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Development of multi-ARMS-qPCR method for detection of hookworms from cats and dogs

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ABSTRACT

Hookworms are blood-sucking nematodes that infect dogs, cats, and humans, causing iron-deficiency anemia, abdominal pain, diarrhea, and skin inflammation. Amplification refractory mutation system (ARMS) is a modified technology based on allele-specific PCR, which is widely used in mutation detection and genotyping. However, no data about ARMS application in hookworm detection. This study aims to establish a multi-ARMS-qPCR method for the detection of three hookworm species from dogs and cats. A universal forward primer and three specific primers (ARMS-Cey, ARMS-Can, and ARMS-Tub) were designed based on the three ITS SNPs (ITS250, ITS78 and ITS153) of *Ancylostoma ceylanicum*, *A. caninum,* and *A. tubaeforme*, respectively. The results showed that the three designed ARMS primers generated specific melting curves for the three hookworms' standard plasmids. The melting temperature (T_m) values were 88.40 °C (*A. ceylanicum*), 83.15 °C (*A. caninum*), and 85.65 °C (*A. tubaeforme*), with good reproducibility of intra- and inter-assay. No amplification was observed with other intestinal parasites. The limit of detection using the established technique was 1, 2, and 104 egg per gram feces (EPG) for *A. caninum*, *A. tubaeforme* and *A. ceylanicum*, respectively. Using multi-ARMS-qPCR assay, 17 out of 50 fecal samples were positive for hookworms, including ten single and seven mixed infections, and single infections were quantified. In conclusion, the used multi-ARMS-qPCR method has the advantages of high efficiency, sensitivity, specificity, and quantitative analysis and can be used for the clinical detection, epidemiological investigation, and zoonotic risk assessment of canine and feline hookworms.

1. Introduction

Hookworms are blood-sucking nematodes of the Ancylostomatidae family that infect dogs, cats, and humans, causing variable symptoms, such as iron-deficiency anemia, diarrhea, abdominal pain, and skin inflammation [1]. The common hookworms infecting dogs and cats globally are *Ancylostoma caninum*, *A. braziliense*, *A. ceylanicum*, *A. tubaeforme*, and *Uncinaria stenocephala*. The first three *Ancylostoma* species are the known zoonotic hookworms, which can be transmitted from animals to human. *A. caninum* and *A. braziliense* primarily cause larva migrans [2], while *A. ceylanicum* can mature into the adult stage in the human intestine, inducing iron-deficiency anemia and abdominal symptoms [3]. Hence, the rapid and accurate detection of hookworms is crucial for the epidemiological investigation and zoonotic risk assessment of hookworms.

The traditional microscopic examination has been widely used for the detection of hookworm infection. However, it is very difficult to discriminate between different hookworm eggs because of their morphological similarities [4]. Recently, several molecular detection methods have been extensively applied for hookworm detection, such as semi-nested PCR, PCR-single strand conformation polymorphism (PCR-SSCP) and PCR-restriction fragment length polymorphism (PCR-RFLP) [5–7]. These techniques not only improve the accuracy of hookworm identification, but also save time and labor compared with the traditional method. However, these methods suffer some disadvantages, such as the high cost, low sensitivity, missed detection, and long detection cycle [8,9]. Therefore, there is an urgent need for a high-throughput, highly efficient, and sensitive molecular detection technique for hookworms.

Amplification refractory mutation system (ARMS), also named allele-specific PCR (ASPCR), is a simple, rapid, reliable, and cost-effective method for detecting any mutation involving single base changes or small deletions [10]. This system principle is that if a primer anneals to a given DNA sequence except for a mismatched 3′ end, DNA polymerase will not extend the reaction [11]. ARMS is based on using an allele-specific primer (AS primer) with 3′-end bases complementary to the

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mutated bases of target DNA. This primer allows amplification of test DNA only when containing the target allele. After an ARMS reaction, the presence or absence of a PCR product is diagnostic for the presence or absence of the target allele [10]. In some occasions, the 3′ end mismatch does not completely prevent the extension reaction. Therefore, an extra mismatch base is artificially introduced at 2–3 bases from the 3′ end of primer to further improve its specificity, and the resulted primer is called the ARMS primer [12]. ARMS-qPCR technology has been widely used in mutation detection and genotyping [13–15]. Real-time PCR (qPCR) detection method has been recorded to be more rapid, sensitive and specific for the assessment of egg count compared to microscopy [16]. To our knowledge, no studies have combined ARMS assay with qPCR to detect and quantify canine and feline hookworms.

This study aims to establish a multi-ARMS-qPCR method for the detection of *A. ceylanicum, A. caninum* and *A. tubaeforme,* based on their three ITS SNPs sequences, as a new technical means for the epidemiological investigation and zoonotic risk assessment of canine and feline hookworms.

2. Materials and methods

2.1. Parasites and fecal samples

Adult hookworms of *A. ceylanicum, A. caninum* and *A. tubaeforme* were obtained from those samples previously identified [17–19] and maintained in our laboratory at −80 °C. The fecal samples from 30 stray dogs and 20 stray cats were collected at animal shelters in Shantou City, Guangdong Province, China and stored at 4 °C until use.

