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Original paper

Toxoplasma gondii and *Toxocara* spp. contamination in university area

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ABSTRACT. *Toxoplasma gondii* and *Toxocara* spp. zoonotic infections may cause severe systemic and ocular illness in infected individuals. Cats play a significant role in environmental contamination and the transmission of parasites. The goal of the present study was to investigate the prevalence of *Toxoplasma gondii* (*T. gondii*) and *Toxocara* spp. infection among stray cats at Ahvaz Jundishapur University of Medical Sciences campus. The current descriptive study began with the collection of 170 fresh cat faecal samples from various sites in the Ahvaz Jundishapur University of Medical Sciences area. Sheather's sugar flotation method was applied to all specimens, and parasites were identified and examined microscopically. Next, a nested-PCR assay, sequencing, and real-time PCR with high-resolution melting curve (HRM) analysis were performed. In this study, out of 170 cat faecal samples microscopically evaluated, 8 (4.70%) and 37 (21.76%) were infected with *T. gondii* oocysts and *Toxocara* eggs, respectively. Using nested PCR, 8 out of 170 samples (4.70%) were found to be infected with *T. gondii*. HRM analysis showed that all isolates could be classified into three genetic lineages. Considerable prevalence, exceeding 50% for *Toxocara* and surpassing 25% for *Toxoplasma* in certain instances, along with genetic diversity, was observed in the present study. Hence, it is suggested that all individuals, including kindergarten children, students, employees, workers, and pregnant women who are in contact with their surroundings, take the necessary precautions.

Keywords: *Toxoplasma*, *Toxocara*, faecal samples, stray cats

Introduction

Toxoplasma gondii (*T. gondii*) infection is a widespread zoonosis among endothermic animals, including humans. According to serological evidence, one-third of the world's population is seropositive for this infection. However, the prevalence varies based on age, immune status, geographical and climatic conditions, nutritional habits, social customs, and the distribution of the definitive host (cats) [1–4]. All nucleated cells are susceptible to *T. gondii* invasion. The parasite possesses three infective stages: fast-replicating tachyzoites (acute infection), slow-replicating bradyzoites (chronic infection), and resistant oocyst forms (environmental transmission). Felines, like cats, are the only definitive hosts for *Toxoplasma*, while other warm-blooded animals such as birds and mammals are considered intermediate host [2,3,5,6].

Human infection may occur congenitally or be acquired during one's lifetime. During congenital infection, invasive tachyzoites parasitize the placenta and inflict severe consequences on the unborn, including abortion, hydrocephaly, and microcephaly, depending on the timing of the infection during pregnancy. Acquired infection occurs through the ingestion of sporulated oocysts in contaminated soil, water, fruits, and vegetables, or via the consumption of raw or undercooked meat containing tissue cysts [5,7.8]. Most individuals with acquired infection are asymptomatic or exhibit lymphadenopathy as the most common symptom, while acute, fatal disease may develop in

immunocompromised patients [9,10].

T. gondii oocysts have been shown to be shed by at least 17 different species of cats [11]. Sporulation of oocysts occurs early in optimum conditions (3 days at 25–30°C); however, in lower temperatures, the sporulation process takes longer [5]. Sporulated oocysts are resistant to many environmental factors, including light, heat, desiccation, bacteria, and fungi. They remain infectious for over 54 months at 4°C in experimental conditions and persist at –10°C (freezing temperature) for about 106 days. Although oocysts are eliminated at 55°C for 2 minutes, sporulated oocysts are resistant to humidity, demonstrating varying degrees of stability against anti-infective agents [12–14]. Cats usually bury their faeces underground, but oocysts can be brought to the surface by rain and the movement of earthworms. Mechanical vectors that come into contact with soil, water, and/or other contaminated sources, such as earthworms, may be responsible for transmitting parasites. These processes lead to environmental contamination by *Toxoplasma* oocysts, which is a primary route of contamination for humans and animals. Rapid identification of *T. gondii* oocysts in environmental samples is a challenging procedure due to the relatively low intensity of oocysts and the presence of assay inhibitors. During the last few decades, polymerase chain reaction (PCR)-based techniques have been developed to accurately identify oocysts in animal or environmental specimens [13].

