Quantitation of bovine cytokine mRNA in milk cells of healthy cattle by real-time TaqMan® polymerase chain reaction

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Abstract

Here we present a novel methodology to quantitate bovine cytokines and growth factors contributing to immunity against bacterial infections of the mammary gland in cattle. Real-time TaqMan® PCR systems were developed to overcome limitations of conventional quantitative PCR methods. The TaqMan® method is based on the cleavage of fluorescent dye-labeled probes by the $5'–3'$ exonuclease activity of the *Taq* DNA polymerase during PCR and measurement of fluorescence intensity by an automated spectrophotometer integrated in a sequence detection system (Applied Biosystems, Foster City, CA). The bovine-specific TaqMan® probes were designed to encompass an intron, thus allowing differentiation between complementary DNA (cDNA) and genomic DNA (gDNA) amplification products. Quantitative analysis of cytokine cDNA was performed in comparison to bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Messenger RNA (mRNA) from the universally expressed housekeeping gene GAPDH proved to be useful as an amplification control and allowed for correction of variations in different numbers of cells in the starting material, in the efficiencies of RNA extraction and reverse transcription. With this method, high-throughput analysis of large numbers of samples was possible within a short time. In addition, decreasing the numbers of working steps shortened the time for analysis and increased accuracy. Profiles of cytokines (interleukin (IL)-2, IL-6, IL-8, IL-12 p40, TNF-α, IFN-γ) and granulocyte–macrophage colony stimulating factor (GM-CSF) were established in normal lactating cattle. Differences of

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cytokine profiles obtained with the real-time TaqMan$^{®}$ PCR system and conventional methods are discussed.

1. Introduction

Understanding the regulation of the immune response during infections of the mammary gland is important for the design of prophylactic vaccines and for the optimization of therapeutic protocols. Certain aspects of immune mechanisms in cattle and their regulation by cytokines and growth factors have already been addressed (Kehrli and Shuster, 1994; Shuster et al., 1996; Taylor et al., 1997; Sordillo et al., 1997). However, there is still a lack in understanding the interplay of the immune compartments involved in the generation of protective immunity against specific bacterial infections. Here, we attempted to introduce a new molecular methodology to address gene transcription in milk cells of normal cows during mid-lactation using real-time TaqMan PCR to define the cytokine profile in milk cells of the healthy mammary gland.

Several methods exist that allow quantitation of cytokine expression at the protein level (ELISA, Elispot, biological assays, intracellular cytokine staining), and at the mRNA level (Northern blots, in situ hybridization, ribonuclease protection assay, reverse transcriptase polymerase chain reaction (RT-PCR)). Protocols to quantitate gene expression in bovines by RT-PCR, Northern blot and in situ hybridization have been published (Aldwell et al., 1996; Ito and Kodama, 1996; Keefe et al., 1997; Lentsch et al., 1997; Taylor et al., 1997; Hsuan et al., 1999; Morsey et al., 1999; Radi et al., 1999). Northern blots and ribonuclease protection assays require large amounts of RNA that may constitute a limiting factor when small amounts of tissue samples or low numbers of cells need to be analyzed. PCR overcomes these limitations due to the exponential amplification of even minute amounts of starting material.

Here we describe a newly developed, highly sensitive and reproducible method to quantitate bovine mRNA transcription by real-time TaqMan PCR (Heid et al., 1996). The signal for bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used to normalize different numbers of cells in the starting material, different efficiencies in RNA extractions, the degree of RNA degradation, and variabilities in reverse transcription (RT) efficiencies. The real-time TaqMan PCR systems described here require less than 1 ng of total cellular RNA per cytokine determination. This technology is a valuable alternative to conventional quantitative RT-PCR systems.

2. Materials and methods

2.1. Animals and preparation of milk samples

Seven Holstein cows (B&G dairy farm, Dixon, CA) were included in this study based upon somatic cell counts (SCC $< 4 \times 10^5$/ml) of the milk. All seven cows were in their second or third lactation and between 51 and 150 days of lactation.

Samples of 1 l composite milk (representing all four quarters) were collected into sterile tubes. Aliquots of 25 ml were fixed and used to determine SCC using a Fossomatic milk cell counter (Siliker Laboratories, Modesto, CA). The remainder of the milk was centrifuged at 700*g* for 20 min at room temperature. The pellets were washed twice in 50 ml phosphate buffered saline (PBS), pelleted and 5×10^6 cells were lysed with 350 μl lysis buffer according to the manufacturer's recommendations (RNeasy mini kit, Qiagen, Valencia, CA) and kept at $-80°C$ until RNA extraction and complementary DNA (cDNA) synthesis.

