC	291
	♣R	GCATTCCTGGTGGATGACTC	
IL-1β	F	TGGGAGATGGAAACATCCAG	231
	R	TTTATTGACTGCACGGGTGC	
IL-2	F	CTACTTCAAGCTCTACGGGG	248
	R	TTGATCTCTCTGGGGTTCAG	
IL-4	F	TGCCCCCAAAGAACACAACTG	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$_{p40}$	F	AGGTCGTGGTAGAAGCTGTG	275
	R	CCTTGTGGCATGTGACTTTG	
IFN-γ	F	AGCCCAGATGTAGCTAAGGG	215
	R	CTCCAGTTTCTCAGAGCTGC	
TNF-α	F	AACAGCCCTCTGGTTCAAAC	315
	R	TCTTGATGGCAGACAGGATG	

*F= Foreword

♣R= Reverse

RNA extraction

Milk samples were first centrifuged at 700 g for 15min and the pellets were washed once with RNAse free phosphate buffer saline (Sigma). Total RNA was then extracted from approximately $5x10^6$ 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 total RNA was eluted using 15-30 µl of 90 °C-heated RNAse free water.

RT-PCR

Approximately 1µl of total RNA was reverse transcribed to Complementary DNA (cDNA) using 20 µl reverse transcription reaction. The mixture containing final concentration of 5 mM $MgCl_2$, 1X of a 10 X PCR buffer, 2.5µM random hexamers, 1 mM of each of dGTP, dATP, dTTP and dCTP, 1U/µl RNase inhibitor and 2.5 U/µl reverse transcriptase (Gene Amp® 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).

PCR amplification

The RT product was brought up to 100 µl by adding 80 µl PCR mixture containing a final concentration of 2mM $MgCl_2$, 1X of a 10X PCR buffer, and 2.5U/100 µl of *Thermus aquticus* DNA polymerase (Ampli Taq® DNA polymerase) and approximately 40-45 pM of forward and reverse primers (Proligo, USA).

PCR mixture of 100 µl was amplified as stated in table-2 using GeneAmp PCR thermocycler system 2400 (Applied BioSystems, USA).

Table (2)

PCR profile times and temperature for amplification of the mouse cytokine cDNA

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

Agarose gel electrophoresis

The RT-PCR products were run on 1% agarose gels [1g agarose (Sigma chemical Co, Spain) in 1x tris-acetate EDTA (TAE) buffer (pH 8) (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).

Results:

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.

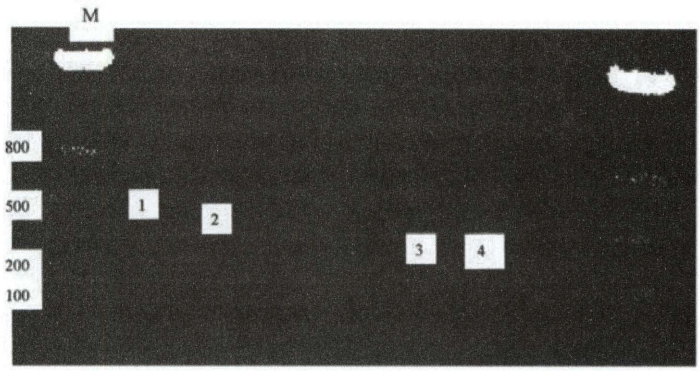

Fig. 1: The gel electrophoresis of cytokines cDNA from normal milk cells. The bands are, **M**= marker, **1**= IL-1α, **2**= IL-1β, **3**= IL-**6**, 4=IL-8

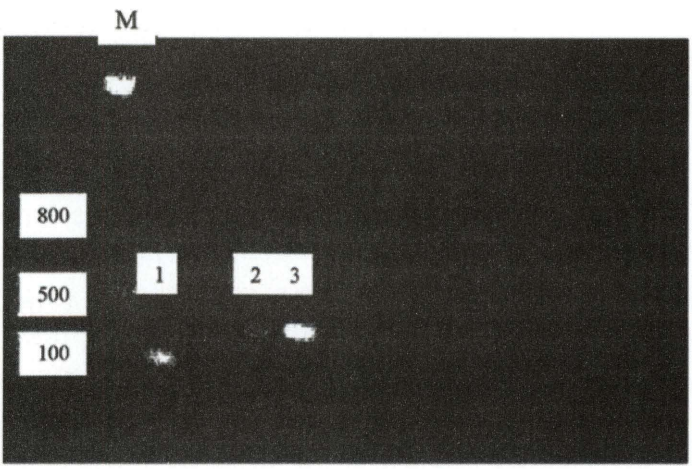

Fig. 2: The gel electrophoresis of cytokines cDNA from normal milk cells. The bands are, **M**= marker, **1**= IL-10, **2**= IFN-γ, **3**= TNF-α.

