Newly developed SYBR Green-based quantitative real-time PCRs revealed coinfection evidence of *Angiostrongylus cantonensis* and *A. malaysiensis* in *Achatina fulica* existing in Bangkok Metropolitan, Thailand

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ABSTRACT

Angiostrongylus cantonensis is a well-known pathogen causing eosinophilic meningitis associated with angiostrongyliasis. Humans, as accidental hosts, are infected by consuming undercooked snails containing third-stage larvae. *A. malaysiensis* is closely related to *A. cantonensis* and has been described as a potential human pathogen. The two species distribution was recently reported to overlap in the same endemic area, particularly in the Indochina Peninsula. Similar morphological characteristics of the third-stage larva in the snail-intermediate host often lead to misidentification of the two species. Thus, we aimed to develop a sensitive and specific method to detect and discriminate *Angiostrongylus* third-stage larva by designing species-specific primers based on the mitochondrial *cytochrome b* gene. We developed the SYBR Green quantitative real-time PCR (qPCR) method for two species-specific detection assays, which could be conducted simultaneously. The method was subsequently employed to detect and identify third-stage larvae of *Angiostrongylus* isolated from infected *Achatina fulica* collected from six public parks in Bangkok Metropolitan, Thailand. The method was also a preliminary applied to detect parasite tissue debris in the patients' cerebrospinal fluid (CSF). SYBR Green qPCRs quantitatively detected approximately 10^{-4} ng of genomic DNA from one larva, facilitating species-specific detection. Based on the pools of third-stage larvae isolated individually from the tissue of each infected *A. fulica* collected from the public parks, the qPCR results revealed that *A. malaysiensis* was the predominant species infecting 5.26% of the collected snails. In comparison, coinfection between *A. malaysiensis* and *A. cantonensis* was 5.97%, and no single infection of *A. cantonensis* was detected in *A. fulica*. Our SYBR Green qPCR method is a useful and inexpensive technique for *A. cantonensis* and *A. malaysiensis* discrimination, and the method has sufficient sensitivity to detect isolated larvae from a snail-intermediate host. The ratio of *A. cantonensis* and *A. malaysiensis* larvae infecting the snails can also be estimated simultaneously. Our qPCRs can be employed in a molecular survey of *A. cantonensis* and *A. malaysiensis* within intermediate hosts and for clinical diagnosis of angiostrongyliasis with CSF specimens in future studies.

1. Introduction

The genus *Angiostrongylus* Kamensky, 1905, contains the zoonotic nematode parasites of vertebrates, commonly known as "lungworms" (Spratt, 2015). 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 (Bhaibulaya and Cross, 1971; Sawabe and Makiya, 1994; Aghazadeh et al., 2016; Ansdell and Wattanagoon, 2018). Among them, *A. cantonensis* is a well-known pathogen that causes eosinophilic meningitis associated with angiostrongyliasis in humans (Barratt et al., 2016), particularly in Thailand (Eamsobhana, 2013). Humans are accidental hosts and are infected by eating undercooked snails, slugs, paratenic hosts, and contaminated fresh vegetables containing the infective third-stage larvae (Wang et al., 2012). The clinical symptoms can range from headaches to coma, depending on the number of parasitic larvae (Wang et al., 2012; Pozio, 2015; Ansdell and Wattanagoon, 2018; Johnston et al., 2019). Many terrestrial and freshwater snails, including, *Achatina fulica*, *Cryptozona* spp., *Pomacea* spp., and *Pila* spp., are reported to be important vectors of *A. cantonensis* (Thiengo et al., 2010; Hu et al., 2018; Vitta et al., 2016). Moreover, the widespread distribution of some invasive vector species (*A. fulica* and *Pomacea* spp.) has promoted parasite transmission (Thiengo et al., 2010; Fontanilla and Wade, 2012; Yang et al., 2013). Rodents, which are the natural-definitive host of *Angiostrongylus*, also facilitated parasite transmission through inhabiting urban areas close to humans (Alicata, 1965; Spratt, 2015; Barratt et al., 2016).

Adults of *A. cantonensis* and *A. malaysiensis* can be morphologically discriminated by the bursal rays of males and the minute protrusion at the posterior end of females (Bhaibulaya, 1979). However, morphological variations between the two species can confound identification. Furthermore, differences between the morphological characteristics of the larval stages have not yet been described. *A. cantonensis* was known as the predominant species widely distributed throughout many countries in Indochina (Rodpai et al., 2016). However, a cryptic species of the Malaysian strain of *A. cantonensis* was recently correctly described as *A. malaysiensis* (Eamsobhana et al., 2015; Dusitsittipon et al., 2017), due to differences in its pathogenicity. It was recently revealed that *A. cantonensis* and *A. malaysiensis* overlap in their distribution, and misidentifications mostly in the specimens from Thailand were discovered (Rodpai et al., 2016; Dusitsittipon et al., 2017, 2018). Thus, an under-estimation of the prevalence of *A. malaysiensis* could result, especially in the Indochina Peninsula, due to the difficulties in the morphological identification of adult and third-stage larva.

