Journal of Water & Health

IWA PUBLISHING

Journal of Water and Health Vol 20 No 4, 620 doi: 10.2166/wh.2022.229

Isolation and identification of potentially pathogenic free-living amoeba in drinking, surface, and stagnant water sources from Alborz Province, Iran

Ehsan Javanmard[a], Maryam Niyyati[b,*], Ali Taghipour[c], Marziye Fatemi[b], Hamed Mirjalali[d] and Panagiotis Karanis[e,f]

[a] Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[b] Department of Medical Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[c] Zoonoses Research Center, Jahrom University of Medical Sciences, Jahrom, Iran
[d] Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[e] Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany
[f] Department of Basic and Clinical Sciences, Nicosia University Medical School, Nicosia 2408, Cyprus
*Corresponding author. E-mail: maryamniyati@yahoo.com; maryamniyati@sbmu.ac.ir

MN, 0000-0003-3158-6350; HM, 0000-0002-2568-9022

ABSTRACT

Free-living amoebas (FLAs) can cause neurological and ocular complications in humans. Water supplies play a critical role in transmitting FLAs to humans. The aim of the present study was to investigate the presence of FLAs in various aquatic sources including drinking water, stagnant water, and surface water in Alborz province, northern Iran, using morphological and molecular techniques. A total of 70 water samples were collected from 34 drinking waters, 23 surface waters, and 13 stagnant waters. Filtration and cultivation were employed to isolate FLAs. PCR assay was applied by using the genus-specific primers on positive samples. Pathogenicity tests (osmo- and thermo-tolerance properties) were performed for *Acanthamoeba* spp., positive sample. Considering the morphological criteria, four positive samples of *Acanthamoeba* sp., three *Vermamoeba* sp., two mixed *Vermamoeba* sp. with Vahlkamfiids, and one mixed *Acanthamoeba* sp. with Vahlkamfiids were isolated. Five *Acanthamoeba* sp. isolates were amplified using the JDP primer pairs. Among them, two genotypes, T4 (three isolates) and T5 (two isolates) corresponding to *A. lenticulata*, were identified. Four *V. vermiformis* samples were confirmed using the sequencing. This study highlighted the occurrence of potentially pathogenic waterborne FLAs in water habitats associated with high human activity. The results of such research on the prevalence of FLAs, as a human hazard, should be communicated to health policymakers.

Key words: free-living amoeba, Iran, pathogenicity, water sources

HIGHLIGHTS

- Water samples were collected from drinking, surface, and stagnant waters.
- According to the morphological assessment, *Acanthamoeba, Vermamoeba*, and *Vahlkamfiids* were detected among samples.
- The genotypes T4 and T5 (corresponding to *A. lenticulata*) were characterized.

INTRODUCTION

Free-living amoebas (FLAs) are ubiquitous parasitic protozoa commonly found in different environmental sources such as water, soil, dust, and air (Landell *et al.* 2013; Plutzer & Karanis 2016; Niyyati & Latifi 2017). FLAs have an amphizoic life cycle and two distinct forms: trophozoite (vegetative form) and cyst (resistant form) (Padzik *et al.* 2018). Under favourable environmental conditions, trophozoites play an active role in feeding and replicating (Khan 2003), while cysts resist harsh environmental conditions such as ultraviolet (UV) radiation, heat, dryness, the presence of antimicrobial agents, and disinfectant solutions (Coulon *et al.* 2010).

Acanthamoeba, *Naegleria*, *Balamuthia*, and *Vermamoeba* are the major genera of the medically important FLAs (Muchesa *et al.* 2016; Fabros *et al.* 2021). These FLAs cause a variety of neurological and ocular complications by attacking the central nervous system (CNS) and cornea (Lorenzo-Morales *et al.* 2011; Hajialilo *et al.* 2015). *Acanthamoeba* sp. is the most

abundant FLA, which is classified into 22 genotypes (T1–T22) based on the small subunit of 18S ribosomal RNA (rRNA) gene (Chelkha *et al.* 2020; Corsaro 2020). However, T3, T4, and T5 genotypes have been detected from various clinical cases of *Acanthamoeba* keratitis (AK) and *Acanthamoeba* encephalitis (Khan 2009; Lackner *et al.* 2010; Mirjalali *et al.* 2013). Among the Vahlkampfiidae family, *N. fowleri*, a thermophilic FLA, is the only human-pathogenic species that can cause the lethal disease, primary amoebic encephalitis (PAM) (Grace *et al.* 2015). *B. mandrillaris* is considered to cause a rare (but deadly) neurological disorder known as granulomatous amoebic encephalitis (GAE) (Mungroo *et al.* 2020). Another FLA, *V. vermiformis* (formerly called *Hartmannella vermiformis*), is less commonly isolated in clinical samples compared to environmental specimens (Delafont *et al.* 2018; Scheid 2019; Siddiqui *et al.* 2021).

The distribution of these potentially pathogenic FLAs in various water sources such as drinking water and surface water poses a health hazard to humans. The Karaj river provides drinking water for Alborz province and some regions of Tehran (capital of Iran). In addition, besides the agricultural development in Alborz, this river is a popular weekend summer resort. All water sources that were investigated in the present study have different uses including agriculture, industry, residential, and recreational activities. Therefore, the purpose of the present study was to investigate the occurrence of FLAs isolated from various aquatic sources including drinking water, stagnant water, and surface water in Alborz province, northern Iran, using morphological and molecular techniques.

