Quantitative real-time PCR for the measurement of feline cytokine mRNA

Christian M. Leutenegger[a],[*], Caroline N. Mislin[a],
Brigitte Sigrist[a], Markus U. Ehrengruber[b],
Regina Hofmann-Lehmann[a], Hans Lutz[a]

[a]*Clinical Laboratory, Department of Internal Veterinary Medicine, University of Zurich,
8057 Zurich, Switzerland*
[b]*Brain Research Institute, University of Zurich, 8057 Zurich, Switzerland*

Abstract

We have developed real-time PCR systems to quantitate feline cytokine gene expression. The method is based on the cleavage of fluorescent dye-labelled probes by the $5'$–$3'$ exonuclease activity of the *Taq* DNA polymerase during PCR and measurement of fluorescence intensity by a Sequence Detection System. The feline-specific TaqMan probes were designed to encompass an intron, thus allowing differentiation of complementary DNA versus genomic DNA amplification products. Quantitative analysis of cytokine cDNA concentrations was performed in comparison to feline 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 the efficiencies of RNA extraction and reverse transcription. GAPDH mRNAs were readily detectable in cDNAs prepared from unstimulated feline peripheral blood mononuclear cells (PBMCs) and from frozen cell pellets, while cytokines (Interleukin (IL)-4, IL-10, IL-12 p35, IL-12 p40, IFNγ, IL-16) were expressed at variable amounts. IFNγ transcription was found to be upregulated in stimulated PBMCs and feline cell lines. The synthesis of cDNA and the performance of the PCR in separate tubes proved to be of superior sensitivity compared to a single-tube based system. The assays described are highly reproducible, require no post-PCR manipulation of the amplicons and permit the analysis of several hundred PCR reactions per day. With this method it is possible to detect and quantify cytokine mRNA expression reliably in small amounts of cells even after storage of samples for at least 5 years. © 1999 Elsevier Science B.V. All rights reserved.

[*] Corresponding author. Present address: Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA 95616, USA. Tel.: +1-530-752-7470; fax: +1-530-752-0414
E-mail address: cmleutenegger@ucdavis.edu (C.M. Leutenegger)

1. Introduction

Cytokines play a central role in the regulation of cell differentiation, proliferation, and cell–cell communication (Balkwill and Burke, 1989). In addition, some cytokines have important effector functions via activation of directly cytotoxic compounds (e.g. perforin, oxygen and nitric oxide radicals). Several studies indicate that variations in cytokine expression are associated with disease activity in immune-mediated or inflammatory disorders (Mosmann and Sad, 1996). Low concentrations of circulating cytokines in blood or plasma make investigation of systemic cytokine patterns a difficult task. Therefore, sensitive detection systems are required to monitor their expression and secretion under various physiological conditions. Several methods exist that allow quantitation of cytokine expression at the protein level (ELISA, Elispot, biological assays, intracellular cytokine staining (Kabilan et al., 1990)) and at the mRNA level (Northern blots, in situ hybridization, ribonuclease protection assay, reverse transcriptase polymerase chain reaction (RT-PCR) (Dallman et al., 1991)) of peripheral blood mononuclear cells (PBMCs), peripheral blood lymphocyte subpopulations and in tissues. Northern blots and ribonuclease protection assays require large amounts of RNA that may constitute a limiting factor when small amounts of tissue samples or blood cell preparations are analyzed. PCR overcomes these limitations due to the exponential amplification of even minute amounts of starting material (Zimmermann and Mannhalter, 1996) and TaqMan procedures for human, bovine and mouse cytokines and chemokines have already been described (Wang et al., 1998; Collins et al., 1999; Fehniger et al., 1999).

Studies of the feline immune system and the use of feline models to study human diseases have been slowed by the relative lack of sequence data and reagents. Previously, consensus sequence primers derived from cytokines of different species have been used to investigate feline cytokine gene expression (Rottman et al., 1995). However, in recent years, many feline cytokine-coding sequences have been characterized and are now available for the design of feline-specific primers and probes (for review see Lutz and Leutenegger, 1998).

Here we describe a newly developed, highly sensitive and reproducible method to quantify cytokine mRNA transcription by real-time TaqMan PCR. The signal for feline glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used to normalize against differences in RNA extractions, the degree of RNA degradation, and variabilities in RT and PCR efficiencies. The TaqMan systems described require less than 1 ng of the total cellular RNA per cytokine determination.

2. Materials and methods

2.1. Lymphocyte isolation, cell culture, and feline cell lines

In vitro-stimulated feline lymphocytes were used as a source of feline cytokine mRNA. Feline lymphocytes were isolated from heparinized venous blood by density gradient

centrifugation through Ficoll-Hypaque (density: 1.077; Pharmacia, Dübendorf, Switzerland). The mononuclear cell fraction was washed twice and resuspended in lymphocyte growth medium (RPMI 1640 medium (Gibco AG, Basel, Switzerland) supplemented with 10% fetal calf serum (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), 100 U/ml recombinant human IL2, and 10 µg/ml ConA (Sigma, Buchs, Switzerland) or 0.075% *Staphylococcus aureus* extract (Pansorbin, Sigma). Freshly isolated cells were incubated for 6, 12 and 72 h. Ten million cells were pelleted ($400 \times g$) for 10 min, frozen as dry pellets on dry ice and stored at -80°C. The two feline kidney cell lines CrFK and NFLK and the feline T-cell line 3201 (kindly provided by Dr. Margret Hosie and Dr. Brian Willett) were used to determine the constitutive production of IFNγ transcription.

2.2. TaqMan primers and probes

For each target gene, three oligonucleotides were selected using the Primer Express software (PE Biosystems, Foster City, CA): two primers and an internal oligonucleotide as a probe. The internal probe was labelled at the $5'$ end with the reporter dye FAM (6-carboxyfluorescein), 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.