To overcome the challenges in the morphological identification of *Angiostrongylus*, several polymerase chain reaction (PCR)-based identification techniques were developed. However, these techniques were aimed at increasing the sensitivity and specificity of detecting *A. cantonensis* in blood, the peripheral tissues of wild Hawaiian rats, and the cerebrospinal fluid (CSF) of patients (Eamsobhana et al., 2013; Jarvi et al., 2015; Qvarnstrom et al., 2016). To date, no technique with both high sensitivity and specificity have been developed for detecting and discriminating between *A. cantonensis* and *A. malaysiensis.*

Studies have used the mitochondrial cytochrome *b* (*Cytb*) gene for species discrimination of *A. cantonensis* and *A. malaysiensis* due to high genetic variation between both species and its ability to provide robust phylogenetic relationships (Dusitsittipon et al., 2017, 2018). The SYBR Green quantitative real-time PCR (qPCR) method was considered suitable for quantitative detection and species discrimination and has been used successfully in many studies to detect, quantify, and discriminate species. For example, *Opisthorchis viverrini* and *Haplorchis taichui* were detected from human stool samples, *Leishmania* was quantified in human samples, and *Salmonella* subspecies were all successfully discriminated (Weirather et al., 2011; Barbau-Piednoir et al., 2013; Lamaningao et al., 2017).

Through the design of species-specific primers to the partial *Cytb* gene sequences of *A. cantonensis* and *A. malaysiensis*, we, therefore, aimed to develop a sensitive and specific method using SYBR Green qPCRs for the detection and species discrimination of *Angiostrongylus* third-stage larvae. We also determined the efficacy of the technique by detecting *Angiostrongylus* genomic material within the CSF of infected patients. The developed qPCRs were subsequently implemented to detect, discriminate, and estimate the ratio between *A. cantonensis* and *A. malaysiensis* third-stage larvae isolated from *A. fulica* collected from Bangkok public parks to explore the prevalence of *A. cantonensis* and *A. malaysiensis* distributing in Bangkok Metropolitan.

2. Materials and methods

2.1. Specimens used

2.1.1. Reference specimens for evaluating sensitivity and specificity

The third-stage larvae of *A. cantonensis*, adults of *A. cantonensis* and *A. malaysiensis*, and CSF of patients with diseases related to eosinophilic meningitis, including neurocysticercosis, gnathostomiasis, and angiostrongyliasis, were used as the reference specimens. Disease diagnoses of the CSF from patients were performed using the immunoblot assay by the immunological diagnostic service unit of the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University. The positive CSF specimens
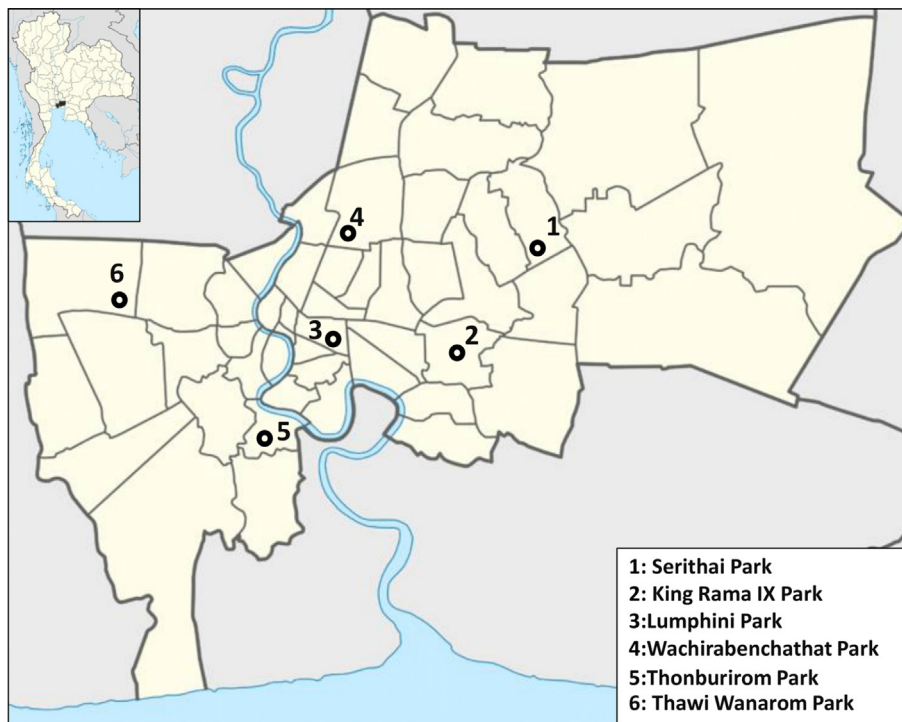
Fig. 1. Map of the six public parks in the Bangkok Metropolitan where wild *A. fulica* were collected.

were kept as the archived research specimen stock of the Department. The active larvae and adult worms of *Angiostrongylus* were preserved in 70% ethanol at −20 °C, while the CSF specimens were kept at −80 °C. The *Angiostrongylus* specimens were used to evaluate the sensitivity, specificity, and reproducibility of the species-specific primers designed for the SYBR Green qPCRs. One sample per disease was used to determine the specificity of the qPCRs.

