Genetics and Molecular Biology, 30, 3, 575-579 (2007)

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Short Communication

Quantification of bovine cytokine gene expression using real-time RT-PCR methodology

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Abstract

T cells produce cytokines that affect host response to infection. This paper reports real-time RT-PCR conditions and validation steps for accurate quantification of *Bos indicus* cytokines, interleukin (IL)-2, IL-4, IL-8, IL12p-35, IL-13, tumoral necrosis factor (TNF)-α, interferon (IFN)-γ, monocyte chemoattractant proteins (MCP)-1 and MCP-2, and the glycoprotein mucin (MUC)-1 in two groups of Nelore cattle, one resistant and the other susceptible to gastrointestinal nematode infections. RPL-19 was shown to be an ideal internal control gene, since its expression was constant across treatments and presented lower variation when compared to the GAPDH gene. The optimized conditions established in the present study can be used to determine the immune response of cattle under different experimental conditions, such as viral, bacterial and parasite infections.

Key words: cytokine gene expression, real-time RT-PCR, cattle, gastrointestinal nematodes, bovine cytokines.

Received: June 27, 2006; Accepted: January 23, 2007.

The vertebrate immune system has evolved to defend the host against several kinds of pathogens, including gastrointestinal nematodes (Finkelman *et al.*, 2004). The T cells, conductors of this response, can be divided into T_H1 and T_H2, and their development is influenced by several cytokines, among them interleukin (IL)-4 and interferon (IFN)-γ. IL-4 promotes T_H2 cell expansion and limits T_H1 cell proliferation. In contrast, IFN-γ induces T_H1 and inhibits T_H2 response (Liew, 2002). T_H1 response has been associated with host susceptibility to nematode infection, while T_H2 response permits the host to build up a protective reaction, facilitating the expulsion of parasites, and has been observed in resistant animals (Finkelman *et al.*, 2004). Consequently, the study of cytokine gene expression is important for understanding the immune response.

Real-time RT - PCR is a highly sensitive method that allows quantification of rare transcripts and small changes in gene expression from a limited amount of sample (Leutenegger *et al.*, 1999; Konnai *et al.*, 2003). However, reliable gene expression quantification using the RT-PCR assay requires specific primers for the target gene and correction for the efficiency of DNA amplification. A control gene that is not affected by the experimental treatment must be used to correct for variations due to differences in RNA quantity, as well as efficiency of reverse transcription and cDNA amplification (Giulietti *et al.*, 2001).

Real-time RT-PCR has been used to measure cytokine gene expression in mammals; however, there are few reports in cattle, and these are limited to *Bos taurus* (Konnai *et al.*, 2003; Gómez-Muñoz *et al.*, 2004; Claerebout *et al.*, 2005). In the present study, we determined optimal real-time RT-PCR conditions to quantify *Bos indicus* cytokines, interleukin (IL)-2, IL-4, IL-8, IL12p-35, IL-13, tumoral necrosis factor (TNF)-α, interferon (IFN)-γ, monocyte chemoattractant protein (MCP)-1 and MCP-2, and the glycoprotein mucin (MUC)-1, in two groups of Nelore cattle, one resistant and the other susceptible to gastrointestinal nematode infections.

Animals and sample preparation

A group of 100 *Bos indicus* bulls (Nelore breed) aged 11 to 12 months were kept together on pasture without anthelmintic treatment for 6 months on a farm located in Londrina, State of Paraná, in southern Brazil (longitude 23°18'37" S, latitude 51°09'46'' O). They were kept on 79 hectares (divided in 4 paddocks) of *Panicum maximum* pastures, naturally contaminated by nematode larvae. Feces

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samples were taken 7 times at a two-weeks interval from all animals, to determine the fecal egg counts (FEC). The mean FEC of each animal was calculated, and the 10 animals with the lowest mean FEC (31.6 ± 43.1; called resistant - R) and 10 with the highest mean FEC (674.6 ± 505.7; called susceptible - S) were selected and slaughtered. Tissue samples from the abomasum, small intestine and abomasum lymph nodules of these animals were collected immediately after slaughter. Total RNA was isolated using Trizol Reagent (Invitrogen Co Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA concentration was assessed by spectrophotometry at 260 nm, and RNA quality was assessed by the OD_{260nm}:OD_{280nm} ratio and electrophoresis on 1% agarose gel stained with ethidium bromide.

