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Original Article

Molecular Identification of Free-Living Amoebae (*Naegleria* spp., *Acanthamoeba* spp. and *Vermamoeba* spp.) Isolated from Unimproved Hot Springs, Guilan Province, Northern Iran

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Abstract

Background: This study was conducted to determine the presence and molecular identify of *Acanthamoeba*, *Naegleria* and *Vermamoeba* in unimproved hot springs.

Methods: From Jul to Aug 2017, 54 water samples were collected from hot springs in different parts of the Guilan Province, North Iran. For the isolation of *Acanthamoeba*, *Naegleria* and *Vermamoeba* approximately 500 ml of the water samples were filtered through a cellulose nitrate membrane with a pore size of 0.45 μ m. The filter was transferred onto non-nutrient agar plates seeded with Gram-negative bacteria (*Escherichia coli*) as a food source. The morphological key of page was used to identify free-living amoebae (FLA) using an inverted microscope, PCR amplification targeting specific genes for each genus and sequencing determined frequent species and genotypes base on NCBI database.

Results: Fifteen of the 54 samples were positive by culture and/or PCR for *Acanthamoeba* and other FLA from unimproved hot springs. By sequencing the positive isolates, the strains were shown to belong to *Acanthamoeba castellanii* (12 case isolates belonged to T4 genotype), 4 cases of *V. vermiformis*, and 3 cases of *N. australiensis*, 2 cases of *N. pagei* and 1 cases of *N. gruberi*.

Conclusion: Although FLA-mediated illnesses are not as high as in environmental distribution, but because of a poor prognosis, more investigations about FLA distribution in hot springs is critical. Hot spring may enhance exposure of the amoebae in individuals. Hence, more attention to unimproved hot springs is needed to prevent free-living amoebae mediated diseases.

Introduction

Free-living amoebae (FLA) as amphizoic amoebae are a group of parasitic protozoa with the growth abilities in different natural environments such as soil and water. In appropriate conditions, they are pathogenic in animals and humans (1). FLA include many genera which cause serious diseases such as cutaneous ulcers, sight threatening keratitis and fatal encephalitis.

Acanthamoeba spp., *Naegleria fowleri* and *Balamuthia mandrillaris* are the most commonly reported causes in the world (2). Other genera in this group, including *Sappinia diploidea*, *Vermamoeba* and *Vahlkampfia* mix infection with other FLA could also lead to severe diseases with a lower incidence around the world (3-5). *Acanthamoeba* strains, especially ones that are potentially pathogenic, can tolerate extremes of temperature, osmolarity, and pH (6). *Vahlkampfia* and *Naegleria* genera are commonly found in warm freshwater.

N. australiensis and *N. italica* can cause infection in experimental animals but *N. fowleri* is able to infect animals and humans with possible life threatening contamination (7, 8). *Acanthamoeba* spp. *Vahlkampfiids* and *B. mandrillaris* can cause serious infections in humans (1). In addition to *Vermamoeba* have as a suitable hosts for pathogenic microorganisms such as *Legionella pneumophila* (9, 10). Other diseases related to FLA are *Vermamoeba* keratitis, and *Vahlkampfia* keratitis (3, 5, 11).

Hot springs are highly regarded for their therapeutic effects and how much of their use in Iran, both as a therapeutically use and as a tourist attraction, is growing; which will increase the chance of exposure to these amoebae (12-14). Guilan Province is a tourist areas of Iran which plays host to millions of Iranian and foreign travelers annually, and one of the tourist attractions of in this province is its hot springs (15). The presence of FLA in the hot springs of the provinces bordering Guilan has been confirmed (14, 16). Parasitological meth-

ods only recognize parasite contamination however, it is not possible to identify the exact species of parasite involved (17).

Hence, we aimed to survey the incidence of waterborne FLA belonging to *Vermamoeba*, *Naegleria* spp. and *Acanthamoeba* spp. isolated from unimproved therapeutic hot springs of Guilan Province using morphological and sequence based methods.

Materials and Methods

Geographical area of study

Guilan Provinces lies along the Caspian Sea. It has a plenty of annual rainfall, humid temperate weather and is known for its mild, moderate and Mediterranean-like climate (15) (Fig. 1).

