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Frequency and genetic diversity of *Acanthamoeba* spp. free living Amoeba in water sources of Urmia, North west Iran

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Abstract

***Background & Aims*:** Free living amoeba that they can cause important diseases such as keratitis and meningoencephalitis, being studied more precisely in the world. In Iran, many studies have been carried out in most parts of the country or are under investigation. Becaus previously no study performed about this parasite in West Azerbaijan (North West Iran), the aim of this study was determination of frequency and genotype of *Acanthamoeba* spp. in water sources.

***Materials and Methods*:** A total of 60 water samples were collected from surface and plumbing waters from five regions of Urmia. Samples after filtration cultured in non-nutrient medium in 30◦ C. and amoeba harvested and DNA extracted. PCR in 18SrDNA fragment performed using primers JDP2 and JDP1, and 11 pruducts sent to sequencing. Results analyzed by bioinformatics software's and submmited in Genbank.

Results: Of the 60 superficial and plumbed water samples, 21 samples were positive, of which four were plumbed water and 17 of them were surface water. Out of the twenty-one positive cases, 10 cases were confirmed in validated centers in terms of gene sequencing. Of the ten cases, one was a T2 genotype and nine were T4.

***Discussion*:** Studies in other parts of the country show that the dominant genotype in Iran is T4, and the frequency and of genotypes of *Acanthamoeba*. Spp in Urmia also partially relates to the parts of country.

***Keywords*:** Frequency, *Acanthamoeba*, spp, Iran

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Introduction

Free-living amoeba belong a large group of amoeba that have facultative life in humans and animals. Three important free-living amoeba, including the genus Negleria, acanthamoeba, and Hartmanella. (1-3).

Acanthamoeba spp. include a wide range of free-living amoeba that live in fresh or sweet waters, moist

soils, corrosive plants, and even in the feces of animals. These amoeba have been known since 1959, and the first human case was reported in 1965. *Acanthomoba* and Negleria, through entering the upper respiratory tract and reaching the CNS, cause meningoencephalitis, so contaminated water is one of the most important transmission routes (4).

Acanthomoba spp. is also a causative agent of Granulomatous amoebic encephalitis (GAE), chronic skin granulomatous and osteomyelitis, which is related to immune system weakness (3, 4). It also causes keratitis and corneal ocular lesions in healthy people, which are most commonly seen in users of soft contact lenses in the eye (5). These amoebae are also said to be carriers of some pathogenic bacteria such as *Legionella*. In the other hand bacteria live as endosymbiosis and amoeba plays a major role in the epidemiology and transmission of dangerous diseases caused by *Legionella* (6, 7).

The classification of *Acanthomoba* spp. based on morphology is always difficult. Morphologically, the size of the cyst and the structure of the *Acanthomoba* spp. are divided into three groups of I,II and III groups. Other studies have provided classifications based on isoenzymic patterns, physiological characteristics, immunological responses, or mitochondrial DNA polymorphisms.The molecular diversity of *Acanthomoba* spp is high, and it seems that through the molecular methods of classification, the sequence of the rRNA gene is more successful and more important (8). By 2001, based on the sequence of 18 srRNAs, 14 types have been identified, which is most importantly the T4 genotype, which includes many strains that cause keratitis and sometimes granulomatous encephalitis (9).

Recent studies have shown that there is a wide variety of *Acanthomoba* spp. due to the characteristics of resistance to biocides that are present in the acanthomoba cysts (3). Genetic diversity and identification can improve the treatment and prognosis of the acanthamobiasis.

For various reasons such as the frequency of amoeba in nature, especially in aquatic resources around human life, heat and chlorine resistance, the ability to survive in the range of PH 4 to 12, as well as the lower profile of the medical and paraeclinic community and the lack of diagnosis as a disease, awareness of the prevalence and the genotype of this parasite will be very important. Prevalence of *Acanthomoba* spp. In water sources which has received a different percentage (2). For example, in a study by sabouri et al. In 2017, the estimated prevalence was obtained as 36% among 2430 samples

However, there is no detailed information on the frequency and genotypic variation of free living amoeba in North West Iran. Therefore, the aim of this study was to determine the frequency of *Acanthomoba* spp. and its genetic diversity in Urmia city in order to provide necessary information to the medical community, prevention and accurate diagnoses.