2.1.2. Third-stage larvae isolated from wild A. fulica to implement with the developed SYBR Green qPCRs

285 *A. fulica* snails were collected by hand from six public parks around Bangkok, Thailand (see Fig. 1). After collection, the snails were transported to the laboratory within an hour and subsequently euthanized at 0 °C for 10 min. The shell of each snail was removed, the foot and mantel parts were dissected and incubated with a digestion solution (1% HCl and 1% pepsin) at 37 °C for 1 h, following the protocol of Vitta et al. (Vitta et al., 2011). According to Ash's criteria, the larvae isolated from each snail were morphologically identified (Ash, 1970). From each *Angiostrongylus*-positive snail, larvae were counted under a stereomicroscope and preserved in 70% ethanol at −20 °C before being used for DNA extraction. The number of *Angiostrongylus*-positive snails and the number of larvae infecting each snail are listed in Table S1. All the larva was taken into the total count, regardless of whether it was active, weak, or had a degraded body. The collection of *A. fulica* was performed with permission from the Animal Care and Use Committee, Faculty of Tropical Medicine, Mahidol University, Bangkok (No. FTM-ACUC 024/2018).

2.2. Genomic DNA extraction

2.2.1. Preparation of reference gDNA and CSF samples

The individual adult *A. cantonensis* and *A. malaysiensis,* and the third-stage larvae of *A. cantonensis* of the described reference specimens were transferred into a 1.7-ml centrifuge tube and washed thoroughly with sterile distilled water to remove the ethanol. The third-stage larvae of *A. cantonensis* were grouped into pools of 1, 5, 10, 50, 100, and 200 larvae. Before DNA extraction, the larval cuticles were homogenized using TissueLyser LT at 50 Hz for 30 s (Qiagen, Hilden, Germany) with 20 mg of 0.1 mm silica beads in 200 μl lysis buffer. For the CSF samples, we centrifuged them separately at 2000 rpm to obtain tissue debris before DNA extraction. Total genomic DNA (gDNA) was extracted from the adult, pooled larval specimens, and CSF samples using the Genomic DNA mini kit (Geneaid Biotech Ltd., Taipei, Taiwan) following the manufacturer's instructions. The eluted gDNA concentration was measured using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, MA, USA). The gDNA was stored at 4 °C until use.

2.2.2. DNA extraction of the isolated third-stage larvae from wild A. fulica

The gDNA of the isolated *Angiostrongylus* larvae from each positive snail was extracted following the third-stage larvae method described above. In instances where the pooled larvae did not exceed 100 per snail, all of the larvae were pooled and extracted

Table 1

Species-specific primers designed for *A. cantonensis* and *A. malaysiensis* using the *CytB* gene.

Species	Primer	Sequencea	Amplicon size (bp)
A. cantonensis	AC4_cytb_F	5′AAT GTT TGT TGA GGC AGA TC 3′	117
	AC5_cytb_R	5′ GCT ACA ACA CCC ATA ACC T 3′	
A. malaysiensis	AM3_cytb_F	5′ CGA GAT ATT TAT TGA GGC TG 3′	141
	AM4_cytb_R	5′GAC AAA ACC TCT ATC AAT AA 3′	

together. In instances where the pooled larvae exceeded 100 per snail, we randomly picked up only 100 larvae. When the pooled larvae were less than 10, we did not perform gDNA extraction and excluded them from the qPCRs to avoid a false negative, resulting from either the degraded or weakened larvae present in the sample pool.

2.3. Development of SYBR Green qPCRs

2.3.1. Design of species-specific primers

Species-specific primers were designed manually based on the partial *Cytb* gene sequences of *A. cantonensis* (GenBank accession numbers KC995188, KC995190, KC995193, KC995268, KC995262, KC995265, KC995248, KC995214, KC995211, KC995226, and KC995223, KX147425) and *A. malaysiensis* (GenBank accession numbers KX147395, KX147380, KX147409, KX147398, KX147455, KX147406, KX147442, KX147445, KX147405, NC_030332, and KT947979). The oligonucleotide primer properties, including GC content, amplicon size, melting temperature, and hairpin formation, were predicted by OligoCalc version 3.27 and Primer3 (Kibbe, 2007; Untergasser et al., 2007). To determine the specificity of the designed primers, *in-silico* PCR was performed using FastPCR 6.6 (Kalender et al., 2014) by testing cross-amplification with the *Cytb* gene sequences of *A. costaricensis* (AP017675), *A. vasorum* (NC_018602) and *A. mackerrasae* (MN793157). The nucleotide sequences of the designed species-specific primers are provided in Table 1.

