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Identification of Three *Toxocara* species, *T. canis* in Dogs, and *T. cati* and *T. malaysiensis* in cats, in Vietnam by PCR-RFLP Analysis and DNA Sequencing

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Abstract

Toxocara canis and *T. cati* are common roundworms parasitizing dogs and cats, respectively. However, a recent study detected *T. malaysiensis*, but not *T. cati*, in cats from some parts of Ha Noi and Nam Dinh Provinces raising the question of whether *T. cati* is present in cats in Vietnam. This study was conducted to determine the composition of *Toxocara* species in dogs and cats in Vietnam. One hundred and twenty-seven *Toxocara* adult worms were collected from dogs and cats and were analyzed by PCR-RFLP assay and DNA sequencing of a partial section of the *cox1* gene. As a result, all samples amplified by PCR showed bands about 430 bp in length. PCR products digested by MseI from isolates from cats and dogs showed four and two restriction patterns, respectively. The six different patterns were chosen to sequence. Based on the restriction patterns and sequencing results, two *Toxocara* species were identified in the cats, namely *T. cati* (110/111 isolates) and *T. malaysiensis* (1/111 isolates) collected from Ha Noi and Hai Duong, respectively; and *T. canis* was identified in the dogs (16/16 isolates). In addition, the present study indicated intra-specific sequence variations of *Toxocara* spp. in dogs and cats. In conclusion, the study confirmed the presence of both *T. cati* and *T. malaysiensis* in cats, and *T. canis* in dogs in Vietnam, and suggests further large-scale investigations to fully understand the distribution and genetic variations of *Toxocara* spp. in cats and dogs in Vietnam.

Keywords

Toxocara roundworm, *Toxocara canis*, *Toxocara cati*, *Toxocara malaysiensis*, PCR-RFLP.

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Introduction

Toxocara canis and *Toxocara cati* are common roundworms parasitizing canids and felids, respectively. Both of them are also

etiological agents causing human toxocariasis, a zoonotic parasitic disease reported worldwide (Magnaval *et al*., 2001). In 2001, a new *Toxocara* species named *T. malaysiensis* was described in cats from Malaysia, and since then, it has been detected in China and Vietnam (Gibbons *et al*., 2001, Li *et al*., 2006; Le *et al*., 2016). While *T. canis* is the unique *Toxocara* species found only in dogs (Nguyen Thi Quyen *et al*, 2016; Le *et al*., 2016), *Toxocara* species composition in cats has been questioned. In Vietnam, *Toxocara* spp. is one of the most common roundworms in dogs and cats (Nguyen Thi Hoang Yen *et al.*, 2020; Nguyen Thi Hoang Yen *et al*., 2022). The prevalence of *Toxocara* spp. in dogs (34.6-47.8%) has generally been found to be greater than in cats (21.0-37.7%) (Anh *et al*., 2016; Nguyen *et al*., 2020; Nguyen *et al*., 2022). *T. cati* has been considered the predominant species in cats in Vietnam (Le *et al*., 2016). However, a recent study that detected *T. malaysiensis*, but not *T. cati*, in domestic cats from Ha Noi and Nam Dinh Provinces (Le *et al*., 2016) raised the question of whether *T. cati* is present in cats from Vietnam.

Traditionally, *Toxocara* species are identified mainly based on their morphological characteristics and predilection sites in a particular host species (Kim *et al*., 2020). However, misidentification may occur because of morphological similarities between species, such as the shape of the esophagus with a ventriculus, dentigerous ridges on the lips, a V-shaped cervical alae, spicules, and position of the vulva opening (Gibbons *et al*., 2001); meanwhile, molecular techniques using ribosomal and mitochondrial DNA markers have helped to obtain more accurate identifications (Jacobs *et al*., 1997; Zhu *et al*., 2001; Gasser *et al*., 2006; Pawar *et al*., 2012; Mikaeili *et al*., 2017; Wang *et al*., 2018; Fava *et al*., 2020). PCR-linked restriction fragment length polymorphism (PCR-RFLP) developed by Fava is a time and cost-saving method for distinguishing the three *Toxocara* species, namely *T. canis, T. cati* and *T. malaysiensis*, because it only requires a single restriction enzyme (MseI) (Fava *et al*., 2020). Thus, in this study, PCR-RFLP and DNA sequencing were performed to differentiate and confirm *Toxocara* species collected from dogs and cats in Northern Vietnam.

