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Genotyping and phylogenetic analysis of free-living amoeba (*Acanthamoeba* and *Naegleria*) in treated and untreated water in the northeastern provinces of Iran

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ABSTRACT

Free-living amoebae (FLA) are widely distributed protozoa in natural or man-made aquatic environments without the need for a host organism for survival. Several strains of FLA are known to be pathogenic. As of date, there is inadequate data on the geographical distribution of FLA in northeastern and northern Iran. This study aimed to investigate the prevalence and genotype distribution of *Acanthamoeba* and *Naegleria* in drinking water and surface water samples in northern and northeastern Iran. A total of 60 water samples were collected and filtered from various sources for the presence of amoebae. DNA extraction was performed, and PCR confirmed the presence of FLA. PCR products were sequenced to identify the species/genotype. Phylogenetic relationships and taxonomic status constructed using MEGA X software. The findings on growth media showed 35% (21/60) and 26% (16/60) were positive for *Acanthamoeba* and *Naegleria*, respectively, while PCR analysis also obtained similar results. All isolates of *Acanthamoeba* were identified as T4 genotype. Poor water quality, as well as insufficient preservation and treatment, might indicate that chlorine disinfection is ineffective in removing contamination of amoebas in treated water samples. Therefore, regular water quality monitoring is essential to control amoeba's growth, reducing the risk of human infections with FLA.

Key words: *Acanthamoeba*, free-living amoebae (FLA), *Naegleria*, water

HIGHLIGHTS

- The presence of FLA was evaluated in treated/untreated drinking water.
- *Acanthamoeba* sp., and *Naegleria* spp., were identified among 21 (35%) and 16 (26%) of samples.
- All *Acanthamoeba* isolates were T4 genotypes.
- *Naegleria* spp., were *N. americana*, *N. dobsoni*, *N. pagei*, and *N. australiensis*.

INTRODUCTION

Different genera of free-living amoebae (FLA), including *Balamuthia*, *Acanthamoeba*, *Naegleria*, *Sappinia* and *Vermamoeba* (*Hartmannella*), can cause disease in humans (Qvarnstrom *et al.* 2009).

FLA is widely distributed in the environment and can be transmitted to humans (Teixeira *et al.* 2009; Bagheri *et al.* 2010).

FLA is also known as amphizoic amoebae because of their ability to live in a host and in different sources, namely soil, air, and water (i.e. sea, lakes, ponds, pools, dams, hospital drinking water sources, and treated water) (Ahmadi *et al.* 2021).

Amoebic cysts are very resistant to temperature variation, pH, chlorine, detergents and common disinfectants. Several species of FLA can cause accidental, opportunistic or non-opportunistic infections (Niyyati *et al.* 2015). These parasites can be found in hemodialysis units, hospitals, and contact eye lens and cleaning solutions along with bacteria or yeast (Greub & Raoult 2003; Niyyati *et al.* 2017).

FLA belonging to the genus *Acanthamoeba* are the causative agents of keratitis in healthy individuals, often among people who wear contact lenses. In opportunistic infections, *Acanthamoeba* species can cause skin infections, fatal granulomatous amebic encephalitis (GAE), and pneumonitis (Marciano-Cabral *et al.* 2000). The phylogenetic analyses based on the nuclear 18S ribosomal RNA (rRNA) gene have recently distinguished at least 22 genotypes (T1–T22) *Acanthamoeb* (Fuerst & Booton 2020). Clinical isolates and environmental samples in Iran and other parts of the world are mainly infected with the T4 genotype (Niyyati *et al.* 2015).

Acanthamoeba parasites are increasing significantly in Iran (Pazoki *et al.* 2020). Despite this fact, a number of regional researchers have focused their molecular discoveries on the discrimination of *Acanthamoeba* sp. (Karamati *et al.* 2016; Spotin *et al.* 2017). In different parts of Iran, due to the clinical importance of Acanthambiasis, genetic analysis of the parasite can be useful to target endangered populations and explain how the parasite spreads in different geographical areas (Spotin *et al.* 2017).

Meanwhile, more than 40 species of *Naegleria* have been identified (Spotin *et al.* 2017); only *Naegleria fowleri*, commonly found in freshwater lakes, rivers and hot springs, is responsible for a rare and fatal brain infection called primary amebic meningoencephalitis (PAM). *Naegleria australiensis* and *Naegleria italica* cause disease in laboratory animals (Di Filippo *et al.* 2017).

Acanthamoeba keratitis and PAM have been documented in Iran (Esboei *et al.* 2020; Ahmadi *et al.* 2021). Although the number of reported cases is low, FLA must not be overlooked as a possible cause of disease. The fact that reported cases are infrequent could be due to the lack of familiarity of physicians and laboratory personnel incorrectly identifying FLA infections (Movahedi *et al.* 2012; Mohammadpour *et al.* 2018).

Limited studies are available about FLA prevalence in the various sources of water samples in northern and northeastern Iran. Therefore, this study aimed to investigate the prevalence and genotype distribution of *Acanthamoeba* and *Naegleria* in drinking water and surface water samples by microscopic identification, staining, and molecular methods.

This information would be helpful for early detection of possible pathogenic FLA contamination in various water sources and further assess the risks of human contact with FLA. Humans can also be exposed to FLA through contact with contaminated water or through open wounds; hence, this study is crucial to fill the gaps of knowledge.

MATERIALS AND METHODS

First, a review of sampling areas and diagnostic methods used in this study is provided (Figure 1).

Ethics statement/permission approval

This cross-sectional review study was conducted in the three north and northeast Iran provinces (Golestan, North Khorasan and Semnan). It was approved by the Mashhad University of Medical Sciences ethics committee (Ethics number: IR.MUMS.MEDICAL.REC.1398.865).

Geographical information

Golestan province with coordinate's 36.8393°N 54.4444°E is located along the Caspian Sea. Moreover, it has a temperate climate. The annual rainfall is similar to other Mediterranean climates. Thousands of domestic and foreign tourists visited the province due to the rivers, hot springs and beautiful beaches. In contrast, North Khorasan province with coordinates of 37.4761°N 57.3317°E and Semnan in the geographical position of 35.5769°N 53.3953°E are semi-desert areas with lower average rainfall (Figure 2).

Sample collection

A total of 60 samples of treated or untreated water were collected at various locations in North Khorasan (20), Golestan (20), and Semnan (20). Each sample was collected in two 200 mL sterile tubes to detect FLA by filtration and centrifugation methods. All samples were transferred to the Parasitology Research Laboratory, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran, and processed the soonest time possible.

