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Identification of *Acanthamoeba* spp. from water and soil of public parks in the north of Iran

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ABSTRACT

Acanthamoeba, a free-living and opportunistic protozoan parasite, is a causative agent of severe human infections of the cornea and brain. The present study evaluated the distribution and genotyping of *Acanthamoeba* spp. in water and soil of recreational places in various areas in Guilan province in northern Iran. Eighty water and 20 soil samples were collected from the study area. Water samples were vacuum filtered through a 0.45 μm pore-size membrane filter. Soil samples were washed with sterile distilled water, and washings were similarly filtered, as mentioned for water samples. The filtered material was cultured on non-nutrient agar plates seeded with heat-killed *Escherichia coli*. Molecular analysis was performed by PCR and sequencing using specific primers for *Acanthamoeba*. Finally, 26 isolates were successfully sequenced. According to culture and PCR methods, 54% of water and 100% of soil samples were contaminated with *Acanthamoeba*. Based on the sequencing data, genotypes T4 (47%), T5 (35.29%), T3 (11.76%), and T11 (5.88%) were identified in water samples. Genotypes T4 (66.6%), T5 (22.2%) and T15 (11.1%) were identified in water samples. Most isolates might present a potential health hazard for humans in this region. To the best of our knowledge, this is the first comprehensive survey of water and soil of recreational areas in northern Iran and the first report on identifying genotype T15 from soil sources.

Key words: *Acanthamoeba*, genotype, polymerase chain reaction, soil, water

HIGHLIGHTS

- *Acanthamoeba* detected in entertainments environmental samples.
- Pathogenic genotypes of *Acanthamoeba* detected in the soil of public entertainment parks.
- Hygiene and policies are necessary to protect humans against *Acanthamoeba* infections.

INTRODUCTION

Acanthamoeba is distributed globally and found in various sources, including soil, water, dust, plants, vegetables and contact lens solutions. Some free-living amoeba of the genera *Acanthamoeba* occasionally invade hosts and cause life-threatening infections such as granulomatous amoebic encephalitis (GAE), amoebic keratitis (AK), and cutaneous lesions. Moreover, *Acanthamoeba* can serve as hosts for some pathogenic microorganisms, which can then cause host infection (Scheid 2018).

Using a contact lens is the predominant risk factor for amoebic keratitis. The incidence of AK has been steadily increasing in direct correlation with contact lens wearing (Varacalli *et al.* 2021). However, AK has also been reported in non-contact lens wearers. Individuals who are not contact lens wearers but have been constantly exposed to dust particles, soil, and contaminated water are also at high risk of infection. It is also important to note that contamination of *Acanthamoeba* can be as simple as an accidental splash of dirty water on the face or bruised skin, making a fast and easy transmission (Bunsuwansakul *et al.* 2019).

Acanthamoeba is a free-living amoeba with two stages in the life cycle, including trophozoites and cysts. Dormant cysts with minimal metabolic activity are much more resistant to harsh conditions, such as extremes in temperature, dryness, and pH (Varacalli *et al.* 2021). Twenty distinct genotypes (T1 to T22) of *Acanthamoeba* have been reported based on rRNA sequences (Tice *et al.* 2016). Notably, genotype T4 is the most frequently isolated from clinical cases. Other isolated genotypes associated with human infections include T2, T3, T5, T6, T11, and T15 (Castro-Artavia *et al.* 2017).

Environmental sources can provide a significant potential risk for human infection, and studying the prevalence of *Acanthamoeba* in different environments could be helpful for the control and prevention of disease in humans. Only a few studies are surveying the contamination with FLA in soil sources of Iran, and in most studies, *Acanthamoeba* has been identified according to its morphological characteristics.

The prevalence of *Acanthamoeba* in different soil and water sources is estimated to be 42.7% in Iran (Kialashaky *et al.* 2018). In a previous study in Guilan province, *Acanthamoeba* was reported in mineral springs, rivers, hot springs and seawater (Mahmoudi *et al.* 2015a, 2015b, 2021; Feiz Haddad *et al.* 2019, 2020).

