Screening of recreational areas of rivers for potentially pathogenic free-living amoebae in the suburbs of Tehran, Iran

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ABSTRACT

A survey was conducted to determine the presence of free-living amoebae (FLA), especially *Acanthamoeba* and *Naegleria*, in river recreation areas in Tehran Province, Iran. All rivers surveyed were associated with human activity, and two were also a source of municipal tap water. Fifty-five water samples from 10 major rivers were screened for FLA and identified by morphological characters, PCR amplification targeting specific genes for *Acanthamoeba* (DF3 region of *Rns* gene) and other FLA (ITS PCR), and homology analysis. The percentage of positive FLA isolates was 27.3%, of which 80% were *Acanthamoeba*, assigned to the T4 and T15 genotype, and 20% were *Naegleria*. Isolation of *Acanthamoeba* T4 genotype (91.7%) from recreation areas could be a health threat and a sanitary risk associated with human activity where young people and tourists congregate in summer. Posting of warning signs and education of high-risk individuals are important for disease prevention. To the best of our knowledge this is the first report of genotype T15 (clustered with *A. jacobsi*) identified in Iran and the first report of the distribution of FLA such as *Naegleria* (*N. pagei*, *N. clarki and N. fultoni*) in recreation areas in rivers of Tehran Province using molecular methods.

Key words | free-living amoebae, recreational area, river sources, Tehran

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INTRODUCTION

Free-living amoebae (FLA) are ubiquitous protozoan organisms distributed in environmental sources such as water, soil, dust and air (Schuster *et al.* 2004; Khan 2006). Several taxa, such as *Acanthamoeba*, *Naegleria fowleri*, *Balamuthia mandrillaris and Sappinia pedata*, are of medical concern (Marciano-Cabral & Cabral 2003; Visvesvara *et al.* 2007; Qvarnstrom *et al.* 2009). The ubiquitous nature of these microorganisms leads to common exposure in humans, and research has shown that 80–100% of healthy people contain serum antibodies against *Acanthamoeba* spp. (Cursons *et al.* 1980). However, those at higher risk could be severely affected by these opportunistic parasites (Khan 2009).

Acanthamoeba have been isolated from water sources worldwide such as tap, fresh, coastal and thermal water, natural hot springs, ponds, lakes, spas and sewage (Ettinger *et al.* 2003; Tsvetkova *et al.* 2004; Lorenzo *et al.* 2006; De Jonckheere 2007; Gianinazzi *et al.* 2010; Marciano-Cabral *et al.* 2010). Fifteen genotypes have been identified based on a highly variable region (DF3) of *Rns* genes within this genus (T1–T15) (Schroeder *et al.* 2001). Recently, two new genotypes have been identified in water samples from Taiwan and France and assigned to T16 and T17, respectively. Neither has been isolated from clinical samples thus far (Corsaro & Venditti 2010; Nuprasert *et al.* 2010). Isolates belonging to T4 genotypes have been identified as a major pathogen in the cornea and can lead to sight-threatening amoebic keratitis (AK) (Di Cave *et al.* 2009; Khan 2009). Recently, other common environmental genotypes such as T5 and T15

doi: 10.2166/wh.2011.068

have also been reported as a causal agent of AK (Spanakos *et al.* 2006; Di Cave *et al.* 2009), and thus environmental genotypes may have the potential for being pathogenic. Studies have revealed that most outbreaks and sporadic cases of AK followed water-related activities such as swimming, bathing or washing eyes while wearing contact lenses (Seal *et al.* 1992; Lorenzo *et al.* 2005). Thus, contamination of water sources with potentially pathogenic FLA could be a risk.

Among vahlkampfiid amoebae, *Naegleria fowleri* and *Paravahlkampfia francinae* are reported as the causative agents of primary amoebic meningoencephalitis (PAM) in young adults, who mainly showed a history of aquatic activity such as diving (Jamerson *et al.* 2009; Visvesvara *et al.* 2009). Recent reports regarding mixed infections of *Vahlkampfia* and *Acanthamoeba* genotype T3 (Niyyati *et al.* 2010) and also *Hartmannella* and *Vahlkampfia* (Aitken *et al.* 1996) have led to increased attention in other FLA as potentially pathogenic to humans, but more investigation is needed to confirm this.

In Iran, the only research regarding the presence of FLA in river sources was conducted in 2004 in southern Iran based solely on morphological criteria (Rezaeian *et al.* 2004). Other research in Iran has been mainly of stagnant waters, soil and animal faeces, with major focus on identification of *Acanthamoeba* (Maghsood *et al.* 2005; Niyyati *et al.* 2009). AK has shown an increasing trend in recent years in Iran (Rezaeian *et al.* 2007), and genotypes of *Acanthamoeba*, including T2, T3, T4 and T11, have been reported as causal agents (Maghsood *et al.* 2005; Niyyati *et al.* 2009). However, there have been no reports of other FLA-related diseases such as encephalitis and PAM in Iran possibly because of low awareness.