2.3.2. SYBR Green quantitative real-time PCRs

The SYBR Green qPCRs were performed separately for *A. cantonensis* and *A. malaysiensis* using the designed species-specific primers. Following the protocol of the Luna® Universal qPCR master mix (New England Biolabs, MA, USA), each 20 μl reaction contained 10 μl of the master mix, 1.0 μM of each pair of species-specific primers, 1 μl of gDNA, and RNAase-free water. The qPCRs of both species were performed simultaneously on a CFX96 TouchTM Real-Time PCR machine (Bio-Rad Laboratories, CA, USA), and the thermal cycling profile 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 60–95 °C and each cycle duration was 5 s with a 0.5 °C increment per cycle. The SYBR Green qPCRs described above were used for standard curve preparation, specificity and sensitivity assays, and reproducibility assay. These qPCRs were also implemented to detect and discriminate species of the third-stage larvae of *Angiostrongylus* isolated from wild *A. fulica*.

2.3.3. Standard curve construction for SYBR Green qPCRs

gDNA of the *A. cantonensis* and *A. malaysiensis* adults were serially diluted in ten-folds (ranging from 10^{-4} to 1 ng/μl) and used as templates for the species-specific SYBR Green qPCRs to construct the standard curve of each species. Three replicates of each gDNA concentration were performed. The precision of the standard curve and the qPCR robustness for both *A. cantonensis* and *A. malaysiensis* were verified by considering the slope values, correlation coefficient, and qPCR efficiency.

2.4. Assessment of the developed SYBR Green qPCRs

2.4.1. Specificity assay

With the thermocycling profile describe above, the species-specific primers developed for *A. cantonensis* and *A. malaysiensis* were evaluated for their specificity to the various target gDNA templates. Triplicates were performed for each gDNA template. The qPCR specificity was also tested with the CSF of patients with gnathostomiasis, cysticercosis, and angiostrongyliasis. PCR products were visualized on 2% agarose gel stained with SYBR™ Safe (Life Technologies, CA, USA) to determine the species-specific band sizes together with positive controls. The purified DNA samples were sequenced by Macrogen (Seoul, South Korea), an external biotechnology company, using the Sanger sequencing method with PCR primers. The obtained nucleotide sequences (query sequences) were confirmed as the target sequences by comparing them with annotated sequences in the NCBI databases using the standard nucleotide BLAST (Johnson et al., 2008). The specificity of qPCR reactions was considered by the melting curve analysis to determine the qPCR amplicons of *A. malaysiensis*- and *A. cantonensis*.

2.4.2. 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. Triplicates were performed for each group, and the quantitation cycle (Cq) values obtained for each group were then estimated with the standard curve to determine the amount of larval gDNA.

2.4.3. Reproducibility assay

To evaluate the reproducibility of the developed SYBR Green qPCRs, inter-and intra- reproducibility experiments were conducted using the same serially diluted gDNA concentrations used previously to construct the standard curve. The intra-reproducibility assay was performed by amplifying three replicates of each gDNA concentration. The inter-reproducibility assay was conducted through three separate sets of qPCR runs, with each set conducted on a different day. The mean, standard deviation (SD), and coefficient of variation (CV) were calculated separately using the Cq values for evaluation. The percentage of the CV of inter-and intra-reproducibility assays were then calculated to determine the precision within and between assays, respectively.

2.5. Implementation of SYBR Green qPCRs to estimate the ratio of A. cantonensis and A. malaysiensis third-stage larvae from wild A. fulica

Using the gDNA of the pooled larvae isolated from each *A. fulica* as a template, the qPCRs were conducted separately with each *Angiostrongylus* species-specific primers. From the Cq values of each species-specific qPCR assay, the approximate number for each species of larva were determined based on the qPCR standard curve of the sensitivity assay. The prevalence of *A. cantonensis, A. malaysiensis,* and the coinfection evidence of both *Angiostrongylus* species in *A. fulica* were then determined by estimating the ratio of *A. cantonensis* to *A. malaysiensis* infected in each *A. fulica*.

3. Results

3.1. Standard curve for SYBR-green quantitative real-time PCRs

The effectiveness of the SYBR Green qPCRs was determined using the *Cytb* species-specific primers for *A. cantonensis* and *A. malaysiensis*. 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 Figs. 2A and B. The qPCR efficiency values for both species-specific primers of *A. cantonensis* and *A. malaysiensis* were 97.78% and 96.4%, respectively.

3.2. Sensitivity and specificity of SYBR Green quantitative real-time PCRs

The sensitivity assay results revealed that approximately 1 ng of gDNA was extracted from 100 larvae, while 10^{-4} ng of gDNA was extracted from 1 larva (Fig. 3). The number of the third-stage larvae was then estimated using the standard curve. From the results, we estimated that the gDNA of a single larva is approximately 10^{-4} ng (average Cq value of 31), while the gDNA of 100 pooled larvae is approximately 1 ng (average Cq value of 16). The Cq value of 35 was defined as the cut-off for no larva detected.

