

# APPLICATION OF PCR METHOD FOR DETECTION AND SPECIES IDENTIFICATION OF TOXOCARA SPP.

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## ABSTRACT

The ascaridoid nematodes of dogs and cats *T. canis* and *T. cati* have wide distribution and are causative agents of Toxocariasis - a disease in human and animals. Human disease has several clinical forms with different clinical manifestations such as visceral, ocular, neuro and covert toxocariasis. The morphological methods used to differentiate the two species, especially to identify their eggs or larvae, may lead to inaccurate diagnosis. This requires the use of more reliable methods, such as PCR, for identification of *Toxocara* species.

The aim of our research was to develop an in-house PCR method for species identification of *Toxocara* and to determine its applicability on different parasites stages.

We applied the method used by Khademvatan et al. (2013), with some modifications for detection of different forms of *Toxocara* - eggs, larvae and adult parasites. We used species-specific oligonucleotide primers from the ITS2 gene sequence of the ribosomal DNA - Tcan1/NC2 for *T. canis* and Tcat1/NC2 for *T. cati*.

The presence of a band with the size of 380 bp, specific for *T. canis*, was found for all stages of the studied parasite.

The described method will allow species differentiation of Toxocariasis causative agents and improve the diagnosis of the disease, as well as

determine the actual spread and reservoirs of these parasites.

**Keywords:** *Toxocara* spp., PCR, ITS-2, molecular identification.

## INTRODUCTION

*Toxocara* are ascaridoid roundworms that are important for both animal and human health. The main representatives of these parasitic helminths are *T. canis* and *T. cati*, and the adult worms of the two species inhabit the lumen of the small intestine of the definitive host, dog and cat, respectively. Humans can be infected via accidental ingestion of embryonated eggs present in the soil or contaminated food, as well as by ingestion of encapsulated larvae present in improperly cooked tissues of paratenic hosts (1, 2). In the human body larvae can not develop to mature adult worms, they migrate via the bloodstream and are localized in different organs (muscle, liver, brain and eyes), which causes the development of various clinical symptoms. The following clinical forms have been described: visceral toxocariasis (3), ocular toxocariasis (OLM), (4), neurotoxocariasis (5), covert or common toxocariasis (CT), (6), and asymptomatic form (7).

The determination of the species specificity of *Toxocara* larvae from animal and human tissues is very difficult because of their small size and few morphological features that can be used for identification (8). Ascaridoid nematodes are usually identified on the basis of their morphological characteristics and predilection sites within hosts, but there are limitations in traditional methods, especially with regard to their stages - eggs and larvae. To overcome the limitations of traditional (morphology-based) determination, various molecular methods based on ribosomal and mitochondrial markers - both for identification of *Toxocara* spp., have been developed (9). Various studies have shown that the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA) is useful genetic marker for accurate identification of different genetic groups (9, 10). To distinguish *T. canis* and *T. cati* from closely related species the high genetic variability inside the molecular markers, such as the internal transcribed spacer 2 (ITS-2), is used (10, 11).

The aim of the present study was to develop an in-house polymerase chain reaction (PCR) method for the identification of *Toxocara* species and to deter-

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mine its applicability to various parasite stages – mature forms, larvae and eggs.

## MATERIAL AND METHODS

### Parasites

Adult parasites of *Toxocara* spp. were submitted for determination to the Department of Parasitology and Tropical Medicine, National Center of Infectious and Parasitic Diseases (NCIPD) by a veterinary clinic in Sofia. One part of the adult *Toxocara* spp. worms were homogenized in phosphate-buffered saline (PBS, pH 7.2) and kept frozen at -20° C until the PCR reaction was performed.

