ISSN (Print) 0023-4001
ISSN (Online) 1738-0006

Korean J Parasitol Vol. 57, No. 1: 9-15, February 2019
https://doi.org/10.3347/kjp.2019.57.1.9

▣ ORIGINAL ARTICLE

Establishment of a T_m-shift Method for Detection of Cat-Derived Hookworms

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Abstract: Melting temperature shift (T_m-shift) is a new detection method that analyze the melting curve on real-time PCR thermocycler using SYBR Green I fluorescent dye. To establish a T_m-shift method for the detection of *Ancylostoma ceylanicum* and *A. tubaeforme* in cats, specific primers, with GC tail of unequal length attached to their 5′ end, were designed based on 2 SNP loci (ITS101 and ITS296) of the internal transcribed spacer 1 (ITS1) sequences. The standard curve of T_m-shift was established using the standard plasmids of *A. ceylanicum* (AceP) and *A. tubaeforme* (AtuP). The T_m-shift method stability, sensitivity, and accuracy were tested with reference to the standard curve, and clinical fecal samples were also examined. The results demonstrated that the 2 sets of primers based on the 2 SNPs could accurately distinguish between *A. ceylanicum* and *A. tubaeforme*. The coefficient of variation (CV) of T_m- values of AceP and AtuP was 0.07% and 0.06% in ITS101 and was 0.06% and 0.08% in ITS296, respectively. The minimum detectable DNA concentration was 5.22×10^{-6} and 5.28×10^{-6} ng/μl samples of AceP and AtuP, respectively. The accuracy of T_m-shift method reached 100% based on examination of 10 hookworm DNA samples with known species. In the clinical detection of hookworm in 69 stray cat fecal sample, the T_m-shift detection results were consistent with the microscopic examination and successfully differentiated between the 2-hookworm species. In conclusion, the developed method is a rapid, sensitive and accurate technique and can provide a promising tool for clinical detection and epidemiological investigation of cat-derived hookworms.

Key words: *Anoylostoma ceylanicum*, *Anoylostoma tubaeforme*, cat, T_m-shift, SNP, ITS1

INTRODUCTION

Hookworms are common parasitic nematodes in the digestive tract of human beings, dogs and cats, which can cause a serious hazard to the host. The main hookworms infecting cats include *Ancylostoma ceylanicum*, *A. tubaeforme*, *A. braziliense*, and *Uncinaria stenocephala*. The recent investigations have shown that the cat's hookworm infection in China is mainly caused by *A. ceylanicum* and *A. tubaeforme* [1,2]. *A. ceylanicum* is globally distributed among dogs and cats, however it is particularly severe in Asia, especially in Southeast Asia and the Pacific. Recently, *A. ceylanicum* has been recorded in many Southeast Asian countries, such as China [3,4], Japan [5], Malaysia [6], Laos [7], Thailand [8]. *A. tubaeforme* mainly infects cats, but it is rarely reported in canines. According to the previous surveys, *A. tubaeforme* has been reported in cats in Australia [9,10], Brazil [11], Italy [12], Qatar [13], Spain [14], the United States [15,16], and China [2]. Currently, *A. ceylanicum* has become the second largest species of hookworm in Asia [17], that can feed on blood and develop into adults in the human body, causing anemia, abdominal pain, diarrhea, and even death. As the number of cat owners increases, it is important to establish a rapid and accurate method that can distinguish *A. ceylanicum* from *A. tubaeforme* to enhance public health and safety.

The fecal examination is a classical method for hookworm detection, but it fails to identify hookworm species. Therefore, it is difficult to judge whether the infection is caused by *A. ceylanicum*. Presently, many molecular detection techniques have been applied to identify hookworms, such as specific polymerase chain reaction (PCR), multiplex PCR, restriction fragment length polymorphism (RFLP) and high-resolution melting (HRM) analysis [18-20]. All the former techniques have certain limitations, some can only detect a small number of

• Received 19 November 2018, revised 25 January 2019, accepted 6 February 2019.
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samples at the same time, and others are complicated and expensive to operate.