2.2. Lymphocyte isolation, cell culture, and stimulation experiments

In vitro-stimulated bovine peripheral blood mononuclear cells (PBMCs) were used as a source of bovine cytokine mRNA to establish and optimize the real-time TaqMan PCR systems. Bovine PBMCs were isolated from EDTA-treated blood samples by density gradient centrifugation through Ficoll-Hypaque (density: 1.083, Sigma, St. Louis, MO). The mononuclear cell fraction was washed twice in PBS and resuspended in lymphocyte growth medium (RPMI 1640 medium, Gibco BRL, Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco), 100 U/ml penicillin, 100 μg/ml strepto-mycin (Gibco), and 10 μg/ml concanavalin A (ConA, Sigma). Freshly isolated PBMCs were incubated for 24 and 48 h. Equal amounts of PBMCs from two individual cattle were cultured for 24 h for mixed lymphocyte reactions (MLRs). After culturing, 10 million cells were pelleted (4000*g* for 10 min), frozen as dry pellets on dry ice and stored at $-80°C$.

2.3. Primers and TaqMan probes

The primers and TaqMan probes were designed as described (Leutenegger et al., 1999a). Briefly, primers and probe targeting a cytokine gene were designed using the Primer Express software (Applied Biosystems, Foster City, CA). The sense and antisense primers were placed in two consecutive exons of the respective gene. The probe spanned the junction of two exons, covered by the forward and reverse primer to ensure discrimination between cDNA and genomic DNA (gDNA). For interleukin (IL)-8, only a short stretch of the bovine sequence was available in GenBank. We therefore placed the 3′-end of the forward primer over the available intron–exon junction. Each probe was labeled at the 5′-end with the reporter dye FAM (6-carboxyfluorescein) and at the 3′-end with the quencher dye TAMRA (6-carboxytetramethyl-rhodamine) and was phosphate blocked at the 3′-end to prevent extension by AmpliTaq Gold DNA polymerase. All primers and probes were synthesized by PE Oligofactory (Applied Biosystems). The sequences of the primers and probes are listed in Table 1.

2.4. RNA and DNA extraction

Total RNA (tRNA) was extracted from lysed cells or PMBCs using the RNeasy mini kit (Qiagen). The extracted tRNA was treated with 10 U/μl of RNase-free DNase I (DNase, Amersham Pharmacia Biotech, Piscataway, NJ) to remove contaminating gDNA at 37°C for 10 min followed by heat inactivation at 95°C for 5 min and chilling on ice.

Table 1
Sequence of PCR primers and TaqMan probes specific bovine GAPDH, cytokines and growth factors[a]

Cytokine	Primer	Sequence (5′–3′)	Length	Probe	Probe sequence (5′–3′)	Accession number
GAPDH	GAPDH.463f	GGCGTGAACCACGAGAAGTATAA	120	GAPDH.489p	ATACCCTCAAGATTG *TC*AGCAATGCCTCCT	AF022183
	GAPDH.582r	CCCTCCACGATGCCAAAGT				
IL-2	IL2.107f	GGATTTACAGTTGCTTTTGGAGAAA	165	IL2.194p	CGTGCCCAAGGTTAA *CG*CTACAGAATTGAA	M12791
	IL2.271r	GCACTTCCTCTAGAAGTTTGAGTTCTT				
IL-6	IL6.271f	TCAGCTTATTTTCTGCCAGTCTCT	105	IL6.192p	CGTCATTCTTCTCACATA *TC*TCCTTTCTTATTGCAGAG	X62501
	IL6.167r	TCATTAAGCACATCGTCGACAAA				
IL-8	IL8.177f	CACTGTGAAAAATTCAGAAATCA*TT*GTTA	113	IL8.214p	AATGGAAACGAGGTC TGCTTAAACCCCAAG	S74436
	IL8.282r	CTTCACCAAATACCTGCACAACCTTC				
IL-12 p40	IL12.623f	TTAATTGAGGTCGTGGTAGAAGCTG	115	IL12.687p	CATCAGGGACATC*AT* CAAACCAGACCC	U14416
	IL12.737r	GGTCTCAGTTGCAGGTTCTTGG				
IFN-γ	IFN.278f	TGGATATCATCAAGCAAGACATGTT	151	IFN.378p	CAGATCATCCA*CC* GGAATTTGAATCAGC	M29867
	IFN.428r	ACGTCATTCATCACTTTCATGAGTTC				
TNF-α	TNF.338f	TCTTCTCAAGCCTCAAGTAACAAGT	103	TNF.367p	AGCCCACGTTGTA *GC*CGACATCAACTCC	Z14137
	TNF.440r	CCATGAGGGCATTGGCATAC				
GM-SCF	GMCSF.170f	ATGAATGACACAGAACTCGTCTCTG	87	GMCSF.197p	AAGTTTGACTCCCA *GG*AACCAACGTGC	U22385
	GMCSF.256r	GCCGTTCTTGTACAGCTTCAGG				

