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Development of Real-time PCR Assays for Detection of *Dirofilaria immitis* from Infected Dog Blood

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심장사상충에 감염된 개의 혈액에서 심장사상충 유전자를 검출할 수 있는 실시간 중합효소연쇄반응 기법 개발

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Dirofilaria immitis is a filarial nematode parasite that causes cardiopulmonary dirofilariasis in dogs. The purpose of this study was the development of real-time PCR assays for efficient detection of *D. immitis*. The *D. immitis*-specific primers confirmed in our previous study and a newly designed TaqMan probe were used for quantitative diagnostics. First, SYBR Green real-time PCR was performed using the specific primers and serially diluted genomic DNA or plasmid DNA, and melting curve analyses were performed after amplification. The melting curve showed one specific peak in each of the genomic and plasmid DNA reactions, suggesting that the primers specifically amplify the *D. immitis* cytochrome c oxidase subunit I gene. Comparison of SYBR Green and TaqMan real-time PCR using serially diluted plasmid DNA showed higher efficiency and specificity with TaqMan real-time PCR. The real-time PCR assays developed in this study will provide improved diagnostic methods to overcome the limitations of conventional diagnostic tools and facilitate more rapid and accurate diagnoses.

Keywords: *Dirofilaria immitis*, Molecular diagnosis, SYBR Green real-time PCR, TaqMan real-time PCR, PCR efficiency

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Introduction

Dirofilaria immitis is the causative agent of cardiopulmonary dirofilariasis. Mosquitoes are the main vectors for accidental infection with *D. immitis*. *D. immitis* larvae develop in the mosquito at 18~34°C and are thereafter transmitted to the new final hosts such as canines, felines, various wild mammals, and some human populations in tropical, subtropical, and some temperate regions in the world. With progressing globalization and climate change, the incidence of infection is increasing [1-9].

Diagnosis is recommended before administration of anti-parasite medication to avoid complications from drug abuse. The most common diagnostic tool for detection of *D. immitis* is microscopic examination of microfilariae from blood samples. However, this approach has limited

sensitivity, and considerable expertise is required to distinguish among filarial parasite species because of their similar morphologies [8,10]. Other methods leveraging antigen detection are limited by their propensity to produce false-negative results during the first 5~8 months of infection because they only detect antigens released from the adult female worm's reproductive tract, leading to errors due to low worm counts, immature infections, and all-male infections [11,12]. To overcome these limitations, methods for PCR-based molecular detection are under development. 5.8S, 16S, or 18S ribosomal RNA (rRNA) genes have been used as target genes to detect filarial nematodes using end-point PCR-based detection from dog bloods or heads of mosquitoes [1,2,6,7].

This study aimed to develop quantitative PCR methods for detecting *D. immitis*. In our previous study, we designed primers targeting a *D. immitis*-specific gene region and developed an end-point PCR method. The specific gene region focused on was cytochrome c oxidase subunit I (*COI*) gene. *COI* is one of mitochondrial DNA (mtDNA) genes which are maternally inherited and generally do not undergo recombination. Amino acid sequences of *COI* gene have changed more slowly than any other mtDNA genes. Therefore, *COI* gene is so-called "barcode" for identifying of species diversity [13-17].

The specificity and sensitivity of the developed assay were very high in our previous study. However, end-point PCR only allows determination of whether a sample is infected. For more precise therapeutic monitoring, therefore, additional development of a method for quantitative analysis is needed.

Materials and Methods

1. Primers and probes

We employed the primers designed in our previous study for SYBR Green and TaqMan real-time PCR assays to detect a 150-bp fragment of *D. immitis* cytochrome c oxidase subunit I (*COI*) (forward: ATT GGG TGC CCC TGA AAT GG; reverse: CCC TCT ACA CTC AAA GGA GGA). For TaqMan real-time PCR, a probe was designed within the fragment and labeled with 6-carboxy-fluorescein (FAM, excitation wavelength 494 nm, emission wavelength 521 nm) at the 5'-end and ZEN™-Iowa Black® FQ quencher at the 3'-end. All primers and probes used in this study were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

2. Extraction of genomic DNA

D. immitis-infected blood samples isolated from random infected source dogs were gifted from Seoul National University. The peripheral blood samples were from 51 blood donation dogs, Beagle species, and were collected in July 2015 in the Republic of Korea. Genomic DNA (gDNA) from blood samples collected in EDTA (ethylenediaminetetraacetic acid) tubes was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration of gDNA was determined with a NanoDrop spectrophotometer (ThermoFisher Scientific, Sunnyvale, CA, USA), and twofold serial dilutions were prepared for analysis.