2.2. DNA extraction

Adult hookworms of the three *Ancylostoma* species preserved in 50% ethanol, were repeatedly washed with double-distilled water (ddH_2O). The genomic DNA (g DNA) of individual worms was extracted using the Wizard® SV Genomic DNA Purification System (Promega, Wisconsin, USA) according to the manufacturer's instructions. The genomic DNA of all fecal samples was extracted by the stool DNA extraction kit (Omega, Georgia, USA) according to the manufacturer's protocols. DNA samples were stored at −20 °C until use.

2.3. PCR amplification of ITS-1

The universal primer NC5 (5′-GTAGGTGAACCTGCGGAAGGATC ATT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′) [20], based on internal transcribed spacer 1 (ITS1) sequence, were synthesized by Shanghai Biotechnology Company (Shanghai, China). The expected amplification fragment was 834 bp in length. The PCR amplification was performed using DNA samples extracted from the three hookworm species as templates. The PCR reaction was done in 25 μL total reaction mixtures containing 9.5 μL of ddH_2O, 12.5 μL of Ex-Taq polymerase (TaKaRa, Dalian, China), 0.5 μL of each primer NC5/NC2 (50 μmol/L), and 2 μL of the DNA sample. Cycling parameters were as follows: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 61.5 °C for 30 s, and extension at 72 °C for 45 s; and then a final extension at 72 °C for 7 min. PCR products were analyzed by ethidium bromide stained agarose gel electrophoresis, re-harvested using a DNA gel extraction Kit (Omega, Georgia, USA) and stored at −20 °C until use.

2.4. Construction of standard plasmids

The purified PCR products were cloned into the PMD-18 vector (TaKaRa, Dalian, China), transformed into *Escherichia coli* DH5α (TaKaRa, Dalian, China) and identified by bacterial liquid PCR. Positive plasmids were sequenced to further validate the hookworm species. After sequencing, 50 μL of positive bacterium liquid was inoculated into 5 mL of lysogeny broth (LB) medium with ampicillin (Amp+), IPTG and X-gal, and then were shaken overnight at 37 °C. Plasmid DNAs were extracted using the Plasmid Mini Kit (Omega, Georgia, USA) and stored at −20 °C. The plasmids (1 μL) were analyzed by the ultramicro-spectrophotometer to evaluate their purity and concentration. Optical density (OD) values (A260/A280) were compared to the reference value (1.8–2.0) [21], and all standard plasmids were stored at −20 °C.

Table 1
Primers for ARMS-qPCR based on three SNPs.

SNP	Primer	Primer sequence (5′-3′)	Length(bp)
ITS 250	Forward	AGCCTTATGGTTCCTTTGATCCTG	24
	ARMS-Cey	CCTGACAGACAAGTGCCG**A**AC	21
ITS 78	Forward	AGCCTTATGGTTCCTTTGATCCTG	24
	ARMS-Can	TAGGGCTGTAACGGTG**A**GT	19
ITS 153	Forward	AGCCTTATGGTTCCTTTGATCCTG	24
	ARMS-Tub	CAGCGAAACGCGAACTT**A**GT	20

ARMS-Cey:Reverse specific primer for *A. ceylanicum* ; ARMS-Can:Reverse specific primer for *A. caninum* ; ARMS-Tub:Reverse specific primer for *A. tubaeforme* ; Bold letter:mismatched base.

2.5. ARMS-qPCR primer design

Based on the three ITS SNPs (ITS78, ITS153, and ITS250) of *A. caninum* (EU159415.1), *A. tubaeforme* (JQ812691.1), and *A. ceylanicum* (KF279133.1) sequences, three specific primers (ARMS-Can, ARMS-Tub, and ARMS-Cey) and a single universal forward primer (Table 1) were designed by the Primer Premier 5.0 (PREMIER Biosoft, California, USA) and Oligo 6 software (Thermo Fisher Scientific, Massachusetts, USA). The primers were synthesized by Shanghai Biotechnology Company (Shanghai, China), diluted with sterile ddH_2O to a final concentration of 10 pmol/μL and stored at −20 °C until use.

2.6. ARMS-qPCR amplification

To test the designed ARMS-primers ability to differentiate between different *Ancylostoma* species, ARMS-qPCR amplification was performed by using each of the constructed *A. ceylanicum*, *A. caninum* and *A. tubaeforme* plasmids as a template. In each PCR, one ARMS primer (ARMS-Can, ARMS-Tub, or ARMS-Cey) and the universal forward primer were used to amplify its corresponding target plasmid (template). The reaction system contained Taq SYBR® Green qPCR Premix (12.5 μL), forward primer (0.5 μL), ARMS-R primer (0.5 μL), ddH_2O (10.5 μL), and DNA template (1 μL). The used qPCR cycling conditions were initial denaturation at 95 °C for 2 min; followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 20 s and extension at 72 °C for 20 s; and melting from 70 °C to 99 °C at 0.5 °C/s intervals.

2.7. Multi-ARMS-qPCR amplification

To evaluate the ability of the developed assay to detect mixed hookworm infection, we used the multi-ARMS-qPCR to amplify *A. ceylanicum, A. caninum* and *A. tubaeforme* plasmids mixture as a template. The optimized reaction system contained 15 μL of Taq SYBR® Green qPCR Premix, 2.5 μL of the universal forward primer, 1.0 μL of ARMS-Can, 1.0 μL of ARMS-Tub, 0.5 μL of ARMS-Cey, 4.0 μL of ddH_2O and 1.0 μL of the plasmid mixture at the ratio of 1:1:1. The used qPCR cycling program was as follows: initial denaturation at 95 °C for 2 min; 40 cycles of denaturation at 95 °C for 10 s, annealing at 62 °C for 20 s, and extension at 72 °C for 20 s; and melting from 70 °C to 99 °C at 0.5 °C/s intervals.