MATERIALS AND METHODS

Sampling, culture, microscopy detection

This cross-sectional study was carried out between September and November 2020. A total of 70 water samples were collected from 34 drinking waters (eight hospitals, seven pharmacies, seven sports clubs, seven shopping centres, three optometry clinics, and two houses), 23 surface waters (public park waters, river waters, and canal waters), and 13 stagnant waters (open and closed reservoir waters) sources at cities in Alborz province, northern Iran (Figure 1). All drinking water samples were collected from tap waters. Surface waters were from artificial streams, which pumped up to parks' pools (in the case of public park waters) and natural waters (in the case of river and canal waters). The north belt of Karaj is limited by mountains and there are rivers and canal waters, which bring waters that originate from these mountains. In some parts, these rivers and canals are near to cities, and we collected samples from these parts. Sampled surface waters are not affected by wastewater, at least at the sampling sites or upstream areas. Stagnant waters were collected from artificially open and closed reservoir waters. The stagnant waters are untreated and are from surface, well, and raining waters that are collected in a container for further purposes such as irrigation.

After collecting samples, 1,000 mL of each water sample was filtered using nitrocellulose filter papers (Millipore sterile, 0.22 mm pore size and 45 mm diameter), cultivated onto 1.2% non-nutrient agar (NNA) covered by heat-killed *Escherichia* coli, as previously described (Niyyati & Rezaeian 2015). Cultivated plates were then incubated at 37 °C and monitored daily for 30 days under a light microscope. Positive samples were sub-cultured onto fresh NNA plates in order to achieve a pure culture of the targeted amoebae. Considering Page keys (Page 1988), isolated FLAs were specified to the genus level using morphological criteria.

Osmo-tolerance assay

At first, 10^3 trophozoites/plate were coated with mannitol-free *E. coli* and transferred onto the centre of the NNA plate as a control. Then, 10^3 of each positive isolate were transported to the centre of the plates by coating the NNA plate with 0.5 and 1 M mannitol concentrations and *E. coli* suspension. Subsequently, the plates were incubated at 30 °C for up to 10 days, and the growth of FLAs at 24, 48, and 72 h was appraised. Trophozoites or cysts were counted from five microscopic areas of approximately 20 mm from the centre of each plate at ×10 microscopic magnification. Finally, the presence of proliferation was evaluated as (+) positive, and the absence of growth (−) as negative (Aykur & Dagci 2021).

Thermo-tolerance assay

For this purpose, 10^3 trophozoites of FLAs were transferred to the centre of *E. coli* coated NNA plates. Then, these plates were incubated at 30 °C as a control, 37, and 42 °C for up to 10 days, and were evaluated after 24, 48, and 72 h of the incubation. Subsequently, proliferation was evaluated as described in the osmo-tolerance assay (Aykur & Dagci 2021).

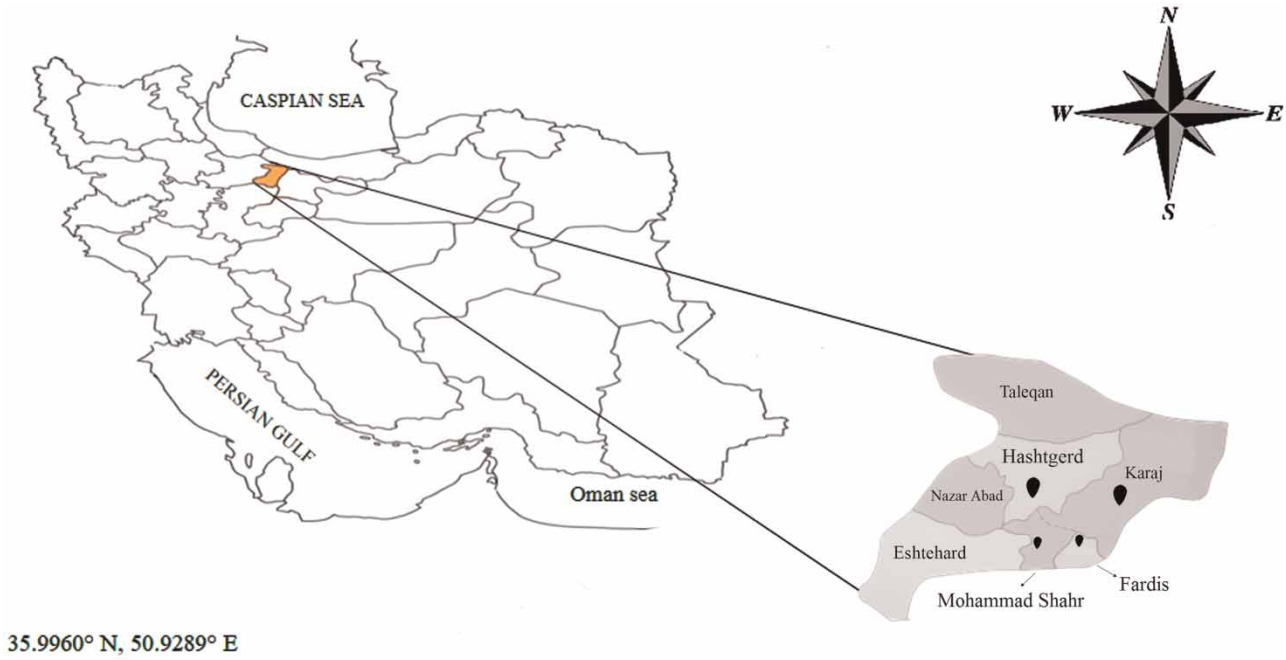

Figure 1 | Map of sampling sites.