Materials and Methods

Collection of roundworms and sample processing

One hundred and twenty-seven adult worms of *Toxocara* spp. were collected from naturally infected dogs and cats (111 from 36 cats and 16 from 6 dogs) at veterinary clinics by anthelminthic expulsion or local abattoirs by necropsy around Ha Noi (Nam Tu Liem and Gia Lam districts), and one *Toxocara* adult worm was collected from a domestic cat in Tien Dong commune, Tu Ky district, Hai Duong province. They were preliminarily identified based on macro-morphology, as *Toxocara* spp. have a ventriculus intercalated between the esophagus and the intestine, and males have a finger-like tail, which is distinguished by tapering to a point in *Toxascaris leonina* males (Bowman, 2014). The roundworms were then thoroughly washed several times in saline solution and kept in 70% ethanol for molecular analyses.

DNA extraction and polymerase chain reaction (PCR)

Total genomic DNA was extracted from adult worms of *Toxocara* spp. by the alkaline lysis method with a few slight changes. Firstly, a piece (about 50mg) of the worm was cut and transferred into a 1.5-mL Eppendorf tube, followed by the addition of 1,800µL of 50mM NaOH. The tubes were incubated at 95°C overnight on a block heater. After that, 200 µl of 1 M Tris-HCl (pH 8.0) was added into each tube. Next, the mixture was vortexed thoroughly and centrifuged at 14,000 *x g* for 10 minutes. Finally, the supernatant was transferred into a new tube and then stored at -20°C until analysis (Nguyen *et al.*, 2016).

The total genomic DNA from each individual worm was subjected to PCR assay using the primer pair ToxCoIF (5′GATTTTACCTGCTTTTGGTATTATTAG-3′) and ToxCoIR (5′-CCAAAGACAGCACCCAAACT-3′) (Fava *et*

al., 2020) to amplify a 426-bp fragment of the mitochondrial *cox1* gene.

All PCR reactions were carried out in a total volume of 50µL using 2µL of template DNA, 25 µl Mastermix 2X_100_tracking dye (Phusa Biochem LTD. Company, Can Tho, Vietnam), 2 µl of each primer (10 pmol), and 19 µl distilled water. The amplification conditions followed those described in a previous study (Fava *et al*., 2020). PCR products were electrophoresed on a 1.0% agarose gel in TAE at 100V for 30 minutes. Gels were stained with GelRed® (Biotium, Fremont, CA) and the bands were visualized under UV light.

DNA sequencing analysis

PCR amplicons were purified and six isolates were chosen for sequencing in both the forward and reverse strands using the same primers employed in the PCR by the Suran Medical and Scientific Solutions Join Stock Company (Hanoi, Vietnam). DNA sequences were aligned using the Geneious Prime Biomatters Company and compared with sequences from the GenBank database via the BLAST search tool.

PCR linked restriction fragment length polymorphism (PCR-RFLP)

Six microliters (6µL) of each PCR product were digested with 4 units of MseI (New England Biolabs, Ipswich, MA) in a total volume of 15µL. The mixture was incubated overnight at 37°C and then electrophoresed on 2% agarose, stained with GelRed®, and visualized using UV light. Digestion products included three restriction patterns: 95, 121, and 210bp (*T. canis*); 22, 44, 172, and 188bp (*T. cati*); and 44, 51, 121, and 210bp (*T. malaysiensis*) (Fava *et al.*, 2020). To estimate the sizes of the fragments, a 100-bp molecular ladder (BioFact, Daejeon, Republic Korea) was used. A negative control (distilled water) was added to each run.

Results

A total of 127 samples were analyzed by PCR assay, and all of them showed a band of around 430bp (**Figure 1**). After digestion by the MseI enzyme, six restriction fragment patterns were observed from the PCR products, four patterns in the *Toxocara* worms from cats, and two other patterns in the *Toxocara* worms from dogs. The first pattern had three clear bands about 210, 120, and 40bp (**Figure 2A**, Tspcat1); the second pattern had three clear bands with two close bands about 190bp and one band about 44 bp (**Figure 2A**, Tspcat2-Tspcat6, Tspcat13; **Figure 2B**, Tspcat52); the third one had four clear bands about 190, 110, 60, and 40 bp (**Figure 2B**, Tspcat22, Tspcat47); and the last one had three clear bands about 190, 120, and 100bp (**Figure 2B**, Tspcat56). According to the restriction patterns reported by Fava *et al*. (2020), the first pattern in this study was expected to be *T. malaysiensis* based on the specific band of 210 bp and the felid host; the following three patterns were expected to be *T. cati* characterized by 190 bp bands. In the case of the dog *Toxocara* worms, two restriction patterns of fragments were observed, one had three bands of 210, 120, and 100bp (**Figure 3**, Tspdog1, Tspdog3); while the other consisted of three bands of about 210, 120, and 50bp (**Figure 3**, Tspdog4). In comparison to the restriction patterns reported by Fava *et al.* (2020), the former pattern was expected to be *T. canis* based on the specific band of 210bp and the canid host; and the latter pattern seemed to be *T. malaysiensis* (Fava *et al*., 2020).

