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Genetic analysis of *Toxocara* spp. in stray cats and dogs in Van province, Eastern Turkey

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Abstract

Introduction: *Toxocara canis* and *Toxocara cati* are roundworms of dogs and cats. The purpose of this study was to investigate the infection caused by these ascarids in cats and dogs, using microscopic and molecular analysis methods. **Material and Methods:** Adult ascarids were gathered from the faeces of dogs and cats in Van province, in 2015–2016. Existing keys and PCR sequencing of the ITS-2 fragment were used to identify the morphological features of the parasite species. **Results:** It was observed that out of 20 adult ascarids, 17 and 3 were found to be *Toxocara canis* and *Toxocara cati,* respectively. The ITS-2 gene region was amplified by PCR to perform molecular analysis. Genotyping indicated that the dogs and cats were infected with *T. canis* and *T. cati*, respectively, and none had *Toxascaris leonina.* **Conclusion:** To the best of our knowledge, this is the first report on the molecular characteristics of adult ascaridoid nematodes from cats and dogs in Turkey. The molecular approaches established in this study enable molecular identification and genetic structure studies of the ascaridoids.

Keywords: dogs, cats, *Toxocara*, genetic marker, Turkey.

Introduction

Toxocara canis and *Toxocara cati* are the most common nematodes living in the small intestine of dogs and cats. Female nematodes may produce up to 200,000 eggs per day, which require an incubation period in the soil before they are infective (5, 23). Therefore, contact with soil contaminated with *Toxocara* eggs is vital for infection to take place. In humans, toxocariasis is mainly caused by two *Toxocara* species, which are the dog nematode, *T. canis* and that of cats, *T. cati*. It is reported that *T. canis* is more frequently detected in human cases and *T. cati* is rarely seen. The most important clinical outcomes in humans are visceral larva migrans (VLM) and ocular larva migrans (OLM) due to the prolonged migration of the larvae of *T. canis* (10, 19). Felines are infected by *T. cati*, *T. malaysiensis*, *T. leonina*, and occasionally *T. canis*. *Toxascaris leonina* also causes infection in canids and felids (4, 14). It is not yet clear whether some of these species have caused disease in humans. Therefore, there is a need for a molecular technique to detect and identify the ascaridoid nematodes accurately (14).

Zoonotic roundworm infections are highly prevalent in many developing countries (9). It was reported that the prevalence rates of *T. canis* and *T. cati* were 4.2%–47.8% and 47%–85% in dogs and cats, respectively, in Turkey as determined microscopically (1, 26). The role of *T. cati* as a zoonotic parasite is not always clearly recognised. Based on epidemiological factors, most of the cases reported in human regarding toxocariasis have been associated with *T. canis* (2), although the distinction of *T. canis* infection from *T. cati* infection has not been achieved yet in serological studies. Toxocara excretory-secretory antigens (TES) shared between *T. canis* and *T. cati* and similarity in the form of infection are the findings indicating that there is no difference in risk to the public health between the two zoonotic diseases. For this reason, the potential role of *T. cati* in human toxocariasis should be paid sufficient attention (20). A study performed in the Netherlands has shown that about 50% of clinical toxocariasis cases were

associated with simultaneous *Ascaris suum* and *Toxocara* spp. infections (25). Also in the Netherlands, Pinelli *et al.* (22) indicated seroepidemiological incidence of *Toxocara* and *Ascaris* among VLM/OLM-suspected patients during 11 consecutive years (1998–2009) and emphasised the potential extent of *A. suum* as a causative agent for VLM/OLM. The traditional methods to identify and differentiate *T. canis*, *T. cati*, and *T. leonina* are based on microscopic analysis. *T. leonina* and *T. canis* are very similar but can be differentiated by the cephalic alae. However, to identify this difference is very difficult because specialised and qualified staff is required for these kinds of analysis (18). Since morphological methods are incapable of distinguishing closely related species, molecular methods should be used for accurate identification. The second internal transcribed spacer (ITS-2) has been used to identify nematodes in dogs, foxes, and cats (8).

The current study performed for the identification of adult ascaridoid nematodes from dogs and cats was based on both microscopic and ITS-2 region analysis. To our knowledge, this is the first molecular characterisation of adult *T. canis* and *T. cati* in Turkey.

Material and Methods

Microscopic analysis. Adult ascaris worms were collected from faeces of dogs and cats in Van province, Eastern Turkey, during 2015–2016. Nematodes were first washed extensively twice in phosphate buffered saline (PBS) and then were identified by microscopy as *T. canis* or *T. cati*, according to existing keys (12, 15). After that, the parasites were fixed in 70% ethanol and frozen at −20ºC until extraction of genomic DNA.

