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Quantitation of Cytokine mRNA Expression in Cobra Snake Venom Stimulated PBMC of Horses Using Real-Time RT-PCR

Surasak Akesowan, Sunutcha Suntrarachun, Suchitra Khunsap and Wichit Thaveekarn
Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, 10330, Thailand

Corresponding Author: Sunutcha Suntrarachun, Department of Research and Development, Queen Saovabha Memorial Institute, Thai Red Cross Society, 1871 Rama IV Road, Patumwan, Bangkok, 10330, Thailand Tel: 66-2-2520161-4/161 Fax: 66-2-2540212

ABSTRACT

Characterization and quantification of cytokine production is essential for understanding the immune response. A SYBR Green real-time quantification Reverse Transcriptase-Polymerase Chain Reaction (RT-qPCR) was used to measure mRNA expression levels of the most common cytokines in horses after cobra snake venom stimulated Peripheral Blood Mononuclear Cells (PBMC) carried out by *in vitro* method. The PBMC from horse was stimulated with cobra snake venom (10 μg mL^{-1}) prior to incubate at 3, 6, 24 and 48 h. Cytokine mRNA expression level, IL-1β, IL-10, IFNγ and TNFα, were measured using SYBR Green real-time RT-PCR. The highest mRNA expression of IL-1β and IL-10 were demonstrated at 3 h after stimulation of cobra snake venom. The levels of IL-1β and IL-10 expression were 0.7 and 2.3 folds. Meanwhile, the mRNA expression of IFNγ and TNFα were peaked at 6 and 24 h with appreciable increment of 13 and 5.4 folds, respectively. However, the mRNA expression of these cytokines was decreased after 24 h. It seems likely that cobra snake venom could stimulate more mRNA expression of IFNγ than IL-1β, IL-10 and TNFα in PBMC of horse. Secretion of IFNγ is likely to be important in early host defense against pro-inflammation and tissue injury from cobra snake venom. Thus, the study of mRNA expression of inflammatory cytokine profiles in this model could provide information useful for understanding the immunopathological mechanism of inflammation from snake venom immunization in horses and led to improve the design and manufacture of snake anitvenom.

Key words: Cytokines, mRNA expression, cobra snake venom

INTRODUCTION

Immune system is responsible for defending the host against pathogenic infection through innate and adaptive immune response. Cytokines are regulatory proteins, which play an important role in the immune system by controlling lymphocyte activation, proliferation, differentiation, survival and apoptosis. Therefore, characterization and quantification of cytokine production is essential for understanding the immune process. They may be useful as immunological markers to understand pathogenesis of the diseases, inflammatory reactions, autoimmune diseases and transplant rejection (Allen *et al*., 2007). Cytokines are low molecular weight and secreted by many different cell types such as lymphocytes, monocytes, antigen-presenting cells, endothelial cells and fibroblasts. They can be classified into different groups including interleukins (IL-1-IL-23), interferons (IFNα and IFNγ), colony-stimulating factors, Tumor Necrosis Factors (TNFs), Tumor Growth Factors (TGF-β) and chemokines (MCP-1, MIP-1) (Giulietti *et al*., 2001; Huggett *et al*., 2005).

Various technologies are routinely applied for cytokine detection and quantification in tissues and blood samples. However, quantification of cytokine at the protein level in tissue samples is too small for detection. Cytokine ELISA kits used for the qualitative and quantitative assessment of cytokines from various biological samples, such as serum or plasma are expensive. Quantification of mRNA expression, real-time quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-qPCR), is becoming the technique of choice to detect low amounts of mRNA copies, because of the exponential increase of the template during thermal cycling. The most widely used methods for fluorescence monitoring of PCR products are intercalating dyes such as SYBR Green and specific probes like TaqMan. Fluorescence monitoring of PCR products eliminates post-PCR analysis of product, removing the errors and time associated with these steps. SYBR Green can be sensitive, reproducible as specific probe and less expensive than specific probes (Forlenza *et al*., 2012; Godornes *et al*., 2007).

