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Identification and genotyping of *Acanthamoeba* spp. in the water resources of western Iran

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ABSTRACT

Background: Acanthamoeba spp. is opportunistic amoeba that resides in water, soil, and air. Some pathogenic genotypes of the genus of *Acanthamoeba* can cause granulomatous amoebic encephalitis (GAE) in people with a defective immune system. The parasite can also cause *Acanthamoeba* keratitis (AK) among contact lens users. This study was conducted to isolate and identify the *Acanthamoeba* genotypes in water resources in Lorestan province, western Iran.

Methods: Collected 72 water samples from surface and groundwater (springs and aqueducts) in Lorestan province. Samples were filtered and cultured in non-nutrient 1.5% agar medium covered with *Escherichia coli (E. coli)* at 25 °C. DNA extraction was done and the PCR reaction was performed to detect the *Acanthamoeba* spp. The positive PCR products were sequenced to determine the genotypes of *Acanthamoeba.*

Results: Out of 72 examined water samples, 23.61% were positive for *Acanthamoeba* sp. by PCR. From PCR-positive samples, 8 (47.05%) samples were T4 genotypes and others were other *Acanthamoeba* genotypes (T1-T23). Therefore, approximately half of the genotypes belong to the pathogenic T4 genotype.

Conclusions: The water examined samples in western provinces of Iran have the potential risk factor for public health. Therefore, the efforts of healthcare providers are needed to identify, train, and prevention from human infections.

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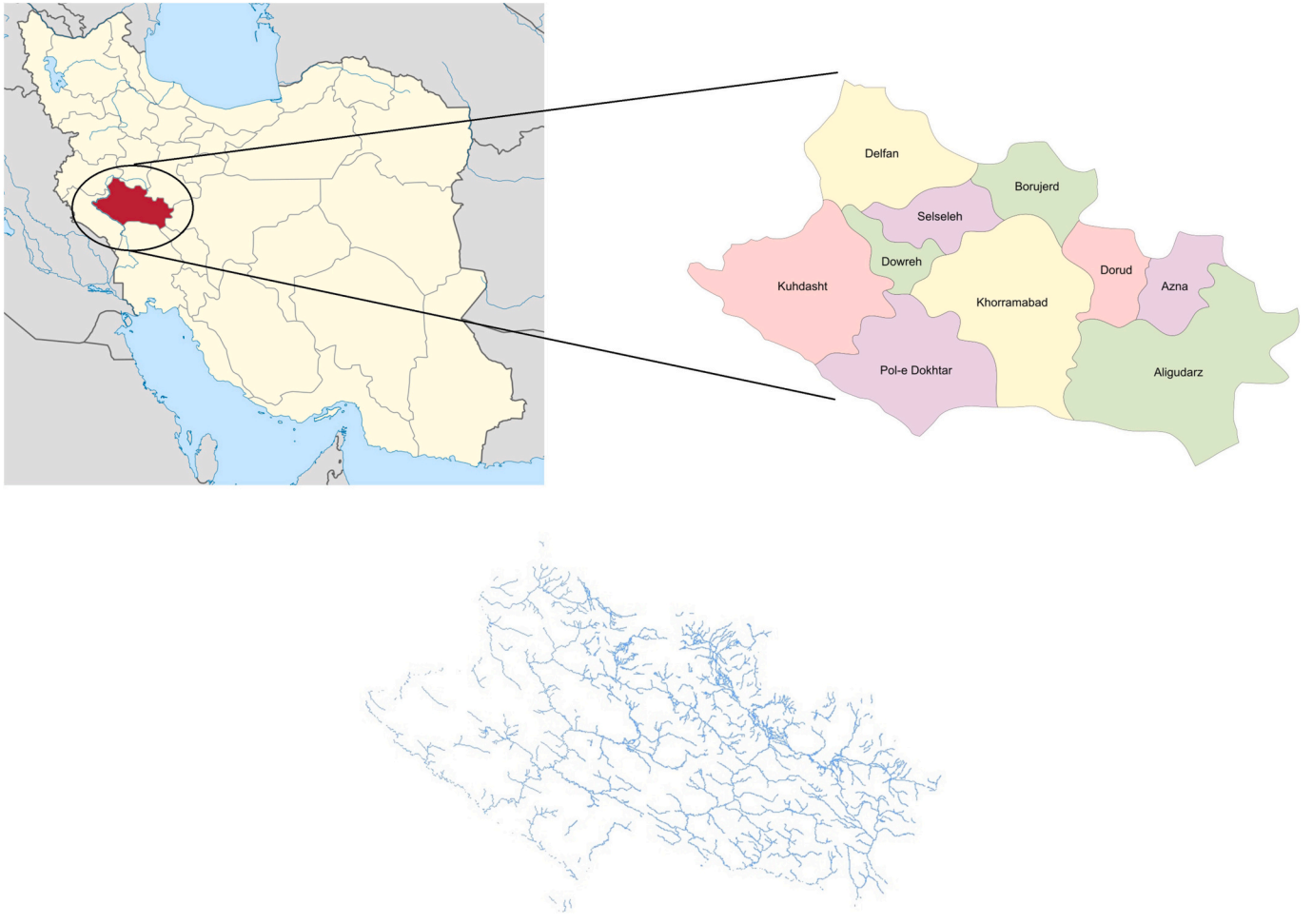

Fig. 1. The geographic map of Lorestan province and its water resources. (https://en.wikipedia.org/wiki/Lorestan_Province#/media/File:IranLorestan-SVG.svg http://abfalorestan.ir/index.aspx?fkeyid=&siteid=1&fkeyid=&siteid=1&pageid=319 http://gisplus.ir/product).