Molecular analysis. Total genomic parasite DNA was extracted using a DNA extraction reagent kit (the GeneJET Genomic DNA Purification Kit, Thermo Scientific, USA), according to the manufacturer's recommendations. The extracts were kept at −20°C. For molecular diagnosis, species-specific primers were chosen from ITS2 region sequences that had previously been described as Tcan1 (5'-AGTATGATGGGCGC GCCAAT-3') and NC2 (5'-TAGTTTCTTTTCCTCC GCT-3') for *T. canis*, Tcat1 (5'-GGAGAAGT AAACTC-3') and NC2 for *T. cati*, and Tleo1 (5'-CGAACGCTCATATAACGGCATACTC-3') and NC2 for *T. leonina* (8). PCR was carried out in a final volume of 20 µL containing: 7.5 µL of DNase- and RNase-free sterile distilled water (Biobasic, Canada), 5 µL of 5× MyTaq Reaction buffer (Bioline, USA), 1 µL of each primer (20 pmol), 5 µL of template DNA (100–200 ng), and 0.5 µL of TaqDNA polymerase (1.25 IU) (MBI Fermentas, USA). The PCR conditions were as follows: 30 s at 94°C (initial denaturation), 35 cycles of 30 s at 94°C, 30 s at 56°C (*T. leonina*) or 58°C (*T. cati*/*T. canis*), 30 s at 72°C, and finally 5 min at 72°C (final extension). The PCR products were separated on agarose gels (1.5%), stained with ethidium bromide, and visualised and photographed by a UV transilluminator. The PCR products were subjected to capillary electrophoretic separation in a specialised laboratory (Sentegen, Turkey), and sequence analyses of the products were performed. Sequence data were compared with other ascarids available at the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), while alignments and phylogenetic analysis were conducted, using the software MEGA and Bioedit (12).

Results

The collected *Toxocara* nematodes were identified microscopically based on morphological features. The male worms had a posterior end curved ventrally, showing a considerable point at the tail-end which was distinguishable from the straight-tailed female worms (Fig. 1. A and B). Some of the samples showed a lance-shaped cephalic alae, while others revealed very broad cephalic alae (Fig. 1 C and D). In female worms, the eggs were brownish and almost spherical (Fig. 1 E).

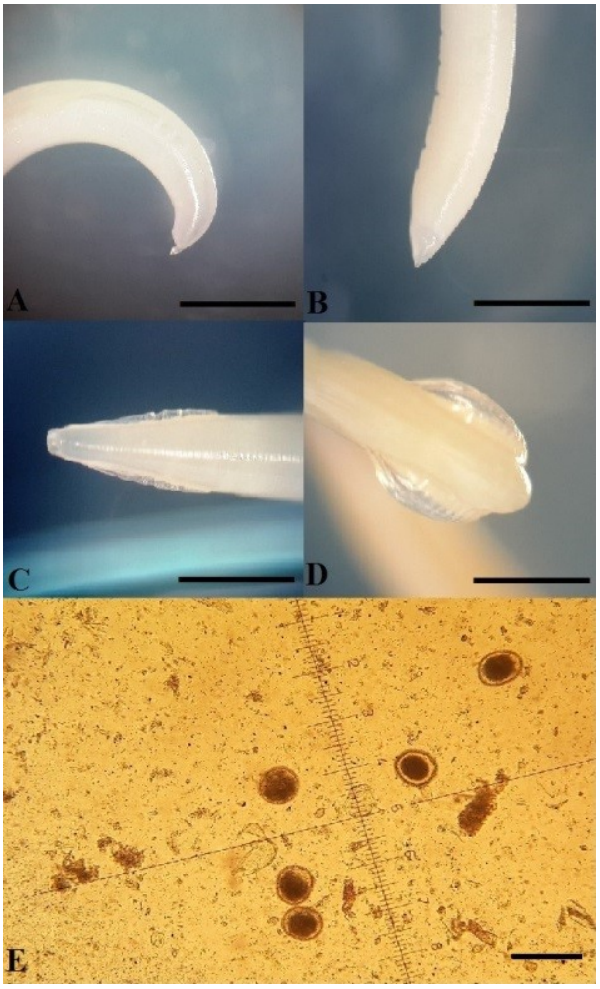

Fig. 1. A – the tail-end of *Toxocara canis* male, B – the tail-end of *Toxocara canis* female, C – the cephalic alae of *T. canis*, D – the cephalic alae of *T. cati*, E – eggs of *Toxocara canis*. A, B, C, D – bars indicate 1,000 µm. E – bars indicate 100 µm

PCR products of about 400 bp were obtained for 17 samples identified as *T. canis* by microscopy and for 3 samples identified as *T. cati* (Fig. 2). Sequencing and alignment of obtained sequences revealed two groups: six identical sequences displaying 100% similarity with a *T. canis* sequence from dogs from Egypt (MG214150) and three identical sequences displaying 100% similarity with a *T. cati* sequence from a cat from Guangdong, China (KY003086). The *Toxocara canis* ITS-2 and *Toxocara cati* sequences obtained were deposited in the GenBank database under accession numbers MH044068–MH044073 and MH043956–MH043958, respectively.

