The Thai Journal of Veterinary Medicine

Volume 47
Issue 1 *March, 2017*

Article 4

3-1-2017

Sequencing characterization and expression analysis of interferon gamma (IFN-ɣ) in Rusa deer (Rusa timorensis) and Thai cervidae

Sirinart Chaichanathong

Worawidh Wajjwalku

Manakorn Sukmak

Boripat Siriaroonrat

Tarasak Nipanunt

See next page for additional authors

Sequencing characterization and expression analysis of interferon gamma (IFN-γ) in Rusa deer (Rusa timorensis) and Thai cervidae

Authors

Sirinart Chaichanathong, Worawidh Wajjwalku, Manakorn Sukmak, Boripat Siriaroonrat, Tarasak Nipanunt, and Nikorn Thongtip

Short Communication

Sequencing characterization and expression analysis of interferon gamma (IFN-γ) in Rusa deer (*Rusa timorensis*) and Thai cervidae

Sirinart Chaichanathong[1,2] Worawidh Wajjwalku[1,3] Manakorn Sukmak[4]

Boripat Siriaroonrat[5] Tarasak Nipanunt[6] Nikorn Thongtip[1,7*]

Abstract

To date, the interferon gamma (IFN-γ) assay has become an important diagnostic tool which is applied alternatively to define infection of intracellular pathogens. In this study, the molecular characteristics of IFN-γ in Rusa deer (*Rusa timorensis*), Hog deer (*Axis porcinus*), Sambar deer (*Rusa unicolor*) and Eld's deer (*Rucervus eldii*) were determined. Among the cervid family, the analysis of IFN-γ cDNA sequences indicated 5 polymorphism nucleotide positions. Deduced IFN-γ amino acid compositions among the cervid group were negligibly different (98-100% homology). Preliminary quantification of IFN-γ mRNA expression in the cervidae by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) was demonstrated. qPCR primers were designed and tested for their ability to quantify IFN-γ mRNA with a suggested detection limit at 10^3 target molecules. Intra- and inter-assay variations of RT-qPCR for IFN-γ were 1.41 and 4.57, respectively. Expression ratio of IFN-γ mRNA in mitogen-stimulated peripheral blood mononuclear cells (PBMCs) relative to non-stimulated cells after normalization against β_2-microglobulin (β2M) in the Rusa deer, Eld's deer and Hog deer were 6.47±3.39, 22.91±7.21 and 17.66±1.30, respectively. This preliminary demonstration may serve as the basic instrument for further development of rapid, simple and reliable immunodiagnostic methods for the study of cervidae in Thailand.

Keywords: cervidae, gene expression, interferon gamma, real-time PCR, sequence characterization

[1]*Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand*
[2]*Center of Excellence on Agricultural Biotechnology (AG-BIO/PERDO-CHE), Bangkok 10900, Thailand*
[3]*Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand*
[4]*Department of Farm Resources and Production Medicine, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand*
[5]*Bureau of Conservation Research and Education, Zoological Park Organization Under the Royal Patronage of H.M. The King, Bangkok 10300, Thailand*
[6]*Department of National Parks, Wildlife and Plant Conservation (DNP), Bangkok 10900, Thailand*
[7]*Department of Large Animal and Wildlife Clinical Science, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand*
[*]***Correspondence:*** *fvetnit@ku.ac.th*

Introduction

Interferon gamma (IFN-γ) is a pleiotropic cytokine which is primarily produced by activated CD4+ T cells, CD8+ T cells and Natural killer (NK) cells. IFN-γ is classified as a Th1 cytokine, which is important for host cell-mediated immune protection (Schroder et al., 2004). The studies of IFN-γ based assays have been widely developed to assess the disease-specific cellular immune responses in various mammal species. To measure the IFN-γ expression, several techniques have been contributed such as enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunospot (ELISPOT) and flow cytometry, which determine IFN-γ at protein level (Chambers, 2013; Schmittel et al., 2001; Asemissen et al., 2001). In addition, the quantitative real-time polymerase chain reaction assay (RT-qPCR) has been developed to determine IFN-γ at mRNA level (Harrington et al., 2006; Harrington et al., 2007).