2.8. Quantification and sensitivity of multi-ARMS-qPCR

For quantification of hookworms, we measured the three

hookworms' standard plasmids by a spectrophotometer and converted their concentrations (ng/μL) into gene copy number (copy/μL). Gene copy numbers were calculated using the formula (DNA concentration (ng) × 10^{-9} × Avogadro constant (6.022×10^{23})/plasmid size (bp) × the average weight of a base pair [22]. Then, the standard plasmids were tenfold serially diluted at concentrations of $1{:}10^1$ - $1{:}10^8$. Multi-ARMS-qPCR was performed using plasmids with different gradient concentration as templates, and a standard curve was constructed. The egg density (EPG) of the three hookworms was calculated according to the number of gene copies for an egg [22]. Briefly, each *Ancylostoma* species eggs were isolated from fresh feces, microscopically counted using a standard counting chamber, preserved in 0.5% formalin at 4 °C. From the stock mixture, three batches of known egg numbers were used in replicates to estimate the gene copy numbers per egg. The gene copy number in each DNA sample was calculated as described above for the multi-ARMS-qPCR assay.

2.9. Evaluation of multi-ARMS-qPCR specificity and stability

The specificity of multi-ARMS-qPCR was evaluated using DNA samples representing the common intestinal organisms of dogs and cats, such as *Dipylidium caninum, Toxocara cati, Trichuris vulpis, Giardia lamblia*, and *Escherichia coli*. The stability of the developed assay was assessed by determining the intra-assay and inter-assay reproducibility. Coefficient of variation (CV) was calculated by analyzing the standard plasmids. The intra-assay and inter-assay reproducibility were measured based on the cycle threshold (C_T values) [23] by testing the standard plasmids at a higher (10^4) and lower (10^1) concentrations, respectively. The intra-assay (n = 3) and inter-assay (n = 3) repeats were performed every other week.

2.10. Fecal samples screening by multi-ARMS-qPCR assay

Fifty fecal samples (2 g feces for each sample) from cats and dogs were examined by the centrifugal flotation technique with saturated zinc sulfate solution. Meanwhile, the egg number per gram of feces (EPG) of each fecal samples was calculated using classical McMaster method. Genomic DNAs were extracted from the fecal samples according to the instructions of stool DNA extraction kit (Omega, Georgia, USA). These DNA samples were detected by using the established multi-ARMS-qPCR assay. The reaction system and amplification conditions were the same as above. Their detecting results were compared with those of PCR-RFLP, where the RFLP analysis was performed referring to Liu et al. [18].

2.11. Data analysis

Rotor-Gene Q Software (Qiagen Com., USA) was used for data analysis, producing graphs, and finding the correlation between C_T value and egg count.

3. Results

3.1. ITS-1 fragment amplification and standard plasmids construction

The amplified fragments from three genomic DNAs of adult *A. ceylanicum, A. caninum*, and *A. tubaeforme* were 834 bp in length without nonspecific bands (Fig. 1), which was in consistent with the expected fragment length. The OD values (A260/A280) of three hookworms' positive plasmids were between 1.8 and 2.0, which were within the acceptable purity level. *A. ceylanicum*, *A. caninum*, and *A. tubaeforme* plasmid concentrations were 81.0, 76.8, and 115.4 ng/μL, respectively.

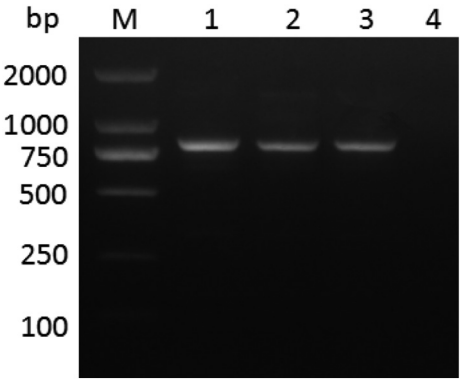

Fig. 1. PCR amplification of the ITS1 fragment of three hookworms. M. DL5000 marker; 1. *A. ceylanicum*; 2. *A. caninum*; 3. *A. tubaeforme;* 4. Negative control.

3.2. ARMS-qPCR amplification

To validate the developed ARMS-qPCR technique, we analyzed *A. ceylanicum*, *A. caninum,* and *A. tubaeforme* plasmids, which previously confirmed to be positive using PCR and sequencing. The ARMS-qPCR melting curves for *A. ceylanicum*, *A. caninum,* and *A. tubaeforme* plasmids (Fig. 2) showed that the used method could distinguish between three hookworm species with the formation of three specific peaks. By the Rotor-Gene Q software analysis, the T_m values were 88.40 °C (*A. ceylanicum*), 83.15 °C (*A. caninum*), and 85.65 °C (*A. tubaeforme*).

