Genotyping determination of *Acanthamoeba* strains: an original study and a systematic review in Iran

Hadi Mirahmadi, Maryam Mansouri Nia, Adel Ebrahimzadeh, Ahmad Mehravaran, Reza Shafiei, Mohammad Taghi Rahimi, Reza Zolfaghari Emameh and Harlan R. Barker

ABSTRACT

This study aimed to detect the presence of *Acanthamoeba* spp. in different water resources of Zahedan, southeast of Iran, and also systematically reviewed all publications regarding *Acanthamoeba* in Iran (2005–2018). Fifty water samples were collected from different water resources in Zahedan. The positive samples were identified morphologically and subjected to polymerase chain reaction (PCR) using fragments of 18S rRNA. In the systematic review, data collection using particular terms was carried out using the following electronic databases including Science Direct, ISI Web of Science, MEDLINE, EBSCO, Scopus, and Google Scholar. A total of 17 (34%) samples were positive for *Acanthamoeba* spp., and nucleotide sequencing indicated that 15 samples (88.23%) belonged to the T4 genotype and the rest belonged to the T5 genotype. A total of 39 studies reported genotyping of *Acanthamoeba* spp. from various geographical areas of Iran and revealed that T4 (35 studies), T5 (19 studies), T3 (11 studies), T11 (8 studies), and T2 (6 studies) genotypes were the most prevalent in Iran. The T4 genotype of *Acanthamoeba* is a prevalent free-living amoeba and widely distributed not only in Zahedan but also in other provinces of Iran. Phylogenetic analysis reveals that *A. castellanii* and *A. griffini* predominantly colocalize with the T4 genotype.

Key words | *Acanthamoeba*, amoeba, epidemiology, genotyping PCR, Iran, parasite

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INTRODUCTION

Acanthamoeba is a cosmopolitan free-living amoeba (FLA) and an opportunistic protozoan parasite which can be found in a variety of environments, including saltwater and freshwater, soil, and decaying plants as well as the skin, cornea, lung, and brain of infected humans (Tsvetkova *et al.* 2004; Khan 2006; Rezaeian *et al.* 2008; Bagheri *et al.* 2010; Magnet *et al.* 2012). Two forms of the parasite, representing distinct stages of its life cycle, have been identified: a vegetative trophozoite and a cyst with a double-layer coat, which is resistant to disinfectants and dryness and can survive for many years in the environment (Schuster & Visvesvara 2004). Contaminated environments are considered as major resources for transmission of

doi: 10.2166/wh.2019.048

Acanthamoeba spp. to humans (Zolfaghari Emameh *et al.* 2016).

Acanthamoeba is very important due to serious and sometimes fatal infections in humans and animals (Marciano-Cabral & Cabral 2003; Schuster & Visvesvara 2004; Visvesvara *et al.* 2007). Some *Acanthamoeba* spp. cause two major diseases: amoebic keratitis (AK) of the eye in contact lens wearers and granulomatous amoebic encephalitis in immune-deficient subjects (Prasher *et al.* 2004; Huang & Hsu 2010; Lass *et al.* 2014). This amoeba has been classified based on the 18S rRNA (ribosomal RNA) sequence of the trophozoite into 20 different genotypes (T1–T20) (Di Filippo *et al.* 2015). Among these, T3, T4, and T5 are the most common pathogenic genotypes. Members belonging to the T4 genotype are the most common causative agents of infections (Maghsood *et al.* 2005; Khan 2006), and more than 90% of AK cases are due to T3 and T4 *Acanthamoeba* (Schuster & Visvesvara 2004). In addition, T2 and T4 genotypes have been reported in Iran's water (Maghsood *et al.* 2005; Niyyati *et al.* 2009b).

Unfortunately, in recent years, there has been an upward trend in the number of immunocompromised individuals (Shafiei *et al.* 2011; Ahmadpour *et al.* 2014; Rahimi *et al.* 2015; Abdollahian *et al.* 2017). In addition, the high potential for resistance and the ability of the parasite to adapt to a variety of environmental conditions presents optimal conditions for the increased incidence of infection and the emergence of this opportunistic parasite as a major health concern. Considering all the abovementioned threats and their potential impacts on human health, early detection of pathogenic *Acanthamoeba* in aquatic environments plays a crucial role in the control and prevention of the disease (Zolfaghari Emameh *et al.* 2018).

Zahedan is located in Sistan and Baluchistan province, in the southeast of Iran, and literature review indicates that, so far, no study has been performed to clarify the situation of *Acanthamoeba* in this area. Therefore, the objective of this study was to investigate and determine the genotype of potentially pathogenic *Acanthamoeba* spp. from different water resources in Zahedan. In addition, we systematically reviewed genotyping studies of *Acanthamoeba* in Iran during 2005–2018.

MATERIALS AND METHODS

Sample preparation

In the present descriptive cross-sectional study, a total of 50 water samples were randomly collected from February to December of 2016 from different resources in Zahedan, where human activity and colonization were identified. Sampling was carried out from tap water, stagnant water of public squares, parks, swimming pools, agricultural channels (each of them had nine samples), and hospitals (five samples). Each sample contains 1,000 mL of water that was collected inside a sterile and contamination-free container and transferred to Parasitology and Mycology Laboratory in the Zahedan University of Medical Sciences, Zahedan, Iran. Samples were filtered using a vacuum pump through a sterile multi-pore nitrocellulose Durapore Membrane filter (pore size, 0.45 μm). The sediments on the membranes were conveyed in the top-down direction in 1.5% non-nutrient agar medium containing amoeba page saline (pH 7.2–7.4) that was overlaid by heat-killed *Escherichia coli* (Niyyati *et al.* 2009b). Plates were incubated (Cole-Parmer Digital Incubator) at 28–30 °C for 7 days. Then, incubating plates were observed daily for outgrowth evaluation of *Acanthamoeba* cysts and trophozoites using an inverted microscope (Zeiss), for up to 1 month. The plates which showed growth of amoeba were transferred to new plates and subcultures were made to receive a plate without any microorganism (fungal or bacteria) contamination. Finally, the plates were subjected to the following procedures (Niyyati *et al.* 2016).