3. Preparation of plasmid DNA

PCR products amplified using the primers were purified and inserted into pLUG-Prime® TA-cloning vector (iNtRON Biotechnology, Republic of Korea), and the plasmid DNA (pDNA) was cloned. The DNA copy number was estimated from the molecular weight of the *D. immitis* pDNA:

$$copy\,number = \frac{amount\,of\,dsDNA(ng)\times 3.0221\times 10^{23}(molecules/mole)}{length\,of\,dsDNA\times 660(\mathrm{g/mole})\times 1\times 10^{9}(ng/g)}$$

DNAs were 10-fold serially diluted for analysis.

4. Real-time PCR

All amplification reactions were performed on a StepOnePlus™ instrument (Applied Biosystems, Foster City, CA, USA) in 20 μL total volume. All analyses were performed in triplicate. Three samples without DNA template were routinely included as a no-template control. The PCR efficiency was calculated from the dilution factor and the slope of the trend line as follows:

$$PCR\,efficiency = -1 + dilution\,factor^{(-1/slope)}$$

1) SYBR green real-time PCR

SYBR Green real-time PCR was performed with *D. immitis COI* primers. The PCR mixture was prepared with 1× SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 500 nM primers, and serially diluted pDNA or gDNA. The PCR protocol included an initial denaturation step at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. The cycle threshold (Ct) was determined automatically. Data collection was performed during each annealing step. Following amplification, melting curve analysis was performed at 95°C for 15 s and 60°C for 1 min, and the temperature was increased from 60 to 95°C with 0.3°C increments to obtain product-specific melting temperatures.

2) TaqMan real-time PCR

TaqMan real-time PCR was performed with *D. immitis COI* primers and TaqMan probe (5'-/56-FAM/TGC TTT ATC /ZEN/TTT TTG GAT TAC TTT TGT TGC GTT GTT GAT GG/3IABkFQ/-3'). The PCR mixture was prepared with 1× TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA), 500 nM primers, 250 nM probe, and serially diluted pDNA or gDNA. The PCR protocol included a UNG (uracil-N-glycosylase) incubation step at 50°C for 2 min, initial denaturation at 95°C for 10 min, and 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min.

Results

In our previous study, we designed primers targeting a *D. immitis*-specific region of *COI* based on multiple sequence alignment of seven filarial *COI* genes. Primer specificities were evaluated by end-point PCR experiments and sequencing.

1. Specificity and sensitivity of the *D. immitis* SYBR green real-time PCR assay

In this study, to confirm primer specificity, we performed melting curve analyses after amplification in the SYBR Green real-time PCR assays. DNA samples were serially diluted as follows: pDNA was 10-fold serially diluted from 5×10^7 copies to 50 copies, and gDNA was twofold serially diluted from 12.5 ng to 0.195 ng. The Ct values for three replicates of serially diluted *D. immitis COI* pDNA and gDNA are listed in Table 1, and the melting curves are presented in Fig. 1. Ct values were analyzed by linear regression analysis and showed good linearity (pDNA: R^2 value, 0.9998; slope, -3.464; PCR efficiency, 94% and gDNA: R^2 value, 0.9984; slope, -0.9684; PCR efficiency, 104%). The gDNA melting curves matched those of pDNA with one specific peak at a melting temperature of 76.74°C. These results suggested that the primers could amplify *D. immitis COI* specifically and efficiently in SYBR Green real-time PCR.

2. Comparison of SYBR green and TaqMan real-time PCR

To improve specificity, TaqMan real-time PCR was developed using the same primers and a TaqMan probe. *D. immitis COI* pDNA was 10-fold serially diluted from 5×10^7 copies to 5×10^2 copies the template for SYBR Green and TaqMan real-time PCR assays. Ct values were analyzed by linear regression analysis, and the results showed good linearity (SYBR Green real-time PCR: R^2 value, 0.9998; slope, -3.4213; PCR efficiency, 96% and TaqMan real-time PCR: R^2 value, 0.9999; slope, -3.4624; PCR efficiency, 94%) (Fig. 2).