For all the sequences studied, the sense and antisense primers were placed in two consecutive exons of the gene. The probe spanned the junction of two exons, covered by the forward and reverse primer. To demonstrate that this junctional specific probes lead to detection of only the complementary DNA (cDNA) but not the genomic DNA (gDNA), one TaqMan system was designed amplifying sequences within a single exon (IFNγ No. 1). The length of the PCR products (amplicon length, see Table 1) was held very short, between 76 and 113 basepairs (bp) (with one exception for IL16: 221 bp), so they are extended in a few seconds and do not require a separate extension step. Therefore, a timesaving two-temperature cycling was used. We compared the feline-specific GAPDH system with three GAPDH systems specific for rat, equine (Leutenegger et al., 1999a) and human GAPDH (obtained from PE Biosystems, cat no 402869). The primer and probe sequences are listed in Table 1.

2.3. RNA and genomic DNA extraction

Frozen cells were lysed in lysis buffer and total RNA was extracted according to the manufacturers' recommendations (RNeasy Blood Kit, Qiagen, Basel, Switzerland). The RNA pellets were dissolved in 30 µl 10 mM Tris-HCl, 1 mM EDTA (pH 7.4) made in diethyl pyrocarbonate (DEPC, Fluka, Buchs, Switzerland)-treated water. Contaminating gDNA was digested with RNase-free DNase I (Promega, Dubendorf, Switzerland) at 37°C for 1 h followed by an inactivation step at 95°C for 5 min.

Genomic DNA was prepared from PBMC purified by Ficoll Hypaque gradient centrifugation from heparin-treated blood samples with a phenol-chloroform standard method (Sambrook et al., 1989). 100 ng of gDNA (determined by photometry at 260/280 nm; Genequant II, Pharmacia, Dübendorf, Switzerland) was used as template for amplification in the TaqMan PCR.

Table 1

Sequence of PCR primers and TaqMan probes specific for feline, rat, equine and human GAPDH and feline cytokines[a]

Cytokine	Primer	Sequence (5′ → 3′)	Length	Probe	Probe sequence (5′ → 3′)
Feline GAPDH	GAPDH.57f	GCCGTGGAATTTGCCGT	82	GAPDH.77p	CTCAACTACATGGTCTACATGTTCCAGTATGATTCCA
	GAPDH.138r	GCCATCAATGACCCCTTCAT			
Rat GAPDH	GAPDH.170f	AAGCTGGTCATCAATGGGAAAC	111	GAPDH.200p	ATCTTCCAGGAGCGCGATCCCG
	GAPDH.280r	GAAGACGCCAGTAGACTCCACG			
Equine GAPDH	GAPDH.74f	AAGTGGATATTGTCGCCATCAAT	88	GAPDH.108P	TGACCTCAACTACATGGTCTACATGTTTCA
	GAPDH.161r	AACTTGCCATGGGTGGAATC			
Human GAPDH	GAPDH.5f	GAAGGTGAAGGTCGGAGT	226	GAPDH.182p	GGCTGAGAACGGGAAGCTTG
	GAPDH.230r	GAAGATGGTGATGGGATTC			
IL4	IL4.143f	GCATGGAGCTGACCGTCAT	81	IL4.169p	TGGCAGCCCCTAAGAACACAAGTGACAA
	IL4.223r	CGGTTGTGGCTCTGCAGA			
IL10	IL10.182f	TGCACAGCATATTGTTGACCAG	76	IL10.209p	ACCCAGGTAACCCTTAAGGTCCTCCAGCA
	IL10.257r	ATCTCGGACAAGGCTTGGC			
IL12 p35	IL12.35.108f	AATGTTCCAGTGCCTCAACCA	113	IL12.35.156P	CAACACGCTTCAGAAGGCCAGACAAACTCTAG
	IL12.35.220r	GATCAATCTCTTCGGAAGTGCA			
IL12 p40	IL12.253f	TGGCTTCAGTTGCAGGTTCTT	81	IL12.283p	CGGTTTGATGATGTCCCTGATGAAGAAGCT
	IL12.333r	TGGACGCTATTCACAAGCTCA			
IFNγ No. 1	IFN.433f	CACCAAGATCTAACCTGAGGAAGC	92	IFN.498p	CGATGCTCTACGGCCTCGAAACAGA
	IFN.524r	TATTGCAGGCAGGATGACCAT			
IFNγ No. 2	IFN.141f	TGGTGGGTCGCTTTTCGTAG	85	IFN.152p	CATTTTGAAGAACTGGAAAGAGGAGAGTGATAAAACAAT
	IFN.225r	GAAGGAGACAATTTGGCTTTGAA			
IL16	IL16.22f	ACTGATTCTACAAACTCGGCTTCTG	221	IL16.179p	TTCCGAGGCTGCCCCTTTGAAAATCCTGTTCA
	IL16.242r	ATTTCATCTCCCGGCTGGAT			

[a] Intro-exon junctions are underlined.

2.4. cDNA synthesis

cDNA was synthesized using 10 U of Avian myoblastosis virus (AMV) reverse transcriptase (Promega, Dubendorf, Switzerland) according to the recommendations of the manufacturer: reverse transcription was performed in a 20 µl final volume containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 8 mM $MgCl_2$, 0.25 mM dNTPs, 4 U RNAsin, 5 mM dithiothreitol (DTT) and 500 ng random hexadeoxyribonucleotide $(pd(N)_6)$ primers (random hexamers; Pharmacia, Dübendorf, Switzerland) or 500 ng oligo(dT) primer (Pharmacia, Dubendorf, Switzerland). The reaction proceeded at 42°C for 1 h. After inactivation at 95°C for 5 min, the reaction volume was adjusted to 100 µl with DEPC treated water. The cDNA was analyzed immediately or stored at −30°C until use.