Analysis of the DNA sequences revealed some variations in the sequences of the restriction sites for MseI in both the cat and dog isolates (**Figure 4**). MseI recognizes sites with the sequence 5'-T*TAA-3'. The sequence of the *cox1* gene of *T. malaysiensis* contained three cutting sites for MseI (black rectangles, **Figure 4**), thus four fragments of 44, 51, 121, and 210bp were collected after digestion. For *T. cati*, the isolate named Tspcat22 contained four cutting sites, and produced five fragments of 22, 44, 63, 109, and 188bp. Two isolates, Tspcat52 and Tspcat56, had three cutting sites but at different positions, giving two different restriction patterns of four fragments 22, 44, 172, and 188bp; and 22, 95, 121, and 188bp, respectively (green rectangles, **Figure 4**). The fragments of 172 and 188bp were very close because they only

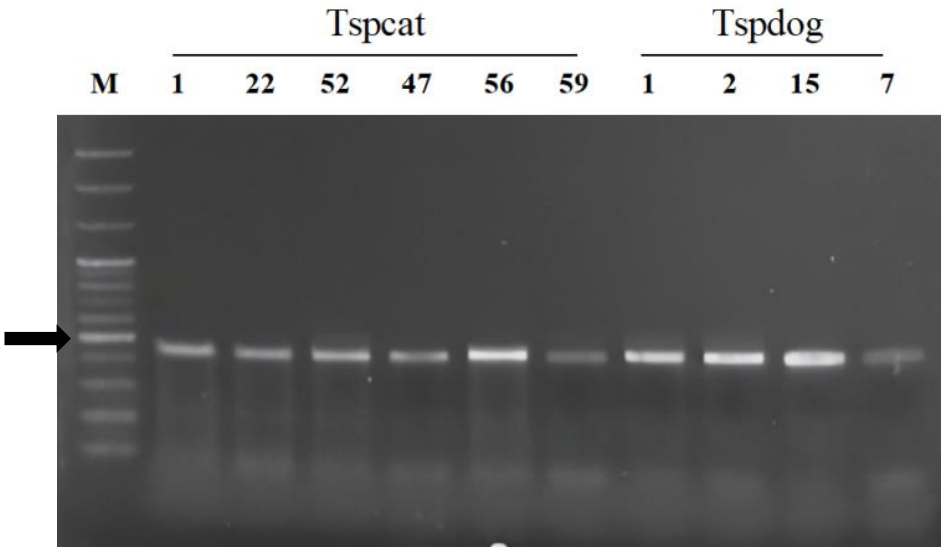

Figure 1. Electrophoretic images of the PCR products of *cox1* amplification of *Toxocara* spp. worms from cats and dogs. Lanes Tspcat1, 22, 52, 47, 56, and 59 are *Toxocara* worms from cats; Lanes Tspdog 1, 2, 15, and 17 are *Toxocara* worms from dogs; and Lane M: Marker. Black arrow shows 500bp position.

