Detection and identification of *Toxocara canis* in infected dogs using PCR

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**Article info**

Received September 26, 2018
Accepted December 18, 2018

**Summary**

Faecal samples were collected from 224 dogs (47 villages) in Ankara. *Toxocara* spp. eggs were diagnosed in faeces using centrifugal flotation and sedimentation methods. A total of 21 dogs (9.38 %) were positive for *Toxocara* spp. eggs. In this study, we used the PCR technique that, in combination with DNA sequencing, allows the detection and identification of *T. canis* eggs in faeces of infected dogs. For this purpose, the ATPase subunit-6 gene (mtDNA) was selected as a target for the amplification of *T. canis*. The primers were used to amplify 217 bp region. Amongst 21 coproscopically detected *Toxocara* isolates from dogs, 5 (23.8 %) samples were PCR-positive for *T. canis*, and the remaining 16 samples were PCR-negative. Results indicate that PCR can detect *Toxocara canis* DNA in faeces of infected dogs, but efficacy was low when compare to sedimentation/flotation. PCR is additional test for diagnosing of this infection. But, the difficulties of identification based on PCR in faecal examinations need to be investigated further.

**Keywords**: *Toxocara canis*; dog; faecal examination; ATPase subunit-6

**Introduction**

Toxocariasis is a zoonosis with worldwide distribution caused by *Toxocara* species of dogs and cats. VLM in humans occurs primarily because of the ingestion of infective eggs (Macpherson, 2013; Strube et al., 2013). The ways of transmission to humans are as follows; soil and sandpits contamination in children, geophagia (Overgaauw & Nederland, 1997; Macpherson, 2005; Bowman, 2009), ingestion of eggs contain infective larvae from dog’s coat (Amaral et al., 2010; Macpherson, 2013; Öge et al., 2014), consumption of unwashed raw vegetables or fruits (Kozan et al., 2005; Lee et al., 2010), consumption of raw or undercooked meat containing arrested infective larvae in paratenic host (Lee et al., 2010; Macpherson, 2013; Strube et al., 2013), low socio-economic level and failure to regularly pick up and dispose of faeces (Overgaauw & Nederland, 1997; Robertson & Thompson, 2002). In different countries, the prevalence of *T. canis* ranged between 4.4 % and 33.8 % in dogs (Habluetzel et al., 2003; Sager et al., 2006; Sowemimo, 2007; Claerebout et al., 2009; Soriano et al., 2010). The prevalence of *T. canis* varied from 4.2 % to 47.8 % in Turkey (Yıldırım et al., 2007; Kozan et al., 2007; Ünlü & Eren, 2010; Çiçek & Yılmaz, 2012). In dogs, routine diagnosis relies mainly on detection of eggs of the parasite in faeces. However, *T. canis* and *T. cati* are not to be clearly distinguishable by microscopy and serological diagnostic methods. The accurate identification of these species and differentiation from each other have an important role for investigating their life-cycles, epidemiology and specific diagnosis of toxocariasis. The seroprevalence of human toxocariasis varies from 2.4 % to 92.8 % (Rubinsky-Elefant et al., 2010). In Turkey, the prevalence was found to be 7.6 – 26.42 % in recent years (Kustimur et al., 2007; Karadam et al., 2008; Akdemir, 2010; Çiçek & Yılmaz, 2012).

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PCR is used for rapid and specific diagnosis, because of their ability to specifically amplify DNA from nematode eggs and thinly sections of larvae or adult stages (Gasser, 2013; Smith et al., 2009). Primer design is the key step in PCR for the identification of parasites. ITS-1 and ITS-2 of nuclear rDNA sequences have been demonstrated to provide reliable genetic markers for the identification and differentiation of species of Toxocara and related nematodes (Jacobs et al., 1997; Zhu et al., 1998; Li et al., 2007). In addition to nuclear ITS-1 and ITS-2 rDNA sequences, recent studies have shown that mtDNA useful alternative genetic markers for investigating of parasitic nematodes (Gasser, 2013). Various mitochondrial DNA regions have been employed for studying the population genetics of parasitic nematodes (Li et al., 2008; Wickramasinghe et al., 2009; Gasser, 2013). However, there is still limited information on the mt genomes of socioeconomically important Toxocara parasites. Therefore, there is need a suitable DNA target region (genetic marker) for the accurate identification of T. canis by PCR technique.

In addition to conventional methods, this coprological study was undertaken to detection and identification of Toxocara canis in infected dogs using PCR in the region of Ankara.

