Isolation and molecular identification of *Acanthamoeba* spp. from hot springs in Mazandaran province, northern Iran

Samira Dodangeh, Elham Kialashaki, Ahmad Daryani, Mehdi Sharif, Shahabeddin Sarvi, Yusef Dadi Moghaddam and Seyed Abdollah Hosseini

ABSTRACT

Acanthamoeba is a free-living protozoan that can be found in natural and artificial environments such as hot tubs, surface water and springs and can cause severe diseases including amoebic keratitis and granulomatous amoebic encephalitis. The present study was conducted owing to the lack of research regarding genotypes of *Acanthamoeba* in hot springs of Mazandaran province in northern Iran. Twenty-four water samples were collected from all hot springs in Mazandaran province. After filtration through nitrocellulose membrane, samples were cultured on non-nutrient agar medium enriched with TYIS-33. The cultures were microscopically examined for the presence of *Acanthamoeba*. Positive cultures were analysed by polymerase chain reaction (PCR) and genotypes were determined by targeting the 18 S rRNA gene. The pathogenic potential of all positive isolates was identified using *thermotolerance* and osmotolerance tests. Eleven (47.8%) samples were positive for *Acanthamoeba*. Based on sequencing analysis, 100% of isolates belonged to the T4 genotype. Thermo- and osmo-tolerance tests showed that four (36.3%) *Acanthamoeba* strains were highly pathogenic. According to our research, the occurrence of *Acanthamoeba* in recreational hot springs could be a hazard for high risk persons. Posting warning signs and regular monitoring of these waters by health planners may therefore be useful for decreasing *Acanthamoeba* spp. infections.

Key words | *Acanthamoeba*, culture, genotype, hot spring, Iran, PCR

Samira Dodangeh
Elham Kialashaki
Ahmad Daryani
Mehdi Sharif
Shahabeddin Sarvi (corresponding author)
Yusef Dadi Moghaddam
Seyed Abdollah Hosseini
Toxoplasmosis Research Center,
Mazandaran University of Medical Science,
Sari,
Iran
E-mail: *shahabesarvi@yahoo.com*

Samira Dodangeh
Elham Kialashaki
Shahabeddin Sarvi
Yusef Dadi Moghaddam
Seyed Abdollah Hosseini
Student of Research Committee,
Mazandaran University of Medical Science,
Sari,
Iran

INTRODUCTION

Free-living amoebae (FLA) encompass a variety of pathogenic and non-pathogenic microorganisms. Among amoebae families, Acanthamoebidae, Vahlkampfiids and Techamoebidae are common pathogenic agents (Visvesvara *et al.* 2007).

Acanthamoeba spp. have two stages in their life-cycle: the trophozoite (which is active and divides by binary fission) (Siddiqui & Khan 2012) and the cyst, which includes cellulose in its cell wall and is resistant to environmentally adverse conditions (Dodangeh & Fakhar 2016). These FLA are usually harmless to humans, but in rare instances, can lead to severe diseases including amoebic keratitis (AK), granulomatous amoebic encephalitis (GAE), otitis, chronic sinusitis, cutaneous lesions and cutaneous ulcers (Marciano-Cabral & Cabral 2003). Most AK cases occur in patients with a history of wearing contact lenses and swimming prior to the onset of symptoms (Khan 2009). These amoebae are also a causative agent of GAE, which is a serious infection of the central nervous system (CNS) in immunocompromised individuals (Marciano-Cabral & Cabral 2003). *Acanthamoeba* spp. can act as natural vectors for pathogenic microorganisms such as yeasts, viruses,

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bacteria and Protozoa (Khan 2009). Medical professionals have become aware of this amoeba mostly because of an increased rate of *Acanthamoeba* infections (Seal 2003).