1. Background

The *Acanthamoeba* belongs to the *Acanthamoebidae* family is among the most abundant opportunistic amoebae in nature (Visvesvara et al., 2007). Different species of *Acanthamoeba* isolated from different environments samples such as soil, surface water (Stockman et al., 2011), cooling and air conditioning devices (Chang et al., 2010), swimming pool and sewage, seawater (Mahmoudi et al., 2012), inside the bottle of mineral water, dental units, dialysis apparatus, air dust, culture media, accessories for contact lenses, ear, and nose secretions, and throat mucus from patients with respiratory distress (Visvesvara et al., 2007). *Acanthamoeba* species have two forms of trophozoites and cysts in their life cycle. The cyst is formed in an unfavorable environment and causes the survival of this parasite in nature (Greub and Raoult, 2003). *Acanthamoeba cysts* can enter the body through water, soil, dust and cause disease (Hosseinbigi et al., 2012). Granulomatous amoebic encephalitis (GAE) usually manifests itself with focal neuropathy and signs of increased intracranial pressure (Shirwadkar et al., 2006). The GAE prognosis is poor and the disease is most often diagnosed after the patient's death (Khan, 2008). *Acanthamoeba* keratitis (AK) usually affects people with an efficient immune system (Khan, 2006). Eye lesions resulting from the *Acanthamoeba* infection mainly occur as corneal ulcers, keratoconjunctivitis, and uveitis (Hosseinbigi et al., 2012). In the absence of treatment, AK leads to ulcers and perforation of the stroma, reduced visual acuity, and ultimately blindness (Rezaeian et al., 2008). Cutaneous acanthamoebiasis is common in people with AIDS; these ulcers are also reported in people without AIDS who suffer from amoebic encephalitis as well as in those who have a transplant or receive immunosuppressive drugs (Hosseinbigi et al., 2012). Up to now, 23 different *Acanthamoeba* spp. (T1-T23) genotypes have been identified (Lamien-Meda et al., 2022; Otero-Ruiz et al., 2022). The genotypes T1, T2, T4, T5, T10, T11, and T12 are the factors of granulomatous amebic encephalitis, whereas T2, T4, T5, T16, and T18 may be the factors of *Acanthamoeba* pneumonia (Kot et al., 2021). *Acanthamoeba* has been reported in different regions and climatic conditions from the equator to the pole (Khan, 2003; Schuster and Visvesvara, 2004; Aykur and Dagci, 2021; Hounkong et al., 2022). Although the literature on *Acanthamoeba* has increased in the last 50 years (Behets et al., 2007), however, information about the prevalence of *Acanthamoeba* in the environment is limited (Tanveer et al., 2013). In some developing countries, *Acanthamoeba* species may not be detected due to a lack of knowledge, expertise, and difficulty in identification. But the most concern is the lack of effective treatment for infections caused by these parasites (Chappell et al., 2001). Studies on various clinical and environmental samples around the world indicate a relatively high prevalence of different species of *Acanthamoeba* (Lorenzo-Morales et al., 2005; Niyyati et al., 2012; Solgi et al., 2012; Niyyati et al., 2013; Tanveer et al., 2013). In Iran, *Acanthamoeba* has also been isolated and reported from different water resources by various diagnostic methods (Rezaian et al., 2003; Rezaeian et al., 2008; Niyyati et al., 2009). The identification of *Acanthamoeba* is usually done by direct microscopy or culture and based on the structural characteristics of the parasite cysts, while these methods have some limitations due to the experience and skills of the examiner person and the effect of cultivation conditions (Chang et al., 2010). In recent years, molecular methods such as PCR have greatly remedied this problem and are appropriate confirmatory tests for the differentiation of *Acanthamoeba* from other free-living amoeba species. This method is a fragmentary reproduction of the 18S rRNA gene as a specific method for identifying the genus of *Acanthamoeba* (Khan, 2008). In most of the studies conducted in Iran, the T4 genotype was the most isolated genotype from different samples (Schroeder et al., 2001; Kilic et al., 2004). The present study was designed to identify *Acanthamoeba* genotypes in surface and groundwater in this region.

2. Materials and methods

2.1. Study area

The name Lorestan means "land of the Lurs", and in the wider sense consists of that part of western Iran coinciding with the province of Ilam and extending for about 650 km on a northwest to the southeast axis from Kermanshah to Fars provinces, with a breadth of 150–180 km. The central range has many summits that almost reach the line of perpetual snow, rising to 4000 m and more, and it feeds the headwaters of Iran's most important rivers, such as the Zayandeh Rud, Jarahi, Karun, and Karkheh. Between the higher ranges lie many fertile plains and low hilly, well-watered districts. The climate is generally sub-humid continental with winter precipitation, a lot of which falls as snow (Köppen Csa). Because it lies on the westernmost slopes of the Zagros Mountains, annual precipitation in Lorestan is among the highest anywhere in Iran south of the Alborz mountains. At Khorramabad, the capital of Lorestan Province, the average annual precipitation totals 530 mm (21 in.) of rainfall equivalent, whilst up to 1270 mm (50 in.) may fall on the highest mountains. (Fig. 1).

2.2. Samples collection

This descriptive cross-sectional study was conducted in Lorestan province from March to September 2016. In this study, 72 water samples were collected from 72 regions including surface and underground waters (springs and aqueducts) such as; Lake Qiu, Mirage Nilofar, Mirage Kahman, Mirage Zaz, Mirage Honam, Wetlands Tang Fani, etc. as 3 times sampling. These mirages and lakes, in addition to being tourist attractions, are sometimes used as a source of drinking water for local people and agricultural purposes. The sample size was 500 to 1000 ml each time.

2.3. Isolation and culture

Approximately 500 ml of each sample was filtered through a cellulose nitrate filter (Millipore Corporation, Bedford, Madison, WI,

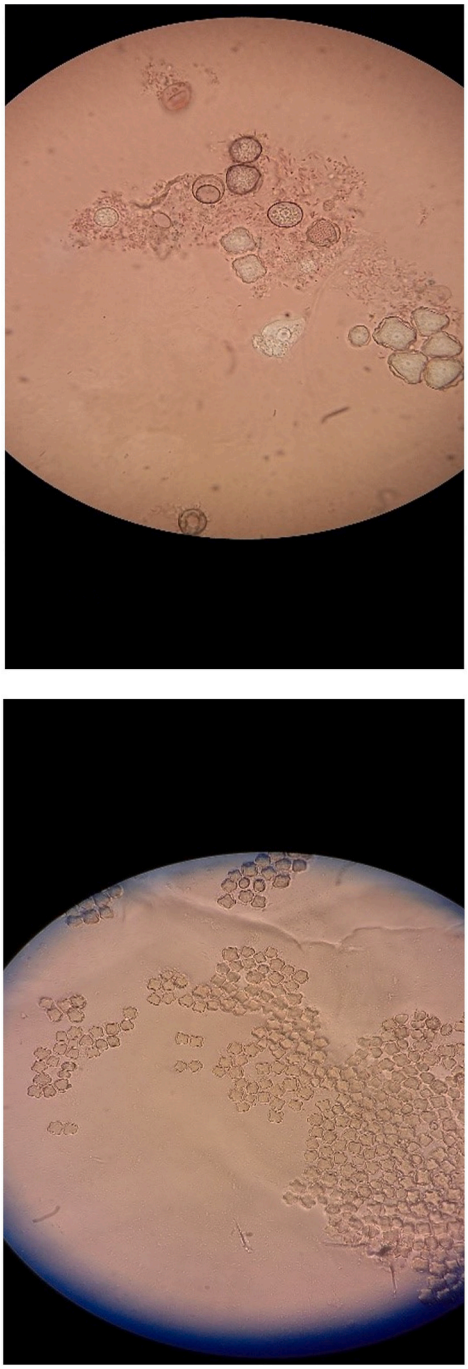

Fig. 2. The free-living amoeba is cultured on 1.5% non-nutrient agar under two different magnifications of the light microscope.