DNA extraction, PCR amplification, and sequencing

Cultivated FLAs were harvested, washed by sterile phosphate-buffered saline (PBS, pH=7.4), and were precipitated by centrifuging for 5 min at 2,000 rpm. Afterwards, DNA extraction was performed by the total DNA extraction kit (Yekta Tajhiz Azma, Iran), according to manufacturer's instructions (YKA, Iran). Four sets of primers were applied in order to detect various FLAs including *Acanthamoeba* spp. (JDP1,2 primer pairs: F5′-GGCCCAGATCGTTTACCGTGAA-3′ and R5′-TCTCACAAGCTGCTAGGGAGTCA-3′) (Schroeder *et al.* 2001), *B. mandrillaris* (Balspec16S primer pairs: F5′-CGCATG-TATGAAGAAGACCA-3′ and R5′-TTACCTATATAATTGTCGATACCA-3′) (Booton *et al.* 2003), Vahlkampfiids (ITS1,2 primer pairs: F5′-GAACCTGCGTAGGGATCATTT-3′ and R5′-TTTCTTTTCCTCCCCTTATTA-3′) (Pélandakis & Pernin 2002), and *V. vermiformis* (NA1, 2 primer pairs: F5′-GCT CCA ATA GCG TAT ATT AA-3′ and R5′-AGA AAG AGC TAT-CAATCT GT-3′) (Lasjerdi *et al.* 2011). PCR reactions were performed in a total volume of 30 μL containing 15 μL of 2X red master mix (Ampliqon, Denmark), 10 ρM of each primer, DNA (10 ng), and distilled water. The thermal cycling profile was set as a pre-denaturation step at 94 °C for 3 min, followed by 35 repetitions at 94 °C for 35 s, and annealing steps were at 56, 56, 56, and 58 °C for 1 min (for *Acanthamoeba* spp., Vahlkampfiids, *Balamuthia* spp., and *Vermamoeba* spp., respectively), and 72 °C for 1 min.

All amplicons were electrophoresed by 1.5% agarose gel, stained with a solution of ethidium bromide, and visualized under a UV transilluminator. The sequencing was conducted using an automated sequencer. To specify genus, species, and genotypes, obtained sequences were compared to the Basic Local Alignment Search Tool (BLAST) against available sequences in the GenBank database.

Phylogenetic analysis

Evolutionary analyses were conducted in MEGA X. The evolutionary analysis was inferred by using the maximum-likelihood (ML) method and the Kimura two-parameter model. Bootstrap with 1,000 replications was employed to test the reliabilities of the tree. Reference sequences retrieved from the GenBank database were also included in the phylogenetic tree.

RESULTS

Morphological characteristics of the detected amoebas

FLAs were detected in 10 (14.30%) out of 70 water samples including drinking waters (seven isolates), surface waters (two isolates), and stagnant waters (one isolate) (Table 1). Considering the FLA morphological characteristics using Page keys

Table 1 | Contamination rate of each water sources (drinking, surface, and stagnant water sources) to free-living amoebae based on morphology

Type of water source	Total sample, *n*	Positive sample (%)
Drinking water	34	7 (20.6)
Surface water	23	2 (8.7)
Stagnant water	13	1 (7.7)
Total	70	10 (14.3%)

(Page 1988), four positive samples of *Acanthamoeba* spp., three *Vermamoeba* spp., two mixed *Vermamoeba* spp., with Vahlkamfiids, and one mixed *Acanthamoeba* spp., with Vahlkamfiids, were grown on plates. All positive samples are summarized in Table 2, according to the sampling location, pH, chlorine concentration, water temperature, and sea level.

Molecular detection and sequencing

All cultured positive samples were surveyed by PCR assay. Among them, five *Acanthamoeba* spp. and four *Vermamoeba* spp. were amplified and showed band sizes of 450 and 700 bp, respectively (Table 3). As shown in Table 3, from three mixed-FLA plates, only two plates were PCR positive. In addition, each of these two plates was positive for one FLA (one plate for *Acanthamoeba* and another for *Vermamoeba*). Despite several attempts, a mixed sample containing *Vermamoeba* spp. and Vahlkamfiids was not successfully sequenced due to high bacterial and fungal contaminations. It is important to mention that after conducting a molecular assay, *B. mandrillaris* and *Naegleria* spp. were not detected in samples.

Pathogenicity assays

The pathogenicity tests were assessed by the cultivation of isolates in different temperatures (37 and 42 °C) and osmolarity ranges (0.5 and 1 M mannitol). The growth of isolates at high temperature (at 42 °C) and high osmolarity (1 M mannitol) was considered as pathogenic potency. Growth at 37 °C and 0.5 M osmolarity were considered as low pathogenicity. The thermo-tolerance and osmo-tolerance properties of the positive samples are listed in Table 3.

Table 2 | Data regarding positive water sources (drinking, surface, and stagnant waters) to free-living amoebae based on the morphology

Isolate code (FLA genus)	Sample sources (location)	City	pH	Chlorine concentration (PPM)	Temperature (°C)	Sea level (M)
JA1 (*Acanthamoeba*)	Drinking water (hospital)	Karaj	7.4	2.1	10.3	1,312
JA2 (*Acanthamoeba*)	Drinking water (pharmacy)	Karaj	7.2	2	10.6	1,312
JA3 (*Acanthamoeba*+Vahlkamfiids[a])	Drinking water (Optometry)	Karaj	7.2	NP	14.8	1,312
JA4 (*Acanthamoeba*)	Drinking water (hospital)	Fardis	7.2	2.1	10	1,360
JA5 (*Acanthamoeba*)	Drinking water (sports club)	Fardis	7.5	3	10.5	1,360
JH1 (*Vermamoeba*+Vahlkamfiids[a])	Drinking water (shopping centre)	Fardis	NP	NP	9.7	1,360
JH2 (*Vermamoeba*)	Drinking water (house)	Karaj (Mohamad Shar)	7.2	NP	10.2	1,312
JH3 (*Vermamoeba*+ Vahlkamfiids[a])	Surface water (park)	Hashtgerd	7.8	NP	15.9	1,310–1,360
JH4 (*Vermamoeba*)	Surface water (park)	Hashtgerd	7.8	2	16.7	1,310–1,360
JH5 (*Vermamoeba*)	Stagenant water	Hashtgerd	7.5	NP	10	1,310–1,360

NP, not provided.
[a]Mixed contamination.

