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Genotyping and phylogenic study of *Acanthamoeba* isolates from human keratitis and swimming pool water samples in Iran

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ABSTRACT

Objective: *Acanthamoeba* keratitis cause severe corneal infection and lead to poor vision and blindness. This disease is caused by a unicellular amphizoic protozoon called *Acanthamoeba* spp. that present in different environments. This study aimed to represent the existence and genotyping of *Acanthamoeba* spp. in patients with keratitis and swimming pool water (SPW) in Tehran Province, Central Iran.

Methods: In this descriptive study, 56 clinical samples were collected from patients with keratitis and 30 water samples were collected from different swimming pools in Tehran Province. All samples were examined based on the morphological and molecular techniques. The genotypes were determined by sequencing the partial of 18S rRNA gene.

Results: Of 56 clinical (corneal) and 30 environmental (SPW) samples, 30.3% and 40.0% were positive for *Acanthamoeba* spp., respectively. According to sequencing analysis, 94.1% of amoebic keratitis isolates were belonged to T4 genotype and only one (5.8%) isolate was belonged to T11 genotype. All genotypes were detected from SPW samples were identified as T4 genotype.

Conclusion: According to our results, use of contact lens and swimming in pool poses the major risk factor for amoebic keratitis in the studied area (Tehran). Moreover, T4 genotype was the predominant genotype of human keratitis and swimming pool samples there. Consequently, essential and practical measures are urgently needed to prevent subjects against this ocular seriously disease.

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1. Introduction

Acanthamoeba spp., *Naegleria fowleri* and *Balamuthia mandrillaris* are the most important Free-living amoebae which can cause of some fatal diseases such as granolumatose amoebic encephalitis (GAE) and primary amebic meningoencephalitis (PAM) (Jeon, 2012). Amoebic keratitis (AK) is a painful corneal infection that mostly happens in contact lens users which can cause vision loss and also blindness in severe cases (Niyyati et al., 2020). The prevalence of *Acanthamoeba* keratitis has increased in the recent years in Iran (Rezeaian et al., n.d.; Spotin et al., 2017). The genus of *Acanthamoeba* spp. are extensively disseminated in the environmental (e.g., air, soil, dust, lake, and recreational and mineral water) and clinical (e.g., hemodialysis, ophthalmology and dentistry units), which can be pathogenic to domestic animals and humans (Khan, 2015; Saberi et al., 2019a).

The water source, such as fresh water, sea water, hot springs and swimming pool are most likely polluted to the potentially pathogenic *Acanthamoeba* spp. and could be a dangerous for immune compromised patients (HIV, graft patients, chemotherapy and corticosteroid consumers, diabetes, lupus patients, cirrhosis) and for patients with eye surgery, eye trauma and whose wear contact lens with a probably history of water activities and also for healthy population (Shanmuganathan, 2019). Trophozoite and cyst are the two stages of *Acanthamoeba* spp. in their life cycle that plays pathogenic and resistance roles, respectively. The cyst form is highly resistant to the chemical materials, wide ranges of pH, temperature changes and dryness (Khan, 2015; De Jonckheere, 1991). Diagnosis of *Acanthamoeba* spp., usually based on microscopically, culture (gold standard) and molecular techniques (Dart et al., 2009; Bacon et al., 1993; Hammersmith, 2006).

Based on our knowledge, 22 genotypes (T1-T22) of *Acanthamoeba* spp. are identified from different environmental and clinical samples based on the 18S ribosomal RNA sequence (Coronado-Velázquez et al., 2020). In Iran, Niyyati et al. (2009) and Mirjalali et al. (2013) reported that T2, T4, T6, T11 and T4 genotypes are the most prevalent in environmental and clinical samples, respectively (Niyyati et al., 2009; Mirjalali et al., 2013).

Given the importance of keratitis disease and its increasing prevalence and the role of contaminated swimming pools in the incidence of this disease, this study aimed to assess the presence of *Acanthamoeba* spp. in clinical and swimming pool water (SPW) samples were genotyped.

