Isolation and identification of *Acanthamoeba* genotypes and *Naegleria* spp. from the water samples of public swimming pools in Qazvin, Iran

Nastaran Paknejad, Elham Hajialilo, Mehrzad Saraei and Amir Javadi

ABSTRACT

Free-living amoeba (FLA), including *Acanthamoeba* and *Naegleria* are facultative parasites in humans. The amoeba have widespread distribution in various water sources. The aim of this study was isolation and molecular identification of *Acanthamoeba* and *Naegleria* isolated from swimming pools and also hot and cold tub waters in Qazvin province. The samples (166 water samples) were cultured to isolate and identify positive specimens. PCR (polymerase chain reaction) amplification, sequencing and phylogenetic analysis were conducted to confirm the isolated species and genotypes of amoeba. According to morphological characterizations, 18.6% of specimens were identified as FLA, which in 71% were *Acanthamoeba* by PCR method. Molecular analysis revealed that 36.3%, 18.1% and 4.5% of *Acanthamoeba* specimens were identified as T3, T4 and T11 *Acanthamoeba* genotypes, respectively. *Protacanthamoeba bohemica* (27.2%) and *Acanthamoeba* sp. (4.5%) were found among the specimens. The results of osmo-tolerance and thermo-tolerance assays demonstrated that 50% of T3 and 25% of T4 genotypes of *Acanthamoeba* were highly pathogenic parasites. The molecular approach showed the presence of *Naegleria lovaniensis* (9%) in hot tub water of swimming pools. This study demonstrated that the swimming pools and hot tub water in Qazvin province were contaminated with *Acanthamoeba* and *Naegleria* species.

Key words | *Acanthamoeba*, free-living amoeba, genotype, Iran, *Naegleria*

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INTRODUCTION

Free-living amoeba (FLA) are opportunistic protozoan parasites and some FLA, such as *Acanthamoeba*, *Naegleria*, *Balamuthia* and *Sappinia* are considered as pathogenic amoeba (Visvesvara *et al.* 2007; Henriquez & Khan 2009; Yousuf *et al.* 2013). The FLA can cause severe and even fatal diseases by invasion and causing damage to the central nervous system (CNS) and other organs. A wide range of diseases including amoebic encephalitis, keratitis and skin ulcerations have been reported in both immunocompetent and immune-deficient individuals (Khan 2006; Henriquez & Khan 2009). Moreover, the number of cases of amoebic keratitis (AK) in Iran has increased in the recent decade which is probably due to poor hygiene among contact lens users (Rezaeian *et al.* 2007). The amoeba can be the reservoir and vehicle of the microbial world, in particular pathogenic bacteria present in nature as they can transfer such microorganisms to humans, leading to enhanced amoeba infection (Rezaeian *et al.* 2008; Siddiqui & Khan 2012; Buse *et al.* 2016).

Amoebae have widespread distribution in different environments including water, soil, dust and air sources. Various water sources, such as pool, river and recreational waters can be contaminated with FLA. Thermal water was described as a suitable niche for *Naegleria* spp., therefore,

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water sources are considered as an important risk factor for amoebic disease (Visvesvara *et al.* 2007; Yousuf *et al.* 2013; Di Filippo *et al.* 2017; Xuan *et al.* 2017). It is obvious that swimming or bathing while wearing contact lenses leads to increased risk of AK.

Vegetative trophozoites and resistant cysts of FLA are widely distributed in nature. In harsh conditions, amoeba can be enclosed by a double-walled cyst structure. The cysts of amoeba withstand different disinfectant solutions, unpleasant environmental conditions such as severe dryness, extremes in temperatures, pH and improper osmolarity (Khan 2006).

