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# Isolation and genotyping of *Acanthamoeba* species and Vahlkampfiidae in the harsh environmental conditions in the centre of Iran

Azam Pourabbasi Ardekan[a], Maryam Niyyati[a,*], Reza Saberi[b], Leyli Zanjirani Farahani[a] and Marziye Fatemi[a]

[a] Department of Medical Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Toxoplasmosis Research Center, Communicable Diseases Institute, Mazandaran University of Medical Sciences, Sari, Mazandaran, Iran
*Corresponding author. E-mail: maryamniyati@yahoo.com

## ABSTRACT

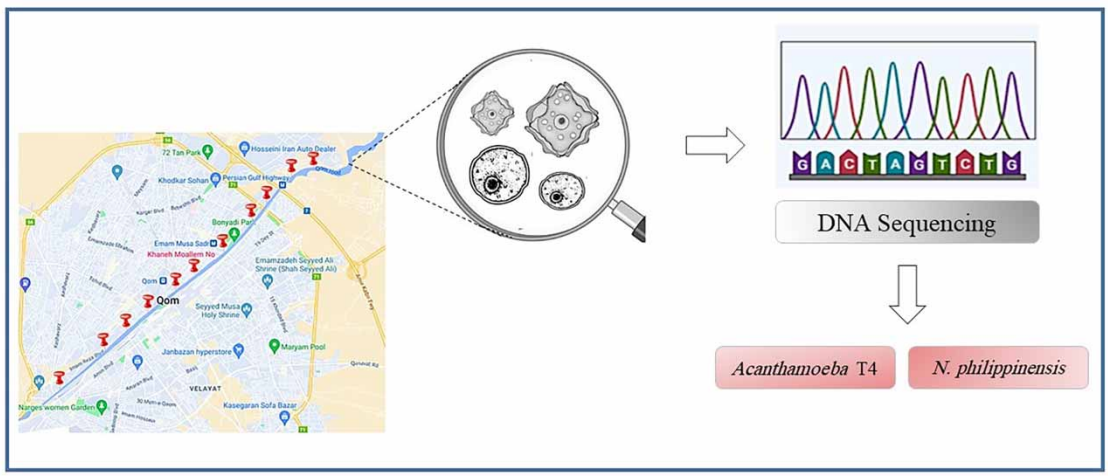
Different species of free-living amoeba (FLA) have been abundantly isolated in harsh environmental conditions such as hot springs and brackish water. The present study aimed to isolate, genotype, and evaluate the pathogenicity of FLAs in Qom Roud, a large river, in the centre of Iran. About 500 mL of water samples ($n = 30$) were collected from each sampling site and were investigated for the presence of FLAs using morphological and molecular characters. Genotype identification was performed using DNA sequencing and a phylogenetic tree was constructed with the MEGA X software. The pathogenic potential of all positive isolates was evaluated using the tolerance ability test. Morphological and molecular analysis indicated that 14 (46.66%) and two (6.66%) water samples were positive for *Acanthamoeba* species and Vahlkampfiidae, respectively. According to sequence analysis, *Acanthamoeba* isolates related to the T4 genotype and Vahlkampfiidae sequences were similar to *Naegleria philippinensis*. In the next step, thermo- and osmotolerance tests indicated four *Acanthamoeba* strains are extremely pathogenic. Our data showed the presence of potentially pathogenic *Acanthamoeba* T4 genotype and *N. philippinensis* in the super harsh Qom Roud. Contamination of water with virulent T4 genotype of *Acanthamoeba* may pose risk factors for contact lens users, children, and immunocompromised people.

**Key words:** *Acanthamoeba*, genotyping, Iran, Vahlkampfiidae

## HIGHLIGHTS

- Pathogenic of *Acanthamoeba* spp. detected in the super harsh environment.
- Fourteen T4 genotypes were identified based on the analysed sequences of the 18S rRNA gene.
- Two (6.66%) of water samples were positive for *Naegleria philippinensis*.

