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Acta Scientiae Veterinariae, vol. 42, núm. 1, enero, 2014, pp. 1-5
Universidade Federal do Rio Grande do Sul
Porto Alegre, Brasil

Acta Scientiae Veterinariae,
ISSN (Printed Version): 1678-0345
ActaSciVet@ufrgs.br
Universidade Federal do Rio Grande do Sul
Brasil

Acta Scientiae Veterinariae, 2014. 42: 1198.

RESEARCH ARTICLE
Pub. 1198

ISSN 1679-9216

Development and Validation of an Efficient Real-time Quantitative Reverse Transcription Polymerase Chain Reaction Assay of Chinese Goose CD4 and CD8α

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ABSTRACT

***Background*:** CD4+ T cells, which are often referred as T-helper cells, play a central role through secreting various cytokines to enhance immune defense to pathogen. CD8+ T cells, which are called cytotoxic T lymphocytes (CTLs), provide potent defenses against virus infection and intracellular pathogens by killing the targets cells directly. In our previous researches, the conventional and semi-quantitative PCR were used to detect the goose CD4 and CD8α. However, the semi-quantitative RT-PCR only detect the relative amount of gene transcription. Quantitative PCR assay was more sensitive than conventional PCR assay, and quantitative PCR assay has a lower limit of sensitivity.

***Materials, Methods & Results*:** Contrast to conventional assays, the detection of amplicons by quantitative RT-PCR could be visualized as the amplification progressed. This effect has provided a great deal of insight into the kinetics of the reaction and it is the foundation of kinetic of real-time qPCR. The analysis of gene transcription by qPCR has proven to be an attractive method due to its potential for increasing laboratory throughput, simultaneous processing of several samples as well as more reliable instrumentation. With those in mind, the real-time quantitative reverse transcription PCR (qRT-PCR) methods for the detection of goose CD4 and CD8α transcripts were reported here for the first time. With this assay, it is possible to carry out a rapid quantitative analysis of goose CD4 and CD8α transcripts over a wide linear range, with an unknown template.CD8 is expressed on the membrane of T cells either as an αα-homodimer or αβ-heterodimer. Since both forms of CD8 have α chain, the transcription levels of CD8 can be monitored by detecting CD8α mRNA expression. Assays were based on the DNA sequence of goose CD4 [GenBank: JX902315], CD8α [GenBank: KC476104], and β-actin [GenBank: M26111]. qPCR was carried out in quadruplicates in a total volume of 20 μL containing 10 μL Ssoadvanced™ SYBR®, 1.5 μL of cDNA, 0.6 μl of each primers (10 μM), and 7.3 μL of sterile water. The amplification program was 94°C for 3 min, followed by 40 cycles of 94°C for 10 s, the annealing temperature of each pair primers for 30 s. The annealing temperatures for real-time qPCR were 61.4°C for CD4 and CD8α, 60°C for β-actin, respectively. After amplification, melting analysis of qPCR revealed specific amplification of each gene that could be visualized clearly as a single peak of melting temperature. The R2 values of the standard curves for all target genes were above 0.999. To further apply the methods we established, the specific real-time qRT-PCR methods were performed for the detection of the biology activity of CD4 and CD8α in the spleen mononuclear cells (MNCs). Fresh spleen monocytes cells (MNCs) were chosen as the responder, PHA was chosen as agonist, and the transcripts of CD4 and CD8α mRNA in spleen MNCs after stimulation were investigated by qRT-PCR assay. Results documented that the transcriptions of goose CD8α and CD4 were significantly up regulated by phytohaemagglutinin (PHA) *in vitro*.

***Discussion*:** This paper expands the application of qPCR to analyze the mRNA transcription profiling of goose CD4 and CD8α, The qRT-PCR method established in this study may provide a better way for the further research on the immunological and biological activity of goose CD4+ and CD8+ T cell *in vitro* and *in vivo*, as well as for the study on gene expression during disease.

Keywords: chinese goose, CD4, CD8α, real-time quantitative PCR.

Received: 25 January 2014 Accepted: 16 June 2014 Published: 23 June 2014

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INTRODUCTION

The cell-mediated immune response is mediated by T lymphocytes. T-cell activation is initiated by the recognition of major histocompatibility complex (MHC) with the T-cell receptor (TCR) [7]. CD4+ T cells respond to antigen in association with MHC class II molecules and CD8+ T cells respond to antigen in association with MHC class I molecules [3].

