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NOTA TÉCNICA

Quantification of canine cytokines using real time reverse transcriptase polymerase chain reaction

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Introduction. Canines are the principal domestic reservoirs of visceral leishmaniasis in both the Old and New World. The development of highly sensitive and quantitative methods, such as real time reverse transcriptase polymerase chain reaction for measurement of canine cytokines has not been exploited in studies of visceral leishmaniasis.

Objective. To standardize the relative quantification of canine IFN-γ, IL-4, IL-10, IL-12p40 and IL-12p35 using real time reverse transcriptase polymerase chain reaction.

Materials and methods. RNA was isolated from PBMCs from 1 year–old foxhounds and cultured with or without Con A, LPS or *Staphylococcus aureus* extract. This RNA was used in one-step real time reverse transcriptase polymerase chain reaction to optimize the concentrations of the cytokine primers and probes, generate standard curves for each cytokine, confirm equivalent amplification efficiency of cytokine and normalizer (18S rRNA) RNA, and to quantify the expression of the cytokine RNA. The comparative Ct method was used to determine the relative levels of gene expression in the samples, expressed as the fold-increase relative to the control samples.

Results. The regression coefficient for the standard curves and the amplification efficiencies of the cytokine and normalizer RNA indicated that the quantification was reliable over a broad concentration range of input RNA. Relative to control cells, activation of PBMCs led to increased expression of IFN-γ (132-fold), IL-4 (8.8-fold), IL-10 (7.2-fold), and IL-12p40 (275-fold). Basal expression of IL-12p35 was also detected.

Conclusion. This approach provides several advantages over conventional assays for cytokine measurement and can be exploited in the study of the immunopathogenesis and immunity in canine leishmaniasis.

Key words: dogs, cytokines, reverse transcriptase polymerase chain reaction, visceral leishmaniasis, T-Lymphocytes**.**

Cuantificación de citocinas caninas mediante reacción en cadena de la polimerasa de transcriptasa reversa en tiempo real

Introducción. Los caninos son el principal reservorio domestico de la leishmaniasis visceral en el Nuevo y Viejo mundo. La reacción en cadena de la polimerasa de transcriptasa reversa en tiempo real para la medición de citocinas caninas no ha sido implementada para el estudio de la leishmaniasis visceral.

Objetivo. Estandarizar la cuantificación relativa de IFN-γ, IL-4, IL-10, IL-12p40 y IL-12p35 caninas utilizando reacción en cadena de la polimerasa de transcriptasa reversa en tiempo real.

Materiales y métodos. Células mononucleares de sangre periférica de perros Fox-Hound fueron estimuladas con ConA, LPS y extracto de *Staphylococcus aureus.* El ARN fue utilizado en la reacción en cadena de la polimerasa de transcriptasa reversa en tiempo real de un solo paso para optimizar las concentraciones de iniciadores y sondas especificas de cada citocina, generar curvas estándar, confirmar la eficiencia de amplificación de las citocinas y del normalizador (18S ARNr) y cuantificar la expresión de ARN. El método comparativo Ct fue

utilizado para determinar los niveles relativos de expresión de ARN en las muestras, expresado como el incremento en el número de veces comparado con los controles.

Resultados. El coeficiente de regresión para las curvas estándar y las eficiencias de amplificación de las citocinas y el normalizador, indicaron que la cuantificación fue confiable en un amplio rango de concentraciones de ARN. La activación de células mononucleares de sangre periférica resultó en un incremento en la expresión de IFN-γ (132), IL-4 (8.8), IL-10 (7,2), y IL-12p40 (275), relativo a células control. La expresión basal de IL-12p35 fue también detectada.

Conclusión. Esta metodología, comparada con los métodos convencionales disponibles para la medición de citocinas, ofrece varias ventajas y podría ser utilizada en estudios sobre inmunopatogenia e inmunidad en leishmaniasis visceral canina.

Palabras clave: perros, citocinas, reacción en cadena de la polimerasa de transcriptasa reversa, leishmaniasis visceral, linfocitos T.

Canines are the principal domestic reservoirs of visceral leishmaniasis in both the Old and New World, and for this reason major efforts to control *Leishmania infantum* (=*L. chagasi*) transmission have targeted the dog. Because dog culling has shown conflicting results with regard to *L. infantum* control, a more recent approach to make canines less efficient reservoirs is to develop vaccines that could significantly decrease the infectivity for sand fly vectors (1). Although there is substantial information on both humoral and cell-mediated immunity of dogs infected with *L. infantum*, new molecular methodologies such as real time reverse transcriptase polymerase chain reaction (RT-PCR) have not been exploited in studies of visceral leishmaniasis. Therefore, the development of sensitive and reliable molecular tools for assessing the immune response of dogs has become of particular interest.