2.5. Quantitation of cytokine transcripts by real-time PCR

TaqMan probes were synthesized that were labelled with a reporter (FAM, (6-carboxyfluorescein) and a quencher molecule (TAMRA, 6 carboxytetramethyl-rhodamine). Upon activation by blue light, the proximity of the quencher and the reporter dye of the intact probe results in suppression of the reporter fluorescence by Förster-type energy transfer (Förster, 1948). Release of the reporter from the vicinity of the quencher dye due to the $5' \rightarrow 3'$ exonuclease activity of AmpliTaq Gold (Holland et al., 1991; Heid et al., 1996) leads to increased reporter fluorescence. Fluorescence intensity is directly related to the amount of input target DNA and can be detected with an automated fluorometer (ABI Prism 7700 Sequence Detection System, PE Biosystems, Foster City, CA). As multiple cytokines from one cDNA sample were analyzed, GAPDH and the cytokines were all amplified in separate tubes due to an economical reason: multiplex PCR (detection of GAPDH and one cytokine in the same tube) would need more GAPDH reactions as when the analyzis of multiple cytokines were carried out in different tubes. The amplification conditions were the same for all cytokines assayed, and therefore, could be combined on the same 96-well plate: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C. Assay compositions were as follows: the 25 µl PCR mixtures contained a comercially available mastermix (TaqMan Universal PCR Mastermix, PE Biosystems, Foster City, CA) containing 10 mM Tris (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625 U of AmpliTaq Gold per reaction, 0.25 U AmpErase UNG per reaction, and 10 µl of the diluted cDNA sample. The primer and probe concentrations for all TaqMan systems were 400 nM and 80 nM, respectively. Final quantitation was done using the comparative C_T method (see 2.7) and is reported as relative transcription or the n-fold differences relative to a calibrator cDNA (i.e. unstimulated PBMC sample).

2.6. One-tube RT-PCR TaqMan system

The above described two-tube PCR system was compared with a one-tube RT-PCR TaqMan system: cellular total RNA was directly subjected to RT and PCR step within the same tube using AMV RT and *Thermus flavus (Tfl)* DNA polymerase. This system was described in detail for the detection and quantitation of feline coronavirus allowing the

detection of 10 copies in the starting reaction (Gut et al., 1999). Briefly, the 25 µl PCR mixture for one reaction contained 5 µl optimized single-buffer 5 × (Access RT-PCR system, Promega, Madison, WI), 2 mM $MgSO_4$, 1 mM deoxynucleotide triphosphates, 2.5 U of AMV reverse transcriptase, 2.5 U *Tfl* polymerase and 10 µl of cDNA. Probe concentrations were used as described above. Primer concentrations were used at 1.2 mM. A reverse transcription step of 45 min at 48°C and a denaturation step of 2 min at 94°C, were followed by 45 cycles, each 1 min at 64°C, 2 min at 68°C, and 30 s at 94°C. Reverse transcription and amplification were carried out in a single tube in an ABI Prism 7700 Sequence Detection System without modifying or moving the samples between RT and PCR. To increase sensitivity, random hexadeoxyribonucleotide $(pd(N)_6)$ primers at a concentration of 50 ng were included in the RT-PCR mix.

2.7. The comparative C_T method

Each well is screened for fluorescence every 7 s and signals were regarded as positive if the fluorescence intensity exceeded 10 × the standard deviation of the baseline fluorescence (threshold cycle, C_T). Among the two methods for quantitation of gene transcription (absolut quantitation by the standard curve method and relative quantitation by the comparative C_T method), the latter was selected due to its ease and speed for setup 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 internal control were determined for each sample. Differences in the C_T for the target and the C_T for the internal control, called ΔC_T, were calculated to normalize for the differences in the amount of total nucleic acid added to each reaction 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 an internal control and relative to the calibrator, was calculated by $2^{-\Delta\Delta C_T}$. Thus, all the 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 the GAPDH standard curve. $2^{\Delta C_T}$ would give a predicted copy number difference between GAPDH and a given cytokine mRNA. As the copy number of GAPDH mRNA is known from the standard curve a predicted copy number for the cytokine mRNA can be calculated.

2.8. Cloning and sequencing of feline GAPDH

Consensus primers for PCR amplification of feline GAPDH were derived from the human and the rat sequences (accession numbers M33197 and M17701). Primers were designated GAPDH.65f 5′GAAGGTGAAGGTCGGAGT3′ and GAPDH.292r 5′GAAGATGGTGATGGG3′. PCR conditions were as follows: Denaturation: 94°C for 30 s; annealing: 55°C for 30 s; primer extension: 72°C for 1 min; 30 cycles. The 228 bp fragment was cloned by TA cloning strategy into a pT7blue-2 vector (Novagen, Madison, WI, USA), propagated in *E. coli* (NovaBlue; Novagen, Madison, WI, USA) and sequenced with a fluorescence based automated sequencing system (ABI 377 DNA

sequencer, Microsynth, Balgach, Switzerland). The sequence of feline GAPDH was deposited in EMBL (accession number AF097177). Dilutions of the sequenced, linearized and purified plasmid were used as standards to test for the analytical sensitivity in the range from 1 to 10^9 copies. Dilutions were done in 300 ng calf thymus DNA (Sigma, Buchs, Switzerland) per 10 µl as carrier. Aliquots of dilutions were frozen at $-20°C$ and used in TaqMan PCR only once.

