Bulgarian Journal of Veterinary Medicine, 2020, **23**, No 4, 487–493
ISSN 1311-1477; DOI: 10.15547/bjvm.2019-0014

Original article

PARASITOLOGICAL AND MOLECULAR STUDY OF *TOXOCARA* SPP. IN *LUMBERICUS TERRESTRICUS* EARTHWORMS

E. RASOULI[1], M. ZIBAEI[2,3], S. BAHADORY[4], H. HOSSEINI[2] & F. FIROOZEH[5,6]

[1]Medical Laboratory Department, Faculty of Paramedicine, [2]Department of Parasitology and Mycology, School of Medicine, [3]Evidence-based Phytotherapy and Complementary Medicine Research Center, [4]Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran; [5]Dietary Supplements and Probiotic Research Center, [6]Department of Microbiology, School of Medicine, Alborz University of Medical Sciences, Karaj, Iran

Summary

Rasouli, E., M. Zibaei, S. Bahadory, H. Hosseini & F. Firoozeh, 2020. Parasitological and molecular study of *Toxocara* spp. in *Lumbericus terrestricus* earthworms. *Bulg. J. Vet. Med.,* **23**, No 4, 487–493.

Soil-associated invertebrates which are coprophagic are capable of acting as paratenic hosts for *Toxocara* species larvae eggs. The aim of the present study was to assess the role of *Lumbricus terrestris* earthworms as potential intermediate host for *Toxocara* species. A total of 240 earthworms were collected from the public places in four areas of Karaj, Alborz Province, Iran. The subjects of the study were digested to detect *Toxocara* species using microscopy and molecular analyses. *Toxocara* larvae and larval eggs were recovered from 1.7% (4/240) of the sampled earthworms. The recovery rate in Shah'Abbasi and Talaghani regions in the studied areas was higher than that of the other regions. In the mentioned area, 8.3% (4/48) of earthworms were revealed to be positive for *Toxocara* species by microscopic observation and only one was confirmed to be positive for *Toxocara canis* by polymerase chain reaction (PCR). This is the first molecular report of *Toxocara canis* infective larvae in *Lumbricus terrestris* earthworms that could be demonstrated in common environment.

Key words: earthworms, *Lumbricus terrestris*, molecular analyses, paratenic host, *Toxocara canis*

INTRODUCTION

Toxocara infection results from zoonotic transmission of the roundworms, *Toxocara canis* (*T. canis*) and *Toxocara cati* (*T. cati*) from dogs and cats, respectively. The infection is highly prevalent in many developing countries and its global importance may be greatly underestimated. After ingestion of the microscopic and thick-shelled-embryonated eggs, the infective larval stage migrates through the

tissue of paratenic hosts including mammals, birds and invertebrates (Dubinský *et al.,* 1995). Humans become infected by consumption of meat of paratenic hosts containing encapsulated larvae as well. In the paratenic hosts including humans, the larvae can migrate through the tissues and cause ocular larva migrans, visceral larva migrans, neurotoxocariasis, and covert toxocariasis (Rubinsky-Elefant *et al.,* 2010).

Lumbricus terrestris is a large and reddish worm species that is widely distributed around the world. It is the largest naturally occurring species of earthworm. This worm is an important soil organism in development and maintenance of nutrient value of soil by converting biodegrade material and organic waste into nutrient rich vermicast (Singh *et al.,* 2016). It can consume a wide range of unstable organic matter such as animal waste and sewage sludge (Subaraja & Vanisree, 2015).

Chickens as paratenic hosts that love earthworms or squigglers of any kind are possible source of *Toxocara* larvae that could be infected via eating soil-associated arthropods, insects, and annelids that have come in contact with *Toxocara* species embryonated eggs (Zibaei *et al.,* 2017). There are several reports indicating infection of humans with toxocariasis after eating raw or under cooked infected chickens (Ito *et al.,* 1986; Nagakura *et al.,* 1989; Salem & Schantz, 1992; Tiara *et al.,* 2003; Yoshikawa *et al.,* 2008; Tiara *et al.,* 2011).

However, there are no reports of molecular detection of *Toxocara* species isolated from the annelids population living in public places. Here, we present the first molecular and parasitological detection of *Toxocara* species in the environment using simultaneously microscopy and molecular analysis.