In Tehran, suburban rivers are increasingly popular, especially for young adults during warm months, and many people enjoy the rivers of suburban Tehran for bathing, fishing and swimming. As there is no current information on the occurrence of potentially pathogenic FLA in rivers of Tehran, this study aimed to evaluate their presence using both morphological criteria and sequence-based methods. To the best of our knowledge this is the first such study.

MATERIALS AND METHODS

Sample collection

Fifty-five water samples from recreational areas of 10 rivers of suburban Tehran Province were collected during warm months of 2010. Five to six sampling sites, in which high levels of activity such as washing, bathing, swimming and fishing were observed, were selected from each river. Water temperature and pH were recorded at each site. At the time of sampling, the temperatures and pHs of water samples ranged from 21.3–33.4 °C and 5.9–7.0, respectively (Table 2). Samples were collected from water approximately 100 cm below the surface, into 500 ml sterile bottles, and transferred immediately to the Department of Parasitology and Mycology, Shahid Beheshti University of Medical Science, Tehran, for further analysis.

Processing of samples

Filtration was performed using a cellulose nitrate filter (pore size 1.2 μm). The filters were inverted on a 1.5% non-nutrient agar plate coated with heat-killed *Escherichia coli*. Outgrowth of amoebae was monitored daily using an inverted microscope. All positive cultures were submitted to cloning to obtain a single cell line and to eliminate bacterial and fungal contamination of plates for subsequent evaluation (Lorenzo *et al.* 2006).

Morphological and molecular analysis

Culture plates containing cloned amoebae and fresh smears were examined for the characteristic morphology of trophozoite and cysts, and classification was determined to genus (Page 1988). If vahlkampfiid amoebae were detected they were subjected to an enflagellation test (Sawyer 1989).

Based on morphological criteria, amoebae were identified as *Acanthamoeba* or vahlkampfiid amoebae. DNA extractions were performed based on the presence of trophozoites and cysts using either the Instagene matrix kit (Chelex; Biorad) or the phenol–chloroform method (Sambrook *et al.* 1989), respectively.

PCR was performed using two primer sets specific for *Acanthamoeba*, JDP1 5′-GGCCCAGATCGTTTACCGT GAA-3′ and JDP2 5′-TCTCACAAGCTGCTAGGGAGTCA-3′ (Schroeder *et al*. 2001), which amplify the most informative region of 18S rRNA. Identification of vahlkampfiid amoebae, including *Naegleria* spp., used internal transcribed spacer (ITS) PCR assay (Pelandakis & Pernin 2002) by forward primer: ITS1 5′-GAACCTGCGTAGGGATCATTT-3′ and reverse primer ITS2 5′-TTTCTTTTCCTCCCCTTATTA-3′. ITS PCR detected all *Naegleria* spp. and also the frequent competitors of these amoebae such as *Willaertia*, *Hartmannella* and *Vahlkampfia.* Amplification of the partial 18S rDNA (ASA.S1) from *Acanthamoeba* and the ITS region of vahlkampfiid amoebae was performed using a total volume of 30 μl Ampliqone (*Taq* DNA Polymerase Master Mix RED, Denmark) as a ready-made mixture. Briefly, 25 μl of *Taq* Master mix were used with 10 ng template DNA, 0.1 μM of each primer and distilled water. The cycling conditions began with the initial denaturing phase of 94 °C for 1 min, followed by 35 repetition cycles at 94 °C for 35 s, annealing at 56 and 58 °C for 45 s for *Acanthamoeba* and vahlkampfiid amoebae, respectively, and at 72 °C for 1 min. PCR products were electrophoresed using 2% agarose gel stained with a solution of ethidium bromide and visualized under UV light. PCR products were resolved using the ABI 3130X automatic sequencer in the Research Center for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran. A homology analysis using the Basic Local Alignment Search Tool (BLASTn) was performed to search for the most similar reference sequences.

Nucleotide sequence accession numbers

The DNA sequences for the new isolates have been deposited in the genetic sequence database at the National Center for Biotechnical Information (NCBI) using the Sequin program (version 10.3) under accession numbers: JF317322–JF317337.