3.3. Multi-ARMS-qPCR amplification

Amplification of the three-hookworm plasmid mixture by the multi-ARMS-qPCR generated three specific melting peaks (Fig. 3a). As well as, *A. ceylanicum*, *A. caninum*, and *A. tubaeforme* plasmids produced specific bands on agarose gel electrophoresis with the size of 94, 170 and 268 bp, respectively (Fig. 3b). These findings indicate that multi-ARMS-qPCR can detect mixed hookworm infection with *A. ceylanicum*, *A. caninum*, and *A. tubaeforme.*

3.4. Multi-ARMS-qPCR sensitivity

Multi-ARMS-qPCR amplification using plasmids with different gradient concentrations demonstrated that the three hookworms' plasmids standard curves had a good linear correlation at the egg concentrations (egg/μL) of 5.4×10^5–5.4×10^{-2} (*A. caninum*), 8.1×10^5–8.1×10^{-2} (*A. tubaeforme*) and 5.7×10^4–5.7×10^0 (*A. ceylanicum*), respectively (Fig. 4). The lowest EPG values required to detect *A. caninum*, *A. tubaeforme*, and *A. ceylanicum* were 1, 2, and 104, respectively.

3.5. Multi-ARMS-qPCR specificity and stability

Multi-ARMS-qPCR assay could specifically amplify the genomic DNA from *A. ceylanicum*, *A. caninum*, and *A. tubaeforme*. No amplification was observed when the multi-ARMS-qPCR was performed on genomic DNA of *Dipylidium caninum, Toxocara cati*, *Trichuris vulpis*, *Giardia lamblia*, *Escherichia coli*, and sterile ddH_2O (Fig. 5). The reproducibility results showed that the coefficient of variation (CV) at C_T values for different concentrations of *A. ceylanicum*, *A. caninum* and *A. tubaeforme* plasmids was less than one (Table 2). Consequently, the intra-assay and inter-assay reproducibility of this method were good.

3.6. Fecal samples analysis by multi-ARMS-qPCR assay

Seventeen out of fifty fecal samples from stray dogs and cats in Shantou City of Guangdong Province were microscopically positive for hookworm eggs. All positive fecal samples were detected to be positive

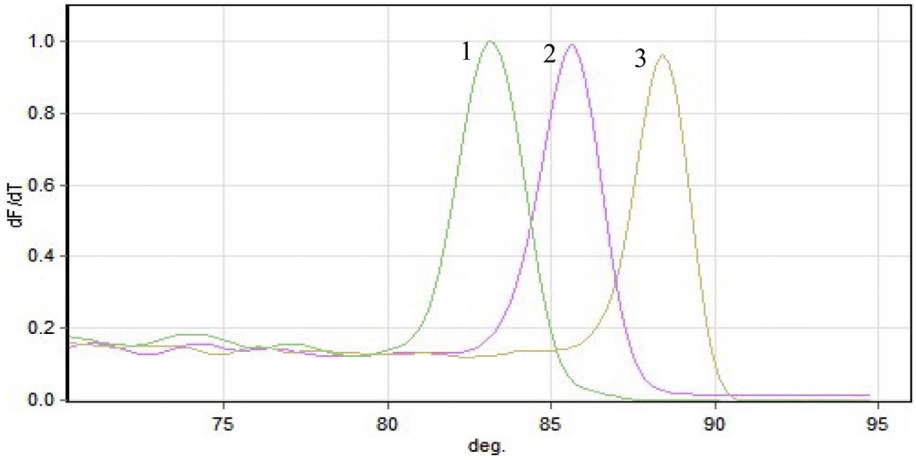

Fig. 2. Melting-curve analysis of standard plasmids. 1. *A. caninum* plasmid; 2. *A. tubaeforme* plasmid; 3. *A. ceylanicum* plasmid.

by using the multi-ARMS-qPCR method, they were identified as *A. caninum* (n = 8), *A. tubaeforme* (n = 2), mixed infection with *A. ceylanicum* and *A. caninum* (n = 5), and mixed infection with *A. caninum* and *A. tubaeforme* (n = 2). Only sixteen positive fecal samples were detected by PCR - RFLP, including *A. caninum* (n = 7), *A. tubaeforme* (n = 2), *A. ceylanicum* (n = 1) and mixed infections of *A. ceylanicum* and *A. caninum* (n = 4), and mixed infection with *A. caninum* and *A. tubaeforme* (n = 2). The sensitivity of the multi-ARMS-qPCR and PCR-RFLP method was 100% and 94.1%, respectively, indicating that the multi-ARMS-qPCR method was more sensitive. Meanwhile, the density of eggs in single infections was quantified by the multi-ARMS-qPCR, shown in Table 3.

4. Discussion

Three hookworms, *A. ceylanicum*, *A. caninum*, and *A. tubaeforme*, have been reported to be the common hookworm species infecting dogs and cats in Guangdong Province of China [17–19]. These hookworms cause blood loss and anemia that can be fatal in young kittens and puppies [2]. Moreover, *Ancylostoma caninum* cutaneous larva migrans in human has been linked to eosinophilic enteritis, eosinophilic pneumonitis, diffuse unilateral neuroretinitis, folliculitis, localized myositis, erythema multiforme, and ophthalmological manifestations [2]. Meanwhile, *A. ceylanicum* can develop into the adult stage in the human intestine, causing abdominal pain and distension and diarrhea, occult blood in the feces, and anemia [3]. Hookworm infections are often caused by exposure to soil or sewage containing infective larva [24–26]. Currently, the hookworm quantitative assessment, in the guidelines for the safe use of wastewater and excreta, mainly relies on egg counting [27]. This method accuracy is limited at low egg density and depends on the inspector's expertise [28]. Although some molecular detection techniques, including HRM and PCR-RFLP, have been applied for hookworm detection [18,29,30], none of these techniques can be used for quantitative detection of hookworm eggs in samples. Gyawali et al. [22] established a qPCR method for quantitative detection of *A. caninum* eggs and evaluation of the DNA copy number per individual worm egg, showing the advantages of high sensitivity and specificity.

