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Molecular characterization of *Toxocara* spp. eggs isolated from public parks and playgrounds in Shiraz, Iran

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Abstract

Human toxocariasis, a worldwide parasitic disease, is caused by the larval stage of intestinal nematodes of dogs and cats, namely *Toxocara canis* and *Toxocara cati.* Human infection occurs by the accidental ingestion of embryonated eggs present in the soil, vegetables or on other contaminated surfaces, as well as via consumption of uncooked paratenic hosts, such as bird meat and giblets. The objective of this study was to evaluate the contamination of soil in public parks and playgrounds in Shiraz using microscopy and molecular methods. A total of 150 soil samples were collected from public parks and playgrounds in various areas of Shiraz, southern Iran. The samples were treated with saturated zinc sulphate solution, and *Toxocara* spp. eggs were detected by microscopic observation followed by nested polymerase chain reaction (PCR). To differentiate *T. canis* and *T. cati* eggs from each other, PCR restriction fragment length polymorphism (RFLP) of the internal transcribed spacer (ITS)-rDNA region by *Sal*I endonuclease enzyme was used. PCR-sequencing was performed to confirm the results of the PCR-RFLP method. Based on the flotation results of the 150 soil samples, six (4%) were found to be positive for *Toxocara* spp. eggs, whereas nested-PCR showed 24 samples to be positive (16%). Based on the PCR-RFLP method and the sequence of the ITS-rDNA region, a total of 23 out of 24 isolates were confirmed as *T. cati* and one out of 24 as *T. canis*. The results showed a higher number of soil samples to be positive for *Toxocara* by the molecular method than microscopy, and higher *T. cati* infection in soil samples, which could have an important role in human infection with toxocariasis in this region.

Introduction

Toxocariasis is a worldwide zoonotic parasitic disease caused by the larval stage of intestinal nematodes of dogs and cats, namely *Toxocara canis* and *Toxocara cati* (Despommier, 2003). Human infection occurs by the accidental ingestion of embryonated eggs present in the soil, in vegetables or on other contaminated surfaces, as well as via consumption of the uncooked meat of paratenic hosts such as chicken, cattle, pigs or earthworms (Ito *et al.*, 1986; Cianferoni *et al.*, 2006; Azizi *et al.*, 2007; Choi *et al.*, 2012; Zibaei *et al.*, 2017). Dogs and cats are definitive hosts of *Toxocara*, whose expelled eggs become embryonated and infective in the soil after three to six weeks, depending on soil type and climatic conditions. Infective *Toxocara* eggs can remain viable in the soil for months to years under optimal conditions (Overgaauw, 1997; Lescano *et al.*, 1998). Therefore, the soil is considered to be the source of transmission of *Toxocara* infection to humans, especially children at play in public parks and playgrounds contaminated with *Toxocara* eggs (Despommier, 2003).

The clinical symptoms of toxocariasis may vary from an asymptomatic infection to severe infection and there are several forms of toxocariasis, namely, visceral larva migrans (VLM), ocular larva migrans (OLM), covert toxocariasis and neurotoxocariasis (Rubinsky-Elefant *et al.*, 2010). VLM is caused by the inflammatory response to the larval migration through vital organs and tissues of the body. OLM can lead to partial or total loss of vision (Schantz, 1989; Tan, 1997; Zibaei *et al.*, 2014). Epidemiological studies have indicated that the prevalence of human toxocariasis in various parts of Iran ranges from 1.39 to 34.48% (Sadjjadi *et al.*, 2000; Akhlaghi *et al.*, 2006; Fallah *et al.*, 2007; Sharif *et al.*, 2007; Nourian *et al.*, 2008; Alavi *et al.*, 2009; Maraghi *et al.*, 2012; Hosseini-Safa *et al.*, 2015; Shahraki *et al.*, 2017).

