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Development and Evaluation of a SYBR Green Real-time PCR Assay for Canine Cytokine Gene Expression

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Abstract : Cytokines are important mediators of the immune response, and quantitating cytokine mRNA is a highly sensitive and attractive method for measuring cytokine production. The objective of the current study was to develop and validate a SYBR green quantitative real-time reverse transcriptase PCR (qRT-PCR) assay for measuring canine cytokine mRNA. The optimal annealing temperatures (T_a) of the designed primers were 62°C for interleukin (IL)-1β, IL-6 and IL-10; 60°C for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and tumor necrosis factor (TNF)-α; and 58°C for high mobility group box 1 (HMGB1). Primer efficiencies of all primers calculated for standard curve samples were between 97.1% and 102.6%. No evidence of secondary structure or primer-dimer formation was seen via melt-curve analysis or gel electrophoresis. The developed qRT-PCR assays are highly specific and sensitive and can be used to quantify gene expression levels of canine cytokines.

Key words **:** canine cytokine, primer design, SYBR green real-time PCR assays.

Introduction

Cytokines play a central role in inflammation, septic shock and other physiological and pathological immune reactions (1,9). Thus, measurement of cytokine levels is an important analytical tool in experimental and clinical settings. Quantitating cytokine mRNA levels is more sensitive and attractive than measuring cytokine protein levels (4), especially for small cell numbers or limited biopsy samples. Of the available methods for quantifying transcript levels from specific genes, real-time reverse transcriptase polymerase chain reaction (qRT-PCR) is one of the most accurate, rapid, and sensitive methods in biological and clinical research (2,3). It is performed using fluorogenic probes or intercalating dyes such as SYBR green, and although fluorescent probes are a more specific technique for determining amplicon accumulation, they are relatively expensive (10). Thus, the simplest technique for detecting newly synthesized PCR products in real-time PCR uses the SYBR green fluorescence dye that binds specifically to the minor groove of double-stranded DNA (8). However, the major drawback of SYBR green is that any double-stranded product, including primer-dimers or secondary structure, is detected, leading to false positives. Therefore, careful reaction design and optimization is essential to maximize sensitivity.

In the current study, we tested the most commonly used six primer sets for potential use in canine samples: interleukin (IL)-1β, IL-6, IL-10, TNF-α, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and high mobility group box 1 (HMGB1). We also explored several aspects of real-time RT-PCR assay design. Our experimental design optimized qRT-PCR conditions from lipopolysaccharide (LPS)-stimulated canine peripheral blood mononuclear cells (PBMCs).

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Materials and Methods

Canine PBMC Culture

All aspects of this study were approved by the Committee on Bioethics at our institution (CBU2008-021). PBMC isolation was performed using a density gradient from blood collected from clinically healthy beagles via venipuncture of jugular veins. Heparinized blood was layered onto Histopaque 1077 (Sigma, USA) and centrifuged at 400 × *g* for 35 min at room temperature. PBMCs from the interface were collected and subjected to red blood cell lysis by adding 83% ammonium chloride solution (pH 7.2). PBMCs were washed twice in PBS and were used for cell culture. Cell concentration and viability were assessed using an impedance cell counter and cells suspended in trypan blue solution, respectively. Cell purity was determined using a conventional Diff-Quik method after Cytospin-centrifuge preparation. Viability was demonstrated to be high at > 98%, and > 95% of cells were PBMCs.

Cells were diluted to 2×10^6 cells/ml with RPMI-1640 (Roswell Park Memorial Institute-1640, Hyclone, Thermo Fisher Scientific Inc., USA) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, defined grade, Hyclone, Thermo Fisher Scientific Inc., USA), L-glutamine (2 mmol/l), 100 U/ml penicillin G and 10 μg/ml streptomycin (Sigma,

USA). For the experiments, cells were seeded into 24-well Nunclon plates (NUNC, Denmark) and grown overnight at 37°C in a 5% CO_2 humidified atmosphere. Mononuclear cells were exposed to 100 ng/ml of lipopolysaccharide (LPS, *Escherichia coli* serotype O111: B4, Sigma, USA) for 3 hours and were harvested for RNA isolation.