2. Materials and methods

2.1. Environmental and clinical samples

During October 2018 to July 2019, fifty-six samples including corneal scrapes, contact lenses or its maintenance solution were collected from keratitis patients referred to the Farabi eye teaching hospital, Tehran city, capital of Iran (Esboei et al., 2019; Rahimi-Esboei et al., 2018). All clinical samples were inspected by both direct microscopic and culture methods. Direct smear were prepared from contact lenses, corneal scrapings or lens solution onto clean glass slides and microscopically examined at 400× of magnification for recognition of cysts or trophozoites (Rezaeian et al., 2007). Thirty water samples were collected from swimming pool in sterile containers in Tehran Province. Water samples were immediately transported to the research laboratory in Toxoplasma Research Center, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran (Niyyati et al., 2009; Niyyati et al., 2015). 250 ml of each water sample were filtered through 0.45 μm pore size filter and the filters were cultured in 2% non-nutrient agar plates overlaid with heat killed *E. coli* as a food source. The plates were incubated at room temperature and monitored daily for the presence of *Acanthamoeba* trophozoites and cysts for up to two weeks. If the culture sample was negative, the samples were kept up to a one month and rechecked (Mirjalali et al., 2013; Coronado-Velázquez et al., 2019).

2.2. DNA extraction and PCR

Acanthamoeba trophozoites and cysts were harvested from culture plates, concentrated using centrifugation at 2500*g* for 5 min. DNA were extracted using DNA extraction kit (Qiagen, Korea). PCR method for diagnosis of *Acanthamoeba* spp. were done by targeting DF3 region of 18S rRNA (rDNA), using specific JDP1 (5′-GGCCCAGATCGTT TACCGTGAA) and JDP2 (5′-TCTCACAAGCTGCTAGG GAGTCA) primers. PCR reactions were completed in 25-μl volumes, containing 12.5 μl Ampliqon (Taq DNA Polymerase Master Mix RED, Denmark), 1 μl forward and reverse primers (10 pmol), 2 μl DNA templates, and 9.5 μl double-distilled water. PCR initiated at 94 °C for 5 min and 32 times repeated 94 °C for 30s, 57 °C for 30s, 72 °C for 40s; with a elongation step of 5 min at 72 °C in final cycle (Hajialilo et al., 2016).

2.3. Sequencing

The PCR products of 17 isolates were purified using the PCR purification kit (High-Pure Purification kit, Roche Diagnostics, Australia) and submitted for sequencing using an ABI 3130× automatic sequencer at the Takapouzist Company, Tehran, Iran. The attained sequences were aligned using BioEdit version 7.2.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The sequences were aligned with *Acanthamoeba* genotype sequences available in the GenBank database, to determine the genotypes using the basic local alignment search tool (BLAST) analysis. Nucleotide sequences were submitted to the GenBank using Bankit. The phylogenetic tree was constructed using the Maximum Likelihood method and Kimura 2-parameter model using the molecular evolutionary genetic analysis (MEGA) software version 7.0 (Tamura et al., 2011).

Table 1
Clinical and laboratory findings belonged to 17 patients with AK in Tehran Province, Iran, during 2018–2019.

No	Code	Age (Year)	Sex	Contact lens type	Source	Direct smear	Culture	PCR	Genotype
1	AK 2	17	F	Soft	CS	+	+	+	T4
2	AK 3	23	F	Soft	CS	+	+	+	T4
3	AK 6	18	M	NL	CS	−	+	+	T4
4	AK 8	31	F	Soft	CS	−	+	+	T11
5	AK 11	26	F	Soft	CL	−	−	+	T4
6	AK 12	24	F	NL	CS	−	+	+	T4
7	AK 13	21	M	Soft	LMS	−	+	+	T4
8	AK 18	22	M	Soft	LMS	+	+	+	T4
9	AK 21	19	F	Hard	CL	+	+	+	T4
10	AK 23	25	F	Soft	LMS	−	−	+	T4
11	AK 26	18	F	Soft	LMS	−	+	+	T4
12	AK 27	21	F	NL	CS	+	+	+	T4
13	AK 29	26	F	Hard	CL	+	+	+	T4
14	AK 33	21	F	Soft	LMS, CL	−	+	+	T4
15	AK 35	17	F	Soft	CL	−	+	+	T4
16	AK 36	20	M	Soft	CS	−	+	+	T4
17	AK 41	24	F	Hard	CL	−	+	+	T4

AK: Amoebic Keratitis, CS: Corneal Scrapes, CL: Contact Lenses, LMS: Lens Maintenance Solution, NL: No Lens.