The family cervidae commonly represents the group of deer. This family consists of 47 deer species distributed in almost every part of the world including South-east Asia (Holmes et al., 2011). Cervids are susceptible to and can be a reservoir for several important intracellular pathogenic diseases, e.g. bovine tuberculosis (BTB), paratuberculosis (Johne's disease) and brucellosis (Harrington et al., 2006). Thus, characterizing IFN-γ in cervidae might be useful for better understanding of disease immune responses as well as development of effective immunodiagnosis.

Thailand has 6 species of endemic wild deer including Hog deer (*Axis porcinus*), Eld's deer (*Rucervus eldii*), Schomburgk's deer (*Rucervus schomburgki*; extinct), Sambar deer (*Rusa unicolor*), Fea's muntjac (*Muntiacus feae*) and Red muntjac (*Muntiacus muntjak*) (Lekagul and McNeely, 1988). In addition, Rusa deer (*Rusa timorensis*) was also introduced to Thailand and widely promoted to commercial farming for venison and velvet production (Sirikhant, 2003). In this study the IFN-γ cDNA sequences of Rusa deer and Thai cervids including Hog deer, Sambar deer and Eld's deer were characterized. Moreover, the IFN-γ assay was preliminarily established to quantify the expression of IFN-γ mRNA by real-time PCR.

Materials and Methods

Sequencing IFN-γ cDNA of Thai cervidae: Heparinized blood samples from adult Rusa deer, Hog deer, Sambar deer, and Eld's deer were collected. PBMCs were isolated and cultured with 10 µg/ml Concanavalin A (Con A) at 37°C, 5% CO_2, for 24 h. Total RNA was extracted from the PBMCs according to Siebert and Chenchik (1993). To remove any contaminated genomic DNA, all samples were treated with DNase I (Thermo scientific). First strand cDNA synthesis was done by RT-PCR using RevertAid First Strand cDNA Synthesis Kit (Thermo scientific) with oligo-(dT) primer.

Completed IFN-γ cDNA sequences were amplified by primers according to Muangkram et al. (2013). PCR was performed using Fusion® Hot start Taq DNA polymerase II (Thermo scientific) following the manufacturer's instructions. PCR products were analyzed and purified for sequencing by ABI 3070 system (Applied Biosystems). Obtained IFN-γ sequences and deduced amino acids were analyzed with other sequences from GenBank database using BioEdit software (Hall, 1999).

Real-time PCR assay: qPCR primers of IFN-γ and β2M were designed based on the obtained sequencing data and sequences available from GenBank (Table 1). Real-time PCR was carried out by Fusion® Hot start Taq DNA polymerase II (Thermo scientific) and EvaGreen® dye (Biotium) was used as a dsDNA-specific binding dye according to the manufacturer's instructions. The reactions were performed on C1000™ Thermal Cycler and analyzed by CFX Manager software (Bio-Rad Laboratories). Melting curve analysis was performed immediately after amplification step by slowly increasing temperature from 75 to 95°C (0.2°C/s).

Table 1 Oligonucleotide primer sequences

Target	Oligonucleotide sequences (5′→3′)	Annealing temperature (°C)	Product size (bp)	References
Completed IFN-γ cDNA	F: GGCCTAACTCTCTCCGAAACA R: TATTGCAGGCAGGATGACCA	55	542	Muangkram et al., 2013
IFN-γ quantification	F: GAAGAATTGGAAAGAGGAGAGTGAC R: AGATCATCCACCGAAATTTGAATCAGC	58	210	This study
β2M quantification	F: TGTGTCTGGGTTCCATCCACCCCAG R: TGCTTACAGGTCTCGATCCCACT	58	223	This study

Primer efficiency and dynamic range of each target gene were determined by generating standard curves using 10-fold dilution series of standard plasmid as previously described by Hein et al. (2001). Real-time PCR efficiencies (*E*) for each target gene were calculated by the following equation: $E = 10^{(-1/\text{slope})}$ (Pfaffl, 2001). Intra-assay variation was determined by triple repeats of standard curve qPCR over a range of different dilutions. Inter-assay variation was examined from triple different runs performed on different days.