Toxocara spp. are roundworms and the most common infection in dogs and cats. The worms lay eggs so that *T. canis* can produce 200,000 eggs per day [15]. The eggs are excreted along with faeces and enter the environment. The eggs require a few weeks to fully develop into larvae. Humans are often infected by accidentally ingesting infected eggs along with contaminated water and food. However, they can also be infected through other modes of transmission, such as by consuming raw or undercooked meat containing the third stage of worms, or through contact with final hosts, as contaminated eggs may attach to their fur [16].

Upon ingestion of embryonated eggs, the larvae hatch and pass through the wall of the small intestine. They then travel through the bloodstream to various organs such as the liver, lungs, and brain, leading to the development of visceral larva migrans (VLM) and ocular larva migrans (OLM). Depending on the affected organ system(s), toxocarosis is often linked to significant variability in clinical presentation. Lung involvement may be associated with shortness of breath and occasionally respiratory failure. Brain involvement may manifest as encephalitis and epileptic seizures. Ocular involvement may be accompanied by endophthalmitis, loss of visual acuity, and occasionally blindness in the affected eyen [17–20]. According to a reported study, 18% of soil samples from the Middle East and North Africa were contaminated with *Toxocara* spp. [21].

Within a university campus, several individuals are at a higher risk of acquiring *T. gondii* oocysts and *Toxocara* spp. The event will involve kindergarten children, gardeners, students, faculty members, employees, and other attendees. Therefore, the present study was designed to determine the contamination rate of *T. gondii* and *Toxocara* spp. in fresh faecal samples of stray cats in Ahvaz Jundishapur University of Medical Sciences. Flotation and molecular techniques were employed as well.

Materials and Methods

Study area and sample collection

A descriptive study was conducted by collecting faecal samples from various locations at Ahvaz Jundishapur University of Medical Sciences, including the professors' lounge, kindergarten, students' self-service area, and several faculties such as dentistry, paramedicine, medicine, hygiene, nursery, and pharmacy. To avoid repetition, sample collection from each location was conducted at one-month intervals.

Each sample was assigned a number and labeled with the date and place of sampling. The samples were then stored at 4°C for future use. The study protocol No: IR.AJUMS.REC.1400.013 was approved by the Ethics Committee on Research at the School of Medicine, Ahvaz Jundishapur University of Medical Sciences.

Floatation method and light microscopy

All faecal samples underwent a flotation method using Sheather's sugar solution, which was prepared by dissolving 1278 grams of sucrose in 1000 ml of distilled water. Parasitic agents were isolated and characterized under a light microscope [22].

DNA extraction and amplification

Initially, all DNA samples were extracted using the DNA Stool Mini Kit (QIAamp, QIAGEN,

Table 1. Distribution of *Toxocara* spp. eggs and *Toxoplasma* oocysts in faecal samples of stray cats in 9 examined districts of Ahvaz Jundishapur University of Medical Sciences

Sampling area	No. of samples	No. of positive *Toxocara* spp. egg samples (%)	No. of positive *Toxoplasma* oocyst samples (%)
Professors' lounge	50	1 (2)	1 (2)
Kindergarten	15	8 (53.3)	4 (26.6)
Dentistry	15	6 (40)	–
Self-Service	15	5 (33.3)	–
Paramedicine	15	2 (13.3)	–
Medicine	15	3 (20)	–
Hygiene	15	2 (13.3)	1 (6.6)
Nursery	15	4 (26.6)	–
Pharmacy	15	6 (40)	2 (13.3)
Total	170	37 (21.76)	8 (4.70)

USA), and the amplification was performed using a nested PCR assay. Two specific primer pairs were used for the amplification process:

F1-Nested PCR1: 5'-TCAAGCAGCGTATT GT CGAG-3'

R1-Nested PCR1: 5'-CCGCAGCGACTTCTA TCTCT-3'

F2-Nested PCR2: 5'-GGAACTGCATCCGTT CATGAG-3'

R2-Nested PCR2: 5'-TCTTTAAAGCGTTCGT GGTTC-3'

The nested-PCR conditions in the current study were as follows: in the first round, initial denaturation was conducted at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 20 seconds, annealing at 53°C for 20 seconds, and extension at 72°C for 20 seconds, as well as a final extension step at 72°C for 4 minutes. In the second round, the initial denaturation was conducted at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 20 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 20 seconds, with a final extension step at 72°C for 4 minutes. Finally, PCR products were run on a 2% agarose gel electrophoresis and visualized using a GelDoc apparatus [23]. For the positive control, *T. gondii* strain RH was obtained from the Laboratory of Toxoplasmosis at Tehran University of Medical Sciences in Tehran, Iran. For the negative control, sterile distilled water was used instead of the template DNA solution.