3. Results

From a total of 56 corneal scrapes specimens, 43 (76.7%) were belonged to female and 13 (23.2%) male subjects. Based on direct smear, culture and PCR methods, 7 (12.5%), 13 (23.2%) and 17 (30.3%) were positive for *Acanthamoeba* spp., respectively.

Positive clinical samples were collected from 7 corneal scrapes, 5 contact lenses, 4 lens maintenance solutions and one sample was from contact lenses and lens maintenance solution sources. Among contact lens users, 78.5% patients used soft and 21.4% used hard type of contact lens. All patients also were in ranged of 16–32 years old, 21 (37.5%) patients were from rural and 35 (62.5%) were from urban areas. As regards of genotype identification, 17 samples were genotyped and 94.1% (16/17) isolates were belonged to T4 genotype and only one (5.8%) isolate was belonged to T11 (Table 1). Moreover, in this study, 40.0% ($n = 12$) of 30 SPW samples were positive for *Acanthamoeba* spp. Based on methods, 2 (6.6%), 11(36.6%) and 12 (40.0%) out of them were positive for *Acanthamoeba* spp. using direct smear, culture and PCR tests, respectively. All SPW samples were belonged to the T4 Genotype (Table 2). Nucleotide sequence under the accession numbers: MT378220– MT378248 deposited in GenBank. (See Figs. 1–3.)

4. Discussion

The status of the acanthamobiasis in patients with keratitis and presence *Acanthamoeba* spp. in some swimming pool in Tehran Province were inspected during October 2018 to July 2019. Fifty six patients with keratitis were assessed that 30.36% of them were positive. Based on many previous studies, poor sanitation and unsterile lens usage are the major risk factors for AK (Rezeaian et al., n.d.; Illingworth et al., 1995).

There are insufficient studies on the rate of AK in Iran. Up to 2015, only 150 cases of amoebic keratitis were reported from Iran, that undoubtedly it was not true incidence rate (Niyyati and Rezaeian, 2015). In 2009, Niyyati et al. reported that 30% out of 50 keratitis samples were positive for *Acanthamoeba* spp. (Niyyati et al., 2009). Hajialilo et al. (2016) revealed that 18 (13%) samples from 138 corneal scrapes and contact lenses samples had amoebic source (Hajialilo et al., 2016). AK, Granulomatous Amebic Encephalitis (GAE) and cutaneous ulcers are the manifestations caused by *Acanthamoeba* spp. but, AK is more probable

Table 2
Characteristics and lab findings of isolated *Acanthamoeba* from swimming pool water samples in Tehran Province, Iran, during 2018–2019.

No	Code	Direct smear	Culture	PCR	Genotype
1	SPW 2	−	+	+	T4
2	SPW 3	−	+	+	T4
3	SPW 4	−	+	+	T4
4	SPW 5	−	+	+	T4
5	SPW 7	+	+	+	T4
6	SPW 9	−	+	+	T4
7	SPW 10	−	+	+	T4
8	SPW 13	−	−	+	T4
9	SPW 15	−	+	+	T4
10	SPW 16	+	+	+	T4
11	SPW 19	−	+	+	T4
12	SPW 25	−	+	+	T4

SPW: Swimming pool water.

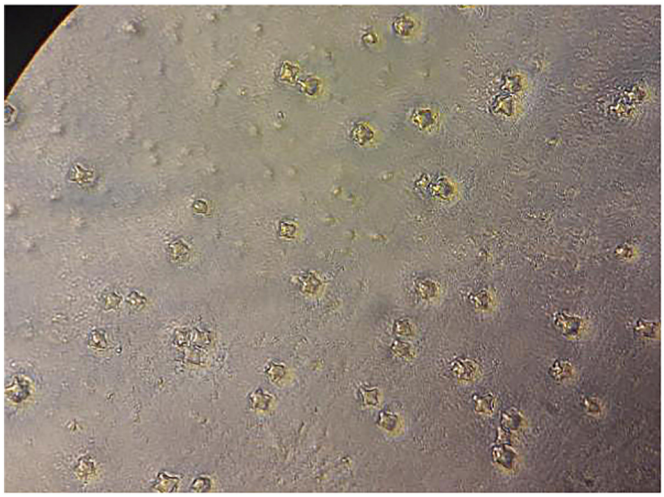

Fig. 1. Microscopically detection of *Acanthamoeba* cyst related to the T4 genotypes, 400 × .