USA, pore size 0.45 μm) using a vacuum pump. The filter papers were upturned onto 1.5% Non-Nutrient Agar, enriched with a layer of *E. coli* (killed by heat as parasite feed). The media were incubated for 1–2 weeks at 25 °C. To identify the amoebae, samples were examined under a light microscope. The *Acanthamoeba* isolates were recognized morphologically based on flattened trophozoites with slender acanthopodia and double-wall cysts. Microscopic diagnosis of *Acanthamoeba* spp. from other free-living amoebas such as *Naegleria fowleri* and *Balamuthia mandrialis*. Was that double-walled and perforated star-shaped cysts were observed that were completely similar to typical *Acanthamoeba* spp. cysts and were consistent with the results of other references. Also, the observed cysts and trophozoites were compared with the cysts and trophozoites of other free-living amoebae, and the presence of *Acanthamoeba* spp. was confirmed. (Fig. 2). Since the wall of the cysts of *Nagelria fowleri* and *B. mandrillaris* do not have pores and are round in shape, therefore the culture media were reported negative for the presence of these amoebae(Lek-Uthai et al., 2009; Duarte et al., 2013; Siddiqui and Khan, 2015; Martínez-Castillo et al., 2016; Sampaotong et al., 2016).

2.4. DNA extraction

To extract the genomic DNA, about 4–5 ml of sterile phosphate-buffered saline (PBS) was added to the medium and the plate completely was washed until the cysts and trophozoites were immersed in the buffer. The solution was transferred to special tubes and centrifuged 3 times for 2.5 min at 2500*g*, then the supernatant fluid was slowly removed. The resulting precipitate contains enough amoebae. The DynaBio TM DNA extraction commercial kit (Takapouzist, Co., Iran) was used to extract the *Acanthamoeba* DNA according to the manufacturer's instructions. The quality and concentration of extracted DNA from each specimen were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, MA, USA) and electrophoresis on 1% agarose gel.

2.5. PCR

The PCR targeting the 18S ribosomal RNA gene of *Acanthamoeba* was performed using 2 μl of DNA, 10 pmol of each primer JDP1 (5′-GGC CCA GAT CGT TTA CCG TGA A-3′), and JDP2 (5′-TCT CAC AAG CTG CTA GGG GAG TCA-3′) (Hooshyar et al., 2013), 1 U de *Taq polymerase* and buffer 1× (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Bioline, London, UK), under the following conditions: Initial denaturation at 94 °C for 4 min and 33 cycles each denaturation at 94 °C (35 s), annealing at 57 °C (45 s) and extension at 72 °C (1 min) followed by a final extension at 72 °C for 5 min. The PCR products presented a fragment of 500 bp on 1.5% agarose gel stained with DNA-safe stain (Cinna colon, Iran).

2.6. Nucleotide sequences

25 μL PCR products which were sent to Takapou Zist Company (Iran). The sequence results were edited by Chromas software version 2.5.0 and analyzed with Nucleotide Blast. The sequences were aligned with relevant sequences related to *Acanthamoeba* spp. from Iran and some other countries deposited in the GenBank. A maximum-likelihood (ML) phylogram based on 18S ribosomal RNA genes was constructed using MEGA X. Bootstrap analyses were conducted using 1000 replicates.

3. Results

3.1. Parasitology and molecular evaluations

The *Acanthamoeba* isolates were recognized morphologically based on flattened trophozoites with slender lobopodium and acanthopodia and double-wall cysts with wrinkled or wavy ectocyst and various endocyst shapes, including star, triangular, square, and round shapes (Fig. 2). This study's principal object was isolation and isolation genotyping of *Acanthamoeba* spp. Although, other free-living amoebae doubted to be *Vannella* and *Hartmannella,* based on morphological features in culture plates, were also found (Table 1). PCR analysis was evaluated using *Acanthamoeba* JDP1 and JDP2 specific primers targeting the 18S ribosomal RNA gene showing that 17 (23.16%) samples were positive for *Acanthamoeba* spp. (Table 2). The 18S ribosomal RNA gene (500 bp) was amplified

Table 1
Frequency and distribution of free-living amoebae taken from surface and groundwater in Lorestan province based on culture results.

	Total		Positive culture		Negative culture	
Culture result	No	%	No	%	No	%
Khorramabad	15	100	15	100	0	0.0
Borujerd	6	100	6	100	0	0.0
Aligudarz	5	100	5	100	0	0.0
Dorud	9	100	9	100	0	0.0
Bayranshahr	8	100	8	100	0	0.0
Aleshtar	8	100	8	100	0	0.0
Nurabad	7	100	7	100	0	0.0
Pol-e Dokhtar	8	100	8	100	0	0.0
Kuhdasht	6	100	6	100	0	0.0
Total	72	100	72	100	0	0.0

by PCR method (Fig. 3).

3.2. Sequencing and determination of Acanthamoeba genotypes

Based on phenotypic and molecular data, all isolates were identified as *Acanthamoeba* spp. The similarity between our *Acanthamoeba* spp. isolates and some other parts of the world was determined by a good bootstrapping value in the phylogenetic tree made based on the 18S ribosomal RNA gene. All isolates positive for the 18S ribosomal RNA gene in the current study were closely related to the sequences from some countries and some other parts of Iran After blasting our samples, most of them had a high identity (98–100%) with the Acanthamoeba sp. and Acanthamoeba genotype T4 and with a lower percentage of genotype T5(96–97%) in the gene bank. As it is clear in the image of the alignment, our amplified part has a high similarity with two other genotypes (T4,T5) and maybe other genotypes. Therefore, we can state that our samples are definitely Acanthamoeba, and it's more like T4 (in one clade in phylogenetic tree) than T5, but the authors believe that it is better and more accurate that reports of the genotype should be checked with other primers and check more variable parts than conserved regions.