The presence of *Acanthamoeba* in aquatic sources and soil could be a dual danger because some are pathogenic and harbour pathogenic microorganisms. Thus, medical and health staff must know the distribution of *Acanthamoeba* in water and soil samples and their control measures.

The present study investigated the prevalence and genotypes of *Acanthamoeba* spp. in soil and water samples collected from different public parks in various counties of Guilan province (northern Iran) by molecular methods. The present study provided more information on FLA diversity in the region and potential sources of FLA infection in Guilan, Iran.

MATERIALS AND METHODS

Study site

This cross-sectional descriptive study was conducted in Guilan province, northern Iran, from May to June 2019. Guilan province (37.2809N, 49.5924E) is located alongside the Caspian Sea. This territory in Iran has a humid subtropical climate with the heaviest rainfall records averaging 190 cm on the southwestern coast. Guilan province consists of 17 counties. Samples were collected from all counties of Guilan province (Figure 1).

Collection and processing of samples

Eighty water samples and 20 soil samples were randomly collected from public parks in all counties of Guilan province. These sampling points were located in public places frequented for recreational activities.

Water samples were collected from water fountains and pond water of parks in 17 counties of Guilan province. Each water sample (500 ml) was vacuum filtered through a 0.45 μm pore-size membrane. Later, filtered material was cultured on non-nutrient agar plates seeded with heat-killed *Escherichia coli*. The plates were incubated at room temperature and examined daily for the presence of cysts and trophozoites for 14 days using a light microscope.

Each soil sample consisted of approximately 50 g of soil collected at a depth of 1–3 cm, without vegetation. The samples were sealed in plastic bags and transported to a laboratory. Soil samples were washed with sterile distilled water, and washings were similarly filtered and cultured, as mentioned for water samples (Mahmoudi *et al.* 2015a, 2015b, 2021).

Subcultures were performed for *Acanthamoeba* positive culture, and then amoebae were harvested from plates and washed using phosphate-buffered saline (PBS pH 7).

DNA extraction and molecular analysis

DNA extraction was performed for all samples with a DNA extraction kit (FAVORGEN Biotech Corporation, founded in Taiwan). According to our previous study, to facilitate the breakdown of the cyst wall, the purified cysts were treated by freezing in liquid nitrogen for 5 minutes and boiling in a water bath for 5 minutes (Mahmoudi *et al.* 2011, 2020). Subsequently, DNA extraction was conducted for each treated sample following the manufacturer's protocol.

In the next step, PCR was performed for all DNA samples and *Acanthamoeba* specific primer set (JDP1: 5′-GGCCCA-GATCGTTTACCGTGAA-3′JDP2: 5′ -TCTCACAAGCTGCTAGGGAGTCA-3′) used to amplify approximately 500 bp of 18S rDNA gene as described previously (Mahmoudi *et al.* 2015a, 2015b, 2021).

The PCR amplification was performed on a thermal cycler (Bio-Rad) using the conditions 94 °C for 3 min; 35 cycles of 94 °C for 35 s, 56 °C for 45 s, 72 °C for 45 s; followed by a final extension at 72 °C for 5 min. The PCR amplification and *Acanthamoeba*-positive DNA sample and distilled water were included as positive and negative control, respectively. Finally,