MATERIALS AND METHODS

Sample collection

Two-hundred and forty *Lumbricus terrestris* earthworms were collected from the four public areas of Karaj (Alborz Province, 35˚ 49' 57″, 50˚ 59' 29″), stored in sealed and labelled polyethylene bags and transferred to the Parasitology Laboratory at Alborz University of Medical Sciences. The studied areas were included: (1) Azimieh, Jahan'Shahr, Baghestan, (2) Hesarak, Mehr'Shahr, (3) Shahrake Banafsheh, Fardis, and (4) Shah'Abbasi, Talaghani.

Preparation

The earthworms were carefully washed five times in PBS to remove debris and were used for further isolation of parasites' eggs or larvae. The animals were fragmented with pointed forceps and examined by dissecting microscope. Thereafter, the tissues were put into digestive solution (pepsin, 5 g; HCl 37%, 10 mL in 1,000 mL water) and kept overnight at 37 ^{o}C to recover the remaining larvae. Sedimental liquid was poured into a centrifuge tube and centrifuged for 2 min at 1,500 rpm, 2 mL sediments were collected, thoroughly mixed, and 0.1 mL samples were viewed for eggs or larvae counts.

DNA extraction

The parasite DNA was extracted as described previously (Zibaei *et al.,* 2017). Briefly, extraction of DNA from worm tissues was done according to standard protocols. Genomic DNA was isolated from samples using Favorprep^{TM} Tissue

Genomic DNA Extraction Mini Kit (Favorgen Biotech, Ping-Tung, Taiwan, China) regarding the manufacturer's instruction.

Polymerase chain reaction (PCR)

During this study, the specific forward primer JW4 (5′-ACTGTCGAGGATGA GCGTGA-3′) was used, which is specific for *T. cati* (Li *et al.,* 2007) and reverse primer NC2 (5′-TTAGTTTCTTTTCCTC CGCT-3′) (Zhu *et al.,* 2002) to amplify partial internal transcribed spacer-1 (ITS-1), complete 5.8S and ITS-2 of rDNA as described previously. The forward primer YY1 (5′-CGGTGAGCTATGCTGGTG TG -3′) was used, which is specific for *T. canis* as previously designed (Jacobs *et al.,* 1997) and combined with reverse primer NC2 (5′-TTAGTTTCTTTTCCT CCGCT-3′) to amplify partial ITS-2 as well. PCR reaction was performed in Super master mix-2× 12.5 µL, Primer (forward) (10 pmol/ µL) 1.5 µL, Primer (reverse) (10 pmol/µL) 1.5 µL, DNA template (optional) 3 µL, DW 6.5 µL. The reaction was carried out under the following conditions: 94 ^{o}C for 30 s, 57.5 ^{o}C for 50 s, 60 ^{o}C for 90 s, and final extension 72 ^{o}C for 7 min.

PCR product electrophoresis

Corresponding PCR products were electrophoresed in 1% agarose gel with 3 µg/ML gel red, and a 100-bp ladder was used as DNA sizemarker for estimating the size of the amplicons and photographed using a gel documentation system (UV Transilluminator).

Statistical analysis

Statistical analysis was done using SPSS software version 18 (IBM, Armonk, USA). Chi-square (χ^2) and Fisher's exact tests were used to evaluate associations of the variables. P-value less than 0.05 was considered statistically significant.

RESULTS

Microscopic investigation

In an attempt to identify the optimal procedure for the diagnosis of visceral larva

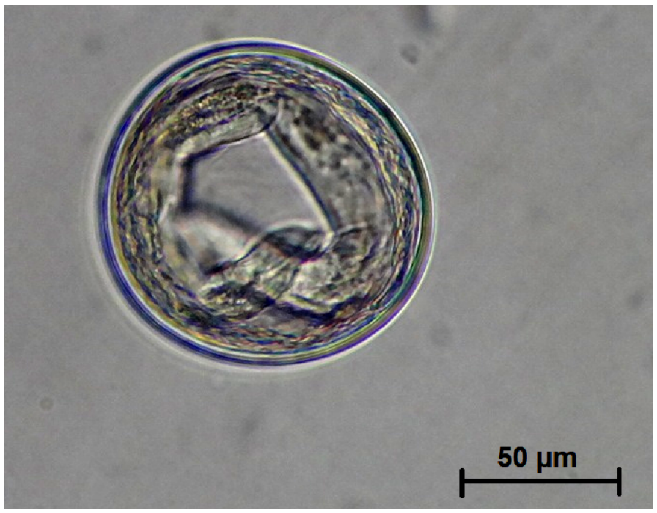

Fig. 1. *Toxocara* spp. larval eggs recovered from *L. terrestris* earthworms taken from public places.