Because the 18S ribosomal RNA gene is a large fragment including many conserved sequences, the amplicon produced by us and many researchers around the world are partial.(Figs. 4,5).

4. Discussion

Free-living amoebae have plenty of abundance in nature. Some species of these amoebae cause serious and sometimes fatal illnesses in people with a healthy immune system as well as in people with immune deficiency. *Acanthamoeba* belonged to the free-living amoeba family and is one of the most common protozoa in nature. This opportunistic parasite is isolated from different sources, and in Iran, it is very important given the prevalence of the diseases caused by this Protozoa, such as *Acanthamoeba* keratitis (Rezaeian et al., 2008; Hosseinbigi et al., 2012). The results of this study showed that all 72 collected water samples from different water resources in Lorestan Province (100%) were positive for free-living amoebae by culture method. The results of this study were consistent with the findings of Hosseinbigi et al., which reported an 80% prevalence of the free-living amoeba in the water of parks and fields in Qazvin City, central Iran (Hosseinbigi et al., 2012) as well as with the result of the study by Mosayebi et al. that reported the frequency of these amoebae in Arak waters was% 61.11 (Mosayebi et al., 2014). The high prevalence of free-living amoebae in different water resource in Lorestan province, indicate the risk of transmission of infection to human. The prevalence of free-living amoebae in immune-deficient patients in Tehran, water resources in Shiraz (Lasjerdi et al., 2011; Solhjoo et al., 2012), and Turkish plumbing waters and Florida household water explained a different prevalence of this amoeba in different sources and areas (Shoff et al., 2008). It seems that the difference between the findings of the above-mentioned studies regarding the prevalence of free-living amoebae with the results of this study is due to different sources of water in various geographic areas and the different diagnostic methods. The detection of the *Acanthamoeba* spp. only based on morphological characteristics is unreliable, due to changes in the cyst form attributable to culture medium conditions and classification is more correct based on more stable molecular characteristics (Khan, 2008; Corsaro and Venditti, 2010). In the present study, PCR was used for the diagnosis and identification of *Acanthamoeba* from cultured specimens. According to the result of the PRC assay, 23.61% (17 samples) of positive cultured water samples were positive for *Acanthamoeba* which is a lower prevalence in comparison whit similar studies in Tehran and Qazvin Province (Rezaeian et al., 2008; Hosseinbigi et al., 2012) that reported 46.35% and 43.18% positivity rate respectively. On the other hand, conducted studies in Egypt and Taiwan have also revealed more abundance of amoeba in these areas (Lorenzo-Morales et al., 2005; Kao et al., 2014). In previous studies, the prevalence of *Acanthamoeba* has been studied in various environmental sources around the world. The prevalence of *Acanthamoeba* in Turkish sources was 22%(Coşkun et al., 2013), Turkey's environmental resources 21% (Kilic et al., 2004), Hungary's public baths 6.7% (Kiss et al., 2014), Japanese clinical specimens 5.6%, Dutch natural water resources samples were 8% (Lass et al., 2014), Seoul's household water samples 7.7% (43) and Florida household water was 2.8% (Boost et al., 2008). Also, in river samples from the US, Jamaica, Germany, and Bulgaria the frequency of *Acanthamoeba* was reported at 7%, 26.4%, 79%, and 94%, respectively (Hoffmann and Michel, 2001; Lorenzo-Morales et al., 2006). Moreover, in Nicaragua, Mexico, Brazil, Jamaica, Spain, and Japan, the frequency of parasites was reported 21%, 22.5%, 26.3%, 36.1%, 59.5%, and 68.7% respectively(Lorenzo-Morales et al., 2005; Bonilla-

Table 2
The frequency and distribution of *Acanthamoeba* spp. in surface and underground water samples in Lorestan province based on PCR results.

PCR result	Total		PCR positive		PCR negative	
	No	%	No	%	No	%
Khorramabad	15	100	5	33	10	66.66
Borujerd	7	100	1	14.28	6	85.71
Aligudarz	5	100	0	0.0	5	100
Dorud	9	100	2	22	7	77.77
Bayranshahr	8	100	4	50	4	50
Aleshtar	8	100	1	12.5	7	87.5
Nurabad	7	100	1	14.28	6	85.71
Pol-e Dokhtar	8	100	3	37.5	5	62.5
Kuhdasht	6	100	0	0.0	6	100
Total	72	100	17	23.61	55	76.38

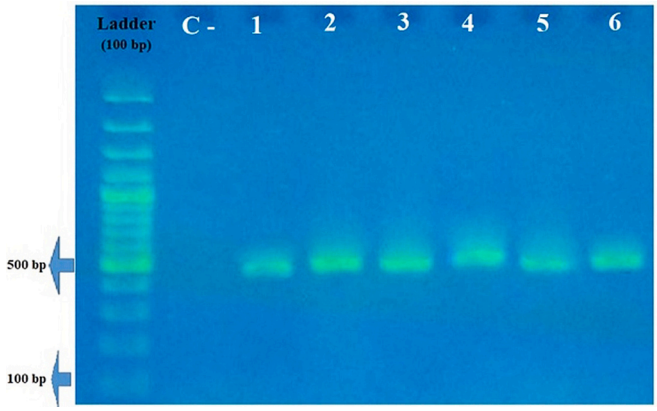

Fig. 3. PCR assay by targeting 18S rRNA to amplify *Acanthamoeba* spp. (500 bp). Ladder: 100 bp DNA ladder marker, C -: Negative control, Lanes: 1–6 *Acanthamoeba* spp.