2.9. Verification of TaqMan PCR specificity

Specificity of TaqMan PCR systems for feline IL4 and IFNγ were verified by cloning and sequencing of TaqMan PCR amplicons. TaqMan systems for feline GAPDH, IL10, IL12 p35, IL12 p40, and IL16 were verified by performing TaqMan reactions with plasmids containing the verified sequence. The accession numbers for the cDNAs were: Feline GAPDH: AF097177; feline IL10: AF060520; feline IL12 p35: U83185; IL12 p40 U83184: feline IL16: AF003701. The cytokine TaqMan systems were at least once tested for the amplification of a single amplicon in agarose gel electrophoresis

3. Results

3.1. Random hexadeoxyribonucleotide (pd(N)₆) versus oligo(dT) primers for reverse transcription

To obtain the highest possible analytical sensitivity, random hexadeoxyribonucleotide $(pd(N)_6)$ and oligo(dT) primers were compared in their efficiencies to reverse transcribe RNA into cDNA. Therefore, the same amount of total RNA was reverse-transcribed with both RT-primers and subsequently tested in real-time PCR for the differences in GAPDH C_T values. Random hexadeoxyribonucleotide $(pd(N)_6)$ primers proved to be superior to oligo(dT) primers in their RT efficiency as shown by the lower C_T value of the random hexadeoxyribonucleotide $(pd(N)_6)$ reverse-transcribed sample (Fig. 1).

3.2. Specificity of feline cytokine primers and TaqMan probes for cDNA templates

All sequences of feline cytokines used for the design of TaqMan systems were available with the exception of feline GAPDH. The feline GAPDH sequence was obtained by PCR cloning and was shown to be 95.6% identical to human GAPDH (accession number M33197). To demonstrate the importance of using species-specific sequences rather than consensus sequence primers for the design of quantitative PCR systems, TaqMan reactions were carried out using cDNA preparations made from cat PBMC's and four different GAPDH TaqMan systems (cat, rat, horse and human). The C_T values of the four GAPDH systems were 17.01 for the cat system, 21.45 for the rat system, 24.89 for the horse system and 29.03 for the human system, respectively. The sequence identities of the forward and reverse primer sequences were for the cat primers 100%/100%, for the rat primers 82%/82%, for the horse primers 96%/70% and for the human primers 79%/72%.
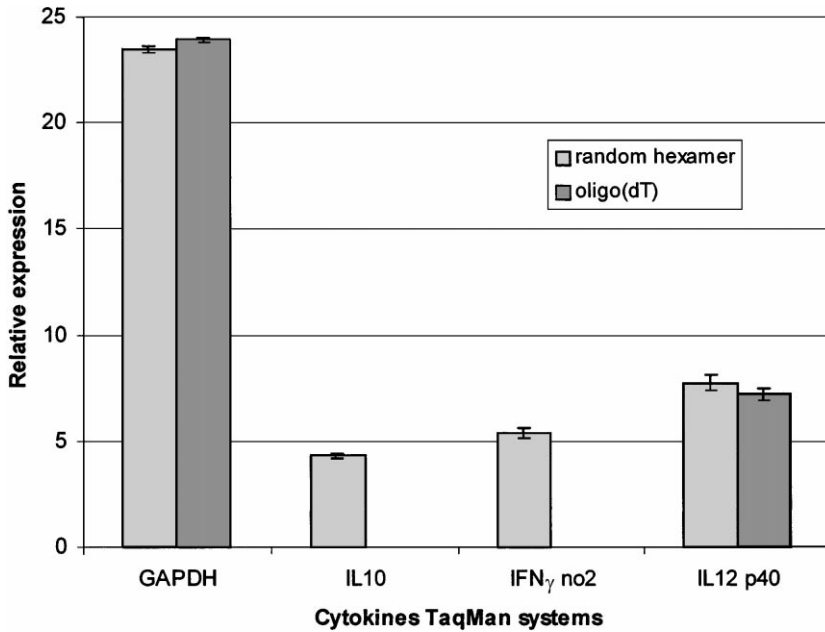
Fig. 1. Comparison of cDNA synthesis using random hexadeoxyribonucleotide (pd(N)$_6$) primer and oligo(dT) primers for RT-priming. Relative expression for each cytokine is given as the difference of the C_T values to 40 (total number of cycles).

To address the question whether contaminating gDNA present in the total RNA samples could influence the cytokine cDNA quantitation, RNA preparations without prior cDNA synthesis were subjected to TaqMan PCR. None of the samples gave a signal for any of the cytokines (results not shown). To investigate whether contaminating genomic DNA was coamplified or interfered with the quantitation of cytokine cDNAs, we amplified either cDNA or 100 ng of genomic DNA. Fig. 2 shows C_T values for all cytokine systems carried out with cDNA and gDNA. Only the IFNγ system No. 1 showed recognition of both, the cDNA and the gDNA; all the other systems were able to differentiate between cDNA and gDNA, confirming the specificity of the primer combinations used for the selective amplification of cDNA. When PCR products were tested in agarose gel electrophoresis (2.5% gel) all cytokine systems produced a single band at the expected length.