Sequencing

Nucleotide sequencing of PCR products using the same forward primer was conducted to definitively confirm the specificity of amplification and identify genetic diversities. The data obtained was analyzed using SPSS-22 software.

Real-time high resolution melting curve analysis

For this purpose, SyBR Master Mix-2X (Bio-Tech Company, South Korea) was used. To prepare a 20 µl reaction for the real-time assay, 10 µl of Master Mix, 2 µl of the forward primer TOXO-F (5 µM, 5′-TCCCCTCTGCTGGCGAAAAGT-3′), 2 µl of the reverse primer TOXO-R (5 µM, 5′-AGCGTTCGTGGTCAACTATCGATTG-3′) [24], each primer, and 2 µl of twice-distilled water were mixed in special microtubes. Subsequently, 4 µl of extracted DNA was added. The PCRs were performed using a LightCycler® 96 Instrument from Roche (USA).

The amplification process included pre-incubation at 95°C for 10 minutes, followed by denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 10 seconds. For the HRM test, the following temperature profiles were used: 95°C for 60 seconds

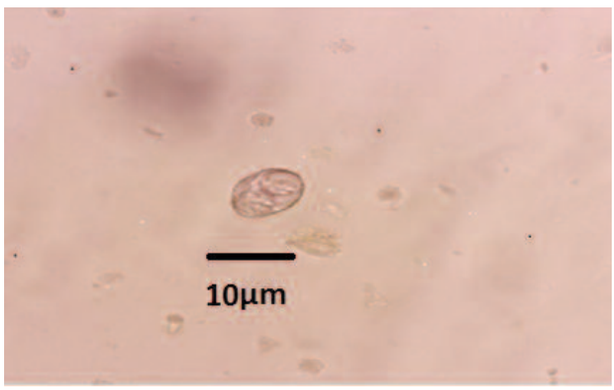

Figure 1. *Toxoplasma* oocyst. Sheather's sugar, magnification at 400×

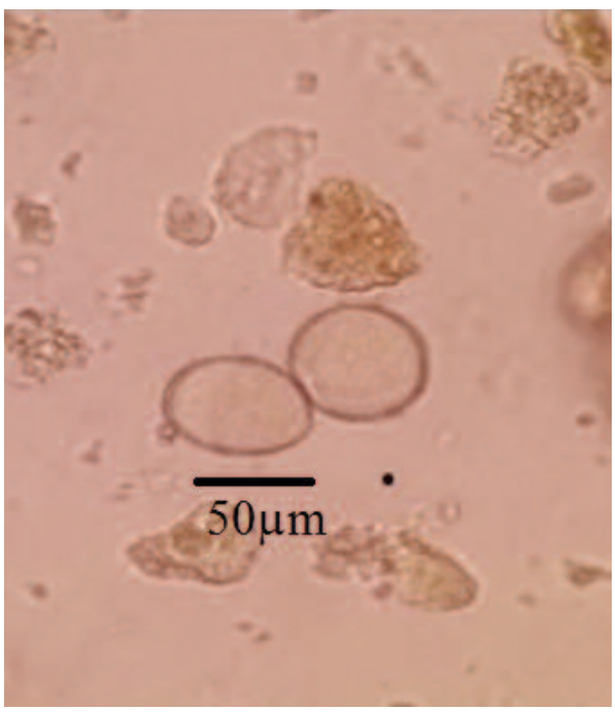

Figure 2. *Toxocara* spp. eggs. Sheather's sugar, magnification at 400×

with a 4.4°C/s ramp, 40°C for 60 seconds with a 2.2°C/s ramp, 65°C for 1 second with a 2.2°C/s ramp, and finally 97°C for 1 second [24,25].

Data analysis

IBM SPSS Statistics version 25 software and a one-way ANOVA test were used to compare mean values among different groups. Significance was set at $P<0.05$.

