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Utilizing a polymerase chain reaction method for the detection of *Toxocara canis* and *T. cati* eggs in soil

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Abstract

A polymerase chain reaction (PCR) technique has been used for the differentiation of *T. canis* and *T. cati* eggs isolated from soil and previously identified from microscopical observations. The method, using specific primers for the identification of the two *Toxocara* species, was assessed in both the field and laboratory. Successful results were obtained when only a single or large numbers of eggs were recovered from 40 g soil samples. The method is sensitive, allows analysis of material independent of the stage of egg development and can be adapted for the recovery of other species of parasites from soil.

Introduction

As the populations of dogs and cats increase, soil contamination with eggs of *Toxocara* spp. is increasing. Infective eggs of *Toxocara* spp. are detected in the soil in public and private locations of city backyards, playgrounds, streets, sandpits and so on, regardless of the season of the year (Mizgajska, 2001). A relationship between the prevalence of toxocariasis amongst children and the degree of soil contamination with *Toxocara* eggs was observed in Poland (Mizgajska, 2001; Mizgajska *et al.*, 2002; Pawłowski & Mizgajska, 2002). Human toxocariasis is caused by either *T. canis* or *T. cati* and to date it has not been established which of the two species is more important in human infections, as there are no routine procedures for distinguishing the two species of nematode.

Furthermore, it is not known which of the species *T. canis* or *T. cati* is more frequently present in soil because the eggs are similar and their differentiation based on morphological features is not conclusive. The present study aims to explore a molecular method for identifying species of *Toxocara* in eggs isolated from soil based on the polymerase chain reaction (PCR) with specific primers constructed by Jacobs *et al.* (1997). The microscopic observations undertaken during routine determination of the degree of soil contamination with geohelminth eggs were confirmed with molecular results. This approach provides an insight into whether or not both *T. canis* and *T. cati* are present in the soil. This method will therefore assist in targeting preventive measures to control *Toxocara* transmission and account for the differences in the behaviour of dogs and cats, the definitive hosts of *Toxocara.*

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Materials and methods

Egg samples

Five soil samples with *Toxocara* eggs were analysed. Two of them were prepared by squeezing eggs from the final part of the uterus of mature worms of *T. canis* from dogs. Samples contained 430 and 10 eggs with blastomers, respectively. Eggs were kept in water for several days. Three other samples contained 1, 1 and 2 eggs with second larval (L2) stages and were isolated by flotation from contaminated soil samples (Mizgajska-Wiktor, 2005) Eggs were measured and identified by morphological features described by Mizgajska & Rejmenciak (1997).

Releasing embryos from eggs

To obtain material for genetic analysis, eggs were crushed by applying pressure to a cover slip on a microscope slide with the fingers. Freed embryos from mechanically destroyed egg shells (fig. 1) were rinsed from the slide and cover slip with distilled water onto a nitrocellulose membrane (Millipore) (Graczyk *et al.*, 1997)

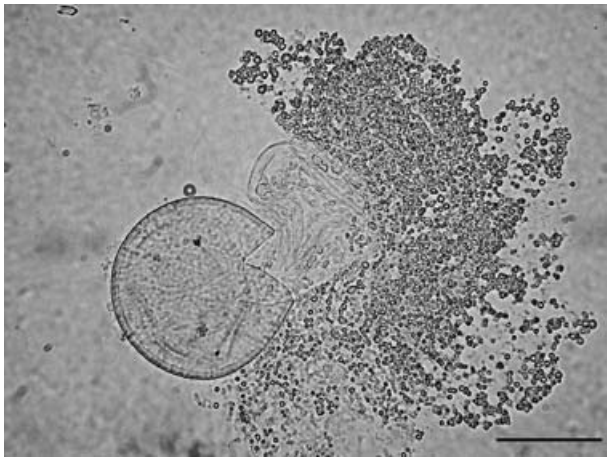

Fig. 1. The developing embryo released from the eggshell of *Toxocara* species (bar = 50 μm).

using a vacuum pump. The membrane was then placed in an Eppendorf tube, dissolved with acetone for 2 min, and the tube centrifuged twice for 20 min at 7000 rpm. The pellet was used for the isolation and purification of DNA.