Melting temperature shift (T_m-shift) method based on single nucleotide polymorphism (SNP) is a new molecular detection technique, which is high-throughput, accurate and inexpensive. This method utilizes 2 allele-specific primers, each of which attach to one of the two SNP loci at its 3′ end, a reverse primer that amplifies both alleles, and a fluorescent dye, SYBR® Green I (Molecular Probes, Eugene, Oregon, USA). Either one or both allele specific primer(s) amplify according the sample genotype. GC rich tails of unequal length are attached to the 5′ end of the allele-specific primers, so that the PCR product has a different melting temperature depending on which of the 2 primers is used. Therefore, genotypes can be distinguished by checking the melting curve on a real-time PCR instrument [21,22]. The T_m-shift method has been applied in many fields, such as medicine, microbiology, and aquatic biology [23-25]. In our laboratory, Pan et al. [26] established a T_m-shift genotyping method for *Giardia lamblia* assemblages A and F in cats, which pioneered a new genotyping method for veterinary parasitic protozoa. In addition, Fu et al. [27] developed a T_m-shift detection method for *A. ceylanicum* and *A. caninum* in dogs and applied it for the first time to identify veterinary parasitic nematode.

The present study aimed to develop and evaluate a T_m-shift method for the identification of *A. ceylanicum* and *A. tubaeforme* in cats, to provide a fast and accurate technique for the clinical detection and zoonotic risk assessment of cat-derived hookworms.

MATERIALS AND METHODS

Source of samples

Adult *A. ceylanicum* and *A. tubaeforme* samples were isolated and identified by Liu et al. [4] and Shi et al. [2], and preserved in 50% ethyl alcohol in our laboratory. Fecal samples were collected from stray cats at the Animal Rescue Station in Shaoguan city of Guangdong province in China and stored at 4℃.

Genomic DNA extraction

The preserved adult hookworms were repeatedly washed with double-distilled water (ddH_2O). After that, the genomic DNA was extracted using the Wizard® SV Genomic DNA Purification System (Promega, Guangzhou, China) according to the manufacturer's instructions, then stored at -20℃.

PCR amplification of ITS1 sequence

The extracted DNA was subjected to polymerase chain reaction targeting *A. ceylanicum* and *A. tubaeforme* internal transcribed spacer 1 (ITS1) sequences using a forward primer AF (5′-CTTTGTCGGGAAGGTTGG-3′) and a reverse primer AR (5′-TTCACCACTCTAAGCGTCT-3′) previously designed by Liu et al. [4]. The target amplification fragment length was 404 bp. PCR was carried out in a final volume of 25 µl, containing 12.5 µl Premix Ex-Taq polymerase (TaKaRa, Dalian, China), 9.5 µl ddH_2O, 0.5 µl of each primer AF/AR (50 µmol/L), and 2 µl DNA template. The utilized PCR program was as follows: 94℃ for 5 min, 35×(94℃ for 30 sec, 61.5℃ for 30 sec and 72℃ for 45 sec), and 72℃ for 7 min. The PCR products were assessed by electrophoresis in agarose gel (1.5%) with ethidium bromide stain (0.2 mg/ml) and visualized on a UV transilluminator. The amplified fragment was recovered from the gel following the protocol of the gel recovery kit (Omega, Guangzhou, China) and kept at -20℃ until further use.

Preparation of standard plasmids

The purified PCR products were cloned in *Escherichia coli* and connected with pMD18-T vector (TaKaRa), then transformed into DH5 Competent Cells (TaKaRa). Positive clones were screened by bacterial PCR and sent to Sangon Biotech (Shanghai, China) for sequencing. The Plasmid Kit (Omega) was used to extract the plasmid DNAs. The plasmids harboring *A. ceylanicum* and *A. tubaeforme* ITS1 sequences were termed as AceP and AtuP. The plasmids (1 µl) were analyzed by the ultramicro-spectrophotometer to evaluate their purity and concentration, where A260/A280 value and optimal concentration should be 1.8-2.0 and 50 ng/µl, respectively.

Establishment of T_m-shift method based on SNP

Based on the 2 SNPs (ITS101 and ITS296), 2 sets of T_m-shift primers were designed using Primer Premier 5.0 software, referencing to the 2 sequences (MG733992, KP072069) downloaded from GenBank. The primer sequences and predicted amplification fragment length are demonstrated in Table 1. The above-mentioned primers were synthesized by Sangon Biotech (Shanghai, China). The synthesized primers were prepared in a template liquid at 100 pmol/µl and stored at -20℃. Then they were re-diluted to a final working concentration of 10 pmol/µl and stored at -20℃ for use after partial shipments. PCR amplification and T_m-shift reaction were performed once in Rotor-Gene Q. The qPCR-T_m-shift reaction mixture had the