We have reported the cloning, tissue distributions, and immunological characteristics of goose CD4 [9] and CD8α [10] *in vivo* previously. In this study, to detect the transcription profiling of goose CD4 and CD8α gene, the rapid and specific real-time quantitative polymerase chain reaction (qRT-PCR) methods were established.

Similar to mammal and chicken lymphocytes, duck lymphocytes can be artificially stimulated by phytohaemagglutinin (PHA) [2]. Furthermore, the biological activity of goose CD4 and CD8α in spleen mononuclear cells (MNCs) were studied after incubated with PHA.

The methods established here make it available to further research on immunological functions of goose CD4 and CD8α.

MATERIALS AND METHODS

Preparation of standard plasmid DNA

The goose CD8α primers amplified an expected 543 bp PCR product which covered nucleotide positions 48 to 590 of the goose CD8α gene (GenBank accession no. KC476104), The CD4 product was 127 bp in length which represented nucleotide positions 937 to 1063 of goose CD4 gene (GenBank accession no. JX902315), and the β-actin product was 446 bp in length which represented nucleotide positions 353 to 798 of goose β-actin gene (GenBank accession no. M26111). The PCR products were ligated into plasmid vectors and confirmed by nucleotide sequencing. The concentrations of recombinant plasmids were determined by taking the absorbance at 260 nm using DU800 Nucleic acid/Protein analyzer[1], and the purity was confirmed using the 260 nm /280 nm ratio [6].

Establishment of the qPCR standard curve

The OD value of the recombinant plasmid was obtained by measuring the absorbance at 260 nm, and then used that to calculate its copy number. Then, the purified plasmid DNA was diluted using sterilized double distilled water to obtain the final plasmid concentration of 1 fmol/uL. Subsequently, the plasmid was serially 10-fold diluted. These dilutions were tested in duplicate and used as the quantification standards to construct the standard curve by plotting the plasmid copy number logarithm against the measured quantification cycle (Cq) values. In addition, the correlation coefficient (R^2) and efficiency of the standard curve were calculated by using the Bio-Rad detection soft ware.

The qPCR for detection of Chinese goose CD8α and CD4

The transcription levels were determined by qPCR using Bio-Rad CFX96 Real-Time Detection System (Bio-Rad CFX96, USA) with the primers of CD8α-F and CD8α-R for CD8α, CD4-F and CD4-R for CD4, β-actin-F and β-actin-R for β-actin (listed in Table 1). qPCR was carried out in quadruplicates in a total volume of 20 μL containing 10 μL Ssoadvanced™ SYBR® Green Supermix[2], 1.5 μL of cDNA, 0.6 μL of each primers (10 μM), and 7.3 μL of sterile water. The amplification program was as follows: 94°C for 3 min, followed by 40 cycles of 94°C for 10 s, 61.4°C (CD8α and CD4) for 30 s (60°C for β-actin). After the amplification phase, a melting curve program (65°C to 95°C with a heating rate of 0.5°C per seconds and a continuous fluorescence measurement) was routinely performed.

Goose β-actin was amplified as the internal control. To exclude the template contamination, the control was performed using H_2O as the template. To evaluate and avoid the variability between each qRT-PCR experiments, the β-actin amplification products were diluted and used as qRT-PCR templates in every reaction, which worked as a board-to-board reference. The cubicle reference data was marked in the same name for calculation to eliminate error between the plates.

Analyzing immunological activities of chinese goose CD4 and CD8α by the established qPCR methods

Preparation of goose spleen MNCs have been described previously [15]. After incubating at 37°C under 5% CO_2 overnight, the cells were stimulated with PHA (50 ug/mL and 100 ug/mL)[3] and collected at 24 h after stimulation. Total RNA of cells were extracted by TRIzol Reagent[4] and reverse transcription was performed using superscript II reverse transcriptase[5]. The transcription levels of target genes were determined by qPCR using the Bio-Rad CFX96.

Statistical analysis

All samples were repeated four times and the data are presented as means with standard deviations. Data were analyzed on a personal computer using Bio-Rad CFX Manager Software and Excel 2003. To avoid DNA contamination, all primers for qPCR were designed as intron-spanning primers.