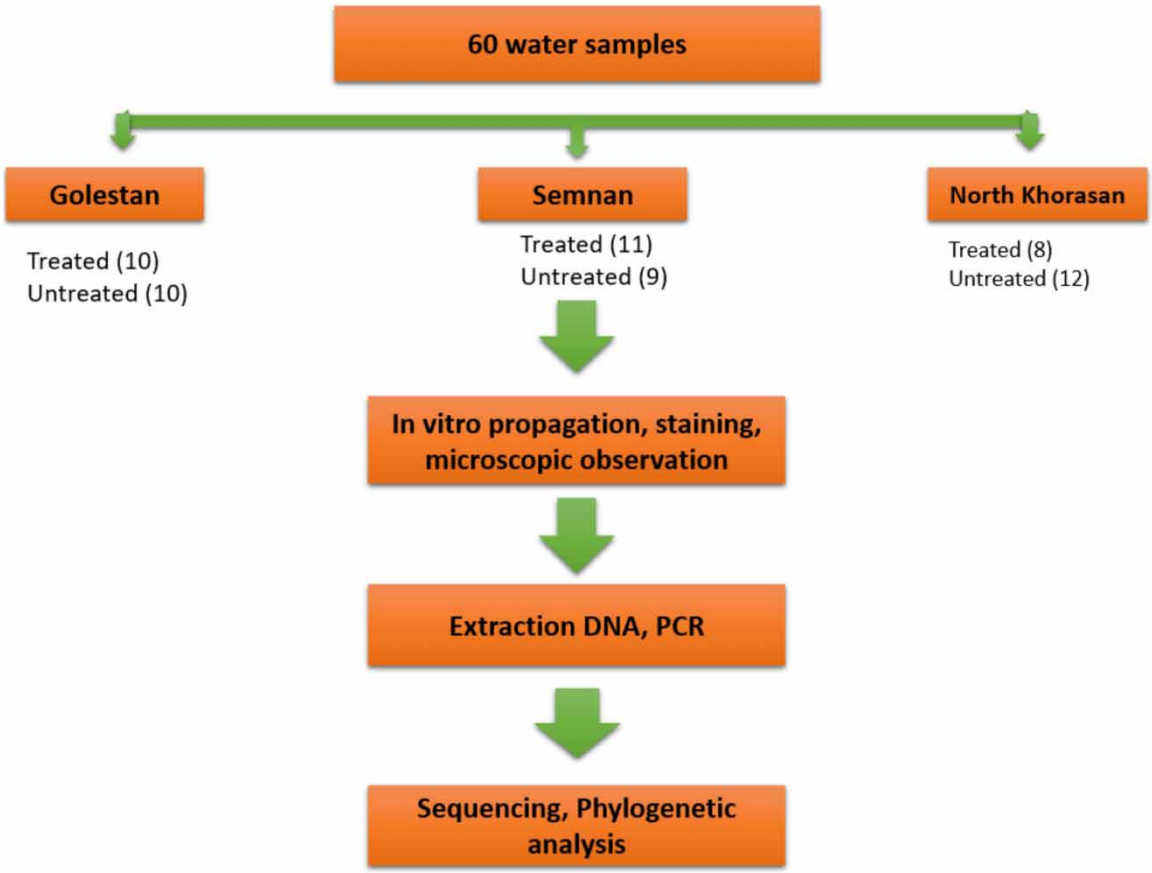

Figure 1 | Overview of the study design.

Isolation of free-living amoebae (FLA)

Firstly, one of the 200 mL tubes of water sample was passed through the cellulose nitrate membrane with 0.22 μm pore size. The filter was then placed on a non-nutrient agar plate coated with *Escherichia coli* (NNA-*E. coli*). In order to compare the filtration with the centrifuge method, all samples were also centrifuged in a volume of 200 mL at 2,000 rpm for 15 min. A few drops of resuspended samples were spread on non-nutrient agar plates coated with a layer of *E. coli*. Then, the media were incubated at room temperature (24–28 °C) for four weeks.

Morphological observation and identification

All the media were checked daily using an inverted microscope (Olympus, CKX53) maximum of 30 days. Giemsa and methylene blue-stained used a suspension of 30 μL from the plates containing the amoebae, and observed with a light microscope (Olympus BX51, Japan). Morphological features identified the differences between amoeba cysts and trophozoites (Page 1967; Page 1988).

DNA extraction

Acanthamoeba spp. cysts and trophozoites were removed from the culture plates by adding PBS and scraping of the media. The eluate was transferred into 1.5 mL sterile tubes. According to the manufacturer's instructions, DNA was then extracted using a commercial DNA kit (Blood Mini Kit, Bioneer, Korea). A nanodrop device (Thermo ND-ONE, USA) was used to determine the quality of DNA and finally stored at −20 °C until further analysis.

PCR amplification

All PCR amplifications were carried out in 20 μL reactions containing 1 μL of genomic DNA template, 10 μL of Amplicon Taq DNA Polymerase Master Mix RED, 0.25 μL each primer (25 μM stock), and 8.5 μL of de-ionized water (ddH20).