3.3. Determination of the linear range and analytical sensitivity for GADPH cDNA

Quantitation of feline GAPDH cDNA cloned into a pT7blue-2 vector showed linearity over eight orders of magnitude (Fig. 3). To obtain linearity even at highest plasmid dilutions, a constant amount of carrier DNA (calf thymus DNA, Sigma, Buchs, Switzerland) was used to prevent plasmid DNA adsorbing to the tube walls. The analytical sensitivity of the cloned GAPDH was 10 molecules (Fig. 3).
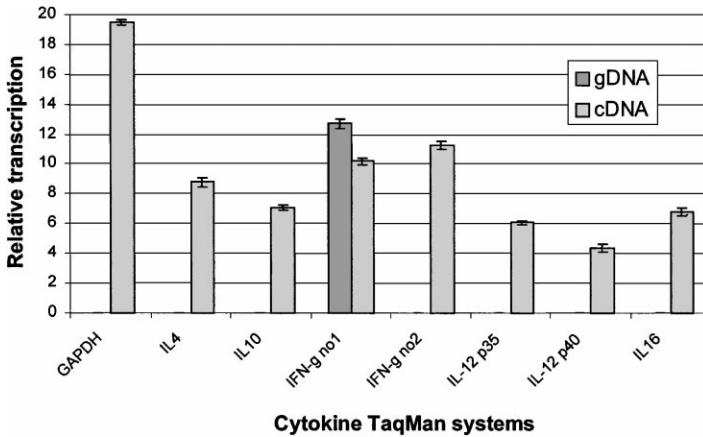
Fig. 2. Differentiation between complementary DNA (cDNA) and genomic DNA (gDNA). 100 ng of gDNA was amplified to test for the ability of the TaqMan systems to discriminate between gDNA and cDNA. Relative expression for each cytokine is given as the difference of the C_T values to 40 (total number of cycles). Dark bars: amplification of gDNA, grey bars: amplification of cDNA or IL16 plasmid.
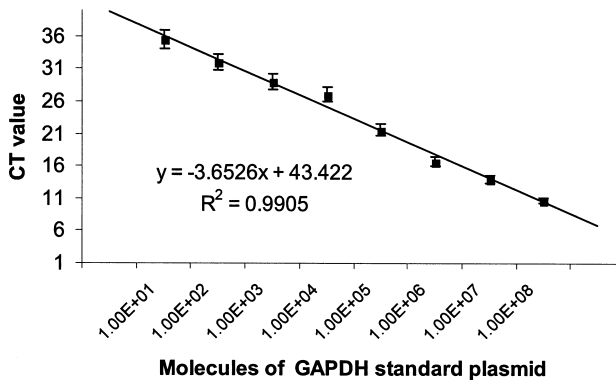

Fig. 3. Linear range from 10 to 10^8 copies of the GAPDH plasmid standard and analytical sensitivity. Each dilution was tested in triplicate.

3.4. Amplification efficiencies of GAPDH and cytokine cDNA

For the comparative C_T method ($\Delta\Delta C_T$ method) to be valid, the efficiency of the target amplification and the efficiency of the reference amplification must be approximately equal. To determine the amplification efficiency of GAPDH and the cytokine cDNAs, different dilutions of cDNA preparations were amplified, the resulting C_T values plotted against the dilution of input total RNA and the regression line was calculated as exemplified with the TaqMan system for IFNγ (Fig. 4). Six or seven dilutions were amplified in triplicate for 40 cycles. Differences of the slopes of cytokines versus

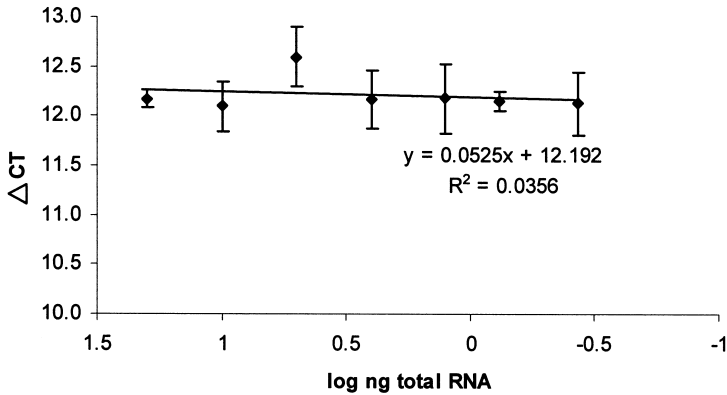Fig. 4. Efficiency determination of the IFNγ TaqMan system. The $\Delta\Delta C_T$ method for relative quantitation requires that efficiencies of target and reference (GAPDH) amplified in different tubes are approximately equal. Standard curves for IFNγ and GAPDH served as basis for the efficiency determination. The figure shows plot of log input amount RNA versus ΔC_T. The absolute value of the slope should be < 0.1.

GAPDH were: IL4: 0.09; IL10: 0.1; IL-12 p35: 0.06; IL12 p40: 0.06; IFNγ no2 (Fig. 4): 0.05; IL16: 0.08, respectively.

3.5. Comparison of the two-tube RT-PCR with the one-tube RT-PCR

Comparison of the two procedures showed that the two-tube PCR was superior to the one-tube RT-PCR with respect to sensitivity and signal detection. Addition of random hexadeoxyribonucleotide $(pd(N)_6)$ for RT-priming to the one-tube RT-PCR system improved the sensitivity however, detection of cytokine signals did not lead to the sensitivity obtained in the two-tube system (Fig. 5). In all the cytokines systems, the two-tube system yielded the best sensitivity, shown by the lowest C_T values.