Results

In the present study, 170 cat faecal samples were collected from 9 districts of Ahvaz Jundishapur University of Medical Sciences. Out of these, 8 samples tested positive for *Toxoplasma* oocysts (Fig. 1). *Toxocara* spp. eggs were observed in 37 specimens (Fig. 2). Table 1 shows the contamination rates of *Toxoplasma* and *Toxocara* spp. in examined stool samples of stray cats. Using nested PCR and 2% gel electrophoresis, a 200 bp band specific to *T. gondii* was detected in 8 faecal samples (Fig. 3).

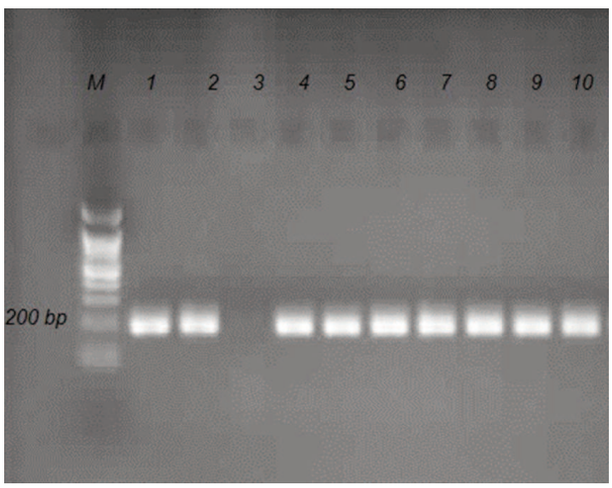

Figure 3. Gel electrophoresis of nested-PCR products using B1-specific primers. M=DNA marker, 1=positive control, 3=negative control, and the other wells contain positive samples from examined faecal samples of stray cats

PCR products obtained from 8 positive samples of *T. gondii* were sent to Spain for sequencing. These sequences were registered in the DNA Databank of Japan (DDBJ) under the accession numbers LC663784-LC663792. Using real-time high-resolution melting (HRM) analysis, eight positive samples were identified, and the *T. gondii* RH strain was used as a positive control. According to the graphs obtained, *Toxoplasma* isolates are classified into three lineages (Figs. 4–7).

Discussion

For the first time, the contamination rate of *T. gondii* oocysts in stool samples of stray cats at Ahvaz Jundishapur University of Medical Sciences was determined. Based on our results, *Toxoplasma* oocysts were detected in 2 out of 170 fresh faecal samples (1.17%) using microscopy. Other epidemiological studies around the world have reported similar prevalence rates, including 1.2% in Iran, 2.3% in Italy, 0.9% in California, 0.6% in Virginia, and 1.3% in Canada. Moreover, the findings from 16 European countries with a large