## GRAPHICAL ABSTRACT



## 1. INTRODUCTION

Free-living amoebae (FLAs) are opportunistic and pathogenic protozoans that live independently in soil or aquatic habitats (Saburi *et al.* 2017; Fabros *et al.* 2021). They belong to the genera *Acanthamoeba*, *Balamuthia*, *Naegleria*, *Vermamoeba*, and *Sappinia* (Trabelsi *et al.* 2012). These amoebae are potentially pathogenic to humans and cause symptoms of meningoencephalitis, encephalitis, and acanthamoeba keratitis (AK) (Lorenzo-Morales *et al.* 2015; Król-Turmińska & Olender 2017). In recent years, most studies have focused on identifying and determining the genotype of *Acanthamoeba* species, because previous studies have shown that the incidence rate of AK has increased in Iran and these cases continue to rise (Haddad *et al.* 2019; Hajialilo *et al.* 2019). In addition to AK, the medical importance of *Acanthamoeba* species is related to the infection of other organs of the brain, urinary tract, and skin (Kot *et al.* 2021; Saberi *et al.* 2021; Park *et al.* 2023). It should be noted that only one primary amoebic meningoencephalitis with the causative agent *Naegleria fowleri* has been reported in Iran (Movahedi *et al.* 2012).

*Acanthamoeba* species were initially divided into three morphological groups (I, II, and III) based on the size and structure of ecto- and endocyst (Pussard 1977). The genus *Acanthamoeba* was classified with 23 genotypes (T1–T23) based on the analysis of the small subunit of the ribosomal RNA gene (SSU-18S-rRNA) (Corsaro 2022). According to shreds of evidence, genotypes of *Acanthamoeba* species in Iran include T2, T3, T4, T5, T11, T13, and T15 (Spotin *et al.* 2017). The T4 genotype is the most common genotype identified in clinical and environmental samples (Maciver *et al.* 2013).

Qom Province, located in the central part of Iran, has several tourist attractions due to the second destination of religious tourism, palaces, historical villages, mosques, gardens, and the famous Salt Lake Desert. The Qom River or Qom Roud is a large river (approximately 400 km) that receives its water from the Zagros Mountains and mounds into the Namak Lake in Qom Province. In the summer season, people go around the Qom Roud for fun and an exciting time. One of the most popular activities during spring and summer is swimming, which brings great joy to children. As regards the amoeba being resistant to harsh conditions, the present research was conducted to identify and determine the genotype of FLAs in the Qom Roud in Qom Province.

## 2. MATERIAL AND METHODS

### 2.1. Sampling and amoeba culture

Water samples ($n = 30$) were collected from different sites such as the shrine parking, recreation and camp, bathing, and swimming places (Figure 1). About 500 mL of water samples were filtered using nitrocellulose filters (pore size: 0.45 μm; Sigma-Aldrich, USA). The filters were cultured onto a 1.5% non-nutrient agar culture medium (Difco, Sparks, MD, USA) enriched with heat-inactivated *Escherichia coli* (ATCC 25922), as a food source for amoebae outgrowth. The plates were incubated at room temperature (23–26 °C) (Khan *et al.* 2001). Later the growth plates by reverse-phase microscope were monitored daily for the detection of growth and proliferation of amoeba for up to a month.

### 2.2. Physicochemical features of water

The School of Public Health's laboratory investigated the physicochemical features of water samples, and they provided us with the values. A pH meter was used to measure pH, a conductivity meter device was used to measure EC and TDS, and a Flame photometer device was used to measure $Na^+$ and $K^+$. It should be noted that the titration method was used to measure TH, $HCa^+$, HMg, $Cl^-$, and ALK. Finally, $NO_3^-$, $SO_4^{2-}$, and $PO_4^{2-}$ parameters were measured using a DR 5000™ UV–Vis Spectrophotometer.

### 2.3. Morphological identification

According to Page (1988), isolated amoebae were identified at the genus level. *Acanthamoeba* cysts were identified by the presence of a double wall and consisted of ectocyst and endocyst, or trophozoites by the presence of acanthopodia. Vahlkampfiidae cysts are spherical, containing a single nucleus and a double wall with pores and wormy-shaped trophozoites.