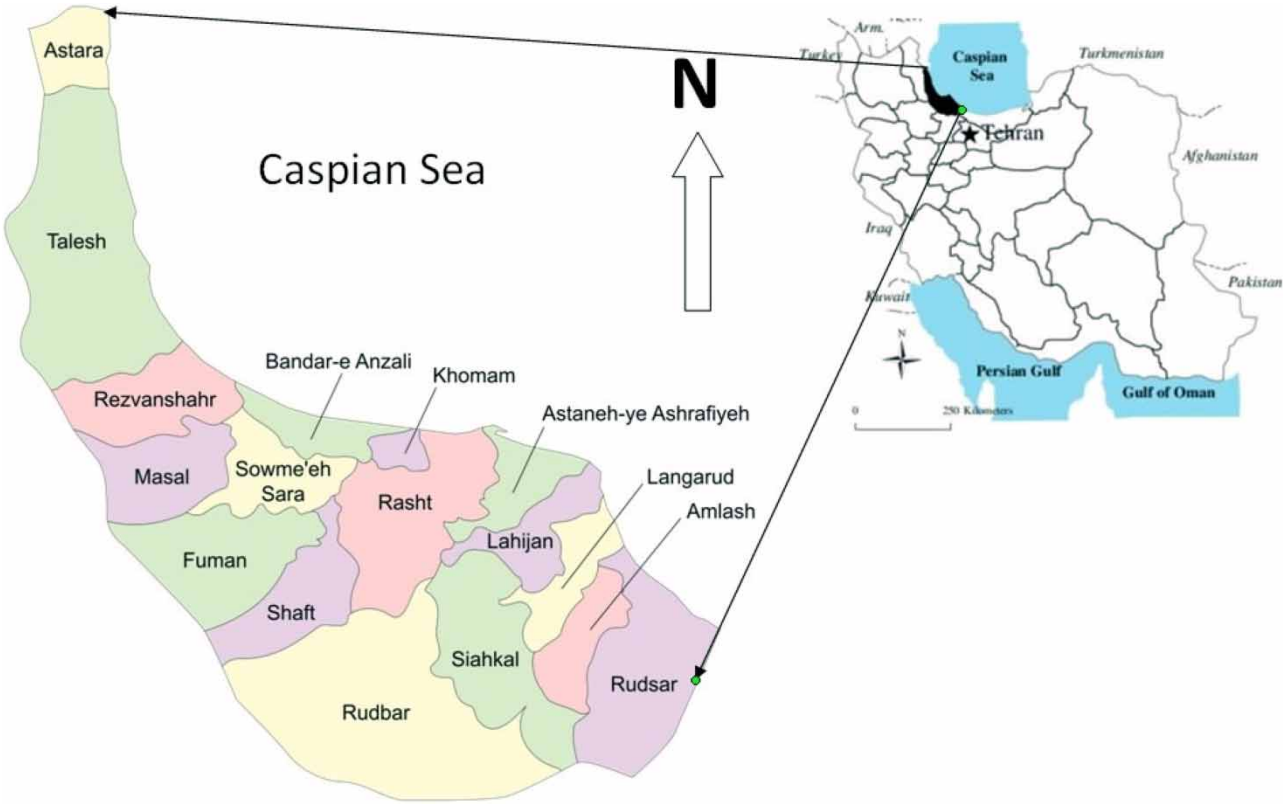

Figure 1 | The map of Guilan province and counties.

PCR was assessed by electrophoresis on 1.5% agarose gels, stained with RedSafe and visualized using the UV transilluminator (Mahmoudi *et al.* 2021).

The PCR products were sequenced using the forward primers used for amplification by a 3130XL Applied biosystems-ABI sequencer (Codon Genetic Group, Iran).

The sequences were compared with genes archived in the gene data bank using BLAST software from the National Center for Biotechnology Information (NCBI) webpage.

RESULTS

Out of 80 water samples collected from the parks, 40 (50%) samples were found to be positive for *Acanthamoeba*; by culture, all of which were confirmed by PCR. All soil samples were positive for *Acanthamoeba* by culture and PCR methods.

Overall, 26 isolates were subjected to genotyping. The most common genotypes were T4 ($n = 14$), T5 ($n = 8$), T3 ($n = 2$), T11 ($n = 1$), and T15 ($n = 1$). At least one isolate of each county was enrolled in the study. Based on the sequencing data, genotypes T3, T4, T5 and T11 were identified in water samples. The genotypes T4, T5 and T15 were identified in soil samples. Derived sequences were deposited in GenBank under accession numbers OM414908 to OM414927 and OM414929 to OM414934. The detected isolates are presented in Table 1.

DISCUSSION

FLA in environmental water samples

In the present study, the frequency of free-living amoeba and *Acanthamoeba* in water samples was 81.73 and 50%, similar to previous studies in Jamaica, 50.6% (Todd *et al.* 2015a, 2015b) and Iran, 59.4% (Golestani *et al.* 2018).