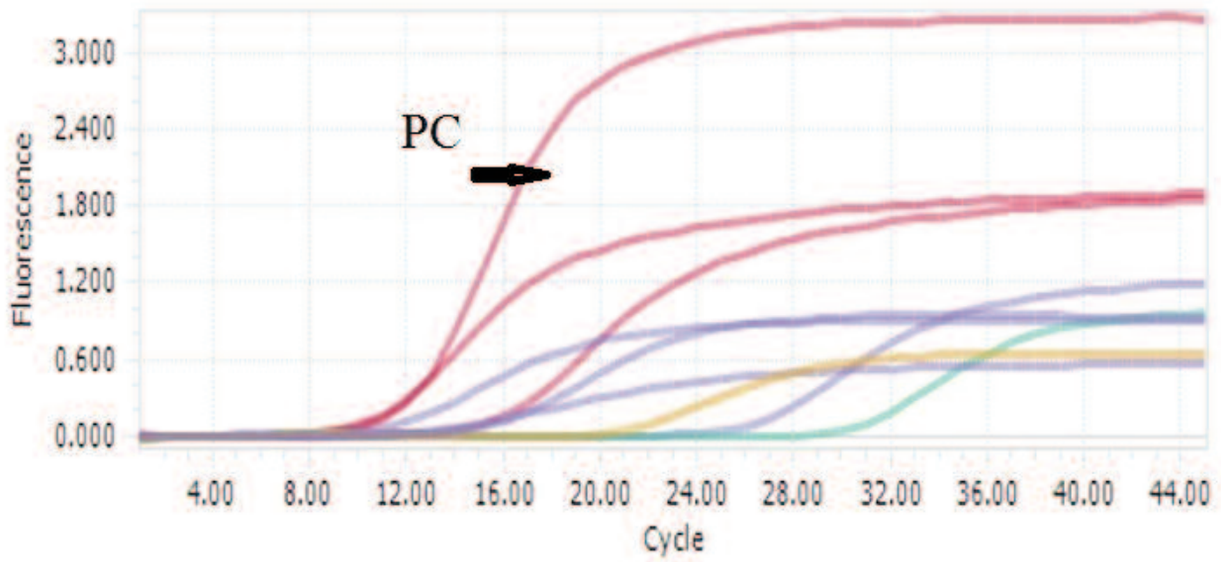

Figure 4. Amplification graph of 8 isolates and the positive control using real-time PCR.
An arrow indicates the positive control (Rh strain *T. gondii*)

sample size (24,106 cat faecal samples) examined through microscopy have documented a prevalence of 0.11% for *Toxoplasma* oocysts. Also, there have been reports mentioning higher prevalence rates, such as a 19.3% prevalence in Thailand using microscopy and PCR [3,23,26]. Such variation in prevalence rates depends on several factors, including the population density of cats, oocyst shedding cycles, and geographical location [23].

Khodaverdi and Razmi [3] in Mashhad reported a 2.28% prevalence rate (4/175) of *Toxoplasma* oocysts in stray cats using flotation and PCR methods, which aligns with the results of the current study. Nasiru Wana et al. [23] in Malaysia identified 7 positive samples out of 200 fresh cat faecal samples examined (3.5%), a rate that is relatively higher than our findings. The lower prevalence in the current study may be attributed to the limited

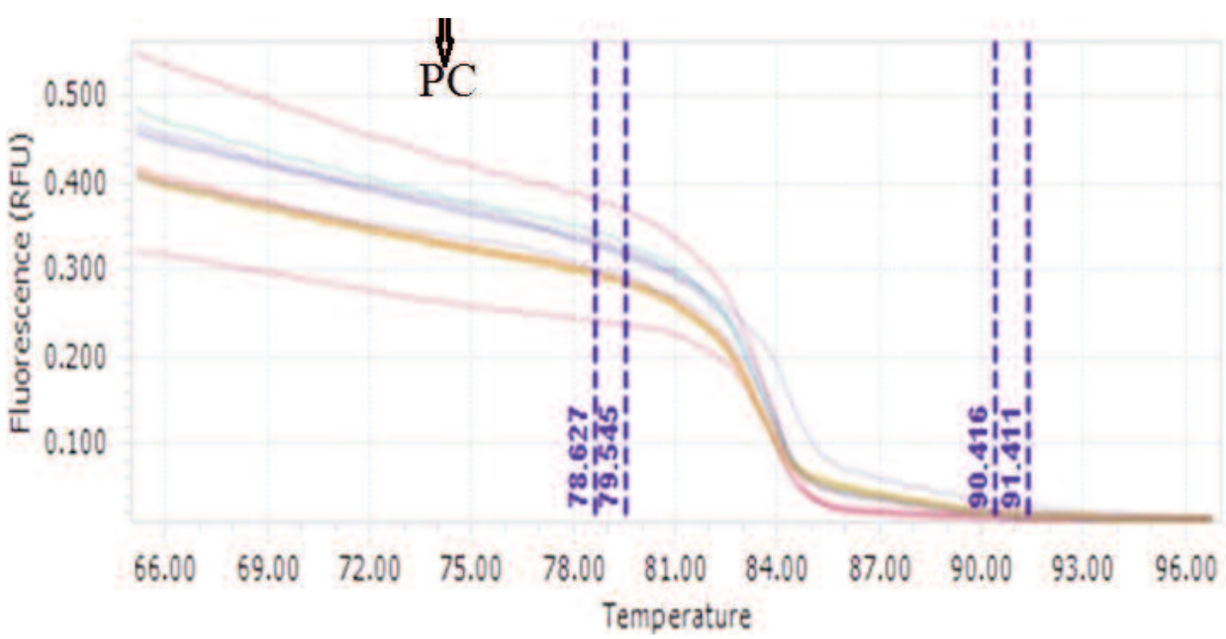

Figure 5. High-resolution melting curves of 8 isolates and a positive control using real-time PCR. An arrow indicates the positive control (Rh strain *T. gondii*)