For preliminary IFN-γ mRNA quantification, the PBMCs from Rusa deer (n=5), Eld's deer (n=3) and Hog deer (n=1) were diluted to 5 x 10^5 cells/well on a 24-well culture plate. The PBMCs were stimulated with or without 10 µg/ml Con A, then cultured at 37°C, 5% CO_2, for 18 h. Total RNA was extracted and cDNA was synthesized. qPCR was performed under the same condition as above. Gene expression data were recorded as qualification cycle (Cq), then were calculated and expressed as relative expression ratio according to the model of Pfaffl (2001).

Results and Discussion

Completed IFN-γ cDNA from Rusa deer, Hog deer, Sambar deer, and Eld's deer were successfully obtained. The coding part of IFN-γ gene was 501-bp, encoding 166 amino acids. Multiple alignments of IFN-γ cDNA sequences among the deer species indicated 5 polymorphic sites (Table 2). The Rusa deer's IFN-γ cDNA sequence was the most homologous with those of Hog deer and Sambar deer, which had 100% homology with each other. This result might be because of the close relationship among the 3-deer species, which were grouped in the same clade by the phylogenetic study of cytochrome *b* (Pitra et al., 2004). The alignment of deduced amino acid sequences of cervid IFN-γ with other ruminants is shown in Figure 1. There were negligible differences in the amino acid compositions of IFN-γ among the cervid group (98-100% homology). Compared to other ruminants, the degrees of homology were considerably high (91-94%). However, the difference in amino acid compositions between taxonomic families resulted in limitation of inter-species activity. Previous studies revealed that IFN-γ immunoassay developed for bovine was inapplicable in cervid due to the lack of antibody cross-reactivity (Rothel et al., 1990; Slobbe et al., 2000).

Table 2 Single nucleotide polymorphism of IFN-γ cDNA of Rusa deer (*R. timorensis*), Hog deer (*A. porcinus*), Sambar deer (*R. unicolor*) and Eld's deer (*R. eldii*), and other sequences of cervid IFN-γ cDNA reported in GenBank

Species	Nucleotide sequence polymorphic positions				
	019	066	186	470	497
Rusa timorensis (KX711963)	A	T	G	G	T
Axis porcinus (KX711965)	.	.	.	A	.
Rusa unicolor (KX711964)	.	.	.	A	.
Rucervus eldii (KX711966)	T	.	C	A	.
Cervus nippon (JQ408441)	.	C	.	A	.
Cervus elephas (L07502)	.	.	.	A	C