Molecular studies

Positive *Acanthamoeba* plates were gently scraped and washed with 5 mL of phosphate-buffered saline (Shokri *et al.* 2016). The solution was transferred to sterile tubes and after centrifugation 5,000 g for 2 min, the sediments having amoebas were collected. The Dyna Bio kit (Takapou Zist, Iran) was used for DNA extraction to extract the total genomic DNA from the collected debris according to the manufacturer's instructions.

A pair of primers, including JDP1: 5′-GGCCCA-GATCGTTTACCGTGAA-3′ as forward and JDP2: 5′-TCTCACAAGCTGCTAGGGGAGTCA-3′ as the reverse, were used to amplify a fragment of approximately 500 bp in *Acanthamoeba* genus-specific 18S rRNA gene (Schroeder *et al.* 2001). The polymerase chain reaction (PCR) mixtures (25 μL) contained 3 μL of extracted DNA solution, 1.25 units of *Taq* DNA polymerase (Cinnagen, Iran), 2.5 μL of 10× PCR buffer, 2 mM of $MgCl_2$, 50 pmol/25 mL reaction mixtures of both forward and reverse primers, 0.4 mM of dNTPs, and 15.5 mL of distilled water. PCR amplification was run on the thermocycler (Eppendorf Mastercycler Gradient) according to the following details: 94°C (3 min), [94°C (35 s), 57°C (45 s), 72°C (1 min): 33 cycles], 72°C (5 min), each of the PCR cycles were followed by cooling at 4°C. PCR products of positive isolates were purified from the agarose gel using a PCR purification kit (Bioneer, Korea) and sequenced in both forward and reverse directions (Applied Biosystems, DNA Analyzers Sequencing, Bioneer, Korea, Sanger method) using the same primers that were used in the PCR (Huang & Hsu 2010).

Phylogenetic analyses

A total of 20 (18S rRNA) sequences were aligned using Clustal Omega (Sievers *et al.* 2011) and the obtained alignment was trimmed on each end to the location where nucleotides were present for at least 75% of entries. With the subsequent edited aligned sequences, a maximum likelihood-based phylogenetic analysis was performed using the IQTree package (Minh *et al.* 2013; Nguyen *et al.* 2015). The best model for analysis was predicted to be TVMe + G4 with Model Finder (Kalyaanamoorthy *et al.* 2017), and the analysis was run with 100,000 bootstrap replicates of the SH-like approximate likelihood ratio test and 100,000 ultrafast bootstrap replicates (parameters set to '-alrt 100,000 -bb 100,000 -nt AUTO' and all other options run as default). The resulting consensus tree had a final log-likelihood value of −2,571.798, and the internal nodes represent the percentage of replicates supporting the configuration at the node. The final figure was visualized using the Python-based ETE3 toolkit library (Huerta-Cepas *et al.* 2016), with representations of sequence data accompanying each leaf of the phylogenetic tree. In this analysis, *Balamuthia mandrillaris* (a single-celled FLA) 18S rRNA sequence was used as the out group.

Systematic review

Along with genotyping of the samples as a major goal of the current study, we systematically reviewed studies of *Acanthamoeba* spp. genotyping from 2005 to 2018 in Iran. A literature search of the current systematic review was carried out through various methods including database search, data collection, and data assessment. The database search was performed using the Medical Subject Heading (MeSH) of specified search terms, alone or in combination, within the following databases: Science Direct, ISI Web of Science, MEDLINE, EBSCO, Scopus, and Google Scholar. The MeSH terms used '*Acanthamoeba*', 'Free-living amoeba', 'Immunosuppressed individuals', 'Keratitis', 'Environment', 'Water', 'Isolation', and 'Genotyping'. References of identified articles were also similarly checked. From those identified studies conducted during 2005–2018 (14 year period), the collected information consists of data such as the year of publication, first author, study areas, sample type, and genotype. Data collection was limited to the study cases that were published in English and/or Persian. It is worth noting that repetitive studies were excluded.

RESULTS

A total of 17 (34%) out of 50 water samples were identified as positive for *Acanthamoeba* spp., through both culture and PCR methods (Figure 1). All 17 positive samples without any contamination were successfully sequenced after two times gel purification and then well typed. The prevalence and frequency of the detected *Acanthamoeba* were summarized in Table 1. In addition, the results of nucleotide sequencing indicated that 15 samples (88.23%) belonged to the T4 genotype and two samples (11.74%) belonged to the T5 genotype.

The process of study design for systematic review was depicted in a flowchart in Figure 2. A list of 39 published studies (2005–2018), identifying the genotypes of *Acanthamoeba* spp. determined in what sample type and in which defined areas of Iran, is presented in Table 2. The different

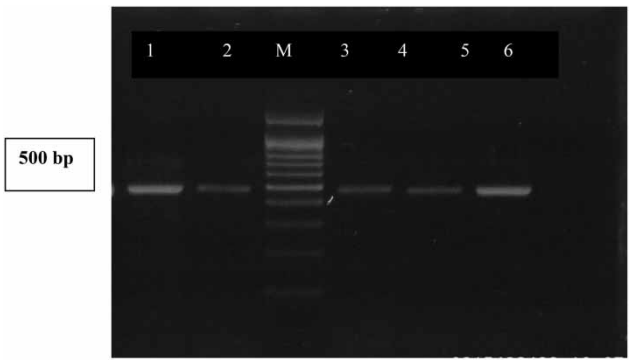

Figure 1 | Gel agarose electrophoresis of the PCR assay using 18S rRNA gene oligonucleotides for the detection of *Acanthamoeba* spp. in the sample: (M) 100 bp ladder marker, (1) positive control, (2–5) *Acanthamoeba* spp. isolates and (6) negative control.

