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ANALYTICAL VALIDATION OF A QUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION ASSAY FOR EVALUATION OF T-CELL TARGETED IMMUNOSUPPRESSIVE THERAPY IN THE DOG

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Abstract

Cyclosporine is an immunosuppressive agent that inhibits T-cell function by decreasing production of cytokines such as interleukin-2 (IL-2) and interferon- γ (IFN- γ). In dogs, there is currently no reliable analytical method for determining effective cyclosporine dosages in individual patients. Our laboratory has developed a quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) assay that measures IL-2 and IFN- γ gene expression, with the goal of quantifying immunosuppression in dogs treated with cyclosporine. This study focuses on analytical validation of our assay, and on the effects of sample storage conditions on cyclosporine-exposed samples. Heparinized whole blood collected from healthy adult dogs was exposed to a typical post-treatment blood concentration for cyclosporine (500 ng/ml) for 1 hour, and then stored for 0, 24, and 48 hours at both room temperature and 4°C. The study was then repeated using a cyclosporine concentration of 75 ng/ml, with sample storage for 0, 24, and 48 hours at 4°C. Cytokine gene expression was measured using RT-qPCR, and assay efficiency and inter- and intra-assay variability were determined. Storage for up to 24 hours at room temperature, and up to 48 hours at 4°C, did not significantly alter results compared to samples that were processed immediately. Validation studies showed our assay to be highly efficient and reproducible and robust enough to be feasible under standard practice submission conditions.

Keywords

Cyclosporine; RT-qPCR; Dog; Pharmacodynamics; Interleukin-2; Interferon- γ

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Conflict of Interest:

The authors declare no financial or commercial conflict of interest exists in relationship to the content of this article.

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1. Introduction

Normal T-cell function is essential for destruction of diseased cells, maintenance of immunological tolerance, provision of an immune memory response and facilitation of the involvement of other white blood cells in immune responses (Bringl and Brenner, 2004; Jiang and Chess, 2004; Luo et al., 2012; Moss et al., 2004). Immunomodulators, including immunosuppressive agents, can be used to target T-cells and thereby modify both cell-mediated and humoral immune responses.

Cyclosporine is an effective immunosuppressive agent used in both human and veterinary medicine to prevent transplant rejection and treat inflammatory and immune-mediated diseases such as atopy, inflammatory bowel disease, immune-mediated hemolytic anemia, and anal furunculosis (Barten et al., 2007; Daigle, 2002; Jensen and Dalhoff, 2001; Plumb, 2008). Cyclosporine is a potent T-cell inhibitor that works by binding to intracellular cyclophilin A, resulting in a cyclosporine-cyclophilin complex that inhibits calcineurin. Calcineurin is required by the T-cell to produce nuclear factor of activated T-cells (NFAT)-regulated cytokines, such as IL-2 and IFN- γ , that help activate T-cells and stimulate lymphocyte proliferation and differentiation (Kumar et al., 2010). The presence of cyclosporine within the T-cell serves to suppress cytokine production and ultimately T-cell function (Archer et al., 2011; Fellman et al., 2011; Rao et al., 1997).

An ideal cyclosporine dosing protocol has not been established in the dog. While measurement of blood cyclosporine concentrations is the standard method of monitoring therapy, published target blood concentrations vary widely from one reference laboratory to the next. Individual dogs may even vary in immune response to the same blood cyclosporine concentration (Archer et al., 2011). Pharmacodynamic assessment of the actual biological effects of cyclosporine on T-cell function would be preferable to simple monitoring of blood drug concentrations, but a clinically validated tool to assess cyclosporine pharmacodynamics in the dog has not been established. As an important step towards development of a suitable pharmacodynamic assay, we evaluated a RT-qPCR assay of NFAT-regulated cytokine gene expression. Our objectives were to perform analytical validation of the assay using established MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines (Bustin et al., 2009; Bustin, 2010), and to determine the effects of sample storage conditions on cyclosporine-exposed samples when blood was incubated with cyclosporine at concentrations comparable to levels that are therapeutically feasible in dogs at standard dose rates.

2. Material and Methods

2.1 Blood Sample Collection and Storage Study

Two different concentrations of cyclosporine were evaluated in our study. We initially evaluated the effects of duration of storage at two different storage temperatures on assay results in samples from 10 dogs when samples were exposed to a concentration of cyclosporine that is considered to be therapeutic (500 ng/mL). In a subsequent smaller study, we evaluated the effects of storage duration at a single storage temperature on assay results

in samples from four dogs when samples were exposed to a concentration of cyclosporine that is considered to be subtherapeutic (75 ng/mL).

