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Source: Journal of Wildlife Diseases, 42(2) : 219-233

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-42.2.219

Journal of Wildlife Diseases, 42(2), 2006, pp. 219–233

CERVINE (*CERVUS ELAPHUS*) CYTOKINE mRNA QUANTIFICATION BY REAL-TIME POLYMERASE CHAIN REACTION

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ABSTRACT: It has been difficult to perform cytokine studies for many wildlife and nontraditional species because of a lack of immunologic reagents at the protein level. Recently, simple and rapid assays for quantifying mRNA expression by real-time reverse transcription–polymerase chain reaction (RT-PCR) have been used for analysis of cytokine profiles in humans and other mammalian species. This report describes the development and application of real time RT-PCR to measure the expression of several important elk (*Cervus elaphus*) cytokine mRNAs, including interleukin (IL)-2, IL-4, IL-10, IL-12p40, interferon-gamma, tumor necrosis factor (TNF)-α, and the enzyme-inducible nitric oxide synthase, all of which are involved in immune responses and regulation. For the broadest potential application of the assay, primers and probes were designed using consensus sequences from several species of interest. To obtain standardized quantitative results, external controls consisting of a DNA template for each target gene were used to generate linear standard curves over a 6 to 8 log range with detection of as few as 10 copies of amplicon per reaction. Sample-to-sample variation in the efficiency of the RT, as well as in the quantity and quality of the starting RNA, was compensated for by normalizing the results to the endogenous housekeeping gene β_2-microglobulin. The assay was evaluated by monitoring the kinetics of cytokine mRNA synthesis induced by mitogenic and antigenic stimulation of peripheral blood mononuclear cells (PBMCs) from *Mycobacterium bovis*–infected elk. Concanavalin A–stimulated PBMCs demonstrated a rapid but transient increase in cytokine mRNA expression following in vitro mitogenic activation with optimal mRNA induction observed after 4 to 16 hr. The PBMCs stimulated with the mycobacterial recall antigen, bovine-purified protein derivative (PPD-bovis), demonstrated variable mRNA induction kinetics for each cytokine. Whereas PPD-bovis optimally induced IL-2 mRNA after 8 hr of in vitro stimulation, longer in vitro stimulation times were necessary for the optimal induction of IL-4 and TNF-α mRNA (up to 48 hr). We demonstrate real-time RT-PCR to be a rapid, sensitive, and reproducible technique, which will make it a valuable tool in the study of immunologic responses and cytokine profiles of cervids and other nontraditional livestock and wildlife species.

Key words: Bovine tuberculosis, *Cervus elaphus*, cytokine, elk, gene expression, *Mycobacterium bovis*, real-time polymerase chain reaction.

INTRODUCTION

Infectious diseases are significant factors affecting the health of wild and captive cervid populations. The North American elk (*Cervus elaphus nelsoni* and *Cervus elaphus manitobensis*) can be a reservoir for several important intracellular pathogens, including *Mycobacterium bovis* (O'Brien et al., 2002), *Mycobacterium avium* subsp. *paratuberculosis* (Manning et al., 1998), and *Brucella abortus* (Rhyan et al., 1997). More recently, chronic wasting disease, a transmissible spongiform encephalopathy that affects deer (*Odocoileus* spp.) and elk, has been diagnosed in wild (Spraker et al., 1997) and captive (Williams and Young, 1980) cervid populations in North America. To better control these diseases in wild and captive cervid herds, improved diagnostic tests, new vaccines, or other tools are required. As is the case for many nontraditional domestic and wildlife species, however, an understanding of the host immune response to infection in exotic ruminants is lacking.

Cytokines are regulatory proteins that play a central role in the immune system by modulating immune responses, including lymphocyte activation, proliferation, differentiation, survival, and apoptosis (Balkwill and Burke, 1989). Cytokines

determine the nature of immune responses to infection and, thus, are key determinants to the prevention or progression of various diseases. For example, the Th1/Th2 paradigm, which originally was reported in terms of different T-helper cell cytokine profiles leading to the induction of different aspects of the immune system (Mosmann and Coffman, 1989), has been shown to play a particularly important role in immunity to infectious agents (Lucey et al., 1996). Type-1 biased responses are mostly involved in protection against intracellular pathogens through cell-mediated immunity. In contrast, type-2 biased responses generally are considered to be protective against extracellular pathogens by promoting protective antibodies (Romagnani, 1997). Knowledge of the regulation of immune responses after infection is critical to understanding the mechanisms of microbial pathogenesis. This knowledge may then prove to be useful for the design of diagnostic tests, for the development of candidate vaccines, and for the monitoring therapeutic effects.

A major hurdle to overcome in conducting such studies for many wildlife and nontraditional domestic species is the difficulty in measuring cytokine expression at the protein level using current techniques, such as enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunospot (ELISPOT), and flow cytometry, because of a lack of species-specific reagents. The requirement that specific antibodies (polyclonal or, preferably, monoclonal) be raised against proteins of interest can be a very laborious and time-consuming process. Accordingly, new cellular and molecular immunologic assays to investigate the immune response of wildlife and nontraditional domestic species are required.

Because several of the genes encoding cervine cytokines recently have been cloned and sequenced (Lockhart et al., 1995; Slobbe et al., 2000), the detection of cytokine mRNA levels provides an alternative approach to functional assays for the detection of cervine cytokines. Recently, reverse transcription (RT) of mRNA into DNA, followed by polymerase chain reaction (PCR) using real-time PCR, has become the method of choice for the rapid analysis and quantification of cytokine mRNA for humans (Blaschke et al., 2000) and other mammalian species (Menne et al., 2002). This technique allows direct monitoring of amplicon accumulation during the PCR process, and it combines amplification, detection, and quantification in a single step, thereby eliminating the need for post-PCR processing. It only requires knowledge of the gene sequence or consensus sequences derived from cytokines of different species, so specific reagents (primers and fluorogenic probes) can then be rapidly produced in the laboratory or purchased. The easy detection of cytokine mRNA transcripts in a limited number of cells, in which the corresponding protein may be difficult to detect, is an additional advantage of the technique (Favre et al., 1997).