Acanthamoeba spp. have been clustered into three distinct morphological groups (I, II and III), on the basis of cyst morphology, which contain more than 24 nominal species (Visvesvara & Schuster 2008). To date, the *Acanthamoeba* genus, based on 18 S rRNA sequencing, is classified into 21 different genotypes (T1–T21) (Corsaro *et al.* 2017). In Iran, T4, T3, T2, T11, T13 and T15 genotypes of *Acanthamoeba* have been reported as the main cause of AK (Niyyati & Rezaeian 2015). During a 10-year survey on 142 AK patients, Rezaeian *et al.* (2007) reported that 49 (34.5%) were infected with *Acanthamoeba*. A review by Niyyati & Rezaeian (2015) concluded that environmental sources such as recreational soil sources, fresh water, tap water and hot springs have been identified as particular risk factors of AK infection. According to a systematic review, most studies of FLA prevalence in Iranian water sources have focused on ponds, taps and swimming pools, with a few conducted on hot springs (Saburi *et al.* 2017). People frequently use hot springs as a natural treatment choice for various common diseases such as skeletal disorders and arthritis (Yazdi *et al.* 2015). Therefore, owing to the presence of many hot springs in Mazandaran province, northern Iran, and the importance of *Acanthamoeba* as a life-threatening pathogen for humans, the present study was conducted to evaluate the presence of *Acanthamoeba* spp. in the hot springs of this province via two approaches, based on morphological and molecular characterization.

METHODS

Sampling location

Mazandaran province is situated within the latitude of 35° 47′–36° 35′ N and longitude of 50° 34′–54° 10′ E. It has an area of 23,833 km² and population of 3,283,582 (2015 census). The province is bordered clockwise by Russia (across the sea) and the provinces of Golestan, Semnan, Tehran, Alborz, Qazvin and Gilan (Mesgari *et al.* 2013). It has a moderate and subtropical climate during the year and an average temperature of 17 °C. This province annually attracts many tourists owing to the presence of recreational sites such as seaside, forest and hot springs.

Sampling and analysing

In this study, water samples were collected from all hot springs (a total of 24) in Mazandaran province between January and March 2017 (Figure 1). The hot springs are located in the towns of Ramsar, Tonekabon, Klardasht, Chalus, Nur, Amol and Babol, with the largest hot spring in Iran being located in Larijan, Amol. These waters emanating from the depths of the Earth are rich in sulphur, calcium, lithium and other minerals. These samples, collected from a depth of approximately 5–10 cm below the surface, were inserted into 500 mL sterile plastic containers and transferred to the Parasitology Laboratory in Mazandaran University of Medical Sciences, Sari, Iran. Physical parameters such as the temperature and pH of water were measured by a portable pH meter. In addition, information about the water types of each hot spring was obtained from the book *Hydrology* compiled by Ghafouri & Mortazavi (1978).

Filtration and cultivation

Approximately 400 mL of each sample was filtered using a nitrocellulose membrane (Millipore; Sigma-Aldrich) with a pore size of 0.45 μm. The filters were cut and placed onto 1.5% non-nutrient agar (NNA) medium covered with TYIS-33 and incubated aerobically at room temperature (Niyyati & Dodangeh 2015). The plates were checked daily up to one month after inoculation.

Microscopic examination and cloning

After incubation at room temperature for 3–5 days, the perimeter around the samples was cut out and transferred to fresh 1.5% NNA medium with lawns of TYIS-33. Amoebae were morphologically identified according to taxonomic criteria using an inverted microscope (Page 1988).

All positive plates were then cloned to obtain bacterium and fungus-free plates. A few trophozoites or cysts were transferred to a new culture agar, and replicates were made in order to obtain plates without contamination (bacterial and fungal).