Isolation and purification of genomic DNA and PCR analyses

The isolation and purification was performed using QIAamp DNA Mini Kit (Qiagen). PCR was performed using specific primers for internal transcribed spacer (ITS-2) of the ribosomal DNA of species of *T. canis* and *T. cati* constructed by Jacobs *et al.* (1997) namely:

Tcan (5′AGTATGATGGGCGCGCCAAT3′),
Tcat (5′GGAGAAGTAAGATCGTGGCACGCGT3′),
NC2 (5′TTAGTTTCTTTTCCTCCGCT3′).

For amplification of DNA the following reagents were used: DyNAzyme II Polymerase (Finnzymes) 1U per 25 μl, reaction buffer 1 × concentrated (10 mM Tris-HCL, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), dNTP 200 μM, primers 1 μM. The reaction was carried out under the following conditions: 94°C, 5 min (denaturation), 55°C, 30 s, 72°C, 30 s, 94°C, 30 s, 55°C, 30 s, (annealing), 72°C, 7 min (extension) and for 30 cycles (Mastercycler Eppendorf).

As each of the two species possesses a pair of specific primers, i.e. *T. canis* hasTcan/NC2 and *T. cati* Tcat/NC2, each sample was analysed twice (Tcan/NC2 and Tcat/NC2) to identify the species. PCR products were separated on a 2% agarose gel and stained with ethidium bromide, transilluminated and photographed.

Sequencing of rDNA

Amplified DNA fragments in the PCR were cloned to the plasmid vector pGEM®-T Easy (according to the protocol) and then sequencing was performed commercially by the Institute of Biochemistry and Biophysics of Polish Academy of Science in Warsaw. Sequence alignments were performed using BLAST® (NCBI).

Table 1. Microscopic and PCR determination of *Toxocara* species in soil samples.

Sample	No. of eggs	Microscopic results	PCR results	
			Tcat/NC2	Tcan/NC2
1	1	*T. canis*	−	+
2	1	*T. cati*	+	−
3	2	*T. canis, T. cati*	+	+
4*	10	*T. canis*	−	+
5*	430	*T. canis*	−	+

+, Band observed; −, band not observed; *samples from uterus.

Results

Of four *Toxocara* spp. eggs isolated from soil samples and morphologically identified, two eggs were recognized as *T. canis* and two as eggs of *T. cati* and this was confirmed by PCR analyses (table 1).

During the electrophoretic analysis of PCR products, bands were observed in the sample with the egg containing the L2 and in the sample with the embryonated egg at the blastula stage. Primers Tcan/NC2 amplified a PCR product of *c*. 380 bp from *T. canis* but not from *T. cati* and primers Tcat/NC2 amplified a product of *c*. 370 bp from *T. cati* but not from *T. canis*. The product for *T. canis* was present in three samples only (fig. 2, lanes 3, 5, 7), and the product for *T. cati* present in one sample (fig. 2, lane 1). There was only one sample with products for both *T. canis* and *T. cati* (fig. 3, lanes 1, 2 for *T. canis* and lanes 3, 4 for *T. cati*).

Sequence data demonstrated a 100% correspondence of *T. canis* sequence to that described by Jacobs *et al.* (1997) (Genebank accession number: Y09489) and Ishiwata *et al.* (2004) (Genebank accession number: AB110034). There was a 99% correspondence of *T. cati* sequence to that

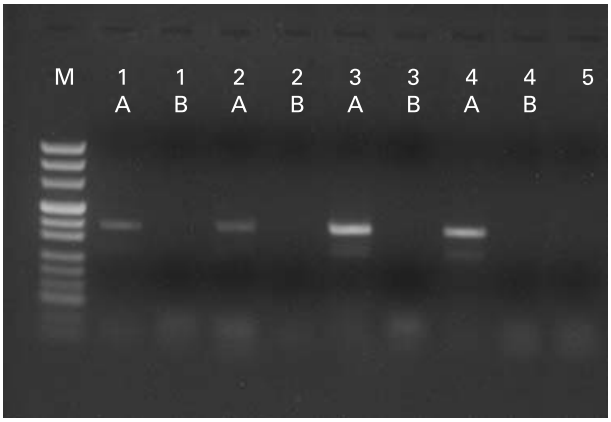

Fig. 2. DNA amplification of eggs of *Toxocara* species by PCR on 2% agarose gel: lane M, pUC Mix Marker 8; lane 1, sample with 1 egg, A – Tcat/NC2 primers, B – Tcan/NC2 primers; lane 2, sample with 1 egg, A – Tcan/NC2 primers, B – Tcat/NC2 primers; lane 3, sample with 10 eggs, A – Tcan/NC2 primers, B – Tcat/NC2 primers; lane 4, sample with 430 eggs, A – Tcan/NC2 primers, B – Tcat/NC2 primers; lane 5, no-DNA control.