Primer design

Primers were designed using Primer3 primer design software (Rozen and Skaletzky, 2000) and cattle-specific GenBank sequences for IL-2 (AF535144), IL-4 (M77120), IL-8 (AF061521), IL-12p35 (AJ271034), IL-13 (NM174089), TNF-α (AF011926), IFN-γ (Z54144), MCP-1 (L32659), MCP-2 (S67956), and MUC-1 (AF399757). The internal control genes tested were ribosomal protein (RP) L-19 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers were analyzed using Oligo Analysis & Plotting Tools software from Operon, in order to avoid secondary structures such as hairpins and loops. Primers were designed on different exons, to differentiate cDNA and genomic DNA amplification. Primer sequences and amplicon lengths are shown in Table 1.

Confirmation of primer specificity

Amplification and melting analysis were performed on a LightCycler (Roche Diagnostics, Mannheim, Germany). Specificity of the RT-PCR products was evaluated by agarose gel electrophoresis, melting curve analysis and direct sequence of amplified products. Melting curve analysis was performed from 70 °C to 95 °C at 0.1 °C/s for all genes studied. Melting temperature (T_M) was defined as the peak of the curve, and if the highest point was a plateau, then the midpoint was identified as T_M. Sequencing was done with the DYEnamic ET DyeTerminator Sequencing Kit (Amersham Bioscience, Piscataway, NJ, USA) and the ABI 3100 Automated DNA Sequencer (Applied Biosystems).

Amplification efficiency

Serial cDNA dilution curves were produced to calculate the amplification efficiency for all genes. A graph of threshold cycle (Ct) versus $\log_{10}$ relative copy number of the sample from a dilution series was produced. The slope

Table 1 - PCR conditions.

Gene	Sequence (5' → 3')	Exon position	Amplicon (pb)	Primer (pMol)	Mg (mM)	Annealing (ºC/s)	Extension (ºC/s)	Melting (ºC)
IL-2	F- TCCAAGCAAAAACCTGAACC r- CAGCGTTTACTGTTGCATCATC	E3 E4	198	2.5	2.0	57 °C / 7	72 °C / 6	82.5
IL-4	F: TGCATTGTTAGCGTCTCCTG R: AGGTCTTTCAGCGTACTTGT	E1 E2	450	2	3.0	56 °C / 6	72 °C / 17	86.0
IL-8	F- TGGGCCACACTGTGAAAAT r- TCATGGATCTTGCTTCTCAGC	E1 E3	203	2.5	3.0	53 °C / 5	72 °C / 9	82.0
IL-12p35	F- GAGGCCTGTTTACCACTGGA r- CTCATAGATACTTCTAAGGCACAG	E1 E4	143	2	3.0	58 °C / 7	72 °C / 7	82.5
IL-13	F: GGTGGCCTCACCTCCCCAAG R: GATGACACTGCAGTTGGAGATGCTG	E2 E4	234	2.5	2.5	60 °C / 7	72 °C / 9	87.0
TNF-α	F- TAACAAGCCGGTAGCCCACG R- TCTTGATGGCAGACAGGATG	E3 E4	279	2	2.5	57 °C / 7	72 °C / 12	88.5
IFN-γ	F- GCCAAATTGTCTCCTTCTACTTC r- GGGTCAAGTGAAATAGTCACAGG	E3 E4	484	2	2.5	61 °C / 7	72 °C / 22	85.0
MCP-1	F- CAAGTCGCCTGCTGCCTATAC r- AGAGGGCAGTTAGGGAAAGC	E2 E3	278	2.5	2.5	56 °C / 5	72 °C / 12	85.0
MCP-2	F- GGGATTCTGTGTCTGCTGCT r- TTTGGTCCAGGAGCCTTATG	E1 E3	241	2	3.0	61 °C / 7	72 °C / 10	87.0
MUC-1	F- TGCTGTTCCCAGTGCTTACA r- GAGACGAGGTGCCATTGT	E1 E2	276	2.5	2.0	55 °C / 5	72 °C / 12	87.0
RPL-19	F- GAAATCGCCAATGCCAAC R- GAGCCTTGTCTGCCTTCA	E1 E2	410	2.5	2.5	60 °C / 7	72 °C / 16	87.5
GAPDH	F- GGCGTGAACCACGAGAAGTATAA R- CCCTCCACGATGCCAAAGT	E1 E2	194	2.5	1.5	59 °C / 7	72 °C / 5	86.0

of the curve was used to determine the amplification efficiency (Pfaffl, 2001): Efficiency = $10^{(-1/slope)}$.