Table 3 | Data of the isolated free-living amoebae from drinking water, surface water, and stagnant water sources in Alborz province, Iran

Isolate code	Morphology	JDP1,2 (*Acanthamoeba*)	ITS1, 2 (Vahlkamfiid)	NA1/2 (*Vermamoeba*)	Bal1/2 (*Balamuthia*)	Genotype/ species	Thermo-tolerance 37/40 °C	Osmo-tolerance 0.1/1 M	Query coverage/ Ref. Acc. No.		Acc. No.
JA1	*Acanthamoeba*	+	–	–	–	T5 (*lenticulata*)	+/–	+/+	100%	MT613720	MZ955617
JA2	*Acanthamoeba*	+	–	–	–	T5 (*lenticulata*)	+/–	+/–	99%	MK217511	MZ955618
JA3[a]	*Acanthamoeba*+ Vahlkamfiids[a]	+	–	–	–	T4	+/+	+/+	70%	LC086295	MZ955619
JA4	*Acanthamoeba*	+	–	–	–	T4	+/+	+/+	98%	KT892924	MZ955620
JA5	*Acanthamoeba*	+		–	–	T4	+/+	+/+	99%	MZ557807	MZ955621
JH1[a]	*Vermamoeba*+ Vahlkamfiids[a]	–	–	+	–	*V. vermiformis*	+/–	N/D	99%	GQ861564	MZ955622
JH2	*Vermamoeba*	–	–	+	–	*V. vermiformis*	+/–	N/D	100%	MK946023	MZ955623
JH3[b]	*Vermamoeba*+ Vahlkamfiids[a]	–	–	–	–	–	+/–	N/D	–	–	–
JH4	*Vermamoeba*	–	–	+	–	*V. vermiformis*	+/+	N/D	100%	JQ271687	MZ955624
JH5	*Vermamoeba*	–	–	+	–	*V. vermiformis*	+/+	N/D	99%	JQ271687	MZ955625

N/D, not determined.

[a]Mixed contamination.

[b]Due to high bacterial contamination of the JH3 sample, PCR and sequencing were not successful.

Phylogenetic analysis

The sequencing results for *Acanthamoeba* sp. revealed the presence of the genotypes T4 (n=3, accession numbers MZ955619, MZ955620, and MZ955621) and T5 corresponding to *A. lenticulata* (n=2, accession numbers MZ955617 and MZ955618) (Table 3). All four *V. vermiformis* samples were also confirmed (accession numbers MZ955622, MZ955623, MZ955624, and MZ955625) (Table 3).

Molecular evolutionary is shown in Figure 2. The phylogenetic tree confirmed the results of BLAST analysis and showed all sequences were grouped together with their reference sequences (Figure 2).

DISCUSSION

This study reports the occurrence of waterborne FLAs belonging to the *Acanthamoeba* sp., T4 and T5 genotypes, and *V. vermiformis* in water sources of Alborz province. In Iran, the previous epidemiological studies have shown that *Acanthamoeba* sp. has a higher prevalence than other FLAs in both clinical and environmental samples (Hajialilo *et al.* 2015; Mahmoudi *et al.* 2015, 2021; Saburi *et al.* 2017). One of the most important reasons for the high occurrence of *Acanthamoeba* sp. is its high capability to adapt to harsh environmental conditions (Aksozek *et al.* 2002). Previous studies have suggested that using that tap water for washing contact lenses is a major risk factor for AK development (Lorenzo-Morales *et al.* 2005; Shoff *et al.* 2008; Koltas *et al.* 2015). The most reported cases of AK are related to people who wear soft contact lenses, which occur due to inappropriate maintenance of their lenses (Lindsay *et al.* 2007). According to the results of this study, seven out of 10 samples, which were contaminated with FLAs, belonged to drinking water. In this regard, cleaning contact lenses with distilled water or non-sterile water is considered a risk factor for AK (Khan 2006; Niyyati & Rezaeian 2015). Based on the published literature, two isolated genotypes in this study (T4 and T5) are considered as the genotypes, which were reported in patients with AK (Ledee *et al.* 2009; Niyyati *et al.* 2010; Omaña-Molina *et al.* 2016). The presence of *A. lenticulata* T5 genotype was associated with acute granulomatous encephalitis in an immunocompetent patient (Lackner *et al.* 2010). Moreover, a fatal case of disseminated acanthamoebiasis caused by *A. lenticulata* (genotype T5) has been reported in a 39-year-old heart transplant recipient (Barete *et al.* 2007). There are reports of the T5 genotype from mucosal tissue of immunocompromised individuals, which may predispose the presence of this genotype in ocular and cerebral involvement (Memari *et al.* 2015, 2017; Niyyati *et al.* 2017; Tananuvat *et al.* 2019). Thereby, immunocompromised patients and people wearing contact lenses need to be fully aware of the transmission and pathogenesis to prevent infections due to *Acanthamoeba* sp.