From another part of the adult parasites (female), *Toxocara* spp. eggs were collected using a scalpel, and washed by centrifugation three times with Tyrode's solution (NaCl - 8 g, KCl - 0.2 g, CaCl<sub>2</sub> anhydrous - 0.2 g, MgCl<sub>2</sub> · 6 H<sub>2</sub>O - 0.05 g, glucose - 2 g, NaHCO<sub>3</sub> - 1 g, distilled water up to 1 l). Portions of the washed parasitic eggs were frozen at -20° C until PCR was performed and the rest were incubated for 1 month to obtain *Toxocara* larvae.

### Cultivation

*Toxocara* spp. eggs were incubated in petri dishes for 1 month in 1% formalin solution at room temperature. Their status was monitored every 72 hours under a microscope to check the state of the eggs and the development of the larvae within them. At the end of the period, the eggs were washed three times with Tyrode's solution and centrifuged at 1000 rpm for three minutes. The protein shell of the *Toxocara* eggs was partially lysed with NaOCL solution and water (in a ratio of 1:1 for 12 h) at room temperature (12). The resulting suspension was washed again at the same speed with Tyrode's solution to obtain a neutral pH. The larvae collected in this way were distributed in mattresses for cultivation in DMEM (Gibco) media (prepared according to the manufacturer's instructions) under anaerobic conditions / 37° C (method described by de Savigny et al. (1975),(13). The vitality of the larvae was monitored daily, and at the end of the first week the nutrient medium was replaced with a new one. After a fortnight incubation, the medium was removed and the sediment containing *Toxocara* larvae was collected and frozen at -20° C until use.

### Isolation of DNA and PCR

DNA from mature forms, parasitic eggs and larvae of *Toxocara* spp. was extracted using a commercial QIAamp DNA Kit (Qiagen), following the manufactur-

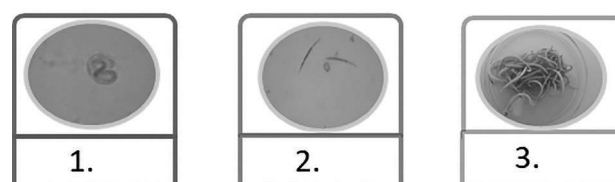
er's instructions. Samples were initially subjected to 3 freeze-thaw cycles for 20 min. Proteinase K digestion was performed overnight at 55°C under continuous shaking conditions (14) on Thermomixer compact (Eppendorf) at 450 rpm.

PCR conditions and primer sets were as previously described (14, 15). Briefly, primers hybridize to the internal transcribed spacer 2 (ITS2) gene and were designated as Tcan1 (5'-AGTATGATGGGCGCGCCAAT-3') and NC2 (5'-TAGTTTCTTTTCTCCGCT-3') for *Toxocara canis* and Tcat1 (5'GGAGAAGTAAACTC-3') and NC2 for *Toxocara cati* (14, 15, 16).

PCR was performed in a final volume of 25 µL of the reaction mixture containing 2.5 µL of 10x PCR buffer, 0.2 mM of each dATP, dTTP, dCTP and dGTP, 25 pmol of each primer, 3 mM MgCl<sub>2</sub>, 1.25 U of Taq DNA polymerase (Fermentas) and 20 ng of template DNA. PCR amplification was done on the GeneAmp PCR System 2700 Amplification Apparatus (Applied Biosystems) under the following conditions: an initial cycle at 94°C for 30 s; followed by 35 cycles including denaturation at 94°C for 60 s; hybridization at 58°C for 30 s and extension at 72°C for 30 s; as well as 1 final extension cycle at 72°C for 10 min. Upon completion of amplification, 20 µl of the PCR products were added dropwise to a 1.8% agarose gel containing ethidium bromide in 1x Tris-acetic acid-EDTA buffer (Fermentas),(17).

## RESULTS

PCR amplification follows the one described by Khademvatan et al. (2013), which we performed with some modifications, presented in detail in Materials and Methods section. The method was developed for the study of parasitic eggs in the environmental fecal samples of stray cats (17), which we applied to the three stages of the parasite - adult forms, eggs and larvae (Fig. 1).