Up to now, 20 genotypes (T1–T20) of *Acanthamoeba* have been found in environmental and clinical specimens, with T4 genotype as the most common genotype that demonstrates higher pathogenicity among patients (Khan 2006; Nuprasert *et al.* 2010; Corsaro *et al.* 2015). To date, several genotypes including T2, T3, T4, T5, T9, T11 and T13 have been reported from different parts of Iran (Nazar *et al.* 2011; Rahdar *et al.* 2012; Solgi *et al.* 2012; Behniafar *et al.* 2015; Hajialilo *et al.* 2016). There are various species of *Naegleria* such as *N. fowleri, N. australiensis, N. lovaniensis*, *N. italica*, etc., but among those only *N. fowleri* is a true pathogen capable of causing primary amoebic meningoencephalitis (PAM) in individuals. In addition, *N. lovaniensis* is the only non-pathogenic thermophilic species of *Naegleria,* with a close evolutionary relationship to *N. fowleri* (Carter 1968; Di Filippo *et al.* 2017). Several species of *Naegleria* including *N. fowleri, N. australiensis, N. pagei, N. gruberi, N. americana, N. dobsoni, N. clarki* and *N. fultoni* have been found in different sources of water and among patients in Iran (Movahedi *et al.* 2012; Niyyati *et al.* 2012, 2015; Javanmard *et al.* 2017; Latifi *et al.* 2017).

Numerous studies have been conducted on different sources of water such as tap water, hot and mineral spring waters, however, few researches were focused on swimming pool water with Jacuzzi tubs. Since no previous study was based on such water sources, the possible presence and distribution of FLA in public swimming pool water sources of Qazvin province, the current study aimed to identify and genotype the FLA isolated from swimming pool water sources and Jacuzzi tubs within Qazvin province.

METHODS

Pool water sampling

This cross-sectional study was carried out from December 2016 to April 2017. In total, 166 water samples were collected from swimming pools and Jacuzzi tubs in Qazvin province. The province is located in the northern margin of central Iran. Water samples were collected from the surface and floor of target swimming pools, hot and cold tubs several times. At the time of study, there were 17 public swimming pools, and among those, 12 voluntarily participated in the study. A total of ten samples were randomly collected from each swimming pool (including five surface water samples and five deep water samples). One water specimen was also taken from hot and cold tubs, separately. The water samples were gathered into 500 mL sterile flasks, and then transferred to the Department of Medical Parasitology and Mycology at the School of Medicine, Qazvin University of Medical Sciences, Iran. Calcium hypochlorite was used on a daily basis for disinfecting the swimming pools.

Isolation of Acanthamoeba and Naegleria

Water specimens were filtered through nitrocellulose membrane filters (0.45 μm pore size). The membranes were cultured on 1.5% non-nutrient agar (NNA) plates, seeded with heated inactivated suspension of *Escherichia coli* bacteria. The plates were incubated at 30 °C for up to 15 to 30 days to grow amoeba, followed by daily examination of the cultures under light microscope to observe the presence of trophozoites and cysts of amoeba (Solgi *et al.* 2012). The positive plates were cloned several times to eliminate bacterial and fungal contamination (Lorenzo-Morales *et al.* 2005; Behnia *et al.* 2017).

Pathogenicity tests' positive plates of *Acanthamoebae* were selected for osmo-tolerance and thermo-tolerance assays. Bactoagar medium with two concentrations of mannitol (0.5 M and 1 M) were used for osmo-tolerance assay while thermo-tolerance assay evaluated the growth ability of *Acanthamoebae* at two different temperatures of 37 and 42 °C. All plates were monitored daily for a week. Controls

were applied to prevent likely errors (Khan 2001; Hajialilo *et al.* 2016).

DNA extraction and PCR amplification

Trophozoites and cysts of amoebae were collected from the plates using phosphate buffered saline (PBS), at a pH of 7.2, followed by treating the FLA with lysozyme and glass beads. High pure polymerase chain reaction (PCR) Template Preparation Kit (Roche, Mannheim, Germany) was used for DNA extraction. PCR reaction was set up with specific primers for *Acanthamoeba*, JDP1 5′-GGCCCAGATCGTTTACCGTGAA-3′and JDP2 5′-TCTCACAAGCTGCTAGGGAGTCA-3′ to amplify the 500 bp length fragment within the 18S rRNA gene region.