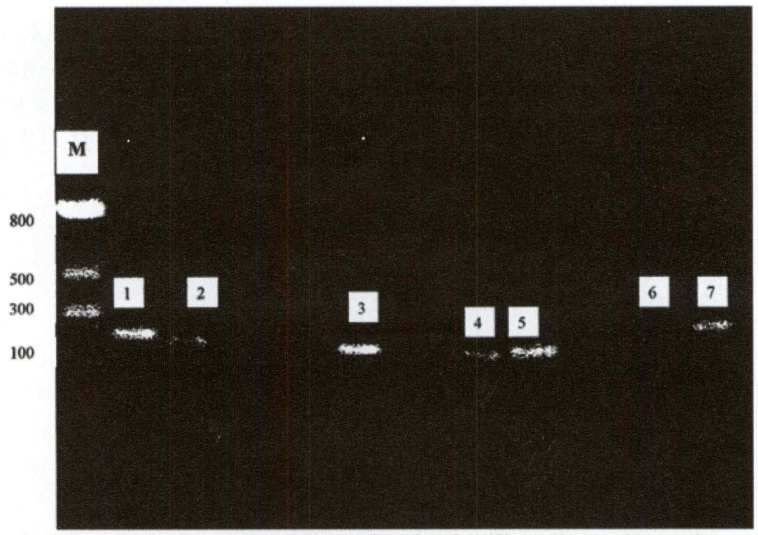

Fig-3: The gel electrophoresis of cytokines cDNA of milk cells at the periparturient period. The bands are, **M**=markers, **1**= IL-1α, **2**= IL-1β, **3**= IL-6, 4=IL-8, 5=IL-10, 6= = IFN-γ, 7=TNF-α. Note that IL-2 and IL-4 bands (between bands 2 and 3) were detected but they were so weak to be visualized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

Discussion:

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; Asai *et al.*, 1998; Burton *et al.*, 2001; (Sordillo *et al.*, 1991). However, the majority of these studies limited their scope to IL-2, IFN-γ and TNF-α. In the present 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.

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 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-α at this period , (Sordillo *et al.*, 1995; Asai *et al.*, 1998).

In this study IL-1α, IL-1β, IL-6, IL-8, IL-10 and IFN-γ were also detected at this period. However, IL-12 cDNA at PP and mid-lactation was not detected. The level of the cytokines in the mammary glands depends greatly on types of

References:

1. Alluwaimi A.M., 2004. The cytokines of bovine mammary gland: prospects for diagnosis and therapy. Research in Veterinary Science 77, 211-222.
2. Alluwaimi A.M. Farver, T. B., and Cullor, J. S., 2003. The transcriptional activity of IL-8 in healthy bovine mammary gland at mid and late stage of lactation period. Pakistan Journal Biological of Science. 6, 729-731.
3. Alluwaimi, A.M., Cullor, J.S., 2002. Cytokines gene expression patterns of bovine milk during mid and late stages of lactation. Journal of Veterinary Medicine B. 49, 105-110.
4. Asai, K., Kai, K., Rikiishi, H., Sugawara, S., Maruyama, Y., Yamaguchi, T., M. Ohta, Kumagai, K., 1998: Variation in CD4+ T lymphocyte subpopulations in bovine mammary gland secretions during lactating and non-lactating periods. Veterinary Immunology and Immunopathology. 65, 51-61.
5. Barber, M., Yang, T. J., 1998. Chemotactic activities in nonmastitic and mastitic mammary secretions: Presence of Interleukin-8 in mastitic but not nonmastitic secretions. Clinical .Diagnostic and Laboratory Immunology, 5, 82-86.
6. Burton J. L, Madsen S. A, Yao J, Sipkovsky S. S., Coussens P. M. 2001. An immunogenomics approach to understanding periparturient immunosuppression and mastitis susceptibility in dairy cows. Acta Veterinary Scandenivian. 42, 407-424.
7. Cai, T., Weston, P., Lund, L., Brodie, B., Mckenna, D.J., Wagner, W.C., 1994. Association between neutrophils functions and periparturient disorders in cows. Amircan Journal of Veterinary Research 55, 934-943.
8. Guidry, A. J., Paape, M. J., Pearson, R. E., 1976. Effects of parturition and lactation blood and milk cell concentrations, corticosteroids and neutrophil phagocytosis in the cow. American Journal of Veterinary Research 37, 1195.
9. Kimura, K. Goff, J. P., Kehrli, M. E.,1999. Effects of the presence of the mammary Gland on expression of neutrophil adhesion molecules and myeloperoxidase activity in periparturient dairy cows. Journal of Dairy Science 82, 2385-2392.
10. Lee E. K., Kehrli M. E., 1998. Expression of adhesion molecules on neutrophils of periparturient cows and neonatal calves. American Journal of Veterinary Research 59, 37-43.
11. Nonnecke B. J,, Kimura K, Goff J. P., Kehrli, M. E., 2003. Effects of the mammary gland on functional capacities of blood mononuclear leukocyte populations from periparturient cows. Journal of Dairy Science 86, 2359-2368.
12. Park, Y. H., Fox, W. C., Hamilton, M. J., Davis, L. K., 1992. Bovine mononuclear leukocyte subpopulations in peripheral blood and mammary gland secretions during lactation. Journal of Dairy Science 75, 998-1006.

13. Riollet, C., Rainard, P., Poutrel, B., 2001. Cell subpopulations and cytokine expression in cow milk in response to chronic *Staphylococcus aureus* infection. Journal of Dairy Science 84, 1077-1084.
14. Shuster, D.E., Kehrli, M.E., Rainard, P., Paape, M., 1997. Complement fragment C5a and inflammatory cytokines in neutrophil recruitment during intramammary infection with *Escherichia coli*. Infection and Immunity 65, 3286-3292.
15. Sordillo, L M., Pighetti, G. M., Davis, M. R., 1995. Enhanced production of bovine tumor necrosis factor-α during the periparturient period. Veterinary Immunology and. Immunopathology 49, 263-270.
16. Sordillo, L. M., Redmond, M. J., Campos, M., Warren, L., Babiuk, L. A., 1991. Cytokine activity in bovine mammary gland secretions during the periparturient period. Canadian Journal of Veterinary Research 55, 298-301.
17. Trinchieri, G., 1995. Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annual Review of Immunology 13, 251-276.

الإفرازات الخلوية (السايتوكاينز) لضرع الأبقار في مرحلة ما قبل الولادة

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الملخص :

لضرع الأبقار حساسية شديدة للعدوى في مرحلة ما قبل الولادة . السايتوكاينز أو الإفرازات الخلوية تعد أحد أهم الوسائل المناعية لتحديد مدى مقاومة الضرع في هذه المرحلة و يمكن أن تستخدم كأحد أهم العوامل المهمة في تحديد الصحة العامة للضرع في هذه المرحلة و مدى الحساسية التي قد يؤدي إلى التهاب الضرع و تقييم العلاج المطبق للتخلص من التهاب الضرع . في هذه الدراسة تم تحديد النشاط الجيني للسايتوكاينز IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFN-γ ، TNF-α في خلايا الحليب لأبقار في مرحلة أسبوعين قبل الولادة و مقارنة هذا النشاط بخلايا من حليب الأبقار في مرحلة الإنتاج (٣- ٤ أشهر بعد الولادة) باستخدام تقنية الـ RT-PCR. لقد تم رصد النشاط الجيني لجميع السايتوكاينز التي تم دراستها إلا IL-12 . و لم يتم رصد أي نشاط جيني للسايتوكاينز IL-12, IL-4, IL-2 في أبقار مرحلة الإنتاج. أكدت النتائج مدى فاعلية هذا الأسلوب في توفير أداة مرنة لدراسة الاستجابة المناعية للضرع في مرحلة ما قبل الولادة .