Table 1. Recovery rate of *Toxocara* spp. larvae and larval eggs from *Lumbricus terrestris* earthworms' visceral body based on microscopic and molecular techniques

Area	Study places	Number of collected worms (%)	Recovered larvae*	
			Microscopy number (%)	Molecular technique number (%)
1	Azimieh, Jahan'Shahr, Baghestan	98 (40.8)	0 (0.0)	0
2	Hesarak, Mehr'Shahr	58 (24.2)	0 (0.0)	0
3	Shahrake Banafsheh, Fardis	36 (15.0)	0 (0.0)	0
4	Shah'Abbasi, Talaghani	48 (20.0)	4 (8.3)	1 (2.1)
	All areas*	240 (100)	4 (1.7)	1 (0.4)

P<0.05 between the studied areas and the recovery rate of *Toxocara* larvae.

migrans in earthworms as paratenic host for *Toxocara* species, the performance and agreement among the two different methods include pepsin digestion and PCR methods, were evaluated.

Toxocara larvae and larval eggs were recovered in 8.3% (4/240) of the subjects collected (Fig. 1). The infected *Lumbricus terrestris* earthworms were found on the Shah'Abbasi and Talaghani regions and *Toxocara* larvae were recovered from the 8.3% (4/48) of worms in Shah'Abbasi and Talaghani regions in the studied areas (Table 1). The difference between the studied areas and contamination rate of *Lumbricus terrestris* earthworms was statistically significant (P<0.05).

PCR assay

All digested samples were subjected to PCR assay using extracted DNA from tissue samples. Analysis of PCR products revealed positive band (330 bp) in the infected earthworms. The DNA of *T. canis* was found in the tissue samples of earthworms in Shah'Abbasi and Talaghani regions in the studied areas (Fig. 2).

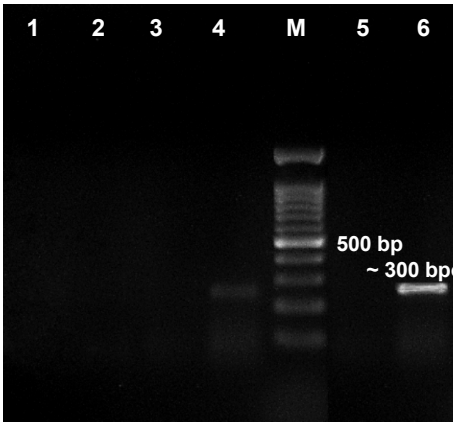

Fig. 2. DNA amplification of *Toxocara* species recovered from *Lumbricus terrestris* by electrophoresis on 1% agarose gels in the analysis of PCR products (earthworm tissues). Lanes 1–4: samples of Shah'Abbasi and Talaghani areas; lane M: 100–1500 bp DNA size marker; lane 5: negative control (no DNA); lane 6: positive control.

DISCUSSION

In a study (Zibaei & Sadjjadi, 2017) the infection of dogs and cats with *Toxocara* species in different parts of Iran has been reported. In the city of Karaj, a study showed that a total of 4 out of 12 public parks were contaminated with *Toxocara* spp. eggs (Zibaei *et al.,* 2017). There are free dogs and cats populations living in semi-wild conditions in public places. The presence of the eggs of *Toxocara* species in soil samples confirmed that the life cycle of the parasite took place in the area. Most previous reports have pointed to the fact that earthworms are intermediate host or paratenic host for many species of parasites (Noble & Glenn, 1961; Murad, 1988). It seems that earthworms provide food for the birds and protective substance for eggs of *Toxocara* species by perpetuating the birds and parasite cycle in contaminated environments. These conditions will indicate a risk between birds as reservoir or paratenic hosts and dogs and cats as definitive hosts.