Lemus et al., 2010). In Iran, in the study of Mosayebi et al. (2014) on Arak water resources, and Eftekhar et al. (2009) on Tehran's surface water, the *Acanthamoeba* prevalence was reported 26.9%, 61% and 27.3%, respectively (Eftekhar et al., 2009; Niyyati et al., 2013; Mosayebi et al., 2014). The results of the present study, which indicate about one-fourth of the specimens were positive for *Acanthamoeba*, could be explained by several reasons; including that the abundance of *Acanthamoeba* in surface and groundwater resources is due to *Acanthamoeba* specificities such as heat resistance, high osmolality, and growth in a wide range of pH (Lorenzo-Morales et al., 2005; Khan, 2006; Shirwadkar et al., 2006; Khan, 2008). The genus of *Acanthamoeba* is more in stagnant waters, such as square water and public parks than rivers. The *Acanthamoeba* species in unrefined water of square ponds and parks are much higher than in domestic refined water. *Acanthamoeba* grows better at 27 °C in environmental resources such as springs and aqueducts than in high-temperature environments (Khan, 2006). The prevalence of *Acanthamoeba* in different environmental samples varies from water, soil, dust, and clinical specimens. The geographical, climatic, and climatic conditions affect the incidence of *Acanthamoeba*. Weather conditions in Lorestan province are moderate. The lack of dust in the air, the high rainfall, and the high environmental water resources provide conditions for the reduction of *Acanthamoeba* cases in the specimens studied. From the limitations of the PCR method, it can be noted that this method does not recognize the *Acanthamoeba* genotypes. Some of the identified genotypes are pathogenic and some are non-pathogenic (Liang et al., 2010). Genotypes of pathogenic and non-pathogenic can be determined by the sequencing of positive specimens of the *Acanthamoeba* genus. Until now, 23 different genotypes of *Acanthamoeba* (T1-T23) have been identified that from pathogen genotypes can refer to T3, T4, and T5 (Khan, 2006; Putaporntip et al., 2021). In the results obtained from the Phylogenetic analysis in the present study, it was found that all amplified product are most similar to T4. But as said in the results of the alignment, it was observed that this part has a high similarity in our samples and two other genotypes and maybe other genotypes. For this purpose and according to the result of the phylogenetic tree, we used the word similarity with the T4 genotype. 3 Various studies performed from 2005 to 2014 on clinical specimens and water resources in different regions of Iran, showed that the T4 genotype was the most prevalent genotype isolated (Maghsood et al., 2005; Niyyati et al., 2009; Zhao et al., 2010; Badirzadeh et al., 2011; Niyyati et al., 2013; Cabello-Vílchez et al., 2014; Kao et al., 2014; Lass et al., 2014). In addition to the T4 genotype, the T5 genotype has been reported as a common genotype in the commentary of Lasjerdi et al. (on Tehran immunodeficiency patients 2011), Niyyati et al. (in dust samples 2009), Nazar and contributors (in Tehran's water resources 2011) in Iran and Reyes et al. (soil resources 2014) from Spanish (Maghsood et al., 2005; Niyyati et al., 2009; Badirzadeh et al., 2011; Cabello-Vílchez et al., 2014; Kiss et al., 2014). The T11 genotype has been reported in Tehran's dust sample, the T2 in the soil sample of Spain, and the T15 in clinical specimens from Japan (Niyyati et al., 2009; Rahman et al., 2013; Reyes-Batlle et al., 2014). Various studies have also been conducted in Turkey on soil samples, surface water, and urban plumbing water(.Aykur and Dagci, 2021) The result of these studies indicates that the T4 genotype is the dominant genotype in Turkey (Kilic et al., 2004). Also, the results of Aykor et al. showed 27 *Acanthamoeba* isolates at the genotype level based on the 18S rRNA gene as T4 (51.85%), T5 (22.22%), T2 (14.81%), and T15 (11.11%) in Izmir, Turkey (Aykur and Dagci, 2021). But a study done in Pakistan on drinking water in 2013, reported seven pathogenic and non-pathogenic *Acanthamoeba* genotypes including T4, T2, T5, T7, T10, T15, T16, and T17 (Tanveer et al., 2013). The T4 genotype can cause dangerous diseases. Among the infections caused by this amoeba are GAE, Skin granulomatosis of *Acanthamoeba,* and AK(Khan, 2003; Marciano-Cabral and Cabral, 2003; Lorenzo-Morales et al., 2005). The GAE prognosis is not good and in most cases causes the death of the patient (Khan, 2008). Also, AK can affect healthy people and people with an efficient immune system, which ultimately can reduce vision and blindness (Khan, 2006; Rezaeian et al., 2008). Different studies indicate that the T4 genotype is the most common strain of AK cases (Ledee et al., 2009; Zhao et al., 2010; Risler et al., 2013). Therefore, considering the pathogenicity of half of the genotypes found in water resources, the efforts of healthcare providers are needed to identify, train, and prevention from contamination. Global studies on the abundance of *Acanthamoeba* in different sources show variable results. For example in the United States (2001), more than 80% of the American population had antibodies against *Acanthamoeba* (Chappell et al., 2001). In a study in Jamaica, water pollution in household water, river water, and seawater varied from 26.4%(in the river) to 49.6% (in the sea) (Lorenzo-Morales et al., 2005). In Iran, *Acanthamoeba* has also been isolated and reported from different water resources (Schroeder et al., 2001; Niyyati et al., 2009). In the study of 55 water