### 2.4. DNA extraction and molecular analysis

All positive morphological plates were subjected to the molecular method. DNA was extracted using a DNA extraction kit (Yekta Tajhiz Azma, Tehran, Iran) according to the manufacturer's instructions. Two sets of specific primers in order to amplify *Acanthamoeba* species (Forward: 5′-GGCCCAGATCGTTTACCGTGAA-3′ and Reverse: 5′-TCTCACAAGCTGCTAGGG AGTCA-3′) (Schroeder *et al.* 2001) and Vahlkampfiidae (Forward: 5′-GAACCTGCGTAGGGATCATTT-3′ and Reverse: 5′-TT

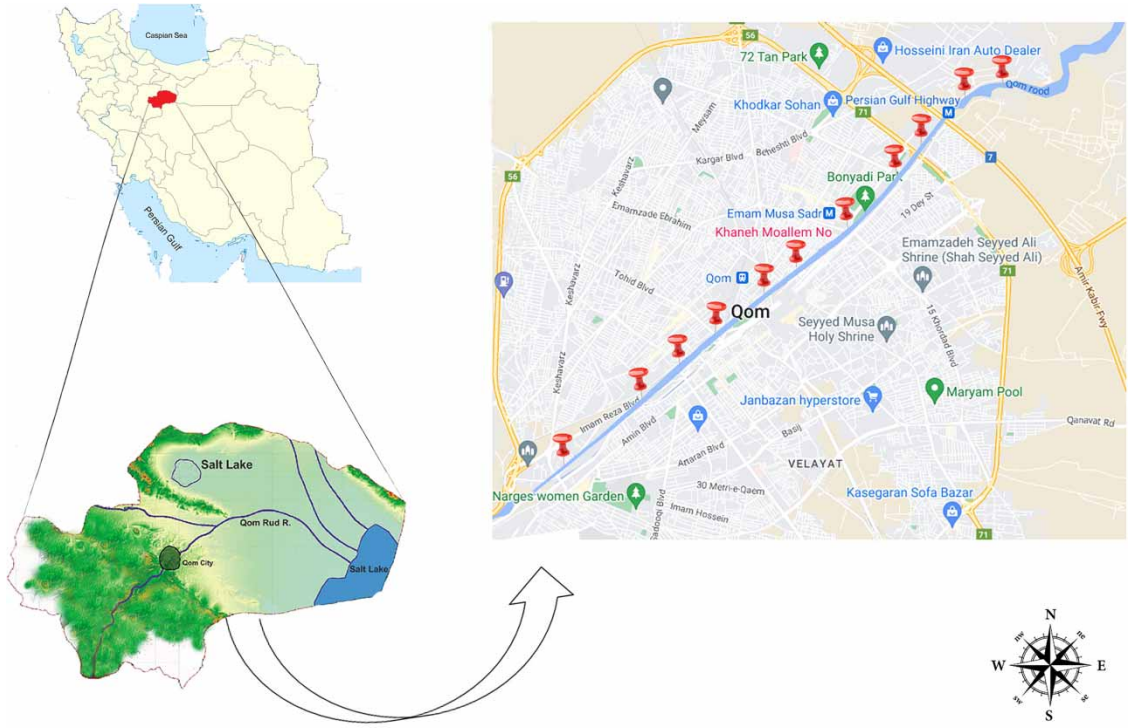



**Figure 1** | Mapping of sampling sites of Qom Roud for detection of free-living amoeba.

TCTTTTCCTCCCCTTATTA-3′) were used as previously described (Pélandakis & Pernin 2002). Amplification reactions were set for a total volume of 25 µl, containing 12.5 µl master mix (Ampliqon Co., Denmark), 1 µl forward and reverse primers (10 pmol), 3 µl DNA templates (70 ng), and 8.5 µl nuclease-free water. The temperature program included a first cycle of denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 45 s, annealing steps at 56 °C for 1 min (for both *Acanthamoeba* and Vahlkampfiidae), and extension at 72 °C for 45 s, then a final extension step at 72 °C for 5 min. The PCR products were confirmed by imaging on 1.5% agarose gel (Invitrogen, Life Technologies GmbH, Leipzig, Germany) stained with SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, MA, USA) (Javanmard *et al.* 2017). Positive and negative controls always monitored the reactions.