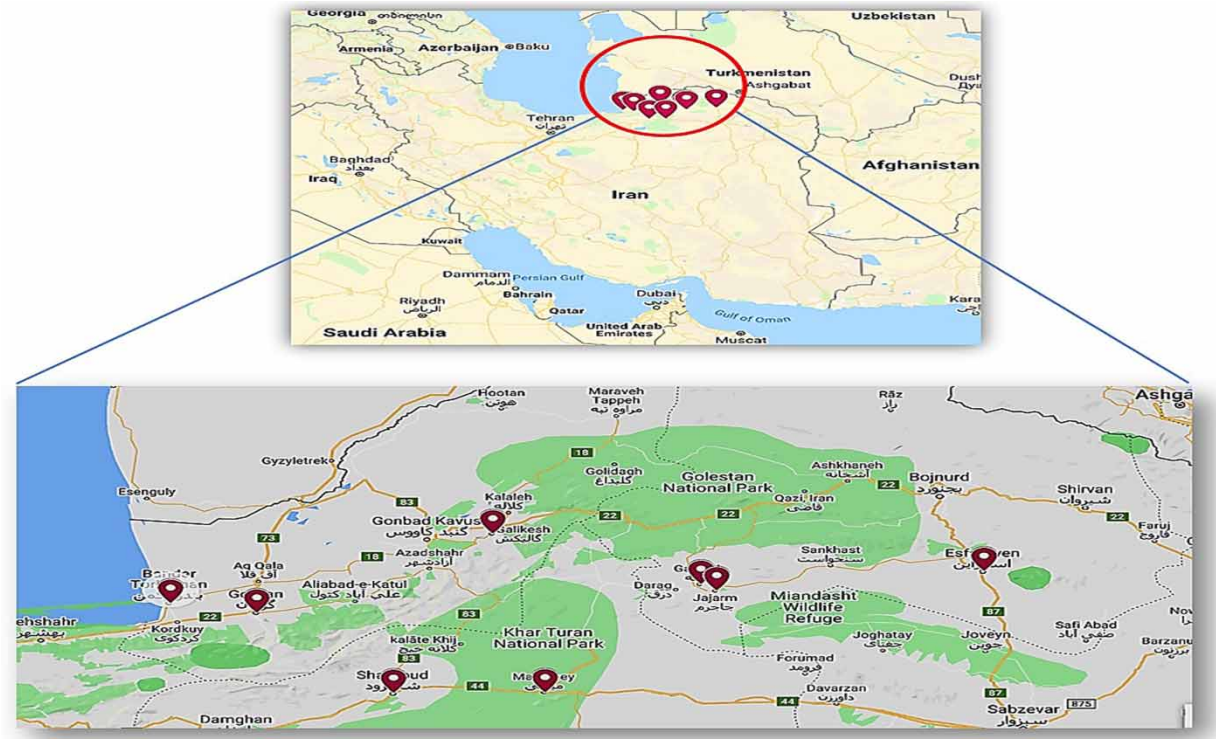

Figure 2 | Location of the sampling area in Iran. The sampled areas are marked with ( ).

A 450 bp fragment of 18S rRNA region of *Acanthamoeba* species was amplified using genus-specific forward primers JDP1 (5′-GGCCCAGATCGTTACCGTGAA-3′) and reverse JDP2 (5′-TCTCACAAGCTGCTAGGGAGTCA) (Booton *et al.* 2002). With the following parameters: the initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 35 seconds, 56 °C for 45 seconds, 72 °C for 1 min, followed by a final expansion at 72 °C for 7 min.

Naegleria species was detected by region-specific amplification ITS (ITS1, 5.8S, and ITS2) using species-specific forward primers: ITS1 forward primer (5-GAA CCT GCG TAG GGA TCA TTT-3) and ITS2 reverse primer (5-TTT CTT TTC CTC CCC TTA TTA-3) (Pélandakis *et al.* 2000). The amplification was performed with an initial denaturation at 94 °C for 2 min, 35 cycles of 94 °C for 35 seconds, 56 °C for 60 seconds and 72 °C for 1 min followed by a final extension at 72 °C for 7 min.

PCR products with positive and negative control of *Acanthamoeba* and *Naegleria* were evaluated by electrophoresis on 1.5% agarose gel at 100 voltage for 45 min. Also, a 100 bp DNA ladder (Yekta Tajhiz Azma, YT8503, Iran) was used to compare the expected amplitude of the gel. Subsequently, it was detected under ultraviolet (UV) light and images were taken (PhotoDoc-It gel imaging system).

Nucleotide sequencing and phylogenetic analysis

Favourable PCR products with good quality were sequenced with an ABI 310 automated fluorescent sequencing system using the primers above. The similarity between FLA sequences was found with other FLA species by the Basic Local Alignment Search Tool (BLAST) software provided by the National Center for Biotechnology Information (NCBI) in GenBank.

The sequences were analyzed by CLC Genomics Workbench 12 software and edited manually. Maximum likelihood reconstructions were performed using the phylogenetic program MEGA software version X. The trees were generated using the neighbour-joining method with the Kimura 2-parameter model. Bootstrap values estimated with 1,000 replicate data sets were indicated at each node. In addition, the samples in our study were compared with similar isolates in Iran and other parts of the world (Tamura *et al.* 2011).

RESULTS

Thirty-five percent (21/60) of water samples, including a sample from North Khorasan (7), Golestan (9), and Semnan (5), were positive for *Acanthamoeba* by culture (as shown in Tables 1 and 2). Similar results were also obtained using molecular analysis.

Table 1 | Occurrence of pathogenic FLA through microscopic examination and PCR in North Khorasan, Golestan, and Semnan

			Free-living amoeba (FLA)			
			Naegleria sp.		*Acanthamoeba* sp.	
Province	Type of water	No. of samples	M (n)	PCR (n)	M (n)	PCR (n)
North Khorasan	Treated[a]	8	ND[c]	ND[c]	3	3
	Untreated[b]	12	5	5	4	4
Golestan	Treated[a]	10	ND[c]	ND[c]	2	2
	Untreated[b]	10	7	7	7	7
Semnan	Treated[a]	11	1	1	2	2
	Untreated[b]	9	3	3	3	3
	Total	60	16	16	21	21

[a]Treated water includes drinking water, tap water and mineral water.
[b]Untreated water includes dams, springs, wells, recreational lake, rivers, waterfalls, canals.
[c]ND, not detected; M, microscopy; n, number of samples; PCR, polymerase chain reaction.

Table 2 | Sample distribution, types of water, and the number of positive samples based on PCR in this study

				No. PCR positive	
Province	City	Type of water	No. of samples	*Acanthamoeba* spp.	*Naegleria* spp.
North Khorasan	Garmeh	Drink	4	1	0
North Khorasan	Garmeh	Dam	4	1	2
North Khorasan	Garmeh	Subterranean	3	2	1
North Khorasan	Jajarm	Drink	2	1	0
North Khorasan	Jajarm	Subterranean	1	0	1
North Khorasan	Jajarm	Recreation areas	3	2	1
North Khorasan	Esfarayen	Drink	2	0	0
North Khorasan	Esfarayen	River	1	0	0
Semnan	Shahrod	Drink	6	1	0
Semnan	Kalpush	Drink	5	1	1
Semnan	Kalpush	Waterfall	5	2	2
Semnan	Kalpush	River	4	1	1
Golestan	Bandar-Torkaman	Sea	1	1	1
Golestan	Bandar-Torkaman	Drink	2	0	0
Golestan	Gorgan	Drink	5	1	0
Golestan	Gorgan	Waterfall	3	2	1
Golestan	Gorgan	River	2	1	2
Golestan	Lowe	Drink	3	1	0
Golestan	Lowe	River	2	2	2
Golestan	Lowe	Waterfall	2	1	1
Total of positive samples			**60**	**21**	**16**

Meanwhile, 26% (16/60) of samples, including samples from North Khorasan (5), Golestan (7) and Semnan (4), were positive for *Naegleria* by culture and PCR analysis (as described in Tables 1 and 2). Only seven *Acanthamoeba* and five *Naegleria* favourable PCR products were sent for sequencing.

The filtration method and centrifugation method showed the same results to detect FLA in media.

In culture, trophozoites and cyst stages were typically produced after 2–3 and 5–6 days of cultivation, respectively. The morphology of all isolates is distinguishable based on the motile stage of trophozoites/flagella.

Acanthamoeba cysts were identified by their star shape and irregular trophozoites with a superior design of acanthapodia in multidirectional motion.

