Journal of Helminthology (2017) **91**, 633–636
doi:10.1017/S0022149X16000559
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The occurrence of *Toxocara* species in naturally infected broiler chickens revealed by molecular approaches

M. Zibaei[1]*, S.M. Sadjjadi[2] and S. Maraghi[3]

[1]Department of Parasitology and Mycology, School of Medicine, Alborz University of Medical Sciences, Karaj, Iran: [2]Department of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran: [3]Institute of Health Research, Thalassemia and Hemoglabinopathy Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

(Received 29 May 2016; Accepted 24 July 2016; First published online 30 August 2016)

Abstract

Consuming raw and undercooked meat is known to enhance the risk of human toxocariasis because *Toxocara* species have a wide range of paratenic hosts, including chickens. The aim of this study was to identify species of *Toxocara* in naturally infected broiler chickens using molecular approaches. A polymerase chain reaction (PCR) method was used for the differentiation of *Toxocara canis* and *Toxocara cati* larvae recovered from tissues and organs, and identified by microscopic observations. Thirty-three 35- to 47-day-old broiler chickens were used for examination of *Toxocara* larvae. The duodenum, liver, lungs, heart, kidneys, skeletal muscles and brain of each chicken were examined using the pepsin method, and DNA from each tissue was extracted as the template for PCR assay. The findings revealed that 5 of 33 (15.2%) broiler chickens were infected with *Toxocara* larvae. Larvae were recovered from the liver ($n = 19$), duodenum ($n = 8$), skeletal muscles ($n = 8$) and brain ($n = 2$) of broiler chickens naturally infected with *Toxocara* spp. The results showed that the frequencies of the species in the chickens were *T. canis* larvae ($n = 5$, 83.3%) and *T. cati* larvae ($n = 1$, 16.7%). Our data from the present study demonstrated the importance of broiler chickens as a paratenic host for the parasite's life cycle in the environment. The implementation of DNA amplification as a routine diagnostic technique is a specific and alternative method for identification of *Toxocara* larvae, and allowed the observation of specific species under field conditions within the locations where broiler chickens are typically raised and exposed to *Toxocara* spp. eggs or larvae.

Introduction

The nematodes *Toxocara canis* and *Toxocara cati* are cosmopolitan parasites of canids and felids, and have a wide range of paratenic hosts, e.g. birds and mammals, where larvae migrate to various tissues and survive for long periods. These parasites are also important zoonotic helminths causing human toxocariasis (Despommier, 2005). Humans are infected by ingestion of *Toxocara* spp. eggs containing third-stage larvae, due to geophagia and pica, as well as the consumption of contaminated raw meat of paratenic hosts containing larvae (Schantz, 1989). There are four clinical forms of toxocariasis – visceral larva migrans (VLM), ocular larva migrans (OLM), covert toxocariasis (CT) and neurotoxocariasis (NT) (Pawlowski, 2001).

*E-mail: zibaeim@sums.ac.ir

https://doi.org/10.1017/S0022149X16000559 Published online by Cambridge University Press

A wide spectrum of paratenic hosts can be infected with *Toxocara* spp. larvae, and patterns of migration have been studied intensively in a variety of animals (Campos-da-Silva *et al.*, 2015). These infections in animals could serve as a model for human toxocariasis and give valuable information about host–parasite interactions (Strube *et al.*, 2013).

The diagnosis of human toxocariasis is based mainly on clinical, epidemiological and laboratory data, which include imaging (computed tomography scans and magnetic resonance imaging), blood examinations, eosinophilia, total IgE level and serological tests (Park *et al.*, 2014).

Experimentally, mice, rats, monkeys, golden hamsters, Japanese quail, pigs, rabbits, chickens and gerbils have been reported as reservoirs for *Toxocara* spp. larvae, due to their susceptibility. The organs affected by migrating larvae, the time points of infection, as well as the relative distribution and survival of larvae, are host species dependent (Zibaei *et al.*, 2010). In chickens infected experimentally with *T. canis*, the highest larval burdens were found in the liver and lungs, and few larvae were recovered from other tissues. Natural infection with *Toxocara* larvae can be assumed to occur in chickens, considering the probable contamination of pasture with faeces from infected farm cats and dogs (Tiara *et al.*, 2012).