Real-time RT-PCR

cDNA was reverse-transcribed from 5.0 µg total RNA using oligo(dT) primer and Superscript II (Invitrogen Co Carlsbad, CA, USA), following the manufacturer's instructions. Real-time PCR was performed using LightCycler (Roche Diagnostics, Mannheim, Germany) and SYBR Green I (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. The PCR conditions were: 7.5 ng of cDNA, $MgCl_2$ at optimal concentration for each gene, 2-2.5 pmol of each primer, 4 µL of SYBR Green I, 0.3 µL of Taq Platinum DNA polymerase (Invitrogen, 5U/µL), 0.4 µL of dNTP (10 mM), and 0.25 µL BSA (20 mg/mL), in a final volume of 20 µL. Reactions were incubated in a LightCycler, following specific conditions for each gene (Table 1). Initial denaturation and denaturation step were the same for all primers, *i.e.*, 95 °C for 5 min and 95 °C for 10 s, respectively. The fluorescence acquisition temperature was 72 °C for all genes. The ΔR_n value (fluorescence variation during the PCR reaction) for each gene was 0.1, which was as low as possible without including any background points.

The RT-PCR conditions established resulted in expected amplicon size and single-band amplification for all genes, indicating that the primers and conditions used were specific for the target genes (Figure 1). Amplification specificity was further validated by melting curve analysis, generated at the end of each PCR reaction. All genes presented a single peak in the melting curve, which indicates absence of primer-dimer formation during the reaction and specificity of the amplification. The melting temperature for all genes is presented in Table 1. Melting curve analysis is a fast method to check for RT-PCR specificity; however, in some cases, RT-PCR products of different lengths have the same melting temperature and therefore are indistinguishable by melting curve analysis. To guarantee specificity, the amplified products were sequenced and gene identity was confirmed (data not shown).

The efficiency, sensitivity and linearity of RT-PCR reactions were examined using 10-fold serial dilutions. The relationship between threshold cycle (Ct) and the log copy number of cDNA for all genes was linear with an R^2 ranging from 0.96 to 0.99, indicating that Ct values changed proportionally to the serial dilution of the samples. Efficiency values ranged from 1.86 to 2.18, indicating efficient amplification near the theoretical optimum level of 2 (Wilkening and Bader, 2004).

A reliable quantitative RT-PCR method requires correction for experimental variations. In the present study, two internal control genes were tested in the two groups of animals, resistant and susceptible to gastrointestinal nematode infections, and in each one of the three tissues collected (Table 2). Ct values in the different tissues and groups ranged from 15.8 to 21.1 for RPL-19 and from 27.5 to 35.4 for GAPDH. The lower Ct value for RPL-19 indicates that this gene reaches the detection threshold with less amplification cycles than GAPDH, indicating that it is more abundant in the abomasum, abomasum lymph nodules and small intestine. The coefficient of variation ranged from 5.6 to 8.9 for RPL-19 and from 7.5 to 18.5 for GAPDH across all tissues and groups. The expression levels of RPL-19 and GAPDH were not different in resistant and susceptible animals ($p > 0.1$), however they were differentially expressed among tissues ($p < 0.01$). These results are in agreement with those of Al-Bader and Al-Sarraf (2005) showing that GAPDH, albumins, actins, tubulins,

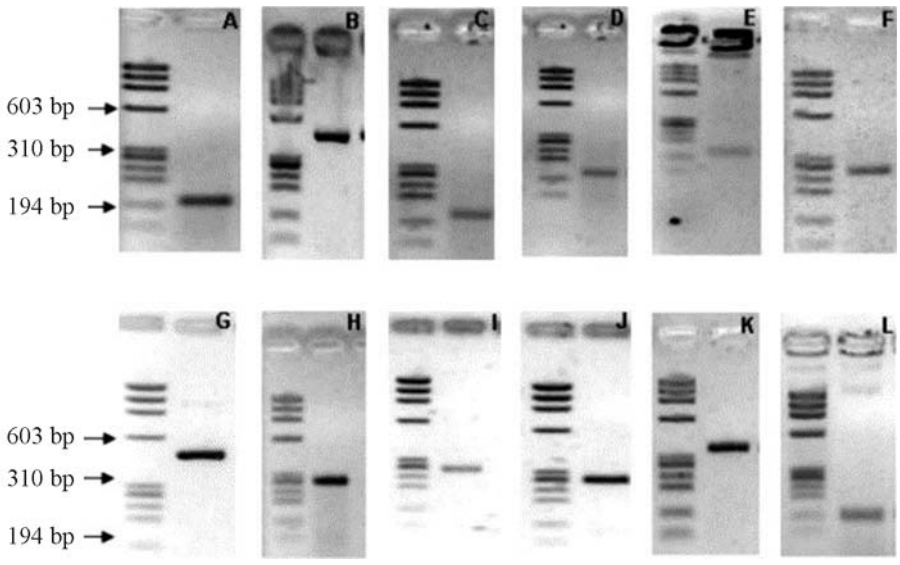

Figure 1 - RT-PCR products. A: IL-2; B: IL-4; C: IL-8; D: IL-12p35; E: IL-13; F: TNF-α; G: IFN-γ; H: MCP-1; I: MCP-2; J: MUC-1; K: RPL19; and L: GAPDH. Phi-X174 *Hae*III was used as a molecular marker.