Ten healthy intact female Walker hounds were used. In our initial study, thirty milliliters of blood was collected from the jugular vein using a Vacutainer collection system and then fractionated in 10 aliquots of 3 mL using heparinized tubes. Five of the 3 mL samples of whole blood were then exposed to cyclosporine at a concentration of 500 ng/mL (Sigma, St. Louis, MO, USA Cat no. C1832). One sample was processed via addition of activators (PMA, ionomycin) immediately post-collection and after 1 hour of cyclosporine exposure, while additional tubes (4) were stored at room temperature or 4°C for either 24 or 48 hours prior to activation. As a control, 5 untreated (no cyclosporine addition) samples were included with one processed via addition of activators immediately post-collection, while additional tubes (4) were stored at the same time and temperature combinations as the cyclosporine treated samples.

A subsequent smaller storage study was then completed using a lower drug concentration and a single storage temperature. Twenty milliliters of blood was collected from the jugular vein from four healthy intact female Walker hounds. Blood was then fractionated into six aliquots of 3 mL using heparinized tubes. Half of the 3 mL samples of whole blood were then exposed to cyclosporine at a concentration of 75 ng/mL (Sigma, St. Louis, MO, USA Cat no. C1832). After a 1 hour incubation period, one sample was processed via addition of activators immediately, while additional tubes were stored at 4°C for either 24 or 48 hours prior to activation. As a control, an untreated sample was included at all time points.

2.2 T-cell Activation

Whole blood samples were activated using a combination of PMA (12.5 ng/mL) (Sigma, St. Louis, MO, USA Cat no. P8139-1MG) and ionomycin (0.8 µM) (Sigma, St. Louis, MO, USA Cat no. I0634-1MG). Samples were then incubated for 5 hours at 37°C with 5% CO₂.

2.3 RNA Isolation and Quality Assessment

Total RNA was isolated from 1 mL of heparinized whole blood using a QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA, USA Cat. No. 52304) according to the manufacturer's instructions and then stored at -80°C until use. An on-column DNase (27.27 Kunitz units) treatment (Qiagen, Valencia, CA, USA Cat. No. 79254) was done for all samples to remove genomic DNA. RNA was quantified using a Nanodrop ND-1000 spectrophotometer using the ND-1000 V3.3.0 software (NanoDrop Technologies, Wilmington DE, USA).

RNA quality was assessed using the Agilent RNA 6000 Bioanalyzer. Twelve samples were analyzed, and an RNA integrity number (RIN) was determined for each.

2.4 Cytokine Gene Expression Quantification

Cytokine RT-qPCR results were analyzed by comparing cyclosporine-treated samples to untreated controls across the various times and temperatures. IL-2 and IFN-γ genes and reference gene GAPDH expression were quantified via RT-qPCR using a SuperScript™ III

Platinum® SYBR® Green One-Step RT-qPCR kit with Rox used as a reference dye (Invitrogen, Grand Island, NY, USA Cat no. 11736-059). Primers for IL-2, IFN- γ , and GAPDH were based on reported GenBank nucleotide sequences as previously published by Kobayashi et al, and are shown in Table 1 (Kobayashi et al., 2007). All reactions were run on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies Corporation, NY, USA Cat no. 4351106) using 7500 software v2.0.6 for analysis. The RT-qPCR was performed in a 20 μ L final volume containing 0.75ng/ μ L of template and 200nM of each primer. Thermal cycling parameters were as follows: 50°C for 3 min, 95°C for 5 min, then 40 cycles of 95°C for 15 sec and 60°C for 30 sec followed by a melting analysis that comprises 95°C for 15 sec, 60°C for 1 min, after which the ramp speed decreases from 1.667°C/sec to 0.01667°C/sec and data is collected continuously until it reaches 95°C, temperature is then held for 30 sec and finally 60°C for 15 sec. All samples were run in triplicate while non-template controls were run in duplicate. A no reverse-transcriptase control, using GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI, USA Cat no. A6001) following the manufacturer's instructions, was also included in one run to ensure there was no contaminating genomic DNA. To calculate the relative change in gene expression for all samples, the 2^{-Ct} method was employed using GAPDH as a reference gene where $Ct = (Ct_{GOI} - Ct_{norm})_{treated} - (Ct_{GOI} - Ct_{norm})_{untreated}$ where GOI is the gene of interest and norm is the reference gene (Livak and Schmittgen, 2001). Cytokine gene expression was presented as a percentage for each time and temperature combination where the untreated control sample represented 100% gene expression for IL-2 and IFN- γ .