The aim of this study was to develop and evaluate a set of SYBR Green RT-qPCR method for detecting cytokines in horses that would provide insights into innate and adaptive immune response. Peripheral Blood Mononuclear Cells (PBMC), from healthy horse, were experimentally induced by snake venom such as cobra venom (*Naja kaouthia*) by using lipopolysaccharide (LPS) as positive control. Various cytokines were selected from their roles in the inflammatory response (IL-1β, TNFa and IFNγ) and in the activation of cellular immunity (IFNγ) and humoral immunity (IL-10). Cytokine mRNA expression level was measured by SYBR Green real-time RT-PCR. The results of this study should provide a basis of future work aimed at understanding the immune response to snake venom in horses and leading to improve the production of snake anitvenom.

MATERIALS AND METHODS

Isolation and stimulation of horse PBMC: Whole blood from a single healthy horse was collected in EDTA collection tubes. Separation of PBMC was performed by Ficoll-Paque™ Plus (GE Healthcare, Uppsala, Sweden). Cell viability was assessed by trypan blue method and PBMC was resuspended in 1640 RPMI (10% fetal bovine serum). The PBMC was cultured 24 h, stimulated at 37°C and 5% CO_2 with various concentrations (1, 10 and 100 μg mL^{-1}) of cobra snake (*Naja kaouthia*) venom and lipopolysaccharide (LPS) (Sigma) as positive control. The optimal concentration of cobra snake venom and LPS was chosen based on the percentage of the viable cells. Different incubation periods, 3, 6, 24 and 48 h, were performed after stimulation with cobra snake venom and LPS. The PBMC without stimulation was negative control.

RNA extraction and reverse transcription: The RNA was extracted from PBMC culture using Trizol reagent (Molecular Research Center, Inc, Cincinnati, Ohio, USA). First strand cDNA was synthesized using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc, USA) following the kit protocol. The reaction volumes were 12 μL, consisting of 1 ng total RNA, 1 μL Oligo (dT) 18 primer and nuclease free water were incubated at 65°C for 5 min and chilled on ice. Then, the following components were added 4 μL 5x Reaction buffer, 1 μL RiboLock RNase Inhibitor (20 U μL^{-1}), 2 μL 10 mM dNTP Mix and 1 μL RevertAid M-MuLV RT (200 U μL). The total reaction volumes were 20 μL with the initial stage of 42°C for 60 min, 70°C for 5 min and 4°C for 30 min. The cDNA was stored at -20°C until use.

Oligonucleotide primers: Primers for cytokine genes were designed based on the sequences of horse cytokines deposited in GenBank: IL-1β, accession NM_001082526.1, IL-10, accession

Table 1: Oligonucleotide primers of IL-1β, IL-10, IFNγ, TNFα and β-actin for DNA sequencing (Designed based on NCBI GenBank database)

Primers	Nucleotides (5'→3')	Product size (bp)
IL1β-F	5'TGTACCTGTCTTGTGGGATGAAA 3'	184
IL1β-R	5' TTCTGCTTGAGAGGTGCTGA 3'	
IL10-F	5' GTCATCGATTTCTGCCCTGT 3'	180
IL10-R	5'GCTTCGTTCCCTAGGATGC 3'	
IFNγ-F	5' TGGACACCATCAAGGAGGAC 3'	107
IFNγ-R	5'GGACCTTCAGATCATTTACCG 3'	
TNFα-F	5' TTACCGAATGCCTTCCAGTC 3'	271
TNFα-R	5' GGGCTACAGGCTTGTCACTT 3'	
β-actin-F	5' ACCAACTGGGACGACATGGACAA3'	380
β-actin-R	5' GTGGTGGTGAAGCTGTAGCC 3'	

NM_001082490.1, IFNγ, accession EU000433.1 and TNFα, accession M64087.1. Primers were designed and optimized using Primer 3. Four primer pairs were selected with the annealing temperature 55°C, primer length 18-24 bp and amplification products 100-300 bp (Table 1).

Real-time quantification PCR: A quantitative polymerase chain reaction assay (Real-time RT-PCR) was carried out with Light Cycler (ESCO, Swift Spectrum 48 Real Time Thermo Cyclers) using RBC ThermOne Real-Time Premix (SYBR Green) for cytokine gene expression in horses. One microliter of cDNA was added to 24 μL of reaction mixture including 2x Thermone premix, 10 pmol of each primer and nuclease free water. The reaction was performed under the following conditions: 95°C, 10 min followed by 40 cyclers of 94°C for 20 sec, 60°C for 30 sec and finally 72°C for 15 sec. This was followed by melting curve analysis. The housekeeping gene, β-actin, was amplified under the same condition as the protocol above.