[a] Intron–exon junctions are italicized.

Genomic DNA was prepared from PBMCs purified by Ficoll-Hypaque gradient centrifugation from EDTA-treated blood samples using DNeasy tissue kit (Qiagen). gDNA of 100 ng was used as template for amplification in the real-time TaqMan PCR.

2.5. RT of total RNA

RT was performed in a 20 μl final volume containing 50 U MuLV reverse transcriptase, 5 mM MgCl$_2$, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.25 μM random hexadeoxyribonucleotide (pd(N)$_6$) primers (random hexamer primer, Amersham Pharmacia Biotech), 0.5 U/μl RNase inhibitor (GeneAmp® RNA PCR kit, Applied Biosystems), and 1 mM dNTPs (Amersham Pharmacia Biotech). The mixture was subjected to 42°C for 60 min and inactivated at 95°C for 5 min. The final volume was adjusted to 100 μl with RNase-free water. The cDNA was analyzed immediately or stored at −20°C until use.

2.6. Real-time TaqMan® PCR for quantitation of cytokine cDNA

Real-time TaqMan PCR systems for bovine GAPDH and the cytokines and growth factors were run in separate wells. The PCR reactions contained 400 nM of each primer, 80 nM of the TaqMan probe and commercially available PCR Mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM MgCl$_2$, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 10 μl of the diluted cDNA sample in a final volume of 25 μl. The samples were placed in 96-well plates and amplified in an automated fluorometer (ABI Prism 7700 Sequence Detection System, Applied Biosystems). Amplification conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C. Final quantitation was done using the comparative C_T method (see Section 2.8) and is reported as relative transcription or the *n*-fold difference relative to a calibrator cDNA (i.e. cytokine transcription in unstimulated PMBCs).

2.7. Analytical specificity

The PCR products of the bovine cytokines and GAPDH were sequenced to verify the analytical specificity using standard sequencing procedures (ABI 377 DNA sequencer, DNA Sequencing Facility, University of California, Davis, CA).

2.8. The comparative C_T method

Among the two methods for quantitation of gene transcription (absolute quantitation by the standard curve method and relative quantitation by the comparative C_T (cycle threshold) method), the latter was selected due to its ease and speed for set-up and analysis. For relative quantitation by the comparative C_T method, values are expressed relative to a reference sample, called the calibrator (unstimulated PBMC sample). First, the C_T for the target amplicon and the C_T for the endogenous control were determined for each sample. Differences in the C_T for the target and the C_T for the endogenous control, called ΔC_T, were calculated to normalize for the differences in the RNA extractions and

the efficiency of the RT step. The ΔC_T for each experimental sample was subtracted from the ΔC_T of the calibrator. This difference is called the $\Delta\Delta C_T$ value. Finally, the amount of target, normalized to the endogenous control and relative to the calibrator, was calculated by $2^{-\Delta\Delta C_T}$. Thus, all experimental samples are expressed as an *n*-fold difference relative to the calibrator. ΔC_T values can also be used to calculate an estimated copy number for every cytokine by using a GAPDH standard curve. $2^{\Delta C_T}$ would give a predicted copy number difference between GAPDH and a given cytokine mRNA. A standard curve can be generated using known amounts of PCR products (from a conventional PCR) or using the cloned target sequence and diluting them 10-fold as described (Leutenegger et al., 1999a).