RESULTS

The melting curve program (65°C to 95°C with a heating rate of 0.5°C per second and a continuous fluorescence measurement) was routinely performed to confirm the presence of the single PCR product (Figure 1). The Ct value of one template, which represents four replications, has a difference of less than 0.25. There were no amplified products observed in the negative control. By using standard templates ranging in concentration from 1.0 × 10-1 to 1.0 × 10-5 copies, the accurate results for a series of samples were obtained, and the data was used to generate the standard curve with the Bio-Rad software (Bio-Rad; USA). The R2 of the associated standard curve was 0.998 and PCR efficiency was 90.1% for CD8α, 0.999 and 93.5% for CD4, 0.994 and 110% for β-actin, which indicated that the crossing threshold values for the standards fell within an accurate range. The slopes were -3.488, -3.581 and -3.104, respectively (Figure 2). Therefore, the $2^{-\Delta\Delta}$Ct methods used for quantitative analysis of goose CD8α and CD4 gene transcripts were valid in this study [1].

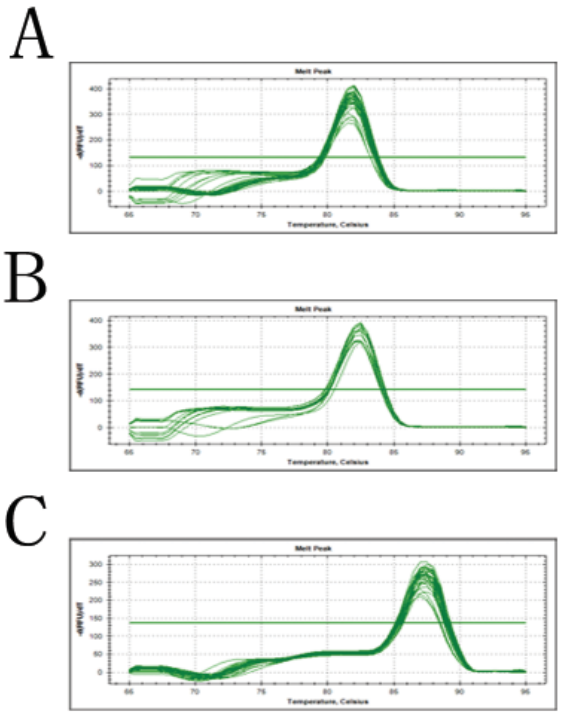

Figure 1. The melting curves and specificity of the qPCR methods. (A): CD4; (B): CD8α; (C): β-actin. By analyzing the melt curve, a single PCR product of qPCR had been confirmed.

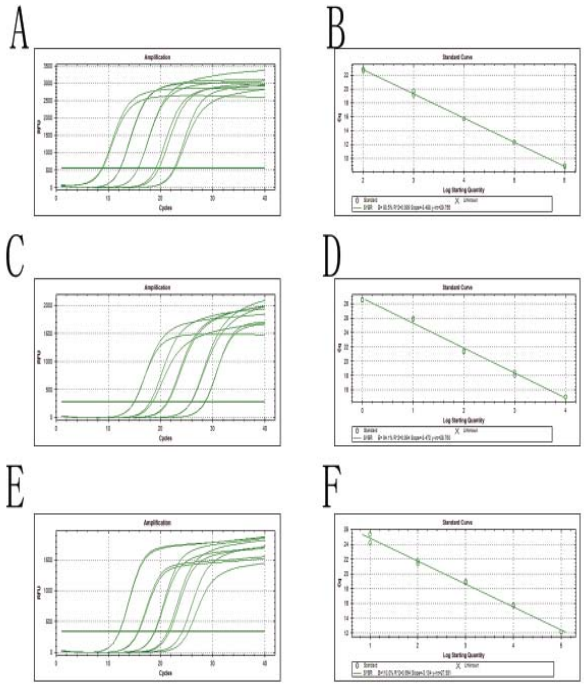

Figure 2. The amplification curves and standard curve of qPCR. Ten-fold dilutions of standard DNA were used, as indicated in x-axis, whereas the corresponding threshold cycle numbers (Ct values) are presented on y-axis. Each curve represents the result of amplifications of each dilution. The correlation coefficient and the slope value of the regression curve were calculated and indicated. (A) and (B) was for CD4; (C)and (D) were for CD8α; (E) and (F) were for β-actin.

