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### Original Article

## Isolation of Free-Living Amoebae from Sarein Hot Springs in Ardebil Province, Iran

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### ABSTRACT

**Background:** Free-living amoebae (FLA) are a group of ubiquitous protozoan, which are distributed in the natural and artificial environment sources. The main aim of the current study was to identify the presence of FLA in the recreational hot springs of Sarein in Ardebil Province of Iran.

**Methods:** Seven recreational hot springs were selected in Sarein City and 28 water samples (four from each hot spring) were collected using 500 ml sterile plastic bottles during three month. Filtration of water samples was performed, and culture was done in non-nutrient agar medium enriched with *Escherichia coli*. Identification of the FLA was based on morphological criteria of cysts and trophozoites. Genotype identification of *Acanthamoeba* positive samples were also performed using sequencing based method.

**Results:** Overall, 12 out of 28 (42.9%) samples were positive for FLA which *Acanthamoeba* and Vahlkampfiid amoebae were found in one (3.6%) and 11 (39.3%) samples, respectively. Sequence analysis of the single isolate of *Acanthamoeba* revealed potentially pathogenic T<sub>4</sub> genotype corresponding to *A. castellanii*.

**Conclusion:** Contamination of hot springs to FLA, such as *Acanthamoeba* T<sub>4</sub> genotype (*A. castellanii*) and Vahlkampfiid amoebae, could present a sanitary risk for high risk people, and health authorities must be aware of FLA presence.

**Keywords:** Hot spring, Free-living amoebae, Iran, *Acanthamoeba castellanii*, Vahlkampfiidae

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## Introduction

Free-living amoebae (FLA) include many genera with high distribution in natural and artificial environment, such as air, water, dust, and soil (1). Several genera of FLA have been concerned in medical sciences, such as *Acanthamoeba* spp., *Balamuthia*, and *Naegleria* (1-2). On the other hand, these FLA could act as a Potential pathogen leading to fatal disease in domestic animals and humans by invasion to central nerve system (CNS) and other organs, especially in those with higher risk (3-4). Amphizotic nature of FLA lead to their presence in different water types, such as fresh, coastal, sea, mineral waters, wastewater, swimming pools, and hot springs. High temperature of hot springs (37-45 °C) can support the growth and distribution of some potentially pathogenic thermophilic FLA (5). To date, *Acanthamoeba* spp., *Naegleria fowleri*, *Balamuthia mandrillaris*, and *Sappinia pedata* are known as causal agents for human disease (6-7).

*Acanthamoeba* is the most prevalent amoebae in the environmental sources, and this genus has been isolated from different water types (5, 7). To date, *Acanthamoeba* have been classified to 17 genotypes (T<sub>1</sub>-T<sub>17</sub>) based on the variable region of 18S rRNA gene (8-10). However, strains belong to the T<sub>4</sub> genotype are the most important causative agent in *Acanthamoeba*-related disease, such as amoebic keratitis (AK), and granulomatous encephalitis (9). Interestingly, most of AK cases showed a history of wearing contact lenses and water activity before the onset of disease (9). In Iran genotypes belonged to T<sub>2</sub>, T<sub>4</sub> and T<sub>6</sub> have been identified in stagnant waters thus far (11-12). However, there are no reports regarding the presence of *Acanthamoeba* spp. in hot springs in Iran.

Among many genera of Vahlkampfiidae, *Naegleria* and *Vahlkampfia* are of medical importance (1, 7, 13). These thermophilic amoebae have global distribution in water sources and can cause FLA-related disease, such as corneal, and brain involvement (1). *Naegleria fowleri* can cause primary amoebic meningoencephalitis (PAM) in young and healthy people with a history of water activity (7, 14-15).

It is worthy to mention that *Acanthamoeba* and Vahlkampfiidae can also act as a carrier of pathogenic bacteria, such as *Legionella*, *Pseudomonas*, and *Helicobacteria*, and this can lead to their expanded threat for human being (16-17).

Sarein is a town of Ardebil Province, which is located in the northwest of Iran, and it is famous for various hot springs and spas as recreation areas and health purposes (Fig.1). Indeed, local people and tourists enjoy water activity in this region through all months of year. The main goal of the current study was to identify the presence of FLA in the hot springs of Sarein town using culturing method and morphological key.