2.9. Determination of amplification efficiency

For the comparative C_T method ($\Delta\Delta C_T$ method) to be valid, the amplification efficiencies of the target and the endogenous control must be approximately equal. To determine the amplification efficiencies of GAPDH and the cytokine cDNAs, six dilutions of cDNA preparations in triplicate were amplified to obtain standard curves. Differences of the slopes between standard curves obtained from GAPDH and the cytokines were plotted against the dilution of input total RNA and the regression line was calculated as exemplified with the real-time TaqMan PCR system for bovine TNF-α.

2.10. Statistical analysis

Statistical analysis of the data was performed in Excel and GraphPad Prism software package version 2.0. Differences between cytokine transcription were analyzed with the Wilcoxon test. Differences were considered significant if $p < 0.05$.

3. Results

3.1. Amplification efficiencies of GAPDH and cytokine real-time TaqMan PCR systems

To determine the amplification efficiencies, six twofold dilutions of cytokines cDNA were amplified in triplicate. Differences of the slopes between standard curves obtained from GAPDH and the cytokine systems were IL-2: 0.07, IL-6: 0.06, IL-8: 0.09, IL-12 p40: 0.1, TNF-α: 0.04, GM-CSF: 0.07, IFN-γ: 0.09. For TNF-α, the differences of the slopes were plotted against the dilution of input total RNA and the regression line was calculated (Fig. 1).

3.2. Specificity of bovine TaqMan PCR systems for cDNA templates

To address the question whether contaminating gDNA present in the total RNA samples could influence the cytokine cDNA quantitation, RNA preparations with and without prior DNase treatment and with or without RT step as well as gDNA samples were subjected to TaqMan PCR (Table 2). All systems generated signals with cDNA derived from ConA stimulated bovine PBMCs or from MLR. None of the systems produced a signal when DNase treated tRNA was used for the amplification. To
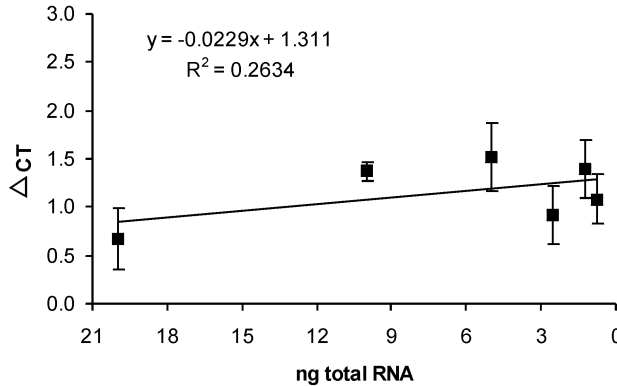
$$y = -0.0229x + 1.311$$
$$R^2 = 0.2634$$

Fig. 1. Determination of amplification efficiency with the TNF-α TaqMan system. The $\Delta\Delta C_T$ method for relative quantitation requires that efficiencies of target and endogenous control (GAPDH) amplified in different tubes are approximately equal. Standard curves for TNF-α and GAPDH were generated to compare amplification efficiencies. The figure depicts input amount of tRNA versus differences of C_T values between endogenous control GAPDH and TNF-α.

investigate whether residual gDNA was co-amplified or interfered with the quantitation of cytokine cDNAs, we amplified 100 ng of genomic DNA extracted from bovine PBMCs. The system for bovine GAPDH recognized gDNA, all other systems did not produce a fluorescent signal when gDNA was used as template. As a confirmation, we also tested non-DNase treated tRNA and obtained the same pattern of fluorescent signals: GAPDH but not the other real-time TaqMan PCR systems produced a signal during PCR. With the IL-8 system, where the 3′-end of the forward primer was placed over an intron–exon junction, full discrimination of cDNA and gDNA was achieved.

3.3. Cytokine and growth factor profiles from in vitro cultured PBMCs

ConA stimulated cells and cells subjected to an MLR were used to establish standard curves and cytokine profiles (Fig. 2). Unstimulated bovine PBMCs transcribed IL-8 and

Table 2
Differentiation between complementary DNA (cDNA) and genomic DNA (gDNA) tested with cDNA, gDNA, DNase I — digested and undigested total RNA (tRNA)[a]

TaqMan system for	cDNA	gDNA	tRNA − DNase I	tRNA + DNase I
GAPDH	21.43	23.58	24.03	40
IL-2	31.26	40	40	40
IL-6	33.08	40	40	40
IL-8	18.92	40	40	40
IL-12p40	21.12	40	40	40
IFN-γ	26.98	40	40	40
TNF-α	21.66	40	40	40
GM-CSF	22.01	40	40	40