Epidemiological surveys have confirmed the infection of dogs and cats with *Toxocara* in Iran (Zibaei & Sadjjadi, 2017). The prevalence of *T. cati* in cats and *T. canis* in dogs has been estimated to vary from 8–78% and 6.3–29%, respectively, in different parts of Iran (Sadjjadi *et al.*, 2001; Changizi *et al.*, 2007; Zibaei *et al.*, 2007; Mikaeili *et al.*, 2013; Emamapour *et al.*, 2015; Sardarian *et al.*, 2015; Hajipour *et al.*, 2016; Vafae Eslahi *et al.*, 2017). There have been some reports of contamination of the soil with *Toxocara* eggs in public areas in Iran. Examination of soil samples from public parks in Shiraz indicated that seven out

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of 112 samples (6.3%) were infected with *Toxocara* eggs (Motazedian *et al.*, 2006). An investigation was carried out for the presence of *Toxocara* spp. eggs in public parks in the city of Urmia and the results showed a contamination rate of 7.8% (Tavassoli *et al.*, 2008). In Khoramabad, the distribution of *Toxocara* spp. eggs in samples collected from public parks was 63.3% (Zibaei *et al.*, 2010). In the city of Tehran 10–38.7% of public places were found to be contaminated with *Toxocara* spp. eggs (Khazan *et al.*, 2012; Tavalla *et al.*, 2012). The contamination rate of *Toxocara* spp. eggs in the public parks of Tabriz city was found to be 9.3% (Garedaghi & Shabestari, 2012). A study of the contamination of soil in public parks in Abadan showed that 29.2% samples were infected with *Toxocara* spp. eggs (Maraghi *et al.*, 2014). In an evaluation of 195 soil samples from Mashhad and 145 soil samples from Khaf city, 18 (9.2%) and 16 cases (11.3%) of contamination with *Toxocara* spp. eggs were detected, respectively (Berenji *et al.*, 2015). Studies in Kermanshah found that 13.5–18% of soil samples were infected with eggs of *Toxocara* spp. (Ghashghaei *et al.*, 2016; Maleki *et al.*, 2016). Pezeshki *et al.* (2017) showed that *Toxocara* spp. eggs were found in 14 (7%) out of 200 soil samples collected from public places in Ardabil city.

The identification of *Toxocara* species is important for planning of prevention and control programmes in human and animal communities (Deplazes *et al.*, 2011; Gawor *et al.*, 2014). The differentiation of *Toxocara* spp. eggs based on morphological features is difficult because of their similarity to other species in terms of morphology and size (Fogt-Wyrwas *et al.*, 2007, Borecka & Gawor, 2008; Zibaei & Sadjjadi, 2010). Therefore, molecular methods such as polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and PCR-sequencing are recommended for the identification of *Toxocara* spp. eggs from soil samples. The study conducted by Borecka (2004) for the differentiation of *Toxocara* spp. eggs isolated from soil by molecular methods showed that internal transcribed spacer 2 (ITS2) PCR products of *T. cati* and *T. canis* were similar in size, and the PCR-RFLP technique produced specific banding patterns, with three fragments for *T. cati* and two fragments for *T. canis*. Therefore, this method is useful for characterization to the species level of *Toxocara* spp. eggs isolated from soil. Khademvatan *et al.* (2014) used microscopy and molecular methods for detection and identification of soil contamination by *Toxocara* eggs in Ahvaz. *Toxocara* eggs were found in 64 (30.4%) and 71 (33.8%) out of 210 soil samples using microscopy and PCR methods, respectively. Based on ITS2 PCR-sequencing, 28% of the samples were diagnosed as *T. cati* and 5.7% as *T. canis*; no mixed contamination was reported in their observations.

Shiraz is a city with a high prevalence of *Toxocara* infection in cats (26.7–52.8%) (Sadjjadi *et al.*, 2001; Zibaei *et al.*, 2007; Mikaeili *et al.*, 2013). However, no previous study has conducted molecular identification of *Toxocara* spp. eggs isolated from the soil in Shiraz. The objective of this study was to evaluate soil contamination in public parks and playgrounds in Shiraz using microscopy and molecular methods simultaneously.