Table 1. Primers for T_m-shift method based on 2 SNPs

Primer	Nucleotide sequence (5′-3′)	Product length (bp)
ITS101AF (Atu)	gattaccg GGCGGCAGTGATTGCTGTA	
ITS101GF (Ace)	gcgggcagggcggg GGCGGCAGTGATTGCTGTG	106
ITS101R	GCCTAATGCTCAACCACCAACA	
ITS296TF (Ace)	gattaccg TTTGCAGAATCGTGACTTT	
ITS296AF (Atu)	gcgggcagggcggg TTTGCAGAATCGTGACTTA	127
ITS296R	TTCACCACTCTAAGCGTCT	

GC tails are underlined.

final volume of 20 μl, containing 2×SYBR® Premix Ex *Taq* TM II (10 μl), long-tail primer (0.4 μl), short-tail primer (0.4 μl), common reverse primer (0.8 μl), ddH_2O (7.4 μl), and plasmid (1.0 μl). The cycling program was as follows: 95℃ for 5 min, 40× (95℃ for 10 sec and 63℃ for 30 sec). The melting process ranged from 70 to 95℃ at the rate of 0.5℃/sec.

Stability, sensitivity and accuracy test

The stability of the established T_m-shift method was detected by repeating the experiments using 2 standard plasmids (AceP and AtuP). Intra-assay was checked 7 times, while inter-assay was checked 3 times for each plasmid. To evaluate the T_m-shift method sensitivity, AceP and AtuP were diluted 10 times and examined following the concentration of 1:10-1:10^8. To assess the T_m-shift method accuracy, 10 samples with known hookworm species were examined. The qPCR-T_m-shift reaction mixture and cycling program were identical to the mentioned above.

Clinical detection

Sixty-nine stray cat fecal samples were analyzed by zinc sulfate flotation technique and T_m-shift method. All fecal samples were suspended in ddH_2O, heated for 5 cycles at 100℃ for 5 min and immediately frozen at -80℃ for 5 min. Genomic DNAs were extracted from the fecal samples using Stool DNA extraction kit (OMEGA, Guangzhou, China) as indicated by the manufacturer's protocols. The qPCR-T_m-shift reaction mixture and cycling program were the same as the mentioned above. All results were further confirmed by DNA sequencing.

RESULTS

Amplification of ITS1 fragment and preparation of standard plasmids

The amplification of the 2-hookworm species ITS1 sequences produced fragments with the length of 404 bp (Fig. 1), which agreed with the expected fragment length. The generated sequence data were submitted to GenBank (accession number: KF279132, JO812691). BLAST analysis showed the highest similarity (100%) with *A. ceylanicum* from Japan (LC036567) and the highest similarity (100%) with *A. tubaeforme* from Guangzhou (MG865903). Thus, 2 hookworms were identified as *A. ceylanicum* and *A. tubaeforme*. The A260/A280 values of positive plasmids of *A. ceylanicum* (AceP) and *A. tubaeforme* (AtuP) were between 1.8 and 2.0. In addition, the concentration of AceP and AtuP was 53.3 and 50.3 ng/μl, respectively, which was consistent with the optimal criteria.

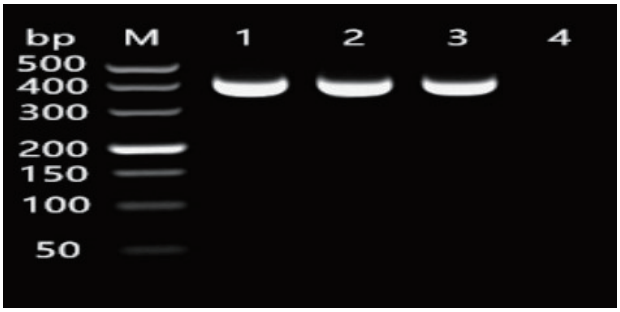


Fig. 1. PCR amplification of the ITS1 sequence of 2 hookworms. M, DL-500 DNA marker; 1, Positive control; 2, *A. ceylanicum*; 3, *A. tubaeforme*; 4, Negative control.

Detection of qPCR-T_m-shift

The T_m-shift standard curves based on the 2 SNPs (ITS101 and ITS296) for standard plasmid AceP and AtuP are revealed in Fig. 2. The result showed that the established T_m-shift method could distinguish *A. ceylanicum* (AceP) from *A. tubaeforme* (AtuP). The analysis of the standard curves by Rotor-Gene Q series software demonstrated that in the ITS101 primer, the T_m values of AceP and AtuP were 88.25℃ and 86.50℃, respectively. In the ITS296 primer, the T_m values of AceP and AtuP were 85.00℃ and 86.60℃, respectively.