The purpose of the present study was to develop, optimize, and validate high-throughput, real-time RT-PCR assays for several important elk cytokines, including interleukin (IL)-2, IL-4, IL-10, IL-12p40, interferon-gamma (IFN-γ), tumor necrosis factor (TNF)-α, and the enzyme-inducible nitric oxide synthase, which are involved in the immune response to infection. A specific set of primers and internal sequence–specific fluorogenic probes were designed and optimized for each target gene. Thereafter, this methodology was applied to monitor the induction of cytokine genes on in vitro polyclonal and antigenic stimulation of peripheral blood mononuclear cells (PBMCs). Because the PBMCs of *M. bovis*–infected animals produce antigen-specific cytokines on stimulation with specific mycobacterial antigens, we measured mRNA production after stimulation of PBMCs from elk experimentally infected with *M. bovis* to demonstrate the

effectiveness of the approach in this animal species.

MATERIALS AND METHODS

Blood collection and mononuclear cell culture conditions

For real-time RT-PCR assay development and validation, blood of wild adult elk from Riding Mountain National Park (51°52′N, 100°36′W; Manitoba, Canada) was collected into heparinized vacutainer tubes. The elk were captured by net-gun as part of a multiagency disease surveillance project sponsored by Parks Canada Agency from December 2003 to February 2004. Blood samples were transported at approximately 20 C and processed within 24 hr from the time of collection. The PBMCs were separated by density-gradient centrifugation using Ficoll-Paque (density, 1.077 g/ml; Amersham Biosciences, Piscataway, New Jersey, USA) and washed twice with RPMI 1640 medium (Invitrogen, Burlington, Ontario, Canada). The PBMCs were diluted to 5×10^6 cells/ml in RPMI 1640 containing 10% fetal calf serum (Invitrogen) and supplemented with penicillin G and streptomycin (100 U/ml and 80 μg/ml, respectively; Invitrogen). Concanavalin A (Con A; Sigma-Aldrich, Oakville, Ontario, Canada) or bovine tuberculin (PPD-bovis; Biologics Production Unit, Ottawa Laboratory Fallowfield, Canadian Food Inspection Agency, Ottawa, Ontario, Canada) was added to cell suspensions at the concentration of 10 μg/ml. Media were added to suspensions acting as "no treatment" controls. A 200-μl volume of each cell suspension was placed into four wells of a 96-well, round-bottom plate (Corning, Acton, Massachusetts, USA) and incubated from 2 to 96 hr at 37 C in a humidified atmosphere containing 5% CO_2.

To test the real-time RT-PCR assay for use in other species, blood was collected and processed, according to the method outlined above, for cattle (*Bos taurus*), sheep (*Ovis aries*), goat (*Capra hircus*), white-tailed deer (*Odocoileus virginianus*), reindeer (*Rangifer tarandus*), and bison (*Bison bison*). Cattle, sheep, and goat blood samples were obtained from control animals housed at the Animal Disease Research Institute, Canadian Food Inspection Agency (Ottawa, Ontario, Canada). Blood samples from white-tailed deer and bison in Riding Mountain National Park were collected and transported as described above for elk. Blood samples of reindeer were collected and kindly provided by staff of the Toronto Zoo (Ontario, Canada).

Total RNA isolation and cDNA synthesis

Total cytoplasmic RNA was extracted from cells following in vitro culture using RLN buffer (Qiagen, Mississauga, Ontario, Canada) and RNeasy® spin columns (Qiagen) according to the manufacturer's instructions. To diminish variability and pipetting errors, the four wells from each treatment and time point were pooled for RNA extraction as one experiment. All RNA samples were treated with on-column RNase-free DNase I (Qiagen) to remove any traces of genomic DNA contamination. The RNA was resuspended in 40 μl of RNase-free water and quantified by spectrophotometry (OD_{260}) and low-mass gel electrophoresis (Invitrogen). Total RNA was reverse transcribed to cDNA with the Omniscript® cDNA Synthesis kit (Qiagen) following the manufacturer's instructions. Briefly, approximately 0.2 μg of total RNA was reverse transcribed in a final volume of 20 μl of reaction mix containing the following components: 1× RT buffer, deoxynucleotide triphosphate mix (5 mM each), ribonuclease inhibitor (10 units/μl; RNase Out, Invitrogen), and 4 U of Omniscript® RT. The samples were incubated at 37 C for 60 min. To control for genomic DNA contamination, every reaction set contained an RNA sample without Omniscript® (RT reaction), which was carried forward into subsequent PCR reactions. The resulting cDNA was stored frozen (−70 C) until assayed by real-time PCR.

Oligonucleotide primers and probes for PCR

Oligonucleotide primers and fluorogenic probes of IL-2, IL-4, IL-10, IL-12p40, IFN-γ, TNF-α, enzyme-inducible nitric oxide synthase, and β_2-microglobulin were chosen based on the bovine and cervine cDNA sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/Entrez/index.html) and designed using Primer3 software (Rozen and Skaletsky, 2000) (http://www-genome.wi.mit.edu/cgib-bin/primer/primer3_www.cgi). Primers and probes were designed for use in multiple species, including elk, cattle, sheep, goat, reindeer, white-tailed deer, and bison. This was accomplished by selecting oligonucleotide sequences from highly conserved coding regions of cDNA in species for which sequence data were available for comparison. No information was available regarding reindeer, white-tailed deer, or bison cDNA sequences in published databases at the time of this study. The default parameters of the Primer3 software were applied except for the following: product size, 70 to 170 bp; primer melting temperature (T_m), 58 to 62 C; maxi-

mum T_m difference, 2.0 C; probe T_m, 68 to 72 C. The presence of a guanidine at the 5′-end was avoided, because this base quenches the reporter signal, even after probe cleavage. Probes contained either the 6-carboxyfluorescein or hexachloro-6-carboxy-fluorescein reporter dye attached covalently at the 5′-end and the Black Hole Quencher molecule covalently attached at the 3′-end (Integrated DNA Technology, Coralville, Iowa, USA). Primer and probe sequences also were chosen to prevent homologies to undesired genes and other coding sequences and checked with BLAST (basic local alignment search tool) software (http://www.ncbi.nlm.nih.gov/BLAST/). For each target gene, different primer concentrations (300–900 nM) were tested to optimize the PCR amplification. The primer/probe sequences and expected PCR product length are shown in Table 1.