In Figure 3(d), *Naegleria* trophozoites are cylindrical, and the cyst is circular with a relatively large central nucleus (Figure 3(c), 3(g) and 3(h)).

The figure also shows *Acanthamoeba* trophozoites with Acanthapodia legs (Figure 3(b)), and a star-shaped cyst (Figure 3(a), 3(e) and 3(f)).

In order to identify the morphology of pathogenic amoebae, Giemsa and methylene blue staining methods were compared to non-stained slides. In both staining methods, two separate layers of cysts (ectocyst and endocyst) were observed for *Acanthamoeba* isolates. *Naegleria* cysts were also observed in the form of a circle with a central nucleus in both stainings (Figure 3).

Using Giemsa and methylene blue staining, the parasite cysts were observed well-contrasted against the background and stained purple and dark blue, respectively. However, there is no distinct demarcation between the nucleus and the cytoplasm (Figure 3(e) and 3(f)).

Meanwhile, for the *Naegleria* cyst stage, trophozoites and flagellation were observed.

Amplification of the 18S rRNA region of *Acanthamoeba* produced a 450 bp PCR product. All isolates of *Acanthamoeba* belonged to the T4 genotype. Five isolates (MW040579, MW040577, MW040576, MW040580, and MW040582) were identified as *A. castellanii*, and two isolates (MW040581, MW040579) were identified as *A. polyphaga* (Table 3, Figure 4).

A PCR product length of about 500 bp was observed for *Naegleria* after amplification of the ITS region. The sequencing analysis showed that *Naegleria* isolates (MW042257 and MW042256) were 100% similar to *N. americana*, and two isolates (MW042259 and MW042258) detected in Semnan province have 96% and 93% similarity to *N. dobsoni* and *N. pagei*, respectively; another isolate (MW042260) was similar to *N. australiensis* (Table 3, Figure 5).

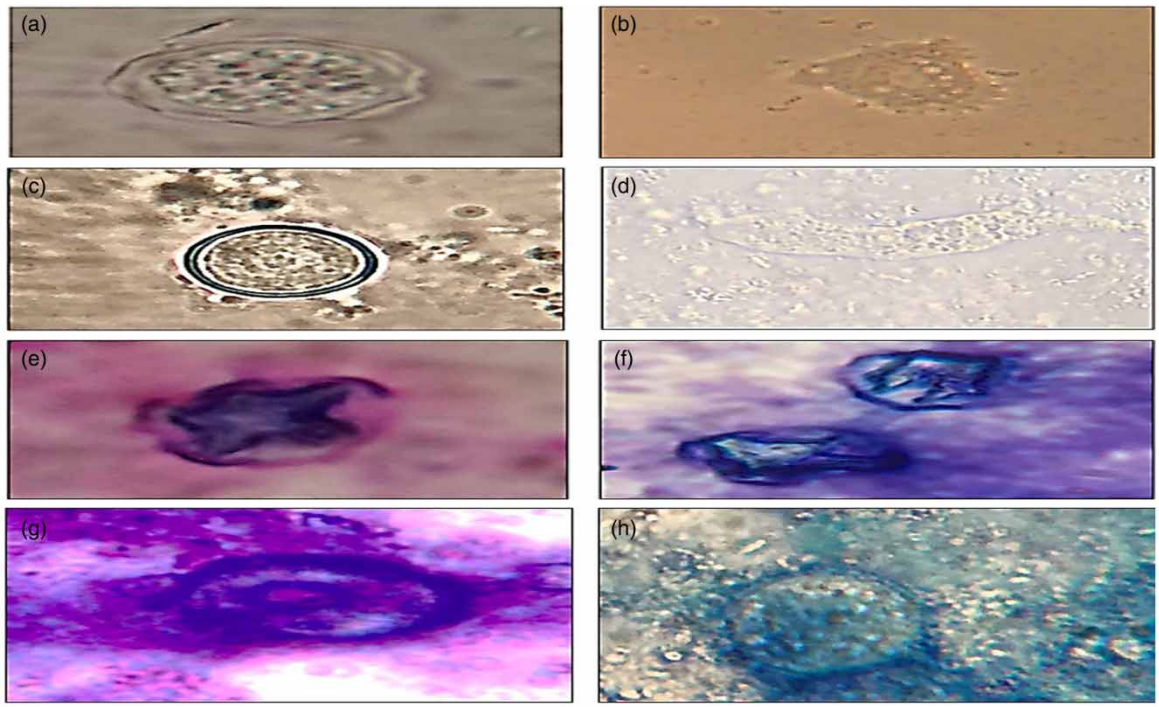

Figure 3 | Morphological features of trophozoites and cysts with a light microscope. (a) Non-stained *Acanthamoeba* star cyst (b) *Acanthamoeba* trophozoite without staining, (c) *Naegleria* circle cyst with a large central nucleus, (d) *Naegleria* cylindrical trophozoite, (e) two walls of *Acanthamoeba* star shape cyst (ectocyst and endocyst) with Giemsa staining, (f) two walls of *Acanthamoeba* star shape cyst (ectocyst and endocyst) with methylene blue staining, (g) *Naegleria* cyst with Giemsa staining, and (h) *Naegleria* cyst with Methylene blue staining.

Table 3 | *Acanthamoeba* genotypes and *Naegleria* species isolated in water samples of the study area

Province	Area	Sampling site	Genus	Species/genotype	Homology/query coverage	Accession number
North Khorasan	Garmeh	Treated water	*Acanthamoeba*	*castellanii*/ T4	100	MW040577
North Khorasan	Jajarm	Treated water	*Acanthamoeba*	*polyphaga*/ T4	97	MW040578
North Khorasan	Garmeh	Dam	*Acanthamoeba*	*castellanii*/ T4	100	MW040579
Semnan	Shahrod	Treated water	*Acanthamoeba*	*castellanii*/ T4	90	MW040580
Semnan	Kalpush	Treated water	*Acanthamoeba*	*polyphaga*/ T4	97	MW040581
Golestan	Gorgan	Treated water	*Acanthamoeba*	*castellanii*/ T4	99	MW040582
Golestan	Bandar Torkaman	Sea	*Acanthamoeba*	*castellanii*/ T4	96	MW040576
Semnan	Kalpush	Treated water	*Naegleria*	*americana*	100	MW042256
Golestan	Garmeh	subterranean	*Naegleria*	*pagei*	93	MW042258
Golestan	Gorgan	River	*Naegleria*	*australiensis*	75	MW042260
Golestan	Bandar Torkaman	Sea	*Naegleria*	*americana*	100	MW042257
North Khorasan	Garmeh	Dam	*Naegleria*	*dobsoni*	96	MW042259

DISCUSSION AND CONCLUSION

The ability of free-living amoebae (FLA) to cause severe infections in humans and their essential role in ecosystems has led to more researches on FLA (Abdul Majid *et al.* 2017). Previously, important studies have been reported on *Acanthamoeba* genotypes isolated from water in Iran (Rezaeian *et al.* 2008; Niyyati *et al.* 2015; Ahmadi *et al.* 2021). However, there is little information about this parasite in northeastern Iran. This study aimed to determine *Naegleria* and *Acanthamoeba* species' existence and molecular identity in treated water and untreated water samples from three northeastern provinces of Iran, especially North Khorasan, Semnan and Gorgan.

