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Research Note

Detection of *Toxocara canis* larvae by PCR in the liver of experimentally infected Mongolian gerbils (*Meriones unguiculatus*)

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Summary

Toxocariasis is a common human zoonosis, which induces a clinically unapparent course of infection. Diagnosis is difficult and relies upon serological testing (searching of specific IgG antibodies by ELISA), laboratory abnormalities and clinical manifestations. The polymerase chain reaction (PCR) technique was adapted for the detection of *Toxocara canis* larvae in a host tissue. Mongolian gerbils (*Meriones unguiculatus*) were used as an animal model for human toxocariasis. 8 animals were inoculated with 1000 *T. canis* eggs, four uninfected were used as control. At 3, 5, 7, and 14 days post-infection, 2 infected and 1 control gerbil were killed and their livers were used for molecular analysis. Specific primer in the PCR reaction allowed identification of *T. canis* larvae, with the parasite gDNA found in the liver of all infected gerbils. The results indicate that the PCR method has a potential as a supporting technique for the diagnosis of human toxocariasis.

Key words: *Toxocara canis*; polymerase chain reaction (PCR); Mongolian gerbils; toxocariasis

Introduction

The dog roundworm, *Toxocara canis* is a worldwide distributed parasite. Humans, especially young children, become infected following accidental ingestion of infective eggs containing L3 stages from the soil or contaminated food. After ingestion, larvae hatch in the small intestine, penetrate the gut wall and migrate via the bloodstream to the liver, where most of them are retained. However, some larvae continue their migration through the heart and lungs to the systemic circulation where they are distributed to various organs (the lungs, heart, eye and brain). At present, four clinical syndromes of human toxocariasis are recognized, namely visceral larva migrans (VLM), ocular larva migrans (OLM), covert toxocariasis (CT), and neurological toxocariasis (NLM) (Magnaval *et al.*, 2001a). The clinical manifestations are dependent on the number of eggs ingested, localization of larvae, host responses and possible reinfections.

In previous decades, mice, rats, rabbits, guinea pigs, sheep, pigs, and baboons had been used as animal models for toxocariasis. Presently, Mongolian gerbils (*Meriones unguiculatus*) are the animals of choice, due to their susceptibility to infection with *Toxocara* spp. as well as the high percentage of ocular cases occuring in comparison with other animal species.

In humans, high peripheral eosinophilia and a positive serologic test results are indicative of active toxocariasis. An additional protocol, useful in distinguishing between active and latent toxocariasis, is measurement of the level of cationic eosinophil protein (Magnaval *et al.*, 2001a; Magnaval *et al.*, 2001b). The most commonly employed diagnostic serologic test is the enzyme-linked immunosorbent assay (ELISA) using excretory-secretory antigens of *T. canis*. A positive ELISA should be confirmed by western blot, which is more specific when lower molecular weight bands, from 24 to 35 kDa are considered (Magnaval *et al.*, 1991). To support the diagnosis, detection of the parasite DNA in a patient tissue sample with molecular technique based on the polymerase chain reaction (PCR) may be involved. Methods designed to discriminate *T. canis* and *T. cati* are based on PCR analysis of genomic DNA extracted from adult worms or embryonated eggs collected from the uteri of female worms (Jacobs *et al.*, 1997; Zhu *et al.*, 1998). We have used a PCR-restriction fragment length polymorphism (RFLP) technique for the differentiation of *T. canis* and *T. cati* eggs isolated from soil (Borecka, 2004).

The present study was undertaken to adapt the PCR technique for the detection of *T. canis* larvae in the liver of experimentally infected Mongolian gerbils, used as an animal model for human toxocariasis.

Materials and methods

Experimental infection
Twelve, five-month-old male Mongolian gerbils (*Meriones unguiculatus*) were used in the experiment. Fertilized eggs of *T. canis* were obtained from the uteri of adult worms collected from feces following the administration of anthelmintics to naturally infected puppies. The eggs were embryonated in 0.1N H_2SO_4 in an Erlenmeyer flask. Each of 8 gerbils was inoculated with 1000 eggs by a stomach tube under light anaesthesia. Four uninfected gerbils were maintained as controls. At 3, 5, 7**,** and 14 days post-infection, each 2 infected and 1 control gerbil were killed (anaesthetized using ketamine and penthobarbital) and the livers were removed for molecular analysis. The time of post-mortem examination was chosen according to Cho et al. (2007).

DNA isolation
The isolation was performed using GeneMATRIX tissue (Eurx, Gdansk, Poland) in accordance to the kit protocol. 25 mg of the liver were homogenized and mixed with buffers and proteinase K solution was added. The samples were incubated at 56 ^{0}C until complete lysis was obtained (3 h). In the next step, incubated samples were centrifuged for 1min at 12 000 rpm (11 000g); supernatants were mixed with SOL-T buffer and incubated for 10 min at 56 ^{0}C. After the incubation, 96 % ethanol was added and the samples were centrifuged for 1 min at 12 000 rpm (11 000g); the obtained supernatants were transferred to spin-columns, placed in collection tubes and centrifuged for 1 min at 12 000 rpm (11 000g). The filters with bound gDNA were washed twice using the Wash-TX buffer. In the next step, the spin-columns were transferred to the new collections of tubes and the elution buffer heated to 70 ^{0}C was added to elute the bound DNA. Samples were incubated for 3 min at room temperature and then centrifuged for 1 min at 12 000 rpm (11 000g).

