Isolation and molecular identification of free-living amoebae (*Naegleria* spp., *Acanthamoeba* spp. and *Vermamoeba* spp.) from mineral springs in Guilan Province, northern Iran

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ABSTRACT

Free-living amoebae (FLA) include many genera which cause serious diseases such as sight-threatening keratitis, cutaneous ulcers and fatal encephalitis. This study was conducted due to the lack of research regarding genotypes *Acanthamoeba*, *Naegleria* and *Vermamoeba* in mineral springs of Guilan Province in northern Iran. Twenty-five water samples were collected from mineral springs in Guilan Province. After filtration through nitrocellulose membrane, samples were cultured on non-nutrient agar plates. The morphological key of Page was used to identify free-living amoebae (FLA) using an inverted microscope. Positive cultures were analyzed by polymerase chain reaction (PCR) and genotypes based on the NCBI database. Eleven (44%) samples were positive for *Acanthamoeba*, *Naegleria* and *Vermamoeba*. By sequencing the positive isolates, the strains were shown to belong to *Acanthamoeba castellanii* (three case isolates belonged to T4 genotype), three cases of *Vermamoeba vermiformis*, and two cases of *N. australiensis,* two cases of *N. pagei* and one case of *N. gruberi*. According to our research the occurrence of *Acanthamoeba*, *Naegleria* spp. and *Vermamoeba* spp. in mineral springs could be hazardous for high risk persons. Regular monitoring and posting warning signs of these waters by health planners could prevent free-living amoebae mediated diseases.

Key words | *Acanthamoeba*, Iran, mineral springs, *Naegleria*, PCR/DNA sequencing, *Vermamoeba*

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INTRODUCTION

Free-living amoebae (FLA) include many genera which cause serious diseases such as sight-threatening keratitis, cutaneous ulcers and fatal encephalitis. *Acanthamoeba* spp., *Naegleria fowleri* and *Balamuthia mandrillaris* are the most commonly reported causes in the world (Hooshyar *et al.* 2013; Javanmard *et al.* 2017; Feiz-Haddad *et al.* 2019a). Other genera in this group, including *Sappinia*, *Vermamoeba* and *Vahlkampfia*, are causative agents of disease with a lower incidence around the world (Abedkhojasteh *et al.* 2015; Javanmard *et al.* 2017). *Acanthamoeba* is widely distributed in a variety of environments including ocean sediments, tap water, ponds, hydrotherapy pools, lakes and hot springs (Feiz-Haddad *et al.* 2019b). There have been several reports of finding the source of *Acanthamoeba* (belonged to T4, T5, T7 and T11 genotype), *Naegleria gruberi* and *Vahlkampfia vahlkampfia* from mineral water in Brazil and Mexico (Rivera *et al.* 1981; Maschio *et al.* 2015). Further, Todd *et al.* (2015) reported *Acanthamoeba* from 50.6 to 17.3% recreational (mineral springs, ponds, lagoons, rivers, beaches and streams) and domestic water, respectively, from Jamaica. Free-living amoebae such as *Acanthamoeba* spp. can act as a Trojan horse and reservoir for pathogenic microorganisms such as Protozoa, bacteria, viruses and yeasts (Khan 2009).

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To date, the *Acanthamoeba* genus, based on 18S rDNA sequencing, is classified into 21 genotypes (T1–T21) (Corsaro *et al.* 2017).

At present, in Iran *Acanthamoeba* genotypes related to keratitis are T4, T3, T2, T11, T13 and T15 (Niyyati & Rezaeian 2015). *Acanthamoeba* keratitis (AK) is mostly seen among healthy individuals and the young, and most of them (80%) have a history of wearing contact lenses (CLs), and it is seen in people who never use CLs as well (Feiz-Haddad *et al.* 2019b). In a study carried out by Shokri *et al.* (2016) in northern Iran on water sources, out of 77 water samples taken from different water sources within the Mazandaran Province in northern Iran (Sari city and suburbs), 83.3% of sequenced isolates belonged to the T4 genotype and the rest belonged to the T2 genotype (Shokri *et al.* 2016). The presence of FLA in the recreational water of the provinces bordering Guilan has been confirmed (Latifi *et al.* 2016, 2017; Feiz-Haddad *et al.* 2019a). Also, there are some studies indicating the presence of *Acanthamoeba* in river water samples in Guilan Province, northern Iran (Mahmoudi *et al.* 2012, 2015a, 2015b). However, there is no information regarding free living amoeba in mineral springs in these areas. Guilan Province has numerous mineral springs in different cities. This study was conducted due to the lack of research regarding the genotypes *Acanthamoeba*, *Naegleria* and *Vermamoeba* in mineral springs of Guilan Province in northern Iran.