than other complications (Marciano-Cabral and Cabral, 2003). AK affects healthy persons and were associated with trauma to the eye (Illingworth and Cook, 1998). Zbiba and Ben Abdesslem (2018) evaluated the prevalence of AK in suspected patients referred to the health center in a region of Tunisia, North Africa. In this study 230 corneas were scraped and the results indicated that 14 (6%) cases of 230 were positive for *Acanthamoeba* keratitis and most cases (10/14 or 71.4%) had a history of contact lenses and similar to our study, 92.8% of cases were between 19 and 27 years old (Zbiba and Abdesslem, 2018). Manikandan et al., (2004) screened the *Acanthamoeba* keratitis during August 1997 to July 2003 in china and 4519 patients were assessed and the results revealed that 32 (0.7%) patients were positive for *Acanthamoeba* keratitis (Manikandan et al., 2004).

Keratitis caused by *Acanthamoeba* spp. is a grievous, devastating infection that can cause corneal damage, loss of vision and maybe blindness. Polluted contact lens is the major risk factors for initiating *Acanthamoeba* keratitis. According to the many previous studies, more than 85% of keratitis happens in contact lens users and the significant association between AK and using contact lenses was proved. In the current study, similar to many previous works in Iran and other parts of the world, most of the AK (64.7%) was happening in soft lens users (Niyyati et al., 2009; Hajialilo et al., 2016). Poor contact lens sanitation, home-made solution, ineffective disinfection systems are the most important risk factors for *Acanthamoeba* keratitis.

The dominant *Acanthamoeba* genotype in Iran and elsewhere in the world in ocular keratitis is T4 genotype, although T2, T3 and T11 genotypes have also been reported in Iran in recent years. Based on studies in other parts of the world, genotypes T2, T3, T4, T5, T6, T11 and in some case studies T15 have also been isolated from ocular keratitis (Niyyati and Rezaeian, 2009). In the present study, the isolates were genotyped from clinical and environmental samples. Of the 12 SPW samples, all were T4 genotypes but, one of the clinical samples was belonged to the T11 and others belonged to the T4 genotype. The results of our study

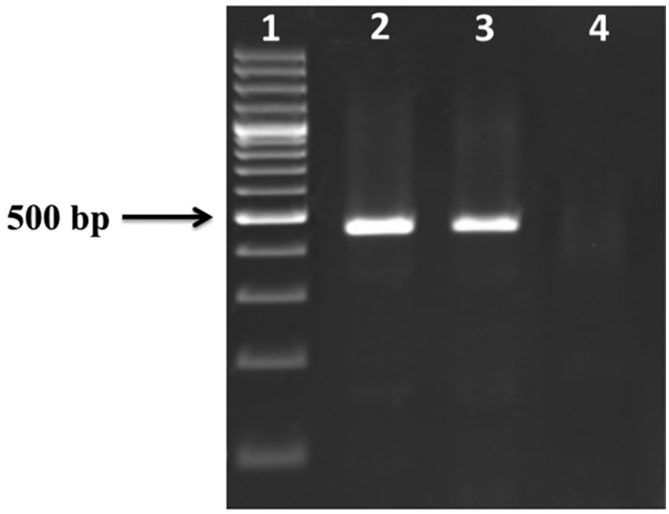

Fig. 2. Agarose gel of PCR products based on 18 s rRNA gene. Lane 1: DNA ladder 100 bp, lane 2: positive sample, lane 3: positive control and lane 4: negative control.