With an increasing number of FLA cases, several studies have been conducted on various water sources as a habitat to gather epidemiological data that would help reduce levels of FLA transmission worldwide (Behniafar *et al.* 2015).

Accordingly, a high percentage of *Acanthamoeba* and *Naegleria* species in water resources have been reported in recent Iranian studies, showing a significant increase for FLA in water resources in Iran, similar to the work done by Rezaian *et al.* In Tehran, 46.25% of the samples contained *Acanthamoeba* (Rezaeian *et al.* 2008). In a study in Shiraz, out of 82 water samples collected from different parts of the city, 48 (58.53%) samples were positive for FLA. *Acanthamoeba* was observed in 39 cases (47.56%). The results (62.96%) showed the T4 genotype (Armand *et al.* 2016b).

In another study, Niyyati *et al.* found that 38.2 per cent of 55 tap water samples collected from Kish Island in Iran were contaminated with FLA (Niyyati *et al.* 2015).

For the first time in Iran, Movahedi *et al.* reported (PAM) caused by *N. fowleri*. (Movahedi *et al.* 2012).

Najafpour *et al.* found several species of *Naegleria* in the water of parks' pools in Mashhad, Iran, including *N. Americana*, *N. clarki*, *N. andersoni*, *N. fultoni*, *N. carteri*, and *N. pagei*, but no *N. fowleri* (Najafpoor *et al.* 2018).

Previous studies in Iran showed that the T4 genotype of *Acanthamoeba* is the dominant type in patients with *Acanthamoeba* keratitis (Maghsood *et al.* 2005; Niyyati *et al.* 2009).

The results showed that the pathogenic *Acanthamoeba* and non-pathogenic *Naegleria* species by both microscopy and PCR techniques in North Khorasan, Semnan and Golestan for the first time in Iran.

Due to the recent advances in molecular detection, conventional PCR has been developed as a reliable method for routine screening of FLA in environmental samples. This is sufficient to distinguish between species of different organisms (Abdul Majid *et al.* 2017).

The classification status of amoebae isolated from water sources is shown in (Table 3) using BLAST software. In this classification, five samples were identified as *A. castellanii* (MW040577, MW040579, MW040580, MW040582, and MW040576), while two were confirmed as *A. polyphaga* (MW040578, MW040581).

Only one family of amoebae pathogenic for humans belonging to the genera of *Acanthamoeba* (Acanthamoebidae) was found following PCR and sequencing.

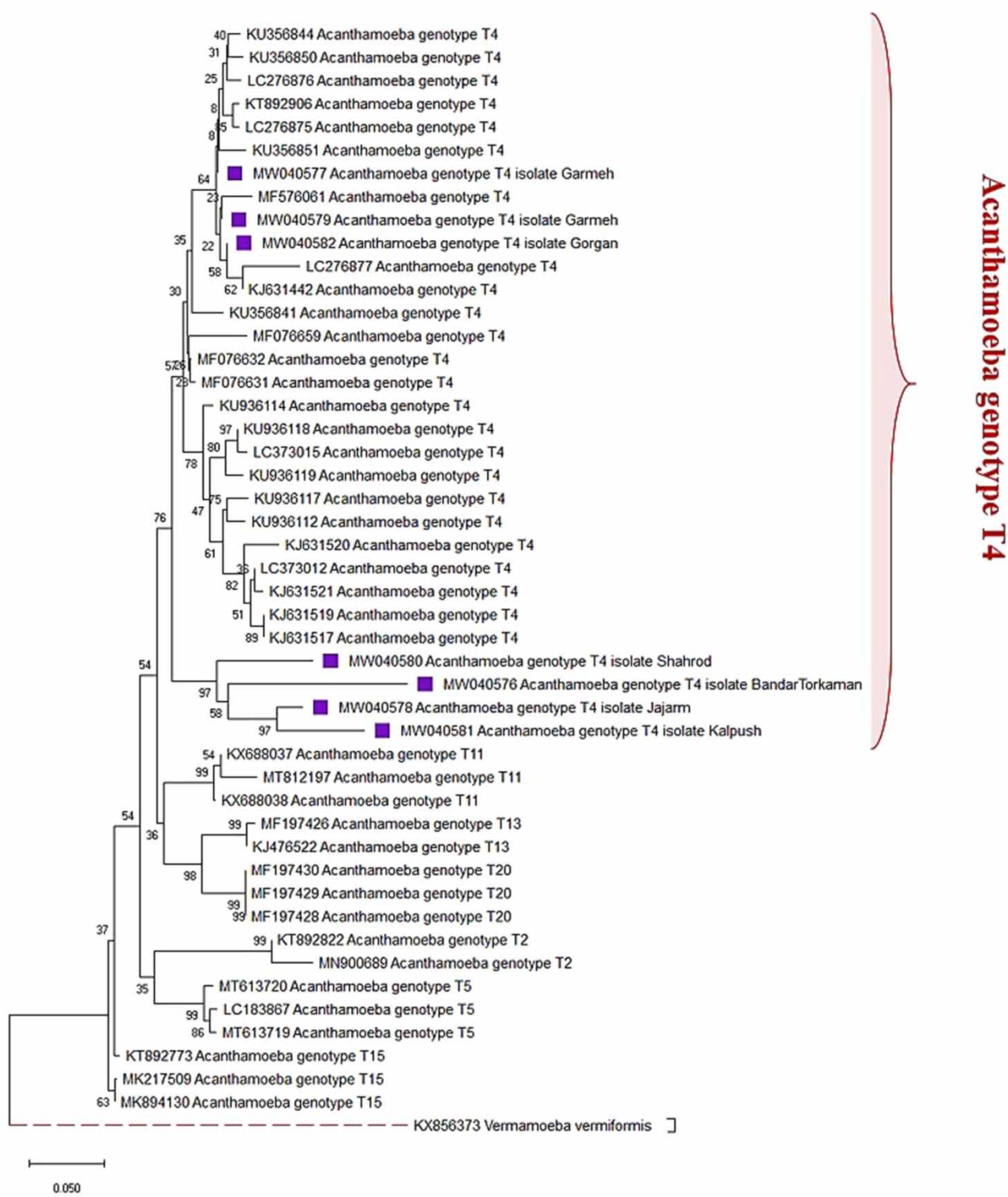

Figure 4 | The taxonomic status of amoebae isolated from water sources in a phylogenetic tree was demonstrated based on the sequences of *Acanthamoeba* 18S rRNA of the gene. Geometric patterns marked the isolates detected in this study.