METHODS

Sampling process

Five mineral springs in Guilan Province, northern Iran, were included in the current study and five samples per site were obtained (Latifi *et al.* 2017). Rudbar, Fuman, Amlash, Rasht and Rudsar (Figure 1) are five cities in this province known for their recreational and therapeutic mineral springs and these are included in the present study (Latifi *et al.* 2017). Briefly, a volume of 500 mL of surface water was collected from mineral springs and transferred within 4 hours to the laboratory to be processed (Latifi *et al.* 2017). Physical parameters such as the pH and temperature of water were measured by a portable pH meter (Digital tester DMT-20) (Dodangeh *et al.* 2018).

Filtration, cultivation and cloning

Samples were filtered using cellulose nitrate membranes with a pore size of 0.45 μm as reported in a previous study (Latifi *et al.* 2017). The filters were then inverted and cultured onto 2% non-Nutrient agar plates covered with a layer of *Escherichia coli*. Incubation of samples was carried out at 30 °C in order to detect *Acanthamoeba* spp. *Naegleria* spp. *Vermamoeba* and 45 °C in order to detect *N. fowleri*. The

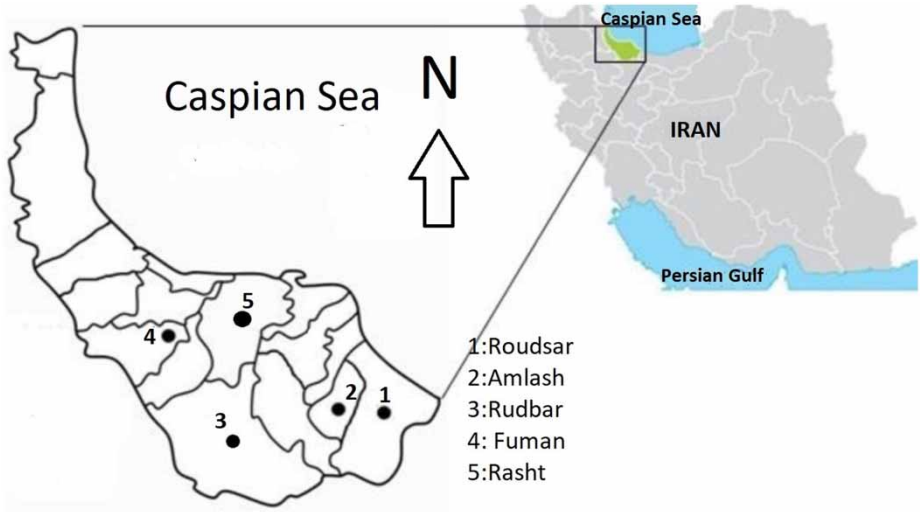

Figure 1 | Map showing the location of mineral springs in Guilan Province, Iran.

morphological key of Page (Page 1988) was used for the identification FLA using both an inverted microscope and wet-mount method. Cloning of the suspected amoebae was performed using the culture replicates method as previously described (Latifi *et al.* 2017).

PCR amplification and gel electrophoresis

In this study, cloned amoebae in plates were harvested using phosphate buffered saline (pH: 7.2) and the amoebae were precipitated by centrifuging for 5 min at 2,000 rpm. DNA was extracted using the modified phenol-chloroform method (Mahmoudi *et al.* 2012; Javanmard *et al.* 2017) and GeNet Bio kit, according to the manufacturer's instructions (GeNet Bio, South Korea). To carry out the polymerase chain reaction (PCR), 25 μL Ampliqone (Taq DNA Polymerase Master Mix RED, Denmark) was combined with DNA (10 ng), 0.1 μM of each primer and distilled water. Four sets of primers were used in order to detect various FLA which are shown in Table 1. In the present study, we used two primer sets, the *Naegleria* genus-specific and *N. fowleri* species-specific, designed from the ITS1-ITS2 region by Pelandakis *et al.* (2000), Latifi *et al.* (2017) and Javanmard *et al.* (2017) who had previously provided successful results. The ribosomal ITS sequence was reported to be a powerful tool for detecting inter- and intra-species differences of various organisms, including *Vahlkampfia* species (Garstecki *et al.* 2005) and *Naegleria* (De Jonckheere 1998). As in *Naegleria*, the differences between species (intra-species of *N. fowleri*) and genus (inter-species) were due to the polymorphism sequence that occurred at the ITS1 and ITS2 regions, respectively (Johan 2002). The ITS region was also being used for the identification of the new *Naegleria* isolates (Sheehan *et al.* 2003; Ithoi *et al.* 2011). The cycling condition was set as pre-denaturation step for 3 min at 94 °C, followed by 35 repetitions at 94 °C for 35 s, annealing steps were at 56, 56, 56 and 58 °C for 1 min (for *Acanthamoeba, Vahlkampfiids*, *Naegleria fowleri* and *Vermamoeba,* respectively), and 72 °C for 1 min (Javanmard *et al.* 2017). The expected sizes of PCR amplicons were revealed by electrophoresis on 1.5% horizontal agarose gel in Trisborate-EDTA buffer and stained with a solution of Safe Stain.