It is well established that cytokines play a pivotal role in the development and maintenance of cellular immunity against the majority of infectious agents. The quantification of cytokine expression in leishmaniasis is especially important since it is a critical determinant of susceptibility and resistance to disease (2) and vaccine-induced immunity (3,4). Recently, semi-quantitative RT-PCR has been used to determine expression of cytokines in normal dogs and during disease evolution in dogs naturally or experimentally infected with *L. infantum* (5-10). RT-PCR was also employed to determine the cytokine profile of asymptomatic dogs experimentally infected with *L. infantum* (11). Collectively, these results indicated that during active disease there is mixed Th1/Th2 cytokine expression and that asymptomatic dogs preferentially expressed Th1-type cytokines, which was associated with parasite containment and no progression towards overt disease.

Because the samples available to be analyzed are often too small for evaluating cytokines at the protein level, the detection of mRNA by RT-PCR is widely used to investigate the cytokine profiles in different tissues. However, this technique requires intensive manipulation (the multiple steps of reverse transcription, PCR, gel electrophoresis and detection, and semi-quantitiative densitometry) to obtain only a semi-quantitative level of analysis. Recently, real time RT-PCR has been shown to be a more accurate and sensitive method for quantifying messenger RNA.

This new approach exploits the 5' nuclease activity of DNA polymerase (12), to cleave a TaqMan probe that is labeled with a reporter fluorescent dye [FAM (6-carboxy-fluorescein)] covalently linked to the 5' end, and a quencher fluorescent dye [TAMRA (6-carboxi-tetramethyl-rodamine)] linked at the 3' end.

When the probe is intact, the reporter dye emission is quenched owing to physical proximity of the reporter and quencher fluorescent dyes, primarily by Foster-type energy transfer (13). During

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the extension phase of the PCR cycle, however, the nucleolytic activity of the DNA polymerase cleaves the hybridization probe and releases the reporter dye from the probe, resulting in increased fluorescence of the reporter only if the probe hybridizes to the target. The probe fragments are then displaced from the target, and polymerization of the strand continues. The fluorescent dye emission is monitored in real time during PCR amplification using a detector.

A computer algorithm compares the amount of reporter dye emission (R) with the quenching dye emission (Q) every 8.5 seconds during the PCR amplification, generating a DRn value (R/Q) which reflects the amount of hybridization probe that has been degraded. The algorithm calculates the cycle at which each PCR amplification reaches a significant threshold (Ct). This Ct value is proportional to the number of target copies present in the sample. Thus, the Ct value is a quantitative measurement of the copies of the target found in any sample (14). The relative expression analysis based on real time analytical reverse transcription (RT-PCR) offers several advantages over other current quantitative methods. It has proved to be rapid, reliable, and highly sensitive method to quantify the simultaneous expression levels of a large number of genes.

Real time RT-PCR has been successfully applied to the quantification of several cytokines in canine intestinal mucosa (15). Here we describe in detail the standardization of real time RT-PCR for quantification of the expression levels of IFN-gamma, IL-4, IL-10, IL-12-p40, IL-12-p35. We validated the methodology by quantifying the cytokine expression in activated canine peripheral blood mononuclear cells (PBMC).

Materials and methods

Dogs

8-12 month-old outbred Foxhound dogs were obtained from Marshall Farms USA, Inc. (North Rose, NY) and maintained in the ALAAC-approved animal facility at UTHSCSA. Animals were handled according to local and federal regulations and research protocols were approved by the UTHSCSA Institutional Animal Care and Use Committee. Dogs were screened and treated for intestinal helminth infection prior to vaccination. Except where noted, there were 4 dogs per experimental group.

Cell isolation and culture for cytokine analysis

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood using standard NycoPrep 1.077/265 animal gradient centrifugation (Accurate Chemical Westbury, NY). In order to determine cytokine expression, several different stimuli known to activate PBMCs were employed.

These cells were cultured at a density of $4x10^6$ cells/mL in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU of penicillin/mL, 100 µg of streptomycin/mL and 10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO). Assays were conducted in 2 mL round-bottomed polypropylene tubes incubated at 37°C, 5% CO2 at different time points. PBMCs were stimulated with either concanavalin A (ConA; Sigma, St. Louis, MO) (2.5, 1.25. 0.625 .0.3125 and 0.1562 µg /mL), lipopolysaccharide (LPS, *E. coli* 0111B4; Sigma, St. Louis, MO) (100 ng/mL) or 0.05% *Staphylococcus aureus* extract (Pansorbin; Calbiochem, San Diego, CA). After each time point the cells were washed once with cold PBS and 180 µL of RNAlater (Ambion Inc. Austin, TX) were added to cellular pellet for RNA preservation. The cells were stored at 4°C until the RNA extraction was carried out.

