Genotyping of *Acanthamoeba* isolated from water in recreational areas of Tehran, Iran

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ABSTRACT

A comprehensive survey assessing the presence of *Acanthamoeba* was conducted on 50 samples from water sources in parks and public squares from 22 municipal districts of Tehran, Iran. The prevalence and genotypes of *Acanthamoeba* were determined by PCR and the PCR fragments of ribosomal RNA genes sequenced. Sixteen (32%) samples were positive for *Acanthamoeba* spp. Sequence analysis revealed that the positive isolates belonged to the T4 and T5 genotypes. Fourteen isolates (87.5%) were T4, and two (12.5%) were T5. *Acanthamoeba* may be a problematic organism for contact lens wearers and for immunocompromised individuals. In Iran, *Acanthamoeba* keratitis has increased in recent years, mainly due to poor hygiene in contact lens wearers. A thorough survey for the prevalence of this amoeba could have a significant role in prevention of disease. This is the first report of the T5 genotype from water in recreational areas of Tehran.

Key words | *Acanthamoeba*, genotypes, public water bodies, recreational areas, survey, Tehran

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INTRODUCTION

Amoebae of the genus *Acanthamoeba* have worldwide distribution and inhabit a wide variety of environments (Ledee *et al.* 2003) including water, dust, soil, and air (Mergeryan 1991; Armstrong 2000). Some species have direct roles as human pathogens causing sight-threatening keratitis, especially in contact lens wearers, and life-threatening granulomatous amoebic encephalitis (Martinez *et al.* 1980). *Acanthamoeba* exists in two forms, trophozoites and cysts. Cysts excyst to yield trophozoites when conditions are favorable (Khan 2003). The genus *Acanthamoeba* is classified into 17 genotypes (T1–T17) based on the 18S rRNA gene. The majority (>90%) of keratitis-causing isolates belong to the T3 and T4 genotypes (Stothard *et al.* 1998; Schuster & Visvesvara 2004; Nuprasert *et al.* 2010); 90% of the *Acanthamoeba* isolates that produce infections belong to the T4 genotype (Maghsood *et al.* 2005; Khan 2006). Of the T4, T3, and T2 genotypes associated with *Acanthamoeba* keratitis (AK), T2 and T4 have been reported in water and T4 in soil in Iran (Maghsood *et al.* 2005; Niyyati *et al.* 2009a). However, few studies of *Acanthamoeba* genotypes have been conducted in recreational areas of Iran. The aim of this study was to determine the presence of *Acanthamoeba*, using morphological and molecular tools, in waters associated with human recreational activity in Tehran, Iran.

MATERIALS AND METHODS

Sample sites

From April to October 2008, 50 water samples (250–400 ml) were randomly collected from 26 public squares and 24 park

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ponds from the 22 municipal districts of Tehran and stored at 4 °C or room temperature until used.

Isolation and culture

Water samples were filtered through a 0.45 μm diameter cellulose nitrate filter (Millipore Corporation, Bedford, Madison, USA). The filters were inverted on heat-inactivated *Escherichia coli* treated, 1.5% non-nutrient agar plates with Page's Amoeba Saline (Khan 2006) and incubated at 30 °C or room temperature. Plates were screened for cysts and trophozoites of *Acanthamoeba* daily for up to one month.

DNA extraction

Acanthamoeba trophozoites and cysts were harvested and washed with phosphate buffered saline, pH 7.4, three times by centrifugation at 500 g for 5 min. Cell pellets were re-suspended in lysis buffer (50 mM NaCl, 10 mM EDTA, 50 mM Tris–HCl, pH 8.0) and submitted to five freeze (liquid nitrogen) thaw (water at 90 °C) cycles. Lysed cells were incubated at 55 °C for 1 hr with 0.25 mg/ml proteinase K. The DNA samples were purified from amoebae isolates by the phenol-chloroform method (Sambrook *et al.* 1989) and DNG-Plus Kit (Cinnagen, Tehran, Iran).