The primers used for *Naegleria* were designed by Beacon Designer7 and Primer-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and included NA1 5′-AACCTGCGTAGGGATCAT-3′ and NA2 5′-TTTTCTTTTCCTCCCCTTAT-3′ which amplified an approximately 400 bp piece. PCR reaction for both *Acanthamoeba* and *Naegleria* were performed in a total volume of 30 µL containing a ready-made mixture of Amplicon (Taq DNA Polymerase Master Mix RED, Denmark), template DNA, 0.1 µM of each primer along with distilled water. The cycling conditions for *Acanthamoeba* were adjusted according to Lasjerdi *et al.* (2011), with some modifications as follows: initial denaturing phase of 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, annealing at 64 °C for 45 s and 72 °C for 45 s, and a final extension step at 72 °C for 7 min (Lasjerdi *et al.* 2011). The thermal cycling conditions used for *Naegleria* were initial denaturing phase at 95 °C for 5 min, followed by 35 cycles at 95 °C for 20 s, annealing phase at 57 °C for 20 s and at 72 °C for 30 s, and a final extension step at 72 °C for 5 min. Electrophoresis and staining with ethidium bromide were performed to visualize the specific band under UV light.

Sequencing analysis

Purification and sequencing of PCR products were conducted using an automatic ABI3130 sequencer machine (Applied Biosystems, USA). The DNA sequences were edited by Chromas (Version 1.0.0.1), and compared to BLAST GenBank database against eukaryotic sequences. The sequences obtained in our experiments were deposited in the GenBank database under the Accession numbers *Acanthamoeba*, *Protacanthamoeba* and *Naegleria*: MH024474-MH024493, MH304644 and MH304645. The phylogenetic tree was constructed using the MEGA7 software; maximum-likelihood algorithm with Tamura-3 parameter substitution model was applied. Bootstrap analysis was performed based on 1,000 replications. *Balamuthia mandrillaris* sequence was used in the dendrogram as an outgroup (Figure 1) (Table 1).

RESULTS

In total, 18.6% (31/166) of water samples collected from the swimming pools were positive for FLA. While both

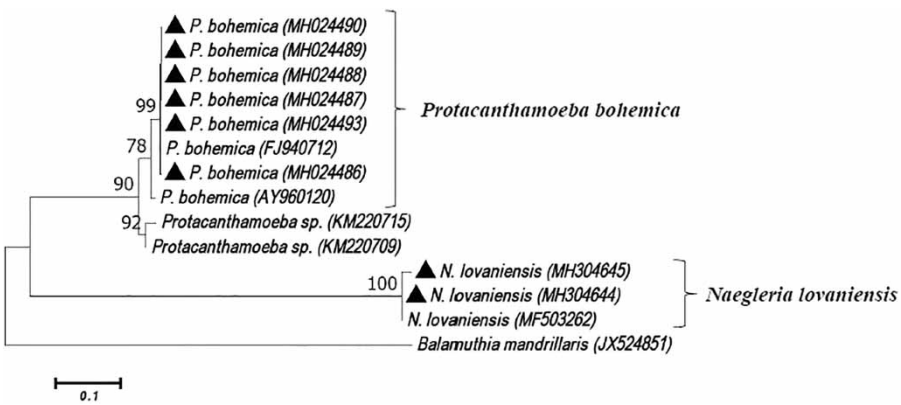

Figure 1 | Phylogenetic tree of *Protacanthamoeba bohemica* and *Naegleria lovaniensis* isolates collected from water sources in Qazvin, Iran. *P. bohemica* and *N. lovaniensis* were clarified in the present study (▲), a close intra-specific proximity was seen among the isolates of *P. bohemica* and *N. lovaniensis* with the reference sequences.

