SYBR-Green-Based Quantitative Real-Time PCR for Discriminating Between Closely Related *Angiostrongylus Cantonensis* and *A. Malaysisensis*

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Research

Keywords: Quantitative real-time PCR, Angiostrongylus cantonensis, A. malaysiensis, Infective stage larva, Achatina fulica, Mitochondrial cytochrome b; Cerebrospinal fluid

Posted Date: June 25th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-37815/v1

Abstract

Background: *Angiostrongylus cantonensis* is a well-known pathogen causing human angiostrongyliasis eosinophilic meningitis. Humans, as accidental hosts, are infected by eating undercooked snails containing third-stage larvae. *A. malaysiensis* is closely related to *A. cantonensis* and has been described as a potential human pathogen. Recently, the two species have been reported to have overlapping distributions in the same endemic area, particularly in the Indochina region. Because of their similar morphological characteristics, misidentification often occurs, particularly of the third-stage larva in the snail intermediate host.

Methods: We designed species-specific primers to mitochondrial *cytochrome b*, which was used as a genetic marker. SYBR-green quantitative real-time PCR (qPCR) was employed to quantitatively detect and identify the third-stage larvae and tissue debris in the cerebrospinal fluid (CSF) of a patient, and to quantify third-stage larvae in the snail *Achatina fulica* collected from the field.

Results: The newly designed primers were highly specific and sensitive, even when using conventional PCR. SYBR green qPCR quantitatively detected around 10^{-4} ng of genomic DNA from one larva and facilitated the specific detection and identification of parasitic genetic material from the CSF of a patient with angiostrongyliasis. The method also estimated the number of larvae in *A. fulica* and revealed that the primary source of *Angiostrongylus* infection in the King Rama IX public park study area was *A. malaysiensis*; although, the two *Angiostrongylus* species each infected 10% of the snails.

Conclusions: Our SYBR green qPCR method is a useful and inexpensive technique for parasite identification and has sufficient sensitivity and specificity to detect a single larva and simultaneously discriminate between *A. cantonensis* and *A. malaysiensis*. The number of larvae infecting or co-infecting the snail intermediate host can also be estimated. In future research, this qPCR method could be employed in a molecular survey of *A. cantonensis* and *A. malaysiensis* occurrence within intermediate and definitive hosts. The technique should also be applied in a study analyzing CSF specimens from patients with eosinophilic meningitis to assess the usefulness of the method for clinical diagnosis.

Background

The genus *Angiostrongylus* Kamensky, 1905, contains the zoonotic nematode parasites of vertebrates commonly known as “lungworms” [1]. Several species in this genus have been reported to be human pathogens, including *A. cantonensis* and *A. costaricensis*, while *A. mackerrasae* and *A. malaysiensis* are considered potential human pathogenic parasites [2–5]. Among these species, *A. cantonensis* is a well-known pathogen that causes angiostrongyliasis eosinophilic meningitis in humans [6], over 2,800 cases of which have been reported worldwide, with a particularly high incidence in Thailand [7]. Humans are accidental hosts and are infected by eating undercooked snails, slugs, paratenic hosts, and contaminated fresh vegetables containing the infective third-stage larvae [8]. The clinical symptoms can range from headaches to coma depending on the number of parasitic larvae [2, 8–10]. Many species of terrestrial

and freshwater snails, for example, *Achatina fulica*, *Cryptozona* spp., *Pomacea* spp., and *Pila* spp., are reported to be critical vectors of *A. cantonensis* [11–13]. Global parasite transmission frequency has been promoted by the widespread distribution of some invasive vector species, for example, *A. fulica* and *Pomacea* spp. [13–15]. The ability of the parasite to spread throughout the world may also be influenced by its natural definitive host, a rodent that inhabits areas of human urbanization [1, 6, 16].

Among species of the genus *Angiostrongylus*, a cryptic species of *A. cantonensis* was recently correctly described as *A. malaysiensis* [17, 18]. Previously, *A. malaysiensis* was known as the Malaysian strain of *A. cantonensis* [1, 5]; however, its pathology differed from the typical pathology of *A. cantonensis* through its ability to migrate to the central nervous system of the monkey host but not cause death [1, 19]. The strain was known to immunize monkeys against the lethal effects of a high number of *A. cantonensis* larvae. Subsequently, the Malaysian strain was species delimited as *A. malaysiensis* [5, 20].

The adult stages of *A. cantonensis* and *A. malaysiensis* can be morphologically discriminated by the structure of the bursal rays of males and the minute protrusions at the posterior of females [21]. The third-stage larvae of *A. cantonensis* are described as using the mellifluous “Q”-movement, which clearly distinguishes them from free-living nematodes [22]. However, differences between the morphological characteristics of the larval stage *A. cantonensis* and *A. malaysiensis* have not yet been described.

Previously, the morphological variations between these two species were never considered, and their similarities were the cause of the misidentification. *A. cantonensis* was known as the predominant species in the genus of *Angiostrongylus*, with distribution throughout many countries in Indochina [23]. In 2016, Rodpai et al. revealed that *A. cantonensis* is widely distributed in Thailand, Lao PDR, Cambodia, and Myanmar, and its distribution broadly overlapped that of *A. malaysiensis* [23]. Dusitsittipon et al. discussed the incongruence between the morphological and molecular identifications and found that most of the misidentified specimens were from Thailand [17, 24].