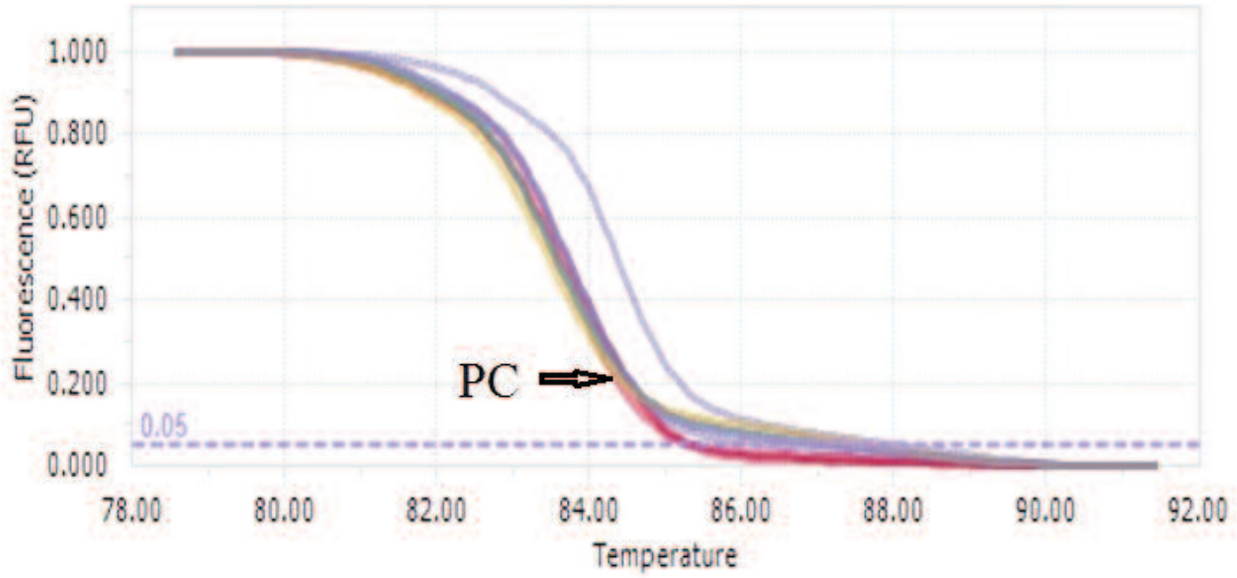

Figure 6. Normalized melting curves of 8 isolates and the positive control using real-time PCR. An arrow indicates the positive control (Rh strain *T. gondii*)

sampling area, which restricts cats' access to potential sources of infection in the environment.

Zamora-Velez et al. [28] in Colombia molecularly examined the faecal samples of 140 cats from 10 districts of the city and demonstrated the absence of *T. gondii* oocysts. Also, in other reports from Spain, China, and Turkey using microscopy, cats were not found to harbor *Toxoplasma* parasites [23]. One reason for the lack of oocyst characterization may be the short duration of oocyst shedding in cats (1–2 weeks), which occurs during the primary infection [29].

Bajalan et al. [30] assessed the prevalence of *Toxoplasma* in 125 stray cats from Khorram Abad, western Iran, using parasitological and serological methods. Accordingly, 80 cats (64%) were shown to possess specific anti-*Toxoplasma* IgG. The prevalence of serum antibodies varies depending on climatic differences, the nutritional status of cats, and their access to waste food. In Iran, the prevalence of *T. gondii* in cats varies from 24.75% in Ahvaz (the current study area in southwest Iran) to 86% in Kashan (located in the central plateau of Iran). From a global perspective, the prevalence rate in cats has been reported to range from 5.4% to 90%. Based on the published literature, the

