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Prevalence of *Toxocara* eggs in soil samples from public areas in Ilam city, Western Iran

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Abstract

Background: Human toxocariasis is a neglected zoonotic disease with a global distribution. This study aimed to assess the level of soil contamination in public spaces within Ilam city by examining the presence of *Toxocara* species eggs, utilizing both microscopic and molecular techniques.

Methods: Fifty soil samples were collected from various regions of Ilam city in western Iran. The samples were concentrated using saturated $ZnSO_4$, and the eggs of the genus *Toxocara* were observed through microscopic examination. Polymerase chain reaction (PCR) and DNA sequencing were then utilized to differentiate between *Toxocara canis* and *Toxocara cati*. Subsequently, multiple alignment and phylogenetic analyses were conducted to determine the genetic relationship species identified in this study with those previously submitted to GenBank.

Results: *Toxocara* eggs were found in 7 (14%) and 6 (12%) out of 50 soil samples using microscopic and PCR methods, respectively. Based on the ribosomal DNA (rDNA) sequence, 5 (10%) samples were diagnosed as *T. canis* and 1 (2%) sample as *T. cati*.

Conclusion: The results of this research revealed a relatively high prevalence of *Toxocara* species in the soil of public places in Ilam city . Since *Toxocara* species are associated with a range of health complications, health authorities must prioritize the development and implementation of health initiatives and preventive strategies.

Keywords: *Toxocara*, Soil, Prevalence, Zoonoses, Iran

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Introduction

Toxocariasis is a zoonotic parasitic disease found worldwide. It is caused by gastrointestinal nematodes in dogs and cats, including *Toxocara canis* or *Toxocara cati* as their definitive hosts, respectively (1,2). Each adult female worm can lay around 200 000 eggs daily, which are then excreted in the feces of dogs and cats. These eggs contaminate various environments such as green spaces, parks, and beaches, where they can develop into infective stages under optimal soil and climate conditions. These contaminated soils serve as the main sources of *Toxocara* eggs (3-5). *Toxocara* infection can be transmitted to humans in several ways. The most common route is through the ingestion of embryonated eggs of *Toxocara* spp that are excreted in contaminated soil, water, fruit, or vegetables (6,7). Another way of transmission is consuming raw or undercooked meat from paratenic hosts, such as birds, cattle, sheep, and pigs, which may contain encapsulated second-stage larvae (5,8). Furthermore, transmission to humans can occur when a person accidentally swallows *Toxocara* eggs through hands contaminated due to contact with the hair of infected animals (9). Finally, the larvae are released into the small intestine of humans, penetrate the intestinal wall, and migrate to the liver, heart, lungs, and other organs, leading to toxocariasis (4,6,7). *Toxocara* infection is commonly asymptomatic in humans. However, if the larvae migrate to or colonize human organs, tissue infection triggers an inflammatory immune response in the body. This can lead to damage and a variety of clinical symptoms, including weight loss, fever, cough, dyspnea, headache, generalized lymphadenopathy, hepatosplenomegaly, and meningoencephalitis (10). Overall, human toxocariasis is categorized into different clinical forms depending on the organs affected. Common toxocariasis, also known as covert toxocariasis, is often described as a self-limited febrile disease. Visceral and ocular larva migrans are severe symptoms of the disease, affecting the internal organs and eyes, respectively. Neurotoxocariasis is another severe form of the disease

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that can occur when *Toxocara* larvae migrate to the brain and spinal cord. However, this manifestation of the disease has been poorly documented (10-14). Despite the increasing awareness of this infection, there has been a rising increase in the known clinical forms of human toxocariasis. As a result, it remains a major public health problem in some impoverished societies (3,5,15,16). The eggs of *Toxocara* species appear to have a high degree of similarity, rendering microscopic techniques inadequate for distinguishing among the various species of these parasites, especially when the eggs are extracted from soil samples. Hence, identifying *Toxocara* species through molecular techniques is essential for health planning and the management of animal-borne diseases. To the best of our knowledge, no study has been conducted on the molecular identification of *Toxocara* species in Ilam province. Therefore, this study investigated the rate of soil contamination with *Toxocara* eggs in public areas in Ilam city , western Iran, using microscopic examination followed by polymerase chain reaction (PCR).