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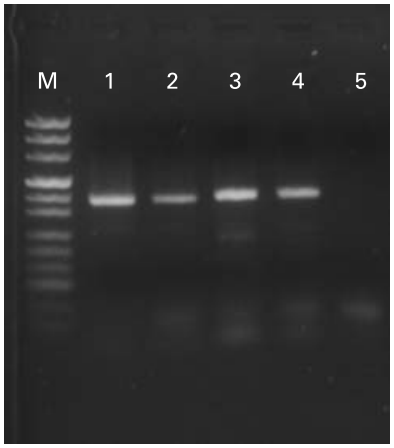

Fig. 3. DNA amplification of one egg of each of *T. canis, T. cati* by PCR on 2% agarose gel: lane M, pUC Mix Marker 8; lanes 1 and 2, Tcan/NC2 primers; lanes 3 and 4, Tcat/NC2 primers; lane 5, no-DNA control.

described by Ishiwata *et al.* (2004) (Genebank accession number: AB110033) and a 98% correspondence to the sequence reported by Zhu *et al.* (1998) (Genebank accession number: AJ002441).

Discussion

Molecular techniques are widely used for detecting pathogenic protozoans in the environment (Majewska & Sulima, 1998; Da Silva *et al*., 1999; Wędrychowicz, 2000). These techniques have been applied in studies on ascaridoid life cycles, systematics and diagnosis (Zhu *et al*., 2001) but they have not been instrumental in determining the contamination of soil with geohelminth eggs. Traditional methods based on morphological features were deemed to be sufficient and the use of relatively expensive genetic methods did not appear to be necessary. But in the identification of species of *Toxocara* molecular techniques are particularly useful in determining the prevalence of *T. canis* or *T. cati* eggs in soil, which in turn will help to elucidate their relative roles in human toxocariasis.

Molecular studies on *Toxocara* have been undertaken over the past decade. Turcekova & Dubinsky (1996) used restriction profiles not only to detect genetic, interspecific differences between *T. canis* and *T. cati* but also sexual differences. Wu *et al.* (1997), using the PCR technique, located primers for the identification of members of the genus *Toxocara*, followed by Jacobs *et al.* (1997) who identified species of *Toxocara* and other zoonotic ascaridoid nematodes by applying two PCR-based techniques, i.e. a two-step process PCR-linked restriction fragment length polymorphism (RFLP) and a more simple PCR using specific primers constructed for *T. canis* and *T. cati*. Zhu *et al.* (1998) applying the PCR–RFLP and also PCR–single stranded conformational polymorphism (SSCP) techniques, confirmed that a nematode previously identified morphologically as *T. cati* was in fact a distinct species. Borecka (2004) used PCR-linked RFLP to identify one larva released from a soil-isolated *Toxocara* egg but this approach was not entirely useful for environmental studies.

The differentiation of *T. canis* and *T. cati* eggs on the basis of morphological features is difficult and inconclusive and reliable methods for identifying species of embryos from soil-isolated eggs during routine environmental studies are equally difficult or lacking. However, in the present study, by applying gentle pressure to a cover slip on a microscope slide, embryonic material was collected for genetic analyses independent not only of the developmental stage of the egg but also of the number of eggs. This is important, as in environmental studies for the detection of geohelminth eggs more than a hundred *Toxocara* eggs at various stages of development can be found in, for example, 40 g soil samples. Therefore this PCR method previously used only for worm tissues obtained from hosts (Jacobs *et al*., 1997) can be used on embryonic material from eggs for studies on environmental contamination.

It should be noted that in the present study, genetic analyses of embryos were carried out after the flotation process and the released material from the slide was rinsed with a large amount of water. This provides at least two advantages by, firstly, confirming morphological identification of eggs and secondly, washing eggs many times is important for the efficiency of DNA detection. The repeated washing of eggs in different fluids during the flotation method and rinsing the embryo with a large amount of water made the removal of soil inhibitors possible. It has also been shown that even with only one egg it is possible to obtain successful results for genetic analyses (fig. 2). This method is recommended as it is sensitive, efficient and confirms the results of microscopic observations undertaken during the routine examination of contaminated soil. With appropriate primers the method can also be adapted for the recovery from soil of geohelminth eggs of other parasite species.

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