Data analysis using the $2^{-\Delta\Delta Ct}$ method (Livak method): At the end of the PCR, the ESCO Swift Spectrum 48 software saved the results, allowing instant manipulation or storage of the data for further analysis. A widely used method to present relative gene expression is the comparative threshold cyclers (Ct) method also referred to as the $2^{-\Delta\Delta Ct}$ method (Forlenza *et al*., 2012). Relative quantification analysis determines the levels of expression of a Gene Of Interest (GOI) and expresses it relative to the levels of an internal control or Reference Gene (RefG). The $2^{-\Delta\Delta Ct}$ method, where, ΔΔCt = ΔCt (Calibrator)-ΔCt (Sample). The ΔCt (Calibrator) = Ct (GOI, Calibrator)-Ct (RefG, Calibrator) and ΔCt (Sample) = Ct (GOI, Sample) -Ct (RefG, Sample). Calibrator is usually an untreated sample. Genes of interest in this study are cytokine genes including IL-1β, IL-10, IFNγ and TNFα. Reference genes are genes that are not affected by the treatment in anyway and are constant under the tested conditions such as b-actin house keeping gene.

RESULTS

In this study, the optimal concentration of cobra snake venom (10 μg mL^{-1}) and LPS (1 μg mL^{-1}) was chosen based on the viability of the PBMC. Cell viability of PBMC was determined by trypan blue dye exclusion resulted in at least 90% of the cells recovered remaining viable after cobra snake venom and LPS stimulation. The results presented an appreciable increase in the IL-10 and IL-1β expression in the early stage of 3 h stimulation at the concentration of 10 μg mL^{-1} cobra snake venom (Fig. 1 and 2). Under this condition, IL-10 and IL-1β mRNA were dramatically increased 2.3 and 0.7 folds, respectively. Meanwhile, the expression level of TNFα and IFNγ were peaked at 24 and 6 h with 5.4 and 13 folds of mRNA expression (Fig. 3 and 4). The results showed that IL-1β,

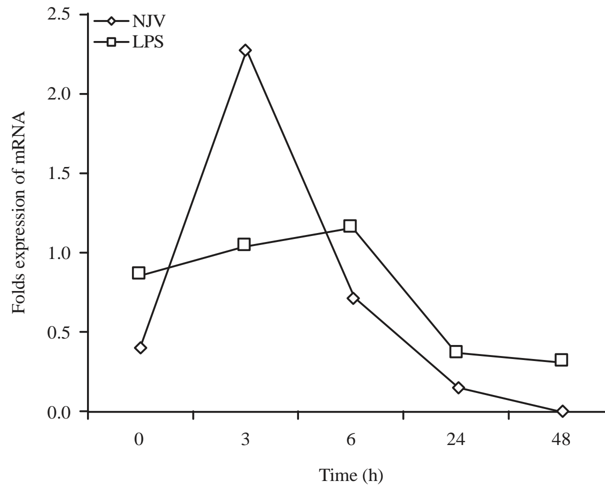

Fig. 1: Relative quantification using real-time RT-PCR for IL-10 cytokine mRNA expression of horse PBMC after stimulation with cobra venom (NJV) and LPS at 0, 3, 6, 24 and 48 h

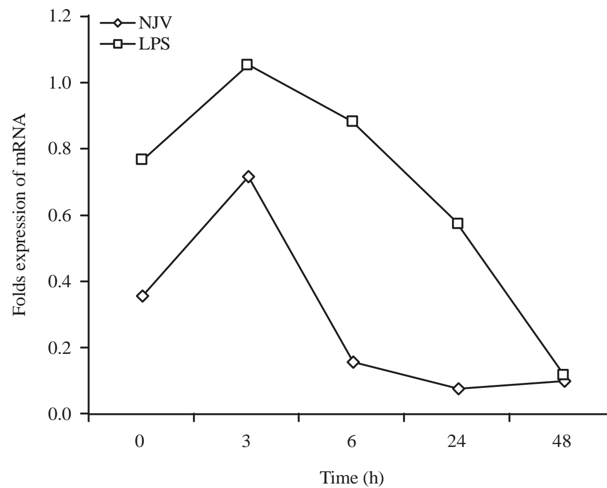

Fig. 2: Relative quantification using real-time RT-PCR for IL-1β cytokine mRNA expression of horse PBMC after stimulation with cobra venom (NJV) and LPS at 0, 3, 6, 24 and 48 h

IL-10 and TNFα were expressed at low levels, less than 10 fold expression of mRNA, after the cobra snake venom stimulation compared with 13 fold mRNA expression of IFNγ. It seems likely that the mRNA expression of all cytokine genes was increased or up-regulation based on time dependent. However, the expression level of these cytokines was decreased after 24 h of induction with cobra snake venom.