In one case (standardization of IL-12p35 PCR) the DH82 canine macrophage cell line (obtained from ATCC; No. CRL-10389) was used. The cells were grown to confluence in complete medium (Minimum Essential Medium Eagle with 15% heat inactivated fetal bovine serum, 1% Hepes buffer, 2 mM L-glutamine, 1 mM Sodium pyruvate, 1500 mg sodium bicarbonate/L, 100 U/ml of penicillin, and 100 mg/ml of streptomycin) and harvested with 0.25 mg/ml trypsin in PBS, washed, and used for RNA isolation.

RNA isolation

Ten volumes of RNA lysis buffer (RLT) (guanidinium based) were used to dilute the samples (in RNAlater) and the RNA was recovered according to the manufacture's protocol (QIAGEN

Inc. Valencia, CA). Finally, total RNA was eluted from the columns in 50 µl of nuclease-free water. To make the DNA more susceptible to DNase digestion the RNA was heated 3 minutes at 95°C then quenched on ice and treated with 5 ml (50 units) of RNase-free DNase and 50 µL of DNAse digestion buffer (Stratagene, Cedar Creek, TX). After 1 hour incubation at 37°C, the RNA was extracted once again and eluted in a final volume of 35 µL of nuclease free water and stored at –70°C until used.

Primers and probes

Except for the endogenous control, ribosomal RNA control reagents (VIC Probe, Applied Biosystems, Foster City, CA), all primers and probes used in this study (sequences listed in table 1) were synthesized at Midland (Texas), and were designed according with Primer Express software (Applied Biosystems, Foster City, CA). In all cases except IFN-g the primers and/or probe were located on different exons. The internal probe was labeled at the 5' end with the reporter dye FAM (6 carboxyfluorescein), and the 3' end with the quencher dye TAMRA (6-carboxy-tetra-methil-rhodamine).

The length of the PCR products (table 1) varied between 67-75 basepairs. Each primer pair was optimized at different temperatures (55 and 60°C) and 3 different concentrations (100, 300, 600 nM), using a fixed amount of target template. The optimal performance was achieved by selecting the primer concentrations that provided the lowest cycle threshold (Ct) and highest magnitude of the signal generated by the PCR (DRn). Similarly, the probe concentration was optimized by 50-200 nM.

Standard curves and amplification efficiencies

Using one step RT-PCR Taq Man reagents (Applied Biosystems), two fold serial RNA dilutions were employed to produce a standard curve and calculate the reaction efficiency for the target genes and the normalizer 18S rRNA. Purified RNA from canine PMBCs stimulated with ConA (2.5 gg/ml) was used for determining the standard curve for IFN-γ, IL-4, and IL-12 p40/35, while LPS was used for IL-10. A graph of threshold cycle (Ct) versus log ng total RNA from the dilution series

Table 1. Oligonucleotides for RT-PCR.

Target	Oligo [1]	Priming site (cDNA) [2]	Oligonucleotide sequence (5' → 3')	Product size	Primer/ probes[3]
	Fw	(553 → 573)	AGGAAGCGGAAAAGGAGTCAG		100nM
IFN-γ	Rv	(599 ← 619)	GGCAGGATGACCATTATTTCG	67 bp	100nM
	Probe	(576 ← 597)	TGCTCTGCGGCCTCGAAACAGA		100nM
IL-12	Fw	(675 → 693)	CACCAGCAGCTTCTTCATCAGA		300nM
p40	Rv	(722 ←741)	CAATGGCTTCAGCTGCAGGT	67 bp	300nM
	Probe	(695 → 720)	ACATCATCAAACCAGACCCACCCACA		100nM
IL-12	Fw	(337 → 354)	TGCCTGGCCTCTGGAAAG		600nM
p35	Rv	(392 ← 410)	TACATCTTCAAGTCCTCAT	74 bp	600nM
	Probe	(363 → 390)	TATGACGGTCCTGTGCCTTAGCAGCATC		100nM
	Fw	(185 → 205)	TCCTCACAGCGAGAAACGACT		300nM
IL-4	Rv	(240 ← 259)	CGCTTGTGTTCTTTGGAGCA	75 bp	300nM
	Probe	(208 ← 233)	ACGTCCTTGACAGTCAGCTCCATGCA		100nM
	Fw	(361→ 378)	CAGGCTGAGACTGAGGCT		300nM
IL-10	Rv	(409 ← 428)	CCACCGCCTTGCTCTTATTC	68 bp	300nm
	Probe	(382 → 407)	ACGCTGTCACCGATTTCTTCCCTGTG		100nM

[1] Fw: forward primer; Rv: reverse primer; probe labeled with 6 FAM at the 5'-end and quenched with TAMRA at the 3'-end.

[2] The position of the primers relative to the published canine cDNA sequences (accession numbers: AF126247 [IFN-g]; U49100 [IL-12p40]; U49085 [IL-12p35]; AF187322 [IL-4]; U33843 [IL-10]).