RNA extraction and cDNA synthesis

The cells were disrupted in RLT buffer (lysis buffer) and homogenized in a QIAshredder spin column (Qiagen, Germany). After centrifugation for 2 min at maximum speed (13,000 rpm), RNA was isolated using an RNeasy Mini Kit (Qiagen, Germany) as per the manufacturer's protocol. Extracted RNA samples were treated with RNase-free DNase (RQ1 RNase-free DNase, Promega, USA) to remove contaminating genomic DNA. RNA concentration and purity (A_{260}/A_{280}) were measured using a spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific, USA). A 260/280 nm absorbance ratio of 1.8-2.0 was regarded as a pure RNA sample.

Reverse transcription (RT) of 0.5-1 μg of total RNA took place in a 20 μL reaction containing 250 ng of random hexamers, 1 μL of 10 mM dNTP mix, 200 U of SuperScript III reverse transcriptase, 40 U of RNaseOUT recombinant RNase inhibitor, 1 μL of 0.1 M DTT and 5× First-Strand Buffer (all materials were from Invitrogen, USA). Total RNA was pre-incubated with random primers and dNTP mixture at 65°C for 5 min and chilled on ice for at least 1 min. After brief centrifugation, RT buffer, DTT, RT enzyme and RNase inhibitor were added, and RT was carried out at 25°C for 5 min, followed by transcription at 50°C for 60 min. Termination was at 70°C for 15 min, then 1 μL of *E. coli* RNase H (Invitrogen) was added at 37°C for 20 min. The resulting cDNAs were stored at −20°C until use.

Primer design for SYBR green real-time PCR

Primer Express Software (version 3.0, Applied Biosystems, CA) was used to design primers for SYBR green real-time PCR. For specific binding to canine cytokine cDNA, template sources were based on consensus sequences from the GenBank database. Target accession numbers and primer sequences are shown in Table 1. The criteria applied in designing templates and primers were:

i. amplify a template region of 75-150 base pairs (bp)

ii. avoid the formation of secondary structures, if possible

iii. use an annealing temperature above the melting temperature for any template secondary structures

iv. avoid templates with > 4 single base repeats

v. maintain a GC content of 50-60%

vi. maintain a melting temperature between 50°C and 65°C

vii. avoid repeats of Gs and Cs longer than three bases

viii. place Gs and Cs at the ends of primers

iv. ensure no 3' complementarity between forward and reverse primers to avoid primer-dimers

x. move primer locations outside the target if necessary to avoid sequence secondary structures

To predict secondary structures that might form at the site of primer binding, the Zuker DNA M-fold server (http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi) was used (11). Primers were re-designed if any regions of the templates were predicted to form secondary structures above the annealing temperature of the PCR reaction. Primer uniqueness and specificity were verified using the Primer-Blast Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast) using GenBank Accession numbers. Primers were commercially synthesized by the Genotech Corporation (Daejeon, Korea).

Quantification of cytokine gene expression by real-time PCR

Real-time PCR was performed in 20 μL reactions containing 300 nM each primer, 1 μL cDNA and 12.5 μL 2× iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) containing antibody-mediated hot-start i-Taq DNA polymerase and nuclease free water (Promega, Madison, WI, USA). Amplification was achieved via the CFX384 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) using a polymerase activation step of 95°C for 3 min, followed by 40 cycles of 15 s at 95°C, 15 s at annealing temperature, and 15 s at 72°C. Assays with cycle threshold (Ct) values over 40 were considered to be negative. A negative (no template) control was included in each run to test for false positives or contamination. The absence of non-specific products or primer-dimers was confirmed by a single melting peak in a melting curve analysis using the CFX Manager Software (version 1.0, Bio-Rad, Hercules, CA, USA). Randomly selected PCR products were subjected to 12.5% native polyacrylamide electrophoresis with ethidium bromide to confirm specific amplification by the observation of predicted molecular weights and the absence of non-specific products or primer-dimers. Standard curves with cDNA were constructed using five-fold serial dilutions of cDNA from 1/5 to $(1/5)^6$ for each amplification reaction mixture. A no-template control of nuclease-free water was included in each run. All samples, controls and standards were analyzed in duplicate.