Stability, sensitivity and accuracy

The stability test results are presented in Table 2. In the prim-

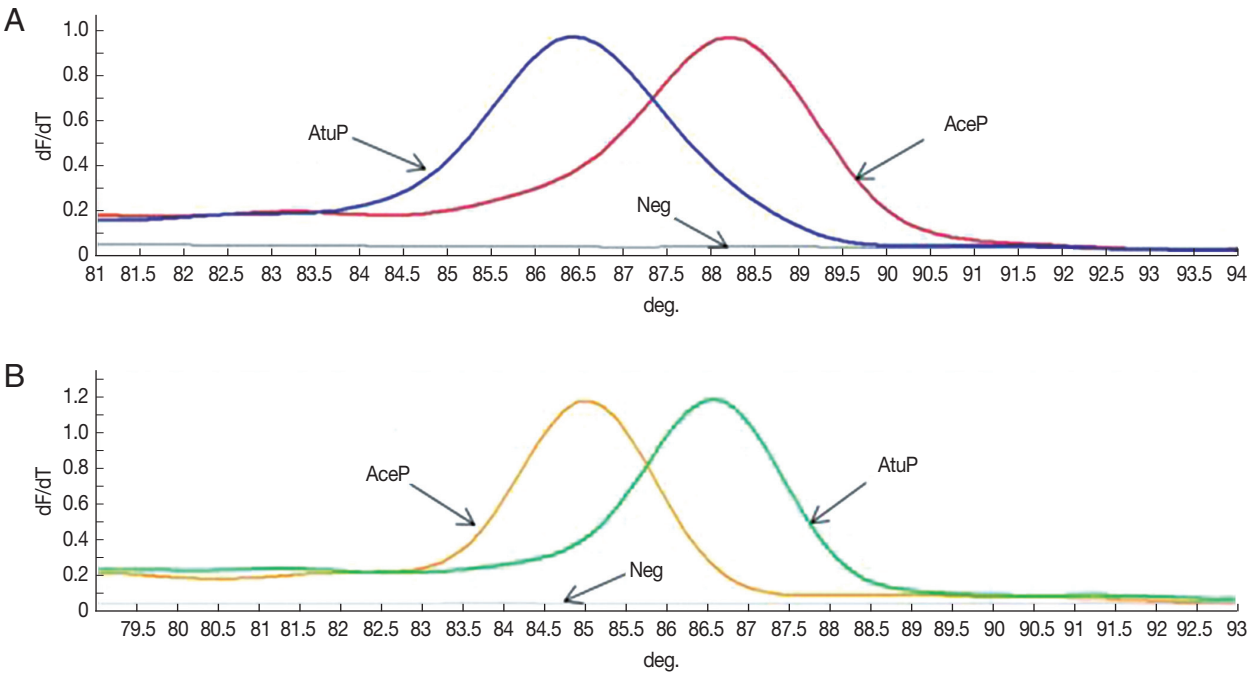


Fig. 2. Standard curves of T_m-shift for 2 hookworm standard plasmids based on ITS101 (A) and ITS296 (B). AceP, *A. ceylanicum* standard plasmid; AtuP, *A. tubaeforme* standard plasmid; Neg, negative control.

Table 2. Stability of T_m-shift method

Repeat	ITS101 (T_m) (°C)		ITS296 (T_m) (°C)	
	AtuP	AceP	AtuP	AceP
First (n=7)	86.49±0.09	88.25±0.10	86.63±0.07	85.01±0.09
Second (n=7)	86.47±0.07	88.21±0.06	86.60±0.10	85.04±0.06
Third (n=7)	86.44±0.09	88.26±0.11	86.59±0.11	85.00±0.10
Average (n=21)	86.47	88.24	85.00	86.60
CV (%)	0.06	0.07	0.08	0.06

er ITS101, the coefficient of variation (CV) of AceP and AtuP melting temperature (T_m) was 0.07% and 0.06%, respectively. In primer ITS296, the CV of AceP and AtuP melting temperature was 0.06% and 0.08%, respectively. The sensitivity test results indicated that the T_m-shift method could still differentiate between *A. ceylanicum* (AceP) and *A. tubaeforme* (AtuP), when they were diluted to $1:10^7$ (5.22×10^{-6} ng/μl and 5.28×10^{-6} ng/μl, respectively) (Table 3). The results of examination of 10 DNA samples by T_m-shift method were identical to their known hookworm species with 100% accuracy (Table 4).