Materials and Methods

Study area

This study was conducted from May to September 2020 in Ilam city , Ilam province, west of Iran, located between 31°58' and 34°15' N and 45°24' and 48°10' E (17). The climate in Ilam is warm and temperate, with an average annual rainfall of 67.35 mm.

Soil sample collection

A total of 50 soil samples were randomly collected from five regions (center, north, south, west, and east) covering public areas in Ilam city . In each designated area, approximately 50 g of soil samples were randomly collected from three different points. The samples were then combined and thoroughly mixed. Each sample was taken from a depth of 3-5 cm within the soil. Out of these samples, 30 were obtained from parks and green spaces, 10 from squares, and 10 from boulevards. The selection of these samples was determined by the size and quantity of the areas, as well as the population density of animals in the regions. The samples were placed in polyethylene bags, labeled by geographical area, and then transferred to the Parasitology laboratory of Ilam University of Medical Sciences for examination. Both microscopic and PCR-based sequencing methods were used to estimate the percentage of soil contamination with *Toxocara* spp in Ilam city(18).

Recovery and microscopic examination of soil samples

The flotation technique, followed by microscopic examination of soil samples, was performed to identify the presence of *Toxocara* ova. Initially, the soil samples were allowed to dry at ambient temperature. Following this, each sample was sieved through a 100 µm mesh to remove gravel particles. A flotation technique was then used to concentrate *Toxocara* eggs from the soil specimens. Fifteen grams of strained soil were placed into a 50 mL tube, mixed with tap water and a few drops of 1% Tween 80, and vigorously vortexed for three minutes. The mixture was then filtered and centrifuged at 4000 rpm for five minutes. The supernatant was discarded, and the pellet was washed repeatedly with distilled water until the supernatant appeared clear. The sediment from the final wash was transferred to a 15 mL tube, suspended in 10 mL of saturated zinc sulfate solution ($ZnSO_4$) with a specific gravity of 1.30, and centrifuged at 4000 rpm for 15 minutes. Subsequently, one milliliter of the supernatant was transferred to a microtube and centrifuged at 7000 rpm for five minutes. After discarding the supernatant, the precipitate was re-suspended in water and centrifuged once more. Upon discarding the supernatant, a drop of sediment was placed on a slide, covered with a coverslip, and examined under a light microscope at magnifications × 100 and × 400 for the presence of *Toxocara* eggs (19). The remaining sediment from the flotation process was then preserved at -20 °C for subsequent DNA extraction.

DNA extraction and PCR

After treatment with zinc sulfate, DNA extraction was carried out on all soil samples. The samples underwent five freeze-thaw cycles using liquid nitrogen and water heated to 37 °C (19,20). Subsequently, total genomic DNA was extracted using a soil DNA isolation kit from Pishgam Biotech Company in Iran, following the manufacturer's instructions with slight modifications. The quality and quantity of the extracted DNA were assessed using a Nanodrop spectrophotometer, which measured light absorption at wavelengths of 260 to 280 nm. To amplify DNA, PCR was performed with species-specific primers targeting the ribosomal DNA (rDNA) region, specifically the partial sequences of the internal transcribed spacer 1(ITS1) and ITS2 genes. The primers used were FM1 (5`- TTGAGGGGAAATGGGTGAC -3′) and FM2 (5`- TGCTGGAGGCCATATCGT - 3′) (18). The PCR reaction was carried out in a final volume of 25 µL, consisting of 12.5 µL of PCR mix (2 × Master Mix; Fazabiotech, Iran), containing 1.25 U Taq DNA polymerase, 200 µM of deoxynucleotide triphosphate (dNTPs), and 1.5 mM $MgCl_2$. Additionally, 1 µL of each primer (10 nmol), 3 µL of template DNA, and 7.5 µL of distilled water were added, and processed in a thermocycler (T100™ Thermal Cycler, BioRAD, California) following a specific temperature profile; one cycle at 94 °C for 10 minutes (initial denaturation), 30 cycles at 94 °C for 45 seconds, 57.5 °C for 45 seconds (annealing step), then, 72°C for 45 seconds (extension), and final extension at 72 °C for 10 minutes. The PCR products were analyzed on a 1% agarose gel, stained with DNA Safe Stain, and

visualized under a UV transilluminator. A 100-base-pair (bp) DNA ladder was utilized to determine the size of DNA fragments.