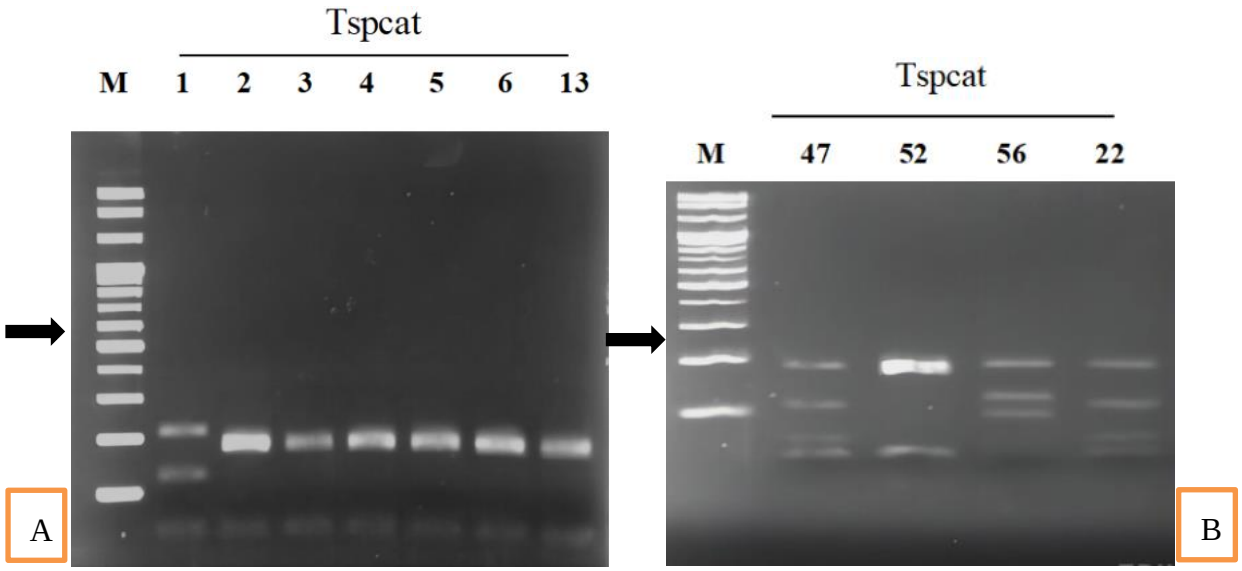

Figure 2. PCR-RFLP band patterns of the *cox1* region using the endonuclease MseI. Lane M: Marker; Lanes Tspcat1, 2, 3, 4, 5, 6, and 13 (A), and lanes Tspcat 47, 52, 56, and 22 (B) show the digested products of *Toxocara* worms from cats. Black arrow shows the 500 bp position.

differed in size by 16bp, while the 22-bp fragment was not clearly observed on the gel electrophoresis.

In the case of *T. canis*, the partial *cox1* gene of the sequence of Tspdog1 contained two cutting sites for MseI, producing three fragments, 95, 121, and 210bp after digestion. The isolate named Tspdog4 had three cutting sites, producing four fragments of 44, 51, 121, and 210bp (red rectangles, **Figure 4**). Of note, we recognized that the restriction fragment pattern of Tspcat1 (*T. malaysiensis*) was similar to that of Tspdogs4 (*T. canis*). Unfortunately, when comparing the PCR-RFLP results and sequencing, we recognized that the 22-bp band was unclearly observed in the electrophoresis results. Positions of the restriction enzyme sites in the amplified *cox1* region for the different species of *Toxocara* and the sizes of the fragments in each isolate are represented in **Table 1**.

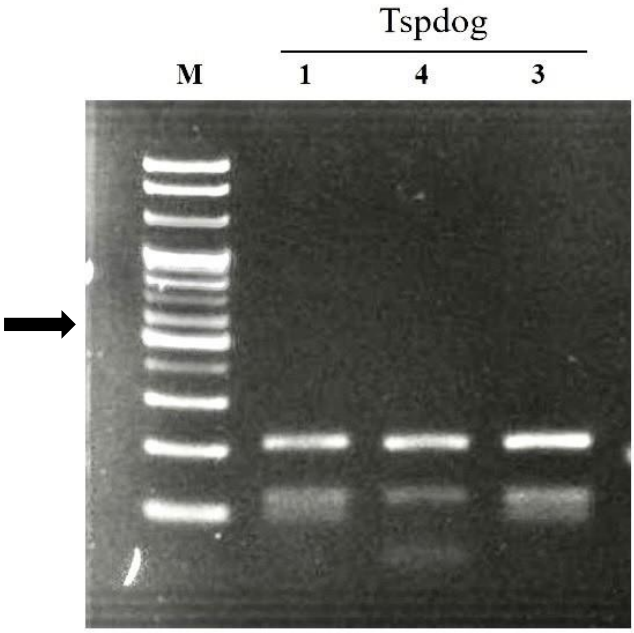

Figure 3. PCR-RFLP band patterns of the *cox1* region using the endonuclease MseI. Lane M: Marker; Lanes Tspdog1, 4 and 3 show the digested products of *Toxocara* worms from dogs. Black arrow shows the 500bp position.