3.6. Quantitation of GAPDH transcription in frozen cells and IFNγ transcription in stimulated cells and cell lines

RNA suitable for RT-PCR analysis could be extracted from cell samples stored at $-80°C$ for up to 5 years. We compared the GAPDH expression in these unstimulated PBMCs to the one of unstimulated PBMCs obtained freshly. In all the cDNAs prepared by reverse transcription of total RNA extracted from 10 million cells, cDNAs coding for GAPDH were readily detectable. The GAPDH C_T values from cDNA of freshly isolated cells and of frozen cells were 18.03 ± 0.76 versus 20.01 ± 1.14, respectively, (n 7, Tab. 3), indicating that GAPDH mRNA levels were decreased by about 3.3-fold in frozen cells. In addition IFNγ transcription was determined upon stimulation of cells with *S. aureus* extracts and ConA. 6 h stimulation with *Staphylococcus aureus* extracts induced a 50-fold IFNγ transcription whereas 72 h stimulation of PBMCs with ConA induced a 130-fold IFNγ transcription compared to unstimulated PBMCs. In comparison to unstimulated PBMCs, CrFK cells showed a 10,000-fold increase of IFNγ transcription.
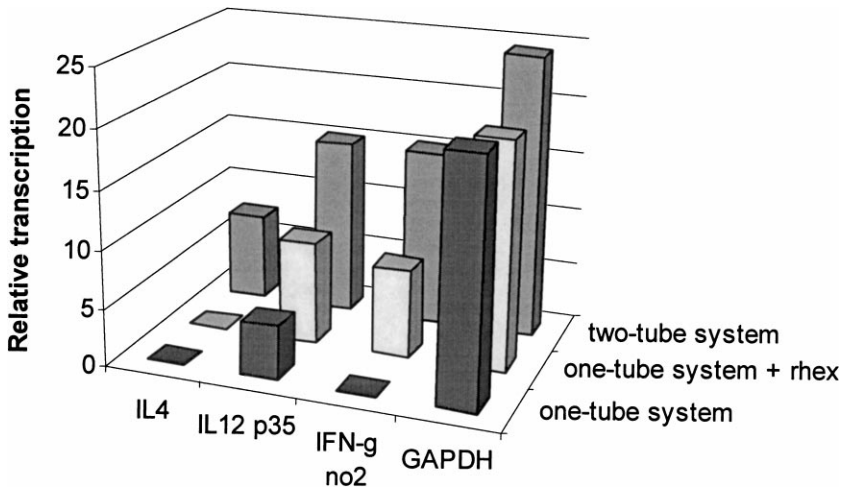
Fig. 5. Comparison of the two-tube TaqMan PCR with the one-tube RT-PCR. Standard deviations for all assays were between 0.08 and 2.3. Light grey bars: Two-tube system, cDNA produced with random hexadeoxyribonucleotide (pd(N)$_6$), white bars: one-tube RT TaqMan PCR, dark grey bars: one-tube RT TaqMan with random hexadeoxyribonucleotide (pd(N)$_6$) (rhex) added for reverse transcription. Relative expression for each cytokine is given as the difference of the C_T values to 40 (total number of cycles).

The T-cell line 3201 and NLFK showed both IFNγ transcription at the level of unstimulated PBMCs.

4. Discussion

In this report we describe the application of a novel assay for the quantitation of feline cytokine mRNA expression in fresh and stored PBMC. Several protocols using quantitative competitive RT-PCR (qc RT-PCR) for feline cytokines have been proposed (Dean et al., 1998; Rottman et al., 1995). One of the most common concepts of qcPCR is the coamplification of two different templates of similar lengths and with the same primer recognition sequences in the same tube, thus ensuring optimised thermodynamics and amplification efficiencies for both template species (Menzo et al., 1992; Siebert and Larrick, 1992). However, this PCR approach has several important drawbacks (Clementi et al., 1995) such as the need for parallel reactions for a single sample, postamplification steps reducing accuracy of the assay, and a narrowed linear range due to shared primer exhaustion (Sundfors and Collan, 1996). The quantitative PCR technique used in this work has several advantages compared to classical quantitative PCR system. The use of fluorescent dye-labelled probes increases the sensitivity of the system, resulting in a linear relationship between copy number and C_T values of at least seven orders of magnitude (Fig. 3). In addition, the use of a third oligonucleotide in the form of the probe adds further specificity to the system comparable to hybridization techniques with blotted PCR products. The elimination of postamplification steps further increases reliability and reproducibility of the assay (Gut et al., 1999; Leutengger et al., 1999b). A major factor

responsible for the accuracy of this method appears to be the determination of the C_T value within the logarithmic phase of the amplification reaction rather than endpoint determination of conventional systems. The Sequence Detection System calculates the C_T value at a time when amplification of PCR products is first detected, i.e., at the beginning of the exponential phase of amplification, when accumulation of inhibitory PCR products is unlikely to occur.

A total of 88 cDNA samples can be quantitated on a 96 well plate together with three non-template controls and 5 dilutions of the plasmid standard. Thus, this methodology offers a significant saving of time: pipetting, amplification and quantitation of 88 cDNA samples to be quantitated was achieved in less than 4 h with hands-on-time of around 60 min. The most pernicious problem facing the diagnostic application of PCR is production of false-positive results attributable to contaminating nucleic acids, particularly in the form of previously amplified material (carry-over). Any contaminant, even the most minute airborne remnant carried over from the previous PCR procedure or from a strongly positive sample (contamination) may be multiplied and can give false-positive results. In the TaqMan system, the problem of carry-over is significantly reduced due to the real-time measuring principle which makes it unnecessary to open the tubes after amplification. In addition, the probability of contamination problems can be further decreased or even eliminated in TaqMan PCR by inclusion of the AmpErase® UNG system (Pang et al., 1992).

Previous quantitative PCR systems used consensus sequence primers derived from cytokines of different species (Rottman et al., 1995). Here we show the influence of primer mismatch of non-species specific primers leading to a 22-fold decrease of PCR signal when the rat GAPDH TaqMan system was used to analyze feline cDNA. The PCR signal dicreased 235-fold and 4153-fold, when the horse or the human GAPDH TaqMan system were used to analyze feline cDNA. These results clearly show the influence of mismatch on the primer sequences and their profound impact of PCR signal generation.

It is known that residual gDNA may still be present in DNase-treated total RNA samples. It is, therefore, important to avoid amplification of contaminating gDNA. This could be achieved by designing junctional-specific probes. As genomic sequences for feline cytokine genes were not available, intron-exon junctions were deduced either from mouse or human sequences. As shown in Fig. 2, all systems except IFNγ No. 1, which was designed within an exon containing no intron/exon boundaries, were able to strictly differentiate between gDNA and cDNA.