In the previous study in Guilan province, a higher incidence of *Acanthamoeba* was reported at 88% and 70.3% in surface water (Mahmoudi *et al.*, 2012, 2015a, 2015b). The public parks' water contamination rate with *Acanthamoeba* in the current study was higher than that reported in other studies in hot springs, 12.9% (Feiz Haddad *et al.* 2019), 44% in mineral springs

Table 1 | Prevalence and genotypes of *Acanthamoeba* in water and soil of entertainments public parks in Guilan province

Sampling	Total samples	Acanthamoeba spp. Culture positive	PCR positive	Genotype			GenBank accession no
Water samples	80	40 (50%)	40 (50%)	T4	8	47%	OM414917 to OM414925, OM414929 to OM414934 and OM414927
				T5	6	35.29%	
				T3	2	11.76%	
				T11	1	5.88%	OM414916
Soil	20	20 (100%)	20 (100%)	T4	6	66.6%	OM414908 to OM414915 and OM414926
				T5	2	22.2%	
				T15	1	11.1%	
Total	100	60 (60%)	60 (60%)				

(Feiz Haddad *et al.* 2019) and 45.3% in seawater (Mahmoudi *et al.* 2021) in Guilan. The difference in detection rates of *Acanthamoeba* in different countries and localities may be influenced by several factors, such as water temperature in distinct geographical regions and various climatic conditions (Tice *et al.* 2016). Also, water stagnation leads to biofilm formation, which augments the number of free-living amoebae (Hassan *et al.* 2012).

The present study revealed that most of the genotyped environmental *Acanthamoeba* isolates were T4 and T5 genotypes (Table 1). This result is consistent with Booton *et al.* (2005) and Tanveer *et al.* (2013). They reported that the T4 genotype is the most common *Acanthamoeba* genotype in the environment.

In previous studies in Guilan, the T4 was the most common genotype of *Acanthamoeba* in hot springs (Feiz Haddad *et al.* 2019) and mineral springs (Feiz Haddad *et al.* 2019). The T4 and T5 were the most common genotypes of *Acanthamoeba* in environmental samples in Iran (Kialashaky *et al.* 2018). The T3 genotype is responsible for multiple cases of keratitis (Booton *et al.* 2005), and Ledee *et al.* (2009) have isolated the T5 genotype from AK cases.

The presence of potentially pathogenic isolates (including genotype T4) in the study area is a hygienic risk for humans, especially immunosuppressed persons and patients with corneal trauma and contact lens wearers engaged in water-related recreation. Moreover, *Acanthamoeba* is likely to be infected by some other pathogenic microorganism; thus, this parasite can help grow and transport waterborne pathogens.

FLA in soil samples

The current research showed that 100% of soil samples are contaminated with *Acanthamoeba*, which almost matches the results of Rezaeian *et al.* (2008), presenting an occurrence of 100% in soil samples in Tehran, Iran.

There are only a few studies on the detection and genotyping of *Acanthamoeba* from soil sources in Iran and the world. In a previous study in Iran, the *Acanthamoeba* contamination rate was 41.6% from soil sources (Karamati *et al.* 2016). Prevalence of *Acanthamoeba* in soil samples was reported at 63.9% and 62.5%, respectively, in Jamaica (Todd *et al.* 2015a, 2015b) and the Canary Islands (Reyes-Batlle *et al.* 2014). A lower detection frequency, 40 and 38%, was recorded in Cairo, Egypt (Tawfeek *et al.* 2016) and South Florida (Booton *et al.* 2005), respectively.

The difference in results may be due to the diversity of environments tested as dry or wet soil and seasonal variation, as mentioned by Kao *et al.* (2013). The prevalence of *Acanthamoeba* in soil samples was higher than in most above mentioned studies in Iran and the world. The higher abundance of *Acanthamoeba* spp. in soil sources of present study regions reflects that they are suitable niches for the outgrowth of amoebae. It may be due to the moister content, organic carbon and soil texture (Rodríguez-Zaragoza 1994; Todd *et al.* 2015a, 2015b; Karamati *et al.* 2016).