PCR analysis

A primer pair (JDP1–JDP2) was used to amplify a fragment of 423 to 551 bp *Acanthamoeba*-specific 18S ribosomal DNA product, designated ASA.S1 (*Acanthamoeba*-specific amplimer S1) (Schroeder *et al.* 2001). The primer sequences for JDP1 were: 5′-GGCCCAGATCGTTTACCGTGAA-3′ and for JDP2: 5′-TCTCACAAGCTGCTAGGGGAGTCA-3′. PCR was performed in a 30 μl volume containing 1.25 U of Taq DNA polymerase (Cinnagen, Tehran, Iran), 2 μl of template DNA, 0.3 mM dNTP, 20 pmol of each primer, 1.5 mM $MgCl_2$, and 10 mM PCR buffer. PCR was performed using an initial 3 min. at 94 °C, 32 amplification cycles (denaturing 94 °C for 45 s, annealing 55 °C for 35 s, and extension 72°C for 1 min), and a final elongation step of 10 min at 72 °C. Amplification products were fractionated by 1% agarose gel electrophoresis stained with a solution of 0.5 mg/ml of ethidium bromide and visualized under UV light.

Nucleotide sequences

The PCR product was submitted for sequencing with an ABI 3130X automatic sequencer at the Research Center for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences. Genotype identification was based on sequence analysis of diagnostic fragment 3 region (DF3) of 18S rRNA gene (Booton *et al.* 2002). The sequences were edited manually with GenRunner v. 3.05 and aligned by using the Genotoul Multalin program.

RESULTS

The *Acanthamoeba* isolates were identified morphologically based on double wall cysts and flattened trophozoite with slender acanthopodia (Page 1988). The main goal of this study was isolation and genotyping of *Acanthamoeba* spp. However, other free-living amoebae suspected to be *Hartmannella* and *Vannella*, on the basis of morphological features in culture plates, were also detected. Morphological and molecular methods used showed that 16 (32%) of the 50 samples were positive for *Acanthamoeba* spp. Gel electrophoresis of the PCR products showed a band of about 430 to 520 bp. Six of the positive samples (37.5%) were taken from park ponds, and 10 (62.5%) were associated with the public squares. Both parks and squares of two districts (district no. 5 and 12) contained *Acanthamoeba* (Table 1), while samples from 8 of the 22 districts were not contaminated. Fourteen (87.5%) of the positive isolates belonged to the T4 genotype, and two (12.5%) were T5 (Table 1).

Comparative database analysis (Blast search) of the genotypes obtained from the 18S rRNA gene showed eight (50%) *Acanthamoeba* spp., two (12.5%) *A. lenticulata*, two (12.5%) *A. quina*, two (12.5%) *A. muritanensis*, one (6.25%) *A. hattchetii*, and one (6.25%) *A. castellani*. Sequence alignment of the T4 and T5 *Acanthamoeba* genotypes identified in the present study in comparison with database T4 (EU934065) and T5 (EU377584) genotypes is shown in Figure 1. Sequences of 13 positive rRNA genes were submitted to the Genbank/EMBL/DDBJ under Accession No.

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Table 1 | Genotypes of *Acanthamoeba* isolates obtained from water in recreational areas, Tehran, Iran

Isolate name	Tehran district	Source of isolate	Species	Genotype	Accession No.
NHE(Ac)1-IR	3[a]	Square	*A. lenticulata*	T5	AB525818
NHE(Ac)3-IR	12	Park	*A. quina*	T4	AB525819
NHE(Ac)4-IR	12	Square	*Acanthamoeba* sp.	T4	AB525820
NHE(Ac)7-IR	16	Square	*Acanthamoeba* sp.	T4	AB525831
NHE(Ac)8-IR	15	Square	*A. lenticulata*	T5	AB525821
NHE(Ac)11-IR	11	Square	*A. muritaniensis*	T4	AB525822
NHE(Ac)12-IR	20	Square	*A. quina*	T4	AB525823
NHE(Ac)14-IR	1	Park	*A. castellani*	T4	AB525824
NHE(Ac)17-IR	6	Park	*A. muritaniensis*	T4	AB525825
NHE(Ac)22-IR	4	Square	*Acanthamoeba* sp.	T4	AB525826
NHE(Ac)33-IR	9	Park	*Acanthamoeba* sp.	T4	AB525827
NHE(Ac)34-IR	10	Park	*A. hattchetii*	T4	AB525828
NHE(Ac)42-IR	6	Square	*Acanthamoeba* sp.	T4	AB525829[b]
NHE(Ac)47-IR	5	Square	*Acanthamoeba* sp.	T4	
NHE(Ac)48-IR	5	Park	*Acanthamoeba* sp.	T4	
NHE(Ac)49-IR	2	Square	*Acanthamoeba* sp.	T4	