An under-estimation of the prevalence of *A. malaysiensis* has occurred because of the difficulty in the morphological identification of adult and third-stage larva. Several polymerase chain reaction (PCR)-based identification techniques were developed, but all were aimed at increasing the sensitivity and specificity of *A. cantonensis* detection in blood, the peripheral tissues of wild Hawaiian rats, and cerebrospinal fluid (CSF) of patients [25–27]. No powerful techniques with high sensitivity and specificity have been developed for detecting and discriminating between *A. cantonensis* and *A. malaysiensis.*

A. cantonensis and *A. malaysiensis* can be distinguished using the mitochondrial *cytochrome b* (*Cytb*) gene as a genetic marker because of the robust phylogenetic relationship between the closely related species [17, 24]. This gene sequence shows sufficiently high genetic variation between species, and species-specific primers can be used to detect and discriminate between the two species with high sensitivity and specificity. The SYBR-green quantitative real-time PCR method (qPCR) was considered suitable for quantitative detection and species discrimination because it is cheaper than TaqMan qPCR, which requires a fluorescent probe. SYBR green qPCR has recently been used in many studies to detect, discriminate, and quantify species as, for example, in the discrimination and quantification of *Leishmania*

in human samples and the discrimination of species and subspecies of *Salmonella*. The technique was also employed for the detection of *Opisthorchis viverrini* and *Haplorchis taichui* from human stool samples [28–30].

In this study, therefore, we designed species-specific primers to *Cytb* partial gene sequences, which have sufficiently high genetic variation between species to prevent cross-amplification [17, 24]. We aimed to develop a highly sensitive and specific method capable of quantitively detecting the third-stage larvae of *A. cantonensis* and *A. malaysiensis* in their intermediate host, *Achatina fulica*, and to determine the efficacy of the method for the detection of *Angiostrongylus* genomic material within the CSF of infected patients.

Methods

Specimens used

Reference specimens for evaluating sensitivity and specificity

Third-stage larvae of *A. cantonensis*, adult worms of *A. cantonensis* and *A. malaysiensis*, CSF of patients with neurocysticercosis, gnathostomiasis, and angiostrongyliasis, and negative CSF of angiostrongyliasis were obtained from archived research specimen stock kept in the Department of Helminthology, Mahidol University. The selected CSF was from patients with diseases related to eosinophilic meningitis. The larvae and adult worms of *Angiostrongylus* were preserved in 70% ethanol at − 20°C, while the CSF specimens were kept at − 80°C. The specimens were used to test the sensitivity and specificity of the species-specific primers designed for the SYBR green qPCR.

Experimental specimens to determining the validity of SYBR green qPCR

Third-stage larvae of *Angiostrongylus* were collected from 48 *A. fulica* collected at King Rama IX Public Park in Bangkok, Thailand (geographical coordinates 13.68N, 100.65E). The snails were transported to the laboratory of the Department of Helminthology within 1 hour. The snails were then euthanized at 0°C for 10 min. Then, the shell of each snail was removed with a meat grinder. The foot and mantel parts of the snail were dissected and individually incubated with a digestion solution (1% HCl and 1% Pepsin) at 37°C for 1 hour. The digestion method used for the snails followed the protocol of Vitta et al.[31]. The larvae collected were identified as *Angiostrongylus* according to the morphological criteria described by Ash [32]. The larvae from each positive snail were approximately counted and preserved in 70% ethanol at − 20°C until used for DNA extraction. The wild snail collection was performed with permission from the Animal Care and Use Committee, Faculty of Tropical Medicine, Mahidol University, Bangkok (No. FTM-ACUC 024/2018).

Preparation of standard genomic DNA

The individual adult *A. cantonensis* and *A. malaysiensis* of the described reference specimens were transferred into a 1.7-ml centrifuge tube and washed thoroughly with sterile distilled water. Total genomic DNA (gDNA) was extracted from each specimen using the Genomic DNA mini kit (Geneaid Biotech Ltd, Taipei, Taiwan) following the manufacturer's instructions. The gDNA was eluted from the column with 30 μl of PCR-grade sterile water and the concentration measured using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, MA, USA). The gDNA was stored at 4 °C until use.

Design of species-specific primers

Species-specific primers were designed manually based on the *Cytb* gene sequences of *A. cantonensis* (GenBank accession numbers KC995211, KC995223, KC995262) and *A. malaysiensis* (GenBank accession numbers KX147380, KX147406, KX147442). The properties of the oligonucleotide primers, including GC content, amplicon size, melting temperature, and hairpin formation, were predicted by OligoCalc version 3.27 and Primer3 [33, 34]. Information on the designed primers is shown in Table S1.

Optimization of PCR conditions using conventional PCR

Optimization of the PCR conditions was performed prior to the main quantitative reaction using conventional PCR, and the sensitivity and specificity of the newly designed primers were evaluated. Afterward, the optimized conditions for the species-specific primers of *A. cantonensis* and *A. malaysiensis* were applied in the SYB-green qPCR.