[a] Numbers are cycle threshold (C_T) values. A value of 40 indicates no specific fluorescent signal.
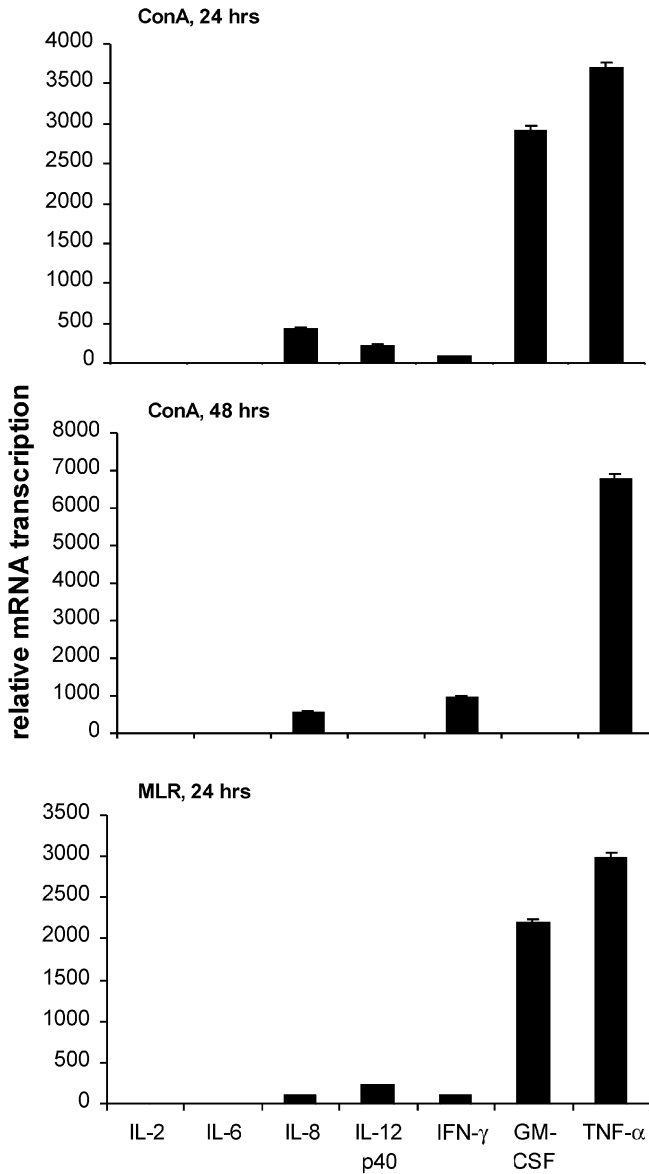
Fig. 2. Cytokine profiles from unstimulated, and ConA stimulated (24 and 48 h) bovine PBMCs and from cells subjected to an MLR for 24 h. Results represent the mean fold increase of cytokines over unstimulated PBMC, which served as a calibrator. The same unstimulated PBMCs served as calibrator for the cells subjected to the MLR.

IL-12 p40 to very low levels. When PBMCs were stimulated with ConA for 24 h, signals for all the cytokines could be detected, although the signals for IL-2 and IL-6 were only five- to twofold upregulated (not visible in Fig. 2). ConA stimulation for 48 h increased the signals for IL-8, IFN-γ and TNF-α. The other signals decreased (IL-12 p40, GM-CSF)
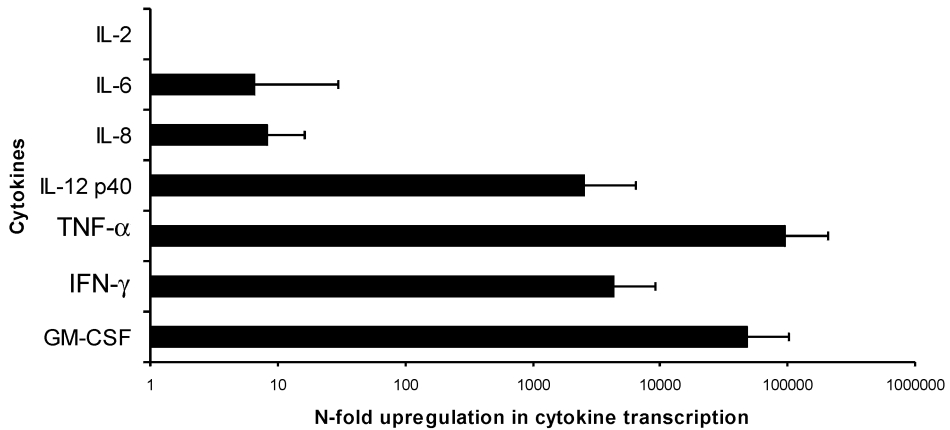
Fig. 3. Cytokine profiles of milk cells from seven healthy Holstein cattle in mid-lactation. Bars represent the means of cytokine transcription. Cytokine transcription is expressed as the *n*-fold differences to the calibrator, which was the lowest detectable cytokine signal. Note the semilogarithmic scale.