In our hands, the two-tube system was superior to the one-tube RT-PCR TaqMan system. Several reasons could attribute to the loss of sensitivity observed in the latter system, such as different enzyme combinations used (AMV and AmpliTaq Gold versus AMV and *Tfl* DNA polymerase), different temperature profiles for the RT step (42°C versus 48°C), different temperature profiles for the PCR step (two-step cycling protocol versus three-step cycling protocol), respectively.

The TaqMan PCR has been shown to achieve high sensitivity (Leutenegger et al., 1999b). The standard curve for GAPDH reveals high sensitivity also for gene transcription. Although we have not tested the analytical sensitivity for each cytokine, we assume that the sensitivity is in the same range as for GAPDH. This assumption is

supported by the fact, that all the systems amplify their target sequence with app-roximately the same efficiency.

Accurate cytokine cDNA quantitation was made possible by normalizing the cytokine signals using the GAPDH signals. This normalization requires that the efficiencies of both the cytokine and the GAPDH amplifications are approximately equal. A sensitive method for comparing the amplification efficiencies of two amplicons is to determine the
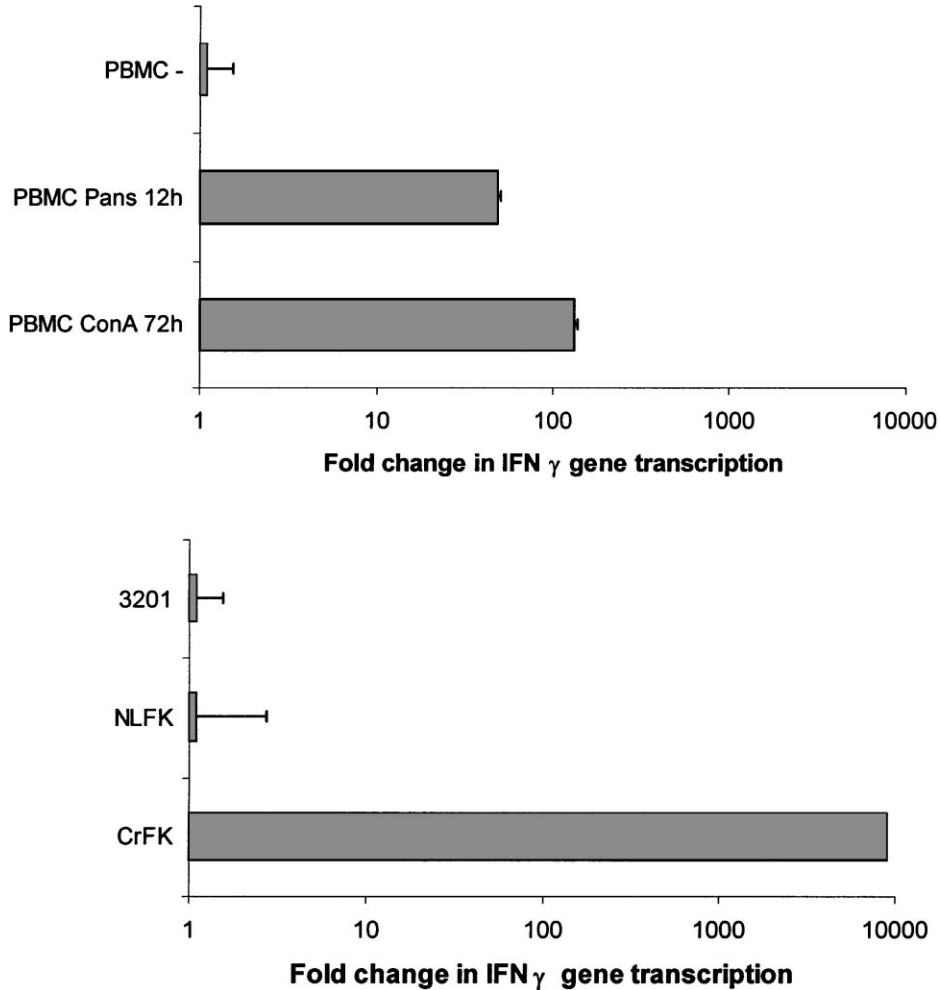

Fig. 6. Levels of IFNγ transcription levels in response to stimulation and of three different feline cell lines. Results represent the mean +/− SEM fold increase over unstimulated PBMC. Note the log scale on the y-axis. (A) PBMCs were stimulated with Pansorbin for 12 h and ConA for 72 h and then harvested for RNA extraction, RT step and TaqMan PCR. IFNγ transcription of stimulated cells was calculated against unstimulated PBMCs serving as calibrator. (B) IFNγ transcription levels of the three feline cell lines CrFK, NFLK and 3201 cells. For direct comparison with unstimulated PBMC of (A), the IFNγ gene transcription of unstimulated PBMCs served as the calibrator.

slopes of C_T values variation obtained from cDNA dilution series done with GAPDH and the respective cytokine (User Bulletin #2, PE Biosystems, 1997). We found that the amplification efficiencies for both GAPDH and cytokine cDNA were approximately equal (<10% difference) thus allowing GAPDH-based normalization of cytokine transcription.

The amount of GAPDH transcripts in freshly obtained cells or cells stored frozen for several years showed a difference of 1.71 C_T values (3.3-fold difference) which is most likely the consequence of RNA degradation. Nevertheless, quantitation of cytokine signals still was possible unless GAPDH signals exceeded C_T values of 24 (data not shown). The possibility of analyzing cells that had been stored for months to years permits the multiple analysis of the same samples such as it is required, e.g., in the monitoring of cytokine mRNA expression during clinical or experimental trials. It further allows the transport of frozen cells or tissue to specialized laboratories.