Sensitivity test

The gDNA of adult *A. cantonensis* and *A. malaysiensis* reference specimens were prepared by 10-fold serial dilution (between 10^{-5} and 1 ng/μl) for use as DNA templates. The 20 μl PCR mixture contained 1 × One PCR™ Plus mixture (GeneDireX Inc., Taoyuan, Taiwan) 10 μM of species-specific primers and 1 μl of gDNA template. The PCR was performed with an initial denaturation at 95°C for 5 min, followed by 34 cycles of denaturation at 95°C for 30 s, gradient primer annealing between 55°C and 60°C for 30 s, extension at 72°C for 30 s, and final elongation at 72°C for 5 min. The reaction mixtures were then held at 12°C until the PCR products were collected. The PCR was conducted on a T100™ thermocycler (Bio-Rad Laboratories, CA, USA). The PCR products were run on 2% agarose gel at 50 V for 1 hour to determine the size of the amplicons.

Specificity test

Ten-fold serial dilutions of the gDNA from the reference specimens *A. cantonensis* and *A. malaysiensis* were used to determine the specificity of the primers. The DNA templates were used in an experimental mixture of species using different ratios of gDNA of *A. cantonensis* and *A. malaysiensis*, as described in Table S2. This was used to evaluate the robustness of further SYBR-green qPCRs. DNA amplification for the specificity test was performed following the PCR conditions described for the sensitivity test. The PCR products were run on 2% agarose gel at 50 V for 1 hour and stained with SYBR™ Safe (Life Technologies, CA, USA) to determine the species-specific band sizes of *A. cantonensis* and *A. malaysiensis*. The PCR

amplicons were then sequenced by the Sanger method with the PCR primers (Macrogen Inc., Seoul, Korea).

SYBR-green Quantitative Real-time PCR

The SYBR-green qPCR reactions were performed separately for each species using the species-specific primers. The qPCR was performed following the protocol of the Luna® Universal qPCR master mix (New England Biolabs, MA, USA). The reactions contained 10 µl of the master mix, 10 µM of each pair of species-specific primers, and 1 µl of gDNA, and RNAase-free water was added to a final volume of 20 µl. The reaction was performed on a CFX96 TouchTM Real-Time PCR machine (Bio-Rad Laboratories, CA, USA) with the standard PCR conditions. The real-time PCR thermal cycle included an initial denaturation at 95°C for 60 s, followed by 45 cycles of denaturation at 95°C for 15 s, and an extension at 60°C for 30 s. A final melting analysis program was applied at 60°C to 95°C and the duration of each cycle was 5 s with a 0.5°C increment per cycle. The SYBR-green qPCR described above was used for standard curve preparation, specificity and sensitivity assays, reproducibility assay, and implementation of the developed method to detect the third-stage larvae of *Angiostrongylus* in *A. fulica*.

Standard curve construction for SYBR-green qPCR

Ten-fold serial dilutions of *A. cantonensis* and *A. malaysiensis* adult worm gDNA were used to construct the SYBR-green qPCR standard curve; gDNA concentrations of 10^{-4} to 1 ng/µl were used as DNA templates for qPCR, and each DNA concentration was amplified three times. The precision of the standard curve and robustness of the qPCR were verified by considering the slope values, correlation coefficient, and qPCR efficiency of both *A. cantonensis* and *A. malaysiensis*.

Specificity assay

The species-specific primers developed for *A. cantonensis* and *A. malaysiensis* were evaluated for specificity to the various gDNA templates described in Table 2 using the SYBR-green qPCR profile described above. The qPCRs were conducted three times for each gDNA template. The specificity of the qPCR was also tested with the CSF of patients with gnathostomiasis, cysticercosis, and angiostrongyliasis. The PCR products were then run on 2% agarose gel at 50 V for 1 hour and stained with SYBR™ Safe (Life Technologies, CA, USA) to determine the species-specific band sizes with the positive controls for each *Angiostrongylus* species. The purified DNA samples were sequenced by Macrogen (Seoul, South Korea), an external biotechnology company, using the Sanger sequencing method with the primers used for the PCR amplification. The obtained nucleotide sequences (query sequences) were confirmed as the specific species target sequences by comparing with annotated sequences in the NCBI databases using the standard nucleotide BLAST [35].

Table 2
Intra- and inter-reproducibility assays of qPCR for the species-specific primers of *A. malaysiensis*.

Category	Genomic DNA standard (ng/μl)	Mean (Ct)	SD	CV(%)
Intra-assay	1×10^{1}	15.65	0.077	0.492
	1×10^{-1}	19.32	0.019	0.098
	1×10^{-2}	22.33	0.052	0.233
	1×10^{-3}	26.12	0.048	0.184
	1×10^{-4}	29.31	0.017	0.058
Inter-assay	1×10^{1}	15.40	0.354	2.299
	1×10^{-1}	18.99	0.467	2.459
	1×10^{-2}	22.24	0.134	0.603
	1×10^{-3}	26.08	0.057	0.219
	1×10^{-4}	29.02	0.410	1.413

Sensitivity assay

To test the sensitivity of the SYBR-green qPCR method, we used gDNA from groups of 1, 5, 10, 50, 100, and 200 third-stage *A. cantonensis* larvae. Before gDNA isolation, the cuticles of the larvae were broken by bead beating with 20 mg of 0.1 mm silica beads in 200 μl lysis buffer using Tissue Lyser LT at 50 Hz for 30 s (Qiagen, Hilden, Germany). Each larval sample was beaten three times and then left on ice to prevent DNA degradation. The gDNA from each group of larvae was then extracted using the Tissue Genomic DNA Mini kit (Geneaid Biotech Ltd, Taipei, Taiwan). Subsequently, each sample was amplified three times with the SYBR-green qPCR profile described above. The threshold cycle (C_t) or the quantitation cycle (Cq) values obtained for each group were estimated with the standard curve for the qPCR to determine the amount of larval gDNA.