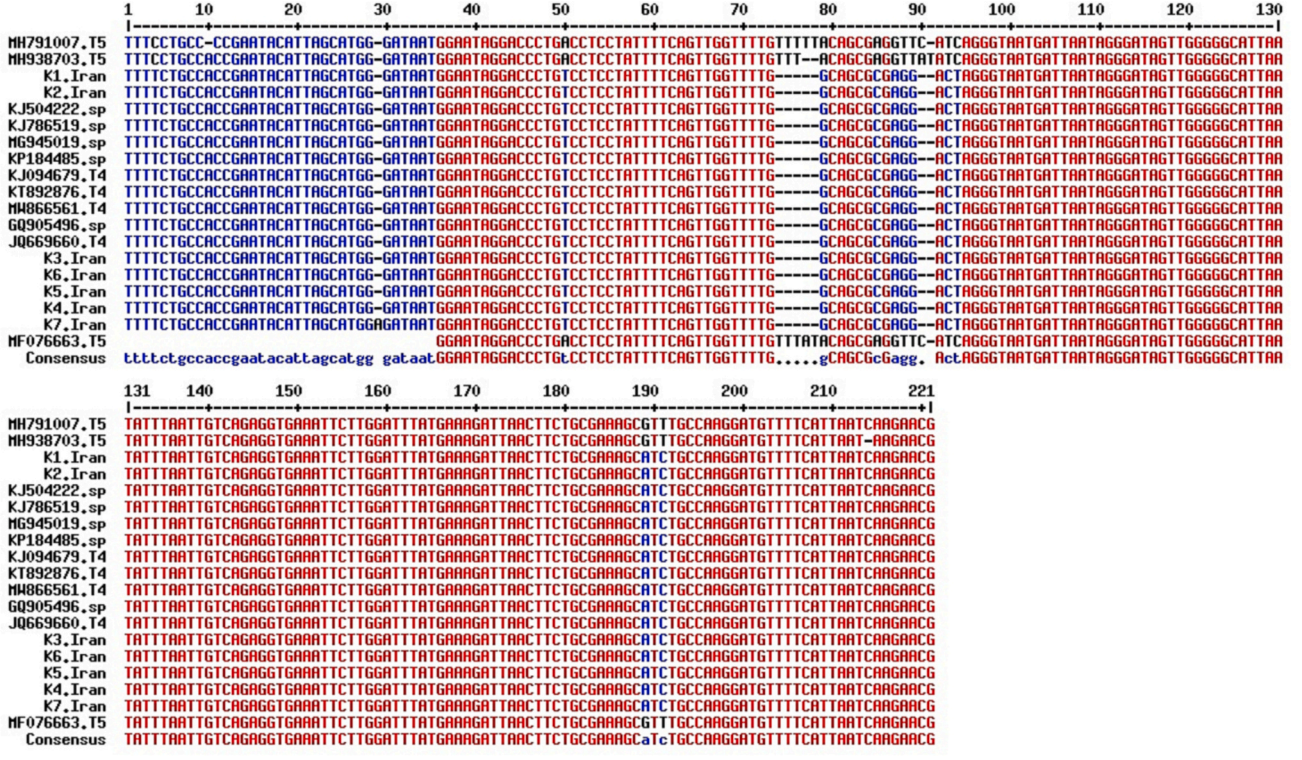

Fig. 4. MultAlin sequence showing the differences and similarities between all isolates and two important genotype T4 and T5. Many similar sequences were removed due to the reduction in image size.

Fig. 5. Maximum-likelihood tree of 18S rRNA gene from western of Iran. The nucleotide sequence of the *Entamoeba histolytica* (GenBank accession no KT253454) detected in Iraq was considered as the outgroup.

samples from 10 recreational rivers around Tehran (Niyyati et al., 2009), 354 water samples from the lake and rivers surrounding Kazeroun city (in southeastern Iran)(Rezaeian et al., 2008), and 40 samples of stagnant surface water in Qazvin city (in the northwest of Iran) were varied from 2.82% (in Kazeroun city) to 35% (in Qazvin city)(Schroeder et al., 2001). Also, the results of the study on 80 samples collected from water, soil, and dental units, showed that 46.25% of the samples were contaminated with *Acanthamoeba* spp. (Hosseinbigi et al., 2012).

5. Limitations

In this study, the lack of access to groundwater and surface water samples in the whole of Lorestan province caused many problems in sampling and high costs.

6. Conclusion

Generally, the results of this study showed that 100% of the samples were positive for Free-living Amoebae by culture method of these, 23.61% of the water samples were *Acanthamoeba* positive, and based on the sequence of genes examined, *Acanthamoeba* genotypes belonged to T4 and other *Acanthamoeba* species respectively. Therefore, to reveal the virulence and genetic markers of different genotypes of this parasite, especially in different environmental resources, more studies and research with specific methods are necessary.

Ethics approval

Ethical approval was received from the Ethics Committee of Lorestan University of Medical Sciences (IR.LUMS.REC.1397.079).

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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References

Aykur, M., Dagci, H., 2021. Evaluation of molecular characterization and phylogeny for quantification of *Acanthamoeba* and Naegleria fowleri in various water sources, Turkey. PLoS One 16, e0256659.

Badirzadeh, A., Niyyati, M., Babaei, Z., Amini, H., Badirzadeh, H., Rezaeian, M., 2011. Isolation of free-living amoebae from sarein hot springs in Ardebil province, Iran. Iran. J. Parasitol. 6, 1.

Behets, J., Declerck, P., Delaedt, Y., Verelst, L., Ollevier, F., 2007. Survey for the presence of specific free-living amoebae in cooling waters from Belgian power plants. Parasitol. Res. 100, 1249–1256.

Bonilla-Lemus, P., Ramírez-Bautista, G.A., Zamora-Muñoz, C., del Rocío Ibarra-Montes, M., Ramírez-Flores, E., Hernández-Martínez, M.D., 2010. *Acanthamoeba* spp. in domestic tap water in houses of contact lens wearers in the metropolitan area of Mexico City. Exp. Parasitol. 126, 54–58.

Boost, M., Cho, P., Lai, S., Sun, W.-M., 2008. Detection of *Acanthamoeba* in tap water and contact lens cases using polymerase chain reaction. Optom. Vis. Sci. 85, 526–530.

Cabello-Vílchez, A.M., Martín-Navarro, C.M., López-Arencibia, A., Reyes-Batlle, M., González, A.C., Guerra, H., Gotuzzo, E., Valladares, B., Piñero, J.E., Lorenzo-Morales, J., 2014. Genotyping of potentially pathogenic *Acanthamoeba* strains isolated from nasal swabs of healthy individuals in Peru. Acta Trop. 130, 7–10.

Chang, C.W., Wu, Y.C., Ming, K.W., 2010. Evaluation of real-time PCR methods for quantification of *Acanthamoeba* in anthropogenic water and biofilms. J. Appl. Microbiol. 109, 799–807.

Chappell, C.L., Wright, J.A., Coletta, M., Newsome, A.L., 2001. Standardized method of measuring *Acanthamoeba* antibodies in sera from healthy human subjects. Clin. Diagn. Lab. Immunol. 8, 724–730.

Corsaro, D., Venditti, D., 2010. Phylogenetic evidence for a new genotype of *Acanthamoeba* (Amoebozoa, Acanthamoebida). Parasitol. Res. 107, 233–238.

Coşkun, K.A., Özçelik, S., Tutar, L., Elaldı, N., Tutar, Y., 2013. Isolation and identification of free-living amoebae from tap water in Sivas, Turkey. Biomed. Res. Int. 2013, 675145.

Duarte, J.L., Furst, C., Klisiowicz, D.R., Klassen, G., Costa, A.O., 2013. Morphological, genotypic, and physiological characterization of *Acanthamoeba* isolates from keratitis patients and the domestic environment in Vitoria, Espírito Santo, Brazil. Exp. Parasitol. 135, 9–14.