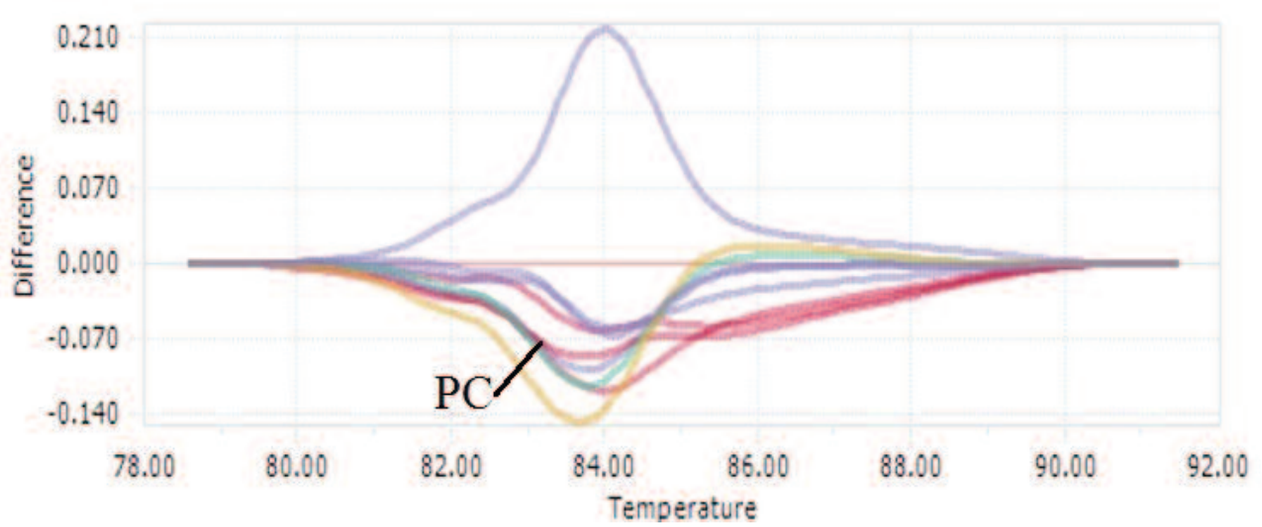

Figure 7. Difference curves were generated for 8 isolates and the positive control using real-time PCR. An arrow indicates the positive control (Rh strain *T. gondii*)

serological prevalence of *T. gondii* infection is much higher among stray cats than in owned cats [30]. Study by Veronesi et al. [26] revealed that, 2 out of 78 (2.56%) collected cat faecal samples tested positive for *T. gondii* using B1 and repeat element (RE) gene amplification by PCR, which aligns with our findings. Concerning the presence of the pathogenic RH strain in the study area, necessary measures should be taken into account. Furthermore, contact with soil in the study area should be considered, especially for pregnant women, particularly those accompanying their children to kindergarten.

Using highly sensitive and specific real-time PCR, 8 isolates were amplified, fully covering the results of nested PCR. Based on HRM real-time analysis, the isolated parasites exhibit genetic diversity and can be categorized into three lineages.

In the present study, *Toxocara* spp. eggs were found in 37 out of 170 cat faecal samples (21.76%). In previous studies on stray cats, prevalence rates of 32%, 16%, 62.5%, and 13% were estimated for examined cats in Tabriz, Tehran, Shahsavar, and Isfahan, respectively. In other parts of the world, the prevalence of *Toxocara* spp. is also observed. Infection rates in cats were 34.8% in England, 3.2% in Australia, 5.7% in Taiwan, 11% in South Africa, 55.2% in Spain, and 25.2% in Brazil [31]. Such a wide range of prevalence may be justified by variations in population density of cats, nutritional status, geographical and traditional differences. Oge et al. [32] examined 224 dogs using flotation and PCR methods and found a 9.38% prevalence (21/224) of *Toxocara canis*, which was lower than our findings. This may indicate appropriate climatic conditions in Ahvaz for egg survival [32]. Spada et al. [29] in Italy demonstrated a 33.1% prevalence of *Toxocara* spp. in 139 examined stray cats [29], which does not imply a statistically significant difference from the current study. Khademvatan et al. [27] conducted a molecular study on faecal samples of cats and reported a 45% prevalence among 140 examined dogs, indicating a higher prevalence than our findings. This could be due to the limited scope of the study area and the cultural significance of the academic disciplines. One of the significant findings of this study is that 26.6% of the samples collected from around the kindergarten were contaminated with *Toxoplasma* oocysts, and 53.3% were infected with *Toxocara* spp. ova. Infection with the two parasites, especially *Toxocara*, may cause visceral larva migrans (VLM), a human disease that involves the liver, heart, lung, muscle, eye, and brain.

This study is the first research that examines the level of *Toxoplasma* and *Toxocara* contamination in various areas of the university campus. The high level of contamination around the kindergarten is concerning and warrants special attention and surveillance. Students should avoid sitting on the grass and serving food to prevent contamination. It is highly recommended that self-service employees prevent soil contamination of edible products to avoid potential infections in food consumers.

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