As shown in this study, one of the most commonly identified FLAs was *V. vermiformis*. However, the pathogenicity of *V. vermiformis* has not yet been fully determined, but some case reports of this FLA have been reported in patients with keratitis (Lorenzo-Morales *et al.* 2007; Abedkhojasteh *et al.* 2013; Scheid *et al.* 2019) and painful ulcers close to the eye (Scheid *et al.* 2019). Lorenzo-Morales *et al.* (2007) reported a mixed infection of *Acanthamoeba* sp. and *Hartmannella* from a patient with keratitis. However, more in-depth studies should be performed to gain a better insight into the status and pathogenicity of this FLA.

B. mandrillaris was not isolated in the collected samples of this study. In accordance with our study, *B. mandrillaris* has not been reported previously in water samples from Semnan, Iran (Javanmard *et al.* 2017). However, in previous studies from Iran, the occurrence of *B. mandrillaris* has been reported in hospital dust, soils of recreational areas, and hot springs (Niyyati *et al.* 2009; Latifi *et al.* 2016). Therefore, further investigations are needed to assess the exact niches of *B. mandrillaris* in the country.

In the present study, none of the 47 species of *Naegleria* spp. were reported using the ITS-based sequencing technique. One of the most important reasons for non-isolation of *Naegleria* spp. in our study can be the incubation temperature of the culture, as most *Naegleria* species are thermophilic and grow in temperatures of 38–40 °C (Jahangeer *et al.* 2020; Saberi *et al.* 2020). Therefore, incubation of plates at different temperatures should be tried to detect *Naegleria* spp. in future studies.

Importantly, the presence of FLAs in water samples, which are routinely used by humans, increases the risk of contamination. In the current study, drinking waters collected from hospitals, drug stores, eye clinics, sports clubs, and shopping centres were contaminated with FLAs. Notably, all drinking waters in these places were collected from tap waters that are used not only for drinking but also for washing hands, dishes, hospital sheets, etc. On the other hand, three isolates JA3, JA4, and JA5, which were obtained from drinking waters from optometry clinics, hospitals, and sports clubs, respectively,

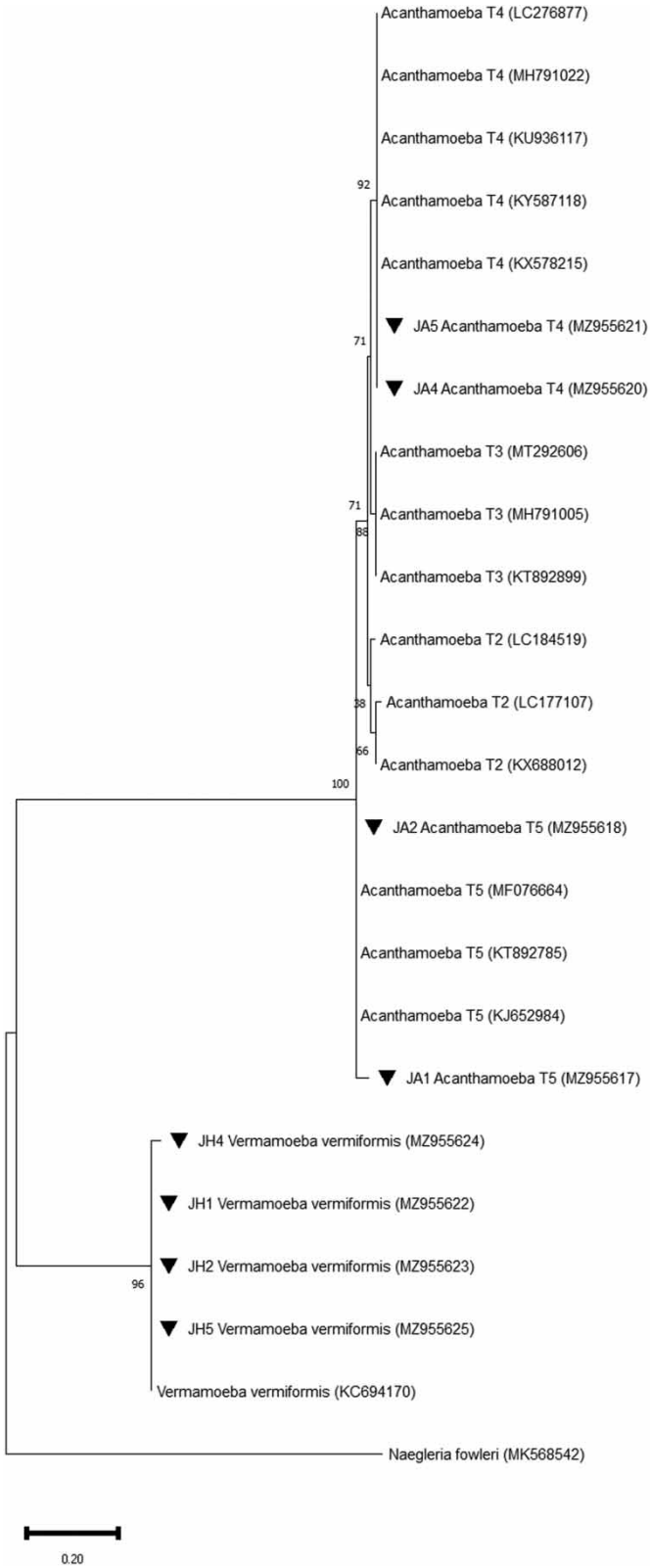

Figure 2 | Phylogenetic tree of the 18S rRNA gene of *Acanthamoeba* spp., and *V. vermiformis* isolated from water samples together with reference sequences. The phylogenetic tree represents that all identified genotypes were clustered with the reference genotypes. The phylogenetic tree was drawn using the ML method and the Kimura two-parameter model. Black-filled triangles indicate FLAs isolated from the current study.

were osmo and thermotolerant. This feature of isolated FLAs signifies the pathogenic potential of isolated strains, which increases the medical and public health importance of FLAs in these places.

