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Development of TaqMan Quantitative PCR Assays for Duplex Detection of *Dirofilaria immitis COI* and Dog *GAPDH* from Infected Dog Blood

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심장사상충에 감염된 개 혈액에서 *Dirofilaria immitis*의 *COI*와 개의 *GAPDH*를 이중 검출하기 위한 정량적 TaqMan PCR 분석법의 개발

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Dirofilaria immitis (*D. immitis*) is a filarial nematode that causes cardiopulmonary dirofilariasis in dogs. In the late stages of infection, infected dogs show one or more symptoms and advanced heart disorder with perivascular inflammation. To detect *D. immitis* specifically and efficiently in the early stages of infection, a duplex TaqMan qPCR assay was developed based on previous studies using primers and probes specialized to detect *D. immitis* cytochrome c oxidase subunit I (*COI*) and dog glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). As positive controls, plasmid DNAs were constructed from *D. immitis COI* or dog *GAPDH* and a TA-cloning vector. Simplex and duplex TaqMan qPCR assays were performed using the specific primers, probes, and genomic or plasmid DNA. The duplex reaction developed could detect *D. immitis COI* and dog *GAPDH* in the same sample simultaneously after optimization of the primer concentrations. The limit of detection was 25 copies for the simplex and duplex assays, and both showed good linearity, high sensitivity, and excellent PCR efficiency. The duplex assays for pathogen detection reduce the costs, labor, and time compared to simplex reactions. Therefore, the duplex TaqMan qPCR assay developed herein will allow efficient *D. immitis* detection and quantification from a large number of samples simultaneously.

Key words: *Dirofilaria immitis*, Duplex detection, TaqMan quantitative real-time PCR

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INTRODUCTION

Dirofilaria immitis (*D. immitis*) is a filarial nematode that causes heartworm disease in canines, felines, various wild mammals, and some human populations. Mosquitoes are the major vectors in the accidental infection of *D. immitis*

in tropical, subtropical, and some temperate regions. With progressing globalization and climate change, the incidence of *D. immitis* infection is increasing [1-10].

The larvae of *D. immitis* develop in the mosquito at temperatures between 18 and 34°C before transmission to the new final host. The larvae remain in the blood until they reach the adult stage, and then they transfer to the heart, where the adult worms cause cardiopulmonary dirofilariasis. Cardiopulmonary dirofilariasis leads to immunopathological and mechanical events associated with progressive damage to the pulmonary blood vessels, the parenchyma, and the right side of the heart and with pathological relaxation of the artery wall, endarteritis, and perivascular inflammation [1-3, 7, 11-13].

Widely used methods for *D. immitis* diagnosis include microscopic morphological examination and antigen detection by immunochromatography or enzyme-linked immunosorbent assay. However, these methods have important limitations. Morphological assays have limited sensitivity and require considerable expertise to distinguish among filarial parasite species because of their rather similar morphology. In addition, antigen detection methods target antigens released from the adult female worm's reproductive tract and produce false-negative results during the first 5~8 months of infection due to low worm counts, immature infections, and all-male infections [3-5, 10-12, 14]. To overcome these limitations, molecular detection by PCR is being developed.

In our previous study, we designed and confirmed primers for detection of *D. immitis* cytochrome c oxidase subunit I (*COI*) and *Canis lupus familiaris* (dog) glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). In addition, we designed a TaqMan probe to specifically detect *D. immitis COI* through quantitative real-time PCR (qPCR) [15]. To extend our previously developed assays, in this study we developed a duplex TaqMan qPCR assay using the previously designed primers and probe and a newly designed dog *GAPDH* probe.

