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Differentiation of *Toxocara* spp. eggs isolated from the soil by the PCR-linked RFLP method

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Summary

PCR-linked RFLP is a method useful in differentiating eggs of *Toxocara* spp. isolated from the soil to a species level. *Toxocara* spp. eggs were extracted from the soil and embryonic material was removed from the eggs by the needle. The region spanning the second internal transcribed spacer (ITS-2) of the ribosomal DNA of each sample was amplified by PCR. The PCR products for ITS-2 of the *T. cati* and *T. canis* were similar in size. Digestion of the purified ITS-2 products of the two *Toxocara* species with RSA I endonuclease produced specific banding patterns, three fragments for *T. cati* and two for fragments for *T. canis*. In the future this method can be used in the diagnostics of human toxocararosis.

Key words: *Toxocara*; rDNA; ITS-2; PCR-linked RFLP; differentiation

Introduction

Dogs and cats harbour two intestinal parasites belonging to the Ascarididae family: *Toxocara canis* and *Toxocara cati*. Both of these species are of medical importance. Their larvae can infect human tissues causing diseases such as visceral larva migrans and ocular larva migrans (Beaver *et al*., 1952; Glickman *et al*., 1979). Humans become infected with ascarids through ingestion of infective eggs present in the environment (Gillespie, 1988). The growing population of dogs and cats in Europe, together with high rates of nematode infections, has resulted in a widespread contamination of the soil with infective eggs. Many studies have been undertaken worldwide to determine the prevalence of *Toxocara* spp., eggs in the soil of public places (Dada and Linquist, 1979; Tsuji *et al*., 1996; Uga *et al*., 1989; Kulišic *et al*., 1998; Oge and Oge, 2000; Alonso *et al*., 2001). Eggs of *Toxocara* spp. are able to survive in the soil for many years (Snow *et al*., 1987).
Identification of closely related taxa such as *T. canis* and *T. cati* is possible by applying molecular techniques. DNA techniques utilising genetic markers in ribosomal DNA have been employed to resolve taxonomic problems relating to various parasitic groups including nematodes (Jacobs *et al*., 1997; Zhu *et al*., 2001). The second internal spacer (ITS-2) of rDNA has proven to be particularly valuable in this context for studying parasitic nematodes (Gasser *et al*., 1994). For example, genetic markers in the ITS-2 region of rDNA have been used for species identification of single strongyle eggs (Campbell *et al*., 1995).
The present study was undertaken to develop a routine PCR-linked RFLP technique for *Toxocara* spp. eggs isolated from the soil with the potential of its applicability identification of ascaridoid larvae in human tissues.

Material and Methods

Isolation of Toxocara spp. eggs from the soil
Toxocara spp. eggs were extracted from the soil using the Dada method (1979). The dried soil samples were sieved through a mesh. 10 g soil samples were trasnsferred to volumetric flasks containing 50 ml of 0.1 % Tween 80 and vortexed for 30 min. The suspensions were transferred to 60 ml centrifuge tubes, then centrifuged at 180 *g* for 10 min, after which the supernatants (which were free of ova) were decanted. A saturated flotation solution-$ZnSO_4$ (specific gravity 1.52), was added and the tubes and were vortexed again, then centrifuged at 180 *g* for 10 min, after which more solution was added to form a meniscus and a coverslip was overlayed. The coverslip was transferred to a glass slide after 15 min. The coverslips were examined under x 100 magnification and the number of *Toxocara* spp. ova counted.

Isolation of genomic DNA
Isolated eggs were suspended in a few drops of destilled water on a slide. Embryonic material was removed from

the eggs by needle. Due to this isolation procedure no problems with soil PCR inhibitors occurred. The embryonic material was incubated at 65°C with Proteinase K (concentration of 10 mg/ml) for 6 h (Mieszczanek and Wędrychowicz, 1999).

PCR method

PCR amplification of ITS2 and subsequent digestion by *Rsa* I provides distinct RFLP patterns able to distinguish *T. canis* and *T. cati*, the method was described by Jacobs *et al*. (1997). These authors extracted genomic DNA either from adult worms collected from dogs, foxes and cats or from embryonated eggs collected from the uteri of female worms. My innovation was adapting the PCR-RFLP method in the categorization of the *Toxocara* eggs obtained from the soil, and this molecular analysis was carried out on single eggs.

The region spanning the ITS-2 was amplified from the gDNA (10 – 15 ng) by PCR using oligonucleotide primers NC13: 5'-ATCGATGAAGAACGCAGC-3' (forward) and NC2: 5'-TTAGTTTCTTTTCCTCCGCT-3' (reverse), designed for the regions of the 5.8S or 28S ribosomal genes. PCR reactions (50 µl) were performed in 10 mM Tris-HCl, pH 8.4; 50 mM KCl; 3.0 mM $MgCl_2$; 250 µM each of dATP, dCTP, dGTP, dTTP; 100 pmol of each primer with 2U Taq polymerase under the following conditions: 94°C, 30 s (denaturation); 55°C, 30 s (annealing); 72°C, 30 s (extension) for 30 cycles (Thermocycler, Biometra). 1 µl (usually equal to 10 – 15 ng) of genomic DNA was added to each PCR reaction. Samples without DNA were included in each amplification run to exclude the 'carry-over' contamination.

RFLP method

Purified ITS-2 PCR products (6 µl or 12 µl) were digested with 10 units (1 µl) of restriction endonuclease (*Rsa* I; Sigma) in 20 µl for 6 h at 37°C. Restriction fragments were separated on 2.5 % agarose gels containing ethidium bromide. The Puc 80 – 1444 marker (produced by BTL) was used to estimate the size of obtained fragments. Gels were transilluminated in the UV-light and photographed.