**Figure 1.** *Toxocara* - eggs (1), larvae (2) and adult worms (3), tested with the developed PCR method.

### PCR of *Toxocara* - eggs

The applied PCR assay using primers Tcan1/NC2 and Tcat1/NC2 in the study of parasitic eggs obtained

from adult female forms by the method described by Khademvatan et al. (2013), revealed the presence of bands of about 380 bp in length, specific for *T. canis* (lines 3, 4, 5 in Figure 2, DNA isolated from *Toxocara* eggs).

To determine the sensitivity of the reaction, DNA was extracted from aliquots of different amounts of parasitic eggs - a sample containing 8 eggs (line 3), a sample with 4 parasitic eggs (line 4) and with only 1 *Toxocara* egg (line 5). Electrophoretic analysis of PCR products showed the presence of a product of approximately 380 bp specific for *T. canis* in all samples containing parasite eggs.

The results showed that PCR was able to detect the presence of *T. canis*-specific DNA in all tested dilutions of the parasitic egg suspension, including the one egg-suspension.

#### PCR of *Toxocara* – larvae

Examination of parasitic larvae obtained after *in vitro* cultivation of *Toxocara* eggs in the applied PCR method showed the presence of the same specific bands (380 bp) found in the test of eggs characteristic of *Toxocara canis* (lines 6, 7 and 8 in Figure 2). The presence of the characteristic band was observed in the reaction mixtures containing 8 (line 6), 4 parasitic larvae (line 7) and a single larva (line 8).

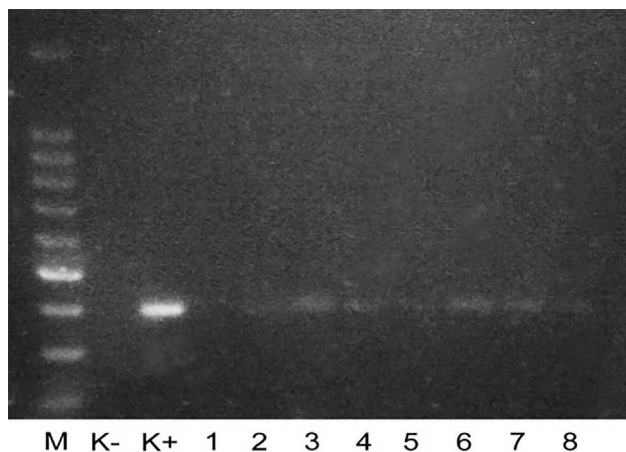
#### PCR of adult *Toxocara* worms

To overcome the strong cuticle sheath of the adult forms of *Toxocara*, two methods of pretreatment were used before the PCR reaction: 1) freezing and thawing at -70° C three times for 20 min and 2) sonication for 3 min.

When the first method (triple freezing and thawing) was applied to the adult forms of the parasites a sample from marital forms examination a very weak band on the agarose gel appeared following electrophoresis in (line 2). Processing the mature forms by ultrasonic disruption resulted in a lack of specific bands (line 1), indicating the need to apply additional processing methods in order to retrieve a larger amount of DNA, and to optimize amplification conditions.

The optimized by us PCR protocol showed for all studied *Toxocara* stages PCR products of approximately 380 bp in length, which identified parasites as *T. canis*.

No amplification was observed in the negative control (Fig. 2).



**Figure 2.** Agarose gel electrophoresis on 1.8% gel of ITS-2-PCR products from *Toxocara* isolates. M, marker (100 bp DNA Ladder); K(-), negative control; line 3, K(+), positive control for *T. canis*; Lines 1, 2 *Toxocara* adult forms; lines 3-5, *Toxocara* eggs; lines 6-8, *Toxocara* larvae, products ~ 380 bp in length.