DNA sequencing of the PCR products

PCR products were submitted to DNA sequencing using an ABI 3130XL automatic sequencer. Blast analysis of the sequences with others available in the GeneBank data was performed using BLAST software from the National Center for Biotechnology Information (NCBI) site. The highest homology was the base of species identification (Latifi *et al.* 2017). The sequences were submitted to the gene bank under the following accession numbers: MK422924, MK441743, MK441744, MK441745-MK441748, MK441750 and MK441752-MK441754.

Results

The pH and temperature of the mineral springs are shown in Table 2. Of the 25 water samples collected from mineral springs in northern Iran, 11 (44%) were positive for *Acanthamoeba* spp., *Naegleria* spp. and *Vermamoeba* spp. based on the morphological criteria of Page (1988). The *Acanthamoeba* cysts were characterized as being double walled; the ectocyst

Table 1 | Primers used in this study

FLA[a]	Primer sequence	References
N. fowleri[b]	F5′-GTGAAAACCTTTTTTCCATTTACA-3′ R5′-AAATAAAAGATTGACCATTTGAAA-3′	Pelandakis *et al.* (2000); Latifi *et al.* (2017)
Vahlkampfiids	ITS1 F5′-GAACCTGCGTAGGGATCATTT-3′ ITS2 R 5″TTTCTTTTCCTCCCCTTATTA-3′	Pelandakis *et al.* (2000); Javanmard *et al.* (2017)
Acanthamoeba spp.	JDP15′-GGCCCAGATCGTTTACCGTGAA-3′ JDP2 5′-TCTCACAAGCTGCTAGGGAGTCA-3′	Mahmoudi *et al.* (2015b)
Vermamoeba	NA1F 5′-TTA CGA GGT CAG GAC ACTGT-3′ NA2R 5′-GAC CAT CCG GAG TTC TCG-3′	Javanmard *et al.* (2017)

[a]Free living amoebae.
[b]*Naegleria fawleri*.

Table 2 | Location and description of mineral springs in Guilan Province

City	Sampling site	Temperature (°C)	pH	*Acanthamoeba*	*Naegleria*	*Vermamoeba*
Rudbar	Damash1	24	6.5	+[a]	–	–
	Damash2	27	6.8	–	–	–
	Damash3	21	6.4	–	+	–
	Damash4	22	6.6	–	–	–
	Damash5	25	6.5	–	–	–
Rasht	Cheshmagol1	22	6.9	–	–	–
	Cheshmagol2	21	7.2	–	–	–
	Cheshmagol3	18	7.0	+	–	–
	Cheshmagol4	16	6.8	–	+	–
	Cheshmagol5	19	6.7	–	–	–
Fuman	Ali Zakhani1	24	6.4	–	–	–
	Ali Zakhani2	22	6.7	–	+	–
	Ali Zakhani3	20	6.4	–	–	–
	Ali Zakhani4	21	6.4	–	+	–
	Ali Zakhani5	26	6.6	–	–	+
Amlash	Lausanne1	24	6.6	–	–	–
	Lausanne2	23	6.4	+	–	–
	Lausanne3	22	6.8	–	–	+
	Lausanne4	27	6.4	–	–	–
	Lausanne5	25	6.1	–	–	–
Rudsar	Sajiran1	23	6.6	–	–	+
	Sajiran2	28	6.7	–	+	–
	Sajiran3	24	6.3	–	–	–
	Sajiran4	21	6.4	–	–	–
	Sajiran5	24	6.8	–	–	–