Previous reports have demonstrated the transmission of *Toxocara* larvae from a paratenic host to others. Free-ranging poultry are especially likely to acquire *T. canis* larvae from contaminated soil while searching for food items, e.g. earthworms which also act as paratenic hosts (Galvin, 1964; Pahari & Sasmal, 1991; Azizi *et al.*, 2007). Tiara *et al.* (2003) observed that *T. canis* migrates through the liver and may survive for up to 3.5 years. This report suggests that poultry meat infected with *Toxocara* spp. constitutes a potential risk of zoonotic transmission.

The current study describes infected broiler chickens as a paratenic host for human toxocariasis, to investigate the possibility of infection with *Toxocara* spp. larvae through consumption of fresh poultry viscera harbouring migrating *Toxocara* larvae. It aimed to evaluate the detection of *Toxocara* species by enzymatic digestion of tissue and DNA amplification.

Materials and methods

Collection and examination of samples

Newly hatched Cobb chicks were obtained from a commercial broiler hatchery. The experiment was conducted on 33 purchased broiler chickens (both sexes), weighing between 700 and 900 g and 35–47 days old. They were kept in an environment where infected dogs and cats occurred. The chickens did not have any clear symptoms of disease before sampling.

The chickens were caught and moved to an animal pathology laboratory for necropsy. After sedation and euthanasia with thiopental, the visceral organs (duodenum, liver, heart, lungs, kidneys, brain) and skeletal muscles of the animals were fragmented with pointed forceps and examined by binocular stereomicroscopy with 20× and 40× ocular objectives. In the case of brain tissue, each piece was compressed between two glass slides and examined for the presence of larvae of *Toxocara* parasites (squash technique). Thereafter, the tissues were put into digestive solution containing 1% pepsin (1:10,000), 1% HCl (37%) in distilled water and were incubated at 46°C for 5 h with constant stirring. After incubation, the digests were filtered through a system of sieves with 20-μm apertures and larvae were collected from the flow-through. To recover the larvae, the filtrate liquid was poured into a centrifuge tube and centrifuged for 2 min at 1500 rpm, 2-ml samples of sediment were collected, thoroughly mixed and 0.1-ml samples were viewed for larval counts (Azizi *et al.*, 2007). After digestion and four washing steps with distilled water on ice, larvae were stored in 5 μl distilled water at –20°C.

Molecular analysis

DNA was extracted from larvae. In brief, 2 μl of Tris–HCl buffer (50 mM, pH 7.4–7.6) was added to tubes containing *Toxocara* larvae in 5 μl water and sealed with a drop of mineral oil. The mixture was incubated for 10 min at 90°C. Then 0.4 μl proteinase K (20 mg/ml) and 2.6 μl water were added and the mixture was incubated for 3 h at 48°C. Proteinase K was inactivated at 90°C for 10 min. Samples were stored at −20°C. Further DNA extractions were performed using QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The polymerase chain reaction (PCR) amplification was evaluated with DNA samples from the larva species. For this purpose, two primer pairs were used. First, JW4 (forward), which is specific for *T. cati*, described by Li *et al.* (2007) (5′-ACTGTCGAGGATGAGCGTGA-3′) and NC2 (reverse) described by Zhu *et al.* (2002) (5′-TTAGTTT CTTTTCCTCCGCT-3′), to amplify partial internal transcribed spacer-1 (ITS-1), complete 5.8S and ITS-2 of rDNA. A second primer YY1 (forward), specific for *T. canis*, was designed by Jacobs *et al.* (1997) (5′-CGGT GAGCTATGCTGGTGTG-3′) and combined with NC2 to amplify partial ITS-2. Reactions were carried out in a total of 25 μl containing 1 U *Taq* polymerase, *Taq* buffer (50 mM KCl, 10 ml Tris–HCl, pH 8.8), 1.5 mM $MgCl_2$, 200 μM deoxyribonucleoside triphosphates (dNTP), 1 μM primers and 0.1% Triton X-100. Amplifications were performed in a DNA thermocycler. The PCR protocol consisted of an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 94°C for 45 s, 58°C for 45 s and 72°C for 90 s, and a final extension step for 10 min at 72°C. The PCR products were analysed by electrophoresis using 1.4% agarose gel with 0.5 μg/ml ethidium bromide, and a 100-bp ladder was used as the DNA size marker for estimating the size of the amplicons. The gels were photographed with UV illumination using a photodocumentation system (Bio-Rad, Hemel Hempstead, UK).