Materials and methods

Sample collection

This cross-sectional study was carried out during June–August 2015 in Shiraz, the capital city of Fars province, in southern Iran. A total of 150 soil samples were collected from 50 public parks and playgrounds from different areas (north, south, east, west and centre) of Shiraz. The soil samples were collected randomly from three sites in each park, from a depth of 10 cm. Samples (200 g each) were placed in labelled containers and transported to the laboratory.

Microscopy method

For detection of *Toxocara* spp. eggs, the soil samples were dried at room temperature for 24 hours and sifted through 80 and 100 µm mesh sieves. The samples were treated with saturated zinc sulphate solution (specific gravity 1.8) according to the method described by Borecka (2004).

Molecular method

DNA extraction and nested PCR

The genomic DNA from all 150 soil samples was extracted using the Tissue Genomic DNA Extraction Mini kit with proteinase (Yekta Tajhiz Azma, Tehran, Iran), according to the manufacturer's instructions, with a minor modification. After zinc sulphate flotation, all contents on the slide were washed with TG1 buffer into a tube. The samples were subjected to three freeze–thaw cycles followed by proteinase K digestion overnight.

The ribosomal DNA region (partial sequence of ITS1 and ITS2) was subjected to analysis using nested PCR reaction. The forward primer (NC5: 5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and reverse primer (NC2: 5′-TTAGTTTCTTTTCCTCCGCT-3′) were used for amplification of the ITS region for the first step of nested-PCR (Li *et al.*, 2007). The first PCR reactions were performed in a final volume of 25 µl. Each reaction contained 12.5 µl of PCR mix (2× Master Mix RED; Ampliqon, Denmark), which included 1.25 U Taq DNA polymerase, 200 µM of deoxynucleotide triphosphate (dNTP) and 1.5 mM $MgCl_2$; 12.5 pmol of each primer and 5 µl of template DNA in an automated thermocycler. The temperature profile was one cycle of 95°C for 6 minutes to denature the double-stranded DNA, followed by 35 cycles of 94°C for 45 s (denaturation), 60°C for 1 minute (annealing), 72°C for 1 minute (extension) and a final extension of 72°C for 6 minutes.

The second step of nested-PCR reactions was performed using forward primer (FM1: 5′-TTGAGGGGAAATGGGTGAC-3′) and reverse primer (FM2: 5′-TGCTGGAGGCCATATCGT-3′) in a 25 µl reaction volume (Mikaeili *et al.*, 2017). 5 µl of the product obtained in the first step of nested-PCR was used as a template for the second step. An initial denaturation step at 94°C for 12 minutes was followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extended at 72°C for 1 minute, with a final extension step at 72°C for 5 minutes. Double-distilled water (DDW) instead of template DNA was included in each set of PCR reactions as negative control. The PCR products were separated by electrophoresis on a 1.5% agarose gel and stained with GelRed nucleic acid gel stain (Biotium, Fremont, USA), 10,000× in water.

PCR-RFLP method

Nested PCR products (5 µl) were digested directly with 0.5 µl restriction enzyme *Sal*I (ER0551, ER0641; Thermo Fisher Scientific, Waltham, USA) in a final volume of 15 µl for 3 h at 37°C. Restriction fragments of amplicons were separated on 2% agarose gel in triethanolamine (TAE) buffer. Gels were transilluminated in ultraviolet light and photographed. Digestion of the

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ITS products of the two *Toxocara* species with *Sal*I endonuclease showed specific banding patterns, i.e. producing two fragments for *T. canis* but no digestion for the ITS PCR products of *T. cati* isolates (Mikaeili *et al.*, 2017).

Sequencing and phylogenetic analysis

PCR-sequencing was performed to confirm the results of the PCR-RFLP method. The PCR products were purified using the EasyPure Quick Gel Extraction Kit (TransGen Biotec, Beijing, China), according to the manufacturer's instructions, and were submitted to sequencing in two directions using the primers employed in the second step of nested-PCR. Sequence results were edited using the Geneious software (www.geneious.com) and compared with GenBank reference sequences using BLAST (http://www.ncbi.nlm.nih.gov/). A phylogenetic tree was constructed with ITS sequences obtained in the present study along with reference sequences deposited in GenBank and reference sequences of *Toxascaris leonina* as outgroup using the maximum likelihood method in the MEGA 5.0 software (Tamura *et al.*, 2011). Bootstrap analyses (using 1,000 replicates) were carried out to determine the robustness of the finding. The sequences reported here were deposited in GenBank.