The phylogenetic tree was reconstructed for the five *Toxocara* species (Fig. 3). It can be clearly observed that the *T. canis* and *T. cati* isolates clustered with the respective reference strains of *T. canis* and *T. cati*.

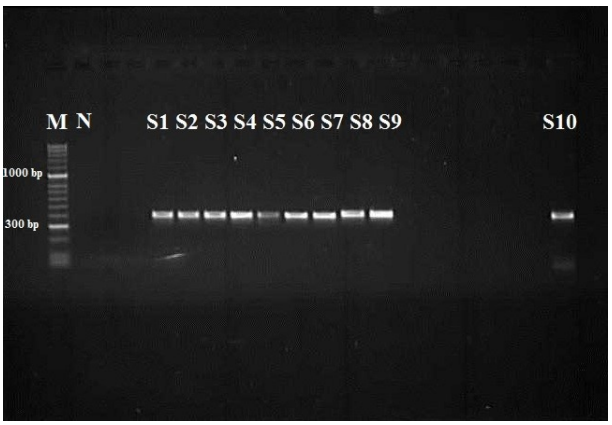

Fig. 2. PCR amplification of ITS-2 (~400 bp) of *Toxocara* genes on 1.5% agarose gel. M – 50 bp DNA marker, N – negative control. Samples from 1 to 7 represent *Toxocara canis*; samples from 8 to 10 represent *Toxocara cati*