Reproducibility assay

The 10-fold serial dilution of the standard DNA, which was used to construct the standard curve, was used in the reproducibility assay. The intra-reproducibility assay was estimated by amplifying three replicates of each DNA concentration using the qPCR profile described above. Variations in the intra-reproducibility assays were assessed. The experiments were repeated on 2 different days to determine the inter-reproducibility. The mean, SD, and coefficient of variation (CV) were calculated separately using the Ct values to evaluate the reproducibility of the developed SYBR-green qPCR. The percentage of the CV of

inter- and intra-reproducibility assays were calculated to determine the consistency of the assay manipulation.

Detection of third-stage larvae of A. cantonensis **and** A. malaysiensis **from naturally infected** A. fulica

The developed SYBR-green qPCR was implemented to detect and identify the third-stage larvae of *Angiostrongylus* collected from *A. fulica*. Before gDNA extraction, the larval cuticles were disrupted as described above. The larvae were then removed separately from each snail, and the DNA was extracted using the tissue genomic DNA mini kit (Geneaid, Taipei, Taiwan) according to the manufacturer's instructions. The extracted gDNA was then used as the template in the developed qPCR method. The standard curves were constructed to estimate the number of larvae and the ratio of *A. cantonensis* to *A. malaysiensis* in each *A. fulica*.

Results

Optimization of the newly designed species-specific primers using conventional PCR

Sensitivity and specificity tests with conventional PCR

The PCR products from the 10-fold diluted gDNA of *A. cantonensis* and *A. malaysiensis* are shown in Figure S1. The different sizes of the species-specific bands were used to discriminate between *A. cantonensis* (117 bp) and *A. malaysiensis* (141 bp). The amplifications were successful for DNA concentrations of 10^{-4} to 1 ng/μl.

The mixed *A. cantonensis* and *A. malaysiensis* gDNA was used to evaluate the specificity of the newly designed species-specific primers. Different ratios of gDNA from the two species, including 10^{-2}:10^{-1} ng, and vice versa, and 1:1 ng were amplified with conventional PCR using the above conditions. The sizes of the species-specific PCR amplicons were used to determine the specificity of the designed primers (Fig. S2). The DNA sequences of those PCR amplicons also confirmed the PCR specificity.

Standard curve for SYBR-green quantitative real-time PCR

The effectiveness of the SYBR-green qPCR was determined using the species-specific primers for *A. cantonensis* and *A. malaysiensis Cytb*. The standard curves for *A. cantonensis* (Slope = − 3.379, R^2 = 0.999) and *A. malaysiensis* (Slope = − 3.412, R^2 = 0.999) are shown in Fig. 1. The efficiency values of the qPCR for both species-specific primers of *A. cantonensis* and *A. malaysiensis* were 94.8% and 96.4%, respectively.

Sensitivity and specificity of SYBR green quantitative real-time PCR

The sensitivities of the species-specific primers used in SYBR-green qPCR were tested using the gDNA extracted from various *A. cantonensis* larvae group sizes from 1 to 200. The Cq values for the amplified larval gDNA were compared with those of the standard curve. Approximately 1 ng of gDNA was extracted from 100 larvae, while 10^{-4} ng of gDNA was extracted from 1 larva (Fig. 2A). The qPCR melting curve and the gel electrophoresis demonstrated the specificity of the primers to the *A. cantonensis* gDNA (Fig. 2B).

The specificity of the SYBR-green qPCR was determined using the gDNA template described in Table S2. Figure S3 shows the specificity of the *A. cantonensis* and *A. malaysiensis*-specific primers. The species-specific primers did not amplify the gDNA across different species. The high specificities of the AC4_cytb_F and AC5_cytb_R primers for *A. cantonensis* were also demonstrated for the artificially mixed *A. cantonensis* and *A. malaysiensis* gDNA, in both the 1:1 ratio and when there was less *A. cantonensis* than *A. malaysiensis* gDNA. These primers did not amplify *Cytb* from gnathostomiasis and neurocysticercosis samples or the heterogeneous sample containing 1 ng of *A. malaysiensis* gDNA. The specificity of the AC4_cytb_F and AC5_cytb_R primers was confirmed by the size of the qPCR amplicon (Fig. 3A). A specificity assay was then performed with AM3_cytb_F and AM4_cytb_R primers for *A. malaysiensis*. They also showed a specificity similar to the primers for *A. cantonensis*. The primers designed for *A. malaysiensis* did not amplify any *Cytb* from the CSF of patients, including those with angiostrongyliasis (Fig. 3B).

Intra- and inter-reproducibility assays of SRBR-green qPCR

Intra- and inter-reproducibility assays were performed similarly to the method used to construct the *A. cantonensis* and *A. malaysiensis* gDNA standard curves. Serial concentrations of gDNA from 10^{-4} to 1 ng were amplified using SYBR-green qPCR. Three replicates of each DNA concentration were conducted for the intra-reproducibility assay, and the qPCRs were repeated over 3 days. The results showed there was consistent amplification between the replicated series of gDNA concentrations in the same assay (inter-reproducibility). The precision of the assay manipulation was considered to be the reproducibility of the qPCR reactions for *A. cantonensis* and *A. malaysiensis*, which is shown in Tables 1 and 2.