For the specificity assay, both species-specific primers did not amplify the gDNA from the different species (*i.e.*, *A. cantonensis* primers only amplified *A. cantonensis* gDNA and not *A. malaysiensis* gDNA). As shown in Fig. 4A, the high specificity of AC4_cytb_F and AC5_cytb_R primers for *A. cantonensis* was also demonstrated in the artificially mixed *A. cantonensis* and *A. malaysiensis* gDNA, in equal concentration for each species, and when there was a lower concentration of *A. cantonensis* than *A. malaysiensis* gDNA. These primers also did not amplify the gnathostomiasis and neurocysticercosis samples, and the heterogeneous sample containing 1 ng of *A. malaysiensis* gDNA. Using the *A. malaysiensis* primers, the specificity was like the *A. cantonensis* primers, where only *A. malaysiensis* was amplified in the artificially mixed gDNA (Fig. 4B). The gnathostomiasis, neurocysticercosis, and angiostrongyliasis samples from the CSF of patients were not amplified. The primer-dimers present for the qPCR reactions of the *A. malaysiensis*-specific primers can be distinguished from the qPCR target by the melting curve analysis. The melting curve of the primer-dimers was around 72 °C and whereas the amplicons for *A. malaysisensis* is 78 °C.

3.3. Intra- and inter-reproducibility assays of SYBR Green qPCRs

The inter-reproducibility results showed consistent amplification between the replicated series of gDNA concentrations in the same assay. Moreover, the low percentage of the CV values of the inter-and intra-reproducibility assays indicated high precision, both within and between amplification (Table S2).

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

From the 285 *A. fulica* collected from the six public parks, 32 snails were positive with the third-stage larvae of *Angiostrongylus* (11.22%), as shown in Table S1. Of the 32 pools of larvae isolated from each positive *A. fulica*, 17 pools had more than 10 larvae, and the species-specific SYBR Green qPCRs were performed on these 17 pools. The qPCR results showed that all positive *A. fulica* was infected with *A. malaysiensis* (Table S3). Five out of 17 *A. fulica* (29.14%) revealed the coinfection of both *A. malaysiensis* and *A. cantonensis*. One *A. fulica* showed relatively high numbers of infections of *A. cantonensis* together with *A. malaysiensis* (Table S3).

4. Discussion

We developed species-specific SYBR Green qPCRs that are simple, economical, highly sensitive, and specific, with the ability to detect, discriminate, and estimate the ratio of the third-stage larvae of *A. cantonensis* and *A. malaysiensis* that were isolated from *A. fulica*. Recent studies reported overlap in the distributions of *A. cantonensis* and *A. malaysiensis* within the terrestrial snail host,
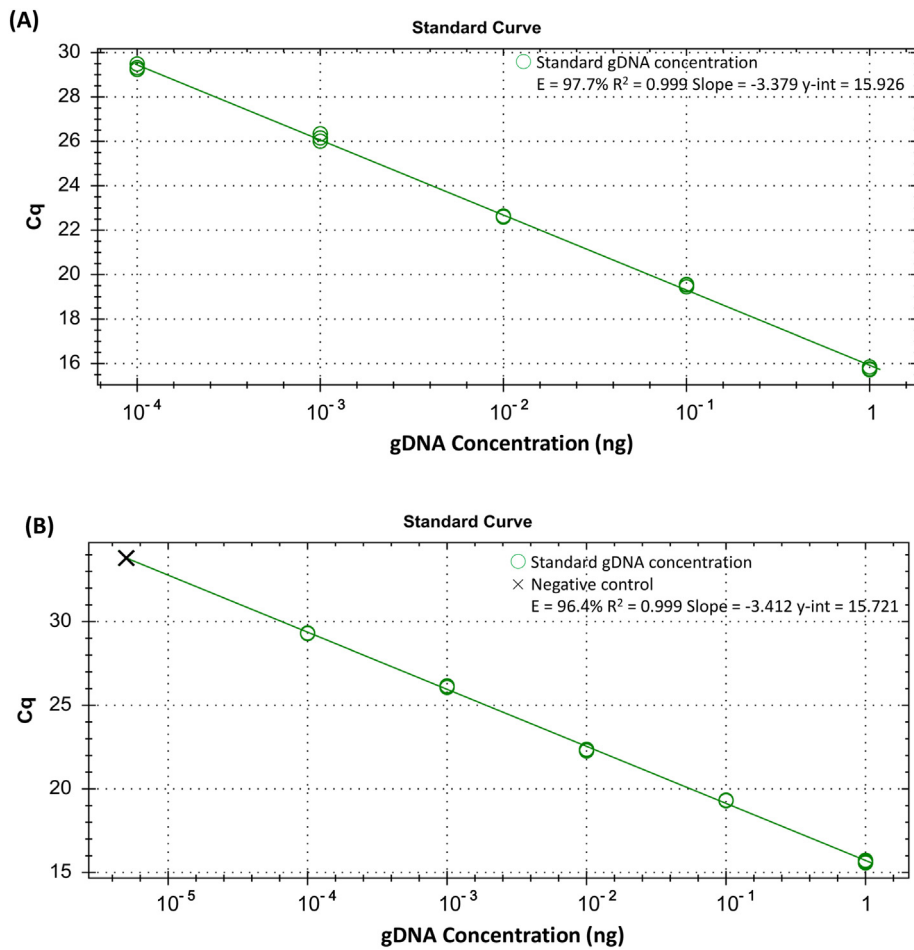
(A)

(B)