Table 1 | Water samples and prevalence of *Acanthamoeba* spp. isolated from the samples in Zahedan (southeast of Iran)

Water sample	Sample count	(Positive count) % of total culture, PCR
Pipe	9	(1) 11.1%
Public squares	9	(5) 55.5%
Park fountain	9	(4) 44.4%
Swimming pool	9	(3) 33.3%
Agriculture channel	9	(2) 22.2%
Hospital drinking water	5	(2) 40%
Total (%)	50	(17) 34%

genotypes detected in the descending order of number of studies were identified: T4 (35 studies), T5 (19 studies) and T3 (11 studies), T11 (8 studies), T2 (6 studies), T15 (2 studies), and T1, T6, T7, T9 and T13 (1 study) isolates were the different genotypes detected from different areas of Iran. Phylogenetic analysis results are presented in Figure 3, which depicts a phylogenetic tree of 17 *Acanthamoeba* 18S rRNA sequences from water samples of different areas of Iran including Zahedan.

Phylogenetic analysis was performed in 18S rRNA sequences. *A. castellanii* and *A. griffini* predominantly colocalize with T4 genotypes, while *A. lenticulata* groups with the only two T5 genotypes. Due to the likely incomplete nature of a number of the sequences used in this analysis, it would be premature to make any final conclusions on the evolutionary relationships between these organisms. Specifically, the single T3 sequence (KT985968.1) appears to be significantly truncated in the 3′-end, as is the sequence for *A. lenticulata*, though to a lesser extent. As a result, the overall tree topology is weakly supported at some nodes, yet leaf pairing is strongly supported in some cases and can point to likely associations between known and unknown genotypes. Regardless, this provides a preliminary glance at the evolutionary relationships of these medically important organisms using the most current sequence data.

DISCUSSION

In the present study, the prevalence and genotype of *Acanthamoeba* spp. was determined in different water resources of Zahedan in the Sistan and Baluchistan province of southeast Iran. Among the water samples, 34% were positive in culture and based on 18S rRNA sequencing, all of *Acanthamoeba* isolates were categorized as T4 and T5, with potentially pathogenic amoebae which are the predominant environmental genotype. Since *Acanthamoeba* spp. are resistant to extreme environmental physical conditions such as temperature, pH, and exposure to various chemicals can increase both their pathogenicity as well as endosymbiont virulence bacteria (Khan 2006). The isolates which belong to the T4 genotype have shown more affinity for host cells and subsequently greater cytotoxicity in comparison with T2, T3, and T11 genotypes (Memari *et al.* 2017).

Based on 18S rRNA sequencing results, most of *Acanthamoeba* which were isolated from keratitis in the world have been categorized as the T4 genotype (Visvesvara *et al.* 2007). Several genotypes of *Acanthamoeba* including T2, T3, T4, and T11 cause AK. T4 is the most predominant genotype in Iran, which leads to AK (Maghsood *et al.* 2005; Niyyati *et al.* 2009b; Hajialilo *et al.* 2015, 2016).

Several studies have reviewed the presence of different genotypes of *Acanthamoeba* spp. in a variety of water resources in different parts of Iran and have reported eight pathogenic and nonpathogenic genotypes of *Acanthamoeba* spp., including T2–T5, T7, T11, T13, and T15.

In some studies including Mazandaran (Dodangeh *et al.* 2018), West Azerbaijan (Haniloo *et al.* 2017), and Ardabil provinces (Badirzadeh *et al.* 2011), T4 (100%) was found in water sources as the predominant genotype of

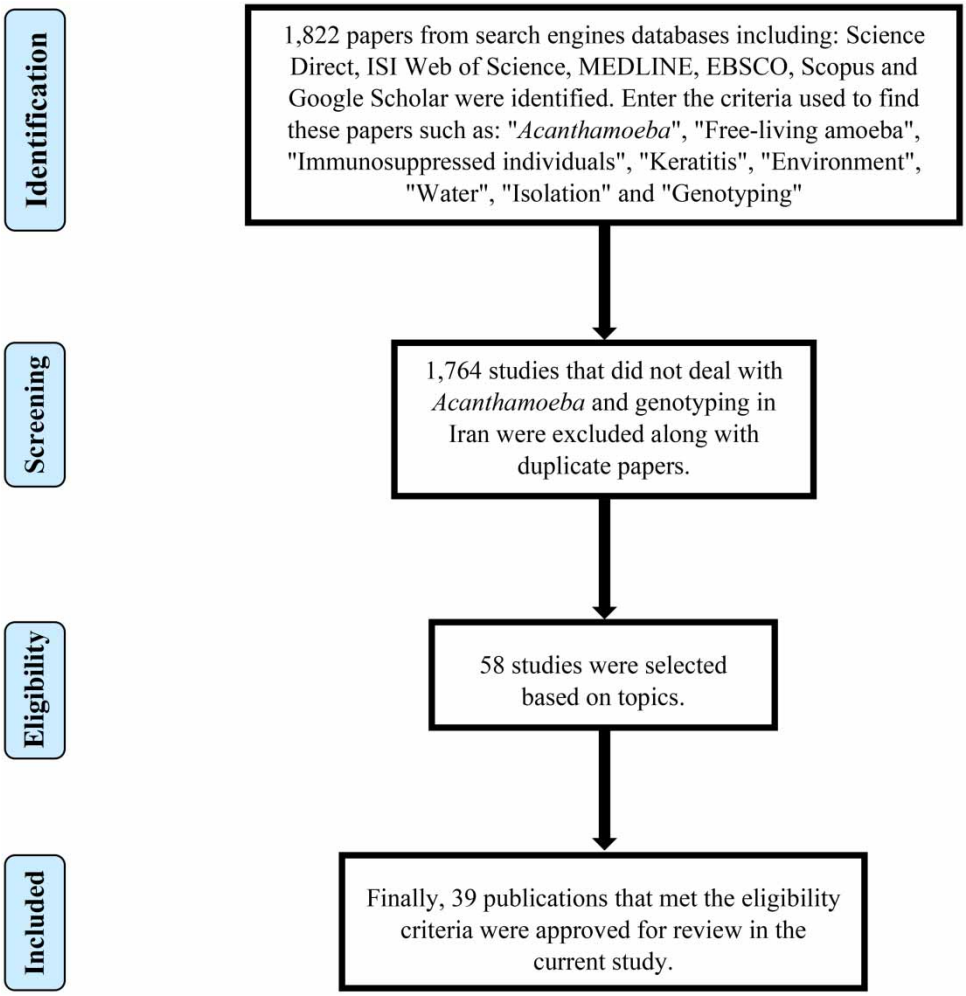

Figure 2 | Flowchart describing the process of the study design.