Table 1
Intra- and inter-reproducibility assays of qPCR for the species-specific primers of *A. cantonensis*.

Category	Genomic DNA standard (ng/μl)	Mean (Ct)	SD	CV(%)
Intra-assay	1×10^{1}	15.63	0.051	0.326
	1×10^{-1}	19.33	0.103	0.533
	1×10^{-2}	22.38	0.012	0.054
	1×10^{-3}	26.21	0.065	0.248
	1×10^{-4}	29.46	0.168	0.570
Inter-assay	1×10^{1}	15.53	0.148	0.953
	1×10^{-1}	19.26	0.099	0.514
	1×10^{-2}	22.35	0.049	0.219
	1×10^{-3}	26.30	0.120	0.456
	1×10^{-4}	29.49	0.035	0.119

Detection of the third-stage Angiostrongylus **larvae from naturally infected** A. fulica

The SYBR-green qPCR results showed that the species-specific primers discriminated between *A. cantonensis* and *A. malaysiensis* third-stage larvae. All of the third-stage *Angiostrongylus* larvae collected from each snail were approximately counted, and DNA was extracted without excluding dead and weakened larvae. Larvae numbers in each snail were estimated as the degree of intensity, and the results are shown in Table 3. The qPCR results suggested there was no cross-amplification. The *Angiostrongylus* larvae detected from four infected snails were mainly *A. malaysiensis*, while two snails had coinfections of *A. malaysiensis* and *A. cantonensis.* There were fewer *Angiostrongylus* larvae counted from each snail than the numbers estimated by qPCR.

Table 3
The SYBR green qPCR results of the *Angiostrongylus* larvae infected in four from fourty-eight *A. fulica* snails collected from King Rama IX Park, Bangkok, Thailand.

A. fulica **snail**	***Angiostrongylus*** **larva intensity**	**Ct values of SYBR green qPCR**		**Estimated larva numbers**
		A. cantonensis **(Ac)**	***A. malaysiensis*** **(Am)**	
AF_P_01	+++	34.32	21.30	Ac ≥ 1 Am ≥ 50
AF_P_02	+		27.97	Am ≥ 5
AF_P_03	+++	35.42	22.97	Ac ≥ 1 Am ≥ 50
AF_P_04	+		29.13	Am ≥ 1

Discussion

In this study, we developed a simple, economical, highly sensitive, and specific assay based on SYBR-green qPCR for the detection, discrimination, and quantification of third-stage larvae of *A. cantonensis* and its closely related species *A. malaysiensis* from the intermediate host *A. fulica*. Recently, in some areas, and particularly areas in Indochina, there have been reports of overlap in the distributions of *A. cantonensis* and *A. malaysiensis* within the terrestrial snail host [23]. Although the gold standard for *Angiostrongylus* identification is the use of morphological characteristics, the characteristics of the third-stage larvae of these two species are very similar [5, 21, 32] and misidentification often occurs.

We designed species-specific primers to the mitochondrial *Cytb* gene of *A. cantonensis* and *A. malaysiensis* based on the genetic variation in the partial sequences. The two species can be differentiated by comparing the size of conventional PCR products for mitochondrial *Cytb* on gel electrophoresis (see Fig. S1). The high sensitivity of conventional PCR allows the amplification of 10^{-4} ng of gDNA. The newly designed primers showed high specificity without cross-species amplification when a heterogeneous gDNA sample containing a small amount of specific DNA template was used (Fig. S2). However, conventional PCR cannot quantify the number of *Angiostrongylus* larvae. Therefore, a qPCR method was developed that could detect a small amount of DNA (10^{-4} ng or less) from both *Angiostrongylus* species. We also established the standard curve and demonstrated the high efficiency of the qPCR reactions (see Fig. 1A and B). The % CV of reproducibility confirmed that the standard curve construction had low system variation in the manipulation assay. The number of larvae was then estimated using a standard curve constructed from 10-fold serial DNA dilutions. Based on the qPCR results, we postulated that DNA damage and loss during the larvae preservation and DNA extraction

process might have resulted in the fluctuation of the Cq value, particularly for a small amount of DNA. In contrast, there was no effect seen when more than 10 larvae were used (Fig. 2A).

We also confirmed the high specificity and absence of cross-amplification of the method using heterogeneous gDNA in the SYBR-green qPCR reactions (Fig. 3). The developed method provides an alternative way of detecting the low amount of *Angiostrongylus* genetic material contained in CSF specimens. We also showed the potential of using SYBR-green qPCR for diagnosis using the CSF of the patients with an *A. cantonensis* infection, and there was no cross-reaction with other diseases that cause eosinophilic meningitis, such as cysticercosis and gnathostomiasis.