[a]Inverted microscope = +.

and endocyst (Figure 2). For *Vahlkampfiids* round cysts with a smooth wall and *Vermamoeba vermiformis* round cysts with a smooth wall but smaller *Vahlkampfiids* refer to Figures 3 and 4 respectively. These 11 isolates were cloned successfully (Figures 2–4). Positive cultures were analyzed by PCR and genotypes base on the NCBI database. In electrophoresis of PCR products, *Acanthamoeba* demonstrated an approximately 500 bp band (Figure 5). *Acanthamoeba* spp. was detected in three of the extracted DNA by PCR, using the JDP primer pairs, which are specific for the *Acanthamoeba* genus. Water pH and temperature were assessed *in situ* by using a portable pH meter (Digital tester DMT-20), so that these parameters of Mineral springs were respectively

Figure 2 | Acanthamoeba castellanii cysts (400×).

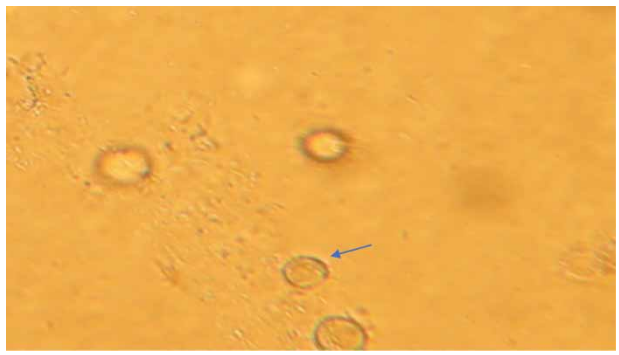

Figure 3 | *Vahlkampfiids* cysts (400×).

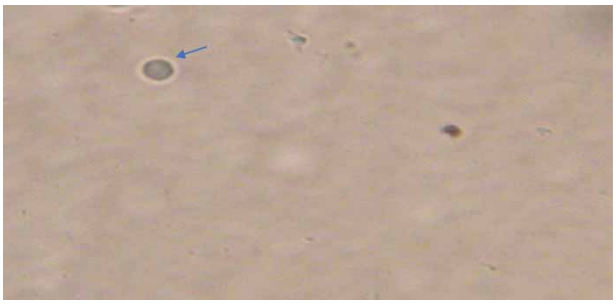

Figure 4 | *Vermamoeba vermiformis* cysts (400×).

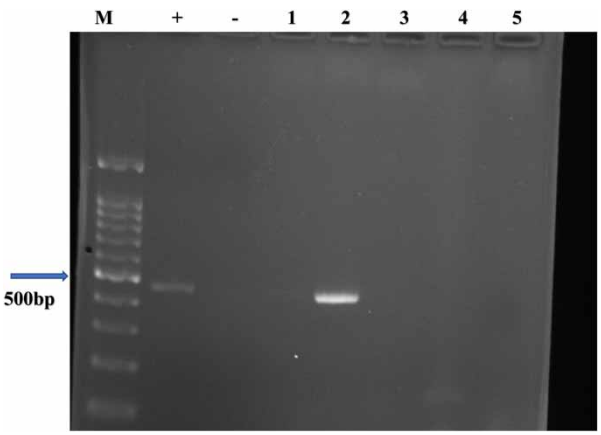

Figure 5 | PCR amplification of the isolated *Acanthamoeba* strains using JDP primers. M marker, − = negative control, + = positive control, two samples.

measured as 16–27 °C and 6.1–7.2 pH (Table 2). By sequencing the positive isolates, the strains were shown to belong to *Acanthamoeba castellanii* (three case isolates belonged to T4 genotype), three cases of *Vermamoeba vermiformis* and two cases of *N. australiensis*, two cases of *N. pagei* and one case of *N. gruberi* (Table 3).

DISCUSSION

The present study is the first study on the mineral springs of Guilan Province, northern Iran, to determine the pathogenic free-living amoeba via molecular methods. This study reports the presence of potentially pathogenic *Acanthamoeba castellanii* (genotype T4), *Vermamoeba vermiformis* and *Naegleria* strains including: *N. australiensis, N. pagei* and *N. gruberi* in the mineral springs of Guilan Province, northern Iran. In the present study pathogenic *N. fowleri* was not detected and there were no significant differences shown between pH value (and temperature) or the presence/absence of *Acanthamoeba*, *Naegleria* and *Vermamoeba* (Table 2). Some studies conducted in these areas have identified waterborne pathogens such as *Acanthamoeba castellanii* and *Vermamoeba verformis* (Mahmoudi *et al.* 2015b; Dodangeh *et al.* 2018).

