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Original Article

Molecular Detection of *Toxoplasma gondii* Oocytes in Soil Samples from Guilan Province, Northern Iran

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Abstract

Background: Soil is an appropriate substrate for the storage and transmission of oocytes of *Toxoplasma gondii*. Ingestion of soil contaminated with *T. gondii* oocysts is a major transmission route of human and animal toxoplasmosis. The present study was carried out to investigate soil contamination with *T. gondii* oocysts in urban and rural areas of Guilan Province, northern Iran.

Methods: Overall, 208 soil samples were collected from 16 cities and villages in Guilan Province, northern Iran from Oct 2020 to Nov 2021. Soil samples were investigated using modified sucrose flotation technique. Real-time polymerase chain reaction was used to detect presence of *T. gondii* DNAs in the samples. Positive samples were further analyzed using nested polymerase chain reaction for GRA6 gene. Moreover, six selected positive samples were used for amplifying and sequencing of the GRA6 gene.

Results: Overall, 31 samples were positive for *T. gondii* with frequency of 14.9% and ranging from 10.9% in rural areas to 16.3% in urban areas. Statistical analysis showed significant differences between the seasons (P=0.003). The phylogenetic analysis illustrated that our six sequences were similar and closely related to Type I strain of *T. gondii*.

Conclusion: Results showed relatively high levels (14.9%) of *T. gondii* oocytes in soil samples of Guilan Province, northern Iran, which provided essential data for the effective prevention and control of toxoplasmosis in the region.

Keywords: *Toxoplasma gondii;* Soil; Northern Iran; Oocytes

Introduction

Toxoplasma gondii is a zoonotic coccidian parasite that infects a wide range of warm-blooded animals, including humans (1). Toxoplasmosis is a parasitic disease caused by *T. gondii*, which is often asymptomatic in immunocompetent individuals. However, the parasite may cause serious complications in seronegative pregnant women and immunocompromised patients, including

cancer patients, transplant recipients and acquired immune deficiency syndrome (AIDS) patients (2-4).

In the life cycle of *T. gondii*, the felidae is the final host and a wide range of mammals and birds, including humans, can act as intermediate hosts (1). Consumption of raw or undercooked meats carrying tissue cysts of *T. gondii*, transfer of the parasite tachyzoites from the infected mothers to the fetuses and digestion of sporulated oocysts of the parasite from the environment are the most common routes of the infection transmission to human (2). Transfer of the infection via digestion of infected oocysts is more significant than other routes of transmission, particularly in children and pregnant women (5). Oocysts originated from the sexual reproduction of *T. gondii* are excreted in soil within the feces of infected cats, which are resistant to harsh environmental conditions. The oocytes can be infectious for months and play significant roles in the epidemiology of *T. gondii* (6). Climate changes cause alterations in the life expectancy of oocysts in soil because of the sporulation period and availability of the infected rodents in various seasons (7, 8).

Studies estimating prevalence of *T. gondii* oocysts in soil have shown seasonal variations in host behaviors such as times of oocyte shedding by cats and human recreational activities (9, 10). Furthermore, studies have reported prevalence rates of *T. gondii* oocysts in soil from 0% in Hawaii, USA, to 50% in northern France (6). In Iran, prevalence of *T. gondii* oocysts in soil samples was studied in several cities and the prevalence rate was reported as 5–87.1% (11-13).

Based on the previous studies, *T. gondii* includes three major genotypes of I, II, III and several atypical genotypes. Genotypes develop various clinical manifestations after infection and may be associated with severity of the disease (14, 15).

The present study was carried out to investigate soil contamination with *T. gondii* oocysts in urban and rural areas of Guilan Province, Iran, and the seasonal models associated with the contamination as well as genotype determination.

Methods

Study area

Guilan Province is located in the north of Iran and south of Caspian Sea with balanced humid climate and mean annual precipitation of 1275 mm. This province is geographically divided into three regions, one with mild climate and high precipitation that decreases to the east; one with mountainous climate on the northern skirt of Alborz Mountain Range that is snow-covered at high altitudes for the majority of the year; and one with a semi-arid climate with annual precipitation of less than 500 mm. The relative humidity is 40%–100% and the mean temperature of the province is approximately 18 °C (16).

Sample collection

Overall, 208 soil samples were collected from 16 cities and villages of Guilan Province, Iran, Oct 2020 to Nov 2021. Parks, public places, rubbish bins and communities in surrounding cities were sampled. At each time, three samples were randomly collected from three various sites through a 10 m2 area. Samples (50 g) were collected at a depth of 2–5 cm below the soil surface using shovel, dried at room temperature for 24 h and sieved using mesh no. 20.