Phylogenetic tree represented similarity of the isolates JA4 and JA5 (which were isolated from hospitals and sports clubs, respectively) with *Acanthamoeba* sp., T4 genotypes, which were reported from contact lens, stagnant waters, and AK (Hajialilo *et al.* 2015; Golestani *et al.* 2018; Hussain *et al.* 2020). This genetic similarity suggests the potency of isolates to cause acanthamoebiasis in hospitalized patients or those people who use contact lenses.

In conclusion, this study highlighted the occurrence of potentially pathogenic waterborne FLAs in different water supplies, especially drinking water, in places with high human activity such as sports clubs, hospitals, and eye clinics. Further studies to evaluate the niches of *B. mandrillaris* and *N. fowleri* are important in Iran. Results of such research on the distribution, relevance, and clinical significance of FLAs should be communicated to health policymakers.

ACKNOWLEDGEMENTS

The authors thank all members of the Department of Parasitology and Mycology and Foodborne and Waterborne Diseases Research Center for their support.

AUTHORS' CONTRIBUTION

M.N., H.M., and E.J. conceived and designed the experiments. E.J. and M.F. performed the experiments. M.N., H.M., and A.T. analysed the data. H.M. contributed reagents/materials/analysis/tools/positive samples. A.T., M.N., H.M., and P.K. wrote the paper. All authors read and approved the final version of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All procedures performed in this study were in accordance with the ethical standards (IR.SBMU.MSP.REC.1399.26) released by the Ethical Review Committee of the Shahid Beheshti University of Medical Sciences, Tehran, Iran.

CONSENT FOR PUBLICATION

All authors declare that they have seen and approved the submitted version of this manuscript.

AVAILABILITY OF DATA AND MATERIAL

All generated data from the current study are included in the article.

FUNDING

This study was financially supported by the Shahid Beheshti University of Medical Sciences under grant no. 20742.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

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

REFERENCES

Abedkhojasteh, H., Niyyati, M., Rahimi, F., Heidari, M., Farnia, S. & Rezaeian, M. 2013 First report of *Hartmannella* keratitis in a cosmetic soft contact lens wearer in Iran. *Iran. J. Parasitol.* **8**, 481.

Aksozek, A., McClellan, K., Howard, K., Niederkorn, J. & Alizadeh, H. 2002 Resistance of *Acanthamoeba castellanii* cysts to physical, chemical, and radiological conditions. *J. Parasitol.* **88**, 621–623.

Aykur, M. & Dagci, H. 2021 Evaluation of molecular characterization and phylogeny for quantification of *Acanthamoeba* and *Naegleria fowleri* in various water sources, Turkey. *PLoS ONE* **16**, e0256659.

Barete, S., Combes, A., De Jonckheere, J. F., Datry, A., Varnous, S., Martinez, V., Ptacek, S. G., Caumes, E., Capron, F. & Francès, C. 2007 Fatal disseminated *Acanthamoeba lenticulata* acanthamebiasis in a heart transplant patient. *Emerging Infect. Dis.* **13**, 736.

Booton, G. C., Carmichael, J. R., Visvesvara, G. S., Byers, T. J. & Fuerst, P. A. 2003 Identification of *Balamuthia mandrillaris* by PCR assay using the mitochondrial 16S rRNA gene as a target. *J. Clin. Microbiol.* **41**, 453–455.

Chelkha, N., Jardot, P., Moussaoui, I., Levasseur, A., La Scola, B. & Colson, P. 2020 Core gene-based molecular detection and identification of *Acanthamoeba* species. *Sci. Rep.* **10**, 1–9.

Corsaro, D. 2020 Update on *Acanthamoeba* phylogeny. *Parasitol. Res.* **119**, 3327–3338.

Coulon, C., Collignon, A., McDonnell, G. & Thomas, V. 2010 Resistance of *Acanthamoeba* cysts to disinfection treatments used in health care settings. *J. Clin. Microbiol.* **48**, 2689–2697.

Delafont, V., Rodier, M.-H., Maisonneuve, E. & Cateau, E. 2018 *Vermamoeba vermiformis*: a free-living amoeba of interest. *Microb. Ecol.* **76**, 991–1001.

Fabros, M., Diesta, X., Oronan, J. A., Verdejo, K. S., Garcia, J., Sophia Romey, M. & Milanez, G. J. 2021 Current report on the prevalence of free-living amoebae (FLA) in natural hot springs: a systematic review. *J. Water Health* **19** (4), 563–574.

Golestani, M. H., Rasti, S., Hooshyar, H., Delavari, M., Mousavi, S. G. A., Iranshahi, L. & Aghajani, A. 2018 Molecular identification and genotyping of *Acanthamoeba* isolated from environmental sources in Kashan, central Iran. *Jundishapur J. Microbiol.* **11** (4), e55582.

Grace, E., Asbill, S. & Virga, K. 2015 *Naegleria fowleri*: pathogenesis, diagnosis, and treatment options. *Antimicrob. Agents Chemother.* **59**, 6677–6681.

Hajialilo, E., Niyyati, M., Solaymani, M. & Rezaeian, M. 2015 Pathogenic free-living amoebae isolated from contact lenses of keratitis patients. *Iran. J. Parasitol.* **10**, 541.