### 2.5. Sequencing and phylogenetic analysis

PCR products of the *Acanthamoeba* and Vahlkampfiidae isolates were purified and sequenced using the Sanger sequencing method in both directions, by Pishgam Biotech Company, Iran. Different software used for genetic data analysis are as follows. Chromas version 2.6, for viewing and editing chromatograms from sequences, BioEdit, version 7.0.5 used for multiple alignments, and BLAST (http://blast.ncbi.nlm.nih.gov) for finding sequence similarity with GenBank sequences, and MEGA X for drawing the phylogenetic tree. To estimate phylogenetic trees, we selected the Neighbor-Joining method using Kimura 2-parameter models in MEGA X. In addition, bootstrap resampling analysis (1,000 replications) was used to assess branch confidence in clades in each tree (Kumar *et al.* 2018). Furthermore, *Balamuthia mandrillaris* was used as an outgroup to construct the phylogenetic tree.

### 2.6. Thermotolerance and osmotolerance tests

To determine the pathogenic potential of the positive isolates, thermal and osmotolerance tests were used following the protocol of Khan *et al.* (2001). Thermotolerance of *Acanthamoeba* was measured as survival at two temperatures (37 and 44 °C) and osmotolerance was determined as the capacity of the culture to D-Mannitol (Merck, Darmstadt, Germany) in two plates at different molarities (0.5 and 1 M).

## 3. RESULTS

### 3.1. Physical and chemical properties

The value of the physicochemical features of water samples in the sampling sites is tabulated in Table 1.

### 3.2. Morphological and molecular identification

Based on the morphological criteria for FLAs, out of 30 water samples, 14 (46.66%) and two (6.66%) samples were positive for *Acanthamoeba* species and Vahlkampfiidae amoeba, respectively. This was subsequently confirmed by molecular test. *Acanthamoeba* DNA was detected in all 14 morphologically positive samples in the PCR assay; a single approximately 500 bp band of the 18S rRNA gene was amplified, which was consistent with the product size of *Acanthamoeba* species, also, a 460 bp of the ITS gene corresponding to Vahlkampfiidae was observed in two samples (Table 2). BLAST analysis demonstrated that *Acanthamoeba* isolates belonged to the T4 ($n = 14$), and two Vahlkampfiidae isolates with codes R4 and R9 were identified as *Naegleria philippinensis*. Sequences were 95–99% similar to the partial sequence *A. culbertsoni* (Acc. No: MH791017), *A. polyphaga* (Acc. No: MK713916.1) and *N. philippinensis* (Acc. No: LC191904.1) isolated from Malaysia, India, and Iran (Figures 2 and 3). Two *N. philippinensis* strains labelled R4 and R9 exhibited high identity with *N. philippinensis* isolated in water from Ahvaz, southwest Iran. The new sequences were submitted to the genetic sequence database using BankIt (Accession numbers: OQ159094–OQ159107 and OQ165120–OQ165121).

### 3.3. Thermotolerance and osmotolerance

Interestingly, four *Acanthamoeba* strains (R23, R24, R26, and R28) possessed the ability to grow at both 37 and 44 °C and both osmolarity (0.5 and 1 M), which these isolates were considered as highly pathogenic amoebae. On the other hand, *Acanthamoeba* species growth at a temperature of 37 °C and 0.5 M osmolarity is classified as a strain with low pathogenicity potential (Table 2).