Toxoplasma gondii oocyst cultivation and DNA extraction

Soil samples were concentrated using modified sucrose flotation based on the method of Matsuo J et al. (17). Briefly, 7 g of the sieved soil were mixed with 50 ml of 0.1% Tween 80 and 0.1% gelatin solution and centrifuged at 1100 g for 10 min. Supernatant was removed and mixed with 5 ml of sucrose solution with 0.1% gelatin and centrifuged at 1100 g for 10 min. Then, supernatant was transferred to another tube and centrifuged after dilution with distilled water (DW) (1:10). This was stored at 4 °C until use. Furthermore, genomic DNA was extracted after sonication using AddPrep Genomic Extraction Kit (Addbio, South Korea) according to the manufacturer's instructions. Concentration of the extracted

DNA was assessed using NanoDrop (Thermo Scientific, USA) and stored at -20 °C until use.

Real-time PCR

SYBR green real-time polymerase chain reaction (real-time PCR) was used for the detection of *T. gondii* DNAs in the soil samples using repetitive sequence (REP-529). The final reaction volume was 25 µl, including 12.5 µl of RealQ Plus 2× Master Mix Green, 0.5 µl of each forward (A.H.S-TOXO-F: 5'-CTGCGTCTGTCGGGATGA-3') and reverse (A.H.S-T OXO-R: 5'-GCGTCGTCTCGTCTGGAT-3') primers, 6.5 µl of DW and 5 µl of DNA template. The real-time PCR was carried out at 95 °C for 10 min followed by 40 cycles of 95 °C for 30 sec, 60 °C for 60 sec and 72 °C for 30 sec. Final extension was carried out at 72 °C for 5 min. The target of real-time PCR included a 529-bp fragment of the RE gene. Negative (DW) and positive (DNA extracted from a virulent RH strain of *T. gondii*) controls were used for the PCR (18).

Nested-PCR targeting GRA6 gene

Positive samples were amplified using nested-PCR and pure products with high quality and quantity for enzymatic cutting were generated. First, samples with CT values less than 35 from real-time PCR were amplified using two pairs of primers (Table 1). The final product was exposed to MseI enzyme overnight at 56 °C and then electrophoresed in 2.5% agarose gels. Moreover, RH standard strain was used as Type I, Tehran strain as Type II and VEG strain as Type III genotypes (19).

Table 1: Primers and their sequences used in nested-PCR

Primer	*Sequence (5'-3')*
GRA6-F	ATTTGTGTTTCCGAGCAGGT
GRA6-R	GCACCTTCGCTTGTGGTT
GRA6-F1	TTTCCGAGCAGGTGACCT
GRA6-R1	TCGCCGAAGAGTTGACATAG

The first step of nested-PCR included 12.5 µl of Taq 2× master mix, 1 µl of each forward and reverse primers and 6.5 µl of sterile DW with 4 µl of the genomic template. The DNA was replicated under conditions of initial denaturation at 95 °C for 5 min and 35 cycles of denaturation at 95 °C for 30 sec, annealing at 56 °C for 30 sec and extension at 72 °C for 30 sec. Sterile DW was used as negative and RH strain as positive controls. For the second step of nested-PCR, the final volume was 25 µl, containing 1 µl of the first-step product diluted 1:20 with 0.75 µl of each primer, 10 µl of sterile DW and 12.5 µl of master mix. In this step, the annealing temperature was set at 60 °C for 45 sec. Then, PCR products were electrophoresed in 2.5% agarose gels dyed with safe stain. Genotypes were assessed using MseI enzyme (Fermentas, Germany) and electrophoresed in 2.5% agarose gels dyed with safe stain.

Gene sequencing

A number of GAR6 gene amplicons were selected and sequenced by Sinacolon Lab (Iran) and using ABI 3730 Sequencer Platform (Applied Biosystems, USA) and similar PCR primers. Sequencing results were confirmed using NEBcutter V2.0 (New England BioLabs, USA) software. Furthermore, Basic Local Alignment Search Tool (BLAST) online program (http://www.ncbi.nlm.nih.gov/blast/) was used to compare the consensus sequences with those from GenBank.

Results

Molecular detection of Toxoplasma gondii using real-time PCR

Overall, 31 samples were positive for *T. gondii* with frequency of 14.9% and ranging from 10.9% in rural areas to 16.3% in urban areas. However, differences were not statistically significant (P>0.05). Moreover, there were no reported statistically significant differences (P=0.14) in the presence of *T. gondii* oocysts within the cities (Table 2).

Results were analyzed during spring, summer, autumn and winter (Fig. 1). Statistical analysis showed significant differences between the seasons with increases in positive cases in summer, compared to other seasons (P=0.003). Prevalence of *T. gondii* oocysts was higher in trash bins than other places. However, differences were not statistically significant (P=0.49) (Table 3).