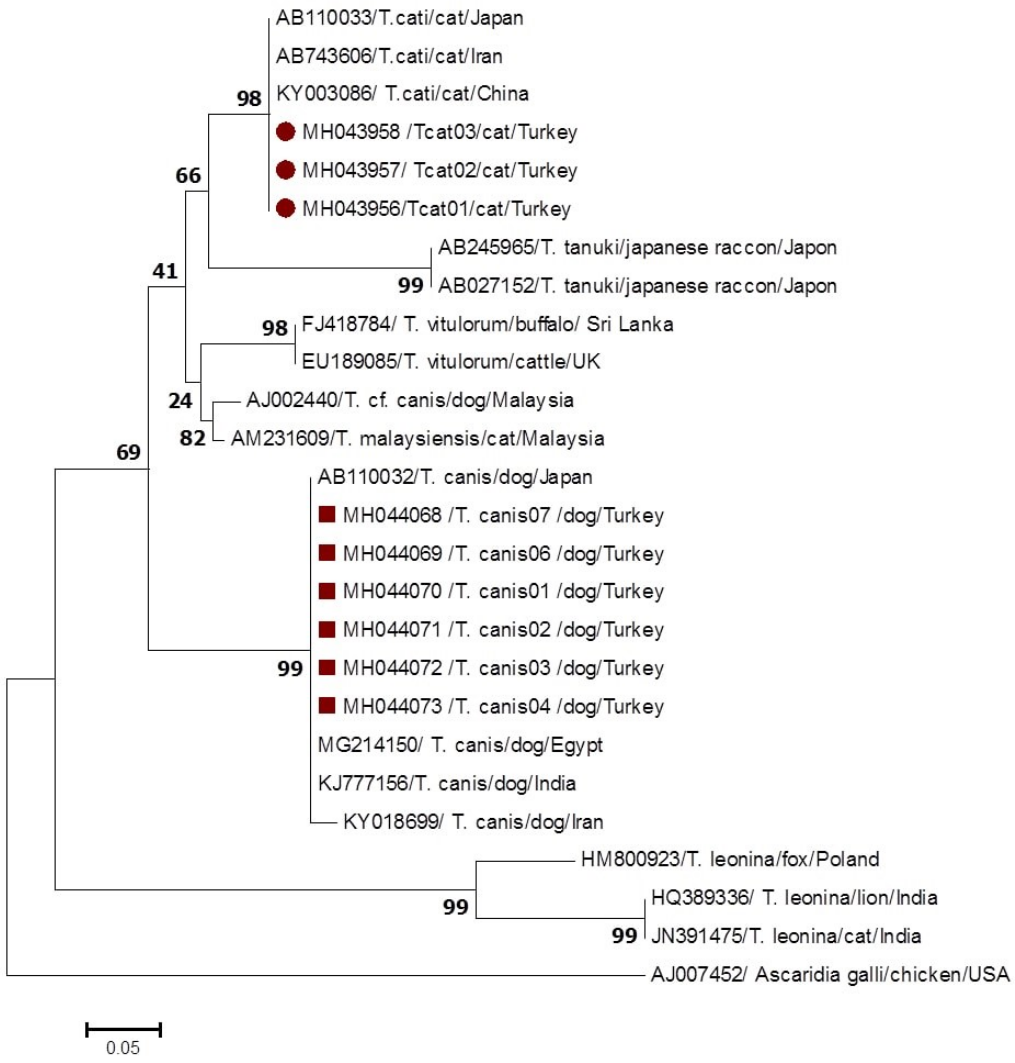

Fig. 3. Neighbour-joining phylogenetic tree (1,000 bootstraps) based on the ITS-2 sequence of ribosomal DNA of *T. canis* and *T. cati* isolated from cats and dogs (marked brown) and other sequences of ascarid retrieved from GenBank. The sequence of *Ascaridia galli* ITS-2 was used as the out-group

Discussion

In previous studies, DNA-based approaches have been applied to identify the nematode species, particularly using ITS fragments (rDNA) (3, 8, 14, 15, 21, 27). The ITS sequences obtained from the present study were compared with those published in GenBank, and the results indicated that the ITS2 sequence of ascarids from dogs was identical to that of *T. canis* published previously (3, 11, 21). The ITS sequence of ascarids from cats was identical to that of *T. cati* (accession numbers KY003086 and AB743606) in GenBank. *T. cati*, *T. canis*, and other members of ascarida formed the basis for the reconstruction of the phylogenetic tree, from which *T. cati* was found to be in the same cluster as the isolates reported from China, Iran, and Japan. The results showed that the roundworm from dogs represented *T. canis* belonging to the *Toxocara* genus. The PCR method is a precise and sensitive method for the detection of ascarid infections in live animals and is based on the principle of detection of DNA originating from the eggs and cells of the parasite. Using ITS-2 sequences as a genetic marker for the PCR approach established in the present study provides positive distinction of *T. cati* from *T. canis*.

Toxocariasis in human is one of the most common helminth infections in the world. The risk of infection in poor people is high (7). The identification and differentiation of *T. cati* and *T. canis* in the past was based on morphological characteristics (17), a not completely satisfactory method because of the difficulties in identifying and discriminating *T. cati*, *T. loenina*, and *T. canis* by morphology, particularly at egg stages. *T. canis* and *T. cati* eggs cannot be distinguished precisely, using microscopic techniques (24). Accordingly, there is a requirement for a molecular approach to detect and accurately identify both ascaridoid nematodes and eggs in the environment.

Having used PCR diagnosis for the Ascarididae family nematodes and different primer pairs targeting various genes for discrimination of the species, He *et al.* (6) reported that cox1 gene sequencing is enough to accurately distinguish and identify *T. cati*. Similarly, in a study performed in Iran on the ascaridoid nematodes, Mikaeli *et al*. (18) noted that mitochondrial gene sequences could be used confidently for the identification of the parasite. In another study, Pawar *et al.* (21) used a DNA-based method to amplify ITS-2, a more appropriate and valid gene region to differentiate *Toxascaris leonina* and *Toxocara cati* from each other. Various mitochondrial and nuclear genes have been efficiently applied in previous studies in the differentiation of other ascarid species. To illustrate, Luo *et al.* (16) investigated genetic characterisation of three mitochondrial genes cox1, cox2, and nad1 in their isolates and confirmed that all the parasites belonged to *Ascaris suum*. In a study on the identification of the characteristics of the ITS gene in *A. suum* using PCR, the sequence homology was 98.4%–99.9%, which is identical with pig isolates reported previously (13). In the present study, the ribosomal ITS-2 gene region was also the clear optimal choice to identify and distinguish the two nematodes accurately. In addition, the ITS-2 sequences showed close resemblance with other *T. canis* and *T. cati* in different parts of the world. The ITS2 rDNA gene should be considered to have a high degree of specificity.

The highly species-specific PCR test for *T. canis* and *T. cati* detailed here should enable more accurate diagnosis than the detection of the worms by microscopy. This study is also the first demonstration of the existence of adult ascaridoid nematodes in cats and dogs in Turkey by the genetic approach using ITS-2 rDNA as a genetic marker.

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