Real-time PCR assay

The real-time PCR reaction mixture contained 5 μl of sample cDNA and 20 μl of a master mix containing probe (200 nM), forward and reverse primers (900 nM each), and the PCR core reagent (Platinum® Quantitative PCR SuperMix-UDG, Invitrogen). The 5-μl volume of cDNA sample was added to the final reaction mixture as a 1:2 dilution of the RT reaction in DNase/RNase-free water. Up to 30 test samples were assayed in triplicate on each plate. Each set of reactions also included positive controls (PCR product) and negative controls (i.e., water instead of sample) for the RT and PCR steps. The wells of the plate were sealed with optical adhesive covers (Bio-Rad Laboratories, Mississauga, Ontario, Canada) and were centrifuged at low speed (e.g., 200 × G, 5 min) to ensure complete mixing. Reactions were run on the iCycler IQ (Bio-Rad

TABLE 1. Oligonucleotide primer and probe sequences for real-time polymerase chain reaction amplification of various cervine target genes.

Target gene[a]	Oligonucleotide sequence (5′→3′)[b]	Product size (bp)[c]	GenBank accession no.	
			Bovine	Cervine[d]
β2M	F: AGACACCCACCAGAAGATGG	98	X69084	N/A[d]
	R: TCCCCATTCTTCAGCAAATC			
	P: FAM-TGGGTTCCATCCACCCCAGA-BHQ			
IL-2	F: ACTAACACTTGCACTCGTTGC	85	M12791	U14682
	R: CAGCAGCAATGACTTCACTTCTT			
	P: FAM-TBCACCTACTTCAAGCTCTACGGG-BHQ			
IL-4	F: CTGCCCCAAAGAACACAACT	164	M77120	L07081
	R: CGTCTTGGCTTCATTCACAG			
	P: FAM-AGGCGTATCTACAGGAGCCA-BHQ			
IL-10	F: TGTCATCGCTTTCTGCCCTG	92	NM_174088	U11767
	R: TTGTAGACACCCTCTCTTGG			
	P: HEX-AGCAGGTGAAGAGCGTCTTC-BHQ			
IL-12p40	F: TCAGGGACATCATCAAACCA	160	U57752	U11815
	R: TTGTTCTTTCCCTGGACCTG			
	P: HEX-CTGGGAGTACCCTGACACGT-BHQ			
IFN-γ	F: GCGCAAAGCCATAAATGAAC	98	M29867	L07502
	R: CTCAGAAAGCGGAAGAGAAG			
	P: HEX-CAAAGTGATGAATGACCTGTGCCA-BHQ			
TNF-α	F: AGAAGGGAGATCGCCTCAGT	89	AF348421	U14683
	R: GGCGATGATCCCAAAGTAGA			
	P: HEX-TCAACCTGCCGGACTACCTGGA-BHQ			
iNOS	F: CAGGAACCTACCAGCTGACG	98	AF340236	N/A
	R: GTTCGACCACTGGATCCTTC			
	P: FAM-CTTCGCCACCAAGCAGGCCT-BHQ			

[a] β2M = β_2-microglobulin; IL = interleukin; IFN-γ = interferon-gamma; TNF-α = tumor necrosis factor-α; iNOS = enzyme-inducible nitric oxide synthase.

[b] Primers were designed based on sequences of bovine and cervine cytokine genes from the EMBL/GenBank databases. F = forward; R = reverse; P= probe; FAM = 6-carboxyfluorescein; BHQ = Black Hole Quencher; HEX = hexachloro-6-carboxy-fluorescein.

[c] bp = base pairs.

[d] N/A = not available.

Laboratories) instrument using the following cycling parameters: Initial activation of AmpErase UNG® (Invitrogen) in the PCR core reagent at 50 C for 2 min; followed by inactivation of the AmpErase UNG®, activation of Platinum® *Taq* DNA polymerase (Invitrogen) in the PCR core reagent, and denaturation of DNA, all at 95 C for 10 min; and then 45 cycles of denaturation at 95 C for 15 sec and annealing with primer extension at 60 C for 45 sec, with the fluorescence being read at the end of this second step. Amplified PCR products were visualized by electrophoresis of 10 µl of the reaction mixture on a 2% agarose gel followed by ethidium bromide staining. The specificities of the amplified bands were confirmed by visualization of a single band of predicted size based on a molecular weight standard (100-bp DNA ladder, Invitrogen) and by sequencing of the PCR product (Guelph Molecular Supercentre, University of Guelph, Guelph, Ontario, Canada).

Optical data obtained by real-time PCR was analyzed using the default and variable parameters available with the iCycler® Optical System Interface V 2.3 (Bio-Rad Laboratories). The PCR threshold cycle number (C_t) for each DNA standard and test cDNA sample was calculated at the point where the fluorescence exceeded the threshold limit. The threshold limit was fixed along the linear logarithmic phase of the fluorescence curves at 10 to 20 standard deviations above the average background fluorescence. The average C_t for triplicate standards and samples was calculated.