## Materials and Methods

### Sampling

During August, September, and October of 2010, 28 water samples were collected from seven hot springs of Sarein in Ardebil Province. Fig.1 shows the map of Ardebil Province and Sarein City (created by Arc GIS version 9.3). Four samples were collected from each water sources including two samples from margin, and two from center of the hot springs. Water temperature was measured using standard thermometer. Samples were transmitted to the Department of

Parasitology and Mycology, Tehran university of Medical Sciences, Tehran, Iran.

#### ***Filtration, cultivation, and cloning***

Samples were filtered using sterile membrane filters (Millipore, pore size 0.45  $\mu\text{m}$ ). Each filters were cultured on non-nutrient agar medium enriched with *Escherichia coli* (NNA medium), and incubation was performed at room temperature for one month. All mentioned plates surveyed daily under light microscope (Carl Zeiss Axiolab) with the magnification of 100X and 400X. Positive samples were then cloned in order to achieve a free-bacteria and fungi plates (12).

#### ***Identification of the cloned amoebae***

The criteria for identification of amoebae were based on morphology of cysts and trophozoites according to the Page, FC (1988) key (18). *Acanthamoeba* were detected based on double wall and wrinkled or wavy ectocyst and characteristic acantopodia of trophozoites. Molecular identification was performed for *Acanthamoeba* positive sample using primers JDP1-2 (JDP1 5'-GGCCCAGATCGTTTACCGTGAA-3' and JDP2 5'-TCTCACAAAGCTGCTAGGGAGTCA-3') (19). DNA extraction was performed using Phenol chloroform method according to our previous study (12) and PCR was achieved in 30  $\mu\text{l}$  Ampliqone (Taq DNA Polymerase Master Mix RED, Denmark) as a ready-made mixture. Twenty-five  $\mu\text{l}$  of Taq Master Mix were used with 5 ng template DNA, 0.1  $\mu\text{m}$  of each primer and distilled water. The thermal cycling conditions were an initial denaturing step of 94 °C for 1

min and 35 repetitions at 94 °C for 35 s, annealing step were 56 °C for 45 s, and 72 °C for 1 min. PCR products were electrophoresed on a 2% agarose gel stained with a solution of ethidium bromide and detected under UV light. Sequencing analysis was performed using BLASTn in the genbank database. Vahlkampfiid amoebae were detected according to spherical and smooth wall cysts and eruptive trophozoites in plates (18).