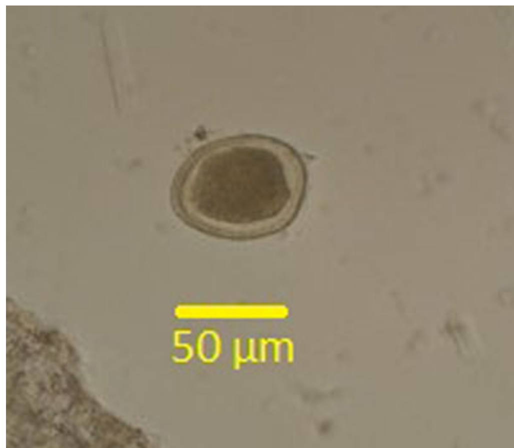

Fig. 1. Egg of *Toxocara* spp. isolated from one of the soil samples taken from public parks and playgrounds in Shiraz, Iran.

Results

Eggs of *Toxocara* spp. were found in six (4%) of the 150 soil samples using the zinc sulphate flotation method (fig. 1). All soil samples were examined by the nested-PCR method using the ribosomal DNA region. From 150 soil samples, six (4%) amplicons of about 1000–1100 bp were successfully produced by the first step of nested-PCR, whereas 24 (16%) samples produced amplicons of about 700 bp only after the second step of nested-PCR; no amplification was observed in the negative controls (fig. 2). Table 1 shows the contamination rate of the soil of public parks and playgrounds in Shiraz according to geographical area.

The nested-PCR products of *Toxocara* spp. were similar in size, therefore the PCR-RFLP differentiated *T. canis* and *T. cati* from each other and showed specific banding patterns. Digestion with *Sal*I endonuclease produced two fragments of 320 and 394 bp for *T. canis* and an undigested band of 736 bp for *T. cati* (fig. 3). Based on the PCR-RFLP method, a total of 23 out of 24 isolates were confirmed as *T. cati* and one out of 24 as *T. canis*.

Sequence analysis was performed for 12 randomly selected nested-PCR products to confirm the PCR-RFLP results. The edited sequences were compared with other available sequences in GenBank using the BLAST system, and 11 isolates were identified as *T. cati* and only one as *T. canis*. The consensus sequences determined in this study were deposited in GenBank with the accession numbers MF592391–MF592402.

The consensus phylogenetic tree indicated that 11 isolates of *T. cati* obtained in the current study based on ITS sequences divided into two haplotypes, and intraspecies variation was 0–0.2%. In this study, the ITS sequence of *T. canis* isolated from soil samples had a 100% homology with *T. canis* isolated from fox in China (accession no. JF837169) and dog in Iran (accession no. KF577855) (fig. 4). Interspecies sequence differences among *T. cati* and *T. canis* isolated from soil samples were significantly higher, being 15–15.3%.

Discussion

The infection of dogs and cats with *T. canis* and *T. cati* in various parts of Iran has been reviewed by Zibaei & Sadjjadi (2017). A study in Shiraz found that a total of 57 out of 108 stray cats (52.8%) were infected with *T. cati* (Sadjjadi *et al.*, 2001). Zibaei *et al.* (2007) reported the prevalence of *T. cati* and other intestinal