Table 2: Detection of *Toxoplasma gondii* oocyst DNAs in soil samples

Region	***N(%)***	***Positive samples N(%)***
Rasht	16	1 (6.2)
Anzali	12	2 (16.7)
Roudbar	12	0 (0)
Fouman	12	3 (25)
Lahijan	12	3 (25)
Astaneh	12	3 (25)
Talesh	12	1 (8.3)
Masal	12	1 (8.3)
Astara	16	5 (31.2)
Rezvanshahr	12	1 (8.3)
Shaft	12	2 (16.7)
Somesara	12	1 (8.3)
Langroud	16	1 (8.3)
Roudesar	16	6 (37.5)
Siahkal	12	1 (8.3)
Amlash	12	0 (0)
Total	208	31 (14.9)

Table 3: Frequency of *Toxoplasma gondii* DNAs in Guilan Province by site of sampling

Area	***Site of sampling***	***N(%)***	***Positive samples N(%)***
Urban	Parks	62	9 (14.5)
	Public places	61	9 (14.8)
	Trash bins	30	7 (23.3)
Rural	Villages	55	6 (10.9)
	Total	208	31(14.9)

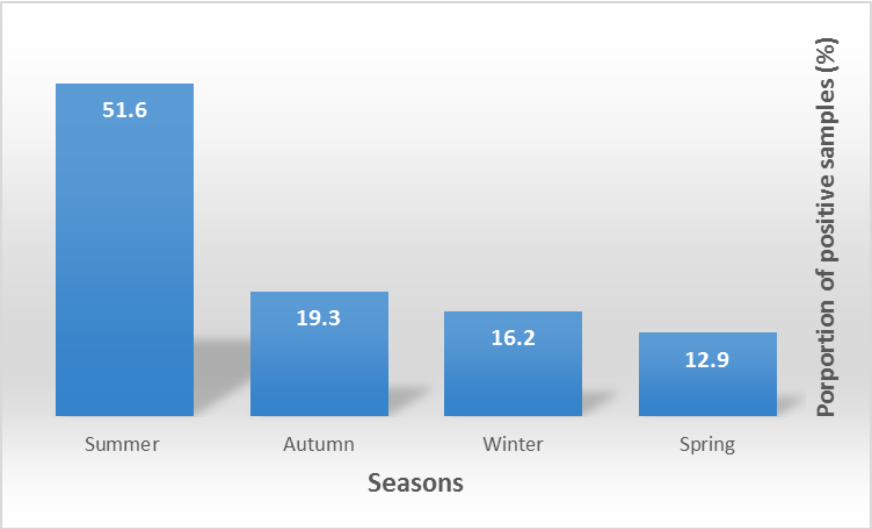

Fig. 1: Frequency of *Toxoplasma gondii* DNAs in soil samples of Guilan Province. Significant differences (*P*=0.003) were seen between summer and other seasons

Assessment of *Toxoplasma gondii* genotypes using RFLP-PCR

Out of 31 samples in genotyping, eight samples included clear bands using electrophoresis of GRA6 amplicons in 2.5% agarose gels (Fig. 2). MseI enzyme restricted 344-bp fragment into incisions based on the known genotype strain. Moreover, all unknown strains included cutting patterns similar to that of Type I. Six amplified GRA6 gene products were sequenced. Nucleotide sequences from this study are available in GenBank Database (accession nos. OM990682–OM990687). The phylogenetic analysis illustrated that our six sequences were similar and closely related to Type I strain of *T. gondii*.

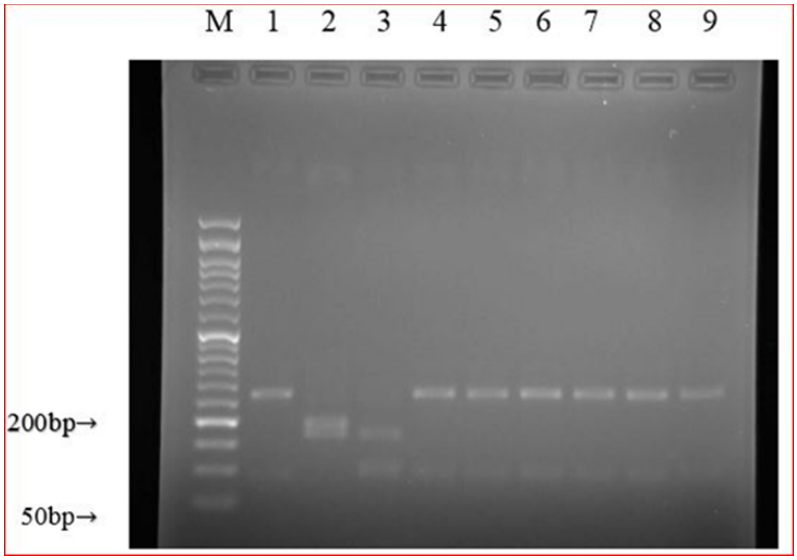

Fig. 2: Electrophoresis of 4 µl of the nested-PCR product using GRA6 marker (344 bp) and MseI digestions in 2.5% agarose gels. Lane M, 50-bp ladder; Lane1 represents RH strain (Type I); Lane2 represents Tehran strain (Type II); Lane 3 represents VEG strain (Type III) and Lanes 4–9, correspond to Type I

Discussion

Nowadays, the importance of soil as a reservoir and its roles in epidemiology of *T. gondii* is clear to researchers (20). Soil contamination by shedding of millions of oocysts from infected stray cats can be a potential threat for human health (8, 21, 22).