Data analysis of SYBR green real-time PCR

Data collection and analysis used CFX Manager Software version 1.0 (Bio-Rad, Hercules, CA, USA). The Ct value was calculated as the cycle at which the fluorescence of the sample exceeded ten standard deviations from the mean of the baseline fluorescence. A graph of Ct vs. log 10 copy number of the sample for the dilution series was produced, and the slope of this graph was used to determine the reaction efficiency. The amplification efficiency, E, was calculated from the slope of the standard curve using the formula $E = 10^{(-1/\text{slope})} - 1$. A slope of −3.32 is indicative of a PCR efficiency of 1 (100%). The coefficients of variation (CV, %) for the slope, R^2, and the E values were used to determine reproducibility. A melting curve was produced by heating samples from 65°C to 95°C in 0.2°C increments with a dwell time of 0.01 s per temperature, during which fluorescence data were collected. Product melt-

Table 1. Primers used for SYBR green real-time PCR

Target gene	GenBank Accession no.	Forward primer					Reverse primer					Amplicon size (bp)
		start[a]	length (bp)	T_m[b] (°C)	GC (%)	forward sequences	start[a]	length (bp)	T_m (°C)	GC (%)	reverse sequences	
IL-1β	Z70047	21	20	59	55	TGAAGTGCTGCTGCCAAGAC	95	19	58	58	GCTGGTGGGAGACTTGCAA	75
IL-6	U12234	10	22	59	50	CCCACCAGGAA-CGAAAGAGA	77	22	58	50	CTTGTGGAGAGGG-AGTTCATAGC	68
IL-10	U33843	245	20	59	55	CAAGCCCTGTCGGAGATGAT	322	21	59	52	CTTGATGTCTGGGTCGTGGTT	78
GAPDH	AB038240	698	19	59	58	TCCCCACCCCCAATGTATC	789	22	58	50	TGCCTGCTTCACTACCTTCTTG	92
TNF-α	NM 001003224	726	20	59	50	CCAAACCGACCCTTTGATCA	808	20	59	55	CCAGCCCTGAGCCCTTAATT	83
HMGB1	NM 001002937	438	19	60	58	CATCCCGGCCTATCCATTG	513	22	59	50	GCTTGTCATCTGCAGCAGTGTT	76

[a]Positions of genes are given according to accession number
[b]Theoretical melting temperature calculated by Primer Express Software

Table 2. Amplification results from primers designed for SYBR Green real-time PCR assays

Target	*E*	slope	R^2	T_a	T_m
IL-1β	99.6%	−3.332	0.999	62°C	82.6°C
IL-6	102.4%	−3.226	0.994	62°C	82.0°C
IL-10	95.7%	−3.430	0.997	62°C	82.5°C
GAPDH	100.2%	−3.317	0.996	60°C	82.6°C
TNF-α	97.1%	−3.393	1.000	60°C	81.8°C
HMGB-1	102.6%	−3.262	0.990	58°C	79.4°C

T_a: annealing temperature, T_m: melting temperature in the melt curve analysis

ing temperatures were determined using the rate of change of fluorescence (−d(*RFU*)/d*T*) vs. temperature graph.

Results

Optimization of SYBR green real-time PCR

For all primers, the melting temperatures of all possible secondary structures from the designed primers were lower than the reaction annealing temperature. Annealing temperatures and primer concentrations were optimized for the temperature and primer concentration that gave the best specificity without a reduction in yield. The optimal annealing temperatures (T_a) are shown in Table 2 and ranged from 62°C for IL-1β, IL-6 and IL-10; to 60°C for GAPDH and TNF-α; to 58°C for HMGB1 at an optimal primer concentration of 300 nM. Primer efficiencies against serial dilution standards for all primers were between 97.1% and 102.6% (Fig 1).