Table 1 | *Protacanthamoeba* and *Naegleria* species from Qazvin province, Iran and origins of sequences used for phylogenetic analyses (Figure 1)

No.	Species	Accession No.	Sources	References
1	*P. bohemica*	MH024486	Water	Present study
2	*P. bohemica*	MH024487	Water	Present study
3	*P. bohemica*	MH024488	Water	Present study
4	*P. bohemica*	MH024489	Water	Present study
5	*P. bohemica*	MH024490	Water	Present study
6	*P. bohemica*	MH024493	Water	Present study
7	*N. lovaniensis*	MH304645	Water	Present study
8	*N. lovaniensis*	MH304644	Water	Present study
9	*P. bohemica*	FJ940712	Water	Gianinazzi *et al.* (2009b)
10	*P. bohemica*	AY960120	*Tinca tinca*	Dyková *et al.* (2005)
11	*Protacanthamoeba* spp.	KM220709	Water	Delafont *et al.* (2014)
12	*Protacanthamoeba* spp.	KM220715	Water	Delafont *et al.* (2014)
13	*N. lovaniensis*	MF503262	Water	Di Filippo *et al.* (2017)
14	*Balamuthia mandrillaris*	JX524851	Human	Roy *et al.* (2015)

Acanthamoeba and *Protacanthamoeba* were found to have contaminated the surface and deep water specimens, no *Naegleria* was detected in water samples of the swimming pools. Out of 14 water specimens collected from hot Jacuzzis, 14% (2/14) were contaminated with *Naegleria* spp. while no amoeba was found in cold Jacuzzis. Double-walled cysts, based on irregular inner layer of cyst (*Acanthamoeba*) and round shape cyst of *Naegleria* are shown in Figure 2. Positive specimens were examined by PCR and showed specific bands of *Acanthamoeba* and *Naegleria*. All 22 culture positive specimens showed bands on agarose gel. According to molecular analysis, 63.6% (14/22), 27.3% (6/22) and 9% (2/22) samples were *Acanthamoeba* (T3, T4 and T11 genotypes, *Acanthamoeba* spp.), *P. bohemica* and *Naegleria* species, respectively. The sequences detected for both *N. lovaniensis* isolates QIN140 (MH304644) and QIN163 (MH304645) demonstrated 100% homology to the reference genes in the GenBank database. According to the results obtained for pathogenicity assays, 35% (7/20) of the *Acanthamoeba* and *Protacanthamoeba* isolates revealed high pathogenicity, among those 50% (4/8) belonged to T3 genotype, 25% (1/4) to T4 genotype and

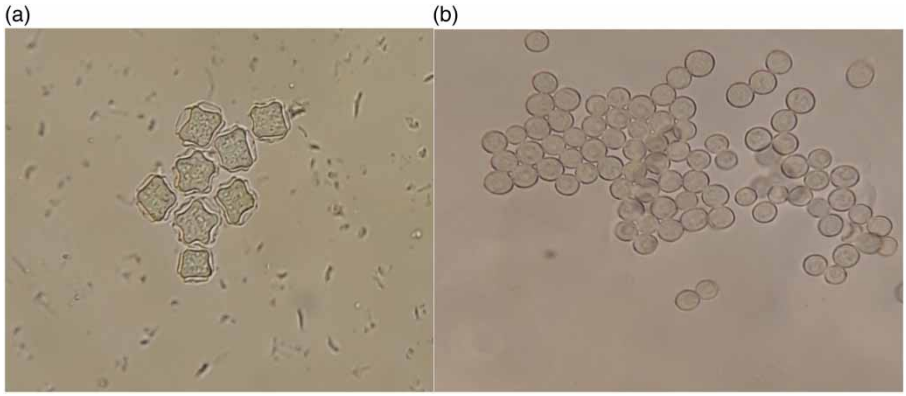

Figure 2 | Light microscopy photographs of (a) *Acanthamoeba* and (b) *Naegleria* cysts (magnification × 400).