Materials and Methods

Sample collection and faecal analysis

Faecal samples taken from 224 dogs in 47 villages were investigated for Toxocara eggs. Age and sex of the dog were determined. Faecal samples were collected from dogs either within the area accessed by free or tethered dogs. For safety reasons, samples were frozen at -80°C for 10 days before examination. Faecal samples were examined by sedimentation-formalin-ethyl acetate and centrifugal flotation with ZnSO₄-solution (Truant et al., 1981). Eggs from all the Toxocara positive samples detected by flotation/sedimentation were examined by one step PCR to determine the T. canis. To concentrate eggs, faecal samples were prepared with improved flotation method (Szell et al., 2014). When Toxocara-type eggs were detected microscopically, 1 ml of the upper part of the flotation from the centrifuge tube was transferred to the 15 ml falcon tube. This step was repeated 4 times. The tube was filled with water and centrifuged at 2000 x g 10 min. The supernatant was discarded, the sediment resuspended in 1 ml water, transferred to a 2ml micro tube and these sediment was used for DNA purification.

DNA isolation and PCR

For the PCR, Toxocara egg DNA was extracted from faeces by QIAmp DNA Stool Mini kit (Qiagen), according to the manufacturer’s instructions with the following modifications: The samples (sediment above defined) were subjected to 95°C, 30 min in Buffer ASL, and proteinase K digestion was performed 70°C, 30 min. Final dilution of DNA were made in 80 µl of elution buffer and stored at -20°C until using. The concentration of DNA in each sample was measured by a spectrophotometer (Thermoscientific Nanodrop-ND 2000) for qualitative and quantitative analyses. The isolates of eggs and adult of T. canis prepared in Özbakış’ work (2015) were used for positive control sample. Also, distilled water was used as a negative-control.

A forward (T.canis ATP-F1: GTTTGTTGTGGTTGGGGCTA) and reverse (T.canis ATP-R1: CCAAAGGACGAGAAACCTCA) primer were used to amplify a 217 bp region of the ATP synthase subunit 6 gene of T. canis (Özbakış, 2015). PCR was carried out in a 30 µl total volume mix containing 10×Taq buffer (1.25 Ml including (NH₄)₂SO₄), 25 mmol/L of MgCl₂, 10mM of dNTP mix, 5U/µl Taq DNA polymerase (Fermentas, Waltham, MA, USA), water (18Mo-hm-cm, AppliChem, Darmstadt, Germany), 10 pmol/µl primers and 10 µl of template DNA. The reaction conditions were: 5 min at 94 °C, followed by 34 cycles of 30 s denaturation at 94 °C, 1 min annealing at 50 °C, 1 min extension at 72 °C and 10 min final extension at 72°C, using a thermal cycler (PX2 Thermo, USA). Amplicons were detected on a 3% agarose TAE gel with ethidium bromide-stained. It was visualized under the UV light with gel imaging system (NDR Bio-Imaging systems Mini Bis Pro).

Sequencing analysis

Toxocara egg products in 3 of PCR positive dogs were subjected to DNA sequencing. Subsequently, amplicons were clean-upped by High Pure PCR Clean-up microcit (Roche, Germany). Nucleotide sequence analysis was performed by Sentegen Biotechnolo-
gy in Ankara and undertaken by BLAST algorithms and databases from the National Centre for Biotechnology (http://www.ncbi.nlm.nih.gov).

Phylogenetic analysis was performed in the Mega software (version 6.0) (Tamura et al., 2013). The tree was constructed using neighbour-joining method (Saitou & Nei, 1987) based on Kimura 2-parameter model (Kimura, 1980) in the software package program. Bootstrap resampling was calculated from 1000 pseudo replicates with random seeds (Felsenstein, 1985).

**Ethical Approval and/or Informed Consent**

For this study formal consent is not required.

**Results and Discussion**

Toxocara eggs were detected in 21 of 224 dogs (9.38%). Toxocara canis was identified in 5 (23.8%) of the 21 Toxocara egg-positive samples by PCR (Fig. 1). But, efficacy of PCR was low when compare to sedimentation/flotation. The conventional parasitological examination is routinely used for diagnosis of toxocariasis in field. PCR has used for identification and differentiation of Toxocara species. The difference between T. canis and T. cati in ITS-1 and/or ITS-2 of nuclear ribosomal DNA (rDNA) have been demonstrated by various authors (Jacobs et al., 1997; Zhu et al., 1998; Li et al., 2007; Borecka et al., 2008; Wickramasinghe et al., 2009; Fahrion et al., 2011). Recent studies have shown that sequences derived from the mtDNA genes provide alternative genetic marker for investigating genetic structures, systematics and phylogeny of parasitic nematodes (Wickramasinghe et al., 2009; Gasser, 2013).