2.5 Validation of qRT-PCR Assay

2.5.1 In Vitro Transcription—The PCR products of the three amplified genes were purified using Wizard SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA Cat no. A9281), cloned into pGEM®-T Easy Vector (Promega Corporation, Madison, WI, USA) and propagated into chemically competent DH5 α premade Z-competent *Escherichia coli* cells (Zymo Research, Irvine, CA, USA Cat no. T3007). Purified plasmid DNAs (PureLink HiPure plasmid miniprep kit, Invitrogen, Grand Island, NY, USA Cat no. K2100-02) were sent for sequencing (Eurofins MWG Operon, Huntsville, Alabama, USA). The plasmid quantification was assessed spectrophotometrically and the number of molecules was determined on the basis of plasmid size and corresponding DNA mass. One microgram of plasmids previously linearized with restriction enzyme *SpeI* (New England BioLabs, Ipswich, MA, USA Cat no. R0133S) were used for the synthesis of the recombinant transcripts using MAXIscript T7 *in vitro* transcription kit (Invitrogen, Grand Island, NY, USA Cat no. AM1312).

2.5.2 Limit of Detection and qRT-PCR Efficiency—The limit of detection (LOD) of all 3 RT-qPCR assays was determined in triplicate using 10-fold serial dilutions of recombinant transcripts representing 10^1 to 10^6 copies of RNA per reaction.

Assay efficiency was assessed using five 10-fold serial dilutions run in triplicate of total RNA isolated from one healthy Walker hound. The slope of the resulting curve was used to calculate assay efficiency using the following equation: Efficiency = $-1 + 10^{(-1/\text{slope})}$

2.5.3 Inter-assay and Intra-assay Variation—Inter-assay variation was determined by running one sample in triplicate on nine different days. Intra-assay variation was calculated using the mean and standard deviation of C_t values for a reaction run in triplicate. This was replicated on nine different plates, all using the same RNA sample, and the coefficients of variation (CV) calculated for each run were averaged together. For all measurements, mean value, standard deviation, and CV were calculated for the threshold cycle (C_t) values.

2.6 Statistical Methods

For the storage study, the data were visually assessed for normality using the UNIVARIATE procedure in SAS for Windows 9.3 (SAS Institute, Inc., Cary, NC) for both the IL-2 and IFN- γ outcomes. Each outcome was found to be approximately normally distributed. A mixed model repeated measures analysis was conducted for each outcome using the MIXED procedure. Separate models were assessed for each storage temperature and treatment combination. Time was included in the models as a fixed effect. The repeated measures of samples taken from the same dog over time were accounted for in a repeated statement using a first order autoregressive covariance structure. A random statement with dog as the random effect was used to account for between-dog variation. Differences in least square means with Dunnett adjustment of p-values were used for comparisons of the 24 hour and 48 hour samples to the 0 hour cytokine gene expression levels if time was found to be a significant fixed effect. An alpha level of 0.05 was used to determine significance in all analyses.

3. Results and Discussion

3.1 RNA Quality

RNA quality was assessed using samples from a variety of storage time and temperature combinations. RIN values had an average of 8.26 and a range of 7.60-8.60 where a RIN value of 1 represents degraded RNA and a value of 10 represents high quality intact RNA.

3.2 Specificity and Sensitivity of the Assay

Specificity was assured by melt-curve analysis demonstrating one amplification peak and sequencing of the amplified products.

Amplified RT-qPCR products that were sent for sequencing were confirmed to be canine IL-2, IFN- γ and GAPDH sequences using the basic local alignment search tool (BLAST).

Regression lines of five 10-fold serial dilutions of total RNA (dilution vs. C_t) had slopes of -3.51 for GAPDH, -3.58 for IL-2, and -3.58 for IFN- γ (Table 2). The resulting amplification efficiencies were 92.86% for GAPDH, 90.33% for IL-2, and 90.27% for IFN- γ (Table 2). The limit of detection of the assays was determined using 10-fold serial dilutions of recombinant transcripts and was found to be 1000 copies of a single RNA transcript for IL-2, IFN- γ , and GAPDH.