[3] Final concentration of primers and probes after titration.

was produced. The difference between the cytokine target gene and the normalizer (18S rRNA) or the delta Ct value was plotted against the log of the ng total RNA concentration to estimate the amplification efficiency. For the amplification of the target and housekeeping genes to have equivalent efficiency the plot of the delta Ct and RNA concentration should have a low regression coefficient (<0.1).

One tube RT-PCR

Each sample was assayed for cytokine mRNA expression and for 18S rRNA as an endogenous control (normalizer) in the same tube in 25 µl RT-PCR mixture. Samples were assayed in triplicate tubes (25 µl each) obtained from a 100 ml reaction mixture that contained a specific concentration of target and normalizer primers (from 100 nM to 600 nM) and probe (100 nM), 50 ml of 2X master mix, 2.5 mL of 40X multiScribe (both in TaqMan one-step RT-PCR master mix reagents, Applied Biosystems) and 10 ng of template.

The samples were placed in Micro Amp optical tubes, (Applied Biosystems) and amplified in an automated fluorometer (ABI Prism 7700 sequence detection system; Applied Biosystems). Amplification conditions were 48°C/30 min (reverse transcription), 95°C/10 min (ampliTaq gold activation), 95°C/15 sec (PCR, denature) and 60°C/1 min (PCR anneal/ extend). The spectral compensation for the post run analysis was made to improve dye spectral resolution. The results were expressed in relative mRNA quantities of any given cytokine to the reference samples (unstimulated PBMCs) using the comparative (DDC_T) method (16).

Relative mRNA expression data calculation

The comparative Ct method (ΔΔCt) for relative quantification of gene expression. This method enables relative quantitation of template and increases sample throughput by eliminating the need for standard curves. The values are expressed relative to a reference sample (called the calibrator, unstimulated PBMC sample). For this method to be successful, the dynamic range of both the target and the reference should be similar. A sensitive method to control this is to look at how ΔCt (the difference between the two CT values of two PCR for the same initial template amount) varies with template dilution.

If the efficiencies of the two amplicons are approximately equal, the plot of log input amount versus DCt will have a nearly horizontal line (a slope of <0.10) and this means that both PCR perform equally efficiently across the range of initial template amount. If the plot shows unequal efficiency, the standard curve method should be used for quantitation of gene expression. It is expected that the normalizer will have a higher expression level that the target (thus, a smaller Ct value).

In this way the Ct for the target and the Ct for the internal control were determined for each sample. Differences in the Ct for the target and the Ct for the internal control, called ΔCt were calculated to normalize for the differences in the amount of total nucleic acid added to each reaction and the efficacy of the RT step. The ΔCt for each experimental sample was substracted from the ΔCt of the calibrator, this difference is called the ΔΔCt value. If the calibrator is representing the minimum level of expression, the ΔΔCt values are expected to be negative. The last step in quantification is to transform these values to absolute values, as follow; 2-(ΔΔCt). Thus, all the experimental samples are expressed as an n-fold difference relative to the calibrator (17).

Results

Determination of optimal primer concentration and annealing temperature

The optimal performance of the real time RT-PCR assay was achieved by selecting the primer concentrations that provided the lowest CT and highest ΔRn for each cytokine (table 1). We found that the optimal (17) and most cost-efective primer/probe concentrations were 100 nM/100 nM for IFN-g, as this concentration gave the greatest signal and breadth of experimental linear amplification and equivalent Ct value (figure 1A). Using the same approach, the optimal primer/probe concentrations were determined for IL-4, IL-10, IL-12p40 and IL-12 p35 (table 1 and data not shown). All the cytokines amplified properly at a 60°C of