Eftekhar, M., NAZEM, A.M.E., HAGHIGHI, A., SHARIFI, K., Nouchi, Z., Athari, A., 2009. Detection of *Acanthamoeba* from Fresh Water Using Polymerase Chain Reaction.

Greub, G., Raoult, D., 2003. Biocides currently used for bronchoscope decontamination are poorly effective against free-living amoebae. Infect. Control Hosp. Epidemiol. 24, 784–786.

Hoffmann, R., Michel, R., 2001. Distribution of free-living amoebae (FLA) during preparation and supply of drinking water. Int. J. Hyg. Environ. Health 203, 215–219.

Hooshyar, H., Hosseinbigi, B., Saraei, M., Alizadeh, S., Eftakhar, M., Rasti, S., Khosro-Shahi, N., 2013. Genotyping of *acanthamoeba* isolated from surface and stagnant waters of Qazvin, Central Iran. Iran Red Crescent Med J 15, 536–538.

Hosseinbigi, B., Sarie SahnehSaraie, M., Alizadeh, S., Rasti, S., Eftakhar, M., Khosro-Shahi, N., Hooshyar, H., 2012. Isolation and molecular identification of *Acanthamoeba* in surface stagnant waters of Qazvin. J. Inflam. Dis. 16, 26–32.

Hounkong, K., Sornying, P., Saechan, V., Sawangjaroen, N., 2022. Isolation and identification of *Acanthamoeba* Spp from water and soil in southern Thailand. Southeast Asian J. Trop. Med. Public Health 53, 9–20.

Kao, P.-M., Hsu, B.-M., Chen, C.-T., Huang, S.-W., Kao, E.-S., Chen, J.-L., Wu, N.-M., Ji, W.-T., 2014. Identification and quantification of the *Acanthamoeba* species and genotypes from reservoirs in Taiwan by molecular techniques. Acta Trop. 132, 45–50.

Khan, N.A., 2003. Pathogenesis of *Acanthamoeba* infections. Microb. Pathog. 34, 277–285.

Khan, N.A., 2006. *Acanthamoeba*: biology and increasing importance in human health. FEMS Microbiol. Rev. 30, 564–595.

Khan, N.A., 2008. Emerging Protozoan Pathogens. Taylor & Francis.

Kilic, A., Tanyuksel, M., Sissons, J., Jayasekera, S., Khan, N.A., 2004. Isolation of *Acanthamoeba* isolates belonging to T2, T3, T4 and T7 genotypes from environmental samples in Ankara, Turkey. Acta Parasitol. 49, 246–252.

Kiss, C., Barna, Z., Vargha, M., Török, J.K., 2014. Incidence and molecular diversity of *Acanthamoeba* species isolated from public baths in Hungary. Parasitol. Res. 113, 2551–2557.

Kot, K., Łanocha-Arendarczyk, N., Kosik-Bogacka, D., 2021. Immunopathogenicity of *Acanthamoeba* spp. in the brain and lungs. Int. J. Mol. Sci. 22, 1261.

Lamien-Meda, A., Köhsler, M., Walochnik, J., 2022. Real-time PCR for the diagnosis of *Acanthamoeba* genotype T4. Microorganisms 10.

Lasjerdi, Z., Niyyati, M., Haghighi, A., Shahabi, S., Biderouni, F.T., Taghipour, N., Eftekhar, M., Mojarad, E.N., 2011. Potentially pathogenic free-living amoebae isolated from hospital wards with immunodeficient patients in Tehran, Iran. Parasitol. Res. 109, 575–580.

Lass, A., Szostakowska, B., Idzińska, A., Chomicz, L., 2014. The first genotype determination of *Acanthamoeba* potential threat to human health, isolated from natural water reservoirs in Poland. Parasitol. Res. 113, 2693–2699.

Ledee, D., Iovieno, A., Miller, D., Mandal, N., Diaz, M., Fell, J., Fini, M., Alfonso, E., 2009. Molecular identification of T4 and T5 genotypes in isolates from *Acanthamoeba* keratitis patients. J. Clin. Microbiol. 47, 1458–1462.

Lek-Uthai, U., Passara, R., Roongruangchai, K., 2009. Morphological Features of *Acanthamoeba* Causing Keratitis Contaminated from Contact lens Cases.

Liang, S.Y., Ji, D.R., Hsia, K.T., Hung, C.C., Sheng, W.H., Hsu, B.M., Chen, J.S., Wu, M.H., Lai, C.H., Ji, D.D., 2010. Isolation and identification of *Acanthamoeba* species related to amoebic encephalitis and nonpathogenic free-living amoeba species from the rice field. J. Appl. Microbiol. 109, 1422–1429.

Lorenzo-Morales, J., Monteverde-Miranda, C.A., Jiménez, C., Tejedor, M.L., Valladares, B., Ortega-Rivas, A., 2005. Evaluation of *Acanthamoeba* isolates from environmental sources in Tenerife, Canary Islands, Spain. Ann. Agric. Environ. Med. 12, 233–236.

Lorenzo-Morales, J., Ortega-Rivas, A., Martínez, E., Khoubbane, M., Artigas, P., Periago, M.V., Foronda, P., Abreu-Acosta, N., Valladares, B., Mas-Coma, S., 2006. *Acanthamoeba* isolates belonging to T1, T2, T3, T4 and T7 genotypes from environmental freshwater samples in the Nile Delta region, Egypt. Acta Trop. 100, 63–69.

Maghsood, A.H., Sissons, J., Rezaian, M., Nolder, D., Warhurst, D., Khan, N.A., 2005. *Acanthamoeba* genotype T4 from the UK and Iran and isolation of the T2 genotype from clinical isolates. J. Med. Microbiol. 54, 755–759.

Mahmoudi, M.R., Taghipour, N., Eftekhar, M., Haghighi, A., Karanis, P., 2012. Isolation of *Acanthamoeba* species in surface waters of Gilan province-north of Iran. Parasitol. Res. 110, 473–477.

Marciano-Cabral, F., Cabral, G., 2003. *Acanthamoeba* spp. as agents of disease in humans. Clin. Microbiol. Rev. 16, 273–307.

Martínez-Castillo, M., Cárdenas-Zúñiga, R., Coronado-Velázquez, D., Debnath, A., Serrano-Luna, J., Shibayama, M., 2016. Naegleria fowleri after 50 years: is it a neglected pathogen? J. Med. Microbiol. 65, 885.