Results and discussion

Human toxocariasis can occur as a result of ingesting infective eggs from the environment or by eating the meat of paratenic hosts containing larvae (Fisher, 2003). Beaver (1956) hypothesized that organs or tissues of infected animals can serve as sources of *Toxocara* infection

https://doi.org/10.1017/S0022149X16000559 Published online by Cambridge University Press

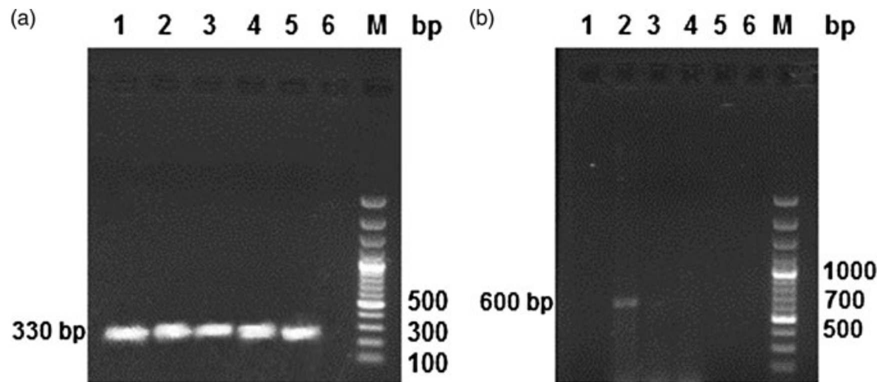

Fig. 1. Analysis of PCR products (*Toxocara* species larvae) by electrophoresis on 1.4% agarose gels with ethidium bromide staining. (a) *Toxocara canis* with YY1/NC2 primers (lanes 1–5); negative (no DNA) control (lane 6); 100–1500 bp molecular weight marker (lane M). (b) *Toxocara cati* with JW4/NC2 primers (lane 2); negative control (lane 6); 100–1500 bp DNA size marker (lane M).

for humans. In the present study, *T. canis* and *T. cati* larvae were recovered ($n = 37$) from 5 of 33 broiler chickens (15.2%). Overall, most larvae were recovered from the liver ($n = 19$), diaphragm muscle ($n = 8$) and duodenum ($n = 8$). Few larvae ($n = 2$) were recovered from the brain tissue of naturally infected broiler chickens.

In recent years, many studies have been published using traditional methods for detection of *Toxocara* larvae in experimentally infected chickens (Galvin, 1964; Maruyama *et al.*, 1994; Tiara *et al.*, 2012; Dutra *et al.*, 2014). Analysing the migration of *Toxocara* larvae using the pepsin digestion method, Tiara *et al.* (2003) found high numbers of *Toxocara* larvae in the duodenum, liver, lungs, heart, brain and muscles of infected chickens. There are several reports indicating infection of humans with *Toxocara* larvae after eating raw or undercooked infected chicken meat (Ito *et al.*, 1986; Nagakura *et al.*, 1989), raw beef liver (Tiara *et al.*, 2011), raw pig liver (Stürchler *et al.*, 1990) and raw lamb liver (Salem & Schantz, 1992). In an early study, Inoue (1987) experimentally inoculated *T. canis* eggs into the gizzard of chickens and observed migrating larvae in the liver. The infected livers were then fed to mice and were capable of infecting them. This study was the first to demonstrate that broiler chickens can be a reservoir for *Toxocara* species larvae.