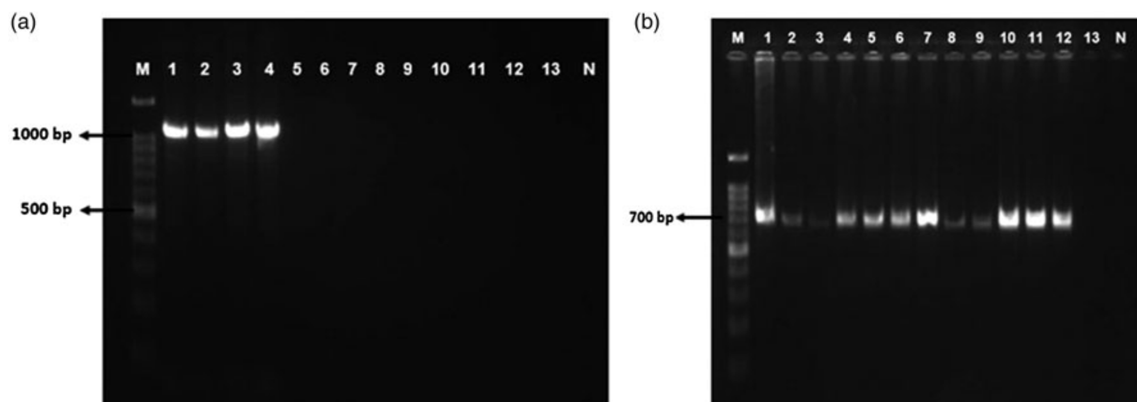

Fig. 2. Agarose gel electrophoresis of (a) the first step and (b) the second step of nested-PCR products. M: 100 bp DNA marker; N: negative control; lane1: positive control; lanes 2–13: soil samples.

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Table 1. Contamination rate of soil samples from public parks and playgrounds in Shiraz, Iran with *Toxocara* spp. eggs, based on microscopy and molecular methods.

Area	No. of samples	Microscopy method		Molecular method	
		No. of positive samples (%)	95% CI	No. of positive samples (%)	95% CI
North	33	0 (0)	0	9 (27.3)	12.2–45.5
West	30	2 (6.7)	0.01–16.7	5 (16.7)	3.3–30
East	30	0 (0)	0	2 (6.7)	0.01–16.7
Centre	30	3 (10)	0.01–23.3	7 (23.4)	10–40
South	27	1 (3.7)	0.01–11.1	1 (3.7)	0.01–11.1
Total	150	6 (4)	1.3–7.3	24 (16)	10–22.7

helminths in stray cats in Shiraz and their results revealed that *T. cati* was one of the most frequently detected intestinal helminths (42.6%). Another study showed that the infection rate of *T. cati* among 30 stray cats in Shiraz was 26.7% (Mikaeili *et al.*, 2013). In a study of intestinal helminths in stray dogs in Mashhad, north-east Iran, the prevalence of infection with *T. canis* was 29% (Emamapour *et al.*, 2015). A similar study investigated the prevalence of zoonotic intestinal parasites in household and stray dogs in rural areas of Hamadan, in western Iran; the results indicated that *T. canis* was the most frequently detected parasite, with a prevalence of 6.3% (Sardarian *et al.*, 2015).

Several studies have been carried out on contamination of soil with *Toxocara* spp. eggs in Iran. Motazedian *et al.* (2006) reported the prevalence of helminth eggs in soil samples from public places and children's playgrounds in Shiraz. In their study, *T. cati* eggs were found in seven out of 112 soil samples (6.3%), using saturated zinc sulphate flotation. However, it is not clear from the methodology how *T. cati* eggs were differentiated from other *Toxocara* spp. eggs, which is difficult using light microscopy. On the basis of their observations, it can be argued that the conclusions reported by Motazedian *et al.* (2006) are not entirely supported by their results (Zibaei & Sadjjadi, 2010). In our survey, using the saturated zinc sulphate flotation method, 4% of the soil samples from public parks and playgrounds were contaminated with *Toxocara* spp. eggs, which was almost the same as