33% (2/6) related to *P. bohemica*. Other isolates of *Acanthamoeba* were found to be of low pathogenicity (Table 2). The *P. bohemica* species of the present study were in a cluster with other species of the *Protacanthamoeba* and reference sequence of *Protacanthamoeba bohemica*, therefore *N. lovaniensis* species were in a separate cluster along with the reference sequence of *Naegleria lovaniensis* (Figure 1) (Table 1).

DISCUSSION

The results of this study demonstrated *Acanthamoeba* T3, T4 and T11 genotypes, as well as *Protacanthamoeba* species in the public swimming pools of Qazvin province. T3 genotype was identified as the dominant genotype of this protozoan parasite in the area. Comparison of molecular studies of FLA in different countries shows geographical variation among the species and genotypes of FLA specimens collected from public swimming pools in different regions of the world. For instance, six species of *Acanthamoeba* including *A. polyphaga, A. mauritaniensis, A. castellanii, A. royreba, A. triangularis* and *A. rhysodes* (Al-Herrawy *et al.* 2014) and three genotypes of *Acanthamoeba* (T3, T4 and T5 genotypes) have been reported from Egypt and Brazil (Caumo & Rott 2011), respectively. *Acanthamoeba* spp. and *Naegleria* spp. were also reported in swimming pools in Malaysia (Init *et al.* 2010). A survey on heated indoor swimming pools in Switzerland detected *A. lenticulata* (Gianinazzi *et al.* 2009a). Genotype diversity is also observed among FLA isolated from different parts of the country. Investigation on fixed and floating biofilms of swimming pools and hot tubs in the city of Tabriz (Iran) found *Acanthamoeba* T3 and T4 genotypes (Poor *et al.* 2018). In another study reported from Semnan province, in the northern half of Iran, the authors showed the presence

Table 2 | Data obtained for *Acanthamoeba* and *Protacanthamoeba* collected from the swimming pool water samples in Qazvin province, Iran

					Osmo-tolerant		Thermo-tolerant	
No.	**Isolate code**	**Accession No.**	**Genotype**	**Identity in sequence reference**	**0.5 M**	**1 M**	**37 °C**	**42 °C**
1	QIA15	MH024474	T3	100%	+	−	+	−
2	QIA16	MH024475	T3	100%	+	−	+	−
3	QIA18	MH024476	T3	99%	+	−	+	−
4	QIA42	MH024477	T3	100%	+	+	+	+
5	QIA44	MH024478	T3	100%	+	−	+	−
6	QIA48	MH024479	T3	99%	+	+	+	+
7	QIA85	MH024480	T3	100%	+	+	+	+
8	QIA164	MH024481	T3	100%	+	+	+	+
9	QIA86	MH024482	T4	98%	+	−	+	−
10	QIA97	MH024483	T4	100%	+	+	+	+
11	QIA105	MH024484	T4	100%	+	−	+	−
12	QIA108	MH024485	T4	100%	+	−	+	−
13	QIA23	MH024486	*P. bohemica*	100%	+	+	+	+
14	QIA25	MH024487	*P. bohemica*	99%	−	−	−	−
15	QIA27	MH024488	*P. bohemica*	99%	+	−	+	−
16	QIA45	MH024489	*P. bohemica*	100%	+	−	+	−
17	QIA46	MH024490	*P. bohemica*	99%	+	+	+	+
18	QIA65	MH024491	*Acanthamoeba* sp.	99%	+	−	+	−
19	QIA88	MH024492	T11	98%	−	−	−	−
20	QIA92	MH024493	*P. bohemica*	99%	+	−	+	−

of *Vermamoeba vermiformis* in water samples of swimming pools (Javanmard *et al.* 2017). The authors of a study carried out on water samples of swimming pools in the city of Shiraz claimed the presence of *Acanthamoeba* T4 and T5 genotypes as well as *V. vermiformis* (Armand *et al.* 2016). *Acanthamoeba* T3, T4 and T5 genotypes were also reported in the water samples collected from pools and ponds in southeast Iran (Aghajani *et al.* 2016). Rezaeian *et al.* (2007) isolated *Acanthamoeba* spp. from water specimens taken from swimming pools in Tehran. Although T4 genotype of *Acanthamoeba* is the most abundant and with higher pathogenicity, compared to other genotypes of amoeba found among *Acanthamoeba* keratitis patients and environmental sources (Niyyati *et al.* 2009; Nuprasert *et al.* 2010; Corsaro *et al.* 2015), our present research revealed the predominance of T3 genotype as the most common genotype among the other isolates with different genotypes. Aligned with our survey, a study on water sources in Osaka, Japan showed T3 genotype of *Acanthamoeba* as the dominant type among the isolates (Edagawa *et al.* 2009).