Recently, infections due to *Acanthamoeba* have increased in the world, which is more likely related to the presence of *Acanthamoeba* in the natural environment, especially in water sources with a variety of prevalence detected (Liang *et al.* 2010; Saburi *et al.* 2017). We isolated one type of *Acanthamoeba* (T4), unlike Edagawa *et al.* (2009) who found T3, and Huang & Hsu (2010) who found that T15 predominated in the waters surveyed. *Acanthamoeba* T4 genotype is associated with AK (*Acanthamoeba*

Table 3 | Data of the free-living amoebae from mineral springs of northern Iran

Code	Name of mineral springs	Number/positive samples	Sequencing	Accession number	PCR (JDP1,2)	PCR (ITS1, 2)	PCR (NA1/2)	PCR for *N. fowleri*
HN1	Rudbar Damash 1	5/2	T4 genotype	MK422924	+	−	−	−
HN3	Rudbar Damash 3		*N. pagei*	MK441745	−	+	−	−
HN8	Rasht Cheshmagol 3	5/2	T4 genotype	MK441743	+	−	−	−
HN9	Rasht Cheshmagol 4		*N. pagei*	MK441746	−	+	−	−
HN12	FumanAliZakhani 2	5/3	*N. gruberi*	MK441747	−	+	−	−
HN14	FumanAliZakhani 4		*N. australiensis*	MK441748	−	+	−	−
HN15	FumanAliZakhani 5		*vermiformis*	MK441752	−	−	+	−
HN17	Amlash Lausanne 2	5/2	T4 genotype	MK441744	+	−	−	−
HN18	Amlash Lausanne 3		*V. vermiformis*	MK441753	−	−	+	−
HN21	Rudsar Sajiran 1	5/2	*V. vermiformis*	MK441754	−	−	+	−
HN22	Rudsar Sajiran 2		*N. australiensis*	MK441750	−	+	−	−

keratitis). *Acanthamoeba* keratitis is seen among people who use non-sterile waters and salt pills for disinfecting or storing their CLs (Feiz-Haddad *et al.* 2019b). Among the 47 known species of *Naegleria,* only *N. fowleri* has been reported to be pathogenic for human (De Jonckheere 2014). *Naegleria fowleri* was not detected in the present study; this may be owing to differences in the incubation temperature of the culture plates and/or the number of springs. Moreover, in contrast to the current study, others have demonstrated the presence of *N. fowleri* in the cooling waters of a Belgian power plant and hot springs in Taiwan (Behets *et al.* 2007; Tung *et al.* 2013). To date, one clinical case of *N. fowleri* has been reported in the country (Movahedi *et al.* 2012). We isolated two cases of *N. australiensis* in the present study (Table 3). *Naegleria australiensis* could be pathogenic to animal models (John & De Jonckheere 1985). Also, we isolated three cases of *Vermamoeba vermiformis* in the present study (Table 3). Keratitis owing to *Vermamoeba* and also a case of mixed infection of *Vermamoeba vermiformis* and *Acanthamoeba* were reported during previous studies (Lorenzo-Morales *et al.* 2007; Abedkhojasteh *et al.* 2015; Hajialilo *et al.* 2015). *Vermamoeba* amoebae are also considered as a reservoir for pathogenic microorganisms such as *Legionella pneumophila* and *Pseudomonas* (Centeno *et al.* 1996). Mineral springs, used therapeutically and as tourist attractions, is growing in popularity; which will increase the chance of exposure to these amoebae. To prevent infection and diseases related to free-living amoebae, mineral springs should be periodically checked, in particular during the summer season when these surface waters are used by thousands of tourists. Furthermore, posting of warning signs at recreational mineral springs could be another option for preventing FLA infections.

CONCLUSIONS

The results of our study approve the presence of potentially pathogenic *Acanthamoeba castellanii* (genotype T4), *Vermamoeba vermiformis* and *Naegleria* strains including: *N. australiensis, N. pagei* and *N. gruberi* in the mineral springs of Guilan Province. Mineral springs may enhance exposure of the amoebae to individuals. Hence, more attention to mineral springs is needed to prevent free-living amoebae mediated diseases.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

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