or disappeared (IL-2, IL-6). Cytokine signals obtained from cells after MLR showed a similar profile as cells stimulated for 24 h with ConA but with sixfold elevated IL-2 levels compared to unstimulated cells (not visible in Fig. 2). Standard deviations of repetitive measurements were within 2%.

3.4. Cytokine mRNA expression in milk cells

The real-time TaqMan PCR systems were used to assess cytokine profiles in cells extracted from milk from healthy cattle in mid-lactation (Fig. 3). All cows showed high levels of transcription for TNF-α, GM-CSF, IL-12 p40 and IFN-γ. Lower but consistent transcription was detected for IL-8. IL-6 transcription was detected in two of seven and IL-2 as not detected in any of the samples. TNF-α was transcribed to highest levels. It was higher than IL-12 p40, IFN-γ and IL-8 (all $p < 0.05$). No significant differences were observed between TNF-α and GM-CSF and IFN-γ expression or between GM-CSF and IFN-γ and IL-12 p40 ($p > 0.05$). However, GM-CSF was significantly higher transcribed than IL-8 ($p < 0.05$).

4. Discussion

This study investigated the use of real-time TaqMan PCR to assess gene transcription in milk cells of healthy cows during mid-lactation. With this new technology, we attempted to overcome limitations of conventional quantitative RT-PCR protocols such as the need of parallel reactions for a single sample, post-amplification steps and a narrowed dynamic range. These drawbacks lead to low efficiency of sample throughput, inaccuracy and high costs. The quantitative real-time TaqMan PCR technique has several advantages compared to conventional quantitative PCR systems. A major factor responsible for the

accuracy of this method is the determination of the C_T value within the exponential phase of the amplification rather than endpoint determination with conventional systems. This leads to a linear relationship between copy number and C_T values in a range of at least eight orders of magnitude (Leutenegger et al., 1999a). In addition, the use of third oligonucleotide (TaqMan probe) adds specificity to the system comparable to hybridization techniques with blotted PCR products. Furthermore, the elimination of post-amplification steps increases reliability and reproducibility of the assay (Gut et al., 1999; Leutenegger et al., 1999b). Overall, the real-time TaqMan PCR offers a quantitative PCR system for high throughput, high analytical sensitivity and specificity, and increased reliability and reproducibility.

Accurate cytokine cDNA quantitation was made possible by normalizing the cytokine signals with the endogenous GAPDH control. Normalization leads to the elimination of differences in numbers of cells in the starting material, efficiencies in RNA extraction, and RT efficiencies. Comparison of signals obtained in different wells require that the amplification efficiencies of both the cytokine and the GAPDH systems are approximately equal. A sensitive method for comparing the amplification efficiencies of two amplicons is to determine the slopes of C_T value variations obtained from cDNA dilution series done with GAPDH and the respective cytokine (User Bulletin #2, Applied Biosystems, 1997). We found that the amplification efficiencies obtained with the GAPDH and the cytokine real-time PCR systems were very similar (<10% difference) and therefore allow GAPDH-based normalization of cytokine transcription.