DISCUSSION

Snake venom contains more than 20 different compounds, mostly proteins and polypeptides. A complex mixture of proteins, enzymes and various other substances with toxic and lethal properties serve to immobilize the prey animal. Some of the proteins in snake venom have very

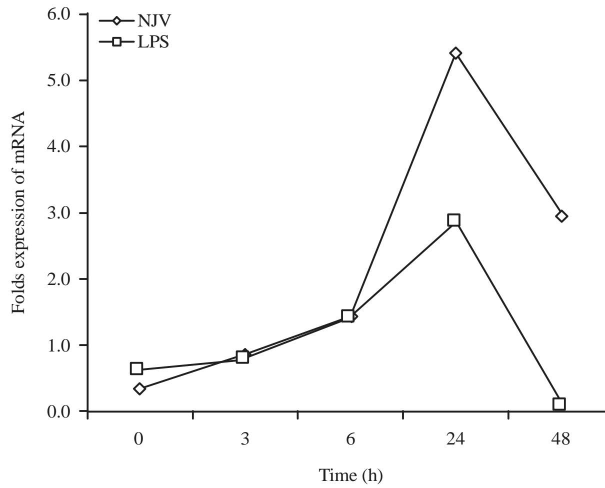

Fig. 3: Relative quantification using real-time RT-PCR for TNFα cytokine mRNA expression of horse PBMC after stimulation with cobra venom (NJV) and LPS at 0, 3, 6, 24 and 48 h

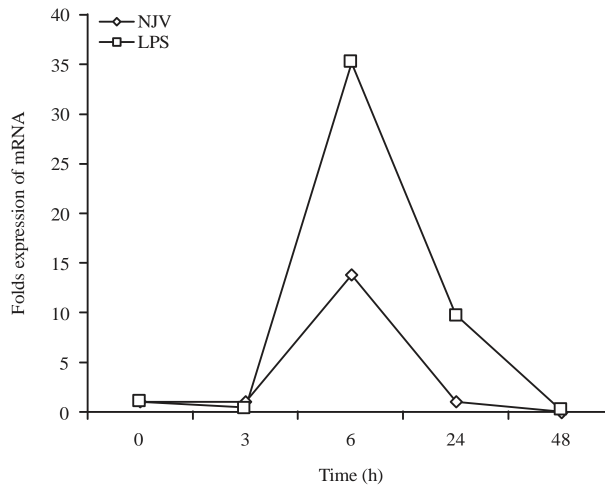

Fig. 4: Relative quantification using real-time RT-PCR for IFNγ cytokine mRNA expression of horse PBMC after stimulation with cobra venom (NJV) and LPS at 0, 3, 6, 24 and 48 h

specific effects on various biological functions including blood coagulation, blood pressure regulation and transmission of the nervous or muscular impulse. These proteins have been developed for use as pharmacological or diagnostic tools or even useful drugs. Snake anitvenom immunoglobulins are the only specific treatment for envenoming by snake bites but also carrier risk of severe allergic reactions such as anaphylaxis. Snake anitvenom production involves the immunization of animals, usually horses with venoms from a single or various snake species. Envenomation by snakes involves the activation of the inflammatory response with the release and activation of pro-inflammatory cytokines. Cytokines are important for the interactions between cells in the immune and inflammatory responses. Cytokines initiate a cascade of events that lead to illness behaviors such as fever, anorexia and as well as a host of physiologic events such as activation of vasodilation and hypotension. The measurement of cytokine expression is fundamental to