MATERIALS AND METHODS

1. Primers and probes

The *D. immitis* and dog genome sequences were obtained from GenBank. The primers and probes for the detection of specific gene regions were designed based on a highly conserved region of the *D. immitis COI* gene (EU159111.1) (150 bp; forward: ATT GGG TGC CCC TGA AAT GG; reverse: CCC TCT ACA CTC AAA GGA GGA) and the dog *GAPDH* gene (NM_001003142.1) (106 bp; forward: CAT GTT TGT GAT GGG CGT GAA; reverse: GAT GAC TTT GGC TAG AGG AGC). The primer specificities were evaluated based on multiple sequence alignment, and the corresponding TaqMan probes were designed between the fragments. The probe targeting *D. immitis COI* was 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. To detect dog *GAPDH* as an internal control, the TaqMan probe was labeled with hexachlorofluorescein (HEX, excitation wavelength 538 nm, emission wavelength 555 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 from dog blood samples

D. immitis-infected blood samples isolated from infected random source dogs were gifted from Seoul National University, Republic of Korea. Peripheral blood samples from healthy volunteers and uninfected dogs were used as negative controls. All animal experiments were in accordance with the guidelines of Eulji university animal care and use committee. Genomic DNA (gDNA) from blood samples collected in ethylenediaminetetraacetic acid (EDTA) 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).

3. Preparation of plasmid DNA

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

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

4. TaqMan qPCR amplification

TaqMan qPCR was performed with the primers and probes described above. The PCR mixture was prepared with 1× TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA), with the concentrations of primers, probes, and pDNA or gDNA adjusted for each experiment. The PCR protocol included a uracil-N-glycosylase incubation step at 50°C for 2 min, an initial denaturation step at 95°C for 10 min, and 40 cycles of denaturation at 95°C for 15 sec and annealing and extension at 60°C for 1 min. All amplification reactions were performed on a StepOnePlus™ instrument (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 μL. All analyses were performed in triplicate. Three samples without DNA were routinely included as a no-template control. The PCR efficiency was calculated from the dilution factor and slope of the trend line as follows:

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

RESULTS

1. Optimization of duplex reaction

Because primer sets compete for reagents such as dNTPs and polymerase, the working concentrations of the primers were optimized for the duplex reactions. With a 1:1 ratio of *D. immitis COI* and dog *GAPDH* primers, the Ct value of *D. immitis COI* was much greater than the true Ct value seen for simplex reactions (data not shown). Therefore, we varied the concentrations of dog *GAPDH* primers and probe with fixed concentrations of *D. immitis COI* primers (500 nM) and probe (250 nM). The optimized ratio of primer concentrations between *D. immitis COI* and dog *GAPDH* was 10:1 (Figure 1).