PCR product standard curves

For each target gene of interest, a standard curve was established using serial dilutions of an external standard consisting of PCR product amplified with each primer set by conventional PCR and then purified from agarose gels following standard procedures (Qiagen Gel Extraction Kit). The PCR-amplified cDNA concentrations were measured by optical density and low-mass gel electrophoresis (Invitrogen). The copy number was calculated using Avogadro's constant, the molecular weight of double-stranded DNA, and the size of the target amplicon (Overbergh et al., 1999). From the calculated molecular concentration of double-stranded DNA, serial dilutions (1:10) of known input copy numbers (10^9 to 10^1) for each target gene were used to generate the standard curves. A master mix was made up and aliquoted into the PCR plate before the addition of the template into each reaction tube individually. Every dilution point was amplified by PCR in triplicate. To check for reproducibility, the standard curves were generated on different days for each target gene of interest. A graph of C_t versus $\log_{10}$ relative copy number of the sample from the dilution series ranging from 10^9 to 10^1 copies was produced (Fig. 1). The slope of this graph was used to determine the reaction efficiency according to the equation $E = 10^{-1/\text{slope}}$, where a value of 2.0 represents the optimal efficiency.

Kinetic study for cytokine mRNAs

To evaluate the usefulness of the real-time PCR analysis, we examined changes of cytokine mRNA expression in PBMCs isolated from *M. bovis*–infected elk. For experimental infection, 6-mo-old elk/red deer hybrids (*C. elaphus nelsoni/Cervus elaphus elaphus*) were obtained from a certified tuberculosis-free herd. Six animals were experimentally inoculated with 1.5×10^3 colony-forming units of a Canadian field strain of *M. bovis* (strain 02/1007, Canadian Food Inspection Agency designation) by the intratonsillar route of inoculation as previously described (Palmer et al., 2002). Animals were housed in secure isolation under biocontainment level 3 conditions (Animal Disease Research Institute, Canadian Food Inspection Agency, Ottawa, Ontario, Canada) in accordance with requirements of the Canadian Council on Animal Care. Five uninfected animals were used as controls. Blood was collected into heparinized vacutainer tubes, and PBMCs were isolated within 4 hr of collection. The PBMCs were incubated at 37 C in a 5% atmosphere either with stimulation (Con A or PPD-bovis) or without stimulation, and mRNA was harvested for cytokine analysis after 0, 2, 4, 8, 16, 24, 48, 72, and 96 hr of culture as described previously. The mRNA levels of IL-2, IL-4, IFN-γ, and TNF-α were then quantified at the different times of culture.

Quantitative and statistical analysis

Quantification of cytokine mRNA levels and tests for statistical significance of differences in mRNA expression were analyzed by the Relative Expression Software Tool (REST) for groupwise comparison and statistical analysis of relative expression results in real-time PCR (Pfaffl et al., 2002). The REST software also indicates coefficients of variance in percentages (mean values are shown in Table 2) and standard deviations based on the C_t of the target gene. For quantification by this method, values are expressed relative to a reference sample (unstimulated PBMC sample), called