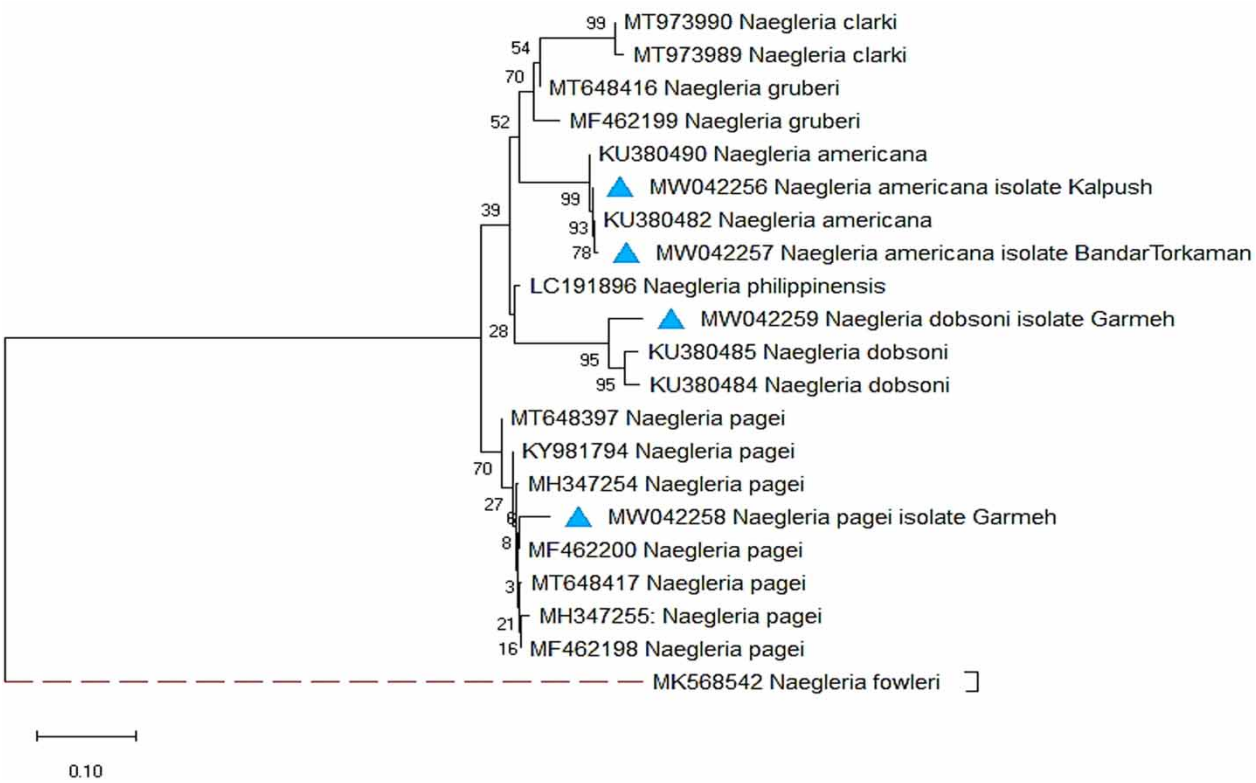

Figure 5 | The taxonomic status of amoebae isolated from water sources in a phylogenetic tree was demonstrated based on the ITS region of *Naegleria* genes. Geometric patterns marked the isolates detected in this study.

The pathogenic strain of *Acanthamoeba* was observed in 35% of water resources in the study area. The highest incidence of FLA was observed in Golestan province (45% *Acanthamoeba* and 35% *Naegleria*) then in north Khorasan FLA (35% *Acanthamoeba* and 25% *Naegleria*) and Semnan (25% *Acanthamoeba* and 20% *Naegleria*).

Based on the 18S rRNA sequence, most *Acanthamoeba* isolated in this study has been identified as T4 genotype, known as the most important causative agent of amoebic keratitis and GAE in the world (Visvesvara *et al.* 2007; Booton *et al.* 2009; Grün *et al.* 2014).

Since the provinces under study have touristic attractions, this isolation in many drinking water sources indicates a severe public health threat for indigenous peoples and tourists. Moreover, the risk of infection with FLA in individuals with immunocompromised states should be considered in these provinces. GAE is frequently related to human immunodeficiency virus and organ transplant. Although, it has also been reported from immunocompetent people (Basher *et al.* 2018).

The phylogenetic analysis also confirms the correct placement of the samples in the T4 genotype (Figure 4). It clearly shows the difference between different genotypes of *Acanthamoeba*. Many previous studies have similarly reported that T4 is the most common genotype among clinical and environmental samples in Iran and other parts of the world (Izumiyama *et al.* 2003; Niyyati *et al.* 2009; Lorenzo-Morales *et al.* 2015; Armand *et al.* 2016b).

Based on one step PCR assay with specific primers targeting the ITS region and 18S rRNA, there was no positive sample for *N. fowleri*. This may result in the proliferation activity of *N. fowleri* at high temperatures (up to 45 °C) (Izumiyama *et al.* 2003).

Although some cases of primary amoebic meningoencephalitis (PAM) caused by *N. fowleri* has been reported in Iran, the diagnosis of PAM should never be ignored because of its severity and fatality. The lack of skills and knowledge of physicians and laboratory personnel regarding the correct diagnosis of this amoeba can cause rare reported cases (Movahedi *et al.* 2012; Mohammadpour *et al.* 2018). *Naegleria* sequence analyses were related to *N. americana* (MW042256, MW042257), *N. australiensis* (MW042260), *N. dobsoni* (MW042259), and *N. pagei* (MW042258) species (Table 3). The phylogenetic analysis also confirms the correct placement of *Naegleria* specimens (Figure 5). It clearly shows the difference between the various species of *Naegleria*. However, these species of *Naegleria* are non-pathogenic to humans, which indicates the importance of poor quality of drinking water and surface water as a severe risk to human health.

Based on the centrifuge methods' findings, in the absence of facilities such as filtration for researchers, the centrifuge method is a promising tool to detect FLA.

In this study, various staining techniques were used in comparison with non-staining samples (Figure 3). We found that Giemsa staining, commonly used for blood parasites, provided a good contrast for identifying *Acanthamoeba* and *Naegleria* cysts. In addition, methylene blue staining had good quality in detecting *Acanthamoeba* and *Naegleria* cysts and distinguishing the outer and the inner layer of the cysts.