Martials & Methods

In this descriptive cross sectional study 30 samples of surface water, including parks, squares and rivers, and 30 samples of plumbing water from five parts of the city of Urmia in 2017-2018 were taken and it was kept at 4 ° C until culture. Samples filtered with cellulose nitrate membrane of 0.45 in diameter and cultured in a non-nutriant medium 1.5% with inactive *E.coli* and saline at 30 ° C. The plates were checked one Month every day for growing of cyst or trophozoite (10-11).

Cells were washed with phosphate saline buffer with PH7.4 three times for 5 minutes at 500 rpm centrifugation. Suspension was placed in a lysis buffer (50mM NACL, 10mM EDTA, 50mM TRIS-HCL, PH 8) five times in a freeze-thawing and melting cycle and the cells were lysed at 55 ° C for one hour, with 0.25 mg / ml protein K, then the DNA was purified by phenol chloroform (SAMBROOK ETAL1989) and DNG plutonium (Sinagene Tehran, Iran).

The primer pair (JDP1-JDP2) for the amplification of the 423 to 551bp fragment refers to the 18SrDNA specific *Acanthomoba* spp., (12) as JDP1 (5-GGCCCAGATCGTTTACCGTGAA) and JDP2 (5-TCTCACAAGCTGCTAGGGGAGTCA).

For PCR in a volume of 30 µl containing 1.25 µg TAG DNA Polymerase Sina gene, 2 µl DNA template, 0.3 µm DNTP, 20 µmol of each primer, 1.5 µg

MgCl2.10 µm of PCR buffer, a 3-min incubation at 94 ° C, 32 cycles of prolongation (denaturing at 94 degrees for 45 seconds, anealing 55 degrees for 35 seconds, and extention for 72 minutes for one minute) and elongation was carried out at a temperature of 72 ° for ten minutes. The final product was electrophoresed with agarose gel and, stained with ethidium bromide, 0.5 mg / ml and it was observed by UV Transiluminator.

Also the product was purified to determine sequence and nucleotide sequences. The obtained sequences analyzed in Mega and Colostal W softwares and new sequences were recorded in the World Bank gene. The results were analyzed by SPSS software ver 16 using T-Test to compare the results.

Results

After four weeks of cultivation from 60 water samples, a total of 21 samples were positive and 39 plates were negative. Of the 30 surface water samples, 17 samples of surface water were positive (28.33%).Of the 39 negative plates, 26 were plumbing samples and 13 were surface water samples. Of the 30 samples collected from urban refined waters from different parts of Urmia, 4 samples (6.66%) were positive.

The city was divided into 5 regions and the results showed that the highest amount of contamination in surface waters was in the east of Urmia with 5 cases (16.66%).

Also, in refined water collected from different urban areas of Urmia, the highest frequency was related to the center with 3 cases (10%) of contamination.

The frequency of *Acanthomoba* spp. in different seasons was studied and results showed that in surface waters the highest frequency was related to autumn (20%) and in refined water with 13.33% in spring and autumn respectively (Table 3).

All positive samples were tested with molecular method using PCR and the special primer jdp1 and jdp2, the band was 500 bp. After final electrophoresis, 11 samples randomly selected with sharp bands were sent for sequencing analysis that a total of 10 positive sequence samples were reported.

The multiple sequence alignment was done in Mega10 software and online software(https://www.genome.jp/tools-bin/clustalw) and Results compared between the obtained sequences. Phylogenetic trees were generated by comparing submitted sequences with reference sequences in GeneBank. The sequences were highly homologous with few differences, corresponding to punctual base substitution. Phylogenetic analyses using character method like Maximum Parsimony showed that the topology is similar among the trees obtained with significant bootstrap support for the clades. (Figure 1).