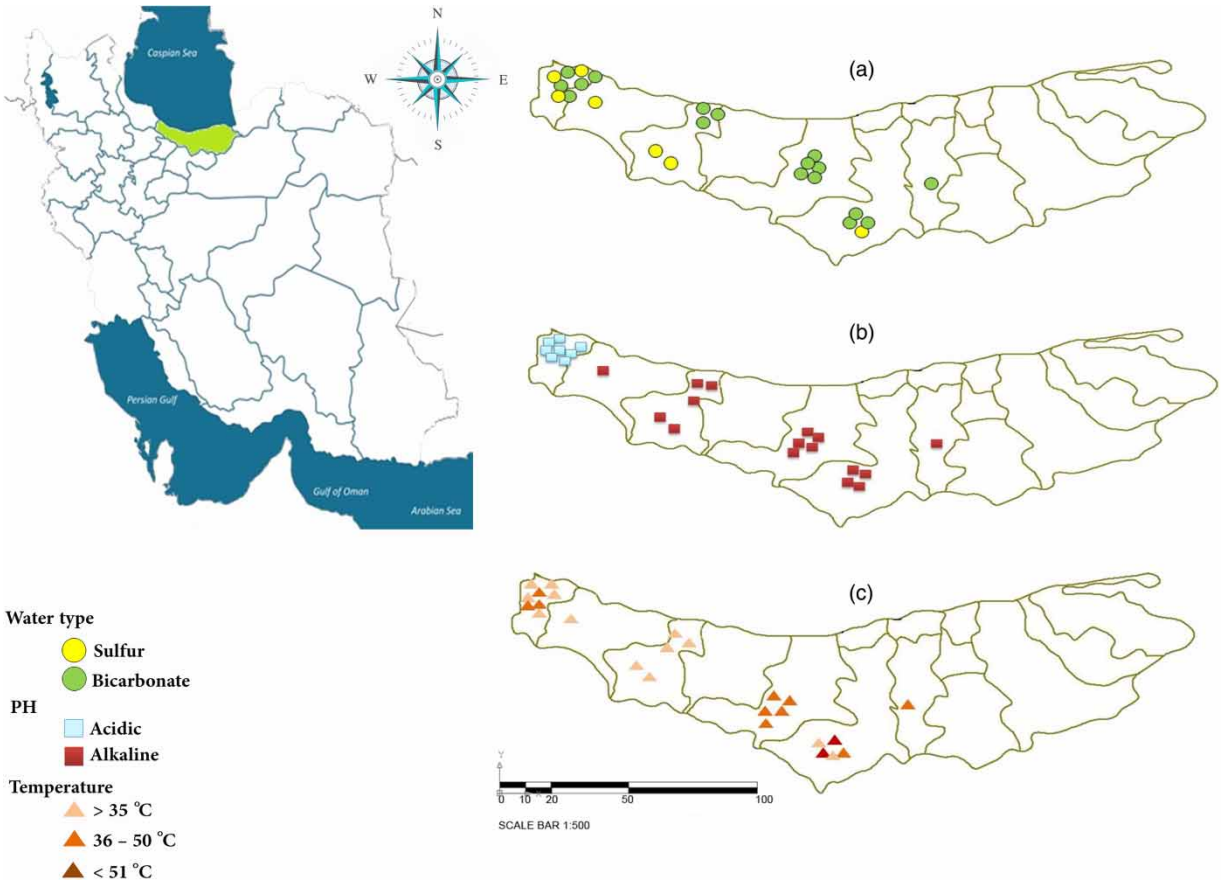

Figure 1 | Map showing the location and description of hot springs in Mazandaran province, Iran.

DNA extraction and PCR analysis

DNA extraction was carried out using the phenol–chloroform method described previously (Shokri *et al.* 2016). Amplification was performed targeting the hypervariable region of Diagnostic Fragment 3 (DF3) of 18 S rRNA (rDNA), using primers JDP1: 5′-GGCCCAGATCGTTTACCGTGAA-3′ and JDP2: 5′-TCTCACAAGCTGCTAGGGAGTCA-3 (Shokri *et al.* 2016).

Polymerase chain reaction (PCR) was carried out in a final volume of 25 μL containing 12.5 μL Ampliqone (Taq DNA Polymerase Master Mix RED, Denmark), 1 μL of each primer, 4 μL template DNA and 7.5 μL distilled water. PCR conditions were: initial denaturation for 1 min at 94 °C followed by 35 cycles of 94 °C for 35 s (denaturation), 56 °C for 45 s (annealing) and 72 °C for 10 min (extension). PCR products were subjected to electrophoresis on a 1.5% agarose gel stained with SYBR Green and visualized under an ultraviolet transilluminator.