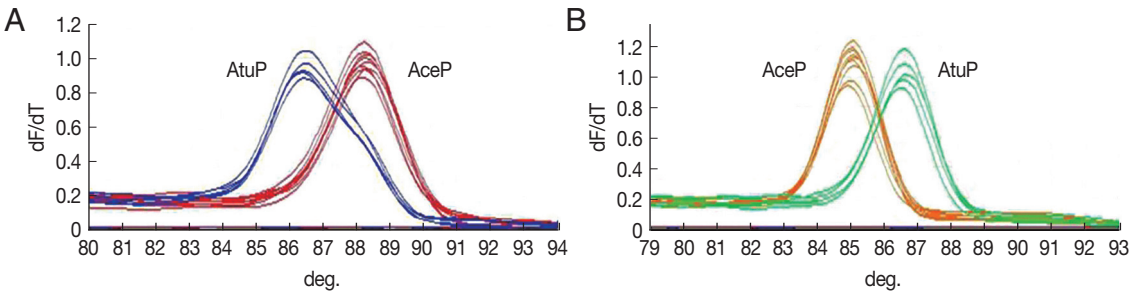
Table 3. Sensitivity of T_m-shift method

Dilution	ITS101 (T_m) (°C)		ITS296 (T_m) (°C)	
	AtuP	AceP	AtuP	AceP
$1:10^1$	86.50	88.25	86.60	85.00
$1:10^2$	86.50	88.25	86.65	85.00
$1:10^3$	86.50	88.35	86.60	84.90
$1:10^4$	86.50	88.35	86.60	85.00
$1:10^5$	86.40	88.15	86.50	85.00
$1:10^6$	86.40	88.25	86.60	85.10
$1:10^7$	86.40	88.25	86.60	85.10
$1:10^8$	-	-	-	-

-, Not detected.

Clinical detection

Twelve out of 69 cat fecal samples were positive for hookworm by the fecal flotation and T_m-shift methods. Using T_m-shift method, 8 and 4 samples were identified as *A. ceylanicum* and *A. tubaeforme*, respectively. The melting curves and detection results are shown in Fig. 3. All detection results were identical to the sequencing results.



Sample No.	Colour	T_m(°C)	Species	Sample No.	Colour	T_m(°C)	Species
AtuP		86.50°C	Ancylostoma tubaeforme	AtuP		86.60°C	Ancylostoma tubaeforme
AceP		88.25°C	Ancylostoma ceylanicum	AceP		85.00°C	Ancylostoma ceylanicum
CSG010		88.25°C	Ancylostoma ceylanicum	CSG010		85.00°C	Ancylostoma ceylanicum
CSG012		88.25°C	Ancylostoma ceylanicum	CSG012		85.10°C	Ancylostoma ceylanicum
CSG018		88.25°C	Ancylostoma ceylanicum	CSG018		85.00°C	Ancylostoma ceylanicum
CSG019		88.35°C	Ancylostoma ceylanicum	CSG019		85.10°C	Ancylostoma ceylanicum
CSG024		86.50°C	Ancylostoma tubaeforme	CSG024		86.50°C	Ancylostoma tubaeforme
CSG025		86.40°C	Ancylostoma tubaeforme	CSG025		86.60°C	Ancylostoma tubaeforme
CSG031		86.50°C	Ancylostoma tubaeforme	CSG031		86.60°C	Ancylostoma tubaeforme
CSG039		86.40°C	Ancylostoma tubaeforme	CSG039		86.65°C	Ancylostoma tubaeforme
CSG052		88.25°C	Ancylostoma ceylanicum	CSG052		85.00°C	Ancylostoma ceylanicum
CSG061		88.15°C	Ancylostoma ceylanicum	CSG061		85.10°C	Ancylostoma ceylanicum
CSG066		88.25°C	Ancylostoma ceylanicum	CSG066		84.90°C	Ancylostoma ceylanicum
CSG068		88.35°C	Ancylostoma ceylanicum	CSG068		85.00°C	Ancylostoma ceylanicum

Fig. 3. Melting curve of T_m-shift based on ITS101 (A) and ITS296 (B) and detection results for 12 hookworm -positive samples.