Figure 4. Alignment of the *cox1* sequences (426 bp) representing the distinct genetic variants among the *Toxocara* isolates from cats and dogs. Rectangles are the locations of the MseI restriction sites: black represents *T. malaysiensis*, green represents *T. cati*, and red represents *T. canis*.

In comparison to sequences deposited in GenBank, only one isolate from cats was identified as *T. malaysiensis* with 99.53% identity to the reference sequence (GenBank

Table 1. Positions of the restriction enzyme sites in amplified in the *cox1* region for different species of *Toxocara* and the sizes of fragments in each isolate

Name of Isolate	Positions of Nucleotides Cut by MseI	Sizes of Fragments
Tspcat1 (*T. malaysiensis)*	44, 96, 217	44, 51, 121, 210
Tspcat22 (*T. cati)*	44, 108, 217, 405	22, 44, 63, 109, 188
Tspcat52 (*T. cati)*	44, 217, 405,	22, 44, 172, 188
Tspcat56 (*T. cati)*	95, 217, 405	22, 95, 121, 188
Tspdog1 (*T. canis)*	95, 217	95, 121, 210
Tspdog4 (*T. canis)*	44, 96, 217	44, 51, 121, 210

accession no. AM412316), and the other isolates showed 92.97% to 98.56% similarity to *T. cati* (accession nos. AM411622 and JF780942). All isolates collected from dogs were identified as *T. canis* with 99.42% to 100% similarity to reference sequences (GenBank accession nos. EU730761 and KC293909).

Discussions

As mentioned in the introduction, there has not been enough molecular evidence for the presence of *T. cati* in cats in Vietnam. *T. malaysiensis* was discovered in Malaysia (Gibbons *et al*., 2001) and China (Li *et al*., 2006), and more recently in Vietnam (Le *et al*., 2016). In a previous study, *T. malaysiensis* was identified as a unique species in the absence of *T. cati* in Vietnamese domestic cats (Le *et al*., 2016). However, the research scale in the previous study was limited to two communes (Thanh Oai and Thuong Tin) in Hanoi and a village (Nghia Hung) in Nam Dinh Province. Similarly, samples used in this study were also collected on a small scale, mainly around Ha Noi. In contrast to a previous report (Le *et al*., 2016), *T. cati* was found to be the predominant species of the *Toxocara* population in cats (110/111 isolates); and the sample collected in Hai Duong Province was the only *T. malaysiensis* isolate. This difference in *Toxocara* spp. in cats may be due to the small sample size from limited areas or may depend on the geographical locations. For the *Toxocara* population in dogs, there was only one species identified in Vietnam as published previously (Le *et al*., 2016; Nguyen Thi Quyen *et al*., 2016).

In addition, we recognized that there were intra-specific sequence variations in the *cox1* region among the isolates (in both cats and dogs). This was the reason why the Tspdog4 sample was initially recognized to be *T. malaysiensis* based on the PCR results. We first expected that there may be cross-infection, hybridization, or back-crossing between *Toxocara* species as the result of the detection of two *T. canis* worms in cats (Fava *et al*., 2020). However, the sequencing results demonstrated there was no cross-infection, and since we only included the *cox1* gene, a mitochondrial marker, we were not able to identify hybrids. Moreover, there was no unification between PCR-RFLP in this study when compared with the research of Fava *et al.* (2020), due to the replacement of nucleotides in restriction sites for MseI (**Figure 4**), resulting in different restriction patterns. Intra-specific sequence variations were reported in *T. cati* around 0-3.6% for *cox1* (He *et al*., 2017), not exceeding 2% and 4% for the ITS gene in *T. cati* and *T. canis*, respectively *(*Fogt-Wyrwas *et al*., 2013). This also explains why there were three and two different restriction patterns in *T. cati* and *T. canis* in this study, respectively. Hence, the results in this study show that it is necessary to use both the PCR-RFLP assay and DNA sequencing techniques to differentiate *Toxocara* species in dogs and cats, as PCR-RFLP helps recognize the similar restriction patterns, which can minimize the number of samples for sequencing.

Conclusions

This study confirmed the presence of two *Toxocara* species (*T. cati* and *T. malaysiensis*) in

cats, and only one *Toxocara* species (*T. canis*) in dogs in Hanoi and Hai Duong provinces, Vietnam. It is necessary to extend the research scale in order to fully evaluate the distribution as well as sequence variations of the *Toxocara* spp. population in cats in this country.

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