The sequenced fragments from 10 randomly selected submitted in the GenBank database with accession numbers: LC426996, LC426997, LC426998, LC426999, LC427000, LC427001, LC427002, LC427003, LC427004, LC427005 respectively (Table4). Analysis of results showed that genotypes in water sources of Urmia city belong to T4 with 90% and T2 10% respectively.

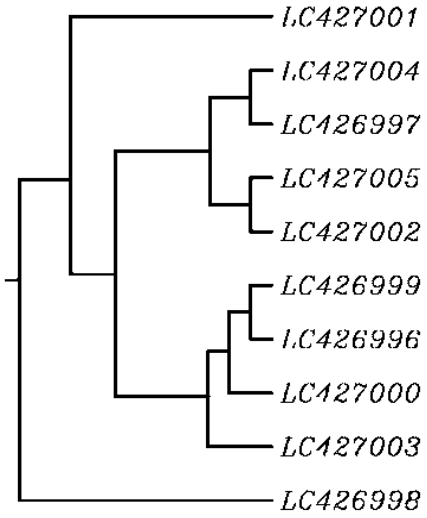

Fig 1. Phyelogentic tree of isolated *Acanthomoba* spp from Urmia city

Table 1. Results of surface water culture in non-nutrient agar media

Sample	N	Positive	%	Negative	%
Surface water	30	17	28.33	13	**28.33**
Tap water	30	4	6.66	26	**13.33**
Sum	60	21	35	39	**41.66**

Table 2. Results of surface and tap water culture in five geographical regions of Urmia

	Surface water			**Refined water**		
Region	*N*	Positive	%	*N*	Positive	%
North	6	3	10	6	0	0
South	6	2	6.66	6	1	3.33
East	6	5	16.66	6	0	0
West	6	4	13.33	6	0	0
Center	6	3	10	6	3	10
Total	**30**	**17**	**56.65%**	**30**	**4**	**13.33**

Table 3. Frequency of *Acanthomoba* spp. in total samples depending on the season of the year

Seasons	**Refined water**			**Surface water**		
	No of samples	Positive	%	No of samples	Positive	**%**
Spring	8	2	6.66	8	4	**13.33**
Summer	7	0	0	7	5	**16.66**
Autumn	10	2	6.66	10	6	**20**
Winter	5	0	0	5	2	**6.66**
Total	30	4	13.33	30	17	**56.65**

Table 4. Results of PCR and sequence of positive samples of Purify water and surface water.

	Isolate Cod	**Samplings site**	**Morphology**	**PCR Result**	**Gene Bank cod**	**Genotype**
Surface Water	E4	Mokhaberat Ave	*Acanthomoba* spp.	+	LC427004	**T4**
	E6	Azarbayjan square	*Acanthomoba* spp.	+	LC427002	**T2**
	W5	Vila Shahr	*Acanthomoba* spp.	+	LC427003	**T4**
	W2	Tokhmorghi park	*Acanthomoba* spp.	+	LC427001	**T4**
	N6	Golmaz village	*Acanthomoba* spp.	+	LC427000	**T4**
	N4	Ghare lar	*Acanthomoba* spp.	+	LC426999	**T4**

	C3	Emam hossin square	*Acanthomoba* spp.	+	LC427005	**T4**
	Ct3	Emam hossin square	*Acanthomoba* spp.	+	LC426998	**T4**
Purified water	St1	Behdari	*Acanthomoba* spp.	+	LC426997	**T4**
	Nt4	Hajipirloo	*Acanthomoba* spp.	+	LC426996	**T4**
	Wt5	Vila Shahr	*Acanthomoba* spp.	+	-	**-**

Discussion

Acanthamoeba spp. belong free living amoeba that can be separated from all environments including tap water resources, surface water, mineral water bottles, eyewash contact lenses, soil, dust, sewage, air conditioning and heating systems (1,2).

The results of this study showed that the frequency of water infection in Urmia is high (35%) and this could be a major contributor to human infection.

Therefore, it is necessary to have effective control methods to prevent pollution of water resources.Despite the importance and frequency of *Acanthamoeba* spp. in Iran, several studies have been carried out in different parts of the country to isolate parasite from water, which has been used low sensitivity method including microscopy (2,13).