Presence of *T. gondii* oocysts in soil samples as well as other factors such as location, season of sampling, and genotypes of the parasite strains were investigated using real time-PCR and nested-PCR techniques with RE marker in the present study. Results showed the prevalence of *T. gondii* oocysts in soil as 14.9%, which was higher than those of Markazi and Tehran Provinces (5%, 8.7% respectively) and lower than those of Mazandaran (78.1%) (11-13). Researchers reported prevalence rates of *T. gondii* oocysts in soil samples from Mexican parks and China as respectively 11.8% and 11.9%; similar to the current study (23, 24).

Climate conditions such as humidity, temperature and rainfall as well as host-linked behaviors are two group parameters that affect spread and survival of the oocysts in soil (25). Relatively, in areas with an average rainfall of more than 25 mm over 10-day periods, increases in the prevalence of *T. gondii* were seen in cats, showing roles of rainfall in accelerating sporulation of the oocysts and exposing cats to the infection (26). Out results showed that the spread of oocysts in soil was more in summer; for which, rapid sporulation and appropriate conditions could increase their infectivity. The highest number of oocysts was isolated from soil samples during summer. Food availability in summer might boost rodent reproduction and cats could be infected by hunting *Toxoplasma* infected rodents as well (27). Hence, this could result in seasonal transmission of *T. gondii*, especially when most of the recreational trips occur in summer with more soil contacts . Technically, foods, drinks, vegetables and soils contaminated with oocysts are more likely to infect pregnant women and children (1,28). *T. gondii* infection was more common in pregnant women, who had direct contacts with soil (29). In the current study, soil contamination with oocysts of *T. gondii* was higher in urban areas than in rural areas. However, differences were not statistically significant. Presence of *T. gondii* oocysts in soil samples around trash bins in cities might reveal association of these locations to human infection. Based on a study in France, geographical distribution of the parasite oocysts in metropolitan environments varies particularly with cat defecation locations (30). Most cats forage near the trash bins in cities and people could be exposed to the infection via direct or indirect contacts with the contaminated dirt around the bins.

Cats have been recorded using the garbage collected by domestic trash collectors for defecation in various urban areas of Guilan Province. Furthermore, cats occasionally defecate on the ground without burying their feces in the soil. After heavy rainfalls and upon the rising of rainwater levels in streets and alleys oocysts could enter to groundwater sources such as wells , especially in the second half of the year, the water of these wells are used for drinking or washing fruits and vegetables in some regions.

In this study, six *T. gondii* strains were isolated from soil samples, closely linked to Type I, using GRA6 marker and MseI enzyme. However, Type I genotyping is preliminary and genotyping with a single marker technically does not identify non-clonal strains. To identify more precisely polymorphisms in the population, use of Multilocus PCR-RFLP and Multi Locus Sequence Typing (MLST) analyses is needed. Based on the published reports, associations exist between *T. gondii* genotype and severity as well as manifestations of the disease in human infections (15, 31). *Toxoplasma gondii* includes three distinct genotypes, known as I, II, III, as well as a number of unknown genotypes. Type I strains are known as highly virulent and Type II and III as relatively non-virulent strains (15). Human toxoplasmosis includes various signs and symptoms, ranging from asymptomatic to life-threatening ones, par-

ticularly in the brain and this variations might be attributed to the strain of the parasite. Investigating genotypes of the dominant strains in environmental reservoirs may help disease management in human populations. For example, mothers infected with Type I strain have almost double chance of having children with mental issues (32). Similarly, Lass et al. reported Type I as the major parasite genotype in Polish cities in an epidemiological study on the occurrence of oocysts in the soil (33). Ferroglio et al. identified Type I as the dominant genotype in a study on animals in northern Italy (34). Moreover, Type I has been reported as the dominant genotype in studies on humans and animals from various regions of Iran (35-40).

It seems that more studies are needed to detect the rate of oocysts and their genotypes in soil samples from different parts of country.

Conclusion

A relatively high level (14.9%) of contamination with *T. gondii* oocysts was detected in soil samples in Guilan Province, northern Iran. Results have revealed the potential risks of soil as an important source of *T. gondii* infection in humans and other animals in northern Iran.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare no conflict of interest.

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