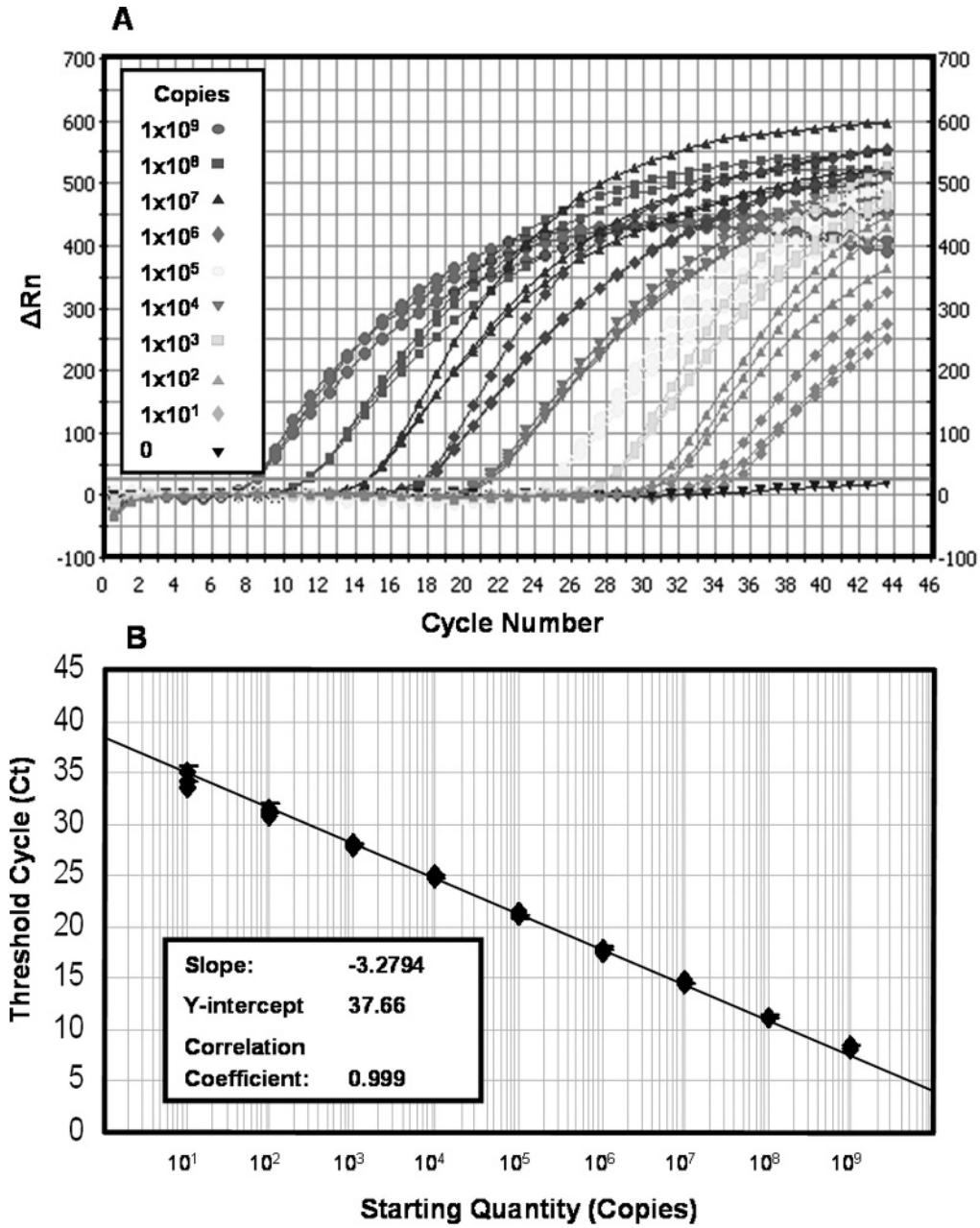

FIGURE 1. Amplification plot and standard curve for cervine interferon-gamma (IFN-γ). Standard curves were constructed with PCR product dilutions ranging from 10^1 to 10^9 input copies (see text for details). (a) A representative real-time polymerase chain reaction (PCR) run in triplicate on the Bio-Rad iCycler is shown. Fluorescence emission is measured continuously during the PCR, and increase in fluorescence emission (relative fluorescence units [RFU] subtracted by the background fluorescence signal) is plotted against cycle number. (B) The standard curve for IFN-γ is generated by plotting cycle threshold (C_t; obtained from the amplification plots in A) against input PCR product copy number.

TABLE 2. Characteristics and validation parameters of cervine target genes by real-time reverse transcription-polymerase chain (PCR) reaction.[a]

	B2M	IL-2	IL-4	IL-10	IL-12p40	IFN-γ	TNF-α	iNOS
Quantification range (test linearity)[b]	10^1–10^8 molecules (r=0.999)	10^1–10^8 molecules (r=0.993)	10^2–10^7 molecules (r=1.0)	10^2–10^8 molecules (r=0.999)	10^2–10^8 molecules (r=0.999)	10^1–10^8 molecules (r=0.999)	10^2–10^7 molecules (r=0.998)	10^1–10^8 molecules (r=1.0)
PCR efficiency[c]	2.01	1.93	1.99	1.96	1.94	1.98	1.84	2.00
Intra-assay coefficient of variation (%)[d]	3.45	0.82	0.65	2.34	0.90	1.34	1.72	1.09
Interassay coefficient of variation (%)[d]	1.50	4.70	3.40	5.30	1.70	4.00	4.50	4.84
Species[e]	*Bos taurus* *Cervus elaphus* *Odocoileus virginianus* *Ovis aries* *Rangifer tarandus* *Bison bison*	*Bos taurus* *Cervus elaphus* *Odocoileus virginianus* *Ovis aries* *Capra hircus* *Bison bison*	*Bos taurus* *Cervus elaphus* *Odocoileus virginianus* *Ovis aries* *Capra hircus* *Rangifer tarandus* *Bison bison*	*Bos taurus* *Cervus elaphus* *Odocoileus virginianus* *Bison bison*	*Bos taurus* *Cervus elaphus* *Odocoileus virginianus* *Ovis aries* *Capra hircus* *Bison bison*	*Bos taurus* *Cervus elaphus* *Odocoileus virginianus* *Rangifer tarandus* *Ovis aries* *Capra hircus* *Bison bison*	*Bos taurus* *Cervus elaphus* *Odocoileus virginianus* *Ovis aries* *Rangifer tarandus* *Bison bison*	*Bos taurus* *Cervus elaphus* *Odocoileus virginianus* *Rangifer tarandus* *Bison bison*

[a] β2M = β_2-microglobulin; IL = interleukin; IFN-γ = interferon-gamma; TNF-α = tumor necrosis factor-α; iNOS = enzyme-inducible nitric oxide synthase.

[b] The quantification range and test linearity (r, Pearson correlation coefficient) are given as molecules per reaction. Assay variation was determined in four repeats (n=4) over the complete quantification range.

[c] The PCR efficiency was calculated according to the equation: $E=10^{-1/\text{slope}}$.

[d] Determination of variation is based on crossing-point variation.

[e] Indicates species with positive cross-reactivity when tested with target gene primer/probe sets.

the calibrator. First, the C_t for the target amplicon and the C_t for the internal control (β_2-microglobulin) were determined for each sample. Differences in the C_t for the target and the C_t for the internal control, called ΔC_t, were calculated to normalize for the differences in the amount of total nucleic acid added to each reaction and the efficiency of the RT step. The ΔC_t for each experimental sample was subtracted from the ΔC_t of the calibrator. This difference is called the $\Delta\Delta C_t$ value. Finally, the amount of target normalized to an internal control and relative to the calibrator was calculated by $E^{-\Delta\Delta Ct}$, where E is the PCR efficiency and 100% efficiency equals a value of 2.0. Thus, all experimental samples are reported as relative transcription or the n-fold difference between the calibrator (unstimulated PBMC sample) and the Con A– or PPD-bovis–stimulated samples following activation (2, 4, 8, 16, 24, 48, 72, and 96 hr).

RESULTS

Real-time PCR assay of PCR product standards

The primers and probes used for real-time PCR assays of cervine cytokine mRNA are shown in Table 1. The PCR product standard curves were generated and used for quantification of the various target genes. In Figure 1B, a representative standard curve from 10-fold serial dilutions of the IFN-γ PCR product is shown. A linear relationship was detected over either orders of magnitude (correlation coefficient $r=0.99$), with an analytical sensitivity of 10 target molecules per reaction. From such standard curves, the overall characteristics of the PCR product target genes are summarized in Table 2. The real-time PCR efficiencies (E) were calculated from the slope of the standard curves and ranged from 1.84 to 2.01. The linear range of detection for cDNA was as low as 10 copies per reaction for most target genes (Table 2).

Assay variation

The reproducibility of the real-time PCR method was examined by intra- and interassay amplification. The PCR consumables were always prepared as a mixture, whereas the cDNA aliquots were added individually to each well. Calculation of test variability was based on the variation of C_t from the mean C_t value. The intra-assay variation was determined in four repeats of a PCR run over a range of different dilutions of the purified PCR product standards (10^1 to $>10^8$ starting molecules). Interassay variation was similarly examined over a wide range of starting molecules from four different experimental runs performed on different days. The various target genes showed minimal intra- and interassay variation, with the coefficient of variation ranging from 0.65% to 3.45% and 1.5% to 5.3%, respectively (Table 2). For the intra-assay experiments, the variability rose as the number of starting template molecules decreased, and in the interassay experiment, it was reversed (data not shown).