Table 2 - Analysis of RPL-19 and GAPDH in abomasum (AB), small intestine (SI) and abomasum lymph nodules (LN) from resistant (R) and susceptible (S) animals.

Gene	(Ct) Threshold cycle			Standard deviation			Coeff. variation (%)		
	AB	SI	LN	AB	SI	LN	AB	SI	LN
RPL-19(R)	22.0a	19.5a	16.31b	1.23	2.11	1.46	5.62	10.8	8.90
RPL-19(S)	21.08a	19.75a	15.82b	1.21	1.10	1.26	5.58	5.6	8.00
GAPDH(R)	35.54a	32.06a	28.51b	6.09	6.02	2.85	17.1	18.46	10.0
GAPDH(S)	32.06a	29.12b	27.51b	2.67	2.42	2.05	8.34	8.31	7.47

Values with different letters in the same row are statistically different ($p < 0.01$).

cyclophilins, 18S rRNA and 28S RNA, used extensively as control genes, may vary under different experimental conditions in different tissues. Although GAPDH is widely used as an internal control gene (Giulietti *et al.*, 2001; Bustin, 2002; Bustin *et al.*, 2005), several reports emphasize problems associated with its use (Ke *et al.*, 2000; Suzuki *et al.*, 2000). Al-Bader and Al-Sarraf (2005) used RT-PCR to measure mRNA levels of several genes, in order to identify those most suitable to normalize expression. These authors concluded that GAPDH was not a suitable control gene, while 18S, RPL-19, BGLU, CY and HH4 were reasonably stable genes that were appropriate for normalization when quantifying mRNA levels. Ontsuka *et al.* (2004) tested the 18S, RPL-19 and β-actin genes in intestinal fractions and concluded that they were stable.

Several methods to quantify cytokines have been developed and RT-PCR is one of the most important techniques for analyzing immune response in bovines and other ruminants. Konnai *et al.* (2003) used this method to quantify cytokine profiles (IL-1, IL-2, IL-4, IL-6, IL-10, IL-12p40, and IFN-γ) in peripheral blood mononuclear cells from bovine and ovine leukemia virus (BLV)-infected animals and concluded that this methodology is a useful tool in the quantitative analysis of various genes in cattle and sheep. Gómez-Muñoz *et al.* (2004) analyzed the immune response during bovine ostertagiosis and observed decreased IL-1 IL-2, IL-4, IL-13, TNF-α and IFN-γ levels and increased IL-10 and TGF-β levels after challenge, concluding that RT-PCR is a rapid and accurate method to classify *Ostertagia ostertagi* as a suppressor of *in vivo* immune response in cattle. Claerebout *et al.* (2005) studied the abomasal (mucosal and lymph nodules) cytokine response (IL-2, IL-4, IL-5, Il-6, IL-10, IL-12p40, IL-13, IL-15, and IFN-γ) in helminthes-naive calves and calves vaccinated with protective antigen from *Ostertagia ostertagi* after challenge with infective third-stage larvae and concluded that RT-PCR is ideal to obtain reliable data under different experimental conditions, i.e, naive and vaccinated calves.

Real-time RT - PCR has significantly extended the use of RT-PCR assays. The primers and conditions optimized in the present study were sensitive, fast and reliable in the detection and quantification of cytokine mRNAs, and the RPL-19 gene was demonstrated to be an ideal internal control gene in bovine experiments. Such real-time PCR assays can be used in *Bos indicus* cattle to investigate the bovine immune response in a variety of situations, such as viral, bacterial and parasite infections.

Acknowledgments

This work was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP). (Lilian Giotto Zaros and Patrízia Ana Bricarello received financial support from FAPESP.) Luiz Lehmann Coutinho is a recipient of a research productivity scholarship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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Internet Resources

Primer3: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi.

Operon Biotechnologies GmbH: https://www.operon.com/oligos/toolkit.php.

GenBank: http://www.ncbi.nlm.nih.gov/Genbank/.

Associate Editor: Pedro Franklin Barbosa