Sequencing and phylogenetic analysis

Nucleotide sequencing was performed on PCR products from positive samples using specific primers designed by Pishgam Biotech Company in Tehran, Iran. The obtained sequences were then analyzed against the GenBank database to determine similarities and differences. Multiple sequence alignments were done with CLUSTAL W software, followed by using Molecular Evolutionary Genetics Analysis version 7 (Mega7) to construct a phylogenetic tree. The genetic relationships of *Toxocara* spp. in Ilam province were compared to reference sequences using the Maximum Parsimony algorithm and the Kimura 2-parameter model, with 1000 bootstrap replicates. *Toxascaris leonina* was the outgroup in this analysis.

Results

Microscopic detection

In microscopic examination, the most important diagnostic characteristics were the size and shape of the eggs. The eggs were spherical and golden, which had a thick shell covered with a pitted surface. They ranged in size from 60 to 85 µm, with some containing a larva (Figure 1). Out of 50 samples, seven (14%) were found to be contaminated with *Toxocara* eggs. Six positive samples (12%) were isolated from parks and green spaces, while one sample (2%) was from boulevards. No positive cases were reported from the squares.

Molecular identification and phylogenetic analysis

The results of the molecular evaluation showed successful amplification of the rDNA gene, resulting in an amplicon size of approximately 700 bp on the agarose gel. Out of the 50 isolates tested, six (12%) were positive for *Toxocara* spp. After DNA sequencing, five isolates were identified as *T. canis,* and a sequence belonged to *T. cati*.

The phylogenetic tree constructed using the Neighbor-Joining method separated the isolates into two distinct clades corresponding to *T. canis* and *T. cati*.

The *Toxocara canis* isolates from Ilam (*T.* canis 1*-5*) clustered closely with reference sequences from Iran, China, Japan, and Iraq, indicating high genetic similarity across geographically distant regions. This clade was supported by strong bootstrap values, confirming the close genetic relationships among these isolates (Figure 2).

Similarly, the *Toxocara cati* isolate from Ilam (*T.* cati 1*) grouped with sequences from China, India, Japan, and other regions of Iran, demonstrating genetic relatedness but also moderate divergence among isolates from different regions. The *T. cati* clade was also supported by reliable bootstrap values, further validating the genetic identity of the isolates (Figure 2).

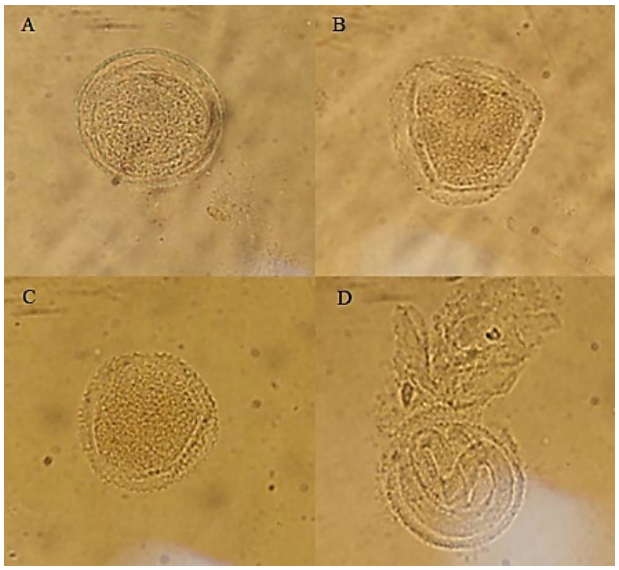

Figure 1. *Toxocara spp.* eggs isolated from soil samples in public areas of Ilam city , western Iran, observed under a light microscope at 400x magnification. The eggs are golden, spherical, and have thick shells with a pitted surface. A, B, and C show unembryonated eggs, while D displays an embryonated egg with a visible larva

The overall phylogenetic analysis confirmed the presence of both *T. canis* and *T. cati* in the soil samples, reflecting the diversity of *Toxocara* species contaminating the environment in Ilam. These findings highlight the potential risk of zoonotic transmission in public areas due to the widespread presence of infective *Toxocara* eggs.