3.3 Cytokine Gene Expression

Combined cytokine gene expression data for all 10 dogs in our initial therapeutic cyclosporine concentration study is shown in Figure 1. The data for all 10 animals at each time and temperature combination were pooled to determine the medians and first and third quartiles. Only one cyclosporine-treated sample was not suppressed below 50% of untreated cytokine gene expression. Cytokine gene expression data for all four dogs in our subsequent subtherapeutic cyclosporine concentration study is shown in Figure 2. Adequate RNA was isolated from all samples in our initial 10 dog study after storage for 24 hours, at both 4°C and room temperature, while at 48 hours adequate RNA was isolated from all 4°C samples, with several room temperature samples having insufficient RNA extracted for gene analysis. Adequate RNA was isolated from all samples in our subsequent four dog study at 4°C.

Statistical analysis of our storage study revealed that storage time had a significant ($p<0.0001$) overall effect in the model only for the IFN- γ assay with room temperature storage using therapeutic concentrations of cyclosporine. When pairwise comparisons were made, IFN- γ levels were not significantly different from 0 hour levels at 24 hours ($p=0.9069$) of storage at room temperature, but were significantly different from 0 hour levels at 48 hours ($p<0.0001$). No other significant differences were detected among time points at either storage temperature for either cytokine assay.

3.4 Inter-assay and Intra-assay Variation

Inter-assay variation for GAPDH, IL-2, and IFN- γ had a CV of 7.07%, 6.97%, and 4.73%, and a SD of 1.22, 2.13, and 1.49 C_t , and intra-assay variation had a CV of 0.24%, 0.33%, and 0.88%, and a SD of 0.040, 0.098, and 0.277 C_t respectively (Table 2).

Optimal cyclosporine treatment protocols have not been established for dogs, in part due to individual to individual variability in immunosuppressive effects despite comparable blood concentrations (Archer et al., 2011). A poor correlation between cyclosporine blood concentrations and clinical response has been demonstrated with both canine atopy and inflammatory bowel disease (Allenspach et al., 2006; Steffan et al., 2004). Cyclosporine dosing in veterinary cases is therefore often empirically adjusted based solely on clinical response.

Cyclosporine blood concentrations similarly do not always predict rejection in human transplant medicine, and this problem has prompted the development of pharmacodynamic assays of the drug's biological effects (Zucker et al., 1996; Caruso et al., 2001; Brunet et al., 2003; Härtel et al., 2003; Barten et al., 2007; Sommerer et al., 2008). While such assays have proven to be valuable for dosing humans, a practical veterinary counterpart has yet to be developed.

Previous work in our laboratory evaluated flow cytometry as a method to measure T-cell cytokine production in dogs in response to oral cyclosporine, and documented suppressed levels of IL-2 and IFN- γ (Archer et al., 2011). Flow cytometry requires immediate sample processing, however, and results can also be influenced by daily variations in machine setup. Quantitative RT-PCR offers an appealing alternative, as samples are potentially stable

enough to allow standard submission from external sources, and extracted RNA can also be frozen and saved for batch analysis.

In this study, we analytically validated a RT-qPCR panel of NFAT-regulated cytokine gene expression designed to measure the effect of drugs such as cyclosporine on T-cell function. Specifically, we evaluated assay repeatability, precision, and accuracy as well sample stability under different storage conditions. Our results suggest that our assay is analytically sensitive and specific, and that blood samples can be kept for up to 48 hours at 4°C, and 24 hours at 4°C or room temperature without loss of interpretable results. Storage at room temperature for 48 hours was less optimal because of a decrease in the amount of RNA that can be extracted, and because IFN- γ assay results became significantly different from results in samples that were processed immediately.

In the initial storage portion of our study, we demonstrated consistently reduced cytokine gene expression in response to an *in vitro* model where whole blood was incubated with cyclosporine at concentrations comparable to clinically relevant blood levels (Figure 1). Under a range of storage times and temperatures, gene expression following incubation with cyclosporine was suppressed below 50% of the degree of expression in untreated samples in all but one sample. The majority of samples suppressed to 25% or less, which is a similar degree of cytokine suppression seen in other studies evaluating cyclosporine at a concentration of 500 ng/mL (Kuzuya et al., 2009; Konstandin et al., 2007). We speculate the single result which suppressed by less than 50% was a laboratory error, since other samples collected from the same animal at the same time and run in parallel exhibited more pronounced suppression of gene expression. Changes in the degree of suppression of gene expression were not seen over time despite exposure to cyclosporine for up to 48 hours at 4°C, suggesting that samples from dogs receiving cyclosporine could be mailed by veterinarians under standard handling conditions without loss of assay validity, despite the presence of cyclosporine in the submitted sample.