Sequencing analysis

Purified PCR products were sequenced with an ABI 3130 automatic sequencer by Bioneer (Daejeon, South Korea) and nucleotide sequences were analysed using BLAST (http://www.ncbi.nlm.nih.gov/Blast). DF3 sequences obtained in this study were submitted to the GenBank database.

Pathogenic tests

Thermotolerance and osmotolerance tests were performed to assay the pathogenicity of the positive isolates (Niyyati *et al.* 2016). For the thermotolerance test, approximately 10^3 trophozoites were separately inoculated onto NNA medium and then each plate was incubated at two different temperatures (37 and 40 °C). For the osmotolerance assay, 10^3 trophozoites were cultivated in NNA medium with two concentrations of Mannitol (0.5 M and 1 M). For both

pathogenic tests, growth of amoebae was monitored at 24, 48 and 72 h by light microscopy.

RESULTS

The temperature and pH of the hot springs are shown in Table 1.

Of the 24 water samples collected from hot springs in northern Iran, 11 (47.8%) were positive for *Acanthamoeba* spp. based on morphological criteria of Page (1988). The *Acanthamoeba* trophozoites were identified by the presence of the acanthopodia and its cysts were characterized as being double walled; the ectocyst and endocyst.

These 11 isolates were cloned successfully (Figure 2). *Acanthamoeba* spp. were detected in all 11 of the extracted DNA by PCR, using the JDP primer pairs, which are specific for the *Acanthamoeba* genus.

Sequencing results for these isolates revealed that all of them were the T4 genotype. As shown in Table 2, the BLAST analysis of the sequences presented a high percentage of identity (98%–100%) and query coverage (91%–99%) in comparison with the deposited genes in the GenBank database. These strains possessed high levels of homology to *A. castellanii*. The recorded accession numbers are MG890617–9, MG890336, MG890338, MG890629, MG890631, MG906986–8 and MG891744.

The results of the tolerance assays are summarized in Table 2. A total of four out of 11 were able to grow at high temperatures (37 and 44 °C) and high osmolarity media (1 M). These isolates were considered as high-potential pathogenic *Acanthamoeba*.

Five isolates demonstrated fast growth at 37 °C and 0.5 M osmolarity, so are classified as low-potential pathogens. However, two isolates did not show osmotolerance to 0.5/1 M and thermotolerance to 37/44 °C (non-pathogenic).

Table 1 | Location and description of hot springs in Mazandaran province

City	Sampling site	Water type	pH	Temperature (°C)	*Acanthamoeba*
Ramsar	Ramak	Bicarbonate	Acidic	31	Positive
	Pol	Sulfur	Acidic	38	Positive
	No. 1	Bicarbonate	Acidic	42	Positive
	Kash and Sangboneh	Sulfur	Acidic	37	Positive
	Siah	Sulfur and radioactive	Acidic	20	Not found
	Madarshah	Bicarbonate	Acidic	35	Positive
	Sadatshahr	Sulfur	Acidic	35	Not found
	Garma	Sulfur	Acidic	35	Not found
Nur	Lavij 1	Sulfur	Alkaline	48	Not found
	Lavij 2	Sulfur	Alkaline	48	Not found
	Lavij 3	Sulfur	Alkaline	48	Not found
	Lavij 4	Sulfur	Alkaline	48	Not found
	Lavij 5	Sulfur	Alkaline	48	Not found
Tonekabon	Tonekabon	Bicarbonate	Alkaline	20	Positive
Chaloos	Kandova	Sulfur	Alkaline	30	Not found
	Dalir	Sulfur	Alkaline	31	Positive
	Allahjar	Sulfur	Alkaline	31	Positive
Klardasht	Telo	Bicarbonate	Alkaline	30	Positive
	Pishtork	Bicarbonate	Alkaline	30	Not found
Babol	Azrud	Sulfur	Alkaline	37	Not found
Strabakoo	Strabakoo	Sulphate	Alkaline	34	Positive
Amol	Ab ask	Bicarbonate	Alkaline	27	Positive
	Larijan Shahabbasi	Sulfur	Alkaline	65	Not found
	Larijan 2	Sulfur	Alkaline	65	Not found