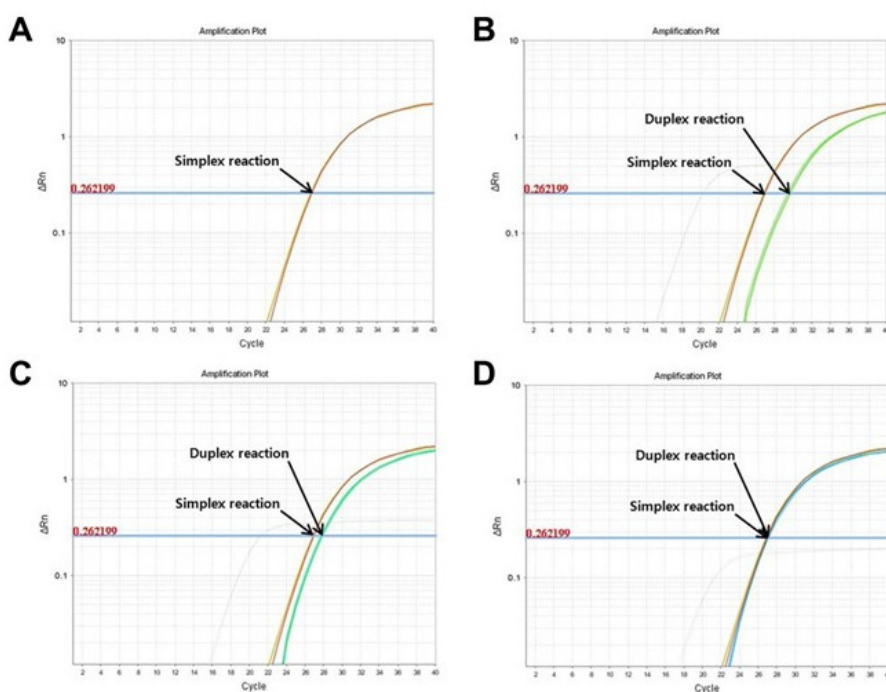

Figure 1. Optimization of duplex TaqMan qPCR. (A) Simplex reaction–amplified *D. immitis COI*, FAM only; 500 nM *D. immitis COI* primers and 250 nM probe. (B~D) Merged plots of the amplification, simplex, and duplex reactions. All of the simplex reaction plots are the same as in (A). Duplex reactions were amplified with *D. immitis COI*, FAM, and HEX. The concentrations of *D. immitis* COI primers (500 nM), *D. immitis COI* probe (250 nM) and dog *GAPDH* probe (100 nM) were fixed, and that of dog *GAPDH* primers was varied as follows: (B) 150 nM, (C) 100 nM, and (D) 50 nM. All amplification reactions were performed using 100 ng genomic DNA extracted from *D. immitis*–infected dog blood.

2. Efficiency of simplex and duplex TaqMan qPCR assays

To test the efficiency of the duplex reaction, simplex and duplex reactions were compared. Ct values analyzed by linear regression analysis showed good linearity (Figure 2). In the detection of *D. immitis COI*, the fit parameters for the simplex reaction were R^2=0.9981 and slope=−3.1424 with 108.1% PCR efficiency, and those of the duplex reaction were R^2=0.9967 and slope=−3.1868 with 106.0% PCR efficiency. For detection of dog *GAPDH*, the fit parameters for the simplex reaction were R^2=0.9929 and slope=−3.2085 with 105.0% PCR efficiency, and those for the duplex reaction were R^2=0.9948 and slope=−3.2293 with 104.0% PCR efficiency.

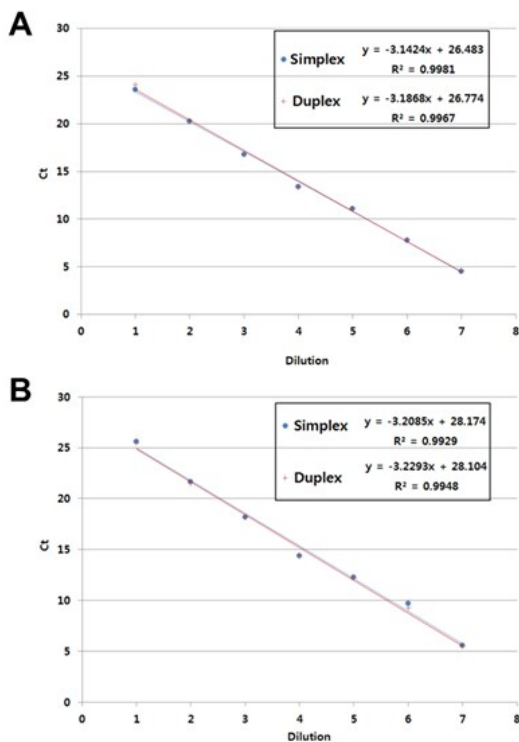

Figure 2. Comparison of simplex and duplex reactions. (A) *D. immitis COI* detection. Simplex: FAM only; 500 nM primers, 250 nM probe, and serially diluted *D. immitis COI* pDNA (3×10^{10} to 3×10^{4} copies). Duplex: FAM and HEX; *D. immitis COI* simplex assay components; 50 nM primers, 25 nM probe, and serially diluted dog *GAPDH* pDNA (3×10^{10} to 3×10^{4} copies). (B) Dog *GAPDH* detection. Simplex: HEX only; 50 nM primers, 25 nM probe, and serially diluted dog *GAPDH* pDNA (3×10^{10} to 3×10^{4} copies). Duplex: FAM and HEX; *D. immitis COI* duplex assay components.

3. Limit of detection

To determine the limit of detection, serially diluted pDNA samples were used as templates. The detection limit was 25 copies for both the simplex and duplex assays. Ct values analyzed by linear regression analysis showed good linearity even for low copy numbers (Figure 3).