It is well known that *Toxocara* species are easily found in earthworms and in other Oligochaetes (Okoshi & Usui, 1968). The importance of earthworms as natural reservoir of *Toxocara* species larvae in the environment has been reported (Dubinsky *et al.,* 1995). In an early study, *Toxocara cati* larvae were recovered from experimental earthworms and cockroaches (Sprent, 1956). In a similar study, 87% of earthworms that were collected from rural areas infected with *Toxocara* species eggs (Mizgajska, 1997). Human toxocariasis acquired by ingestion of living earthworms was reported in the People's Republic of China (Yu *et al.,* 2012). Recently, a case of visceral larva migrans (VLM) associated with earthworm ingestion has been reported (Cianferoni *et al.,* 2018).

The digestion method is one the best methods for detection of *Toxocara* larvae in the body cavity of earthworms. In the present study, *Toxocara* larvae were recovered from 1.7% from the tissue of naturally infected earthworms. The rate of infected worms with *Toxocara* larvae in Shah'Abbasi and Talaghani regions was higher than other areas. This fact might be the consequence of a relatively higher number of stray cats and dogs in this region.

Molecular studies on *Toxocara* have been undertaken over two decades. Molecular based technique provided a rapid, reliable and sensitive means by which one establishes the exact etiology of such parasitic infection so that optimal therapy can be promptly started (Zibaei *et al.,* 2013). The DNA-dependent strain identification still presents one of the gold standards for molecular identification of *Toxocara*. The differentiation between *T. canis* and *T. cati* eggs and larvae on the basis of morphological features is difficult and inconclusive, and reliable methods for identifying species of larvae from tissues during histopathological studies are equally difficult or lacking. In the current survey, PCR based upon the amplification of ITS-1 and ITS-2 fragments revealed that 1 of 240 (0.4%) earthworms showed a positive reaction to *T. canis*. This PCR method, which was used previously only for worms from tissue obtained from experimentally infected paratenic hosts, can be used for studies on natural infections.

To our knowledge, this is the first molecular report that reveals *Toxocara* infective larvae in *Lumbricus terrestris* earthworms as paratenic host. The finding of this study showed that earthworms can be natural paratenic hosts for the larva of *T. canis* and they may consider the role of these animals in the establishment emer-

ging pathogens and as reservoir of other parasitic infection and contribute to maintain disease in the environment. These results also represent that the molecular method has potential for the detection and identification of *Toxocara* species larvae in the earthworms of *Lumbricus terrestris*, which has to be evaluated in more samples in order to validate the method.

ACKNOWLEDGEMENTS

The technical assistance of Mrs. L. Moshki of the Parasitology Laboratory, School of Medicine, Alborz University of Medical Sciences, Iran is acknowledged with pleasure. This research was supported by the Vice Chancellor for Research of Alborz University of Medical Sciences (Project No. Abzums. Rec. 1396. 183). Furthermore, the authors would like to thank Prof. S. M. Sadjjadi for his guidance.

REFERENCES

Cianferoni, A., L. Schneider, P. M. Schantz, D. Brown & L. M. Fox, 2018. Visceral larva migrans associated with earthworm ingestion: clinical evolution in an adolescent patient. *Pediatrics,* **117**, 336–339.

Dubinský, P., K. Havasiová-Reiterová, B. Petko, I. Hovorka & O. Tomasovicová, 1995. Role of small mammals in the epidemiology of toxocariasis. *Parasitology,* **110**, 187–193.

Ito, K., K. Sakaei, T. Okajima, K. Ouchi, A. Funakoshi, J. Nishimura, H. Ibayashi & M. Tsuji, 1986. Three cases of visceral larva migrans due to ingestion of raw chicken or cow liver. *The Journal of the Japanese Society of Internal Medicine*, **75**, 759–766.

Jacobs, D. E., X. Zhu, R. B. Gasser & N. B. Chilton, 1997. PCR-based methods for identification of potentially zoonotic ascaridoid parasites of the dog, fox and cat. *Acta Tropica,* **68**, 191–200.

Li, M. W., R. Q. Lin, H. H. Chen, R. A. Sani, H. Q. Song & X. Q. Zhu, 2007. PCR tools for the verification of the specific identity of ascaridoid nematodes from dogs and cats. *Molecular and Cellular Probes,* **21**, 349–354.

Mizgajska, H., 1997. The role of some environmental factors in the contamination of soil with *Toxocara* spp. and other geohelminth eggs. *Parasitology International,* **46**, 67–72.

Murad, B. M. M., 1988. The invertebrates. Higher Education Press, Baghdad.