Therefore, the use of molecular methods in diagnosis and identifying infectious agents isolated from the environment is recommended. In this study, in addition to studying the frequency of *Acanthamoeba* spp. molecular methods were used to identify and diagnosis of from surface waters and purified water.

The presence of *Acanthamoeba* spp. in rivers and surface waters around Urmia accounts for 56.66% of the risk factor for those who spend their leisure time and leisure time along the rivers and the children playing in these rivers.

In this study, *Acanthomoba* isolated from pond water and surface waters (56%), which is hazardous to children and those who drink or even swim in the water during these warmer months in this area. The presence of *Acanthomoba* in rivers and surface waters around Urmia (56.66%) is a risk factor for people who have spent their leisure hours along these rivers and children who play in these rivers.

In a study by Mafi et al. In 2012 -2013, the *Acanthomoba* species detected in surface waters of Tehran using Real Time PCR and from 70 samples, 11 of parks and 3 of the swimming pools were positive (total 20%) respectively and isolate were mostly related to the T4 genotype (14). In the present study, *Acanthomoba* species was isolated from the plumbing and refined water (13.33%), which is considered to be a significant risk factor for those who use waters for their everyday life or even drink

In recent years, the use of eye lenses among adolescents and young people is very common. There is some chance that some people will wash their contact lens with a lack of familiarity and non-compliance with their health and get rid of amoebic keratitis. (15) In a study in Turkey, conducted by Ackhelin et al in 2013 on Sivas drinking waters of Turkey, the frequency of *Acanthomoba* species genotypes was studied. The results of this study showed that T4 genotypes had the highest frequency in drinking water. (16).

One of the interesting findings of the present study was the difference in the frequency of *Acanthomoba spp.* in geographical regions of Urmia, with the highest frequency in the east (16.66%) and the lowest in the southern region with 6.66%.

In a similar study conducted by Nazer *et al* 2011 in the central regions of Iran in 2008, non-drinking water was studied in recreational areas in 22 areas of Tehran. Results showed that 32% of samples were positive and 87.5% were T4 genotypes and two were genotypes T5 (17).

Another finding of the present study was to investigate the frequency of amoebae of surface waters and plumbing in Urmia city in different seasons of the year. Considering that proper temperature is one of the effective factors in the growth of these parasites, the present findings were expected.

One of the important challenges in identifying and differentiating *Acanthamoea spp.* is the use of morphological characteristics of cysts in the culture medium. Studies have shown that even a particular strain may show itself, depending on the conditions in the culture medium.

Based on the shape and size of cysts, they are classified into three groups (I, II, and III), which, given the possible changes in morphology and cyst size, will not be helpful in classifying the species.Therefore, a more reliable method for determining the species and species of *Acanthamoea* seems necessary. At present, researchers are looking for ways to find and track reliable amoeba with high sensitivity (2).

Therefore, the use of molecular methods for the genotype of these parasites is necessary.

In this research, the nucleotide sequencing of each sample was analyzed according to the highest homology, and these sequences compared with gene bank database and have from 96 to 100 percent homology results were in accordance another studies (17) and 90% of the isolates were related to the genotype T4.

Also, in a study in southwest of Iran, the isolation and genotyping of *Acanthamoea spp.* from natural resources of Ahwaz city by Rahdar et al. In 2012, 71.6 samples of water and 26 soil samples were positive and 86.6% of T4 genotypes and 6.6% T2 and 6.6% T5 It was reported (18).

In conclusion, water infection to *Acanthamoea spp.* is high in the area and considering the abundance of water resources around the city and frequency of T4 genotype as pathogenic form of the parasite medical and research centers should be well aware of diagnostic methods such as microscopic and molecular methods with this parasite.

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Author's contribution

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Ethical standards

This study was approved by Ethical Committee of Urmia University of Medical Sciences, Urmia, Iran (no: IR.umsu.rec.13.55.12).

Conflict of interest

The authors declare no potential conflicts of interests with respect to the research, authorship and/or publication of this article.

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