Mosayebi, M., Ghorbanzadeh, B., Eslamirad, Z., Ejtehadifar, M., Rastad, B., 2014. The isolation and detection of *Acanthamoeba* in rural water sources of Arak, Iran. Med. Lab. J. 7, 66–71.

Niyyati, M., Lorenzo-Morales, J., Rezaie, S., Rahimi, F., Mohebali, M., Maghsood, A.H., Motevalli-Haghi, A., Martín-Navarro, C.M., Farnia, S., Valladares, B., 2009. Genotyping of *Acanthamoeba* isolates from clinical and environmental specimens in Iran. Exp. Parasitol. 121, 242–245.

Niyyati, M., Lasjerdi, Z., Nazar, M., Haghighi, A., Nazemalhosseini Mojarad, E., 2012. Screening of recreational areas of rivers for potentially pathogenic free-living amoebae in the suburbs of Tehran, Iran. J. Water Health 10, 140–146.

Niyyati, M., Ebrahimi, M., Haghighi, A., Haydari, S., 2013. Isolation and genotyping of *Acanthamoeba* spp. from recreational soil of parks in Tehran, Iran. Armaghane Danesh 18, 530–538.

Otero-Ruiz, A., Gonzalez-Zuñiga, L.D., Rodriguez-Anaya, L.Z., Lares-Jiménez, L.F., Gonzalez-Galaviz, J.R., Lares-Villa, F., 2022. Distribution and current state of molecular genetic characterization in pathogenic free-living amoebae. Pathogens 11, 1199.

Putaporntip, C., Kuamsab, N., Nuprasert, W., Rojrung, R., Pattanawong, U., Tia, T., Yanmanee, S., Jongwutiwes, S., 2021. Analysis of *Acanthamoeba* genotypes from public freshwater sources in Thailand reveals a new genotype, T23 *Acanthamoeba* bangkokensis sp. nov. Sci. Rep. 11, 1–13.

Rahman, M.M., Yagita, K., Kobayashi, A., Oikawa, Y., Hussein, A.I., Matsumura, T., Tokoro, M., 2013. Genetic characterization of clinical *Acanthamoeba* isolates from Japan using nuclear and mitochondrial small subunit ribosomal RNA. Korean J. Parasitol. 51, 401.

Reyes-Batlle, M., Todd, C.D., Martín-Navarro, C.M., López-Arencibia, A., Cabello-Vilchez, A.M., González, A.C., Córdoba-Lanús, E., Lindo, J.F., Valladares, B., Piñero, J.E., 2014. Isolation and characterization of *Acanthamoeba* strains from soil samples in Gran Canaria, Canary Islands, Spain. Parasitol. Res. 113, 1383–1388.

Rezaeian, M., Niati, M., Farnia, S., MOTEVALI, H.A., 2008. Isolation of *Acanthamoeba* spp. from Different Environmental Sources.

Rezaian, M., Bagheri, F., Farnia, S., Babai, Z., 2003. Isolation of pathogenic amoeba (naegleria and acanthameoba) from water sourcesand margin soils of reveirs and lakes in kazerun. J. School Public Health Inst. Public Health Res. 1, 41–48.

Risler, A., Coupat-Goutaland, B., Pélandakis, M., 2013. Genotyping and phylogenetic analysis of *Acanthamoeba* isolates associated with keratitis. Parasitol. Res. 112, 3807–3816.

Sampaotong, T., Roongruangchai, J., Roongruangchai, K., 2016. Viability and morphological changes of *Acanthamoeba* spp. cysts after treatment with effective microorganisms (EM). J. Parasit. Dis. 40, 369–373.

Schroeder, J.M., Booton, G.C., Hay, J., Niszl, I.A., Seal, D.V., Markus, M.B., Fuerst, P.A., Byers, T.J., 2001. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *acanthamoeba*e from humans with keratitis and from sewage sludge. J. Clin. Microbiol. 39, 1903–1911.

Schuster, F.L., Visvesvara, G.S., 2004. Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. Int. J. Parasitol. 34, 1001–1027.

Shirwadkar, C.G., Samant, R., Sankhe, M., Deshpande, R., Yagi, S., Schuster, F.L., Sriram, R., Visvesvara, G.S., 2006. *Acanthamoeba* encephalitis in patient with systemic lupus, India. Emerg. Infect. Dis. 12, 984.

Shoff, M., Rogerson, A., Kessler, K., Schatz, S., Seal, D., 2008. Prevalence of *Acanthamoeba* and other naked amoebae in South Florida domestic water. J. Water Health 6, 99–104.

Siddiqui, R., Khan, N.A., 2015. Balamuthia mandrillaris: morphology, biology, and virulence. Trop. Parasitol. 5, 15.

Solgi, R., Niyyati, M., Haghighi, A., Taghipour, N., Tabaei, S.J.S., Eftekhar, M., Nazemalhosseini Mojarad, E., 2012. Thermotolerant *Acanthamoeba* spp. isolated from therapeutic hot springs in Northwestern Iran. J. Water Health 10, 650–656.

Solhjoo, K., Ghadar-ghadr, S., Zia-Jahromi, S., 2012. Identification of *Acanthamoeba* species in water sources by PCR. J. Jahrom Univ. Med. Sci. 10, 33–41.

Stockman, L.J., Wright, C.J., Visvesvara, G.S., Fields, B.S., Beach, M.J., 2011. Prevalence of *Acanthamoeba* spp. and other free-living amoebae in household water, Ohio, USA—1990–1992. Parasitol. Res. 108, 621–627.

Tanveer, T., Hameed, A., Muazzam, A.G., Jung, S.-Y., Gul, A., Matin, A., 2013. Isolation and molecular characterization of potentially pathogenic *Acanthamoeba* genotypes from diverse water resources including household drinking water from Khyber Pakhtunkhwa, Pakistan. Parasitol. Res. 112, 2925–2932.

Visvesvara, G.S., Moura, H., Schuster, F.L., 2007. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., Balamuthia mandrillaris, Naegleria fowleri, and Sappinia diploidea. FEMS Immunol. Med. Microbiol. 50, 1–26.

Zhao, G., Sun, S., Zhao, J., Xie, L., 2010. Genotyping of *Acanthamoeba* isolates and clinical characteristicsof patients with *Acanthamoeba* keratitis in China. J. Med. Microbiol. 59, 462–466.