In the current study we found *P. bohemica* among the specimens. *Protacanthamoeba* is classified in the order amoebida and a member of the family *Acanthamoebaidae* (Fuerst *et al.* 2015). *P. bohemica* has been introduced as the species of *Protacanthamoeba* (Dyková *et al.* 2005). The species was reported in samples obtained from water sources in Switzerland (Gianinazzi *et al.* 2009b). Neither of these two amoebae were detected in humans. Four isolates (QIA42, QIA48, QIA85 and QIA164) of *Acanthamoeba* that belong to the T3 genotype were demonstrated to be highly pathogenic, compared to the QIA97 isolate as the sole T4 genotype which was pathogenic in osmic-thermotolerance pathogenicity assay. The results of pathogenicity tests clarified that T3 genotype can be pathogenic to humans, although more research including *in vivo* experiments is needed to confirm the exact pathogenicity of various genotypes of amoeba. The present research found no *Acanthamoeba* in hot Jacuzzi water samples, whereas in a study reported from Brazil the presence of *Acanthamoeba* was reported in water samples of hot tubs (Fabres *et al.* 2016). As expected, the only *Naegleria* identified was isolated from a hot Jacuzzi with no FLA in cold Jacuzzi, indicating that a cold Jacuzzi is an inappropriate environment for the growth of FLA. This study showed that there is no tropism in the distribution of FLA in both surface and deep water samples of swimming pools.

The species of the genus *Naegleria* can cause fatal central nervous system infections (De Jonckheere 2014). A case of primary amoebic meningoencephalitis with *N. fowleri* infection in a five-month-old male infant was found in Iran (Movahedi *et al.* 2012). *Naegleria* is considered as a thermophilic amoebae with hot springs or warm waters as the suitable habitat for this amoebae (De Jonckheere 2014). The pond water of parks in Mashhad city were contaminated with some *Naegleria* spp. (Najafpoor *et al.* 2018). In the present study, *Naegleria* (*N. lovaniensis)* was found in hot tubs of swimming pools whereas no contamination was identified in cold tubs, implying that low temperature is an unsuitable condition for growth of FLA. Our study is the first report of *N. lovaniensis* in the country. Although several species of *Naegleria* have been identified (Di Filippo *et al.* 2017), it is only *N. fowleri* that is proven to be pathogenic for humans. Currently, the non-pathogenic *N. lovaniensis is* considered as an amoeba with close evolutionary relationship to *N. fowleri* (Di Filippo *et al.* 2017).

Swimming in contaminated water while wearing contact lenses is an important risk factor of AK (Khan 2006) and an increase in the rate of AK across the country could be due to improper cleaning of swimming pool water which is a major public health concern (Rezaeian *et al.* 2007). The standard concentration of free chlorine is around 1–3 ppm (Rasti *et al.* 2012) and both swimming pools and hot and cold tubs in our study were found to be within the standard range of chlorine concentration. Our study demonstrated that the recommended concentration of free chlorine failed to prevent the growth of FLA. In conclusion, T3 and T4 genotypes were found to be the most common genotypes of *Acanthamoeba* in the swimming pools of the study area with the potential to threaten the health of individuals who swim in these pools. The genotypes and species of the amoeba may have pathogenic potential and need re-evaluation, so this study highlights the necessity for taking strict measures over the sanitation of recreational water sources, specifically those of swimming pools, to protect individuals against FLA infection.

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