Discussion

Toxocariasis is a neglected zoonotic disease with a global distribution (10). Human habitats often contain significant amounts of *Toxocara* eggs, primarily due to frequent fecal contamination from domestic and stray dogs or cats (21). Once deposited into the environment, these eggs become infectious within 3 to 6 weeks at temperatures ranging from 12 to 37 °C and can survive in soil for several years (22). Consequently, soil in public areas, such as beaches, parks, and children's playgrounds, which are used for human recreation, serves as a primary source for spreading *Toxocara* infections (2,12,23-26). Therefore, assessing soil contamination with *Toxocara* eggs becomes an important public health concern. In the present study, microscopic analysis of soil samples displayed that 14% of public places in Ilam city contained *Toxocara* eggs, while molecular testing indicated a 12% positivity rate.

The contamination rate identified through microscopy was higher than that determined by PCR analysis. This difference may be attributed to the lower specificity of the direct microscopic method in differentiating *Toxocara* eggs from those of other ascarids and pollen grains. In

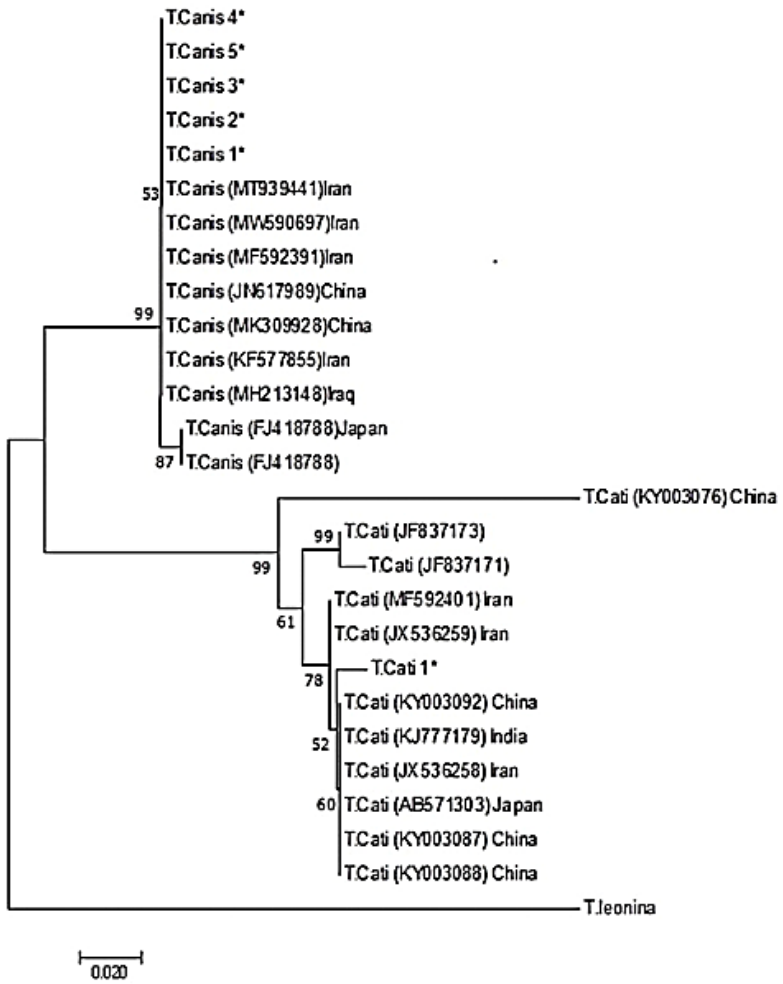

Figure 2. The phylogenetic tree based on rDNA gene sequences shows relationships between *Toxocara canis* and *Toxocara cati* isolates from Ilam, Iran, and reference sequences from other countries. Bootstrap values > 50% are shown. *Toxocara leonina* served as the outgroup. The scale bar represents nucleotide substitutions per site

contrast, the PCR method utilized primers specifically designed to identify *Toxocara* species. Notably, the highest levels of contamination were observed in parks and green spaces, increasing the risk of exposure to *Toxocara* eggs for individuals, especially children who often visit these recreational areas. The absence of fencing surrounding parks and green spaces in Ilam likely allows stray animals to enter freely, contaminating these zones with *Toxocara* eggs. A study conducted in Turkey has shown a notable difference in soil contamination levels between fenced and unfenced parks (27).

The global prevalence of *Toxocara cati* is estimated at approximately 17% (28), while *T. canis* has a reported prevalence of 11.1% worldwide (29). It is estimated that soil contamination with *Toxocara* eggs is present in nearly 21% of locations worldwide. The prevalence rates range from 13% in North and Central America to 35% in the Western Pacific (21).