After proving the efficacy and consistency of the developed qPCR assay, we implemented it in the molecular discrimination of *A. cantonensis* and *A. malaysiensis* third-stage larvae from *A. fulica* collected in a suburban public park in Bangkok, Thailand. The number of larvae can be ascertained using the developed qPCR method and comparing the results to the standard curve (see Fig. 2A). We obtained relatively fewer numbers from the larvae count compared with the qPCR estimate because the larvae collected from the snails included those that were dead and dying. Therefore, when applying this method to a molecular survey of *Angiostrongylus* larvae, the report should indicate that the numbers obtained are approximate or only indicate the ratio between species. When small numbers of larvae are being studied and species discrimination without quantification is adequate, we suggest that conventional PCR is used with the species-specific primers.

Although the developed method required the design of two species-specific primers, which were tested using two separate DNA templates, the two sets of primers can be used together with the same qPCR conditions and are sensitive enough to detect a single target species. This method does not require the design of a separate probe, such as with the TaqMan qPCR technique, which can be complicated and expensive. Moreover, SYBR green qPCR appears to be more sensitive than the TaqMan probe-based qPCR [29, 36]. The specificity of SYBR-green qPCR may also provide information regarding the amount of DNA amplification by using the melting curve analysis [37].

Previously, the TaqMan probe real-time PCR was developed using ribosomal internal transcribed spacer 1 as the genetic marker [38] for detecting third-stage *A. cantonensis* larvae in mollusks, and it was more sensitive than 18S rDNA-based conventional PCR [38, 39]. However, the TaqMan qPCR was not designed to estimate larval numbers or the level of infection in the snails [38]. The technique of detecting the DNA of *A. cantonensis* was applied to the diagnoses of patients with eosinophilic meningitis [26]. Although the newly developed SYBR-green qPCR method, using the mitochondrial *Cytb* gene as a marker, has not yet been proven to be suitable for molecular diagnosis, it may provide an alternative method in the future. The new technique has the potential to confirm the occurrence of *A. malaysiensis* infection in patients living in areas where the distributions of *A. cantonensis* and *A. malaysiensis* overlap.

Although the sensitivity assay for the developed qPCR was limited because of the lack of a verified source of *A. malaysiensis* larvae, such as a reference strain, an assessment of the amount of DNA from various numbers of *A. cantonensis* was used instead. The high precision of the technique was clear, as

the mean Ct values for *A. cantonensis* and *A. malaysiensis* were similar between manipulation assays, even for low amounts of gDNA (Tables 3 and 4). This evidence can be used to infer that the approximate amount of gDNA from the various *A. cantonensis* larva numbers can be used to represent *A. malaysiensis*. The approximate number of *A. malaysiensis* larvae may be determined from the estimated number in the sensitivity test of *A. cantonensis* larvae (Fig. 2A). The developed method has not yet been employed to detect larvae in *A. fulica* tissue quantitively. We suggest that larvae should be isolated from the snails before gDNA extraction to determine the accuracy of the larvae number estimated by the qPCR method.

Conclusions

The developed SYBR-green qPCR method is a useful and inexpensive alternative technique for molecular parasite detection. It has sufficiently high sensitivity and specificity to simultaneously detect a single larva of *Angiostrongylus* and discriminate between larvae of *A. cantonensis* and *A. malaysiensis*. The developed qPCR method can also be used to estimate the number or ratio of larvae infecting or co-infecting the snail intermediate host. In further studies, the developed qPCR method may be used to perform a molecular survey of the incidence of *A. cantonensis* and *A. malaysiensis* in the natural intermediate host and the definitive rodent host, particularly in Indochina, which has been found to be an area with overlapping distributions of *A. cantonensis* and *A. malaysiensis*. However, the suitability of the method for diagnosis in the clinical setting needs to be evaluated by testing mixtures of unknown parasitic and free-living nematode larvae. In the future, the usefulness of the developed SYBR-green qPCR technique for clinical diagnosis using CSF specimens from patients with eosinophilic meningitis should also be assessed.

Declarations

Ethics approval and consent to participate

Ethical clearance was provided by the Animal Care and Use Committee, Faculty of Tropical Medicine, Mahidol University, Bangkok (No. FTM-ACUC 024/2018)

Consent for publication

Not applicable

Availability of data and material

All data generated during this study are included in the published article

Competing interests

Authors declare that they have no competing interests

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, and not-for-profit sectors.

Authors' contributions

UT, KC, NS, YL conceived the study. WJ performed molecular works. WJ, KC, SD, VC, UT collected the samples. All authors reviewed the manuscript draft and read and approved the final manuscript.