Extraction of tRNA by silica-based methods leads to co-purification of genomic DNA. Although DNase treatment is efficient, trace amounts of residual gDNA may still be present in DNase-treated total RNA samples. It is therefore important to avoid amplification of residual gDNA. This is achieved by designing junctional-specific TaqMan probes or primers for the real-time TaqMan PCR systems. Since genomic sequences for bovine cytokine genes were not available, intron–exon junctions were deduced either from mouse or human sequences based on the assumption that the position of introns is highly conserved among species. In our experience, this is true for most of the real-time TaqMan PCR systems established in earlier protocols (Leutenegger et al., 1999a; Leutenegger et al., 1999c). Only the real-time TaqMan system for bovine GAPDH recognized gDNA and generated a specific signal. The same observation was made with a deduced intron within the gene encoding canine GAPDH (unpublished observations). The generation of a fluorescent signal with the GAPDH real-time TaqMan PCR system can be explained by a lack of the intron sequence or by appearance of pseudogenes as described for other mammals (Galland et al., 1990; Garcia-Meunier et al., 1993). We therefore needed to ensure that DNase treatment of total RNA efficiently removed contaminating gDNA. As shown in Table 2, DNase treatment reduced the amount of gDNA completely. Equivalent amounts of tRNA after DNase treatment were tested with the GAPDH system. Fluorescent signals were detected only within a small number of samples (<5%). In addition, the signals derived from residual gDNA contributed less than 0.1% to the cDNA signal. The other TaqMan systems for bovine cytokines and growth factors showed full discrimination between cDNA and gDNA.

The TaqMan systems for bovine cytokines IL-2, IL-6, IL-8, IL-12 p40, TNF-α, IFN-γ, and GM-CSF were used to determine the cytokine transcription in purified milk cells

from healthy cows. Transcription of IL-8, TNF-α, IFN-γ, and GM-CSF mRNA were detected in all and IL-12 p40 was detected six of seven samples, respectively. However, IL-12 p40 and IL-8 mRNA expression were significantly lower than the TNF-α, GM-CSF and IFN-γ. IL-6 was only detected in two of seven and IL-2 mRNA transcription was not detected at all, which is in agreement with an earlier report (Taylor et al., 1997). The main source for IL-6 transcription are macrophages and occurs mainly following an inflammatory stimulus (Adams and Czuprynski, 1994). In the healthy lactating mammary gland, total SCC are $<10^5$/ml milk. However, Holstein cows are bred for high milk production causing a permanent state of stress and therefore a certain amount of tissue damage and possibly low-grade bacterial presence is common. This assumption of the apparent healthy state of the animals is important to understand the expression of cytokines indicating a certain level of inflammation. Interestingly no TNF-α transcription could be detected in an earlier study using a conventional RT-PCR system (Taylor et al., 1997). Low sensitivity and a suboptimal conventional RT-PCR system could be the reasons for the lack of detection.

Milk somatic cells consist of several cell types, including neutrophils, macrophages, lymphocytes and a small number of epithelial and natural killer (NK) cells. The presence of these cells at a certain localization involves the migration upon a chemotactic signal. Migration of neutrophils and to a certain extent T-lymphocytes from the blood stream to the site of recruitment is IL-8 dependent (Ribeiro et al., 1991; Wang et al., 1996). In addition, IL-8 is also an important factor for activating neutrophils during inflammatory processes (Galligan and Coomber, 2000). An earlier report described the lack of IL-8 mediated chemotaxis in non-mastitic milk. The absence of IL-8 mediated chemotaxis can be explained by a lack of IL-8 expression or by the existence of a low molecular weight inhibitor (Ormrod and Miller, 1992). Our observation suggests that IL-8 expression is also regulated at the post-translational level possibly by upregulation of IL-8 specific inhibitors.

Using conventional quantitative PCR systems, IL-12 p40 mRNA was only detected in mastitis cows but not in healthy cows (Taylor et al., 1997). However, here we demonstrated that the majority of the samples from healthy cows showed IL-12 p40 transcription. Macrophages are the predominant cell type in the milk in the apparent healthy state of lactating cows. Phagocytosis and bactericidal activities as nonspecific defense mechanisms are the main functions of this cell type. In addition, macrophages play a key role in antigen processing and presentation during the initiation of a specific immune response. Once activated, macrophages are the main source for IL-12 although NK cells also produce this cytokine which in turn induces IFN-γ transcription (Collins et al., 1999; Tuo et al., 1999).

GM-CSF transcription was so far not described in milk cells from normal cows. Currently, mastitic milk secretions are analyzed to characterize whether the transcription of this growth factor is upregulated in comparison to normal milk samples.

In summary, this study revealed differences between cytokine profiles determined either with conventional quantitative RT-PCR systems or with a novel real-time TaqMan PCR system. The real-time TaqMan PCR system described in this work is a promising method to overcome limitations of conventional quantitative PCR systems and could lead to a better understanding of immune-regulatory cytokines and their potential use for diagnosis, prophylaxis and immune therapy.

Acknowledgements

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