[a]The 22 municipal districts of Tehran are defined by numbers.
[b]The four last isolates showed identical sequences under Accession No.: AB525829.

AB525818–AB525833. Three of the samples belonging to the T4 genotype showed 100% homology with the NHE(Ac)42-IR isolate.

DISCUSSION

Acanthamoeba may be a problematic organism for contact lens wearers and for immunocompromised individuals. In Iran, AK has shown an increase in recent years, mainly due to poor hygiene in contact lens wearers. Our findings will serve as additional evidence for the presence of *Acanthamoeba* genotypes in habitats associated with human populations, representing a risk for human health. In this study, T4 was the most commonly identified genotype (87.5%). A study by Maghsood *et al.* (2005) of 12 pool and waterfall samples showed 58.3% T2 and 33.3% T4. Because of the high prevalence of the T4 genotype in recreational areas of Tehran and the association of this genotype with AK (Ledee *et al.* 1996; Walochnik *et al.* 2000; Khan & Paget 2002), more attention to the hygienic maintenance of water in public areas is recommended. The ten-year study on patients with amoebic keratitis has shown that AK continues to rise in Iran. This is due to increasing number of lens wearers (Rezaeian *et al.* 2007). Therefore contact lens wearers should be aware of the risks associated with *Acanthamoeba* in such waters. The T4 genotype in seven soil-related samples and two animal-origin isolates, as well as T2 and T6 in two pool samples, has been reported in Iran (Niyyati *et al.* 2009a). A study of 13 dust sources (trees, laboratory ventilation systems, laboratory desks, domestic air conditioning, and a floral arrangement, among others) showed 84.6% T4, 7.6% T5, and 7.6% T11 genotypes (Niyyati *et al.* 2009b). This study revealed T4 as the most common genotype in the dust sources.

The T5 genotype was identified in two isolates. What is of particular interest is that T5 is dramatically underrepresented in AK cases due to study reported by Ledee *et al.* (2009) in the USA. A study on five patients have shown that one out of five cases of AK studied in Greece presents T5 sequence type (*Acanthamoeba lenticulata*), which is the second most frequent genotype found among environmental samples (Spanakos *et al.* 2006). A drug-resistant severe AK caused by T5 Acanthamoeba genotype (*A. lenticulata*) was also reported