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Figures

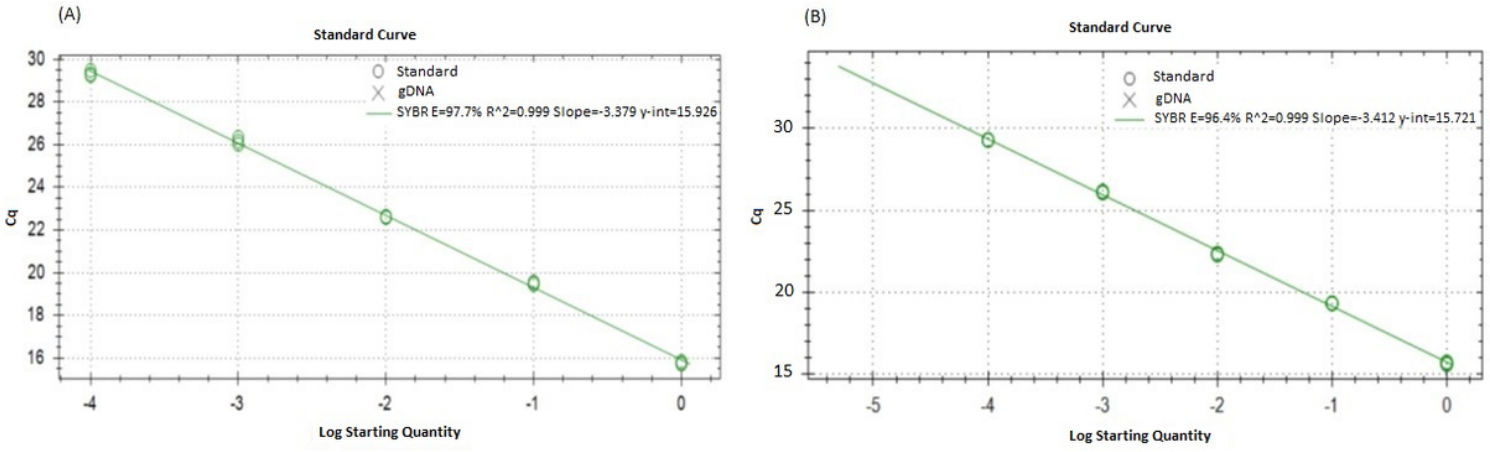

Figure 1

Amplification plots indicating the standard curve of the SYBR green qPCR with each species-specific primer for A. cantonensis (A) and A. malaysiensis (B) Cytb. The standard curves are plotted from 10-fold serial dilutions of gDNA from 10−4 to 1 ng/µl.

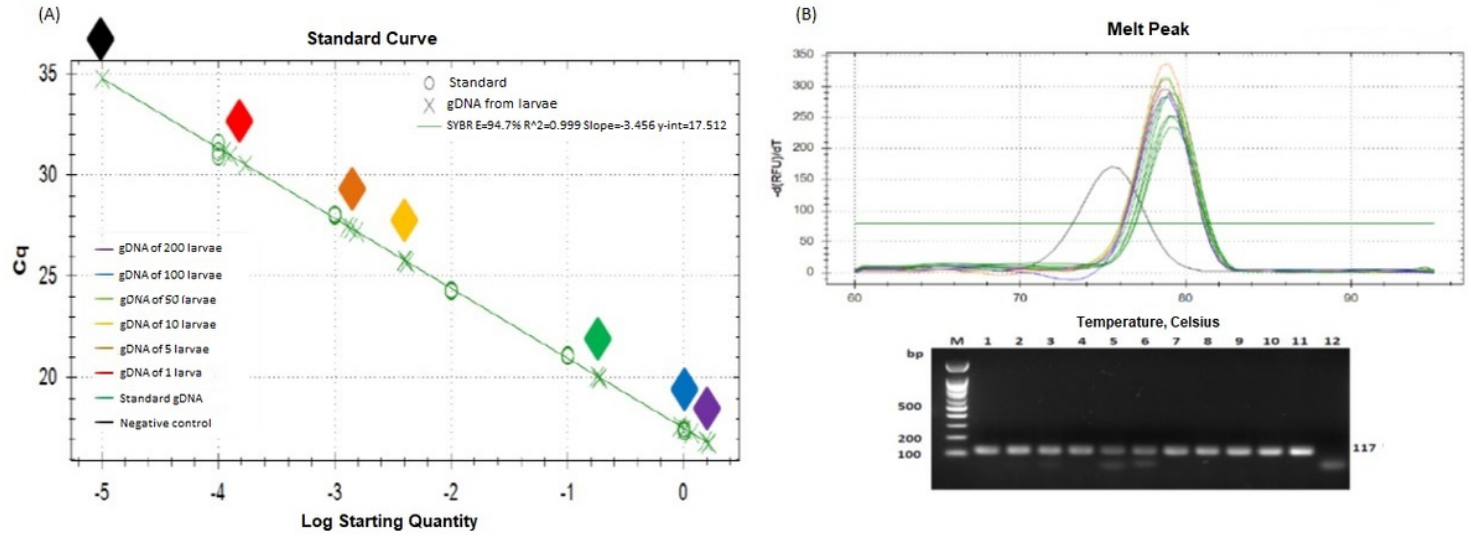

Figure 2

Sensitivity test for species-specific primers using an SYBR-green qPCR approach. (A) Quantitative estimation of the gDNA from third-stage larva of A. cantonensis. The gDNA of various larvae numbers, 1, 5, 10, 50, 100, and 200, were amplified and plotted on the standard curve constructed from the 10-fold serial dilution (10−4–1 ng). (B) The melting curve of A. cantonensis was specific at 79□C (left). Gel electrophoresis showing the results of qPCR; lanes 1–5 are the PCR products of 10-fold serial dilution of gDNA from 10−4 to 1 ng/µl, respectively; lanes 6–12 are the PCR products amplified from gDNA extracted from 1, 5, 10, 50, 100, and 200 larvae and the negative control (right).