Acanthamoeba. Also in Semnan province on drinking water (Javanmard *et al.* 2017) and in East Azerbaijan province on surface resting waters (Fallah *et al.* 2017), T5 (100%) was the only found genotype, while T4 and T5 genotypes were detected in some studies by Niyyati *et al.* and Mahmoudi *et al.* in Guilan, Mazandaran, Alborz, West Azerbaijan, and Tehran provinces on different water sources (Nazar *et al.* 2011; Mahmoudi *et al.* 2015; Niyyati *et al.* 2015a, 2015b; Khezri *et al.* 2016). Based on our results, the prevalence of T4 as *A. castellanii* was higher than T5 as *A. griffini* which is in accordance with previous findings that suggested T4 as the most prevalent genotype not only in Iran but also in the world.

Moreover, in other studies, other genotypes of *Acanthamoeba* were detected in Iran. T2, T7, and T11 genotypes were reported from Isfahan province (Golestani *et al.* 2018), and T3 was found from stagnant water of Zabol in Sistan and Baluchistan province (Aghajani *et al.* 2016). Considering the fact that both Zabol and Zahedan are located in Sistan and Baluchistan province, T3 genotype was not detected in our samples.

T2 as *A. palestinensis* was reported from Qazvin, Mazandaran, and Ilam provinces (Hooshyar *et al.* 2013; Niyyati *et al.* 2016; Shokri *et al.* 2016). The T2 genotype has a pathogenic potential and it can grow at high temperature and osmolality and has been reported as a causative agent of keratitis in Iran and worldwide (Maghsood *et al.* 2005; Niyyati *et al.* 2016; Golestani *et al.* 2018).

The T15 genotype is frequently isolated from warm water such as hot springs (Huang & Hsu 2010), but the

Table 2 | A list of studies that reported *Acanthamoeba* spp. and their genotype from different samples and areas of Iran

No.	First author	Year	Area (Province)	Positive samples	Sample	Reported genotype	References
1	Meighani M	2018	Markazi	16/48	Soil	T4 (36.4%), T5 (18.2%), T6 (9.1%)	Meighani *et al.* (2018)
2	Dodangeh S	2018	Mazandaran	11/24	Water	T4 (100%)	Dodangeh *et al.* (2018)
3	Golestani MH	2018	Isfahan	66/122	Stagnant water	T4 (76%), T5 (13.8%). T2 (3.4%), T7 (3.4%), T11 (3.4%)	Golestani *et al.* (2018)
4	Ghaderifar S	2018	Razavi Khorasan	19/90	Pond water of parks	T4 (100%)	Ghaderifar *et al.* (2018)
5	Behnia M	2017	Tehran	6/90	Water treatment facilities	T4 (83%) and T11 (17%)	Behnia *et al.* (2017)
6	Memari F	2017	Tehran	1	Mucosal tissue of HIV patient	T4 (100%)	Memari *et al.* (2017)
7	Niyyati M	2017	Tehran	7/187	Oral cavity of hemodialysis patients	T1 (14.29%), T4 (85/71%)	Niyyati *et al.* (2017)
8	Javanmard E	2017	Semnan	1/16	Drinking water	T5 (100%)	Javanmard *et al.* (2017)
9	Haniloo A	2017	West Azerbaijan		Water sources	T4 (100%)	Haniloo *et al.* (2017)
10	Fallah E	2017	East Azerbaijan	28/50	Surface resting waters	T5 (3.57%)	Fallah *et al.* (2017)
11	Aghajani A	2016	Sistan	38/93	Pools and stagnant water	T3 (2.63%), T4 (89.47%), T5 (7.9%)	Aghajani *et al.* (2016)
12	Khezri A	2016	West Azerbaijan	14/24	River sources and tap water	T4 (92.85%), T5 (7.15%)	Khezri *et al.* (2016)
13	Shokri A	2016	Mazandaran	20/43	Water samples from lakes, rivers, waterscapes, sea, tap waters, pools, waterholes, rice fields, and fishponds	T2 (16.7%), T4 (83.3%)	Shokri *et al.* (2016)
14	Karamati SA	2016	East Azerbaijan	25/60	Soil	T3 (8%), T4 (52%), T5 (4%), T11 (4%)	Karamati *et al.* (2016)
15	Armand B	2016	Fars	48/82	Surface and tap water	T4 (62.96%), T5 (33.33%), T15 (3.71%)	Armand *et al.* (2016)
16	Hajialilo E	2016	Tehran	18/183	AK patients	T4 (78%), T9 (11%), T11 (11%)	Hajialilo *et al.* (2016)
17	Memari F	2016	Tehran	11/90	Oral cavity of immunosuppressed individuals	T3 (18.18%), T4 (72.72%), T11 (9.09%)	Memari *et al.* (2016)
18	Niyyati M	2016	Ilam (Southwest)	16/40	Geothermal rivers and pools	T4 (93.7%), T2 (6.25%)	Niyyati *et al.* (2016)
19	Behniafar H	2015	East Azerbaijan	17/67	Drinking and recreational water	T3 (23.52%), mixed T3/T4 (5.88%), T4 (58.82%), T5 (5.88%), and T13 (5.88%)	Behniafar *et al.* (2015)
20	Hajialilo E	2015	Tehran	9/62	Contact lenses of Keratitis patients	T4 (11.1%)	Hajialilo *et al.* (2015)
21	Memari F	2015	Tehran	21/80	Nasal swabs of cancer patients	T4 (50%), T3 (2.8%), T5 (5.6%)	Memari *et al.* (2015)

(*continued*)