Assay application in other species

Although the quantification assays were optimized and validated to measure cytokine expression from elk (*C. elaphus*) PBMCs, primers and probes also were designed, using conserved regions of cDNA, to react with other species of relevant interest, including cattle, sheep, goat, reindeer, white-tailed deer, and bison. For all these species, the immune response target genes examined in this study demonstrated a high degree of homology (>95% when cDNA sequences were available from public databases for comparison). To assess the cross-species application of the method, the assay was tested on Con A–stimulated PBMCs from these additional species. Positive reactivity is indicated in Table 2. For some species and target genes for which 100% homology of primer/probe sets was not possible, mismatches of up to 1 or 2 bp were found to amplify target sequences. For example, the IL-2 primer/probe set contained a single mismatch in the forward primer yet demonstrated positive reactivity for the species listed in Table 2.

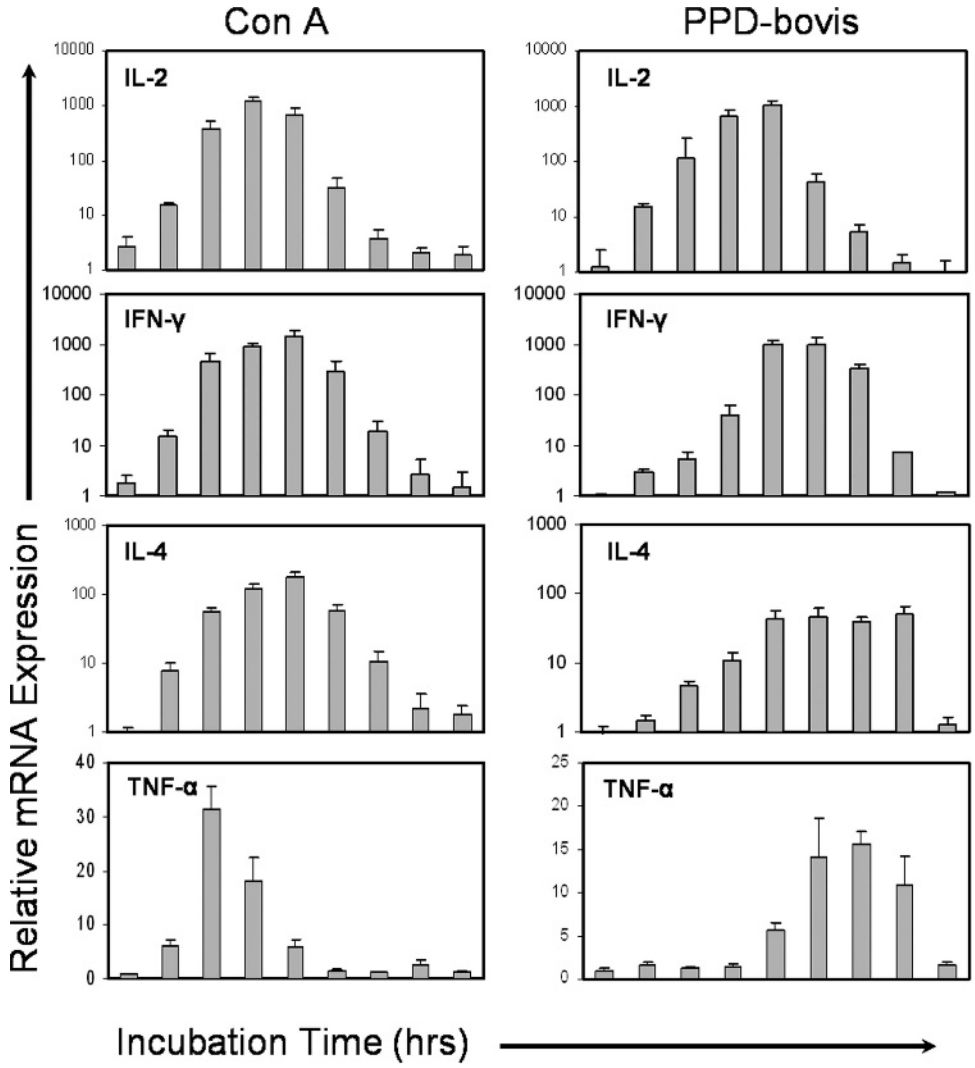

FIGURE 2. Real-time polymerase chain reaction (PCR) analysis of kinetic changes in cervine cytokine gene expression in concanavalin A (Con A)– and bovine-purified protein derivative (PPD-bovis)–stimulated peripheral blood mononuclear cells (PBMCs). Isolated PBMC from six *Mycobacterium bovis*–infected elk hybrids were stimulated with Con A (10 μg/ml) or PPD-bovis (10 μg/ml). The RNA was extracted from these PBMCs at 0, 2, 4, 8, 16, 24, 48, 72, and 96 hr and analyzed by real-time PCR in four independent experiments. The results are expressed as mRNA expression relative to nonstimulated (NS) cells after normalization against β_2-microglobulin. The mean±SEM (error bars) of the four experiments is represented.

Kinetics of mitogen- and antigen-induced cytokine mRNA expression

To determine whether the quantitative real-time RT-PCR method is suitable for analysis of cervine mononuclear cell responses, a kinetic study of mRNA expression for selected target genes (IL-2, IL-4, IFN-γ, and TNF-α) was performed. The PBMCs isolated from six *M. bovis*–infected elk hybrids were stimulated with either mitogen (Con A) or a well-established recall antigen (PPB-bovis). Cell cultures were harvested for mRNA extraction after 0, 2, 4, 8, 16, 24, 48, 72, and 96 hr of mitogen or antigen stimulation.

Figure 2 illustrates the capacity of this system to quantify cytokine mRNAs. Cytokine mRNAs were rapidly and tran-

siently induced after incubation of PBMCs from both the *M. bovis*–infected and noninfected elk hybrids with the mitogen Con A. Cytokine mRNA expression for the different target genes reached a peak at 4 to 16 hr poststimulation, whereas expression was low or less than the detectable level before cultivation.