PCR based upon the amplification of ITS-1 and ITS-2 fragments revealed that 5 of 33 chickens showed a positive reaction to *T. canis* and *T. cati*. In the present study, two primer sets were selected to detect *Toxocara* spp. During electrophoretic analysis of PCR products, bands were observed in samples of the larvae. Under the optimized cycling conditions, primer set JW4/NC2 amplified a product of 600 bp solely from *T. cati*, ($n = 1$, 16.7%) and primer set YY1/NC2 amplified a product of 330 bp uniquely from *T. canis* ($n = 5$, 83.3%) (fig. 1). One broiler chicken was co-infected with both *Toxocara* species. The results of nucleotide sequences of the PCR products of genes were identical to those deposited in GenBank.

Molecular studies on *Toxocara* have been undertaken over two decades. Restriction profiles have been used not only to detect genetic, interspecific differences between *T. canis* and *T. cati* but also sexual differences (Gasser, 2013). Using PCR techniques, Wu *et al.* (1997) 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. 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* 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. However, in the present study, by applying a digestive technique, larvae were recovered for genetic analysis, independent not only of the developmental stage of the larvae but also of the number of larvae. This is important, as in experimental studies for the detection of larvae more than 100 *Toxocara* larvae at various stages of development can be found in tissue samples. Therefore, this PCR method, which was used previously only for worms from tissue obtained from experimentally infected hosts, can be used for studies on natural infections (Zibaei *et al.*, 2013).

In conclusion, the results of this study demonstrate that broiler chickens can be natural paratenic hosts for the larvae of *Toxocara* species, and these larvae in poultry may be agents of human toxocariasis when humans consume raw, undercooked chicken meat. These findings also indicate that the molecular method has considerable potential for the detection and identification of *T. canis* and *T. cati* larvae in poultry tissues.

Acknowledgements

We wish to thank the staff of the Parasitology Laboratory, Lorestan University of Medical Sciences for their help and technical assistance.

Financial support

This research received no specific grant from any funding agency, commercial or not-for-profit sectors.

https://doi.org/10.1017/S0022149X16000559 Published online by Cambridge University Press

Conflict of interest

None.

Ethical standards

This study was carried out according to the ethical framework for research animal welfare and ethics of Lorestan University of Medical Sciences and was approved by the Ethical Committees of Lorestan University of Medical Sciences.

References

Azizi, S., Oryan, A., Sadjjadi, S.M. & Zibaei, M. (2007) Histopathologic changes and larval recovery of *Toxocara cati* in experimentally infected chickens. *Parasitology Research* **102**, 47–52.

Beaver, P.C. (1956) Parasitological reviews: larva migrans. *Experimental Parasitology* **5**, 587–621.

Campos-da-Silva, D.R., da Paz, J.S., Fortunato, V.R., Beltrame, M.A., Valli, L.C. & Pereira, F.E. (2015) Natural infection of free-range chickens with the ascarid nematode *Toxocara* sp. *Parasitology Research* **114**, 4289–4293.

Despommier, D. (2005) Toxocariasis: clinical aspects, epidemiological, medical ecology and molecular aspects. *Clinical Microbiology Reviews* **16**, 265–272.

Dutra, G.F., Pinto, N.S., de Avila, L.F., Dutra, P.C., Telmo Pde, L., Rodrigues, L.H., Silva, A.M. & Scaini, C.J. (2014) Risk of infection by the consumption of liver of chickens inoculated with low doses of *Toxocara canis* eggs. *Veterinary Parasitology* **203**, 87–90.

Fisher, M. (2003) *Toxocara cati*: an underestimated zoonotic agent. *Trends in Parasitology* **19**, 167–170.