Sampling

This cross sectional study (Code of ethics: IR.AJUMS.REC.1396.501) was performed from July to August 2017. To run this study, 54 water samples were collected from hot springs in different parts of the Guilan Province, North Iran (Fig. 1). Six hot springs in Guilan, North of Iran were included in this study and nine samples per site (reservoir-margin-center) were obtained. Samples were collected from the surface of the waters (<5 cm below) (14).

Filtration, cultivation and cloning

Approximately 500 ml water samples were filtered using cellulose nitrate membranes with a pore size of 0.45 µm. Filters were transferred to non-nutrient agar (NNA) plates seeded with *Escherichia coli* as a food source (14, 15). The morphological key of page was use for the identification FLA using an inverted microscope. Cloning of the suspected amoebae was performed using culture replicates method (11, 18).

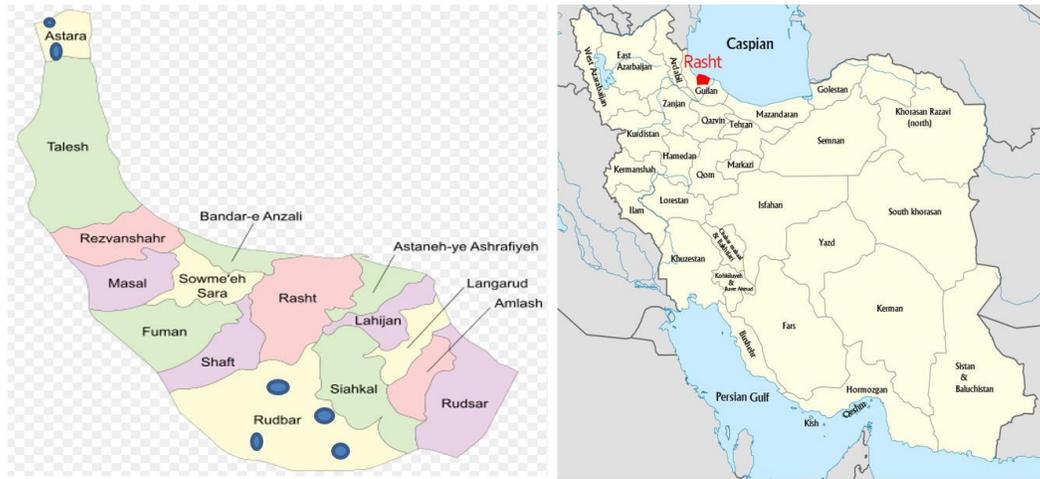


Fig. 1: Map of the investigated Guilan greater area (left) and its location in Iran (right)
The sampling points are indicated by blue circles (●)

DNA extraction, PCR analysis and Sequencing

DNA was extracted using GeNet Bio kit, according to manufacturer's instructions (GeNet Bio, South Korea). Four sets of primers were used in order to detect various FLA shown in Table 1. To carry out the PCR reaction, 25 ml of red master mix (Denmark) was combined with DNA (10 ng), 0.1 μM of each primer and distilled water. The cycling condition was set as pre-denaturation step for 3 min

at 94 °C, followed by 35 repetitions at 94 °C for 35 sec, annealing steps were at 56 °C, 56 °C, 56 °C and 58 °C for 1 min (for *Acanthamoeba*, *Vahlkampfiids*, *N. fowleri* and *Vermamoeba*, respectively), and 72 °C for 1 min. All sequences were edited manually and analyzed with reference sequences by Chromas software program. The sequences were submitted to gene bank under following accession numbers: MH347242-MH347263.