Stimulation of PBMCs with ConA and *S. aureus* extracts showed inducible IFNγ transcription. Interestingly, NFLK and CrFK, both feline kidney cell lines, showed a clear cut difference in their ability to transcribe message for IFNγ. The reason for this is unknown but different cell types for the production of these cell lines could explain this difference (Fig. 6).

In summary, we have adapted a highly sensitive real-time PCR system for the quantitation of feline cytokine mRNA expression. Due to the high sensitivity, this assay is suitable for analyzing the cytokine mRNA present in small volumes such as PBMCs collected from cats or from cell culture systems and to determine early events in the kinetics of cytokine mRNA expression and Th1/Th2 differentiation.

Acknowledgements

References

Balkwill, F.R., Burke, F., 1989. The cytokine network. Immunol. Today 10, 299–304.

Clementi, M., Menzo, S., Manzin, A., Bagnarelli, P., 1995. Quantitative molecular methods in virology. Arch. Virol. 140, 1523–1539.

Collins, R.A., Howard, C.J., Duggan, S.E., Werling, D., 1999. Bovine interleukin-12 and modulation of IFN production. Vet. Immunol. Immunopathol. 68, 193–207.

Dallman, M.J., Montgomery, R.A., Larsen, C.P., Wanders, A., Wells, A.F., 1991. Cytokine gene expression: Analysis using northern blotting, polymerase chain reaction, polymerase chain reaction and in situ hybridization. Immunol. Rev. 119, 163–179.

Dean, G.A., Higgins, J., LaVoy, A., Fan, Z., Pedersen, N.C., 1998. Measurement of feline cytokine gene expression by quantitative-competitive RT-PCR. Vet. Immunol. Immunopathol. 63, 73–82.

Fehniger, T.A., Shah, M.H., Turner, M.J., VanDeuse, J.B., Whitman, S.P., Cooper, M.A., Suzuki, K., Wechser, M., Goodsaid, F., Caligiuri, M.A., 1999. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: Implications for the innate immune response. J. Immunol. 162, 4511–4520.

Förster, V., 1948. Zwischenmolekulare Energiewanderung und Fluoreszenz. Ann. Phy. 2, 55–57.

Gut, M., Leutenegger, C.M., Huder, J., Pedersen, N.C., Lutz, H., 1999. One-tube fluorogenic reverse transcription-PCR for the quantitation of feline coronaviruses. J. Virol. Meth. 77, 37–46.

Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M., 1996. Real time quantitative PCR. Genome Res. 6, 986–994.

Holland, P.M., Abramson, R.D., Watson, R., Gelfand, D.H., 1991. Detection of specific polymerase chain reaction product by utilizing the 5'–3' exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Am. Sci. USA. 88, 7276–7281.

Kabilan, L., Andersson, G., Lolli, F., Ekre, H.P., Olsson, T., Troye-Blomberg, M., 1990. Detection of intracellular expression and secretion of interferon-gamma at the single-cell level after activation of human T-cells with tetanus toxoid in vitro. Europ. J. Immunol. 20, 1085–1089.

Leutenegger, C.M., Klein, D., Hofmann-Lehmann, R., Mislin, C., Hummel, U., Böni, J., Boretti, F., Guenzburg, W.H., Lutz, H., 1999a. Rapid feline immunodeficiency virus provirus quantitation by polymerase chain reaction using the TaqMan fluorogenic real-time detection system. J. Virol. Meth. 78, 105–116.

Leutenegger, C.M., von Rechenberg, B., Huder J.B., Zlinsky, K., Mislin, C., Akens, M.K., Auer, J., Lutz, H., 1999b. Quantitative real-time PCR for equine cytokine mRNA in nondecalcified bone tissue embedded in methyl methacrylate. Calcif. Tissue Int. (in press).

Lutz, H., Leutenegger, C.M., 1998. Feline Cytokines. In: Griebel P., Bazin, H., Govaerts, A., Pastoret, A.A. (Ed.) Handbook of Vertebrate Immunology, Feline Immunology, Academic Press New York, pp. 297–301.

Menzo, S., Bagnarelli, P., Giacca, M., Manzin, A., Varaldo, P.E., Clementi, M., 1992. Absolute quantitation of viremia in human immunodeficiency virus infection by competitive reverse transcription and polymerase chain reaction. J. Clin. Microbiol. 30, 1752–1757.

Mosmann, T.R., Sad, S., 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol. Today 17, 138–146.

Pang, J., Modlin, J., Yolken, R., 1992. Use of modified nucleotides and uracil-DNA glycosylase (UNG) for the control of contamination in the PCR-based amplification of RNA. Mol. Cell. Probes 6, 251–256.

Rottman, J.B., Freeman, E.B., Tonkonogy, S., Tompkins, M.B., 1995. A reverse transcription-polymerase chain reaction technique to detect feline cytokine genes. Vet. Immunol. Immunopathol. 45, 1–18.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: A Laboratory Manual, 2nd, ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Siebert, P.D., Larrick, J.W., 1992. Competitive PCR. Nature 359, 557–558.

Sundfors, C., Collan, Y., 1996. Basics of quantitative polymerase chain reaction: 2. electrophoresis and quantitation of polymerase chain reaction products. Electrophoresis 17, 44–48.

Wang, S.C., Klein, R.D., Wahl, W.L., Alarcon, W.H., Garg, R.J., Remick, D.G., Su, G.L., 1998. Tissue coexpression of LBP and CD14 mRNA in a mouse model of sepsis. J. Surgical Res.76, 67–73.

Zimmermann, K., Mannhalter, J.W., 1996. Technical aspects of quantitative competitive PCR. Biotechniques 21, 268–272.