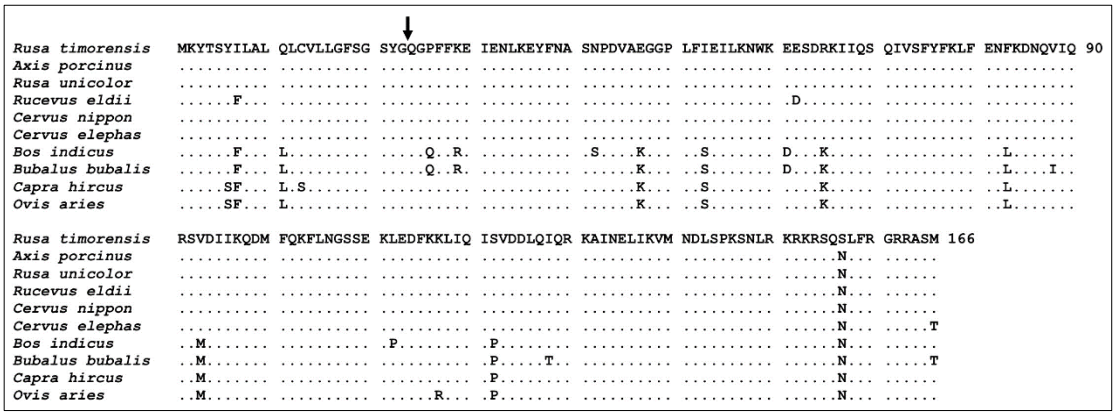

Figure 1 Multiple alignment of deduced IFN-γ amino acid sequences of Rusa deer and Thai cervids with other ruminants including Sika deer (*Cervus nippon*; JQ408441), Red deer (*Cervus elaphas*; L07502), Cattle (*Bos indicus*; AF533686), Buffalo (*Bubalus bubalis*; AF484688), Goat (*Capra hircus*; U34232) and Sheep (*Ovis aries*; NM001009803). The dots indicate the conserved amino acid sequences relative to Rusa deer IFN-γ. The arrow indicates the signal peptide cleavage site.

The standard curves of IFN-γ and β2M quantification were generated. The characterization and validation parameters of RT-qPCR are indicated in Table 3. The melting curve analysis revealed that the melting temperature (T_m) of IFN-γ and β2M in all analyzed species were 82.20°C and 86.40°C, respectively (Figure 2B). Gel electrophoresis showed RT-qPCR products at sizes of 210 bp for IFN-γ (Figure 2C) and 223 bp for β2M (data not shown).

In cervids, RT-qPCR using hydrolysis (*TaqMan*) probe has been primarily developed to detect IFN-γ mRNA in Elk (*C. elaphas*), and has been evaluated for cross-detection in Red deer, White-tailed deer and Reindeer (Harrington et al., 2006; Harrington et al., 2007). The present study preliminarily demonstrated RT-qPCR using EvaGreen® to detect IFN-γ mRNA in deer species found in Thailand. The qPCR primers were designed to be universally used for all cervids and were successfully demonstrated in Rusa deer, Hog deer and Eld's deer. IFN-γ mRNA in Con A-stimulated PBMCs was quantified and reported as mRNA expression ratio relative to non-stimulated PBMCs after normalization against β2M. In Rusa deer, the IFN-γ relative expression ratio was 6.47±3.39, lower than those of Eld's deer and Hog deer, which were 22.91±7.21 and 17.66±1.30, respectively, similar to that reported in Red deer (Harrington et al., 2006). Both primer design and detection system can affect the sensitivity of qPCR (Wong and Medrano, 2005; Curry et al., 2002). A previous study in Red deer indicated that RT-qPCR using hydrolysis probe could provide more sensitivity than using DNA-binding dye (Hein et al., 2001; Harrington et al., 2006; Harrington et al., 2007). In addition, studying different deer species might require different PBMC's stimulated conditions, which possibly leads to difference in IFN-γ mRNA expression results. Thus, further studies of the validation of stimulated condition as well as base-line IFN-γ mRNA expression profiles of each species, or even individuals, of a defined method should be initially evaluated.

Basic molecular genetic knowledge is necessary for developing a disease-specific cytokine detection method. This study firstly characterized IFN-γ cDNA sequences of cervidae found in Thailand, and then subsequently used the obtained data to set up the preliminary quantification of IFN-γ mRNA in Rusa deer, Hog deer and Eld's deer. Therefore, IFN-γ assay by RT-qPCR applied for disease-specific detection should be further evaluated in order to develop rapid, simple and reliable diagnostic tools for the study of cervidae in Thailand.

Table 3 Characterization and validation parameters of real-time PCR quantification for IFN-γ and β2M in cervidae

Target	Quantification range	Correlation coefficient (*r*)	PCR efficiency (*E*)	Intra-assay variation (%)	Inter-assay variation (%)
IFN-γ	10^3-10^{10}	0.994	1.91	1.41	4.57
β2M	10^1-10^8	0.994	2.00	1.74	4.86