Hussain, R. H., Isa, N. S., Kamaruddin, K. A., Ghani, M. K., Khan, N. A., Siddiqui, R. & Anuar, T. S. 2020 Morphological and molecular characterization of *Acanthamoeba* isolated from contact lens paraphernalia in Malaysia: highlighting the pathogenic potential of T4 genotype. *Asian Pac. J. Trop. Med.* **13** (12), 542–549.

Jahangeer, M., Mahmood, Z., Munir, N., Waraich, U. E. A., Tahir, I. M., Akram, M., Ali Shah, S. M., Zulfqar, A. & Zainab, R. 2020 *Naegleria fowleri*: sources of infection, pathophysiology, diagnosis, and management; a review. *Clin. Exp. Pharmacol. Physiol.* **47**, 199–212.

Javanmard, E., Niyyati, M., Lorenzo-Morales, J., Lasjerdi, Z., Behniafar, H. & Mirjalali, H. 2017 Molecular identification of waterborne free living amoebae (*Acanthamoeba*, *Naegleria* and *Vermamoeba*) isolated from municipal drinking water and environmental sources, Semnan province, north half of Iran. *Exp. Parasitol.* **183**, 240–244.

Khan, N. A. 2003 Pathogenesis of *Acanthamoeba* infections. *Microb. Pathogen.* **34**, 277–285.

Khan, N. A. 2006 *Acanthamoeba*: biology and increasing importance in human health. *FEMS Microbiol. Rev.* **30**, 564–595.

Khan, N. 2009 *Acanthamoeba. Biology and Pathogenesis*. Caister Academic Press, UK, vol. 54, ISBN: 978-1-904455-43-1.

Koltas, I. S., Eroglu, F., Erdem, E., Yagmur, M. & Tanır, F. 2015 The role of domestic tap water on *Acanthamoeba* keratitis in non-contact lens wearers and validation of laboratory methods. *Parasitol. Res.* **114**, 3283–3289.

Lackner, P., Beer, R., Broessner, G., Helbok, R., Pfausler, B., Brenneis, C., Auer, H., Walochnik, J. & Schmutzhard, E. 2010 Acute granulomatous *Acanthamoeba* encephalitis in an immunocompetent patient. *Neurocrit Care* **12**, 91–94.

Landell, M. F., Salton, J., Caumo, K., Broetto, L. & Rott, M. B. 2013 Isolation and genotyping of free-living environmental isolates of *Acanthamoeba* spp. from bromeliads in southern Brazil. *Exp. Parasitol.* **134**, 290–294.

Lasjerdi, Z., Niyyati, M., Haghighi, A., Shahabi, S., Biderouni, F. T., Taghipour, N., Eftekhar, M. & Mojarad, E. N. 2011 Potentially pathogenic free-living amoebae isolated from hospital wards with immunodeficient patients in Tehran, Iran. *Parasitol. Res.* **109**, 575–580.

Latifi, A., Niyyati, M., Lorenzo-Morales, J., Haghighi, A., Tabaei, S. S. & Lasjerdi, Z. 2016 Presence of *Balamuthia mandrillaris* in hot springs from Mazandaran province, Northern Iran. *Epidemiol. Infect.* **144**, 2456–2461.

Ledee, D., Iovieno, A., Miller, D., Mandal, N., Diaz, M., Fell, J., Fini, M. & Alfonso, E. 2009 Molecular identification of T4 and T5 genotypes in isolates from *Acanthamoeba* keratitis patients. *J. Clin. Microbiol.* **47**, 1458–1462.

Lindsay, R. G., Watters, G., Johnson, R., Ormonde, S. E. & Snibson, G. R. 2007 *Acanthamoeba* keratitis and contact lens wear. *Clin. Exp. Optometry* **90**, 351–360.

Lorenzo-Morales, J., Ortega-Rivas, A., Foronda, P., Martínez, E. & Valladares, B. 2005 Isolation and identification of pathogenic *Acanthamoeba* strains in Tenerife, Canary Islands, Spain from water sources. *Parasitol. Res.* **95**, 273–277.

Lorenzo-Morales, J., Martínez-Carretero, E., Batista, N., Álvarez-Marín, J., Bahaya, Y., Walochnik, J. & Valladares, B. 2007 Early diagnosis of amoebic keratitis due to a mixed infection with *Acanthamoeba* and *Hartmannella*. *Parasitol. Res.* **102**, 167–169.

Lorenzo-Morales, J., Martín-Navarro, C. M., Martínez-Carretero, E., Piñero, J. E. & Valladares, B. 2011 *Encephalitis due to Free Living Amoebae: An Emerging Issue in Human Health, Non-Flavivirus Encephalitis, Sergey Tkachev*. IntechOpen. Available from: https://www.intechopen.com/chapters/23069.

Mahmoudi, M. R., Rahmati, B., Seyedpour, S. H. & Karanis, P. 2015 Occurrence and molecular characterization of free-living amoeba species (*Acanthamoeba*, *Hartmannella*, and *Saccamoeba limax*) in various surface water resources of Iran. *Parasitol. Res.* **114**, 4669–4674.

Mahmoudi, M. R., Zebardast, N., Masangkay, F. R. & Karanis, P. 2021 Detection of potentially pathogenic free-living amoebae from the Caspian Sea and hospital ward dust of teaching hospitals in Guilan, Iran. *J. Water Health* **19**, 278–287.

Memari, F., Niyyati, M., Haghighi, A., Tabaei, S. J. S. & Lasjerdi, Z. 2015 Occurrence of pathogenic *Acanthamoeba* genotypes in nasal swabs of cancer patients in Iran. *Parasitol. Res.* **114**, 1907–1912.