Table 2 | continued

No.	First author	Year	Area (Province)	Positive samples	Sample	Reported genotype	References
22	Niyyati M	2015	Hormozgan	14/21	Water sources of tap water	T3 (14.2%), T4 (57.1%), T5 (21.42%), T11 (7.1%)	Niyyati *et al.* (2015a, 2015b)
23	Niyyati M	2015	Tehran	16/75	Recreational water sources	T4 (85.71%), T5 (14.29%)	Niyyati *et al.* (2015a, 2015b)
24	Mahmoudi MR	2015	Guilan, Mazandaran, Alborz and Tehran	18/38	Surface water resources	T4 (88.8%), T5 (11.2%)	Mahmoudi *et al.* (2015)
25	Lasjerdi Z	2015	Multiple cities, Iran	18/42	Ophthalmology wards in reference hospitals	T4 (92.3%), T5 (7.7%)	Lasjerdi *et al.* (2015)
26	Niyyati M	2014	Tehran	6/90	Volunteer-provided contact lenses	T3 (16.6%), T4 (66.8%), T5 (16.6%)	Niyyati *et al.* (2014)
27	Hooshyar H	2013	Qazvin	14/40	Surface and stagnant waters	T4 (78.6%), T2 (21.4%)	Hooshyar *et al.* (2013)
28	Mirjalai H	2013	Tehran and Ardabil	3/36	Hot spring and window dust and corneal	T4 (100%)	Mirjalali *et al.* (2013)
29	Niyyati M	2013	Tehran	9/55	Soil	T4 (100%)	Niyyati *et al.* (2013)
30	Rahdar M	2012	Khuzestan	56/110	Water and soil	T4 (86.6%), T2 (6.6%), T5 (6.6%)	Rahdar *et al.* (2012)
31	Solgi R	2012	North west	12/60	Therapeutic hot springs	T4 (83.3%), T3 (16.7%)	Solgi *et al.* (2012)
32	Niyyati M	2012	Tehran	13/55	River recreation areas	T4 (91.7%), T15 (8.3%)	Niyyati *et al.* (2012)
33	Badirzadeh A	2011	Ardabil	1/35	Water	T4 (100%)	Badirzadeh *et al.* (2011)
34	Nazar M	2011	Tehran	16/72	Water of parks and squares	T4 (87.5%), T5 (12.5%)	Nazar *et al.* (2011)
35	Lasjerdi Z	2011	Tehran	37/70	Immunodeficiency wards of hospitals	T4 (96.9%), T5 (3.1%)	Lasjerdi *et al.* (2011)
36	Niyyati M	2010	Tehran	1/1	Soft contact lens wearer	T3 (100%)	Niyyati *et al.* (2010)
37	Niyyati M	2009	Multiple cities, Iran	15/50	Keratitis and environmental	T3 (6.7%), T4 (86.7%), T11 (13.3%)	Niyyati *et al.* (2009b)
38	Niyyati M	2009	Iran	13/13	Dust sources	T4 (84.6%), T5 (7.6%), T11 (7.6%)	Niyyati *et al.* (2009a)
39	Maghsood AH	2005	Tehran, Hamedan, Mazandaran	25/25	Keratitis patients, pool and waterfall samples	T2 (40%), T3 (8%), T4 (48%)	Maghsood *et al.* (2005)

T15 genotype was detected in two occasions from cold rivers of Tehran (Niyyati *et al.* 2012) and surface and tap water of Fars province (Armand *et al.* 2016).

In our study, the highest and lowest prevalence rate of the amoeba in the examined samples was observed in public square (55.5%) and pipe (11.1%) water samples, respectively. But 40% of the hospital drinking water samples were positive for *Acanthamoeba*. A similar investigation on tap water at hospitals in 13 cities of Iran detected 48% *Acanthamoeba* spp. in the examined samples that our findings are in agreement with this study (Bagheri *et al.* 2010). In fact, the isolation of potentially pathogenic *Acanthamoeba* spp. from either hospital tap water or hospital wards can put patients particularly immunodeficient ones in danger of acquiring the infection.

In the current study, *Acanthamoeba* was detected in 33.3% of swimming pool samples. In a study in Zabol from Sistan and Baluchistan province in the southeast of Iran, 93 samples of pools and stagnant water were evaluated

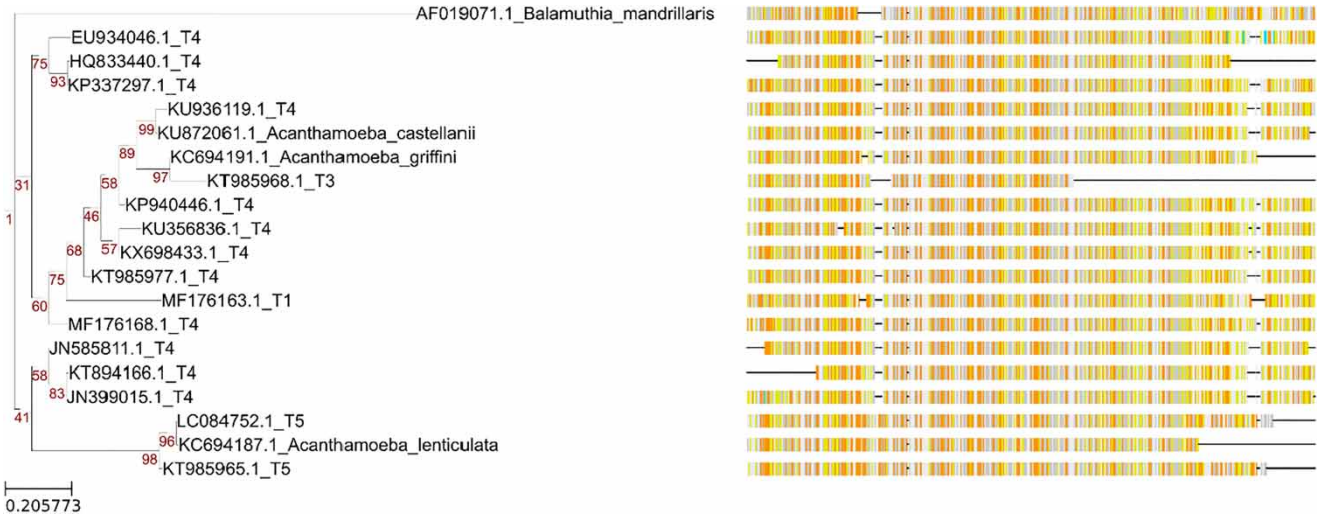

Figure 3 | Phylogenetic tree resulting from the maximum likelihood analysis of 20 18S rDNA sequences using the IQTree software, with 100,000 ultrafast bootstrap replicates. *B. mandrillaris* sequence is used as an out group. Sequences for corresponding tree leaves are represented as an alignment (right). Support numbers for nodes are presented as the percentage of bootstrap replicates supporting the current configuration.