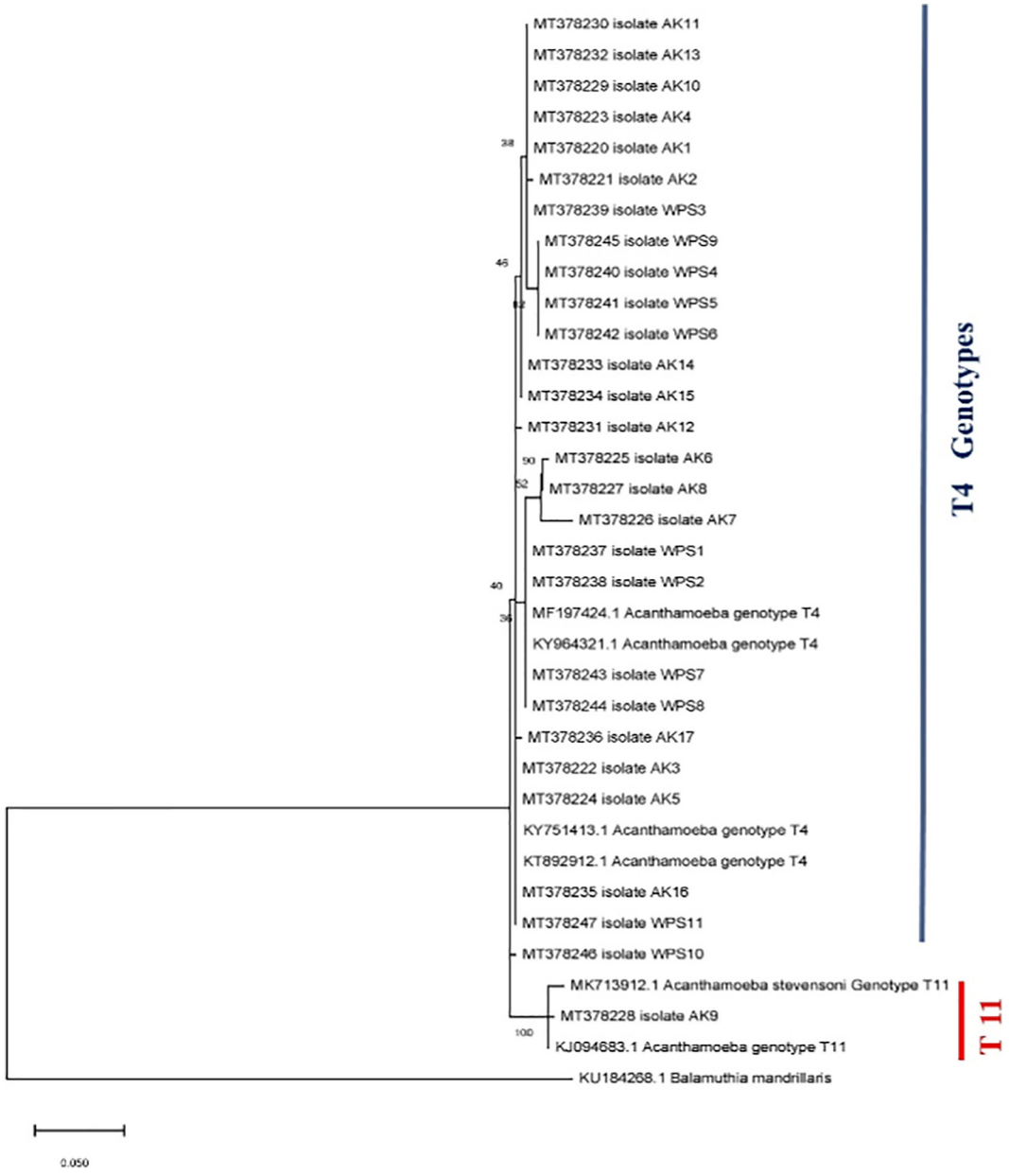

Fig. 3. The Relationships between genotypes of *Acanthamoeba* spp. isolated from AK and swimming pool water (SPW) sample of present study and other *Acanthamoeba* T4 and T11 genotypes obtained from GenBank. *Balamuthia mandrillaris* was used as out-group reference. Phylogenetic tree based on 18srRNAgene using Maximum Likelihood method and Kimura 2-parameter model in MEGA-7 software. The bootstrap consensus tree was inferred from 1000 replicates.

inconsistent with other studies revealed that T4 genotype is dominant isolated in all clinical samples that it should be assessed more than other genotypes and more precautionary measures should be taken to prevent this genotype.