Table 1: Primers used in this study

FLA ¹	Primer sequence	Reference
<i>Acanthamoeba</i> spp.	JDP15'-GGCCCAGATCGTTTACCGTGAA-3'	19
	JDP2 5'-TCTACAAGCTGCTAGGGAGTCA-3'	
<i>Vahlkampfiids</i>	ITS1 F5'-GAACCTGCGTAGGGATCATTI- 3'	2
	ITS2 R 5'TTTCTTTTCCTCCCCITATTA-3'	
<i>N. fowleri</i> ²	F5'-GTGAAAACCTTTTTTCCATTTACA-3'	14
	R5'-AAATAAAAAGATTGACCATTTGAAA-3'	
<i>Vermamoeba</i>	Hv1227F 5'-TTA CGA GGT CAG GAC ACTGT-3'	2
	Hv1728R 5'-GAC CAT CCG GAG TTC TCG-3'	

1. Free living amoebae: FLA 2. *Naegleria fowleri*

Results

Of 54 water samples, 15 (27.7%) cases were positive for outgrowth of free-living amoebae. Indeed, the positive samples included 7 cases

of *Acanthamoeba*, 2 cases of *Vahlkampfiids*, 1 case of *Vermamoeba*, 2 mixed cases of *Acanthamoeba*, *Vahlkampfiids* and *Vermamoeba*, 2 mixed cases of *Acanthamoeba* and *Vahlkampfiids*, and 1 mixed case of *Acanthamoeba* and

Vermamoeba. Accordingly, *Acanthamoeba* were found in 12 (54.5%) samples as the most prevalent amoebae in the tested samples (Tables 1, 2).

For morphological determination of *Acanthamoeba castellanii*, the following characters were regarded; a double walled with a smooth wrinkled outer cyst wall and stellate endocyst (Figs. 2 and 3) for *Vahlkampffids* round cysts with smooth wall and *V. vermiformis* round cysts with smooth wall but smaller *Vahlkampffids* (Fig. 3 and 4).

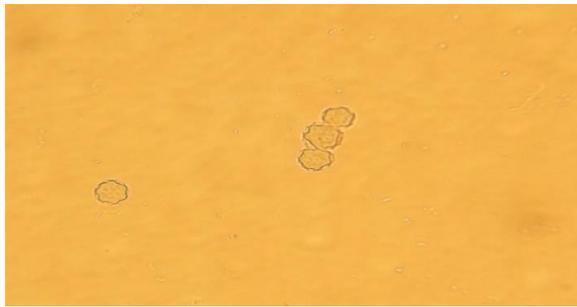


Fig. 2: *Acanthamoeba castellanii* cysts (400 X)



Fig. 3: Mixed *Vahlkampffids* cysts (1) (400 X) and *Acanthamoeba castellanii* trophozoite (2) (400 X)

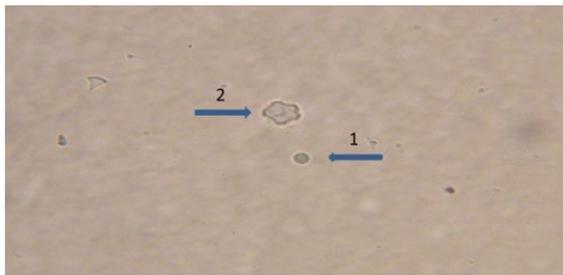


Fig. 4: Mixed *Vermamoeba vermiformis* cysts (1) (400 X) and *Acanthamoeba castellanii* cysts (2) (400 X)

In electrophoresis of PCR products, twelve cases of *Acanthamoeba* demonstrated an approximately 500 bp band (Fig. 5). Sequencing analysis of 12 positive cases showed T4 (100%) genotypes with homology analysis of NCBI website revealing 95%-100% similarity.

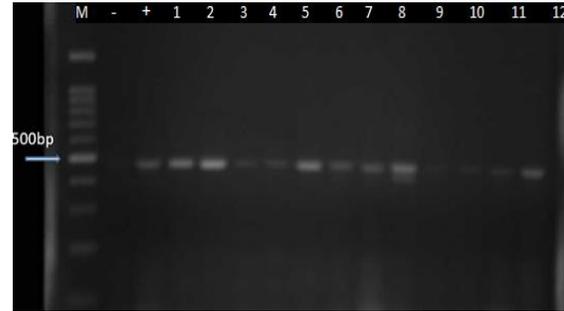


Fig. 5: PCR amplification of the isolated *Acanthamoeba* strains. M marker, -=Neg Control, +=Pos Control, 1-12 samples

Taken together, 6 cases of *Vahlkampffids* (2 single and 4 mixed with *Acanthamoeba*) and 4 cases of *Vermamoeba* (1 single and 3 mixed with *Acanthamoeba*) were determined. Six cases of *Vahlkampffids* exhibited an approximately 400 bp band, and 4 samples of *Vermamoeba* demonstrated a nearly 500bp band during electrophoresis (Figs. 6 and 7).