## 4. DISCUSSION

Since the first discovery of the genus *Acanthamoeba* over nine decades ago (Castellani 1930), subsequently increasing number of species of *Acanthamoeba* have been globally reported. The clinical significance of *Acanthamoeba* species should be considered in particular (Marciano-Cabral & Cabral 2003). AK is a painful and sight-threatening infection that negatively affects a patient's quality of life (Varacalli *et al.* 2021). Recently, *Acanthamoeba* species have been detected in the bronchoalveolar lavage fluid from immunocompetent patients with chronic respiratory disorders, and urine samples

**Table 1** | The amounts of each recorded physicochemical feature in the studied location during sampling

| Parameter | Unit | Result | Optimal maximum | Maximum allowed |
|---|---|---|---|---|
| pH | µS/cm | 7.37 | – | 6.5–8.5 |
| EC | mg/l | 1,540 | 250 | 400 |
| TDS | mg/l $CaCO_3$ | 4,616 | 1,000 | 1,500 |
| TH | mg/l $CaCO_3$ | 1,600 | 200 | 500 |
| $HCa^+$ | mg/l $CaCO_3$ | 960 | 300 | – |
| HMg | mg/l | 640 | 30 | – |
| $NO_3^-$ | mg/l | 1.1 | – | 50 |
| $SO_4^{2-}$ | mg/l | 1,380 | 250 | 400 |
| $Na^+$ | mg/l | 1,100 | 200 | 200 |
| $K^+$ | mg/l | 14.3 | – | 12 |
| $Cl^-$ | mg/l | 1,540 | 250 | 400 |
| ALK | mg/l $CaCO_3$ | 248 | – | – |
| $PO_4^{2-}$ | mg/l | 0.8 | – | – |
| Saltiness | mg/l | 2,782 | – | – |

**Table 2** | Data regarding the positive strains of *Acanthamoeba* and Vahlkampfiidae isolated from Qom Roud in Qom Province, Iran

| Code | Genera | Genotype/species | Thermotolerance 37/41 | Osmotolerance 0.5/1 | Temperature (°C) | Sampling location |
|---|---|---|---|---|---|---|
| R3 | *Acanthamoeba* | T4 | +/− | +/− | 28 | Out of reach |
| R4 | *Naegleria* | *N. philippinensis* | +/− | −/− | 30 | Recreation and camp |
| R5 | *Acanthamoeba* | T4 | +/+ | −/− | 32 | Recreation and camp |
| R6 | *Acanthamoeba* | T4 | +/+ | +/− | 29 | Recreation and camp |
| R8 | *Acanthamoeba* | T4 | −/− | −/− | 28 | Children's swimming |
| R9 | *Naegleria* | *N. philippinensis* | +/− | +/− | 27 | Recreation and camp |
| R14 | *Acanthamoeba* | T4 | +/− | −/− | 30 | Out of reach |
| R15 | *Acanthamoeba* | T4 | +/− | −/− | 32 | Recreation and camp |
| R18 | *Acanthamoeba* | T4 | +/+ | +/− | 29 | Adjacent to the shrine parking |
| R20 | *Acanthamoeba* | T4 | +/+ | −/− | 29 | Recreation and camp |
| R23 | *Acanthamoeba* | T4 | +/+ | +/+ | 26 | Recreation and camp |
| R24 | *Acanthamoeba* | T4 | +/+ | +/+ | 28 | Children's swimming |
| R26 | *Acanthamoeba* | T4 | +/+ | +/+ | 30 | Adjacent to the shrine parking |
| R27 | *Acanthamoeba* | T4 | +/− | +/− | 32 | Recreation and camp |
| R28 | *Acanthamoeba* | T4 | +/+ | +/+ | 29 | Children's swimming |
| R30 | *Acanthamoeba* | T4 | +/+ | −/− | 28 | Adjacent to the shrine parking |