DISCUSSION

The qPCR is an attractive method to study gene transcriptional levels because of its low inter- and intra-assay variability and its equivalent or greater analytical sensitivity compared with traditional methods [5]. And the qPCR is a powerful tool for quantitative analysis of target cDNA due to its ease of performance, together with excellent specificity and rapid, more reliable instrumentation and improved protocols [4]. The method is based on the detection of a fluorescent signal produced and monitored during the amplification process, without the need for post-PCR processing, which makes the procedure short, and decreases the risk of contamination. Here, we described a real-time qPCR assay to detect the transcription levels of chinese goose CD4 and CD8α. Quantification of goose CD4 and CD8α transcripts is essential for gaining more insight into the biological activity and immune function of these genes.

In this study, qPCR was performed to monitor the kinetics of goose CD4 and CD8α gene transcripts *in vitro*. The optimized qPCR reaction system was used to detect the relative transcription levels of CD4 and CD8α in goose spleen MNCs after incubation with PHA. Results demonstrated that the goose CD4 and CD8α mRNA expression were significantly up-regulated ($P < 0.05$) by 50 μg/mL and 100 μg/mL PHA after incubation for 24 h (Figure 3) which is similar to studies reporting PHA-mediated lymphocyte proliferation in duck and in mammals [8].

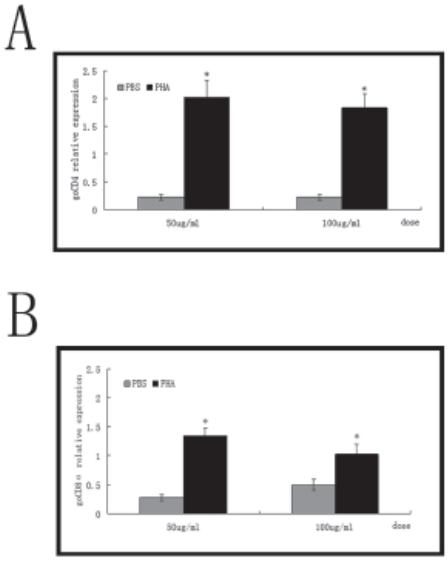

Fig 3. Effects of PHA on CD4 (A) and CD8α (B) mRNA transcriptions in goose spleen MNCs. The cells were treated with PHA for 24 h. CD4, CD8α and β-actin mRNA levels were quantified by qRT-PCR. CD4 and CD8α mRNA expression was normalized by β-actin and expressed as fold change compared with the mean value of the control group without drug treatment. Data presented are expressed as Mean ± SEM (n = 4), and groups denoted by stars (*) represent a significant difference at $P < 0.05$ (ANOVA followed by Fisher's LSD test). *$P < 0.05$ versus the time-matched group.

CONCLUSIONS

In summary, efficient methods for the quantization assay of goose CD4 and CD8α mRNA level have been established, and have been successfully applied in measuring goose CD4 and CD8α transcript levels in PHA–stimulated lymphocytes. Evidently, the assay described here for quantifying CD4 and CD8α gene transcriptions, which can be applied in the further researches on the molecule immunology of goose.

SOURCES AND MANUFACTURERS

[1]Beckman Coulter, Brea, CA, USA.

[2]Bio-Rad, Hercules, CA, USA.

[3]L1668, Sigma-Aldrich, St. Louis, MO, USA.

[4]Invitrogen, Carlsbad, CA, USA.

[5]Promega, Madison, WI, USA.

***Authors' contributions*.** SC and FL conceived, designed and performed the whole experiment. QZ and YQ performed the experiments of verification. YQ analyzed the data. RJ, ML, DZ and XC contributed reagents/materials/analysis tools. QZ and SC drafted the manuscript. MSW and ACC revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

***Funding*.** This work was funded by National Natural Science Foundation of China (31201891), Sichuan Provincial Cultivation Program for Leaders of Disciplines in Science (2012JQ0040), Major Project of Education Department in Sichuan Province (12ZA107), Innovative Research Team Program in Education Department of Sichuan Province(No.12TD005), Science and Technology Support Programs of Sichuan Province,China (2011JO0040, 2011ZO0034) and China Agricultural Research System (CARS-43-8), and Sichuan province research program (2013TD0015).

***Ethical approval*.** All experiments were carried out in strict accordance with the Regulation of Animal Experimentation of Sichuan province, China following Regulation on the Management of Lab Animal Quality Control (MOST 1997-593), which is similar to the Institutional Animal Care and Use Committee (IACUC). All experiments were performed in accordance with animal ethics guidelines and approved protocols.

***Declaration of interest*.** Authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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