In comparison with cytokine gene expression induced by polyclonal activation using Con A, the kinetics of induction by antigen (PPD-bovis) was somewhat different for each cytokine. For example, whereas Con A optimally induced IFN-γ mRNA expression as early as 8 hr after in vitro stimulation, 16 to 24 hr were required for optimal induction following PPD-bovis stimulation (Fig. 2). In contrast, the induction of IL-2 mRNA by PPD-bovis was optimal after 8 hr of in vitro stimulation, which closely mirrored the response to Con A. Longer in vitro stimulation times were necessary for the optimal induction of IL-4 and TNF-α mRNA (up to 48 hr). These results indicate that optimal detection of antigen-induced cytokine mRNA depends on both the cytokine being measured and the antigen used for stimulation.

When comparing the amplitude of the response for each cytokine, the induction of IL-2 and IFN-γ mRNA were the most pronounced. The maximum increase of mRNA expression of these two genes in the presence of bovine tuberculin was approximately 1,000-fold, whereas the maximum increase of the other target genes did not exceed 50-fold.

DISCUSSION

With the emergence of infectious diseases, such as tuberculosis and chronic wasting disease, in free-ranging and captive cervids, it has become necessary to develop assays to detect infected animals, and vaccination protocols ultimately may be required to limit the spread of infection. For these advances to occur, however, a better understanding of the host's immune response to infection will be critical. Real-time RT-PCR is a powerful technique for studying cytokine gene expression and is particularly appealing for the study of wildlife and nontraditional domestic species, because it circumvents the current lack of protein-based reagents and assays.

In this study, we designed, optimized, and validated a two-step, real-time RT-PCR for the quantification of cervine cytokine mRNA expression from cultured PBMCs. Assay optimization is as critical for obtaining reproducible results using real-time RT-PCR as it is for conventional methods. The amplification of PCR products was linear over a wide range of input copies (10^1 to $>10^8$) and was sensitive to as few as 10 molecules. The specificity of the assay was virtually guaranteed by the use of three oligonucleotides, including a fluorogenic-bound oligonucleotide complementary to the sequence of the PCR product and localized between two primers that defined the endpoints of the amplicon.

Several factors potentially impact the reproducibility of the RT-PCR reaction, including the RNA extraction process, the variable efficiency of the RT reaction, and the exponential nature of the PCR amplification. This latter factor means that trivial variations in reaction components can greatly influence the final yield of the amplified product (Wu et al., 1991; Bishop et al., 1997). Traditionally, real-time RT-PCR has corrected for differences in input RNA quantity and in the efficiency of cDNA synthesis by normalization to an appropriate housekeeping gene (Karge et al., 1998). The expression levels of such reference genes should, ideally, remain constant between the cells of different tissues and under different experimental conditions (Thellin et al., 1999). If these requirements are not fulfilled, then normalization to varying internal references may lead to erroneous results (Bustin, 2000). Recently, it has become clear that housekeeping genes, such as β-actin and

glyceraldehyde-3-phophate dehydrogenase, may be inappropriate as internal references because of their variability (Bustin, 2002). The choice of β_2-microglobulin as the housekeeping gene for this study was based on invariance demonstrated in previous studies (Hamalainen et al., 2001) and was verified in our experimental system, with an average fold-change of less than two (data not shown). When the reproducibility of the real-time RT-PCR protocol was evaluated, the inter- and intrarun coefficients of variation for the C_t data were less than 6%, which is consistent with those of previous studies (Heid et al., 1996; Gerard et al., 1997). To characterize the RT-PCR variation, an average variation coefficient was calculated over the whole range of the calibration curve, which reflects the realistic PCR variation over the complete quantification range. This variability associated with real-time RT-PCR is significantly better than the 14% reported with conventional RT-PCR (Zhang et al., 1997).

Total RNA can be quantified using either absolute or relative quantification. Absolute quantification, in which the copy number for each mRNA transcript is determined, requires specific in vitro–transcribed RNA standards of known molecular weight to determine the absolute copy number for target RNA transcripts in a sample. This is often useful and convenient when applied to a single marker (Matsumura et al., 2001). Large numbers of such quantitative RNA standards, however, can be unusually difficult and laborious to prepare, control for quality, and maintain (Bustin, 2000). This is relevant to the present situation, in which a panel of different markers was tested. In contrast, relative quantification uses arithmetic formulae to calculate relative expression levels, expressed as an *n*-fold difference relative to the internal control (nontreated) sample, and is adequate for investigating physiologic changes in gene expression levels (Livak and Schmittgen, 2001). Advantages of using this system of quantification are that no standards have to be constructed and that greater throughput can be achieved, thus saving both time and money. The relative quantification approach, however, has certain limitations, including not permitting the comparison of the amount of mRNA between different cytokines. Hence, only values obtained for the same cytokine can be compared among samples.

Our goal is to apply the real-time RT-PCR methodology to the study and diagnosis of wildlife diseases, such as tuberculosis, brucellosis, and chronic wasting disease. It generally is accepted that cell-mediated immunity, including the activation of macrophages, is of greatest importance in the control of intracellular pathogens, such as tuberculosis. Such antimycobacterial immune responses are directed through the production of type-1 cytokines, including TNF-α, IL-12, and particularly, IFN-γ (Raupach and Kaufmann, 2001). Immune responses are utilized in the diagnosis of tuberculosis and have been based on tuberculin skin testing and measurement of a delayed-type hypersensitivity response, which also is mediated by type-1 immunity (Cher and Mosmann, 1987). More recently, the measurement of IFN-γ has been used to establish the diagnosis of tuberculosis in humans and cattle (Katial et al., 2001; Wood and Jones, 2001). As a first step toward applying real-time RT-PCR to the study of cytokine profiles in cervids, we characterized the kinetics of cytokine mRNA expression of selected genes from PBMCs of *M. bovis*–infected elk hybrids that were stimulated in vitro with mitogen (Con A) or the heterogeneous mycobacterial antigen bovine tuberculin (PPD-bovis).