3. Limit of detection

To determine the detection limit, SYBR Green and TaqMan real-time PCR assays were performed with serially diluted pDNA, from 5×10^8 to 50 and 25 copies. The minimum detection level was less than 25 copies in both assays. The Ct

Table 1. Ct values for SYBR Green real-time PCR using serially diluted plasmid DNA and genomic DNA

Plasmid DNA			Genomic DNA		
Copies	Ct values	CV (%)	Ng	Ct values	CV (%)
50	29.51	0.56	0.195	29.23	0.94
5×10^2	25.95	0.52	0.391	28.51	0.47
5×10^3	22.35	0.17	0.781	27.33	0.64
5×10^4	18.88	0.42	1.563	26.45	0.17
5×10^5	15.48	0.32	3.125	25.37	0.29
5×10^6	12.09	0.50	6.25	24.48	0.08
5×10^7	8.71	0.59	12.5	23.52	0.19

Abbreviation: CV, coefficient of variation.

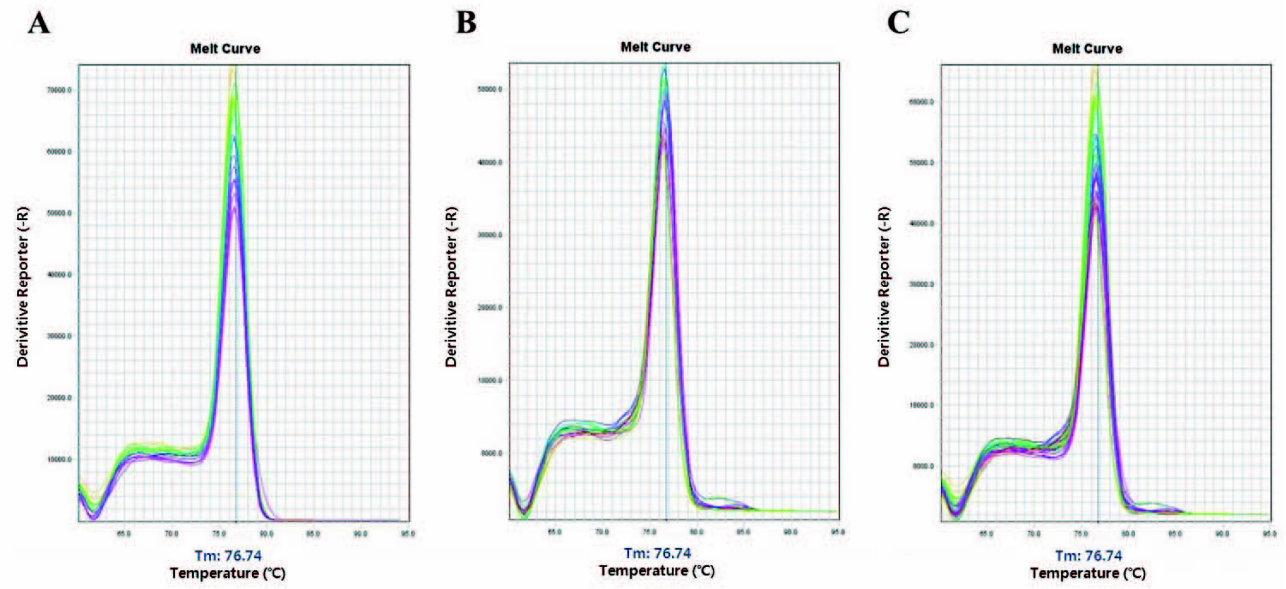

Fig. 1. Melting curves for products of SYBR Green real-time PCR. The templates were (A) 10-fold serially diluted plasmid DNA (5×10^7 to 50 copies) and (B) twofold serially diluted genomic DNA (12.5 ng to 0.195 ng). (C) Overlay of curves from (A) and (B). The curves show one specific peak at 76.74°C.

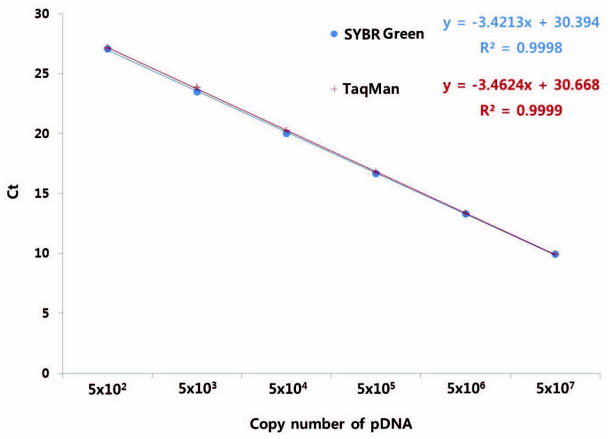

Fig. 2. Comparison of SYBR Green and TaqMan real-time PCR assays. The graph showed the results of linear regression analysis using Ct values of SYBR Green and TaqMan real-time PCR using serially diluted pDNA, respectively. Blue circles represent the Ct values of the SYBR Green real-time PCR assay, and the blue line is the trend line for those values. Red crosses show the Ct values of the TaqMan real-time PCR assay, and the red line is the trend line for those values.

values are listed in Table 2.