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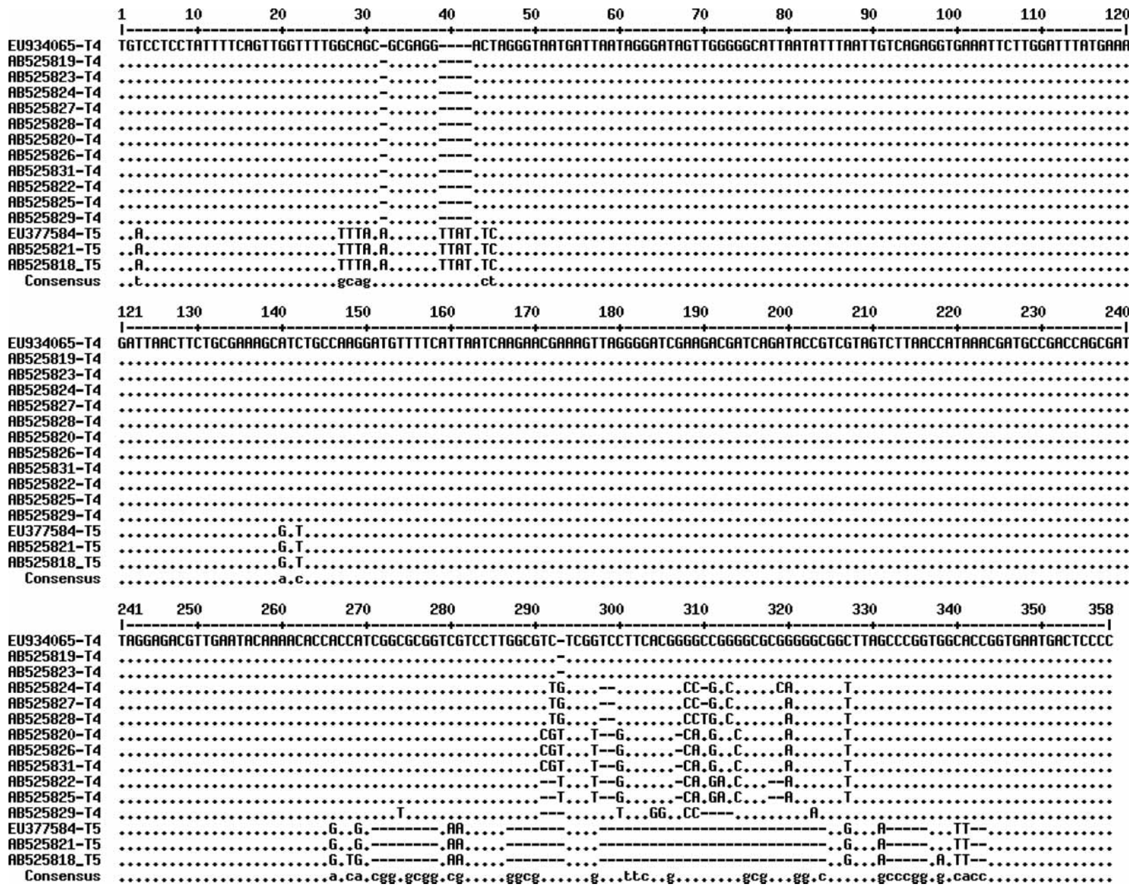

Figure 1 | Sequence alignment of T4 and T5 *Acanthamoeba* isolates in comparison with database T4 (EU934065) and T5 (EU377584) genotypes. The alignment shown is partial 18S rRNA gene including DF3 that contains the highly variable and informative section of the sequence. The genotype accession numbers of the submitted genes are shown. Gaps are represented as dashes.

by Iovieno *et al.* (2010). As the number of people who wear contact lenses continues to rise, the risk of developing T5-induced keratitis may increase and this genotype may play an important role in ophthalmic disease. Acanthamoeba and other naked amoebae have been reported in tapwater (Shoff *et al.* 2008). Another study on water storage tanks have shown that these water supplies promote colonization of domestic water with free-living amoebae, including Acanthamoeba, and hence increase the risk of AK. This accounts for the significantly greater incidence of AK in the UK and supports advice to avoid using tap water in contact lens care routines (Kilvington *et al.* 2004). A study by Lorenzo-Morales *et al.* (2006) in the Nile delta region of Egypt showed Acanthamoeba in 16 of 37 samples of freshwater of genotypes T1, T2, T3, T4, and T7. Five species, *A. lenticulata, A. quina, A. muritanensis, A. hattchetii*, and *A. castellani* were identified using database comparative analysis (Table 1). Although 8 isolates from 16 positive samples belonging to 5 species were recognized, further molecular studies are recommended for confirmation, as well as to verify the species and frequency of *Acanthamoeba* in Iran. In conclusion, identification of *Acanthamoeba* genotypes should be carried out due to the increasing prevalence of AK in Iran and worldwide. These data would provide information about the distribution of various genotypes of *Acanthamoeba*, such as the T5 genotype in water sources, which is reported for the first time in Iran. There is a clear need for more detailed knowledge about the distribution of each genotype in Iran.

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