for *Acanthamoeba*. Overall, 40.8% of the samples were positive that the following genotypes were detected: T4 (89.47%), T5 (7.9%), and T3 (2.63%) (Aghajani *et al.* 2016). Another study by Maghsood *et al.* (2005) on 12 pool and waterfall samples showed *Acanthamoeba* T4 (33.3%) and T2 (58.3%) genotypes. Although the result of our study was almost similar to the findings of the two abovementioned surveys, T2 and T3 genotypes were not observed in our swimming pool specimens. An acceptable justification for the contamination of swimming pool waters might be associated with cyst form which has a double-layer coat that is resistant to disinfectants despite normal chlorination/disinfection procedures. Therefore, we could consider this fact as an alarm and precaution for soft contact lens wearers. They should not wear lenses while they swim in pools due to the probable danger of acquiring Acanthamoebiasis corneal infection.

The T4 genotype is the most common genotype detected in water samples and patients in Iran. Moreover, many studies have been undertaken to identify *Acanthamoeba* in natural resources and patients in other countries. The results show that the T4 genotype is the dominant genotype in the examined specimens (Zhao *et al.* 2010; Coskun *et al.* 2013; Lass *et al.* 2014). Even though in many studies, T4 is the most common genotype, other genotypes including T3, T5, T11, and T13 are common genotypes in drinking water (tank and tap water) (Behniafar *et al.* 2015), T2, T3, T5, and T15 in surface and groundwater (Mahmoudi *et al.* 2012; Niyyati *et al.* 2012; Rahdar *et al.* 2012; Behniafar *et al.* 2015), T3 in recreational water (pools, springs, and the sea) (Badirzadeh *et al.* 2011; Solgi *et al.* 2012; Behniafar *et al.* 2015), and T2, T3, T5, and T15 in stagnant water (ponds, fountains, and streams) (Hooshyar *et al.* 2013; Armand *et al.* 2016).

Overall, the occurrence of T4 and T5 genotypes of *Acanthamoeba* as potentially pathogenic agents in different water resources in Zahedan of Iran reflects an urgent need to improve water treatment procedures in the city to prevent *Acanthamoeba*-related infections, especially for immunodeficient patients.

CONCLUSION

Authors from the current study draw the conclusion that *Acanthamoeba* spp. is present in the water sources of Zahedan and the predominant genotype is T4, which is pathogenic particularly for high-risk individuals. Furthermore, the current systematic review elucidates that the most common genotypes isolated from clinical and environmental samples from Iran were T4 and T5. Phylogenetic analysis

on 18S rRNA sequences indicates that *A. castellanii* and *A. griffini* predominantly colocalize with the T4 genotype.

ACKNOWLEDGEMENTS

This study was a part of the MSc thesis of Maryam Mansouri Nia and supported financially by the Cellular and Molecular Research Center of the Zahedan University of Medical Sciences, Zahedan, Iran (Grant No. 7139).

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICAL STATEMENT

This manuscript does not report on animal or human data.

CONSENT FOR PUBLICATION

This manuscript does not contain individual-level data or identifying information for any person.

REFERENCES

Abdollahian, E., Shafiei, R., Mokhber, N., Kalantar, K. & Fata, A. 2017 Seroepidemiological study of *Toxoplasma gondii* infection among psychiatric patients in Mashhad, Northeast of Iran. *Iran. J. Parasitol.* **12** (1), 117–122.

Aghajani, A., Dabirzadeh, M., Maroufi, Y. & Hooshyar, H. 2016 Identification of *Acanthamoeba* genotypes in pools and stagnant water in ponds in Sistan region in southeast Iran. *Turkiye Parazitol. Derg.* **40**, 132–136.

Ahmadpour, E., Daryani, A., Sharif, M., Sarvi, S., Aarabi, M., Mizani, A., Rahimi, M. T. & Shokri, A. 2014 Toxoplasmosis in immunocompromised patients in Iran: a systematic review and meta-analysis. *J. Infect. Dev. Ctries.* **8** (12), 1503–1510.

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

Badirzadeh, A., Niyyati, M., Babaei, Z., Amini, H., Badirzadeh, H. & Rezaeian, M. 2011 Isolation of free-living amoebae from sarein hot springs in Ardabil Province, Iran. *Iran. J. Parasitol.* **6**, 1–8.

Bagheri, H. R., Shafiei, R., Shafiei, F. & Sajjadi, S. A. 2010 Isolation of *Acanthamoeba* spp. from drinking waters in several hospitals of Iran. *Iran. J. Parasitol.* **5**, 19–25.

Behnia, M., Hatam-Nahavandi, K., Hajialilo, E., Niyyati, M., Tarighi, F., Bakhtiar Akram, A., Salimi, M. & Rezaeian, M. 2017 Occurrence of *Acanthamoeba* genotypes in wastewater samples in Tehran, Iran. *Iran. J. Parasitol.* **12**, 516–521.

Behniafar, H., Niyyati, M. & Lasjerdi, Z. 2015 Molecular characterization of pathogenic *Acanthamoeba* isolated from drinking and recreational water in east Azerbaijan, northwest Iran. *Environ. Health Insights* **9**, 7–12.

Coskun, K. A., Ozcelik, S., Tutar, L., Elaldi, N. & Tutar, Y. 2013 Isolation and identification of free-living amoebae from tap water in Sivas, Turkey. *Biomed. Res. Int.* 675145.

Di Filippo, M. M., Santoro, M., Lovreglio, P., Monno, R., Capolongo, C., Calia, C., Fumarola, L., D'Alfonso, R., Berrilli, F. & Di Cave, D. 2015 Isolation and molecular characterization of free-living amoebae from different water sources in Italy. *Int. J. Environ. Res. Public Health* **12**, 3417–3427.