Previous reports concerning polyclonal-stimulated PBMCs pointed to the very early appearance of IL-2, IFN-γ, and IL-12 mRNA but also to their rapid decrease after reaching optimal values (Fan et al., 1998; Stordeur et al., 2003). We observed a similar early induction of cytokine

mRNA following polyclonal activation with Con A. In contrast, the optimal culture times for cytokine mRNA expression following mycobacterial antigen stimulation were, in general, longer and different for each cytokine. Early (4–8 hr) induction of the type-1 cytokine mRNAs of IL-2 and IFN-γ was seen. Following maximal induction, IL-2 mRNA decreased rapidly, whereas IFN-γ mRNA remained high for 48 hr after antigenic stimulation before decreasing. Longer kinetics were observed for the mRNAs of the type-2 cytokine IL-4 and for TNF-α, a cytokine necessary for containment of tuberculosis infection (i.e., granuloma formation) (Flynn et al., 1995).

Low levels for the constitutive expression of most cytokines were detected in freshly isolated PBMCs, and most cytokines were induced at low levels simply by the act of in vitro culture. Similar findings have been reported in other species (Rottman et al., 1995; Dozois et al., 1997). These results emphasize that because of the extreme sensitivity of RT-PCR, cytokine mRNA expression of a sample must always be interpreted in the context of mRNA expression of the same cytokine in a negative control sample (in this case, no stimulation). The specificity of the cytokine mRNA production was high, because the mRNA levels remained low in both nonstimulated cells from the *M. bovis*–infected animals and PPD-bovis–stimulated cells from the noninfected control animals (data not shown). This indicates that only previously primed T cells, and not naïve T cells, are able to respond in this assay.

During the early stages of mycobacterial infections, type-1 immune responses are dominant, but as disease progresses, a shift can occur from type 1 to type 2, with an associated anergy of cellular responses and the development of enhanced humoral responses (Ritacco et al., 1991; Fifis et al., 1994). Because skin testing and IFN-γ–based diagnostics tests are cell-mediated immunity assays, alterations in the immune balance associated with disease progression have implications regarding the diagnosis and control of tuberculosis. Our results underline the importance of determining the kinetics of cytokine mRNA production relative to the antigen of interest if the type-1/type-2 cytokine balance is to be examined for a particular model. The robust IL-2 and IFN-γ mRNA responses in PPD-bovis–stimulated PBMC cultures appear to be promising indicators of mycobacterial sensitization, and mRNA from these cytokines should be extracted after 16 to 20 hr of in vitro stimulation.

Because cytokine production is controlled at both the transcriptional and posttranscriptional levels, the expression of cytokine mRNA does not guarantee the production of a functional protein. Several studies, however, have shown a good correlation between cytokine mRNA expression and protein production or biologic activity (Rottman et al., 1995; Giguere and Prescott, 1999; Listvanova et al., 2003). The recent availability of a commercial ELISA for cervid IFN-γ (Pfizer Animal Health, Kalamazoo, Michigan, USA) would allow this question to be examined for this experimental system. Studies are currently in progress to evaluate the relationship between disease progression and the type-1/type-2 immune bias in the developing antimycobacterial immune response of elk to chronic *M. bovis* infection.

The potential broad value of the approach was demonstrated by the ability to detect cytokine mRNAs from other species (Table 2). This was achieved by designing primer/probe sets using highly conserved coding regions for species selected on the basis of their known or potential infection status under Canadian conditions. Although we did not examine the kinetics of cytokine response to mycobacterial infection in these other species, it is apparent that at least for IFN-γ, as detected by commercial ELISAs (Wood and Jones, 2001; Palmer et al., 2004), the type-1 immune response to

mycobacterial antigens is a well-conserved biologic response.

Recent advances in genomic information and technology have made possible large-scale transcriptional profiles for the main domestic animals of economic importance and their response to infectious challenge. Such a global approach promises to reveal details of the host-pathogen interaction and may lead to the identification of expression "signature" patterns specific for various pathogens. Using bovine cDNA microarrays, recent gene expression profiling studies of cattle infected with *M. avium* subsp. *paratuberculosis* indicates that transcriptional profiles of PBMCs from *M. paratuberculosis*–infected cattle differ from the profiles of PBMCs from uninfected controls (Coussens et al., 2005). This would suggest that the freshly isolated and unstimulated PBMCs from *M. bovis*–infected elk also may have differing levels of mRNA relative to noninfected elk, and this will be explored in future studies. Despite the current limitations of using microarray technology for nontraditional and wildlife species, such studies will undoubtedly suggest new candidate genes for exploring the immune response by quantitative real-time RT-PCR.

In summary, a convenient and reproducible method for quantifying mRNA expression of numerous cervine cytokines by real-time PCR was developed and validated. The technique was fast, sensitive over a large dynamic range, and suitable for analyzing cytokine mRNA present in small volumes, such as that from cell culture systems. This assay was used to measure changes in mRNA levels from PBMCs after polyclonal and antigen-specific activation, thus demonstrating that it could be used to monitor immune responses in vitro. The RT-PCR described here could be modified readily to quantify the mRNA expression of other wildlife and nontraditional species. The technique will be particularly relevant to researchers wishing to quantify cytokine mRNA for which protein-based reagents are not yet available, and it should facilitate disease diagnosis and characterization of molecular events and their kinetics during both acute and chronic infections.

ACKNOWLEDGMENTS

The study described in this paper was funded by the Canadian Food Inspection Agency. The authors acknowledge the assistance Jim Algire and the Animal Care Staff for blood collection and animal handling of elk/red hybrids at the Ottawa Laboratory Fallowfield. We also acknowledge the Parks Canada Agency staff from Riding Mountain National Park for blood collection and animal handling of free-ranging elk. The technical assistance of Wendy Monagle, Anna Romanaowska, and Erin Trottier is appreciated.

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Received for publication 31 March 2005.