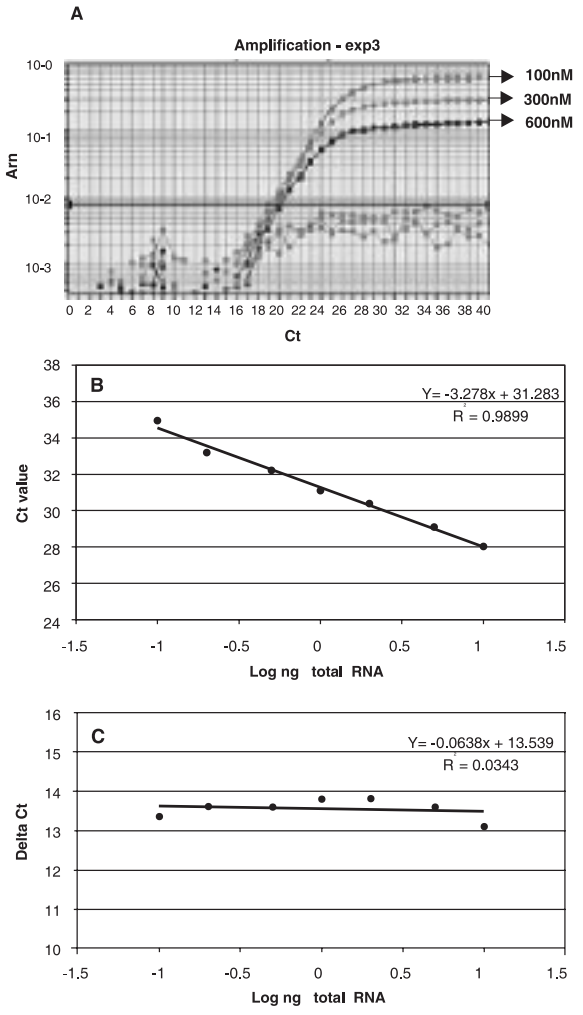

Figure 1. Standard curve and amplification efficiency for canine IFN-γ real time RT-PCR. (A) identification of optimal primer concentrations. Real time RT-PCR was set up with 3 different concentrations of primers: 100, 300 and 600 nM. Shown is the real time amplification tracing with the 100 nM concentrations showing the greatest fluorescence intensity (**Δ**Rn). (B) IFN-γ standard curve. Two-fold serial dilutions of RNA from PBMCs from different animals stimulated with ConA for 48 hr were amplified. The average Ct values of triplicate samples are plotted against the log of the ng total RNA and the slope of the line with regression coefficient is shown. The standard deviations were so narrow they are not included in the graph. (C) Determination of amplification efficiency. The comparative Ct method for relative quantification requires that the efficiency of target (IFN-gamma) and normalizer (18S rRNA) amplified in the same tube are approximately equal (the absolute value of the slope should be approximately 0.1). Standard curves of IFN-γ and 18S rRNA were used to determine the amplification efficiency. Shown is the plot of log input amount of RNA versus the delta Ct and the absolute value of the slope was 0.034.

annealing temperature, except for IL-12 p35, which amplified at 55°C.

Standard curves and amplification efficiencies

The regression coefficient (R^2) for the standard curves of the different cytokines was determined by plotting the Ct data against the log of total RNA. We found a high R^2 value for all of the cytokines, i.e., IFN-g: 0.989 (figure 1B); IL-4: 0.979, IL-10: 0.989, IL-12–p40: 0.941, IL12-p35: 0.977 (curves not shown), indicating that there was a linear amplification over a broad range of concentrations. The efficiency (Eff) of the reaction can be calculated by the formula:

$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1$$

The efficiency of the PCR should be 90 - 100% (– -3.6 > slope > – -3.1). A number of variables can affect the efficiency of the PCR, these factors include length of the amplicon, secondary structure and primer quality. The amplification efficiencies for each of the cytokines were acceptable with each of the regression coefficients being approximately 0.1: IFN-γ: 0.03 (figure 1C); IL-4: 0.03; IL 10: 0.18; IL-12–p40: 0.04; IL12-p35: 0.11 (curves not shown). This indicates that over a broad concentration range of input RNA the rate of amplification of normalizer and cytokine template remains the same.

Relative quantification of mRNA

Once the real time RT-PCR was standardized it was validated by determining the relative expression of cytokines in resting and activated canine PBMCs. Total RNA from the PBMCs was subjected to single tube RT-PCR using the cytokine and the normalizer (18S) primers. To detect the possibility of genomic DNA contamination in the samples, a control tube without reverse transcriptase was included. No amplification for cytokine cDNAs was observed in the absence of reverse transcriptase.

The relative IFN-γ mRNA expression was determined after stimulation of PBMCs with different concentrations of ConA and for different times (0, 6, 12, 24, and 48 h). The greatest expression of this cytokine was found 6h after stimulation (figure 2A) and at a concentration of 2.5 µg/mL of ConA (figure 2B). Since in lymphoproliferative assays