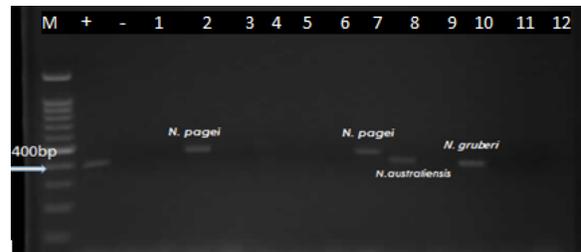


Fig. 6: PCR amplification of the isolated *naegleria* strains. M marker, -=Neg Control, +=Pos Control, (2, 7,8 and 10 samples)

Water PH and temperature were assessed in situ by using a portable pH meter (Digital tester DMT-20), so that these parameters of hot springs were respectively measured as 23.4%-31.6 °C and 6.5-7.2 (Table 3).

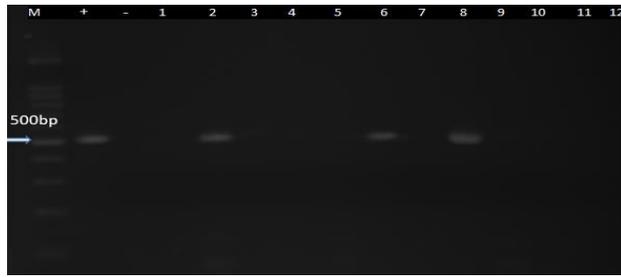


Fig. 7: PCR amplification of the isolated *Vermamoeba*. M marker, - = Neg Control, + = Pos Control, (2,6 and 8 samples)

Moreover, sequencing analysis revealed 95%-100 % similarity with *Vahlkampfiids* and *V. vermiformis*. Accession numbers of nucleotide sequences were deposited in the GenBank database, and have been demonstrated in Table 3. Moreover, six hot springs, 83.4% (n=5), 66.6% (n=4) and 50% (n=3) were respectively positive for *Acanthamoeba*, *Vahlkampfiids*, and *Vermamoeba* amoebae.

Table 2: Data regarding isolated of free-living amoebae in Guilan Hot Springs, and Samples Sites

City	Sam-pling Site	No. of Samples			Num-ber/ positive samples	Acan-thamoeba	Vahlkam pfid	Ver-ma-moeba	Mixed Acan-thamoeba, Vahlkampfiid and Ver-mamoeba	Mixed Acan-thamoeba and Vahlkampfiid	Mixed Acan-thamoeba and Ver-mamoeba
Roud bar	Kolour	3	3	3	9/2	1	1	0	0	0	0
	Louye	3	3	3	9/4	2	0	0	1	1	0
	Mastkhor	3	3	3	9/4	3	0	0	0	1	0
Astara	Kalashatar	3	3	3	9/2	1	0	0	0	0	1
	Alidashi	3	3	3	9/3	0	1	1	1	0	0
	Kou-tekoume	3	3	3	9/0	0	0	0	0	0	0
Total	6	1	1	1	54/15(27.7%)	7	2	1	2	2	1

R: Reservoir C: Center M: Margin

Discussion

The present study is the first study on unimproved hot springs of Guilan Province, Northern Iran to determine the pathogenic free-living amoeba via molecular methods. In this investigation waterborne free living amoebae belonging to the *Acanthamoeba* T4 genotype, *Naegleria* (*N. pagei*, *N. australiensis* and *N. gruberi*) and *Vermamoeba vermiformis* were found in the unimproved hot springs of Guilan Province, Northern Iran and the present study is the second report of *N. gruberi* in the country. *Acanthamoeba* was detected in surface water of Guilan, previously (15).

No significant differences were shown between pH value (and temperature) and the

presence/absence of *Acanthamoeba*, *Naegleria*, *Vermamoeba*. In previous studies, the T4 genotype was reported to be isolated from samples such as soil, hospital wards, surface waters, recreational water areas, dust sources and also hot springs in Iran (13, 19-23). In contrast with results reported by other studies that reported T15 and T3 as predominate genotypes in waters surveyed (11, 24), the founding of PCR analysis and sequencing in the present work confirmed that the T4 genotype was the predominant type (Table 3).