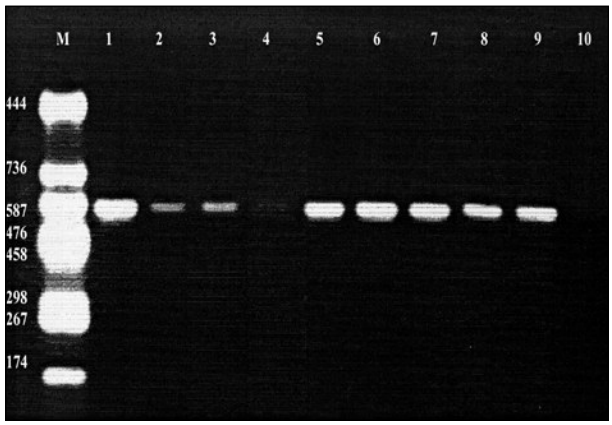

Fig. 1. PCR performed with DNA extracted from adult *T. cati*, adult *T. canis* and eggs of *Toxocara* spp. isolated from the soil: M- molecular-weight marker; lane 1 represents PCR carried out in the presence of 11 ng of *T. cati* DNA; lane 2 represents PCR with 14 ng of *T. canis* DNA, isolated from a dog; lane 3 represents PCR with 10 ng of *T. canis* DNA, isolated from a fox; lanes 4 – 9 represents PCR with DNA extracted from a few eggs of *Toxocara* spp. isolated from soil samples; lane 10 is a no-DNA control

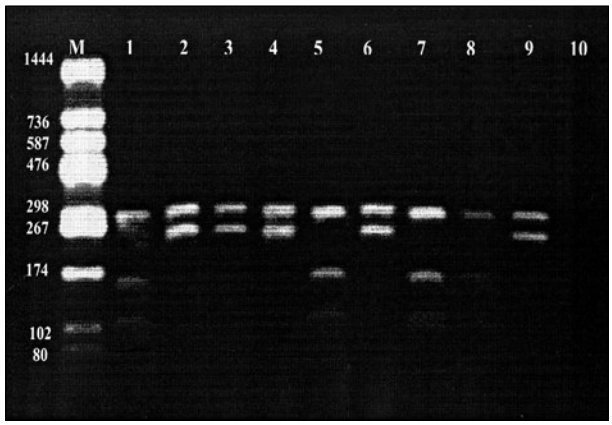

Fig. 2. Results of PCR- linked RFLP of the ITS-2 of two ascaridoid species: M- molecular-weight marker; *T. cati* (lane 1); *T. canis* isolated from a dog (lane 2); *T. canis* isolated from a fox (lane 3); eggs of *Toxocara* spp. extracted from soil samples (lanes 4 – 9); no-DNA control (lane 10)

Results

The PCR product for ITS-2 of *T. cati* and *T. canis* were similar in size. The banding patterns for all samples were identical (Fig. 1). The PCR method alone was not able to differentiate the eggs of *Toxocara* spp. to the species level. The PCR products of *T. cati* and *T. canis* were therefore digested with the endonuclease Rsa I. Digestion of the ITS-2 products of the two *Toxocara* species with the endonuclease RSA I produced specific banding patterns, composed of three fragments for *T. cati* and two fragments for fragments for *T. canis* (Fig. 2.). This method thus appeared useful for species delineation of *Toxocara* spp. eggs isolated from soil to the species level.

Discussion

The aim of this research work was the application of a PCR-RFLP method in the differentiation of *Toxocara* eggs obtained from the soil. In this study primers specific for the *Toxocara* genus designed by Jacobs *et al*. (1997) were used.

In the beginning when small samples (1 g of soil) were examined I had problems with obtaining a sufficient number of eggs from soil samples. Increasing the quality of soil samples up to 10 grams ensured adequate numbers of eggs for analysis.

However, the greatest problem encountered was connected with the extraction of embryos from eggs. The application

of very low temperatures (-70°C), boiling for 10 minutes, and a incubation at 65°C with Proteinase K (10 mg/ml) for two hours did not lead to the desintegration of egg shells. For this reason embryonic material was removed from eggs by needle.

Until now, differentiation of *Toxocara* eggs isolated from the soil was conducted using morphometric methods (Mizgajska and Rejmenciak, 1997), but the results obtained were not credible. Adapting molecular methods, e.g. PCR or PCR-RFLP, to the delineation of *Toxocara* eggs has the increased the correctness of obtained results. Before me Wu *et al.* (1997) and Turčeková and Dubinský (1996) studied the delination of *T. canis* and *T. cati* with molecular methods.

T. canis and/or *T. cati* can be involved in human infection, but many veterinarians and medicians are not aware of this problem. The PCR-RFLP method could be useful in detecting human infection with nematodes of the *Toxocara* genus and determining which of the two species (or both?)- *T. canis* and/or *T. cati*, cause the disease diagnosed in humans. Toxocarosis was recognized for the first time in the middle of the last century (Wilder, 1950; Nichols, 1956), but until now it is not known to which extent the two species implicate toxocarosis or produce worse symptoms. Probably the first differentiated larvae by Nicholson (1956) and isolated by Wilder (1950) from human eyes was attributed to *T. canis.* Consequently, many authors who write about toxocarosis have accepted *T. canis* as the etiological factor.

In conclusion, the results of my work indicate that the described PCR-RFLP method has a considerable potential to become a technique for the differentiation of *Toxocara* larvae isolated from the soil or from human tissues.

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