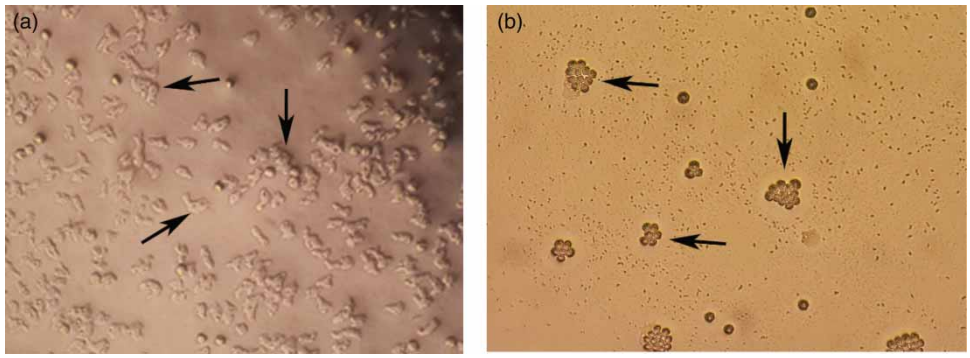

Figure 2 | Photograph of cloned *Acanthamoeba* T4 genotype in NNA. Trophozoites (a) and cysts (b); magnification: ×10; bars = 30 μm.

Table 2 | Data regarding the *Acanthamoeba* genotypes isolated from hot springs in Mazandaran province

Code	Sampling site	Temperature tolerance (37/44 °C)	Osmotolerance 0.5/1 m	Genotype	Max identity/query coverage (%)	Accession No.
KD1	Ramsar (Ramak)	+/+	+/+	T4	99/98	MG890617
KD2	Ramsar (Pol)	+/+	+/+	T4	98/91	MG890338
KD3	Ramsar (No. 1)	+/+	+/+	T4	99/96	MG890618
KD4	Ramsar (Kash and Sangboneh)	+/−	+/−	T4	99/95	MG906988
KD6	Ramsar (Madarshah)	+/−	+/−	T4	99/96	MG890619
KD14	Tonekabon	−/−	−/−	T4	100/99	MG906987
KD16	Chaloos (Dalir)	+/−	+/−	T4	99/95	MG890629
KD17	Chaloos (Allahjar)	+/−	+/−	T4	99/95	MG890336
KD18	Klardasht (Telo)	+/−	+/−	T4	99/95	MG890631
KD21	Strabakoo	+/+	+/+	T4	99/93	MG906986
KD22	Amol (Ab ask)	−/−	−/−	T4	99/94	MG891744

DISCUSSION

Acanthamoeba is one of the most commonly isolated FLAs, possessing a diverse distribution in environmental samples. The presence of a high percentage of *Acanthamoeba* has been documented in samples surveyed from human-related water habitats (Saburi *et al.* 2017). The presence of antibody titres in 80% of surveyed humans suggests that exposure to *Acanthamoeba* spp. is ubiquitous (Chappell *et al.* 2001).

Owing to their specific physical and chemical characteristics, natural hot springs are used all over the world for therapeutic purposes and bathing (Yazdi *et al.* 2015).

Mazandaran province in northern Iran has numerous hot springs in different cities (Figure 1) and attracts many tourists annually. Among the cities, Ramsar has received special attention owing to the presence of its recreational and therapeutic hot springs, and may therefore possess possible hazards of exposure to FLAs.

This study is the first survey regarding the frequency of *Acanthamoeba* genotypes in all hot springs of Mazandaran province. Some studies conducted in these areas have identified other waterborne pathogens such as *Balamuthia mandrillaris* and *Naegleria* spp. (Latifi *et al.* 2016, 2017).

The findings of this study using culture and PCR methods have demonstrated that a high percentage (47.8%) of hot springs in Mazandaran province were contaminated with *Acanthamoeba* spp.