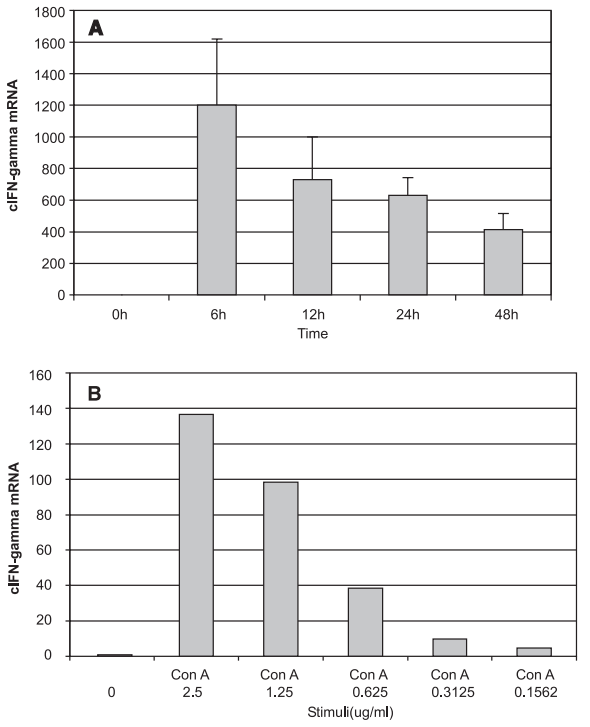

Figure 2. Expression of canine IFN-γ mRNA in stimulated PBMCs: canine PBMCs were cultured at 37°C in 5% CO_2 with ConA for different times and at various concentrations. (A) Time course of IFN-g expression. PBMCs were stimulated with 2.5 µg/mL Con A for 6, 12, 24 and 48 h. After each time point the cells were collected for RNA isolation and the level of expression of IFN-γ mRNA was determined by real time PCR. (B) Dose response of ConA-induced IFNg expression. PBMCs were stimulated with ConA at 2.5, 1.25, 0.625, 0.3125 and 0.1562 µg/ml for 48 h. The data are shown as the fold-increase normalized IFN-g mRNA in stimulated compared to unstimulated PBMCs.

using ^{3}H incorporation, at least 48h are needed to see a significant cell proliferation, we stimulated PBMCs from 4 different canine donors with 2.5 µg/mL ConA, and evaluated the samples at that time point. The expression level of IFN-γ was significantly greater than unstimulated PBMCs (figure 3A), but this was lower compared to the peak expression at 6h (see figure 2A). The stimuli also upregulated the mRNA expression of IL-4 (8.8 fold) when compared with unstimulated PBMCs (figure 3B).

To quantify canine IL-10 expression, PBMCs were cultured in medium alone or stimulated with LPS (100 ng/ml) for 3, 6, 12, 24 and 48 h. LPS induced a clear but transient expression of IL-10 mRNA in purified PBMCs, with a peak (7.2 fold) at 6h after stimulation compared with unstimulated PBMCs (figure 3C). To quantify canine IL-12 expression we cultured PBMCs and stimulated them with a suboptimal concentration of ConA (1.25 µg/mL), SAC (0.05%), or both for 3, 6, or 12 h. Under these conditions IL-12p40 peaked at 6 h after stimulation, reaching higher expression levels (275 fold) than those seen in cells stimulated with ConA alone (figure 3D). The constitutive basal expression of IL-12p35 was detected in the canine DH82- macrophage like cell line (data not shown).

Discussion

We have designed and tested primers and probes suitable for real-time RT-PCR quantification of canine cytokines that are potentially involved in protection (IL-12p35, IL-12p40, IFN-γ) or susceptibility (IL-4, IL-10) to *L. infantum*.

The standardization method we used has been described here in detail with the aim of making these protocols available to other researchers who could either use these validated real-time PCR primers and probes or design additional primers for the study of dog cytokines. Primer concentrations were identified that gave optimal signal to noise ratios and broad dynamic amplifications ranges.

Different approaches to quantify the amount of template can be performed (17). We chose the comparative Ct method because it does not use known amounts of standard but compares the relative amount of the target sequence to any of the reference values chosen and the result is given as relative to the reference value (in our case the expression level of resting PBMC). Additionally, it is not necessary to have cDNA plasmids for the target gene, which are required standards for absolute quantification.

Our validation experiment showed that efficiencies of target and reference were approximately equal, which is important for the Ct calculation to be valid. This enables relative quantification of template without having to set up a standard curve for each run, and therefore increases sample throughput. We successfully used two different