A previous study by Abdul Majid *et al.* (2017) stated that all stages (cysts and trophozoites) of FLA were defined better in cellular features and organs using Giemsa staining (Abdul Majid *et al.* 2017).

The findings showed that methylene blue and Giemsa staining could identify and diagnose FLA for clinical epidemiology and public health purposes.

However, the classification of FLA based on morphological criteria is insufficient and requires more precise identification, such as molecular PCR methods. Also, we can claim that this is probably the first time that methylene blue staining has been used to identify *Acanthamoeba*.

Despite conventional water treatment methods (filtration and disinfection) being used throughout Iran, treated waters were contaminated with FLA in all three provinces. It seems the FLA cyst stage is resistant to biocides and chlorination.

This finding increased knowledge about the distribution of FLA isolates in North Khorasan, Golestan and Semnan. Local authorities should not overlook the high prevalence of amoebae in the provinces. Appropriate precautions must be taken to ensure high water quality. It is suggested to add some other disinfectants such as chlorinated cyanuric acid, Baquacil and chlorine dioxide to water to control the growth of FLA (Marciano-Cabral 1988; Abd Ghani *et al.* 2010). Water used for recreational purposes (i.e. river, sea, dam) should also be closely monitored because untreated water may contain large amounts of FLA to transmit infections to humans (Ma *et al.* 1990; Szénási *et al.* 1998; Niyyati *et al.* 2015; Esboei *et al.* 2020).

Therefore, our results, in line with the results of other Iranian researchers who have studied in other cities of Iran (Rezaeian *et al.* 2008; Niyyati *et al.* 2015; Armand *et al.* 2016a; Esboei *et al.* 2020; Ahmadi *et al.* 2021) show the abundance of FLA in various water sources in Iran.

The results show the significance of an urgent need to seriously consider FLA where human activity has been observed in understudy water resources. Polluted surface waters investigated can act as potential sources for the distribution and transmission of *Acanthamoeba*, especially recreational waters and hot springs. They need periodic monitoring, particularly during the summer when millions of people use this surface water. Future research on FLA occurrence in treated and untreated water with larger sample sizes should be taken.

According to the results of this study and the pathogenic role of these protozoa in immunocompromised patients and the ability of these microorganisms to carry other pathogens such as *Legionella*, further studies are needed. Importantly, to provide a safe drinking water system in hospitals, you must be extremely careful to prevent nosocomial infections in immunocompromised patients, such as HIV patients.

As a result, the present study indicates the high presence of *Acanthamoeba* and *Naegleria* in water resources, including drinking water in northeastern and northern Iran.

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DATA AVAILABILITY STATEMENT

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

ETHICAL STATEMENTS

The Ethics Committee of Mashhad University of Medical Sciences in Iran approved this study.

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REFERENCES

Abd Ghani, M. K., Roslee, N. F., Nordin, A., Suboh, Y. & Ab Rahim, N. 2010 Pemencilan *Acanthamoeba* spp. daripada persekitaran akuatik. *Jurnal Sains Kesihatan Malaysia* **8**, 15–19.

Abdul Majid, M. A., Mahboob, T., Mong, B. G., Jaturas, N., Richard, R. L., Tian-Chye, T., Phimphila, A., Mahaphonh, P., Aye, K. N. & Aung, W. L. 2017 Pathogenic waterborne free-living amoebae: an update from selected Southeast Asian countries. *PloS one* **12**, e0169448.

Ahmadi, O., Sharifi, Y., Khosravinia, N., Moghaddas, E., Akhoundi, M., Fotouhi-Ardakani, R., Asadi, J., Mohamadzade, A. H., Sangani, G. S. & Mirjalali, H. 2021 Molecular identification and phylogenetic analysis of free-living amoeba (*Naegleria* and *Acanthamoeba*) from treated and untreated drinking water. *Gene Reports* **25**, 101328.

Armand, B., Motazedian, M. & Asgari, Q. 2016a Isolation and identification of pathogenic free-living amoeba from surface and tap water of Shiraz City using morphological and molecular methods. *Parasitology Research* **115**, 63–68.

Armand, B., Motazedian, M. H. & Asgari, Q. 2016b Isolation and identification of pathogenic free-living amoeba from surface and tap water of Shiraz City using morphological and molecular methods. *Parasitology Research* **115**, 63–68.

Bagheri, H., Shafiei, R., Shafiei, F. & Sajjadi, S. 2010 Isolation of *Acanthamoeba* spp. From drinking waters in several hospitals of Iran. *Iran Journal of Parasitology* **5**, 19–25.

Basher, M. H. A., Ithoi, I., Mahmud, R., Abdulsalam, A. M., Foead, A. I., Dawaki, S., Atroosh, W. M. M., Nissapatorn, V. & Abdullah, W. O. 2018 Occurrence of *Acanthamoeba* genotypes in Central West Malaysian environments. *Acta Tropica* **178**, 219–228.

Behniafar, H., Niyyati, M. & Lasjerdi, Z. 2015 Molecular characterization of Pathogenic Acanthamoeba isolated from drinking and recreational water in East Azerbaijan, Northwest Iran. *Environmental Health Insights* **9**, 7–12.

Booton, G., Kelly, D., Chu, Y.-W., Seal, D., Houang, E., Lam, D., Byers, T. & Fuerst, P. 2002 18S ribosomal DNA typing and tracking of *Acanthamoeba* species isolates from corneal scrape specimens, contact lenses, lens cases, and home water supplies of *Acanthamoeba* keratitis patients in Hong Kong. *Journal of Clinical Microbiology* **40**, 1621–1625.

Booton, G. C., Joslin, C. E., Shoff, M., Tu, E. Y., Kelly, D. J. & Fuerst, P. A. 2009 Genotypic identification of *Acanthamoeba* sp. isolates associated with an outbreak of *Acanthamoeba* keratitis. *Cornea* **28**, 673–676.

Di Filippo, M. M., Novelletto, A., Di Cave, D. & Berrilli, F. 2017 Identification and phylogenetic position of *Naegleria* spp. from geothermal springs in Italy. *Experimental Parasitology* **183**, 143–149.

Esboei, B. R., Fakhar, M., Saberi, R., Barati, M., Moslemi, M., Hassannia, H., Dadimoghadam, Y. & Jalallou, N. 2020 Genotyping and phylogenic study of *Acanthamoeba* isolates from human keratitis and swimming pool water samples in Iran. *Parasite Epidemiology and Control* **11**, e00164.

Fuerst, P. A. & Booton, G. C. 2020 Species, sequence types and alleles: dissecting genetic variation in *Acanthamoeba*. *Pathogens* **9**, 534.