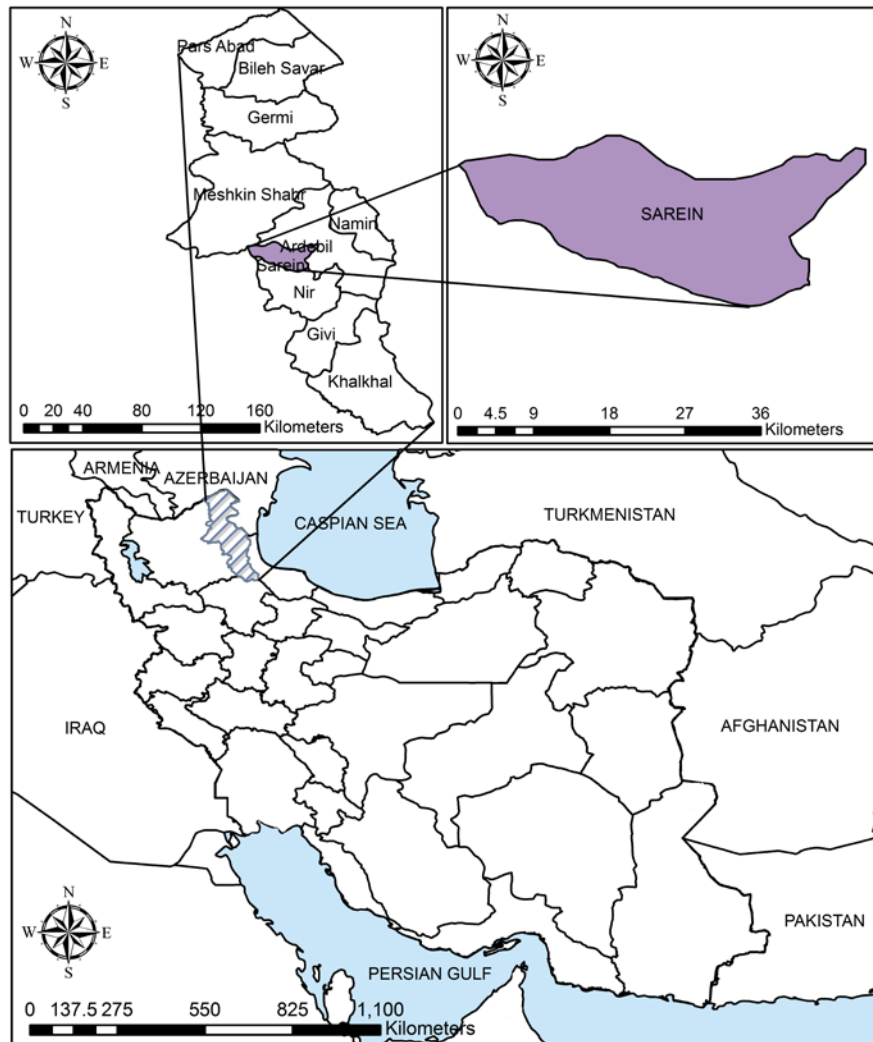
## **Results**

Overall, 12 out of 28 (42.9%) samples were positive for outgrowth of FLA (Table 1). *Acanthamoeba castellanii* and Vahlkampfiid amoebae were found in one (3.6%) and 11 (39.3%) samples, respectively (Fig.2, 3). Only one *A. castellanii* strain was isolated in Pehenloo hot spring from the margin site, which was further confirmed using PCR based method. Sequencing analysis of this isolate in the GenBank database was revealed a 100% similarity with the T4 genotype corresponding to *A. castellanii* species. Vahlkampfiid amoebae were detected according to spherical and smooth wall cysts and eruptive trophozoites (Fig. 3). Contamination of hot springs to Vahlkampfiid amoebae where detected in Pehenloo, Ghavmishgholi, General, Abdarmanieh Sabalan, Sarisuei, Beshbajilar, and Ghahvesuei. The mean temperature of surveyed hot springs was 43.6°C (Table 1). Both collected samples of margin and center of the springs were positive.

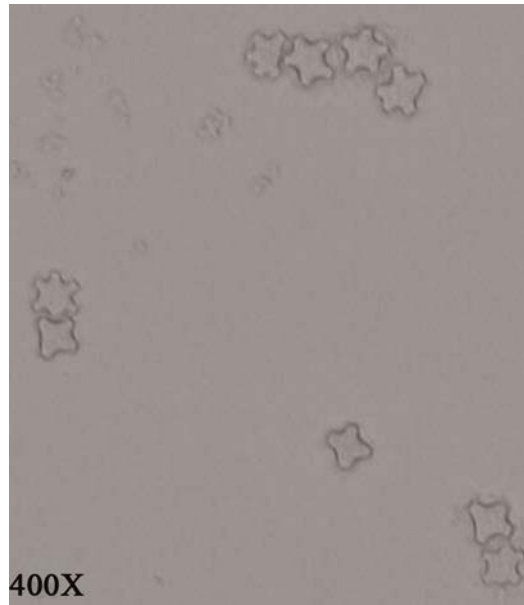
**Table 1:** Frequency of free-living amoebae in Sarein Hot Springs, and Samples Sites

Sampling Site	Temp (°C)	No. of Samples			Total Samples	<i>Acanthamoeba castellanii</i>	Vahlkampfiid amoebae
		Oct	Sep	Aug			
Pehenloo	43	2	1	1	4	1	2
General	43	2	1	1	4	-	2
Ghavamishgholi	46	2	1	1	4	-	1
Abdarmanieh	41	2	1	1	4	-	1
Sabalan							
Sarisuei	46	2	1	1	4	-	2
Beshbajilar	41	2	1	1	4	-	2
Ghahvesuei	43	2	1	1	4	-	1
Total	43.6*	14	7	7	28	1	11