Following our initial storage study, we conducted a smaller study using a lower concentration of cyclosporine to ensure that prolonged drug exposure over 1-2 days of storage did not cause a progressive decrease in cytokine gene expression that could be misinterpreted as evidence of therapeutic efficacy. We conducted this second study at 4°C only because, based on our initial study, we determined that 4°C was the preferred temperature for sample submission without excessive loss of extractable RNA. Compared to exposure to a cyclosporine concentration of 500 ng/mL, exposure of samples to 75 ng/mL of cyclosporine caused a less consistent reduction in cytokine gene expression. Importantly, however, sample storage did not increase the degree of cyclosporine-mediated suppression of gene expression (Figure 2).

In order to validate our assays, amplification efficiency, LOD, and inter- and intra-assay variability were determined. For PCR, amplification efficiency documents how well the target is amplified by the assay, and amplification efficiencies between 90 and 110% are generally considered acceptable (Taylor et al., 2010). Our assay efficiencies fell within this range, confirming adequate performance. The LOD assesses the sensitivity of an assay, and has a minimum theoretical limit of three copies per PCR reaction (Bustin et al., 2009). The

LOD for our assay was determined to be 1000 copies, similar to LODs reported by other groups developing similar assays (Spackman et al., 2002). Although our LOD is higher than ideal, our target genes are highly expressed, and typical samples will have sufficient cells with ample gene expression to ensure a much higher copy number. We therefore expect that the impact of a higher than optimal LOD on the clinical applicability of our assay will be minimal. LOD values lower than this have been shown to be vulnerable to the Monte-Carlo effect, which can result in inaccurate data (Karrer et al., 1995). The repeatability and reproducibility of our assay was evaluated by calculating intra-assay and inter-assay variation. For quantitative assays, a CV value greater than 20% is thought to result in a significant loss of precision (Reed et al., 2002). For our assays, CV values for intra- and inter-assay variability were less than 10%, suggesting a high level of repeatability and reproducibility.

In conclusion, our RT-qPCR assays of NFAT-regulated cytokine gene expression appear to be sensitive, efficient, precise and robust enough to permit evaluation of the effects of cyclosporine on T-cell function in dogs. Furthermore, samples analyzed using our assays provide stable results for 24-48 hours under standard handling conditions, and can therefore be routinely shipped without loss of sample viability.

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Figure 1.

Cytokine gene expression in samples exposed to 500 ng/mL cyclosporine as percentage of expression in unexposed samples. Figures presented as median, first and third quartiles (n = 10 dogs except for 48hr RT n = 5 dogs). A IL-2. B IFN- γ . * Significant difference from 0 hour results.

Figure 2. Cytokine gene expression in samples exposed to 75 ng/mL cyclosporine as percentage of expression in unexposed samples. Figures presented as median, first and third quartiles (n = 4 dogs). A IL-2. B IFN- γ .

Table 1

Sequences of primer sets used in the quantitative reverse-transcription PCR assay

Primer Set		Primer Sequence (5' - 3')	GenBank Accession Number
GAPDH	Forward	AACTCCCTCAAGATTGTCAGCAA	AB038240
	Reverse	CATGGATGACTTTGGCTAGAGGA	
IL-2	Forward	CCTCAACTCCTGCCACAATGT	U28141
	Reverse	TGCGACAAGTACAAGCGTCAGT	
IFN- γ	Forward	GCATTCCAGTTGCTGCCTACT	AF126247
	Reverse	ACCAGGCATGAGAAGAAATGCT	

Primers used published by Kobayashi et al., 2007

Table 2

Assay amplification efficiency, slopes of regression lines, and inter-assay and intra-assay variation for reference gene and cytokine genes

Gene	Efficiency (%)	Amplification Factor	Slope	Inter-assay Variation		Intra-assay Variation	
				CV (%)	SD (C _t)	CV (%)	SD (C _t)
GAPDH	92.86	1.93	-3.51	7.07	1.22	0.24	0.015 - 0.091
IL-2	90.33	1.90	-3.58	6.97	2.13	0.33	0.037 - 0.244
IFN-γ	90.27	1.90	-3.58	4.73	1.49	0.88	0.039 - 0.382

Assay efficiency for reference gene and cytokine genes determined using five tenfold dilutions (10^1 - 10^6) of total RNA isolated from a healthy intact female Walker hound

Inter-assay variation determined by running one sample in triplicate on nine different days. CV was calculated using the mean and standard deviation of C_t values for one sample run on nine different days.

Intra-assay variation was calculated using the mean and standard deviation of C_t values for a reaction run in triplicate. This was replicated on nine different plates all using the same RNA sample, and the CVs calculated for each run were averaged together.

CV, coefficient of variation SD, standard deviation