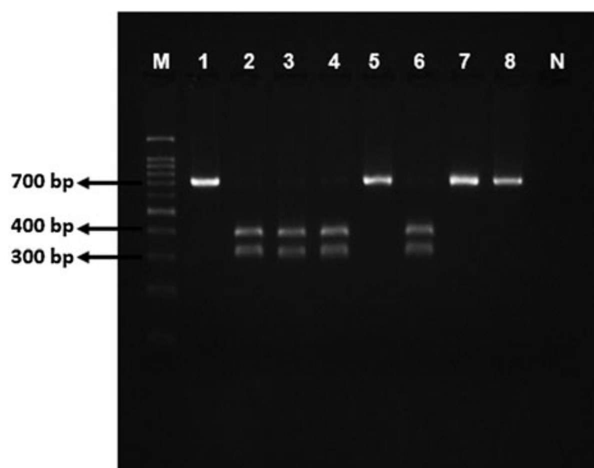

Fig. 3. PCR-RFLP pattern of *Toxocara* after digestion with *Sal*I restriction enzyme. M: 100 bp DNA marker; N: negative control; lane 1: positive control (*T. cati*); lanes 2, 3, 4: positive control (*T. canis*); lanes 5, 6, 7, 8: soil samples.

the findings of Motazedian *et al.* (2006) for Shiraz. In another study, a total of 580 soil samples were collected from different areas of Sari, northern Iran. The soil contamination with *Toxocara* spp. eggs was reported to be 3.73%, using the saturated sucrose flotation method (Hezarjaribi *et al.*, 2016). Saraei *et al.* (2012) reported a low rate of soil contamination with *Toxocara* eggs (3.15%) in public parks of Qazvin, Iran. The rate of contamination of soil with *Toxocara* eggs in our study was lower than in some previous studies conducted in different areas of Iran. A study undertaken in Khorram Abad found that the distribution of *Toxocara* spp. eggs in samples collected from public parks was 63.3%, using the saturated sucrose flotation method (Zibaei *et al.*, 2010). In a study conducted in Tehran city during 2008–2009 on 150 soil samples collected from various sites, *Toxocara* eggs were observed in 38.7% of the samples using sodium nitrate flotation (Tavalla *et al.*, 2012). A study carried out in Khuzestan found that 29.2% of the soil samples were infected with *Toxocara* spp. eggs using the modified flotation method using saturated sucrose (Maraghi *et al.*, 2014).

Studies carried out in various countries have indicated that the contamination rate varies markedly in different regions of the world. A total of 5.71% of soil samples in Thailand (Wiwanitkit & Waenlor, 2004), 11.9% of soil samples in the Czech Republic (Dubná *et al.*, 2007), 0.8% of soil samples in Costa Rica (Paquet-Durand *et al.*, 2007), 9% of soil samples in Japan (Zibaei & Uga, 2008), 16.4% of soil samples in Spain (Dado *et al.*, 2012), 4.75% of soil samples in India (Thomas & Jeyathilakan, 2014), 77% of soil samples in the Philippines (Paller & de Chavez, 2014) and 22.2% of soil samples in Iraq (Taher, 2017) have been reported to be positive for *Toxocara* spp. eggs.

The differences in contamination rate depend on a variety of factors, including soil type, climate, soil collection method, flotation method, number of infected stray cats and dogs in the region, culture, and people's interest in keeping cats and dogs as pets.

Soil contamination with *T. canis* and *T. cati* eggs is considered to be the main source of human toxocariasis (Nijsse *et al.*, 2015). In most studies, soil contamination with *Toxocara* spp. eggs has been investigated using microscopy and the results have been reported only for the genus *Toxocara* (Zibaei *et al.*, 2010; Maraghi *et al.*, 2014; Hezarjaribi *et al.*, 2016). *Toxocara* spp. eggs are similar in size and shape. Although it is possible to differentiate them by electron microscopy, their identification by light microscopy is not possible (Holland & Smith, 2006; Zibaei & Sadjjadi, 2010). Molecular methods such as PCR-RFLP, PCR-sequencing and PCR using species-specific primers are the most suitable techniques for the differentiation of *Toxocara* spp.