#### DISCUSSION

*T. canis* and *T. cati* are species of human and animal health significance. Besides them, other Ascaridoid nematodes, such as *T. malaysiensis* and *Toxascaris leonine* cause gastrointestinal infections in mammals of the families Canidae and Felidae (18) and their zoonotic potential is still unclear. To overcome the limitations of traditional (morphology-based) taxonomy various molecular methods based on ribosomal and mitochondrial markers for identification of *Toxocara* spp. and diagnosis of toxocariasis have been developed (9), including conventional and real-time PCR techniques for amplification of different targets (11, 16, 19).

Sequences of ITS1 and ITS2 rDNA regions were demonstrated to be reliable markers for distinguishing *T. canis*, *T. cati*, *T. malaysiensis*, and *Toxascaris leonine* (16, 20). ITS-2 sequence-based analyses were found useful for differentiation between adult worms of *T. canis*, *T. cati*, and *Toxascaris leonina*, as well as other ascaridoids that can be found in human tissues (first demonstrated by Jacobs et al. (16)). Molecular techniques for diagnosis of toxocariasis in bronchoalveolar lavage have been developed in experimentally *Toxocara* spp. infected mice, in order to improve the diagnosis in patients with pulmonsnary signs and symptoms (21). Data published on toxocariasis show a widespread prevalence of this helminthosis, which is still a neglected and poorly evaluated problem (22). Our studies among healthy individuals in Bulgaria show 8.0% prevalence of

Toxocariasis found in ELISA, and and 4.0% in WB (23). Studies on the prevalence of *T. canis* in animals in our country show that this is one of the most common infestations in dogs (12%) (24), especially in small puppies. The exact determination of environmental contamination with the *Toxocara* eggs is important for effective prevention and control of the disease. However, distinguishing ascarid eggs of different species based only on microscopic examination is difficult, due to their similar morphology and size. In addition, invasions in cats with adult *T. canis* have been reported (25). Khademvatan et al. (2013) found *T. canis* eggs in 4 (6.34%) of 63 *Toxocara*-positive fecal samples from stray cats (17), and Fahrion et al. (2011) showed that dogs shed significant amounts of *T. cati* eggs, probably as a result of coprophagia (26). Therefore, the need for more sensitive and specific techniques to overcome the above mentioned disadvantages is obvious.

In our study, PCR based on amplification of the ITS-2 gene was used for identification of *Toxocara* eggs, larvae, and marital forms to species level. The methodology was applied by Khademvatan et al. (2013) for the characterization of *Toxocara spp.* eggs in feces of stray cats (17). With some modifications and optimization in our conditions, it was applied for species determination of *Toxocara* at all stages - eggs, larvae, and adult forms of the parasite. In all cases, the species *T. canis* was distinguished by the characteristic band of approximately 380 bp in length. The electrophoresis following PCR reaction on the adult forms of the parasite visualized a very weak band, indicating the need for additional sample processing and optimization of the amplification conditions in order to retrieve a larger amount of DNA. Our results show that the PCR method applied and optimized by us can be used for species identification of different forms of *Toxocara* parasites found in tissues of paratenic hosts, including humans. It can be also used for confirmatory diagnosis of toxocariasis at the species level, as well as for determining the causative agents of environmental pollution (soils, sands and sludge sample).

## CONCLUSION

Toxocariasis remains one of the most widespread and economically important zoonoses. The parasites of genus *Toxocara* have an exceptional capacity to survive for a long time in the environment and in the

host, and are adapted to a number of domestic and wild definitive and paratenic hosts.

The limitations of morphological methods for determination of *Toxocara* species require the use of more specific methods. The present work is a pilot first in the country examination of the applicability of PCR technique for species-specific identification of the eggs and larvae of *T. canis* and *T. cati*. This molecular assay could be a useful tool not only for diagnosis of toxocariasis in humans and animals, but also for contamination assessment of environmental samples such as sand, soil, as well as wastewater treatment plant (WWTP) sludge.

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