Melt curve analysis

All melting curves described a single distinctive peak, indicating the formation of one specific amplicon and the absence of non-specific amplification and primer-dimer formation (Fig 2). Polyacrylamide gel electrophoresis confirmed the absence of non-specific products or dimers (data not shown). All controls lacking a template or enzyme were negative.

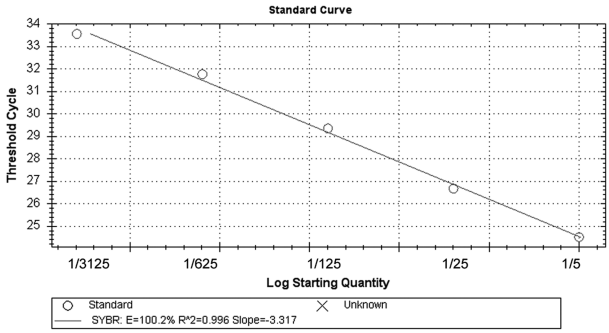

Fig 1. Representative dilution series for GAPDH using SYBR Green real-time PCR. cDNA from canine PBMCs stimulated with LPS for 3 h were serially diluted in 1/5 increments for qRT-PCR, producing a 100.2% efficiency. The slope was −3.317 with an R^2 of 0.996. Primer Express Software was used for primer design, and amplification targets were characterized using the Zuker M-fold server to predict secondary structures. A graph of Ct vs. log 10 copy number of the sample for the dilution series was produced, and the slope of this graph was used to determine the reaction efficiency.

Discussion

Real-time PCR is a powerful and effective technique for the accurate quantification of gene expression. Assays for the detection of a single gene require the careful choice of primers, target sequence, and method for detecting the amplified products. This study describes the steps used to optimize and validate a reliable set of primer pairs for qRT-PCR assays using the fluorogenic DNA binding dye SYBR green. SYBR green che-

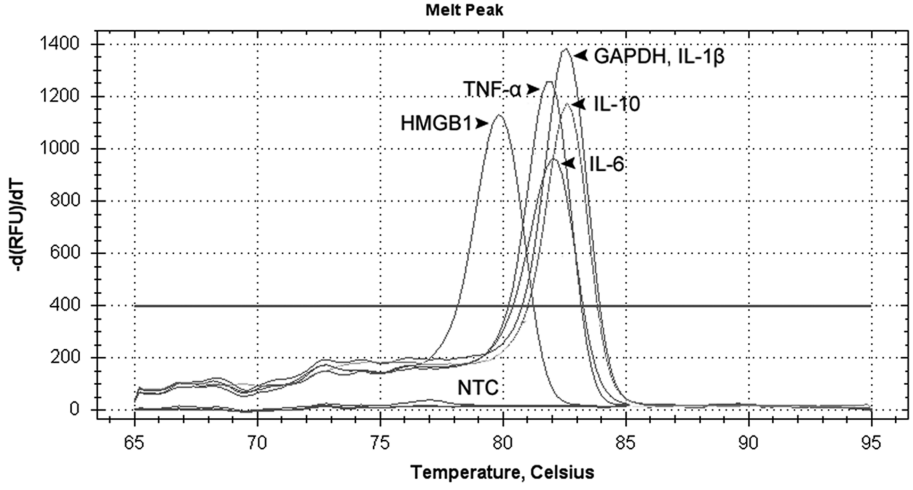

Fig 2. Melt curve analysis of the designed primers. Single peaks indicate the absence of primer-dimers and other non-specific PCR products. Melting temperatures ranged from 79.4°C to 82.6°C.

mistry was used in this study instead of Taqman probe chemistry, even though the latter may have given a higher specificity (6). Unlike SYBR green methods, designing assays using TaqMan probe chemistry is not always possible, because this technique requires that the primers and probes satisfy rigid constraints that are not always easily achieved, especially with the gene sequences selected in this study. Using SYBR green techniques, however, we obtained highly reliable and accurate quantification of canine cytokine gene expression, using properly validated methods. The costs associated with SYBR green quantification are considerably less than those associated with fluorescently-labeled oligonucleotide probes used in hydrolysis methods, like TaqMan, or hybridization and hairpin techniques, like molecular beacon technology (5).