Table 4. The accuracy of T_m-shift method

Sample	Species	ITS101 (T_m) (°C)	ITS296 (T_m) (°C)	GenBank
M6	*A. ceylanicum*	88.25	85.00	KF279134
M55	*A. ceylanicum*	88.25	85.00	KF279135
M58	*A. ceylanicum*	88.25	85.00	KF279136
M60	*A. ceylanicum*	88.35	85.00	KF279137
M76	*A. ceylanicum*	88.25	85.10	KF279138
C1	*A. tubaeforme*	86.50	86.60	MG904956
C2	*A. tubaeforme*	86.50	86.60	MG904957
C3	*A. tubaeforme*	86.40	86.60	MG904958
C4	*A. tubaeforme*	86.50	86.60	MG904959
C5	*A. tubaeforme*	86.40	86.50	MG904960

Sample M6, M55, M58, M60, M76 were isolated and identified by Liu et al. [4]. The sequences of sample C1, C2, C3, C4, and C5 have been uploaded to GenBank.

DISCUSSION

Ancylostoma ceylanicum and *A. tubaeforme* are the 2 most common parasitic nematodes in cats. The former is the only animal-derived hookworm that can cause severe infection in humans. In Malaysia, the infection rate of *A. ceylanicum* is as high as 23.4%, which has become the second largest hookworm in local people [28]. In Laos, one-third of human hookworm infections are caused by *A. ceylanicum* [29]. Also, there are reports of human infection with *A. ceylanicum* in Taiwan [30]. In recent years, many cases of cat infection with *A. tubaeforme* have been detected in Guangzhou, China [2]. With the economic development and life quality improvement, more and more people are raising cats. Hence, it is of great significance to establish a T_m-shift detection method for cat-derived *A. ceylanicum* and *A. tubaeforme* for the prevention and control of zoonotic hookworm disease.

Common molecular biological methods for hookworm species identification include polymerase chain reaction (PCR), multiplex PCR, restriction fragment length polymorphism (RFLP), and high-resolution melting curve (HRM) [18-20]. Although the first 3 methods are specific and sensitive, they require gel electrophoresis after PCR, which makes the samples can be easily contaminated. In addition, the long-term use of ethidium bromide may seriously endanger the health of the operator. As a new molecular detection method, T_m-shift method is simple and quick to operate compared with the above-mentioned methods. The T_m-shift method is completed at one time on a closed fluorescent PCR instrument

minimizing the contamination risk. As well as, it can examine 36 or 72 samples at the same time. Compared with HRM, the T_m-shift method does not require a relatively complicated HRM program. The used fluorescent dye in T_m-shift method is the ordinary SYBR Green I fluorescent dye, which is less expensive than the saturated dye required for HRM. Furthermore, the T_m-shift method can complete the amplification of the target fragment by using a 2-step method instead of 3 steps in HRM, which greatly saves the experiment time and improves the detection efficiency.

Even though the T_m-shift detection method has clear benefits over the other methods, it has some limitations during application. For example, the T_m-shift method has higher requirements for the design of 2 specific primers. The T_m value of the specific primer should be between 59˚C and 62˚C, and the primer length should be controlled in 15-22 bp. For a higher *Taq* polymerase amplification efficiency, the difference of T_m value between the reverse and specific primer (before adding sequences at the 5′ end) should be ranged from 4 to 5˚C. In our study during the establishment of T_m-shift detection methods for cat-derived *A. ceylanicum* and *A. tubaeforme*, the primers were optimized where the short chain "gcggcg" was replaced with "gattaccg". The results showed that this operation increased the T_m value of 2 melting curves to some extent. The difference between the peak of the melting curve for the 2 hookworms was 1.75˚C at ITS101, while it was 1.60˚C at ITS296. The established T_m-shift detection method was applied to examine 69 stray cat fecal samples in Shaoguan, Guangdong. The examination results of clinical fecal samples showed that 12 samples were positive for hookworm, from which 8 samples were *A. ceylanicum* and 4 samples were *A. tubaeforme*. The positive rate of T_m-shift method was consistent with the microscopic examination results, and this method could distinguish *A. ceylanicum* from *A. tubaeforme*. These results indicated that the designed T_m-shift method is rapid, accurate, efficient and sensitive.

It could be concluded that the developed T_m-shift method based on the 2 SNPs loci of ITS1 sequence can successfully identify *A. ceylanicum* and *A. tubaeforme* species. As well as, it can provide a suitable technical mean for clinical diagnosis and zoonotic risk judgment of cat-derived hookworms.

ACKNOWLEDGMENT

This work was supported by a grant from National Natural Science Foundation of China (grant no. 31672541) and the Science and Technology Planning Project of Guangdong Province, China (grant no. 2014A020214005).

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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