Fig. 2. Amplification plots of the SYBR Green qPCR standard curves for *A. cantonensis* (A) and *A. malaysiensis* (B). The SYBR Green qPCRs were performed separately using the species-specific *Cytb* primers with the 10-fold serial dilutions of gDNA from 10^{-4} to 1 ng/μl as a template. The standard curve was plotted based on the Cq values of each gDNA concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
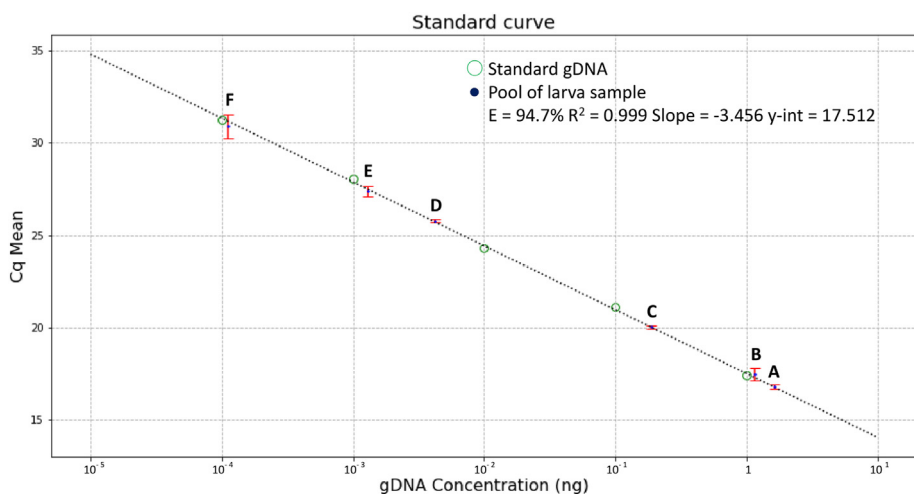

Fig. 3. The Mean Cq values for the pooled larvae samples of *A. cantonensis*. Error bars represent standard deviation. The unfilled-green circles are the Cq values for each gDNA concentration. The numbers of *A. cantonensis* larvae used for estimating gDNA concentration based on the mt*Cytb* target gene are (A) 200 larvae, (B) 100 larvae, (C) 50 larvae, (D) 10 larvae, (E) 5 larvae, and (F) 1 larva. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
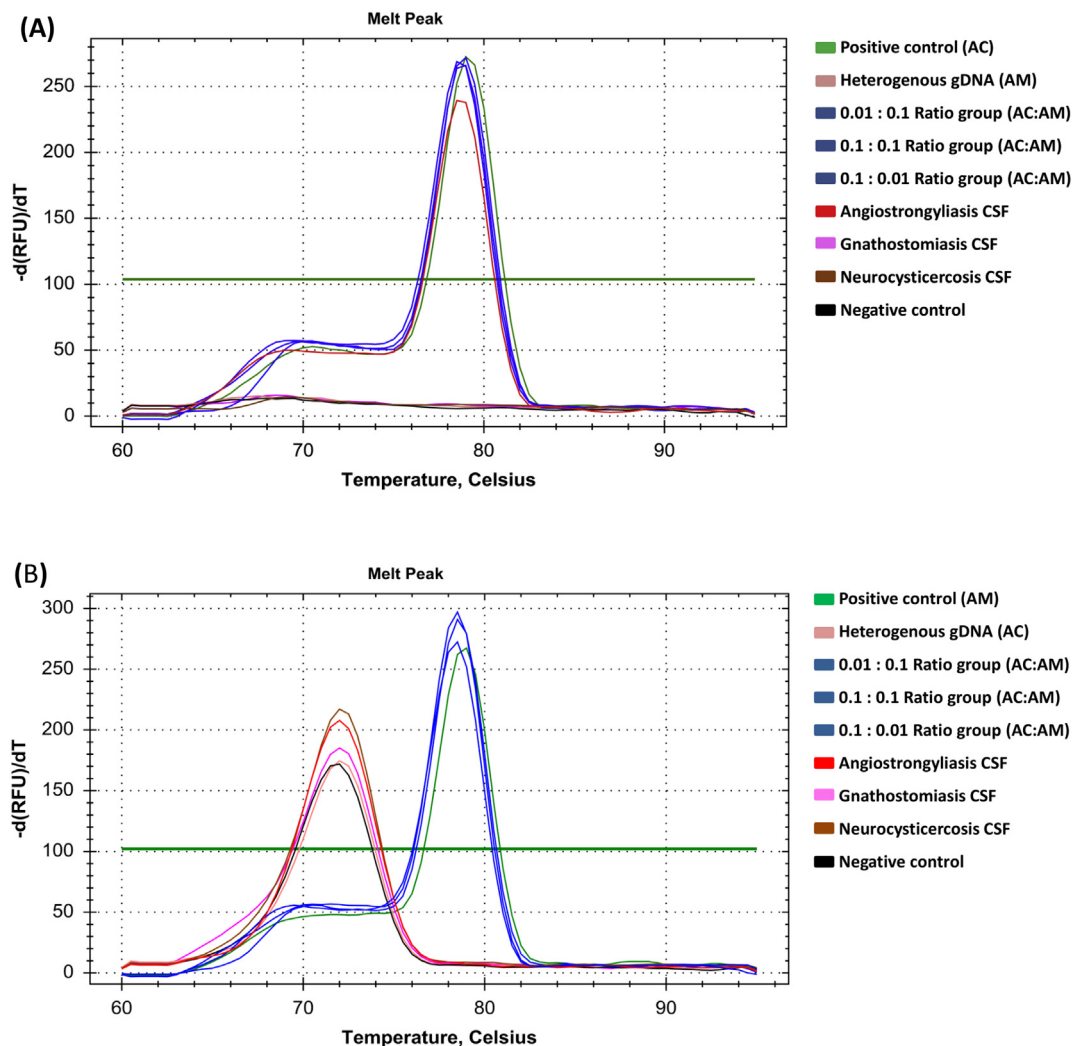
Fig. 4. The SYBR Green qPCR melting curve of *A. cantonensis* (AC) and *A. malaysiensis* (AM) at 79 °C and 78 °C, respectively, showing primer specificity for *A. cantonensis* (A) and *A. malaysiensis* (B) to the CSF of angiostrongyliasis, gnathostomiasis, and neurocysticercosis, the various artificially mixed gDNA containing AC and AM and heterogeneous and homogenous gDNA as the control for each qPCR assay. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