In previous studies, the presence of *Acanthamoeba* spp. was reported in different surface water samples in

Mazandaran. For example, Mahmoudi *et al.* (2015) reported the presence of *Acanthamoeba* in 12/14 water sources, including river, sea and hot springs of this area. They reported four (two in Ramsar and two in Nur) out of six hot spring samples were contaminated with *Acanthamoeba* spp. Those results are somewhat similar to our results, although in our study, the hot springs present in Nur were not positive for *Acanthamoeba* spp. In addition, reports from other water sources of Mazandaran showed similar results to our findings. Shokri *et al.* (2016) isolated *Acanthamoeba* spp. in 55.8% of 77 samples from several parts of Sari city and its suburbs, based on morphological criteria and molecular methods.

This amoeba has been reported in different aquatic environments, such as drinking water, sea, surface water, rivers, creeks, ponds, swimming pools and hot springs, with a variety of prevalence detected (Saburi *et al.* 2017). For example, contamination of hot spring resorts with *Acanthamoeba* was estimated to be almost 16% in Swiss (Gianinazzi *et al.* 2010) and 21.2% (T1–T6 and T15) in southern Taiwan thermal springs (Huang & Hsu 2010). Solgi *et al.* (2012) reported the contamination by *Acanthamoeba* in hot springs in north-western Iran as 20%, with isolates belonging to T4 and T3.

All samples from hot springs contain mineral compounds, while their temperature range demonstrates the resistance of *Acanthamoeba* spp. to high temperature ranges (42 °C in the present study). The *in vitro* growth of an *Acanthamoeba* isolate at high temperature or at high osmotic stress can be relevant to virulence, since virulence is partly associated with an isolate's capacity to adapt and survive in the tissues of a mammalian host (Khan & Tareen 2003). However, the thermo- and osmo-tolerance tests are not sufficient for determination of pathogenicity of the collected *Acanthamoeba* isolates and hence *in vivo* tests are also needed to determine the pathogenic potential of the isolates.

In addition, as shown in Table 1, the *Acanthamoeba*-positive samples were observed in both alkaline ($n = 6$) and acidic ($n = 5$) waters. A study by Kao *et al.* (2012) demonstrated a significant association between pH and the presence of *Acanthamoeba* in thermal spring samples in southern Taiwan. The *Acanthamoeba*-positive samples demonstrated higher mean pH values compared with the *Acanthamoeba*-negative samples.

Our results demonstrate that *Acanthamoeba* species isolated from hot springs of the Mazandaran is limited to only one T4 genotype, which has certain properties that make it highly virulent. Several studies have shown T4 to be the most common keratitis-causing genotype in Iran and worldwide (Mirjalali *et al.* 2013). However, whether the ability of T4 isolates for causing keratitis is due to their high pathogenicity, high prevalence, or both, is somewhat unclear. Some researchers have hypothesized that the universal distribution and highly transmissible nature of this genotype could be a reason for their predominance in nature (Mirjalali *et al.* 2013). In this study, we found a high percentage of potentially pathogenic *Acanthamoeba* in hot springs, thus posing a risk to humans, particularly for immunocompromised patients and contact lens wearers.

In these environmental sources, *Acanthamoeba* primarily feed on bacteria, although algae, yeast, fungi, and other amoebae could also serve as sources of nutrition (Marciano-Cabral & Cabral 2003). The presence of bacteria in these areas is probably a major factor in stimulating excystment. Therefore, in future studies it would be useful to survey the contamination of hot springs with bacteria.

Finally, the knowledge of prevalence of this opportunistic protozoan in the environment may help clinicians to adequately manage the care of particularly susceptible patients. Thus, it may be useful to titre antibodies in immunocompromised patients. Hot springs need regular monitoring, especially during the summer when they are more used by tourists. In addition, posting of warning signs at recreational hot springs could be another option for preventing FLA infections.

CONCLUSION

The findings of our study confirm the presence of potentially pathogenic *Acanthamoeba* spp. in the hot springs of Mazandaran province. The only identified *Acanthamoeba* genotype in the hot springs was T4, which is associated with AK. Our findings emphasize the necessity for regular inspection of hot springs, in order to reduce health risks and to prevent infections caused by pathogenic strains in humans.

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