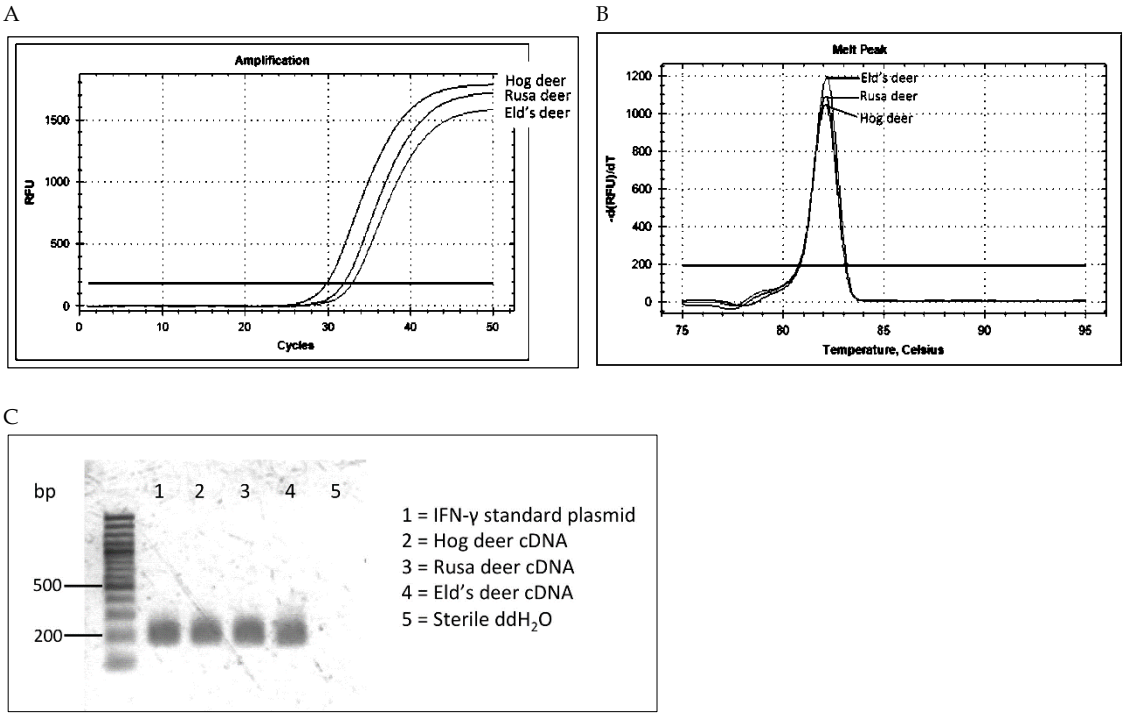

Figure 2 IFN-γ real-time PCR amplification plots and melting curve analysis of multiple cervid species including Hog deer, Rusa deer and Eld's deer. (A) Real-time PCR amplification plots of IFN-γ cDNA obtained from Con A-stimulated PBMCs. RFU stands for reactive fluorescence units. (B) Melting curve analysis RT-qPCR with quantitative IFN-γ primers using cDNA templates of Hog deer, Rusa deer and Eld's deer. (C) Gel electrophoresis of RT-qPCR products.

Acknowledgements

This study was supported by Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus and Center of Excellence on Agricultural Biotechnology: (AG-BIO/PERDO-CHE). We acknowledge the Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Sean Campus for laboratory facility. We gracefully acknowledge Zoological park organization and Department of National Parks, Wildlife and Plant Conservation (DNP), Thailand for samples and collaboration.

References

Asemissen AM, Nagorsen D, Keilholz U, A Letsch, Schmittel A, Thiel E and Scheibenbogen C 2001. Flow cytometric determination of intracellular or secreted IFNγ for the quantification of antigen reactive T cells. J Immunol Methods. 251(1–2): 101-108.

Chambers MA 2013. Review of the diagnosis of tuberculosis in non-bovid wildlife species using immunological methods-an update of published work since 2009. Transbound Emerg Dis. 60(Suppl. 1): 14–27.

Curry JD, McHale C and Smith MT 2002. Factors influencing real-time RT-PCR results: application of real-time RT-PCR for detection of leukemia translocations. Mol Biol Today. 3: 79-84.