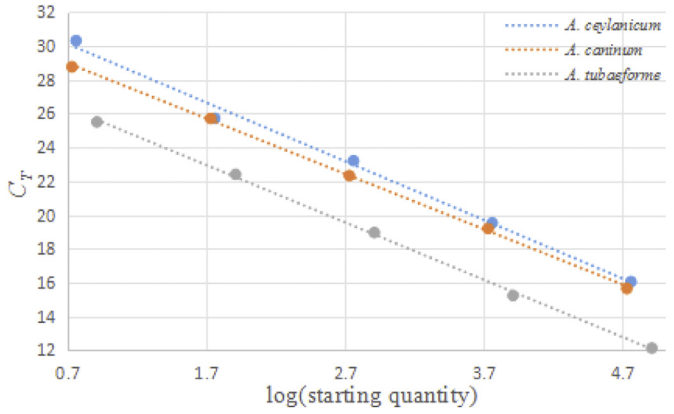

Fig. 4. Standard curves of multi-ARMS-qPCR. *A. ceylanicum* standard equation: $C_T = -3.469x + 32.54$, $R^2 = 0.994$, Efficiency = 94%; *A. caninum* standard equation: $C_T = -3.273x + 31.265$, $R^2 = 0.999$, Efficiency = 102%; *A. tubaeforme* standard equation: $C_T = -3.391x + 28.712$, $R^2 = 0.999$, Efficiency = 97%.

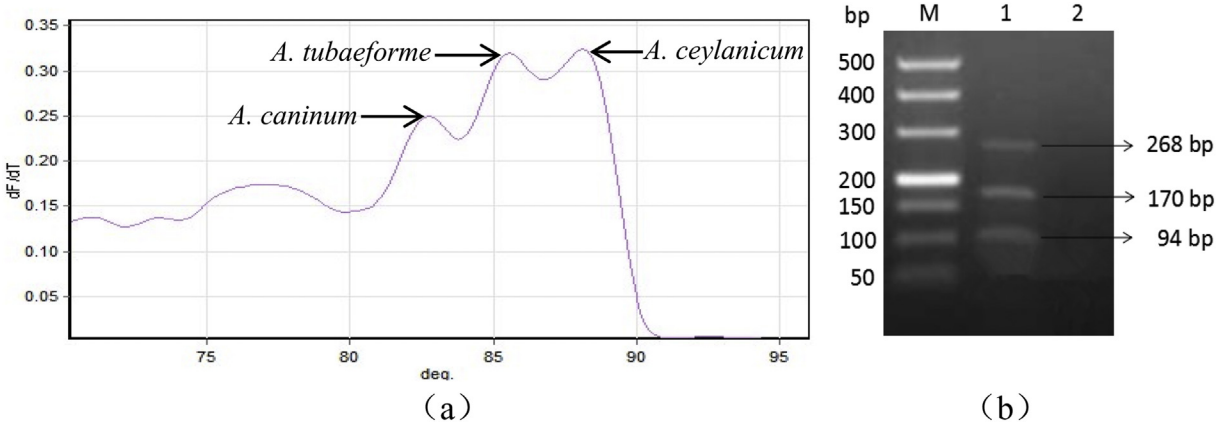

Fig. 3. Multi-ARMS-qPCR melting curve (a) and PCR amplification (b). M. DL 500 Marker; 1. Three hookworm plasmids; 2. Negative control.

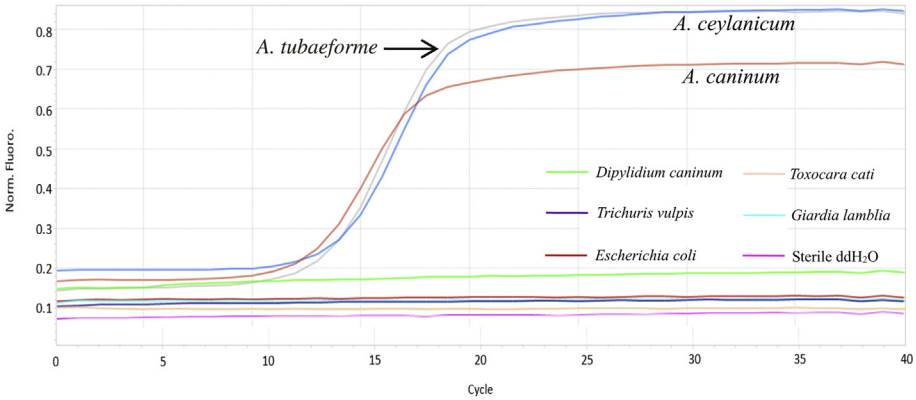

Fig. 5. Specificity of multi-ARMS-qPCR for amplifying the genomic DNA from *A. ceylanicum, A. caninum, A. tubaeforme* and other control samples.