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Fig. 4. Phylogenetic relationship of ITS sequences of *Toxocara cati* and *Toxocara canis* isolates obtained in this study and reference sequences retrieved from GenBank. *Toxascaris leonina* (AN: KF577861) was used as the outgroup.

eggs isolated from the soil (Borecka, 2004; Fahrion *et al.*, 2011). The differentiation of *Toxocara* spp. eggs is essential for serological studies and the planning of prevention and control programmes in human and animal communities. For the first time, Borecka (2004) used a PCR-linked RFLP method for the differentiation of *Toxocara* spp. eggs isolated from the soil. PCR-RFLP was useful for differentiating eggs of *Toxocara* spp. isolated from the soil to species level. The PCR products for ITS2 of *T. cati* and *T. canis* were similar in size. Digestion of the purified ITS2 products of the two *Toxocara* species with *Rsa*I endonuclease produced specific banding patterns. In our study, molecular identification of *Toxocara* spp. eggs isolated from the soil was performed using PCR-RFLP and PCR-sequencing methods. The binding patterns of the nested-PCR for all samples were similar in size and this method alone could not differentiate *Toxocara* spp. eggs. PCR-RFLP with *Sal*I endonuclease produced two fragments of 320 and 394 bp for *T. canis* and an undigested band of 736 bp for *T. cati.* Otero *et al.* (2017) used molecular analysis and sequencing of the 18S rRNA gene to discriminate between *Toxocara* species in soil samples collected from public parks and playground sandpits in Portugal, and their results showed that 53% of soil samples were positive for *Toxocara* spp. and only *T. cati* eggs were found in soil samples Other authors who detected *Toxocara* spp. eggs in the environment used molecular methods with species-specific primers (Khademvatan *et al.*, 2014; Ozlati *et al.*, 2016; Gao *et al.*, 2017; Studzińska *et al.*, 2017).

In the current study, the molecular method indicated that 16% of soil samples were contaminated with *Toxocara* spp., which was higher than the percentage indicated by the microscopy method (4%), while Ozlati *et al.* (2016) found *Toxocara* spp. eggs in 57 (31.6%) and 14 (7.7%) soil samples using the microscopy and PCR methods, respectively, which is inconsistent with our findings. Khademvatan *et al.* (2014) used the microscopy and molecular methods for detection and identification of soil contamination by *Toxocara* eggs in Ahvaz, southwestern Iran. Nucleotide sequencing was performed to confirm the results of the PCR method. *Toxocara* eggs were found in 30.4% and 33.8% of soil samples using the microscopy and PCR methods, respectively, and there was no statistically significant difference between the two methods (PV = 0.279).

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Based on PCR-RFLP and PCR-sequencing methods, we found a total of 23 out of 24 isolates were confirmed as *T. cati* and one out of 24 as *T. canis* without any mixed contamination. Similar to our study, Khademvatan *et al.* (2014) indicated that *T. cati* was more prevalent than *T. canis* (28% and 5.7%, respectively), and no mixed contamination was observed. In contrast to our study, Ozlati *et al.* (2016) identified *T. canis* in 15.5% of soil samples, *T. cati* in 27.2% of soil samples and mixed infections in 12.2% of soil samples.

In conclusion, this study revealed that a higher contamination rate was observed using the molecular method (16%) in comparison to the microscopy method (4%). PCR-RFLP and PCR-sequencing methods are the most suitable technique for the differentiation of *Toxocara* spp. eggs.

As soil contamination with *Toxocara vitulorum* eggs is possible, and the morphological identification of *Toxocara* spp. eggs is difficult, molecular methods have been used for the characterization of species (Gasser *et al.*, 2006; Fahrion *et al.*, 2011; Pawar *et al.*, 2012). In the present study, the rate of soil contamination with *T. cati* eggs was much higher than that with *T. canis* eggs, which is in accordance with reports of the infection rate of *T. cati* among stray cats in Shiraz (Sadjjadi *et al.*, 2001; Mehrabani *et al.*, 2002; Zibaei *et al.*, 2007; Mikaeili *et al.*, 2013). Soil contamination with *Toxocara* spp. eggs is considered to be the main source of toxocariasis in humans, and children are the main group exposed to contamination because of their high contact with soil when playing in public parks and playgrounds; therefore, prevention and control programmes in human and animal communities are needed. Our examination of 150 soil samples collected from various sites in 50 parks in Shiraz gives an indication of the level of soil contamination with *Toxocara* eggs in the town. However, the higher rate of infection of soil samples with *T. cati* eggs may play the most important role in human infection with toxocariasis in this urban environment.

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Conflict of interest. None.

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