Nagakura, K., H. Tachibana, Y. Kaneda & Y. Kato, 1989. Toxocariasis possibly by ingesting raw chicken. *Journal of Infectious Diseases,* **160**, 735–736.

Noble, E. R. & A. Glenn, 1961. The Biology of Animal Parasites. Lea & Febiger Press, Philadelphia.

Okoshi, S. & M. Usui, 1968. Experimental studies on *Toxascaris leonina*. VI. Experimental infection of mice, chickens and earthworms with *Toxascaris leonina*, *Toxocara canis* and *Toxocara cati*. *Nihon Juigaku Zasshi*, **30**, 151–166.

Rubinsky-Elefant, G., C. E. Hirata, J. H. Yamamoto & M. U. Ferreira, 2010. Human toxocariasis: diagnosis, worldwide seroprevalence and clinical expression of the systemic and ocular forms. *Annals of Tropical Medicine and Parasitology,* **104**, 3–23.

Salem, G. & P. Schantz, 1992. Toxocaral visceral larva migrans after ingestion of raw lamb liver. *Clinical Infectious Diseases,* **15**, 743–744.

Singh, S., J. Singh & A. P. Vig, 2016. Earthworm as ecological engineers to change the physicochemical properties of soil: Soil vs vermicast. *Ecological Engineering,* **90**, 1–5.

Sprent, J. F., 1956. The life history and development of *Toxocara cati* (Schrank 1788) in the domestic cat. *Parasitology,* **46**, 54–78.

Subaraja, M. & A. J. Vanisree, 2015. Cerebral ganglionic variations and movement behaviors of *Lumbricus terrestris* on exposure to neurotoxin. *Annals of Neurosciences,* **22**, 199–207.

Taira, K., A. Permin & C. M. Kapel, 2003. Establishment and migration pattern of *Toxocara canis* larvae in chickens. *Parasitology Research*, **90**, 521–523.

Taira, K., Y. Saitoh & & C. M. Kapel, 2011. *Toxocara cati* larvae persist and retain high infectivity in muscles of experimentally infected chickens. *Veterinary Parasitology,* **180**, 287–291.

Yoshikawa, M., M. Nishiofuku, K. Moriya, Y. Ouji, S. Ishizaka, K. Kasahara, K. Mikasa, T. Hirai, Y. Mizuno, S. Ogawa, T. Nakamura, H. Maruyama & N. Akao, 2008. A familial case of visceral toxocariasis due to consumption of raw bovine liver. *Parasitology International,* **57**, 525–529.

Yu, T., L. N. Zhao, M. J. Fan, H. Wu & Q. K Chen, 2012. Visceral larva migrans associated with earthworm and gecko ingestion: A case report. *Journal of Medical Case Reports,* **6**, 210.

Zhu, X. Q., S. D'Amelio, H. W. Palm, L. M. Paggi, M. George-Nascimento & R. B. Gasser, 2002. SSCP based identification of members within the *Pseudoterranova decipiens* complex (Nematoda: Ascaridoidea: Anisakidae) using genetic markers in the internal transcribed spacers of ribosomal DNA. *Parasitology,* **124**, 615–623.

Zibaei, M., S. M. Sadjjadi, M. Karamian, S. Uga, A. Oryan & S. H. Jahadi-Hosseini, 2013. A comparative histopathology, serology and molecular study, on experimental ocular toxocariasis by *Toxocara cati* in Mongolian gerbils and Wistar rats. *Bio-Med Research International,* **2013**, 109580.

Zibaei, M. & S. M. Sadjjadi, 2017. Trend of toxocariasis in Iran: A review on human and animal dimensions. *Iranian Journal of Veterinary Sciences,* **18**, 233–242.

Zibaei, M., S. M. Sadjjadi & M. Maraghi, 2017. The occurrence of *Toxocara* species in naturally infected broiler chickens revealed by molecular approaches. *Journal of Helminthology,* **91**, 633–636.

Paper received 30.01.2019; accepted for publication 08.05.2019

Correspondence:

Mohammad Zibaei, MSPH, Ph.D
Professor of Parasitology,
Department of Parasitology and Mycology,
School of Medicine,
Alborz University of Medical Sciences,
Karaj 3149779453, Iran,
tel.: +98 26 32663316;
fax: +98 26 32563325
e-mail: zibaeim@sums.ac.ir
https://orcid.org/0000-0003-2265-7460