One of the important reasons for the study of *Acanthamoeba* infection in environmental samples is the high probability of transmission of this disease from the environment for patients. Niyyati et al., for the first time in the world analyzed *Acanthamoeba* isolated from dust samples and showed that the only genotype isolated from 13 Iranian dust samples was T4 genotype (Niyyati et al., 2009). Furthermore, in the study conducted by Saberi et al. (2019), *Acanthamoeba* spp. was detected in dust phenomenon in Ilam Province, western Iran. In mentioned study, T4 and T2 genotypes were reported (Saberi et al., 2019b). In the study of Lorenzo et al., 114 soil samples were studied, the only known genotype being T4 (Lorenzo-Morales et al., 2013). Abdi et al. (2019) evaluated the status of *Acanthamoeba* spp. in water sources of Urmia, northwestern Iran, that 35% of superficial and plumbed water samples were positive for *Acanthamoeba* spp. The genotyping results indicated that 10% and 90% of cases were belonged to the T2 and T4 genotypes, respectively (Abdi et al., 2020). In the study of Maghsoud et al. (2003) from 12 clinical

samples, T2 was the dominant identified genotype (58.3%) and the second genotype was T4 which was reported in 33.3% of all clinical samples (Maghsood et al., 2005). The results of the study conducted by Saberi et al. indicated that 17.5% of hydraulic system samples and 50% of dust samples in Hemodialysis Units from Iran were positive for *Acanthamoeba* with T3, T4 and T5 Genotypes (Saberi et al., 2019a).

In case of swimming pools, 40% (12/30) of swimming pools from Tehran province were positive using PCR technique. In a similar study conducted by Faraji et al., (2017) revealed that 41.7% of Swimming Pools of Khoramabad city, western Iran were polluted with *Acanthamoeba* spp. by using of PCR method (Faraji et al., n.d.). Mafi et al., (2015) evaluated the prevalence of the free living amoebae using morphological methods in Swimming Pools and Park Ponds of Tehran and the results showed that 24% were positive for free living amoebae (Mafi et al., 2017). The prevalence rate of infection in the study of Mafi et al. was lesser than current work that it may be due to the use of a low sensitive method.

Based on the study of Gorink et al., (2013) and Shoff et al., (2008), 12 and 19.4% of the drinking water from Turkey and Florida were polluted to the *Achanthamoeba* spp., respectively (Shoff et al., 2007; Górnik and Kuźna-Grygiel, 2004). It means that the uses of conventional techniques for removal of the pathogens are not well designed and unable to delete.

Trophozoites of *Achanthamoeba* spp. transform to the cyst during unfavorable conditions which is more resistant to pH, dehydration, osmolarity, freezing, ultraviolet radiation (UV), irradiation and chemical disinfection agents such as chloromethane, trihalomethane, bromomethane, ozone and sodium hypochlorite (NaOCl). Spw needs to be hygienic to protect swimmers from infectious pathogens; therefore, it's vital to assess the pool contaminations as one of the important sources of *Acanthamoeba*.

According to these studies and other studies, there are a large number of water sources contaminated with *Acanthamoeba* spp. infection that, given the importance of this parasite in human health, the necessary measures should be taken to eliminate it. Different genotypes of *Acanthamoeba* spp. have been identified, some of which are pathogenic and others non-pathogenic. Identification of genotypes in each environment and geographical region and determination of its dominant genotype is very valuable (Niyyati et al., 2009; Niyyati et al., 2015; Hajialilo et al., 2016; Niyyati and Rezaeian, 2015; Badirzadeh et al., 2011; Mataji Bandpei, 2016; Orosz et al., 2018). The results of the studies on environmental samples have shown that T4 genotype is highly dispersed in the environment, these samples can be considered as important risk factors for AK.

5. Conclusion

According to our results, use of contact lens and swimming in pool poses the major risk factor for amoebic keratitis in the studied area (Tehran). Moreover, T4 genotype was the predominant genotype of human keratitis and swimming pool samples there. Consequently, essential and practical measures are urgently needed to prevent subjects against this ocular seriously disease. Also, increasing the awareness of the contact lens user regarding the route of transmission the *Acanthmanoeba* is required. Further studies are recommended to be developed in this area on other clinical and environmental samples.

Declaration of Competing Interest

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

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