This study is the first to establish a multi-ARMS-qPCR method for both qualitative and quantitative detection of *A. ceylanicum*, *A. caninum*, and *A. tubaeforme*. The amplification of the three-hookworm plasmid mixture by the multi-ARMS-qPCR generated three specific melting curves, which were in consistent with the PCR amplification results of the three hookworm plasmids. These results suggest that the developed method can detect mixed infection with *A. ceylanicum*, *A. caninum*, and *A. tubaeforme* and distinguish between these hookworm species. Regarding specificity, this method could specifically amplify the three target hookworms, and no cross-reaction with the common intestinal parasites, such as *Toxocara cati*, *Trichuris vulpis, Dipylidium caninum*, and *Giardia lamblia*, of dogs and cats was observed. The present study compared the detection efficiency of the multi-ARMS-qPCR method with that of PCR-RFLP. The multi-ARMS-qPCR method found 5 mixed infections with *A. ceylanicum* and *A. caninum,* one positive sample more than those found by PCR-RFLP. However, PCR-RFLP detected one sample with *A. ceylanicum* single infection, which was not found by the multi-ARMS-qPCR. Perhaps, *A. caninum,* in one mixed infection with *A. ceylanicum*, could not be detected by PCR-RFLP. Moreover, a previous study found that SYBR Green I dye molecules had a priority in binding to different DNA, where it preferred to bind to higher G + C content and longer amplicons [31,32]. In addition, the concentration of SYBR Green I also affects the formation of specific peaks [33]. Therefore, our study increased the Taq SYBR® Green qPCR Premix volume from 12.5 μL to 15 μL. Additionally, we adopted three hookworms' plasmids with low concentration to ensure that all DNA templates can bind completely to SYBR Green I in the reaction system.

To evaluate the quantitative detection of the three hookworms, we established the multi-ARMS-qPCR standard curves for three hookworm species plasmids. The results showed that the limit of detection for *A. caninum*, *A. tubaeforme*, and *A. ceylanicum* was 1, 2, and 104 egg(s) per gram of feces, respectively. While fecal egg counts using the McMaster method in this study recorded a detection limit of 20 EPG for the three hookworm species. Thus, the sensitivity of the established multi-ARMS-qPCR method is good. Nevertheless, the sensitivity for the detection of *A. ceylanicum* is significantly lower than that of *A. caninum* and *A. tubaeforme*. This difference may be attributed to the presence of few optional SNPs and existing mismatches at ITS250 in the design of *A. ceylanicum* specific primers. When the template concentration is very low, it will interfere with the amplification. Therefore, we hypothesize that the developed multi-ARMS-qPCR detection method can be used to quantify *A. ceylanicum* only under moderate or severe infection intensity. While it can effectively detect and quantify *A. caninum* and *A. tubaeforme* under very low infection intensity.

Fecal samples screening by the multi-ARMS-qPCR assay recorded single or mixed infection with *A. caninum* and *A. ceylanicum* in canine fecal samples, and that with *A. tubaeforme* and *A. ceylanicum* in feline fecal samples. Besides, the multi-ARMS-qPCR assay could quantify the single hookworm infection. The obtained results indicate that *A. caninum* is the dominant hookworm species among dogs in Shantou City, Guangdong Province, which agreed with the previously recorded prevalence of canine and feline hookworm in Guangzhou City, Guangdong Province [17,29]. Whereas, our study reported *A. tubaeforme* as the common feline hookworm species in Shantou City, similar to previous results reported from stray cats in Shaoguan and Foshan, Guangdong Province [19,30]. These data suggest that *A. tubaeforme* is the most common hookworm species among cats in Guangdong Province, China.

In summary, this study established a new multi-ARMS-qPCR method for the detection of *A. ceylanicum*, *A. caninum*, and *A. tubaeforme* single and mixed infections as well as quantification of single infections. This method may provide a new technical means for further studies on the epidemiological investigation and assessment of the zoonotic risk from canine and feline hookworms.

Declaration of Competing Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Table 2
Stability evaluation of multi-ARMS-qPCR for three hookworm plasmids (C_T value).

Batch	*A. ceylanicum*		*A. caninum*		*A. tubaeforme*	
	5.7×10^1	5.7×10^4	5.4×10^1	5.4×10^4	8.1×10^1	8.1×10^4
First (n = 3)	26.08 ± 0.09	16.32 ± 0.12	25.75 ± 0.05	15.65 ± 0.03	22.43 ± 0.02	12.11 ± 0.05
Second (n = 3)	25.89 ± 0.12	16.41 ± 0.08	25.82 ± 0.06	15.73 ± 0.03	22.16 ± 0.21	12.00 ± 0.02
Third (n = 3)	25.82 ± 0.06	16.14 ± 0.16	25.78 ± 0.03	15.70 ± 0.05	22.39 ± 0.08	12.16 ± 0.04
Average	25.93	16.29	25.78	15.70	22.33	12.09
CV(%)	0.35	0.74	0.18	0.23	0.46	0.30

Table 3
Qualitative and quantitative detection of fifty fecal samples by multi-ARMS-qPCR.