characterizing the inflammatory responses. The most widely used to quantify cytokine expression is Real-Time RT-PCR because it only required threshold cycler (Ct) numbers and estimated PCR amplification efficiencies of both target and housekeeping gene in control and treated samples. The relative fold change of gene expression is normalized through a housekeeping gene to account for minor differences that exist in starting amount of RNA, quality of RNA, as well as RT and PCR amplification efficiencies. The accurate estimation of PCR amplification efficiency has a tremendous impact on mRNA quantification. Thus, a constant challenge for accurate relative mRNA quantification through real-time RT-PCR was to determine a stable housekeeping gene (Giguere and Prescott, 2000). For cultured PBMC, the expected ΔCt (difference in Ct of a housekeeping gene between control and stimulated PBMC) should be near 0 indicating a good stability of the housekeeping gene. b-actin is expressed among almost all cell types. It is widely used to normalize results in real-time RT-PCR. It should not be affected by the treatment in any way and are constant under the tested conditions (Salyers and Whitt, 1994; Wagner and Freer, 2009). Hence, β-actin was chosen as house keeping gene or reference gene in this study.

This study presents the validation of real-time RT-PCR based on SYBR Green to quantify the relative mRNA expression of cytokine genes in PBMC of horse after cobra snake venom stimulation. This method allows rapid quantification of low concentrations of DNA. Real-time RT-PCR could be characterized the expression of a wide range of cytokines involved in the immune response of horses. The cytokines were selected based on their roles including IL-1β and TNFα which play role in inflammatory response, IFNγ in the activation of cellular immunity and IL-10 in humoral immunity. The cytokine gene expression occurred by stimulating PBMC indicated the type of immune response that has been activated. This information is useful for establishing immunological markers of inflammation, infection and also can be used to develop the effective immunization and therapies (Overbergh *et al*., 1999).

Cobra snake venom could stimulate more mRNA expression of IFNγ than IL-1β, IL-10 and TNFα in PBMC of horse. Secretion of IFNγ is likely to be important in early host defense against pro-inflammation and tissue injury from cobra snake venom. The IFNγ was produced by T helper cell, cytotoxic T lymphocyte and NK cell. T lymphocytes become the major source of IFNγ in the adaptive immune response (Odbileg *et al*., 2005; Overbergh *et al*., 2003; Ramos-Payan *et al*., 2003). Other inflammatory cytokines, including IL-1β, IL-10 and TNFβ, are also important in pro-inflammatory response and tissue injury after inflammation and infection. The release of cytokines can stimulate B lymphocytes to proliferate and differentiate into memory B cells and plasma cells. Plasma cells generate the isotype of antibodies. During a first envenomation, anitvenom IgG appears after anitvenom 1 g antibodies but reach higher plasma concentration which persist long time after envenomation. After a second exposition to the same snake venom, memory B cells are activated and produce IgG in levels that increase faster and reach higher titers than those occurring after the first venom exposition (Leon *et al*., 2011).

Previous studies (Chaves *et al*., 2005; Clissa *et al*., 2001), on the quantification of inflammatory cytokines induced by snake venoms, were using Cytokine ELISA assay. Cytokine ELISA assay required high volumes of serum or plasma compared to the relative quantification of cytokine gene expression using Real-Time RT-PCR. Moreover, cytokine ELISA kits were more expensive than individual reagents for SYBR Green reagent for Real-Time RT-PCR. Some studies on inflammatory cytokines and immune response to snake venoms, using Real-Time quantitative RT-PCR, have been reported only from the poisonous snakes of Latin America such as *Bothrops* spp. and *Crotalus* spp. (Petricevich *et al*., 2000; Favoretto *et al*., 2011). Sanchez-Matamoros *et al.* (2013) have developed

SYBR Green Real-Time RT-PCR assay for evaluation of cytokine gene expression in horse. This method gave good efficiency for all cytokine genes tested in response to exposure to economically important pathogens. However, none of the studies have been reported concerning the measurement of cytokine mRNA expression in animals affected by Southeast Asia's snakes, such as cobra snake (*Naja kaouthia*), which is widely distributed in Southeast Asia. This study demonstrated for the first time, the determination of the cytokine mRNA expression upon cobra snake venom stimulation of PBMC from horse. It could be possible that mRNA expression of inflammatory cytokine profiles in this horse model will provide information useful for understanding the immunopathological mechanism of inflammation from snake venom immunization in horses. The results of this study provided a basis of future work aimed to improve the production of snake antivenom.

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