4. Clinical sample evaluation using duplex TaqMan qPCR

Clinical samples were evaluated by duplex TaqMan qPCR assay and quantified using the standard curve (Figure 3). The results for the positive control, a sample at

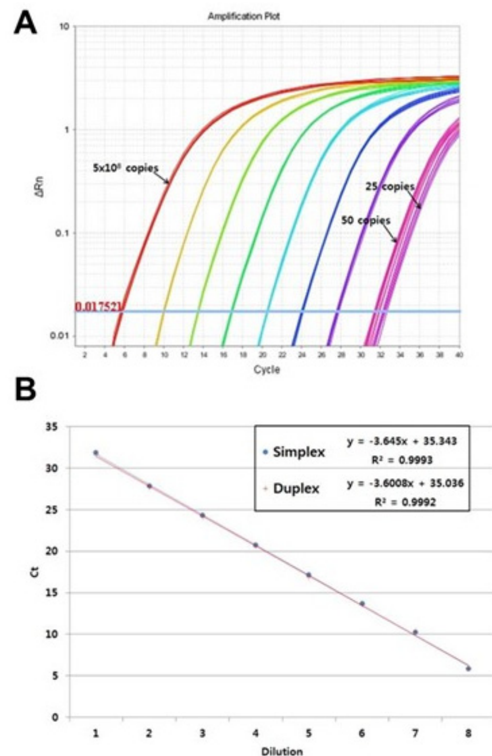

Figure 3. Limit of detection and standard curve. Eight points of a 10-fold dilution series (from 5×10^{8} to 50 copies of *D. immitis COI* pDNA) and 25 copies of *D. immitis COI* pDNA were analyzed by TaqMan qPCR for *D. immitis COI* (500 nM primers and 250 nM probe) and dog *GAPDH* (50 nM primers and 250 nM probe). In the duplex assay, all templates contained 5×10^{5} copies dog *GAPDH* pDNA. (A) Merged image of amplification plots of simplex (*D. immitis COI*) and duplex assay (*D. immitis COI* and dog *GAPDH*). (B) Linearity plots of Ct values for simplex and duplex assays. Blue circles show the Ct values for the simplex assay, and the blue line is the trend line for those values. Red crosses represent the Ct values of the duplex assay, and the red line is the trend line for those values. Both trend lines showed good linearity even at low copy numbers.

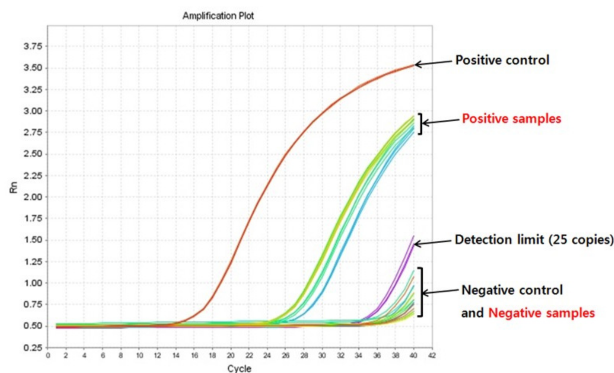

Figure 4. Clinical sample test using duplex TaqMan qPCR. Plasmid DNAs were used as positive control templates (5×10^6 copies *D. immitis COI* pDNA and 5×10^5 copies dog *GAPDH* pDNA), negative control templates (0 copies *D. immitis COI* pDNA and 5×10^5 copies dog *GAPDH* pDNA), and detection limit samples (25 copies *D. immitis COI* pDNA and 5×10^5 copies dog *GAPDH* pDNA). Genomic DNA (100 ng) extracted from *D. immitis*-infected or healthy dog blood samples was used as the template for positive and negative samples, respectively.

the detection limit, the negative control, and the positive and negative clinical samples are shown in Figure 4. The Ct value of the positive control was 14, and that of the sample at the detection limit was 34. Ct values of the negative control and negative samples exceeded the detection limit (>36). The Ct values of positive clinical samples were 24 ~26, which were corresponded to the Ct values of 5×10^3 copies for the standard curve.