PCR method
The extracted DNA was used for PCR, in which amplification of internal transcribed spacer 2 (ITS-2) was performed according to Jacobs et al. (1997). The region spanning ITS-2 was amplified from the gDNA (5-10ng) by PCR using oligonucleotide specific for *T. canis*: *Tcan1* and NC2. 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, and dTTP; 100 pmol of each primer with 2U Taq polymerase under the following conditions: 94 ^{0}C for 30 s (denaturation), 55 ^{0}C for 30 s (annealing), and 72 ^{0}C for 30 s (extension) for 30 cycles (Thermocycler, Biometra, Germany). A sample of 1 µl of genomic DNA was added to each PCR reaction. Samples without DNA were included in each amplification run to exclude the 'carry-over' contamination. The obtained fragments were separated on 2.5 % agarose gels containing ethidium bromide. The pUC Mix marker (BTL, Poland) was used to estimate the size of the obtained fragments. Gels were transilluminated in UV-light and photographed.

Results and discussion

The gDNA of *T. canis* was found in the liver of all infected animals at 3, 5, 7, and 14 days PI (Fig. 1). In previous investigations (Jacobs *et al.*, 1997; Zhu *et al.*, 1998) genomic DNA was extracted from adult worms and embryonated eggs. Our innovation was to adapt the PCR for detection of *T. canis* gDNA extracted from a tissue of experimentally infected animals. Cho *et al.* (2007) found that in 3, 5, 7 and 14 days PI, 10 %, 5 %, 1 % and 1 % of infective larvae were presented in the liver tissue. Therefore, using 1000 *Toxocara* eggs for infection, in appointed days in the liver were retained 100, 50, 10 and 10 larvae, respectively. For gDNA isolation 25 mg of the tissue was used, which is 0.9 % of the liver (average weight 2.8 g). For this reason in examined pieces 0.8, 0.4, 0.09 and 0.09 larvae were possibly present. It seems that the method is able to detect <1 larva per sample, demonstrating high sensitivity. Species specific primers for *T. canis* and *T. cati* employed in the PCR technique allow gDNA of both ascarid species to be accurately diagnosed with elimination of false positive and false negative results.

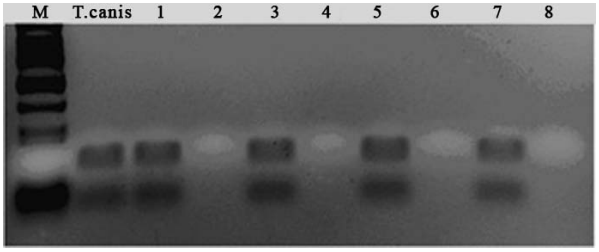

Fig. 1. The PCR of *T. canis*: lane M, pUC Mix Marker; lane *T. canis*, positive control, gDNA isolated from adult worms; lanes 1, 3, 5 and 7, products of PCR of *T. canis* DNA extracted from the liver of infected gerbils killed on 3, 5, 7**,** and 14 day PI, respectively; lanes 2, 4, 6 and 8, negative control, PCR for *T. canis* DNA extracted from the liver of control gerbils killed on 3, 5, 7**,** and 14 day

Toxocariasis is reported to be one of the most prevalent helminthiasis in industrialized countries (Magnaval *et al.*, 2001a). The seroprevalence in adult humans ranges from 1 – 4 % in central Europe (Obwaller, 1998), but may be much higher in young children, up to 20 % in developed countries and 60 % in developing countries (Thompson *et al.*, 1986). The infection with *Toxocara* spp. is usually accompanied with non-specific signs and symptoms such as hepatomegaly, splenomegaly, lymphadenopathy, fever, abdominal pain and headache, which can present significant diagnostic difficulties. The clinical manifestations are not always apparent, and depend on the infective dose, internal distribution of larvae, host susceptibility and possible reinfections. Serological testing is the primary diagnostic tool in patients suspected for toxocariasis, but these tests are insufficient, as cross-reactions may occur. For example, cross-reactivity has been reported between *T. canis* and *Trichinella spiralis*, and *T. canis* and *Ascaris lumbricoides* (Kennedy *et al.*, 1989; Żarnowska *et al.*, 1994).

In our study**,** gerbils were inoculated with high doses of *T. canis* eggs**,** simulating massive infections, which can be found in small children. The present results indicate that the PCR method has potential to be used for the detection of *T. canis* and *T. cati* larvae in humans, in particular in ocular cases difficult for diagnosis.

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