Hall TA 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 41: 95-98.

Harrington NP, Surujballi OP and Prescott JF 2006. Cervine (*Cervus elaphus*) cytokine mRNA quantification by real-time polymerase chain reaction. J Wildl Dis. 42(2): 219-233.

Harrington NP, Surujballi OP, Waters WR and Prescott JF 2007. Development and evaluation of a real-time reverse transcription-PCR assay for quantification of gamma interferon mRNA to diagnose tuberculosis in multiple animal species. Clin Vaccine Immunol. 14(12): 1563-1571.

Hein J, Schellenberg U, Bein G and Hackstein H 2001. Quantification of murine IFN-γ mRNA and protein expression: impact of real-time kinetic RT-PCR using SYBR Green I dye. Scand J Immunol. 54(3): 285-291.

Holmes K, Jenkins J, Mahalin P and Berini J 2011. "Cervidae." [online]. Available: http://animaldiversity.org. Accessed May 16, 2016.

Lekagul B and Mcneely JA 1988. Mammal of Thailand. 2nd ed. Bangkok Kurusapa Ladproa press, Bangkok.

Muangkram Y, Wajjwalku W, Pinyopummintr T, Thongtip N, Kaolim N, Chaichanathong S, Insee J, Kamolnorranath S, Siriaroonrat B, Tipkantha W and Sananu S 2013. Characterization of interferon gamma (IFN-γ) sequence in Asian tapir and relationship within order Perissodactyla. In: Proceeding of the 6th Asian Meeting on Zoo and Wildlife Medicine/Conservation in Singapore in 2013. The Institute of Technical Education (college west), Singapore: p. 216-218.

Pfaffl MW 2001. A new mathematical model for relative quantification in real-t ime RT-PCR. Nucleic Acids Res. 29(9): 2002-2007.

Pitra C, Fickel J, Meijaard E and Groves C 2004. Evolution and phylogeny of old world deer. Mol Phylogenet Evol. 33(3): 880-895.

Schmittel A, Keilholz U, Bauer S, Kuhne U, Stevanovic S, Thiel E and Scheibenbogen C 2001. Application of the IFN-γ ELISPOT assay to quantify T cell responses against proteins. J Immunol Methods. 247(1–2): 17-24.

Schroder K, Hertzog PJ, Ravasi T and Hume DA 2004. Interferon-γ: an overview of signals, mechanisms and functions. J Leukoc Biol. 75(2): 163- 189.

Siebert PD and Chenchik A 1993. Modified acid guanidinium thiocyanate-phenol-chloroform RNA extraction method which greatly reduces DNA contamination. Nucleic Acids Res. 21(8): 2019-2020.

Sirikhant C 2003. A financial analysis of Rusa deer farming investment in Thailand. M.S. Thesis, Kasetsart University.

Slobbe L, Lockhart E, Kelly J and Buchan G 2000. The production and biological assessment of cervine interferon gamma. Cytokine. 12(8): 1211-1217.

Wong ML and Medrano JF 2005. Real-time PCR for mRNA quantitation. Biotechniques. 39(1): 75-85.

บทคัดย่อ

การหาลำดับนิวคลีโอไทด์ และการวิเคราะห์การแสดงออกของอินเตอร์เฟอรอน แกมมา (IFN-γ) ในกวางรูซ่า (*Rusa timorensis*) และสัตว์ตระกูลกวางในประเทศไทย

สิรินารถ ชัยชนะทอง[1,2] วรวิทย์ วัชชวัลคุ[1,3] มานะกร สุขมาก[4]
บริพัตร ศิริอรุณรัตน์[5] ตรศักดิ์ นิภานันท์[6] นิกร ทองทิพย์[1,7]*