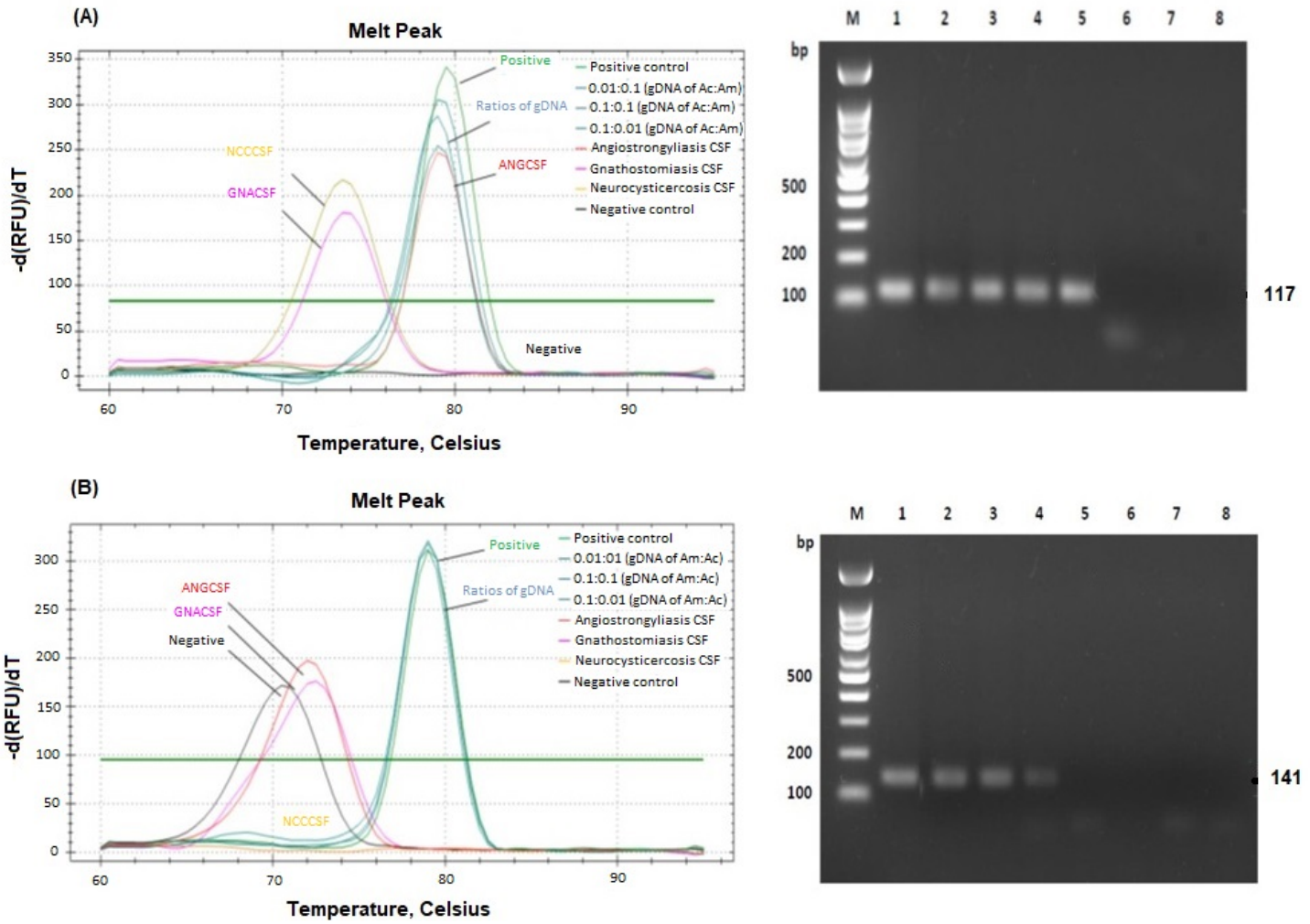

Figure 3

Specificity of species-specific primers for A. cantonensis and A. malaysiensis. (A) The specificity of AC4_cytb_F and AC5_cytb_R primers to Cytb of A. cantonensis, and the CSF from patients of gnathostomiasis (GNA), neurocysticercosis (NCC), and angiostrongyliasis (ANG) using the SYBR green qPCR method. Gel electrophoresis showing the results of SYBR-green qPCR. Lane 1 is the PCR product from 1 ng gDNA of A. cantonensis. Lanes 2–4 are PCR products from the different ratios (10−2:10−1 ng, 10−1:10−1 ng, and 10−1:10−2 ng) of the gDNA of A. cantonensis and A. malaysiensis, respectively. Lane 5 is the PCR product of CSF from a patient with angiostrongyliasis. Lanes 6 and 7 show no PCR product from patients with neurocysticercosis and gnathostomiasis. Lane 8 is the negative control. (B) The specificity of AM3_cytb_F and AM4_cytb_R primers to Cytb of A. malaysiensis and the CSF from patients with gnathostomiasis (GNA), neurocysticercosis (NCC), and angiostrongyliasis (ANG) using SYBR-green qPCR. Gel electrophoresis showing the results of SYBR-green qPCR. Lane 1 is the PCR product from 1 ng of A. malaysiensis gDNA. Lanes 2–4 are PCR products from the different ratios (10−1:10−2 ng, 10−1:10−1 ng, and 10−2:10−1 ng) of A. malaysiensis and A. cantonensis gDNA, respectively. Lanes 5–7 show no PCR product for the CSF of patients with angiostrongyliasis, neurocysticercosis, and gnathostomiasis. Lane 8 is the negative control.

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