particularly in Indochina (Rodpai et al., 2016). Although the gold standard for *Angiostrongylus* identification is based on morphological characteristics, the third-stage larvae of these two species are morphologically similar (Ash, 1970; Bhaibulaya and Cross, 1971; Bhaibulaya, 1979), resulting in misidentification. The SYBR Green qPCR method was then used to explore the distribution of *Angiostrongylus* in Bangkok Metropolitan to reflect the risk of human contact to angiostrongyliasis.

4.1. Development of SYBR Green qPCRs

Using the species-specific primers, the SYBR Green qPCRs developed could detect a small amount of DNA ($<10^{-4}$ ng) for both *Angiostrongylus* species. We also established the standard curve and demonstrated high qPCR efficiency (see Figs. 2A and B). The CV percentage of reproducibility confirmed that the standard curve construction had low system variation in the manipulation assay. However, the Cq values were slightly fluctuated, especially the values estimated from the small larval pool. We postulated that DNA damage and loss during the larvae preservation and DNA extraction processes might have resulted in fluctuating Cq values, particularly for a small amount of DNA. In contrast, no fluctuation effect was observed when the larvae quantity exceeded 10 (Fig. 3).

We also confirmed the high specificity and the absence of cross-amplification of the developed method using heterogeneous gDNA in the species-specific SYBR Green qPCRs for both primer sets (Figs. 4A and B). Furthermore, the developed method showed the preliminary result to detect low amounts of *Angiostrongylus* genetic material in the CSF specimens. We demonstrated the potential of the method for diagnosing angiostrongyliasis by no cross-reaction with other diseases that also cause eosinophilic

meningitis. Although the species-specific primers sometimes could amplify non-specific amplicons, the melting curve analysis can distinguish the target amplicon from the non-specific ones. As the diagnostic capability of the qPCRs was tested only will a small number of CSF specimens, further investigation is required with larger sample size.

Cost-effectiveness in terms of time, specificity, and the cost was also achieved through the developed species-specific SYBR Green qPCR method. Although two separate PCR mixtures had to be conducted for each species-specific primer, the reactions can be conducted simultaneously using the same qPCR thermocycling conditions, saving time for the user. This method does not require the design of a separate probe for saving the cost of the qPCRs (Maeda et al., 2003; Tajadini et al., 2014; Zhang et al., 2016).

The TaqMan probe real-time PCR was previously developed using the ribosomal internal transcribed spacer 1 (ITS1) as the genetic marker to detect third-stage A. cantonensis larvae in mollusks, and it was more sensitive as compared to the 18S rDNA-based conventional PCR (Qvarnstrom et al., 2007, 2010). The TaqMan assay targeting ITS1 region was also applied for the diagnostics of patients with eosinophilic meningitis by detecting A. cantonensis DNA (Qvarnstrom et al., 2016). However, it was designed to detect only A. cantonensis detection (Qvarnstrom et al., 2010). Our newly developed SYBR Green qPCRs serve as an alternative to the TaqMan assays that have already been developed and can detect both A. cantonensis and A. malaysiensis. Although the newly developed SYBR Green qPCRs using the mitochondrial Cytb gene as a marker has not been proven suitable for molecular diagnosis yet, it may be an alternative method in the future. The new method also 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.