Discussion

Development of molecular diagnostic tools is essential for improving the clinical benefit of pathogen detection. In particular, real-time PCR assays produce quantitative results with improved specificity, sensitivity, and reproducibility and reduced risk of carry-over contamination. Furthermore, real-time PCR requires less time and labor than conventional PCR. Therefore, real-time PCR assays are promising tools for detecting pathogen gDNA from biological fluids such as blood, urine, and sputum [18-23].

Researchers developed methods to differentiate filarial nematodes using high resolution melt (HRM) real-time qPCR which were performed PCR amplification and then melting curve analysis which showed different melting peaks among filarial nematodes. However, those developed methods could

Table 2. Ct values and detection limits of TaqMan and SYBR Green qPCR assays

pDNA (copies)	SYBR Green real-time PCR		TaqMan real-time PCR	
	Ct values	CV (%)	Ct values	CV (%)
25	31.7	1.19	32.33	1.71
50	30.66	0.61	31.04	0.88
5×10^2	27.09	0.55	27.16	0.26
5×10^3	23.5	0.15	23.87	0.02
5×10^4	20.02	0.55	20.24	0.14
5×10^5	16.66	0.24	16.77	0.03
5×10^6	13.3	0.35	13.32	0.23
5×10^7	9.94	0	9.94	0.32

Abbreviation: CV, coefficient of variation; pDNA, plasmid DNA.

be used for only detection and differentiation [10,24].

In this study, the SYBR Green real-time PCR assay using primers targeting *D. immitis COI* showed high PCR efficiency, specificity, and sensitivity. The Ct values of gDNA (12.5 ng to 0.195 ng) were similar to those of pDNA (5×10^3 to 50 copies), and the melting curves for products from both templates had one specific peak. This assay provides a diagnostic tool that could be used to detect *D. immitis* levels quantitatively in real-time. Furthermore, a TaqMan real-time PCR assay was developed using the specific primers and a TaqMan probe designed to specifically detect the sequence of *D. immitis COI* between the forward and reverse primers. The results from TaqMan real-time PCR showed efficiency similar to SYBR Green real-time PCR with high specificity and sensitivity. The detection limit was 25 copies for both assays. Thus, the TaqMan real-time PCR assay exhibited greater specificity in less time than the SYBR Green real-time PCR assay.

The assays developed herein will allow *D. immitis*-specific real-time monitoring from the early stage of infection before administration of medication. Additionally, further development of these real-time PCR-based assays will facilitate efficient therapeutic control of *D. immitis* infection.

요 약

선형 사상충의 일종인 심장사상충은 개의 심폐 사상충증을 유발한다. 이에 본 연구의 목적은 심장사상충을 효과적으로 검출할 수 있는 실시간 중합효소연쇄반응 기법을 개발함에 있다. 연구에 있어서 사용된 프라이머 및 프로브는 선행연구에서 제작된 심장사상충 특이 프라이머 및 새롭게 제작된 TaqMan 프로브를 이용하였다. 선행연구에서 제작된 프라이머 및 농도별로 희석된 게놈유전자와 플라스미드유전자가 SYBR Green 실시간 중합효소연쇄반응 수행에 이용되었으며, 중합효소연쇄반응 과정 중 증폭 이후의 녹는 곡선의 결과를 분석하였다. 분석결과 사용된 프라이머는 각각 게놈유전자 및 플라스미드 유전자에서 특이 녹는 곡선을 나타냄에 따라 심장사상충 특이 사이토크롬 C 산화효소 유전자만을 증폭하고 있음을 확인 할 수 있었다. 새롭게 제작된 TaqMan 프로브는 SYBR Green 실시간 중합효소연쇄반응과의 결과를 농도별로 희석된 플라스미드 유전자를 이용하여 비교 분석하였고, 분석결과 TaqMan 프로브를 이용한 실시간 중합효소연쇄반응이 검출효율 및 특이도에 있어서 우수함을 확인할 수 있었다. 본 연구를 통하여 개발한 실시간 중합효소연쇄반응은 기존의 전통적인 진단기법의 한계를 극복할 수 있는 신속하고 정확한 향상된 진단기법을 제시한다.

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Conflict of interest: None

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