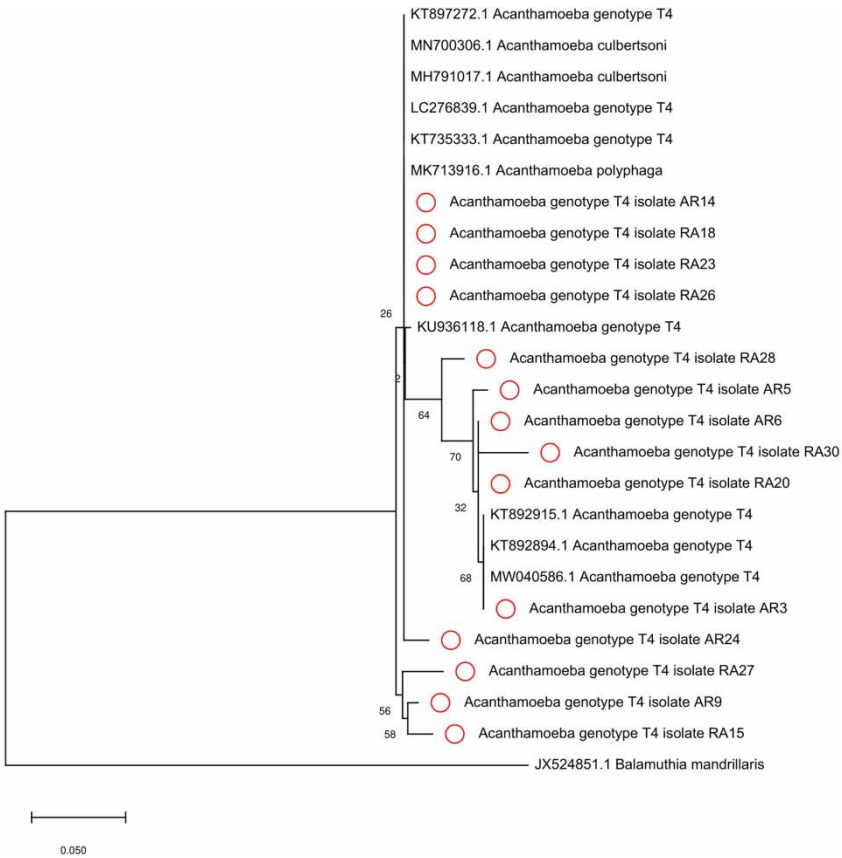



**Figure 2** | Phylogenetic tree of sequences of *Acanthamoeba* T4 genotype in the present study (red circles) and other reference sequences obtained from GenBank.

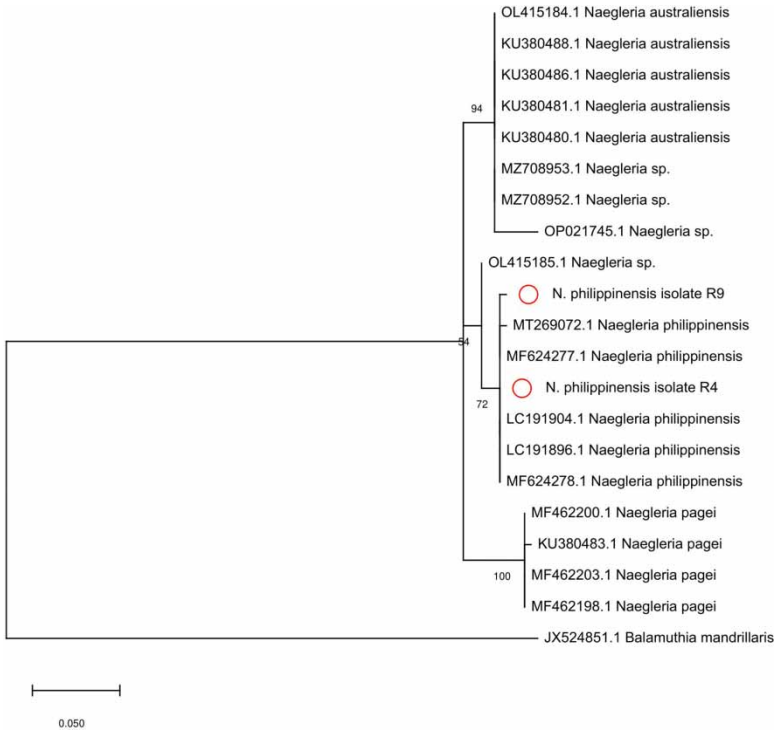



**Figure 3** | Phylogenetic tree of sequences of *N. philippinensis* in the present study (red circles) and other reference sequences obtained from GenBank.