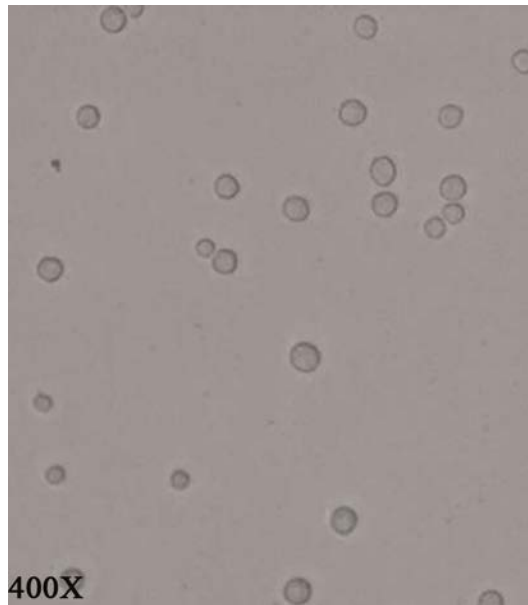
\*Mean temperature of 7 hot springs



**Fig. 1:** Map of the Ardebil Province and Sarein city, Iran (Created by Arc GIS version 9.3)



**Fig. 2:** *Acanthamoeba castellanii* cysts (400 X)



**Fig. 3:** Vahlkampfiidae cysts (400 X)

## Discussion

This study is the first survey regarding the presence of FLA in hot springs of Iran. To date, hot springs are famous for health purposes and traditional treatment option, worldwide. Sarein in northwest of Iran con-

tain numerous hot springs and this region attract many tourists annually.

The results of the present study revealed, hot springs of Sarein were contaminated with opportunistic FLA, such as *Acanthamoeba*,

and Vahlkampfiid amoebae, and this may lead to severe disease in high-risk people, such as contact lens wearers. This is in support with researches conducted worldwide (15, 17, 20-22). In a study in Spain, 12 natural hot springs examined in which 8 springs were contaminated to different taxa including *Acanthamoeba* (3) and Vahlkampfiid amoebae (7, 20). In another survey in Thailand, 28 out of 69 samples were found positive for FLA, which 13.0% (9/69) were *Acanthamoeba*, and 34.8% (24/69) were *Naegleria* (5). Moreover, in another study in Thailand, 56 samples were collected from 28 natural water resources that six samples were *Naegleria* spp., 3 samples were *Acanthamoeba*, 5 were mixed, and 2 samples were unidentified (22).

Our results confirmed that *Acanthamoeba* is more sensitive to growth in high temperature in contrast to Vahlkampfiid amoebae which hot water may lead to the favorable condition for their proliferation. This is in support with other studies, which showed higher isolation of Vahlkampfiids compared to *Acanthamoeba* in hot springs (5, 22). More researches are needed for identification of different vahlkamfiids in Iran using molecular based methods.

Another interesting finding of the present study was the isolation of potentially pathogenic *A. castellanii* belonged to the T<sub>4</sub> genotype. T<sub>4</sub> strains have been proved to contain certain properties that render them to be more virulent (11). T<sub>4</sub> genotype was also a predominant type found in AK patients in Iran (12). In another study by Rezaeian et al, 354 samples of water and soil were collected from rivers and lakes of Parishan and Dashte Arzhan in Kazerun city. They found 10 cases of FLA based on morphological criteria (2). Furthermore, the number of AK cases, which have been diagnosed up to 2004, were more than 3000 cases worldwide and more than 70 percent were contact lens wearers (23). In Iran, 45 AK cases have been

diagnosis during 1995-2005 in School of Public Health at Tehran University of Medical Sciences (24).

In conclusion, contamination of hot springs to FLA, such as *Acanthamoeba* and Vahlkampfiid amoebae, could present a sanitary risk for high-risk people, and health authorities must be aware of FLA presence. Education to high-risk people, and improved disinfection methods must apply for preventing FLA-related disease in this region.

## Acknowledgments

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## Erratum

### **The Problem of Mixing up of *Leishmania* Isolates in the Laboratory: Suggestion of ITS1 Gene Sequencing for Verification of Species (Iranian J Parasitol: Vol. 6, No.1, 2011, pp. 41-48)**

There was an error in a paper published recently in **Iranian J Parasitol**: Vol. 6, No.1, 2011, pp. 41-48 entitled "The Problem of Mixing up of *Leishmania* Isolates in the Laboratory: Suggestion of ITS1 Gene Sequencing for Verification of Species".

The error is the international code of the parasite No. 1 in the Table 1 in Page 44. The incorrect form is: "MHOM/IR/75/ER" and the correct form is "MRHO/IR/75/ER". In other words, "HOM" should be replaced by "RHO".

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