Galvin, T.J. (1964) Experimental *Toxocara canis* infections and pigeons. *Journal of Parasitology* **50**, 124–127.

Gasser, R.B. (2013) A perfect time to harness advanced molecular technologies to explore the fundamental biology of *Toxocara* species. *Veterinary Parasitology* **193**, 353–364.

Inoue, H. (1987) Studies on visceral larva migrans. Infectivity of *Toxocara canis* larvae from paratenic host and antibody titers in rats. *Medical Journal of Hiroshima University* **35**, 1417–1429.

Ito, K., Sakaei, K., Okajima, T., Ouchi, K., Funakoshi, A., Nishimura, J., Ibayashi, H. & Tsuji, M. (1986) Three cases of visceral larva migrans due to ingestion of raw chicken or cow liver. *Nihon Naika Gakkai Zasshi* **75**, 759–766.

Jacobs, D.E., Zhu, X., Gasser, R.B. & Chilton, N.B. (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., Lin, R.Q., Chen, H.H., Sani, R.A., Song, H.Q. & Zhu, X.Q. (2007) PCR tools for the verification of the specific identity of ascaridoid nematodes from dogs and cats. *Molecular and Cellular Probes* **21**, 349–354.

Maruyama, S., Nino, T., Yamamoto, K. & Katsube, Y. (1994) Parasitism of *Toxocara canis* larvae in chickens inoculated with the ascariid eggs. *Journal of Medical Sciences* **56**, 139–141.

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

Pahari, T.K. & Sasmal, N.K. (1991) Experimental infection of Japanese quail with *Toxocara* larvae through earthworms. *Veterinary Parasitology* **39**, 337–340.

Park, B.M., Jeong, S.O., Park, H.S., Jung, S.S., Kim, S.Y., Kim, J.O. & Lee, J.E. (2014) Differences in the clinical and radiological characteristics of lung-involved toxocariasis between toxocariasis with eosinophilia and those without eosinophilia. *Journal of Thoracic Disease* **6**, 1757–1764.

Pawlowski, Z. (2001) Toxocariasis in humans: clinical expression and treatment dilemma. *Journal of Helminthology* **75**, 299–305.

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

Schantz, P.M. (1989) *Toxocara* larva migrans now. *American Journal of Tropical Medicine and Hygiene* **41**, 21–34.

Strube, C., Heuer, L. & Janecek, E. (2013) *Toxocara* spp. infections in paratenic hosts. *Veterinary Parasitology* **193**, 375–389.

Stürchler, D., Weiss, N. & Gassner, M. (1990) Transmission of toxocariasis. *Journal of Infectious Diseases* **162**, 571.

Tiara, K., Permin, A. & Kapel, C.M. (2003) Establishment and migration pattern of *Toxocara canis* larvae in chickens. *Veterinary Parasitology* **9**, 521–523.

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

Tiara, K., Saitoh, Y., Okada, N., Sugiyama, H. & Kapel, C.M. (2012) Tolerance to low temperatures of *Toxocara cati* larvae in chicken muscle tissue. *Veterinary Parasitology* **189**, 383–386.

Wu, Z., Nagano, I., Xu, D. & Takahashi, Y. (1997) Primers for polymerase chain reaction to detect genomic DNA of *Toxocara canis* and *T. cati*. *Journal of Helminthology* **71**, 77–78.

Zhu, X.Q., D'Amelio, S., Palm, H.W., Paggi, L., George-Nascimento, M. & Gasser, R.B. (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., Sadjjadi, S.M. & Uga, S. (2010) Experimental *Toxocara cati* infection in gerbils and rats. *Korean Journal of Parasitology* **48**, 331–333.

Zibaei, M., Sadjjadi, S.M., Karamian, M., Uga, S., Oryan, A. & Jahadi-Hosseini, S.H. (2013) A comparative histopathology, serology and molecular study on experimental ocular toxocariasis by *Toxocara cati* in Mongolian gerbils and Wistar rats. *BioMedical Research International* **2013**, e109580.

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