Number	Host	Hookworm	C_T vaule	EPG
1	Stray dog	*A. caninum*	31.06	24
2	Stray dog	*A. caninum*	26.45	602
3	Stray dog	*A. caninum*	27.85	226
4	Stray dog	*A.caninum* & *A.ceylanicum*	+	+
5	Stray dog	*A.caninum* & *A.ceylanicum*	+	+
6	Stray dog	*A. caninum*	30.85	56
7	Stray dog	*A. caninum*	26.43	612
8	Stray dog	*A. caninum*	31.08	23
9	Stray dog	*A.caninum* & *A.ceylanicum*	+	+
10	Stray dog	*A. caninum*	30.86	27
11	Stray dog	*A.caninum* & *A.ceylanicum*	+	+
12	Stray cat	*A. caninum*	29.14	91
13	Stray cat	*A.caninum* & *A.tubaeforme*	+	+
14	Stray cat	*A.caninum* & *A.tubaeforme*	+	+
15	Stray cat	*A. tubaeforme*	30.26	7
16	Stray cat	*A. tubaeforme*	31.47	3
17	Stray cat	*A.caninum* & *A.ceylanicum*	+	+

" + " represent mixed infection, which the density of eggs can not be quantified.

2014A020214005).

References

[1] S. George, B. Levecke, D. Kattula, V. Velusamy, S. Roy, P. Geldhof, R. Sarkar, G. Kang, Molecular identification of hookworm isolates in humans, dogs and soil in a tribal area in Tamil Nadu, India, PLoS Negl. Trop. Dis. 10 (2016) e4891.

[2] D.D. Bowman, S.P. Montgomery, A.M. Zajac, M.L. Eberhard, K.R. Kazacos, Hookworms of dogs and cats as agents of cutaneous larva migrans, Trends Parasitol. 26 (2010) 162–167.

[3] R.J. Traub, *Ancylostoma ceylanicum*, a re-emerging but neglected parasitic zoonosis, Int. J. Parasitol. 43 (2013) 1009–1015.

[4] R. Ngui, Y.A. Lim, K.H. Chua, Rapid detection and identification of human hookworm infections through high resolution melting (HRM) analysis, PLoS ONE 7 (2012) e41996.

[5] R.B. Gasser, N.B. Chilton, H. Hoste, I. Beveridge, Rapid sequencing of rDNA from single worms and eggs of parasitic helminths, Nucleic Acids Res. 21 (1993) 2525–2526.

[6] J.M. Hawdon, Differentiation between the human hookworms *Ancylostoma duodenale* and *Necator americanus* using PCR-RFLP, J. Parasitol. 82 (1996) 642–647.

[7] R.B. Gasser, J.R. Monti, Q. Baozhen, A.M. Polderman, P. Nansend, N.B. Chiltonet, A mutation scanning approach for the identification of hookworm species and analysis of population variation, Mol. Biochem. Parasitol. 92 (1998) 303–312.

[8] A.V. Koehler, R.S. Bradbury, M.A. Stevens, S.R. Haydon, A.R. Jex, R.B. Gasser, Genetic characterization of selected parasites from people with histories of gastrointestinal disorders using a mutation scanning-coupled approach, Electrophoresis 34 (2013) 1720–1728.

[9] L.P. Tan, X.G. Yu, A.Y. Abdullahi, S. Wu, G.C. Zheng, W. Hu, M.R. Song, Z. Wang, B. Jiang, G.Q. Li, Development of a rapid HRM genotyping method for detection of dog-derived *Giardia lamblia*, Parasitol. Res. 114 (2015) 4081–4086.

[10] S. Little, Amplification-refractory mutation system (ARMS) analysis of point mutations, Current Protocols in Human Genetics, John Wiley & Sons, Inc., 2001.

[11] X.Y. Fan, Z.Y. Hu, F.H. Xu, Z.Q. Yan, S.Q. Guo, Z.M. Li, Rapid detection of rpoB gene mutations in rifampin-resistant *Mycobacterium tuberculosis* isolated in Shanghai by using the amplification refractory mutation system, J. Clin. Microbiol. 41 (2003) 993–997.

[12] C.R. Newton, A. Graham, L.E. Heptinstall, S.J. Powell, C. Summers, N. Kalsheker, J.C. Smith, A.F. Markham, Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS), Nucleic Acids Res. 17 (1989) 2503–2516.

[13] R.M. Ferrie, M. Schwarz, N.H. Robertson, S. Vaudin, M. Super, G. Malone, S. Little, Development multiplexing and application of ARMS tests for common mutations in the CFTR gene, Am. J. Hum. Genet. 51 (1992) 251–262.

[14] S. Ochsenreither, M. Reinwald, E. Thiel, T. Burmeister, Melting point assay for the JAK2 V617F mutation, comparison with amplification refractory mutation system (ARMS) in diagnostic samples, and implications for daily routine, Mol. Diag. Ther. 14 (2010) 185–190.

[15] H.K. Chaudhary, M. Shrestha, P. Chaudhary, B.H. Poudel, Amplification refractory mutation system-polymerase chain reaction for rapid detection of rpoB gene mutations in *Mycobacterium tuberculosis*, Int. J. Appl. Sci. Biotech. 5 (2017) 81–85.

[16] M.A. Dang-Trinh, J.M.M. Angeles, K.J. Moendeg, A.M. Macalanda, L. Higuchi, C. Oto, M. Kirinoki, Y. Chigusa, S.I. Kawazu, Utilization of real time PCR for the assessment of egg burden in the organs of *Schistosoma japonicum* experimentally infected mice, Exp. Parasitol. 189 (2018) 61–65.