were collected from patients presenting with recurrent urinary tract infections (UTIs) (Saberi *et al.* 2021, 2022). The prevalence of *Acanthamoeba* species in the water represents a sanitary risk for humans. Numerous studies have shown that the *Acanthamoeba* species has been reported abundantly in different environmental sources in Iran (Karamati *et al.* 2016; Javanmard *et al.* 2017; Norouzi *et al.* 2021). The present study shows the presence of *Acanthamoeba* species and Vahlkampfiidae in harsh environmental conditions. The Qom Roud is a large river in Iran that receives its water from the Zagros Mountains and mounds into Namak Lake. The name Namak Lake is derived from the high salinity of its water. Therefore, the results of this research indicate the presence of *Acanthamoeba* species and Vahlkampfiidae in difficult environmental conditions with high salinity.

It seems that *Acanthamoeba* is the most predominant protozoa present in the environment. We reported that 46.66 and 6.66% of isolates were positive for *Acanthamoeba* species and Vahlkampfiidae, respectively. This finding is in line with the Latifi *et al.* study, in which Vahlkampfiidae (45.45%) and *Acanthamoeba* (40.9%) were isolated from hot springs of Mazandaran province, northern Iran (Latifi *et al.* 2014). A similar observation was made earlier, where *Acanthamoeba* species (50%) were detected in geothermal rivers in southwestern Iran (Niyyati *et al.* 2016). It seems that the relatively high abundance of *Acanthamoeba* species in harsh environmental conditions, such as hot springs or high salinity, is due to the presence of a resistant cyst form in the life cycle of the parasite (Aksozek *et al.* 2002).

Genotyping of *Acanthamoeba* helps to identify the different species of this protozoan (Megha *et al.* 2023). In the current study, the isolates were identified as T4 genotypes by phylogenetic analysis, an approach proven to be useful for the molecular characterization of *Acanthamoeba* species. Moreover, the phylogenetic analysis of the current *Acanthamoeba* T4 genotype and *N. philippinensis* sequences was similar to those of reference isolates. The sequences in the phylogenetic tree were grouped distinctly into two main clusters, one containing the *Acanthamoeba* and *N. philippinensis* sequences, as we expected. Interestingly, the current sequences of the T4 genotype showed several different nucleotides in distinct *Acanthamoeba* isolates. This aspect indicates the genetic variation of the T4 genotype (Megha *et al.* 2023). Generally, *Acanthamoeba* genotyping offers valuable information in studying their taxonomy, drug susceptibility, molecular epidemiology, and clinical studies (Ledee *et al.* 2009; Fuerst 2014).

In the end, according to thermo- and osmotolerance tests, four *Acanthamoeba* isolates also demonstrated pathogenic potential. Thermo- and osmotolerance experiments are important in comprehending the environmental and physiological features of *Acanthamoeba*, as well as their potential to cause infections (Khan *et al.* 2001). However, it is suggested that more research should be carried out to determine pathogenicity (Mirjalali *et al.* 2013).

## 5. CONCLUSION

The current findings serve as a document for the presence of *Acanthamoeba* species and Vahlkampfiidae amoeba in the salty water of Qom Roud. *Acanthamoeba* isolates belonging to the T4 genotype, which is implicated in the majority of *Acanthamoeba* infections. This river is related directly to human populations and further investigation is needed for genotype distribution, identification, training, and prevention.

## AUTHORS CONTRIBUTION

All authors contributed to the study conception and design. A.P.A, L.Z.F., and M.F. carried out the experiment. R.S. wrote the manuscript with support from M.N. All authors read and approved the final manuscript.

## DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

## CONFLICT OF INTEREST

The authors declare there is no conflict.

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