In the present study, genotypes T4, T5, T11 and T15 were detected, and all showed pathogenic potential using thermotolerance assay.

A previous genotyping analysis in soil samples in Iran resulted in identifying genotypes T3, T4, T5 and T11 (Karamati *et al.* 2016). Mahmoudi *et al.* (2021) reported T2, T4 and T11 in hospital ward dust samples from Guilan province.

In a meta-analysis study of *Acanthamoeba* prevalence and genotyping in Iran, the T4 (81.2%) and T5 (16.04%) were the most common genotypes of *Acanthamoeba* in environmental samples in Iran (Kialashaky *et al.* 2018).

The T4 genotype is the primary etiological agent of *Acanthamoeba*-related infections such as GAE, AK and cutaneous infections in Iran and worldwide. Moreover, researchers reported an enhanced rate of keratitis due to *Acanthamoeba* spp. with T4 genotype. This genotype has become increasingly important because of its wide distribution in environmental sources and the resistance of cysts to disinfectants (Kialashaky *et al.* 2018).

Todd *et al.* (2015a, 2015b) reported T4, T5 and T11 genotypes in soil samples in Jamaica, West Indies. Genotypes belonging to T2, T5 and T4 were reported in the soils of Gran Canaria, Canary Islands, Spain (Reyes-Batlle *et al.* 2014).

In the present study, T4 and T5 were the most common genotypes of *Acanthamoeba* in soil and water samples. Soil contamination can contribute to water pollution and dust from soil and vice versa, and water is one of the standard routes for transporting soil pollutants. Since T4 was reported as the dominant genotype in soil and water in the current research, and considering that this genotype is one of the leading causes of amoebic keratitis, brain encephalitis and skin infections, therefore the soil is regarded as one of the risk factors and can be considered important in the transmission of this disease in the study area.

The results of this study show that soil pollution in parks, which are places for children to play and gather, can be a health risk to the children who are most exposed to it.

Soil contamination by *Acanthamoeba* cysts can cause contamination of water resources and also cause contamination of humans through the dust.

Therefore, with the necessary warnings, health officials should be given warning signs to prevent *Acanthamoeba* infections.

To the best of our knowledge, this is the first comprehensive survey of water and soil of recreational areas in northern Iran and the first report on identifying genotype T15 from soil sources in the study area. Genotype T4 was the more prevalent genotype in the soil and water of public parks in the study area. Our results indicate that the public parks in Guilan province are commonly contaminated with *Acanthamoeba* and potential risks to humans. The current findings show a high abundance of *Acanthamoeba* spp. in soil and water samples of recreational public parks, reflecting that they are suitable niches for the outgrowth of amoebae. This may be due to appropriate environmental conditions in the study area, representing a risk for human health. Thus, the implication of alarming signs in recreational areas and education to high-risk people, such as contact lens wearers, is significant.

CONCLUSIONS

The current findings serve as a document for the presence of pathogenic *Acanthamoeba* isolates in the soil and water of parks in the study area. These places are related directly to human populations and could be reservoirs and sources of the T4 genotype. Therefore, soil and water of parks are considered risk factors for sensitive hosts, including contact lens users, children, and people with immune system defects in the study area. The predominance of the T4 genotype and its wide distribution in the environment can increase the possibility of infection with cysts of this pathogenic genotype by humans.

ETHICAL APPROVAL

Ethical approval was obtained from the Ethical Committee Board of the Guilan University of Medical Sciences (Ref. No. approved this study: IR.GUMS.REC.1400.055).

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AUTHORS' CONTRIBUTIONS

M.R.M. drafted the manuscript, evaluated the results, and provided administrative and technical support; M.M. acquisition of data; B.R. and N.Z. analysed and interpretation of data; K.A. and M.S. acquisition of data and evaluated the results; P.K. drafted the manuscript and performed critical revision of the manuscript, assessed the effects, and provided administrative and editorial support.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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