The molecular prevalence observed in this study was lower than that reported in the Amadiya district of Duhok, Iraq. Despite geographic and climatic similarities with Ilam, differences in soil composition or socio-economic conditions, including waste disposal practices and stray animal management, may explain these discrepancies (30). In Karaman, Turkey, the prevalence of *Toxocara* eggs in children's playgrounds was 19.4%, significantly higher than that in Ilam. This disparity could be linked to factors such as inadequate stray animal control, lower awareness of zoonotic risks, or differences in public health policies (31). Additionally, in both Iraq and Turkey, the evaluations were performed solely using microscopic techniques, which may account for variations in reported prevalence rates. In this study, the molecular prevalence of *Toxocara* eggs in soil samples was consistent with previous findings from Ilam and Tehran (2,24). The contamination rate with *Toxocara* species in Ilam exceeded the levels observed in cities such as Qazvin, Ardabil, and Mashhad (32-34), but was lower than those reported in Shiraz, Yazd, Ahvaz, and Khorram Abad (18,19,35,36). Different prevalence rates in these regions may be influenced by factors such as humidity, temperature, and soil texture, which are critical determinants for the survival and dispersion of *Toxocara* eggs. Cities with warmer climates and higher humidity, such as Shiraz and Ahvaz, tend to have higher

contamination rates due to favorable conditions for the persistence of helminth eggs. In contrast, regions like Qazvin and Mashhad, which have colder climates or more developed urban infrastructure, might experience reduced contamination levels.

The phylogenetic analysis confirmed the presence of both *T. canis* and *T. cati*. The clustering of Ilam isolates with sequences from various countries, such as China, Japan, and Iraq, highlights the genetic similarity between isolates from different geographic regions, suggesting the potential for widespread environmental dissemination of these species.

The strong bootstrap support for the *T. canis* clade, which included isolates from Iran, China, Japan, and Iraq, suggests that *T. canis* is genetically conserved across regions, despite geographical distances. This may reflect global patterns of canine movement, environmental contamination, and zoonotic transmission, contributing to the widespread presence of *T. canis*. The clustering of *T. cati* isolates from Ilam with sequences from India, China, and Japan also indicates genetic relatedness across regions, although some degree of divergence was observed. This could be attributed to host specificity, environmental factors, or local evolutionary pressures that may influence the genetic diversity of *T. cati*.

The detection of both *T. canis* and *T. cati* in public areas poses a significant public health concern. These soil samples represent potential sources of human exposure to infective *Toxocara* eggs, which can lead to toxocariasis, particularly in children who are at greater risk due to their frequent contact with contaminated soil (37).

Gene sequencing analyses revealed a higher prevalence of *T. canis* compared to *T. cati* in the soil samples from Ilam. These increased levels of contamination may be due to the large population of stray dogs in Ilam, which tend to defecate in open spaces, unlike cats that typically bury their waste.

Conclusion

The results of this study indicate that dogs may play a more important role in transmitting this zoonotic infectious disease to individuals, especially children who play on the ground or with contaminated utensils. Overall, public areas, particularly parks, playgrounds, and other communal spaces, serve as reservoirs for the transmission of *Toxocara* eggs, emphasizing the need for targeted interventions such as deworming programs for pets, proper waste disposal, and public health education to reduce the risk of environmental contamination.

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Authors' contributions

Conceptualization: Razi Naserifar and Hamid Hasanpour.
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Investigation: Tayebeh Eydi and Hamid Hasanpour.
Methodology: Tayebeh Eydi and Hamid Hasanpour.
Project administration: Razi Naserifar and Hamid Hasanpour.
Resources: Hamid Hasanpour.
Software: Hamid Hasanpour.
Supervision: Razi Naserifar.
Validation: Hamid Hasanpour.
Visualization: Razi Naserifar, Hamid Hasanpour, Asad Mirzaei, and Nahid Maspi.
Writing–original draft: Nahid Maspi and Hamid Hasanpour.
Writing–review & editing: Nahid Maspi and Omid Raiesi.

Competing interests

The author(s) declare that there are no conflicts of interest.

Ethical issues

This study was approved by the Ethical Committee of Ilam University of Medical Sciences in Iran (approval No. IR.MEDILAM.REC.1399.229).

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