Mitochondrial DNA (mtDNA) markers can be used for investigating the taxonomy and genetics of Toxocara species. Conserved primers can be rationally and selectively designed in mitochondrial genome. Wickramasinghe et al. (2009) reported that the mitochondrial ATPase 6 genes were well conserved in Toxocara species and can be used for discrimination of species and for molecular phylogenetic. The important finding in this study was that only 5 out of 21 microscopically positive samples were PCR-positive T.canis. The other 16 dogs that were PCR negative were microscopically positive for Toxocara eggs. PCR efficacy depends on the number of eggs in faeces. PCR may not be able to detect DNA of ascarids as a result of low DNA concentration. We detected that the number of eggs in these faecal samples (epg) was very low in dogs (< 50). Toxocara species have the host specificity, i.e. T. cati for felids and T. canis for canids. But, Roth & Schneider (1971) reported the findings of T. canis adults in the intestines of dissected cats. Some studies have suggested that coprophagy in dogs may be responsible for finding eggs of dog-typical (Sager et al., 2006) as well as dog-atypical (Fahrion et al., 2011). Dogs may consume their own faeces, faeces of other dogs and/or faeces of other species (Nijss et al., 2014). Looking at the PCR results, in some dogs might have T. cati parasites instead of T. canis. But, this situation generally can not be explanation for the low PCR efficacy. That’s why the result of faecal examination must be interpreted with carefully. The development of molecular diagnostic tests for identifying T. canis is important. Mainly T. cati resemble T. canis very closely in routine microscopic diagnosis and this may be lead to miss-identification. Toxocara cati might play a role in human toxocariasis than estimated rate, as there is no difference in the zoonotic potential of T. canis and T. cati (Oge et al., 2014). The phylogenetic tree based on ATPase subunit-6 gene sequencess was able to distinguish between ascarid nematode samples and was used a Haemonchus contortus for an out group (Fig. 2). 244-407 bp were used in reference to FJ418787 accession number of T. canis gene data compared to phylogenetic tree. When the sequences data were compared with obtained T. canis sequences from GenBank database (Access. no: KJ777173, KJ777174, FJ418787, EU730761, JN593098) on ATPase subunit 6 gene between 98.2 % and 99.4 % homology exhibited. The identity between our T.canis samples and the reference T.canis samples was

![Analysis of PCR products amplified of T. canis from faecal samples by agarose gel electrophoresis.](image)

**Fig. 1.** Analysis of PCR products amplified of T. canis from faecal samples by agarose gel electrophoresis.

M: Marker, PK: Positive control, NK: no DNA control, 1 - 21: Dog isolates
low (98.2 %) considering it was such a small fragment (164 bp). Nucleotide sequence identities with each other of our samples (11, 12 and 2 number dog isolates) were found 100 %, 100 % and 94.4 %, respectively.

Based on faecal analysis, Toxocara spp. eggs were found in the faeces of 9.38 % of investigated dogs. The low prevalence of Toxocara infection in dogs in these villages could be attributed to the fact that the majority of dogs which were older than 2 years when looked at the raw data. Toxocara egg has been found in both young (5/43-11.62 %) and adult (16/181-8.83 %) animals. Adult dogs may still pose a risk to human health as they are susceptible to Toxocara infection.

The potential role of Toxocara parasites in human toxocariasis should not be ignored or underestimated. The presence of Toxocara spp. eggs was found in the soil, raw vegetables and dogs’ faeces and hair in Ankara (Table 1). These situations are significant as the eggs have the potential to develop to infective larval stage and responsible for most VLM cases in humans. In the current study, the prevalence of Toxocara eggs in faeces is not high when compared with similar studies is not high when compared with similar studies (Öncel, 2004; Orhun & Ayaz, 2006; Kozan et al., 2007; Ünlü & Eren, 2007; Balkaya & Avcıoğlu, 2011) but may be important.

The findings and considerations presented here indicate that we may have an imprecise image of true prevalence of patent infections with Toxocara spp. in dogs. The difficulties of identification based on PCR in faecal examinations need to be investigated further.

Conflict of Interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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