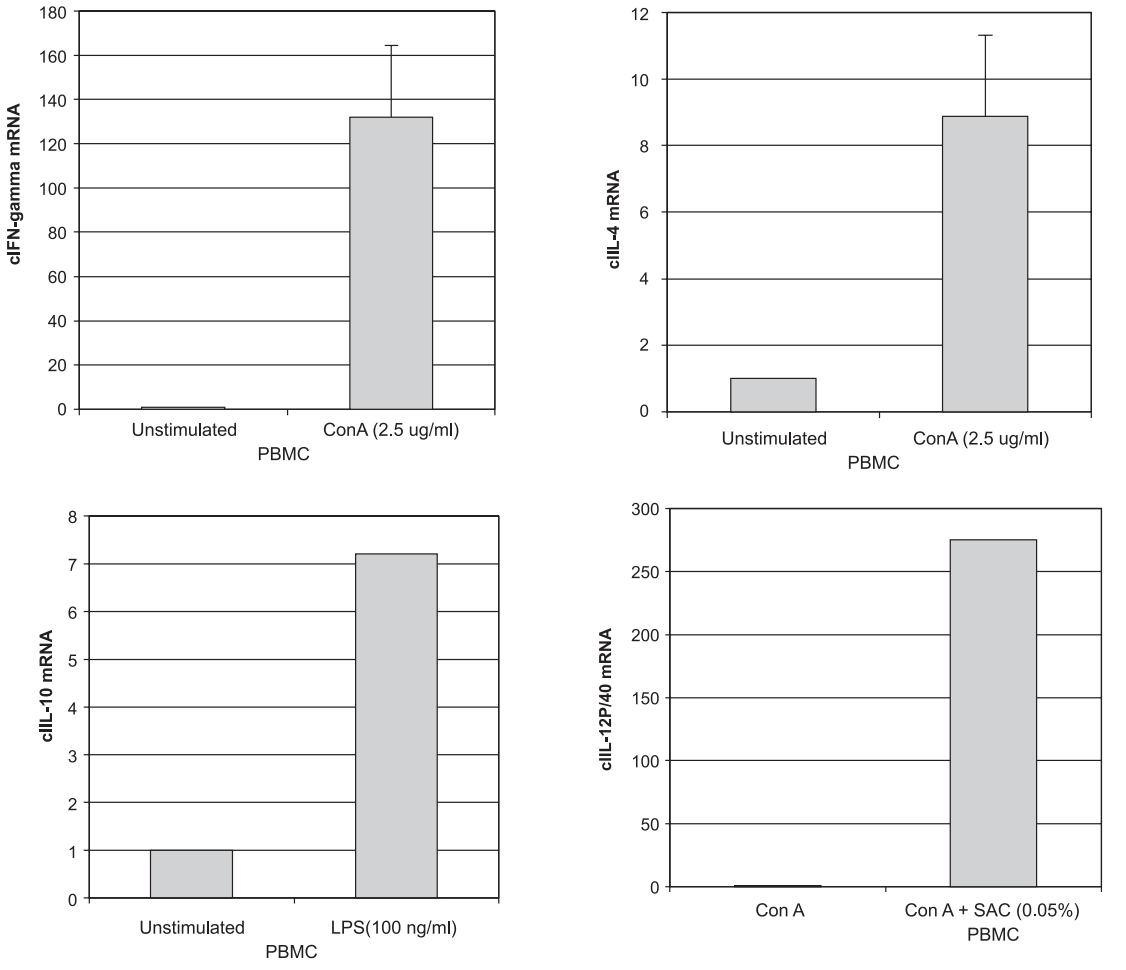

Figure 3. Expression of cytokines in activated canine PBMCs. (A) IFN-γ expression at 48 hrs after stimulation with 2.5 µg/ml ConA. (B) IL-4 expression at 48 hrs after stimulation with 2.5 µg/ml ConA. (C) IL-10 expression at 6 hrs after stimulation with 100 ng/ml LPS. (D) IL-12p40 expression at 6 hr after stimulation with suboptimal ConA (1.25 µg/ml) and *S. aureus* Cowan extract (0.05%). The data are shown as the fold-increase (normalized to 18s) of mRNA in stimulated compared to unstimulated PBMCs (IFN-γ, IL-4, IL-10) or maximally stimulated (ConA + SAC) compared to suboptimally stimulated (ConA) in the case of IL-12p40.

dyes with distinct emission wavelengths (FAM-for the cytokines and VIC for the 18S rRNA) in the same tube after spectral compensation for the post run analysis was applied.

The cytokines presented here are well known to be transcriptionally regulated so measurement of mRNA expression is a valid approach. Furthermore, antibody reagents are not readily available for most of the canine cytokines, so examination of mRNA is often the only approach that can be used.

There is a wide range of methods that could be used to quantify mRNA expression, such as Northern blotting, *in situ* hybridization, RNase protection assays, cDNA arrays, and RT-PCR. RT-PCR has the ability to quantify cytokine mRNAs that have low expression levels. Real time RT-PCR is becoming widely used in the research and clinical fields (18). Real-time RT-PCR is advantageous over standard RT-PCR (17), because: 1) there is a requirement of 1,000-fold less RNA than conventional assays; 2) there is a broad dynamic range of detection; 3) there is no-post PCR

processing required such as electrophoretical separation of amplified DNA; 4) it provides a constant record amplification of an amplicon, and 5) as opposed to traditional PCR, which is measured at end-point (plateau), real-time PCR collects data in the exponential phase (17).

The real-time RT-PCR has been recently applied to determine the local cytokine response in skin biopsies from *Leishmania* infected dogs. Although both Th1 and Th2 cytokine subsets were found to be part in the local immune response, the expression of Th2 cytokines like IL-4 were associated with severe clinical signs and high parasite burden in the skin lesions (19). The capacity to determine expression of cytokines that are considered to be markers of Th1 or Th2 responses is essential for studying the pathogenesis of visceral leishmaniasis in the dog model, and more importantly, for evaluating candidate vaccines. In separate immunogenicity studies related to the testing of anti-*Leishmania* DNA vaccines we have used the primers and methods described herein to assess the relative expression of cytokines such as IL-4 and IFN-γ following vaccination (20). In this study we observed that intramuscular vaccination induced a strong *in vitro* antigen specific IFN-γ but not IL-4 response. Similarly, we were able to perform relative quantification of IL-12p35/p40 in the canine DH-82 macrophage cell line transfected with a canine IL-12 heterodimeric cDNA adjuvant construct (21).