[17] Y.J. Liu, G. Zheng, M. Alsarakibi, X.H. Zhang, W. Hu, P.Y. Lu, L.Q. Lin, L.P. Tan, Q. Luo, G.Q. Li, Molecular identification of *Ancylostoma caninum* isolated from cats in southern China based on complete ITS sequence, Biomed. Res. Int. 2013 (2013) 1–6.

[18] Y.J. Liu, G.C. Zheng, P. Zhang, M. Alsarakibi, X.H. Zhang, Y.W. Li, T. Liu, S.N. Ren, Z.X. Chen, Y.L. Liu, S.J. Li, G.Q. Li, Molecular identification of hookworms in stray and shelter dogs from Guangzhou City, China using ITS sequences, J. Helminthol. 89 (2015) 196–202.

[19] X.L. Shi, Y.Q. Fu, A.Y. Abdullahi, M.W. Wang, F. Yang, X.G. Yu, W.D. Pan, X.X. Yan, J.X. Hang, P. Zhang, G.Q. Li, The mitochondrial genome of *Ancylostoma tubaeforme* from cats in China, J. Helminthol. 92 (2018) 22–33.

[20] T. Yoshinaga, R. Kinami, K.A. Hall, K. Ogawa, A preliminary study on the infection of Anisakid larvae in juvenile greater amberjack *Seriola dumerili* imported from China to Japan as mariculture seedlings, Fish Pathol. 41 (2006) 123–126.

[21] P. Desjardins, D. Conklin, NanoDrop microvolume quantitation of nucleic acids, J. Vis. Exp. 45 (2010) e2565.

[22] P. Gyawali, W. Ahmed, J.P. Sidhu, P. Jagals, S. Toze, Quantification of hookworm ova from wastewater matrices using quantitative PCR, J. Environ. Sci. (China) 57 (2017) 231–237.

[23] J.H. Ruan, N. Zhang, C.Q. Huang, W.J. Wang, Y.D. Jin, Establishment and application of a real-time qPCR for the rapid detection of *Salmonella* spp. in imported animal-derived feed stuff, Chinese Vet. Sci. (2018), https://doi.org/10.1101/324798.

[24] N. Tomono, M.T. Anantaphruti, P. Jongsuksuntigul, P. Thongthien, P. Leerapan, Y. Silapharatsamee, S. Kojima, S. Looareesuwan, Risk factors of helminthiases among schoolchildren in southern Thailand, SE Asian J. Trop. Med. 34 (2003) 264–268.

[25] R.J. Traub, I.D. Robertson, P. Irwin, N. Mencke, T.R.C. Andrew, The prevalence, intensities and risk factors associated with geohelminth infection in tea-growing communities of Assam, India, Trop. Med. Int. Health 9 (2004) 688–701.

[26] T. Liabsuetrakul, P. Chaikongkeit, S. Korviwattanagarn, C. Petrueng, S. Chaiya, C. Hanvattanakul, P. Kongkitkul, C. Sinthuuthai, N. Kalong, D. Ongsawang, S. Ungsathapornpon, A. Ameeroh, P. Bavonnarongdet, A. Buadung, Epidemiology and the effect of treatment of soil-transmitted helminthiasis in pregnant women in southern Thailand, SE Asian J. Trop. Med. 40 (2009) 211–222.

[27] WHO, Guidelines for the Safe Use of Wastewater, Excreta and Greywater in Agriculture, WHO Library Cataloguing-In-Publication Data, Geneva, 2006.

[28] P. Gyawali, J.P. Sidhu, W. Ahmed, P. Jagals, S. Toze, Rapid concentration and sensitive detection of hookworm ova from wastewater matrices using a real-time PCR method, Exp. Parasitol. 159 (2015) 5–12.

[29] S. Tun, I. Ithoi, R. Mahmud, N.I. Samsudin, H.C. Kek, L.Y. Ling, Detection of helminth eggs and identification of hookworm species in stray cats, dogs and soil from Klang Valley, Malaysia, PLoS ONE 10 (2015) e0142231.

[30] M.W. Wang, X.X. Yan, J.X. Hang, X.L. Shi, Y.Q. Fu, P. Zhang, F. Yang, W.D. Pan, G.Q. Li, Molecular differentiation of three canine and feline hookworms in South China through HRM analysis, J. Helminthol. (2018), https://doi.org/10.1017/S0022149X18000068.

[31] C. Schneeberger, P. Speiser, F. Kury, R. Zeillinger, Quantitative detection of reverse transcriptase-PCR products by means of a novel and sensitive DNA stain, PCR Meth. Appl. 4 (1995) 234–238.

[32] F. Vitzthum, G. Geiger, H. Bisswanger, H. Brunner, J. Bernhagen, A quantitative fluorescence-based microplate assay for the determination of double-stranded DNA using SYBR green I and a standard ultraviolet transilluminator gel imaging system, Anal. Biochem. 276 (1999) 59–64.

[33] S. Giglio, P.T. Monis, C.P. Saint, Demonstration of preferential binding of SYBR green I to specific DNA fragments in real-time multiplex PCR, Nucleic Acids Res. 31 (2003) e136.