Greub, G. & Raoult, D. 2003 Biocides currently used for bronchoscope decontamination are poorly effective against free-living amoebae. *Infection Control & Hospital Epidemiology* **24**, 784–786.

Grün, A. L., Stemplewitz, B. & Scheid, P. 2014 First report of an *Acanthamoeba* genotype T13 isolate as etiological agent of a keratitis in humans. *Parasitology Research* **113**, 2395–2400.

Izumiyama, S., Yagita, K., Furushima-Shimogawara, R., Asakura, T., Karasudani, T. & Endo, T. 2003 Occurrence and distribution of *Naegleria* species in thermal waters in Japan. *Journal of Eukaryotic Microbiology* **50** (Suppl), 514–515.

Karamati, S. A., Niyyati, M., Lorenzo-Morales, J. & Lasjerdi, Z. 2016 Isolation and molecular characterization of *Acanthamoeba* genotypes isolated from soil sources of public and recreational areas in Iran. *Acta Parasitologica* **61**, 784–789.

Lorenzo-Morales, J., Khan, N. A. & Walochnik, J. 2015 An update on Acanthamoeba keratitis: diagnosis, pathogenesis and treatment. *Parasite* **22**, 10.

Ma, P., Visvesvara, G. S., Martinez, A. J., Theodore, F. H., Daggett, P. M. & Sawyer, T. K. 1990 Naegleria and *Acanthamoeba* infections: review. *Reviews of Infectious Diseases* **12**, 490–513.

Maghsood, A. H., Sissons, J., Rezaian, M., Nolder, D., Warhurst, D. & Khan, N. A. 2005 *Acanthamoeba* genotype T4 from the UK and Iran and isolation of the T2 genotype from clinical isolates. *Journal of Medical Microbiology* **54**, 755–759.

Marciano-Cabral, F. 1988 Biology of Naegleria spp. *Microbiology Reviews* **52**, 114–133.

Marciano-Cabral, F., Puffenbarger, R. & Cabral, G. A. 2000 The increasing importance of *Acanthamoeba* infections. *Journal of Eukaryotic Microbiology* **47**, 29–36.

Mohammadpour, M., Rahimi, F. & Khorrami-Nejad, M. 2018 Total necrosis of cornea, iris and crystalline lens with exposure of vitreous hyaloid face in the context of recalcitrant *Acanthamoeba* keratitis. *Journal of Current Ophthalmology* **30**, 377–380.

Movahedi, Z., Shokrollahi, M. R., Aghaali, M. & Heydari, H. 2012 Primary amoebic meningoencephalitis in an Iranian infant. *Case Reports in Medicine* **2012**, 782854.

Najafpoor, A. A., Zarrinfar, H., Ghaderifar, S., Alidadi, H., Esmaily, H., Hajialilo, E., Farash, B. R. H. & Ahmadi, E. 2018 *Naegleria* species population found in pond water of parks in Mashhad city, can the physicochemical factors affect it? *MethodsX* **5**, 1427–1430.

Niyyati, M., Lorenzo-Morales, J., Rezaie, S., Rahimi, F., Mohebali, M., Maghsood, A. H., Motevalli-Haghi, A., Martín-Navarro, C. M., Farnia, S., Valladares, B. & Rezaeian, M. 2009 Genotyping of *Acanthamoeba* isolates from clinical and environmental specimens in Iran. *Experimental Parasitology* **121**, 242–245.

Niyyati, M., Lasgerdi, Z. & Lorenzo-Morales, J. 2015 Detection and molecular characterization of potentially pathogenic free-living amoebae from water sources in Kish Island, Southern Iran. *Microbiology Insights* **8** (MBI), S24099.

Niyyati, M., Arab-Mazar, Z., Lasjerdi, Z., Lorenzo-Morales, J., Espotin, A., Yadegarynia, D., Gachkar, L. & Roodsari, S. R. 2017 Molecular characterization of *Acanthamoeba* strains isolated from the oral cavity of hemodialysis patients in Iran. *Parasitology Research* **116**, 2965–2969.

Page, F. C. 1967 Taxonomic criteria for *limax* amoebae, with descriptions of 3 new species of *Hartmannella* and 3 of *Vahlkampfia*. *The Journal of Protozoology* **14**, 499–521.

Page, F. 1988 *A New Key to Freshwater and Soil Gymnamoebae*. Freshwater Biological Association, Ambleside.

Pazoki, H., Niyyati, M., Javanmard, E., Spotin, A., Mirjalali, H. & Behravan, M. R. 2020 Isolation and phylogenetic analysis of free-living amoebae (*Acanthamoeba*, *Naegleria*, and *Vermamoeba*) in the farmland soils and recreational places in Iran. *Acta Parasitologica* **65**, 36–43.

Pélandakis, M., Serre, S. & Pernin, P. 2000 Analysis of the 5.8 S rRNA gene and the internal transcribed spacers in *Naegleria* spp. and in *N. fowleri*. *Journal of Eukaryotic Microbiology* **47**, 116–121.

Qvarnstrom, Y., da Silva, A. J., Schuster, F. L., Gelman, B. B. & Visvesvara, G. S. 2009 Molecular confirmation of Sappinia pedata as a causative agent of amoebic encephalitis. *The Journal of Infectious Diseases* **199**, 1139–1142.

Rezaeian, M., Niati, M., Farnia, S. & Motevali, H. A. 2008 *Isolation of Acanthamoeba spp. From Different Environmental Sources*.

Spotin, A., Moslemzadeh, H. R., Mahami-Oskouei, M., Ahmadpour, E., Niyyati, M., Hejazi, S. H., Memari, F. & Noori, J. 2017 Phylogeography, genetic variability and structure of *Acanthamoeba* metapopulations in Iran inferred by 18S ribosomal RNA sequences: a systematic review and meta-analysis. *Asian Pacific Journal of Tropical Medicine* **10**, 855–863.

Szénási, Z., Endo, T., Yagita, K. & Nagy, E. 1998 Isolation, identification and increasing importance of 'free-living' amoebae causing human disease. *Journal of Medical Microbiology* **47**, 5–16.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. 2011 MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731–2739.

Teixeira, L. H., Rocha, S., Pinto, R. M. F., Caseiro, M. M. & Costa, S. O. P. d. 2009 Prevalence of potentially pathogenic free-living amoebae from *Acanthamoeba* and *Naegleria* genera in non-hospital, public, internal environments from the city of Santos, Brazil. *Brazilian Journal of Infectious Diseases* **13**, 395–397.

Visvesvara, G. S., Moura, H. & Schuster, F. L. 2007 Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. *FEMS Immunology & Medical Microbiology* **50**, 1–26.

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