Among the 47 known species of *Naegleria*, only *N. fowleri* has been reported to be pathogenic for human (8). An investigation on hot springs sources in Iran reported an increased occurrence of *Naegleria* genus in the tested

samples (14). The pathogenic *N. fowleri* was not found in this study. To our knowledge, so far, there is no report on the presence of

pathogenic *N. fowleri* in environmental sources of Iran.

Table 3: Data of the free living amoebae from unimproved hot springs, Guilan Province, Iran

Name Hot Spring	Isolate code	Morphology	PH	Temperature °C	PCR (JDPL2)	PCR (ITS1, 2)	PCR for <i>N. fowleri</i>	PCR (Hv1227F, Hv1728R)	Sequencing	Accession Number
Kolour	RN2	<i>Acanthamoeba</i>	7.1	31.6*	+	-	-	-	T4 genotype	MH347242
	RN3	<i>Vahlkamfiiid</i>	6.8	31.2	-	+	-	-	<i>N. pagei</i>	MH347254
Louye	RN14	<i>Acanthamoeba</i>	6.5*	30.3	+	-	-	-	T4 genotype	MH347243
	RN16	<i>Acanthamoeba</i>	7	30	+	-	-	-	T4 genotype	MH347244
	RN17	<i>Acanthamoeba, Vahlkamfiiid</i>	6.8	30.5	+	+	-	+	T4 genotype	MH347245
		<i>Vermamoeba</i>							<i>N. Pagei</i>	MH347255
Mastkhor	RN18	<i>Acanthamoeba, Vahlkamfiiid</i>	7.1	23.4*	+	+	-	-	<i>V. vermiformis</i>	MH347260
	RN20	<i>Acanthamoeba</i>	6.6	23.6	+	-	-	-	T4 genotype	MH347246
									<i>N. gruberi</i>	MH347256
	RN21	<i>Acanthamoeba, Vahlkamfiiid</i>	6.8	23.4	+	+	-	-	T4 genotype	MH347247
									<i>N. australiensis</i>	MH347248
	Kalashtar	RN22	<i>Acanthamoeba</i>	7.2*	30.6	+	-	-	-	T4 genotype
RN23		<i>Acanthamoeba</i>	6.6	30.1	+	-	-	-	T4 genotype	MH347250
RN30		<i>Acanthamoeba, Vermamoeba</i>	6.8	27.6	+	-	-	+	T4 genotype	MH347251
	<i>V. vermiformis</i>								MH347252	
Alidashi	AN43	<i>Acanthamoeba, Vahlkamfiiid</i>	6.6	27.8	+	+	-	+	T4 genotype	MH347261
									<i>N. australiensis</i>	MH347253
	AN44	<i>Vahlkamfiiid</i>	7	30.4	-	+	-	-	<i>N. australiensis</i>	MH347258
	AN45	<i>Vermamoeba</i>	7.2	28.9	-	-	-	+	<i>V. vermiformis</i>	MH347262

* = maximum and minimum water PH and temperature

However, a clinical case of *N. fowleri* has been reported in the country (25).

In the present study, *N. australiensis* was the most prevalent species, which can be pathogenic to mouse (26). *V. vermiformis* was also one of the most detected FLA, based on molecular assays (Table 3). A case of *Vermamoeba* keratitis and a case of mix infection of *V. vermiformis* and *Acanthamoeba* were reported during previous studies (4, 5, 27).

To prevent infection and diseases related to free-living amoebae, hot springs should be periodically checked, in particular, during the summer season, when these surface water are

used by thousands of tourists (28). The disease could originate to possess a seasonal mode of frequency in the region and serious monitoring for proper preparation against the disease should be in place (29).

Conclusion

Although FLA-mediated illnesses is not as high as in their environmental distribution, because of a poor diagnosis, more investigations about FLA distribution in hot springs is critical. Hot spring may enhance exposure of the amoebae to individuals. Hence, more attention

to unimproved hot springs is needed to prevent free-living amoebae mediated diseases.

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Conflict of interests

All the authors declare that they have no conflict of interest.

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