The most commonly used methods for quantifying PCR data are an absolute method using a standard curve and a comparative C_T method. The latter is a mathematical model that calculates relative fold-changes in gene expression between the experimental and calibration samples. The comparative C_T method can be used only if the amplification kinetics of the target and housekeeping genes are approximately equal (7), as determined by a validation experiment. If the efficiencies of the two amplicons are not approximately equal, then the analysis should be performed using an absolute quantification method with standard curves. However, this method requires a standard template source, such as purified cloned cDNA, or PCR-amplified cDNA that must included in each run, limiting the number of samples that can be assayed on the same plate. In our experiments, little difference was observed between primers, with slopes ranging from −3.226 to −3.430. Efficiency can be improved by proper primer design, including ensuring that primers are specific for the desired target sequence, binding at positions that avoid secondary structures, and minimizing the occurrence of primer-dimer formation. Optimization of the detection method, Mg^{2+} concentration, annealing temperature, enzyme concentration, and PCR product length are also critical for efficiency. In this study, 3 mM Mg^{2+} yielded the highest performance, with higher or lower concentrations giving a lower efficiency (data not shown). Thus, appropriate reaction conditions are required for robust and efficient amplification. Poor assay design can result in problems including non-specific products and inefficient primer association with the target, which can dramatically affect data quality.

Conclusions

Development of a SYBR green real-time PCR assay for canine cytokine gene expression was successfully achieved by proper primer design, annealing temperature and melt curve analysis. The information in this study should help investigators implement qRT-PCR assays for the cytokine genes quantified here, without having to repeat the laborious validation process, as long as the conditions validated by our trials are maintained. In addition, this study outlines the procedures and techniques for developing a straightforward validation system for establishing primers to be used in qRT-PCR assays.

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SYBR Green 실시간 역전사 중합효소연쇄반응을 이용한 개 싸이토카인 유전자 발현의 정량

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요 약 : 싸이토카인은 염증 및 면역 반응의 평가에 있어서 매우 중요한 요소이다. 따라서 이들의 mRNA 수준을 정량하고 평가하는 것은 염증 및 면역 반응을 평가하는데 있어서 그 민감도가 매우 높은 방법으로 알려져 있다. 본 연구의 목적은 SYBR green dye를 이용하여 개의 싸이토카인 mRNA를 정량적 실시간 역전사 중합효소연쇄반응(real-time reverse transcriptase PCR; qRT-PCR)으로 분석을 할 수 있도록 함에 있다고 할 수 있다. 제작된 시발체(primer)의 최적화된 붙임 온도(annealing temperature; T_a)는 인터루킨(interleukin; IL)-1β, IL-6, IL-10이 각각 62°C, glyceraldehyde 3-phosphate dehydrogenase (GAPDH)와 tumor necrosis factor (TNF)-α가 60°C 그리고 high mobility group box 1 (HMGB1)이 58°C였다. 표준 정량 곡선을 이용하여 측정한 시발체의 효율성은 97.1%에서 102.%로 매우 높았고, 2차 구조물(secondary structure)과 시발체-이합체 형성(primer-dimer formation)은 융해곡선(melt-curve)분석과 전기영동을 통해 확인하였다. 이렇게 정립된 qRT-PCR 분석법은 민감도와 특이도가 매우 높은 개 싸이토카인 유전자 정량법으로 활용될 수 있을 것이다.

주요어 : 개 싸이토카인, 시발체 제작, SYBR green 실시간 역전사 중합효소연쇄반응