DISCUSSION

Cardoso et al. [11] reported that suspect or symptomatic dogs show one or more clinical features, including hair loss, appetite loss, skin ulcers, cyanosis, dermatitis, diarrhea, dry chronic cough, difficultly breathing, nosebleed, exercise intolerance, hemorrhagic disorders, hyperthermia, joint inflammation, limping, lethargy, lymph node enlargement, neurologic signs, ocular lesions, bent claws, pale mucous membranes, excessive thirst, vomiting, weakness, and weight loss. Healthy or asymptomatic dogs had no signs or historical abnormalities. Nationally, 3.6% of healthy dogs tested positive for *D. immitis* antigen; however, in some regions, up to 40% of healthy dogs were *D. immitis* antigen-positive. Thus, many dogs infected with *D. immitis* remain asymptomatic for several months or a few years [11].

Development of methods for molecular diagnosis of *D. immitis* infection is needed to detect parasites from the early stage of infection easily, accurately, and efficiently. qPCR assays provide high sensitivity and specificity and require less time and labor to complete than conventional PCRs [16-21]. In addition, duplex qPCR assays can detect two genes in the same sample simultaneously and are therefore more cost-effective than simplex assays for detecting each gene separately. Furthermore, duplex qPCR assays can accommodate a large number of samples simultaneously [17].

In this study, we developed duplex TaqMan qPCR assays to detect and quantify the *D. immitis*-specific *COI* gene fragment in dog blood simultaneously with amplification and detection of the dog *GAPDH* gene as the internal control. The specificity and applicability of the method were confirmed in our previous study using multiple sequence alignment from the GenBank DNA database. To optimize the duplex reaction, primer concentrations were varied, and the optimized conditions yielded specificities and sensitivities similar to those of the simplex assays.

The *D. immitis COI* detection limit of duplex qPCR assays was 20 copies lower than that of previously developed assays [15]. The low detection limit of the duplex qPCR assay provides a clinically useful tool to identify pathogens with a low infection rate or in the early stage of infection, thereby reducing the potential for false-negative results [17]. In addition, these results showed overall superior performance compared with our previously developed assays using end-point PCR and real-time PCR. TaqMan qPCR-based clinical diagnostics for pathogen detection provide clear results even for samples contaminated with other pathogens or impurities because the primers and probes used in the assays are specific to the *D. immitis COI* gene region and broadly covered 54.7% of the gene sequence (150 bp) without cross-reaction with gDNA from dog blood.

Toward overall therapeutic monitoring, this study allows for additional development of multiple-pathogen

detection using specific primers and probes in the same sample. Such an extension of the method warrants further investigation.

요 약

Dirofilaria immitis (*D. immitis*)는 개의 심폐사상충증을 일으키는 선형사상충이다. 이 기생충에 감염된 개는 감염 후기 단계에서 하나 이상의 증상과 혈관 주위의 염증을 동반한 심화된 심장 질환을 보인다. 감염 초기단계에 특이적이고 효율적으로 *D. immitis*를 검출하기 위해서, 선행연구에서 밝혀낸 *D. immitis*의 cytochrome c oxidase subunit I (*COI*)와 개의 glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)를 검출하는 특이적인 프라이머와 프로브를 이용하여 이중 TaqMan qPCR 방법을 개발했다. 양성 대조군인 플라스미드 유전자는 TA-cloning vector와 *D. immitis*의 *COI*나 개의 *GAPDH*로 구성되었다. 단일과 이중 TaqMan qPCR 방법은 특이적인 프라이머와 프로브, 그리고 게놈 유전자나 플라스미드 유전자로 수행했다. 프라이머의 농도를 최적한 후, 본 연구에서 개발한 이중 반응은 *D. immitis*의 *COI*와 개의 *GAPDH*를 동일 시료에서 동시에 검출했다. 검출 한계는 단일과 이중 방법 모두 25 copies였고, 두 방법 모두 좋은 선형성과 높은 민감도, 그리고 우수한 PCR 효율을 보여주었다. 병원체를 검출하기 위한 이중 방법은 단일 방법에 비해 비용과 노동력, 시간이 적게 든다. 따라서 이중 TaqMan qPCR 방법의 개발은 많은 수의 시료로부터 동시에 효율적으로 *D. immitis* 검출과 정량이 가능하게 할 것이다.

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Conflict of interest: None
Author's information (Position): Oh IY[1], Researcher; Kim KT[2], Researcher; Gwon SY[1,4], Graduate student; Sung HJ[1,3], Professor.

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