ในปัจจุบัน การตรวจอินเตอร์เฟอรอนแกมมา (IFN-γ) เริ่มมีบทบาทที่สำคัญในการใช้เป็นเครื่องมือทางเลือกสำหรับวินิจฉัยการติดเชื้อก่อโรคที่อาศัยอยู่ภายในเซลล์ การศึกษาครั้งนี้ได้ศึกษาลักษณะจำเพาะทางอณูชีววิทยาของยีน IFN-γ ในกวางรูซ่าและสัตว์ตระกูลกวางในประเทศไทย ได้แก่ เนื้อทราย กวางป่า และละมั่ง จากการศึกษาลำดับนิวคลีโอไทด์ของ cDNA ของยีน IFN-γ ในกลุ่มสัตว์ตระกูลกวาง พบว่ามีความแตกต่างของลำดับนิวคลีโอไทด์ทั้งหมด 5 ตำแหน่ง เมื่อเปรียบเทียบองค์ประกอบของกรดอะมิโน พบว่ามีความแตกต่างกันเพียงเล็กน้อย (98-100% homology) ทำการศึกษาเบื้องต้นในการวัดเชิงปริมาณการแสดงออกของ IFN-γ mRNA ด้วยเทคนิค Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) โดยใช้ไพร์เมอร์ที่ออกแบบและทดสอบประสิทธิภาพสำหรับการวัดปริมาณ IFN-γ mRNA จาก peripheral blood mononuclear cells (PBMCs) โดยปริมาณ IFN-γ mRNA วัดได้ต่ำสุดที่แนะนำ คือ 10^3 โมเลกุล Intra-assay variation และ Inter-assay variation สำหรับการวัดปริมาณ IFN-γ mRNA โดยวิธี RT-qPCR มีค่าเท่ากับ 1.41 และ 4.57 ตามลำดับ อัตราการแสดงออกสัมพัทธ์ของ IFN-γ mRNA จากตัวอย่าง PBMCs ที่ได้รับการกระตุ้นด้วยสารกระตุ้นที่ไม่จำเพาะต่อตัวอย่างที่ไม่ได้รับการกระตุ้น เทียบกับการแสดงออกของ β_2-microglobulin (β2M) mRNA ในกวางรูซ่า ละมั่ง และเนื้อทราย มีค่าเท่ากับ 6.47±3.39, 22.91±7.21 และ 17.66±1.30 ตามลำดับ การศึกษาเบื้องต้นในครั้งนี้อาจใช้เป็นข้อมูลพื้นฐานสำหรับพัฒนาวิธีการตรวจวินิจฉัยทางภูมิคุ้มกันในสัตว์ตระกูลกวางในประเทศไทย ที่ให้ผลรวดเร็ว แม่นยำ และสะดวกต่อการใช้งานต่อไป

คำสำคัญ: กวาง การแสดงออกของยีน อินเตอร์เฟอรอนแกมมา เรียลทามพีซีอาร์ การหาลำดับดีเอ็นเอ

[1]ศูนย์เทคโนโลยีชีวภาพเกษตร มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตกำแพงแสน จ.นครปฐม 73140
[2]ศูนย์ความเป็นเลิศด้านเทคโนโลยีชีวภาพเกษตร สำนักพัฒนาบัณฑิตศึกษาและวิจัยด้านวิทยาศาสตร์และเทคโนโลยี สำนักงานคณะกรรมการการอุดมศึกษา กรุงเทพฯ 10900
[3]คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตกำแพงแสน จ.นครปฐม 73140
[4]ภาควิชาเวชศาสตร์และทรัพยากรการผลิตสัตว์ คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตกำแพงแสน จ.นครปฐม 73140
[5]สำนักอนุรักษ์ วิจัย และการศึกษา องค์การสวนสัตว์ในพระบรมราชูปถัมภ์ กรุงเทพฯ 10300
[6]กรมอุทยานแห่งชาติ สัตว์ป่า และพันธุ์พืช กรุงเทพฯ 10900
[7]ภาควิชาเวชศาสตร์คลินิกสัตว์ใหญ่และสัตว์ป่า คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตกำแพงแสน จ.นครปฐม 73140
***ผู้รับผิดชอบบทความ E-mail:** fvetnit@ku.ac.th*