Although real-time RT-PCR for cytokine detection could be used in a variety of canine infectious and non-infectious diseases (15,22), in the particular case of visceral leishmaniasis, the implementation of this method will complement and improve other methods aimed at characterizing the immune response during asymptomatic infection, disease evolution, and following vaccination.

Conflict of interests

The authors of the present article declare that there are no conflicts of interest that may have influenced the results of this work.

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References

1. **Reithinger R, Coleman PG, Alexander B, Vieira EP, Assis G, Davies CR.** Are insecticide-impregnated dog collars a feasible alternative to dog culling as a strategy for controlling canine visceral leishmaniasis in Brazil? Int J Parasitol 2004;34:55-62.
2. **Solbach W, Laskay T.** The host response to *Leishmania* infection. Adv Immunol 2000;74:275-317.
3. **Handman E.** Leishmaniasis: current status of vaccine development. Clin Microbiol Rev 2001;14:229-43.
4. **Melby PC.** Recent developments in leishmaniasis. Curr Opin Infect Dis 2002;15:485-90.
5. **Grone A, Frisk AL, Baumgartner W.** Cytokine mRNA expression in whole blood samples from dogs with natural canine distemper virus infection. Vet Immunol Immunopathol 1998;65:11-27.
6. **Frisk AL, Baumgartner W, Grone A.** Dominating interleukin-10 mRNA expression induction in cerebrospinal fluid cells of dogs with natural canine distemper virus induced demyelinating and non-demyelinating CNS lesions. J Neuroimmunol 1999;97:102-09.
7. **Pinelli E, Van der Kaij SY, Slappendel R, Fragio C, Ruitemberg EJ, Bernardina W *et al*.** Detection of canine cytokine gene expression by reverse transcription- polymerase chain reaction. Vet Immunol Immunopathol 1999;69:121-6.
8. **Pinelli E, van der Kaaij SY, Broeren CP, Ruitenberg EJ, Rutten VP.** Measurement of dog cytokines by reverse transcription-quantitative competitive polymerase chain reaction. Immunogenetics 1999;49:696-9.
9. **Quinnell RJ, Courtenay O, Shaw MA, Day MJ, Garcez LM, Dye Ch *et al*.** Tissue cytokine responses in canine visceral leishmaniasis. J Infect Dis 2001; 183:1421-4.
10. **Santos-Gomes GM, Rosa R, Leandro C, Cortes S, Romao P, Silveira H.** Cytokine expression during the outcome of canine experimental infection by *Leishmania infantum*. Vet Immunol Immunopathol 2002;88:21-30.
11. **Chamizo C, Rubio JM, Moreno J, Alvar J.** Semi-quantitative analysis of multiple cytokines in canine peripheral blood mononuclear cells by a single tube RT-PCR. Vet Immunol Immunopathol 2001;83:191-202.
12. **Holland PM, Abramson RD, Watson R, Gelfand DH.** Detection of specific polumerase chain reaction product by utilizing the 5'®3' exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci 1991;88:7276-80.
13. **Foster VT.** Zwischenmolekulare energiewanderung und fluoreszenz. Ann Phys 1948;2:55-75.

14. **Gibson U, Heid CA, Williams PM.** A novel method for real time quantitative RT-PCR. Genome Res1996;6:995-1001.

15. **Peters IR, Helps CR, Calvert EL, Hall EJ, Day MJ.** Cytokine mRNA quantification in histologically normal canine duodenal mucosa by real-time RT-PCR. Vet Immunol Immunopathol 2005;103:101-11.

16. **Pfaffl MW**. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;1:29:e45.

17. **Dorak MT**. Real time PCR 2005. [Consulted: 7-15-05] Available in: dorakmt.tripod.com/genetics/realtime.html.

18. **Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C.** An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods 2001; 25:386-401.

19. **Brachelente C, Muller N, Doherr MG, Sattler U, Welle M.** Cutaneous leishmaniasis in naturally infected dogs is associated with a T-helper biased immune response. Vet Pathol 2005;42:166-75.

20. **Saldarriaga OA, Travi BL, Park W, Perez LE, Melby PC.** Immunogenicity of a multicomponent DNA vaccine against visceral leishmaniasis in dogs. Vaccine 2006;24:1928-40.

21. **Saldarriaga OA, Perez LE, Travi BL, Melby PC.** Selective enhancement of the type 1 cytokine response by expression of a canine interleukin (IL)-12 fused heterodimeric DNA. Vet Immunol Immunopathol 2006;110:377-88.

22. **Aihara Y, Kasuya H, Onda H, Hori T, Takeda J.** Quantitative analysis of gene expressions related to inflammation in canine spastic artery after subarachnoid hemorrhage. Stroke 2001;32:212-7.