4.2. Distribution of Angiostrongylus in Bangkok metropolitan

We implemented the developed species-specific qPCRs for the molecular discrimination of A. cantonensis and A. malaysiensis third-stage larvae isolated from A. fulica collected from Bangkok's public parks. The qPCR results are indicative that A. malaysiensis is the predominant population, and no single infection of A. cantonensis was detected from the pooled larvae (Table S3). The coinfection of A. cantonensis and A. malaysiensis in A. fulica was relatively substantial (5.29%) in the representative population. However, the numbers of larvae determined by the qPCRs were smaller than the number counted under the stereomicroscope, possibly due to inactive and dead larvae and the DNA extraction process starting with a TissueLyser homogenizing step for breaking down the larval cuticle, resulting in discrepancies. Thus, we suggest using a ratio instead of larval numbers for coinfection evidence and parasite intensity.

Until now, A. cantonensis is the species of human pathogen distributing worldwide. However, in the Indochina Peninsula, coexistence between A. cantonensis and its closely related A. malaysiensis have been reported (Barratt et al., 2016; Rodpai et al., 2016; Dusitsittipon et al., 2017). A. malaysiensis is a potential human pathogenic species in Thailand and Southeast Asia, causing neurological disease in experimental monkeys (Cross, 1979). However, A. malaysiensis infection in humans has not been reported, even though the parasite (Liat, 1974) has been reported in snail-intermediate hosts in the Indochina region (Rodpai et al., 2016; Dusitsittipon et al., 2017). Recently, the coinfection evidence in the same snail intermediate hosts and overlapping distribution of A. cantonensis and A. malaysiensis lead to the question of the parasitic ability of A. malaysiensis to be a human pathogen. Comparative studies on the pathogenicity between A. cantonensis and A. malaysiensis in a mice-nonpermissive host of A. cantonensis (OuYang et al., 2012) are required to predict human pathogenicity differences between these closely related species. Our species-specific qPCRs developed may support further research on pathogenicity study by detecting and isolating the A. malaysiensis from A. cantonensis in the wild snail populations before in vivo experiments with the mice.

A fulica is one of the harmful-invasive species originating from East Africa and is highly predacious and can reproduce quickly. The presence of A. fulica in the ecosystem resulted in the extinction of native fauna and decreased biodiversity (Fisher et al., 2006). Modification of environments due to increasing human activity could have aided in the deployment and dissemination of A. fulica. Therefore, this snail is abundant in areas with high population density in urban areas (Ohlweiler et al., 2010; Albuquerque et al., 2008). Not only have A. cantonensis and A. malaysiensis been reported in A. fulica, but it is also a crucial vector known to harbor A. cantonensis and A. costaricensis (Carvalho et al., 2003; Caldeira et al., 2007). Notably, the distribution of A. fulica may be useful to predict the host-parasite association of parasite transmission (Pavanelli et al., 2017).

In this study, we revealed that A. malaysiensis, instead of A. cantonensis, is the predominant species in Bangkok Metropolitan. Although A. cantonensis was previously recognized as the main Angiostrongylus species distributed throughout Thailand (Dusitsittipon et al., 2015), the underestimation of A. malaysiensis in Thailand could have occurred because of morphological similarity. The coinfection of both A. cantonensis and A. malaysiensis presented in the A. fulica population in Bangkok. The coinfection evidence may support the investigation of the F1 hybrid in Bangkok city, which was revealed previously by the microsatellite genotyping (Dusitsittipon et al., 2017).

4.3. Limitations

We could not design probes from the available Cytb sequences in GenBank for both Angiostrongylus species due to high intraspecific variation for A. cantonensis and A-T rich region for A. malaysiensis. Also, we could not develop a duplex SYBR Green qPCR due to the closeness of the melting curve between A. cantonensis (79 °C) and A. malaysiensis (78 °C). However, we overcame this limitation by separating the PCR reaction between the two species. Moreover, the specificity of the two species-specific primer sets should be further evaluated with real specimens of other Angiostrongylus such as A. costaricensis, A. vasorum, and A. mackerrasae to confirm the predicted in silico results.

5. Conclusions

The developed species-specific SYBR Green qPCRs are a useful and alternative technique with high sensitivity and specificity. They can detect a single larva of *Angiostrongylus* and discriminate between *A. cantonensis* and *A. malaysiensis* simultaneously, rendering the method comparable to the probe-based approach. The developed method can also be used to estimate the ratio of larvae infecting the snail-intermediate host. *A. malaysiensis* was found to be the predominant species infecting *A. fulica* in the Bangkok Metropolitan. The coinfection evidence of both *A. malaysiensis* and *A. cantonensis* larvae in the same *A. fulica* host was detected through our method. Our newly developed qPCRs can be used to perform a molecular survey of *A. cantonensis* and *A. malaysiensis* in the natural intermediate host, particularly in Indochina Peninsula, to investigate parasite prevalence and to increase our understanding of host-parasite relationships. The newly developed method will be require further testing with various environmental samples to evaluate the specificity of the method to render it valuable for field applications. Moreover, the suitability of the method for clinical diagnosis using CSF from patients will also require further evaluation to assess the usefulness of the method for implementation.

Ethical approval statement

Ethical clearance was provided by the Animal Care and Use Committee, Faculty of Tropical Medicine, Mahidol University, Bangkok (No. FTM-ACUC 024/2018 and No. FTM-ACUC 025/2020).

Availability of data and materials

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

Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fawpar.2021.e00119.

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