Dodangeh, S., Kialashaki, E., Daryani, A., Sharif, M., Sarvi, S., Moghaddam, Y. D. & Hosseini, S. A. 2018 Isolation and molecular identification of *Acanthamoeba* spp. from hot springs in Mazandaran province, northern Iran. *J. Water Health* **16**, 807–813.

Fallah, E., Jafarpour, Z., Mahami-Oskouei, M., Haghighi, A., Niyyati, M., Spotin, A. & Khezri, A. 2017 Molecular characterization of *Acanthamoeba* isolates from surface resting waters in northwest Iran. *Iran. J. Parasitol.* **12**, 355–363.

Ghaderifar, S., Najafpoor, A. A., Zarrinfar, H., Esmaily, H. & Hajialilo, E. 2018 Isolation and identification of *Acanthamoeba* from pond water of parks in a tropical and subtropical region in the Middle East, and its relation with physicochemical parameters. *BMC Microbiol.* **18**, 139.

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**, e55582.

Hajialilo, E., Niyyati, M., Solaymani, M. & Rezaeian, M. 2015 Pathogenic free-living amoebae isolated from contact lenses of keratitis patients. *Iran. J. Parasitol.* **10**, 541–546.

Hajialilo, E., Behnia, M., Tarighi, F., Niyyati, M. & Rezaeian, M. 2016 Isolation and genotyping of *Acanthamoeba* strains (T4, T9, and T11) from amoebic keratitis patients in Iran. *Parasitol. Res.* **115**, 3147–3151.

Haniloo, A., Pezeshki, A., Mahmmodzadeh, A. & Kadkhodamohammadi, E. 2017 Genotyping of *Acanthamoeba* spp. from water sources from northwestern Iran. *Acta Parasitol.* **62**, 790–795.

Hooshyar, H., Hosseinbigi, B., Saraei, M., Alizadeh, S., Eftakhar, M., Rasti, S. & Khosro-Shahi, N. 2013 Genotyping of

Acanthamoeba isolated from surface and stagnant waters of Qazvin, central Iran. *Iran. Red Crescent Med. J.* **15**, 536–538.

Huang, S. W. & Hsu, B. M. 2010 Isolation and identification of *Acanthamoeba* from Taiwan spring recreation areas using culture enrichment combined with PCR. *Acta Trop.* **115**, 282–287.

Huerta-Cepas, J., Serra, F. & Bork, P. 2016 ETE 3: reconstruction, analysis, and visualization of phylogenomic data. *Mol. Biol. Evol.* **33**, 1635–1638.

Javanmard, E., Niyyati, M., Lorenzo-Morales, J., Lasjerdi, Z., Behniafar, H. & Mirjalili, H. 2017 Molecular identification of waterborne free living amoebae (*Acanthamoeba*, *Naegleria* and *Vermamoeba*) isolated from municipal drinking water and environmental sources, north half of Iran. *Exp. Parasitol.* **S0014-4894**, 30419-8.

Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., Von Haeseler, A. & Jermiin, L. S. 2017 Modelfinder: fast model selection for accurate phylogenetic estimates. *Nat. Methods* **14**, 587–589.

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 Parasitol.* **61**, 784–789.

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

Khezri, A., Fallah, E., Mostafazadeh, M., Spotin, A., Shahbazi, A., Mahami-Oskouei, M. & Hazratian, T. 2016 Molecular and morphometric characterization of *Acanthamoeba* spp. from different water sources of northwest Iran as a neglected focus, co-bordered with the country of Iraq. *Jundishapur J. Microbiol.* **9**, e38481.

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

Lasjerdi, Z., Niyyati, M., Lorenzo-Morales, J., Haghighi, A. & Taghipour, N. 2015 Ophthalmology hospital wards contamination to pathogenic free living amoebae in Iran. *Acta Parasitol.* **60**, 417–422.

Lass, A., Szostakowska, B., Idzinska, A. & Chomicz, L. 2014 The first genotype determination of *Acanthamoeba* potential threat to human health, isolated from natural water reservoirs in Poland. *Parasitol. Res.* **113**, 2693–2699.

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. *J. Med. Microbiol.* **54**, 755–759.

Magnet, A., Galvan, A. L., Fenoy, S., Izquierdo, F., Rueda, C., Fernandez Vadillo, C., Perez-Irezabal, J., Bandyopadhyay, K., Visvesvara, G. S., da Silva, A. J. & del Aguila, C. 2012 Molecular characterization of *Acanthamoeba* isolated in water treatment plants and comparison with clinical isolates. *Parasitol. Res.* **111**, 383–392.

Mahmoudi, M. R., Taghipour, N., Eftekhar, M., Haghighi, A. & Karanis, P. 2012 Isolation of *Acanthamoeba* species in surface waters of Gilan province-north of Iran. *Parasitol. Res.* **110**, 473–477.

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.

Marciano-Cabral, F. & Cabral, G. 2003 *Acanthamoeba* spp. as agents of disease in humans. *Clin. Microbiol. Rev.* **16**, 273–307.

Meighani, M., Eslamirad, Z., Hajihossein, R., Ahmadi, A. & Saki, S. 2018 Isolation and genotyping of *Acanthamoeba* from soil samples in Markazi province, Iran. *Open Access Maced. J. Med. Sci.* **6**, 2290–2294.

Memari, F., Niyyati, M., Haghighi, A., Seyyed Tabaei, S. J. & 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., Lorenzo-Morales, J. & Jonaydi, Z. 2016 Isolation and molecular characterization of *Acanthamoeba* strains isolated from the oral cavity of immunosuppressed individuals in Tehran, Iran. *Acta Parasitol.* **61**, 451–455.

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**, 143–147.

Minh, B. Q., Nguyen, M. A. & von Haeseler, A. 2013 Ultrafast approximation for phylogenetic bootstrap. *Mol. Biol. Evol.* **30**, 1188–1195.

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**, 530–535.