Memari, F., Niyyati, M. & Joneidi, Z. 2017 Pathogenic *Acanthamoeba* T4 genotype isolated from mucosal tissue of a patient with HIV infection: a case report. *Iran. J. Parasitol.* **12**, 143.

Mirjalali, H., Niyyati, M., Abedkhojasteh, H., Babaei, Z., Sharifdini, M. & Rezaeian, M. 2013 Pathogenic assays of *Acanthamoeba* belonging to the T4 genotype. *Iran. J. Parasitol.* **8**, 530.

Muchesa, P., Leifels, M., Jurzik, L., Barnard, T. & Bartie, C. 2016 Free-living amoebae isolated from a hospital water system in South Africa: a potential source of nosocomial and occupational infection. *Water Sci. Technol.* **16**, 70–78.

Mungroo, M. R., Khan, N. A. & Siddiqui, R. 2020 *Balamuthia mandrillaris*: pathogenesis, diagnosis, and treatment. *Expert Opin. Orphan Drugs* **8**, 111–119.

Niyyati, M. & Latifi, A. 2017 Free living amoeba belonging to *Vannella* spp. isolated from a hotspring in Amol City, Northern Iran. *Novel Biomed.* **5**, 85–88.

Niyyati, M. & Rezaeian, M. 2015 Current status of *Acanthamoeba* in Iran: a narrative review article. *Iran. J. Parasitol.* **10**, 157.

Niyyati, M., Lorenzo-Morales, J., Rezaeian, M., Martin-Navarro, C. M., Haghi, A. M., Maciver, S. K. & Valladares, B. 2009 Isolation of *Balamuthia mandrillaris* from urban dust, free of known infectious involvement. *Parasitol. Res.* **106**, 279–281.

Niyyati, M., Lorenzo-Morales, J., Rezaie, S., Rahimi, F., Martín-Navarro, C. M., Mohebali, M., Maghsood, A. H., Farnia, S., Valladares, B. & Rezaeian, M. 2010 First report of a mixed infection due to *Acanthamoeba* genotype T3 and Vahlkampfia in a cosmetic soft contact lens wearer in Iran. *Exp. Parasitol.* **126**, 89–90.

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. *Parasitol. Res.* **116**, 2965–2969.

Omaña-Molina, M., Vanzzini-Zago, V., Hernandez-Martinez, D., Gonzalez-Robles, A., Salazar-Villatoro, L., Ramirez-Flores, E., Oregon-Miranda, E., Lorenzo-Morales, J. & Martinez-Palomo, A. 2016 *Acanthamoeba* genotypes T3 and T4 as causative agents of amoebic keratitis in Mexico. *Parasitol. Res.* **115**, 873–878.

Padzik, M., Baltaza, W., Conn, D. B., Szaflik, J. P. & Chomicz, L. 2018 Effect of povidone iodine, chlorhexidine digluconate and toyocamycin on amphizoic amoebic strains, infectious agents of *Acanthamoeba* keratitis: a growing threat to human health worldwide. *Ann. Agric. Environ. Med.* **25**, 725–731.

Page, F. C. 1988 *A New Key to Freshwater and Soil Gymnamoebae: With Instructions for Culture*. Freshwater Biological Association, Ulverston, Cumbria, pp. 1–122.

Pélandakis, M. & Pernin, P. 2002 Use of multiplex PCR and PCR restriction enzyme analysis for detection and exploration of the variability in the free-living amoeba *Naegleria* in the environment. *Appl. Environ. Microbiol.* **68**, 2061–2065.

Plutzer, J. & Karanis, P. 2016 Neglected waterborne parasitic protozoa and their detection in water. *Water Res.* **101**, 318–332.

Saberi, R., Seifi, Z., Dodangeh, S., Najafi, A., Abdollah Hosseini, S., Anvari, D., Taghipour, A., Norouzi, M. & Niyyati, M. 2020 A systematic literature review and meta-analysis on the global prevalence of *Naegleria* spp. in water sources. *Transboundary Emerging Dis.* **67**, 2389–2402.

Saburi, E., Rajaii, T., Behdari, A., Kohansal, M. H. & Vazini, H. 2017 Free-living amoebae in the water resources of Iran: a systematic review. *J. Parasitc Dis.* **41**, 919–928.

Scheid, P. L. 2019 *Vermamoeba vermiformis* – a free-living amoeba with public health and environmental health significance. *Parasitol Res.* **118** (6), 1999–2004.

Scheid, P. L., Lâm, T.-T., Sinsch, U. & Balczun, C. 2019 *Vermamoeba vermiformis* as etiological agent of a painful ulcer close to the eye. *Parasitol. Res.* **118**, 1999–2004.

Schroeder, J. M., Booton, G. C., Hay, J., Niszl, I. A., Seal, D. V., Markus, M. B., Fuerst, P. A. & Byers, T. J. 2001 Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoeba* from humans with keratitis and from sewage sludge. *J. Clin. Microbiol.* **39**, 1903–1911.

Shoff, M., Rogerson, A., Kessler, K., Schatz, S. & Seal, D. 2008 Prevalence of *Acanthamoeba* and other naked amoebae in South Florida domestic water. *J. Water Health* **6**, 99–104.

Siddiqui, R., Makhlouf, Z. & Khan, N. A. 2021 The increasing importance of *Vermamoeba vermiformis*. *J. Eukaryotic Microbiol.* **68** (5), e12857.

Tananuvat, N., Techajongjintana, N., Somboon, P. & Wannasan, A. 2019 The first *Acanthamoeba* keratitis case of non-contact lens wearer with HIV infection in Thailand. *Korean J. Parasitol.* **57**, 505.

First received 6 September 2021; accepted in revised form 7 March 2022. Available online 18 March 2022