Nazar, M., Haghighi, A., Niyyati, M., Eftekhar, M., Tahvildar-Biderouni, F., Taghipour, N., Abadi, A., Nazemalhosseini Mojarad, E. & Athari, A. 2011 Genotyping of *Acanthamoeba* isolated from water in recreational areas of Tehran, Iran. *J. Water Health* **9**, 603–608.

Nguyen, L. T., Schmidt, H. A., von Haeseler, A. & Minh, B. Q. 2015 IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* **32**, 268–274.

Niyyati, M., Lorenzo-Moralesm, J., Rahimi, F., Motevalli-Haghi, A., Martín-Navarro, C. M., Farnia, S., Valladares, B. & Rezaeian, M. 2009a Isolation and genotyping of potentially pathogenic *Acanthamoeba* strains from dust sources in Iran. *Trans. R. Soc. Trop. Med. Hyg.* **103**, 425–427.

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. 2009b Genotyping of *Acanthamoeba* isolates from clinical and environmental specimens in Iran. *Exp. Parasitol.* **121**, 242–245.

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., Lasjerdi, Z., Nazar, M., Haghighi, A. & Nazemalhosseini Mojarad, E. 2012 Screening of recreational areas of rivers for potentially pathogenic free-living amoebae in the suburbs of Tehran, Iran. *J. Water Health* **10**, 140–146.

Niyyati, M., Ebrahimi, M., Haghighi, A. & Haydari, S. 2013 Isolation and genotyping of *Acanthamoeba* spp. from recreational soil of parks in Tehran. *Iran. Armaghane Danesh* **18**, 530–538.

Niyyati, M., Rahimi, F., Lasejerdi, Z. & Rezaeian, M. 2014 Potentially pathogenic free-living amoebae in contact lenses of the asymptomatic contact lens wearers. *Iran. J. Parasitol.* **9**, 14–19.

Niyyati, M., Lasgerdi, Z. & Lorenzo-Morales, J. 2015a Detection and molecular characterization of potentially pathogenic free-living amoebae from water sources in Kish island, southern Iran. *Microbiol. Insights* **8**, 1–6.

Niyyati, M., Mafi, M., Haghighi, A. & Hakemi Vala, M. 2015b Occurrence of potentially pathogenic bacterial-endosymbionts in *Acanthamoeba* spp. Iran. *J. Parasitol.* **10**, 181–188.

Niyyati, M., Saberi, R., Latifi, A. & Lasjerdi, Z. 2016 Distribution of *Acanthamoeba* genotypes isolated from recreational and therapeutic geothermal water sources in southwestern Iran. *Environ. Health Insights* **10**, 69–74.

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

Prasher, P., Sachdeva, P., Nath Bhatnagar, R. & Walia, S. 2004 *Acanthamoeba* keratitis: a review. *North Zone Ophthalmological Society India (NZOS)* **14**, 1–7.

Rahdar, M., Niyyati, M., Salehi, M., Feghhi, M., Makvandi, M., Pourmehdi, M. & Farnia, S. 2012 Isolation and genotyping of *Acanthamoeba* strains from environmental sources in Ahvaz city, Khuzestan province, Southern Iran. *Iran. J. Parasitol.* **7**, 22–26.

Rahimi, M. T., Mahdavi, S. A., Javadian, B., Rezaei, R., Moosazadeh, M., Khademlou, M., Seyedpour, S. H. & Syadatpanah, A. 2015 High seroprevalence of *Toxoplasma gondii* antibody in HIV/AIDS individuals from north of Iran. *Iran. J. Parasitol.* **10**, 584.

Rezaeian, M., Niyyati, M., Farnia, S. H. & Haghi, A. M. 2008 Isolation of *Acanthamoeba* spp. from different environmental sources. *Iran. J. Parasitol.* **3**, 44–47.

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

Schuster, F. L. & Visvesvara, G. S. 2004 Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. *Int. J. Parasitol.* **34**, 1001–1027.

Shafiei, R., Riazi, R., Sarvghad, M. R., Galian Sharifdini, M., Mahmoodzadeh, A. & Hajia, M. 2011 Prevalence of IgG and IgM anti-*Toxoplasma gondii* antibodies in HIV positive patients in northeast of Iran. *Iran. J. Pathol.* **6**, 68–72.

Shokri, A., Sarvi, S., Daryani, A. & Sharif, M. 2016 Isolation and genotyping of *Acanthamoeba* spp. as neglected parasites in north of Iran. *Korean J. Parasitol.* **54**, 447–453.

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., Thompson, J. D. & Higgins, D. G. 2011 Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* **7**, 539.

Solgi, R., Niyyati, M., Haghighi, A., Taghipour, N., Tabaei, S. J., Eftekhar, M. & Nazemalhosseini Mojarad, E. 2012 Thermotolerant *Adcanthamoeba* spp. isolated from therapeutic hot springs in northwestern Iran. *J. Water Health* **10**, 650–656.

Tsvetkova, N., Schild, M., Panaiotov, S., Kurdova-Mintcheva, R., Gottstein, B., Walochnik, J., Aspock, H., Lucas, M. S. & Muller, N. 2004 The identification of free-living environmental isolates of amoebae from Bulgaria. *Parasitol. Res.* **92**, 405–413.

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 Immunol. Med. Microbiol.* **50**, 1–26.

Zhao, G., Sun, S., Zhao, J. & Xie, L. 2010 Genotyping of *Acanthamoeba* isolates and clinical characteristics of patients with *Acanthamoeba* keratitis in China. *J. Med. Microbiol.* **59**, 462–466.

Zolfaghari Emameh, R., Barker, H. R., Tolvanen, M. E., Parkkila, S. & Hytonen, V. P. 2016 Horizontal transfer of beta-carbonic anhydrase genes from prokaryotes to protozoans, insects, and nematodes. *Parasit. Vectors* **9**, 152.

Zolfaghari Emameh, R., Barker, H. R., Hytonen, V. P. & Parkkila, S. 2018 Involvement of beta-carbonic anhydrase genes in bacterial genomic islands and their horizontal transfer to protists. *Appl. Environ. Microbiol.* **84** (15), e00771-18.

First received 10 March 2019; accepted in revised form 24 May 2019. Available online 19 July 2019