RESULTS

Of 55 water samples from sites in rivers of suburban Tehran, 15 (27.3%) were positive for FLA based on morphological observations, PCR targeting specific genes and sequence-based analysis (Table 1). Detection rates were 13 (80%) for

Table 1 | Distribution of *Acanthamoeba* and *Naegleria* isolates in recreation areas of rivers in Tehran

Isolate	Locality	ITS PCR	JDP PCR	Genera	Species/ genotype	Accession no.
TRW1[a]	Fasham	–	+	*Acanthamoeba*	T4	JF317322
TRW2	Fasham	–	+	*Acanthamoeba*	T4	JF317323
TRW3	Jajrood[b]	+	+	*Acanthamoeba*	T4	JF317324
				Naegleria	*N. fultoni*	JF317337
TRW10	Jajrood	–	+	*Acanthamoeba*	T4	JF317325
TRW12	Jajrood	–	+	*Acanthamoeba*	T4	JF317326
TRW14	Karaj[b]	–	+	*Acanthamoeba*	T4	JF317327
TRW15	Karaj	–	+	*Acanthamoeba*	T4	JF317328
TRW16	Lar	–	+	*Acanthamoeba*	T4	JF317329
TRW22	Damavand	–	+	*Acanthamoeba*	T4	JF317334
TRW25	Damavand	–	+	*Acanthamoeba*	T4	JF317330
TRW37	Boomehen	–	+	*Acanthamoeba*	T15 *(A. jacobsi)*	JF317331
TRW39	Boomehen	–	+	*Acanthamoeba*	T4	JF317332
TRW42	Roodehen	–	+	*Acanthamoeba*	T4	JF317333
TRW12	Darband	+	–	*Naegleria*	*N. pagei*	JF317336
TRW38	Karaj	+	–	*Naegleria*	*N. clarki*	JF317335

[a]TRW: Tehran River Water.
[b]Source for municipal tap water.

Table 2 | Data of percent positive samples in each water source

Sampling site[a]	No. of samples from each river	Positive samples *n* (% positivity)	Mean temp (°C)	Mean pH
Fasham	6	2 (33.3)	30.5	6.1
Jajrood	6	3 (50)	27.7	5.9
Karaj	6	3 (50)	33.4	6
Damavand	5	2 (40)	25.1	6.3
Lar	6	1 (16.7)	23.1	5
Roodehen	6	1 (16.7)	25.7	6.3
Boomehen	5	2 (40)	26	7
Darband	5	1 (20)	29.1	5.5

[a]Two rivers were negative for FLA.

Acanthamoeba, and three (20%) for vahlkampfiid amoebae. *Thecamoeba* were also found mixed with *Acanthamoeba* and vahlkampfiid amoebae.

Morphological determination of *Acanthamoeba* was based on double walled and wrinkled or wavy ectocysts and characteristic acanthopodia of trophozoites (Figure 1). Sequence analysis of the DF3 region of *Rns* genes of these isolates revealed T4 (92.3%) and T15 (7.7%) genotypes in positive samples, with homology analysis revealing 95–100% similarity (Table 1). This is the first report of T15 in Iran. This genotype clustered with *A. jacobsi*, with 99% identity and query coverage to genes in the gen data banks (accession numbers: GU573867.1 and GU573859.1).

Most *Acanthamoeba* isolates were recovered from waters at temperatures 21.3–27.7 °C (Table 2). The culture plate of one sample (isolate TRW3) showed various amoebae including *Acanthamoeba*, vahlkampfiid amoebae, *Thecamoeba* and some unidentified *miniamoebae*. Sequence analysis confirmed T4 genotype and *Naegleria fultoni* on this plate.

Identification of vahlkampfiid amoebae was based on eruptive trophozoites and spherical cysts and were found in three plates (Tehran River Waters: TRW3, TRW12, TRW38; Figure 2). None produced flagellate forms. Sequence analysis of tree isolates demonstrated 100%

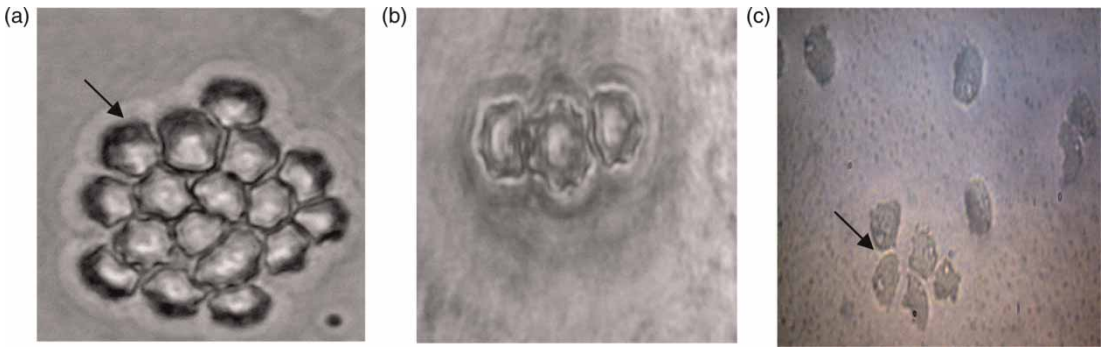

Figure 1 | Light micrograph of *Acanthamoeba* cysts (a, b) and trophozoite (c) in non-nutrient agar ×400.

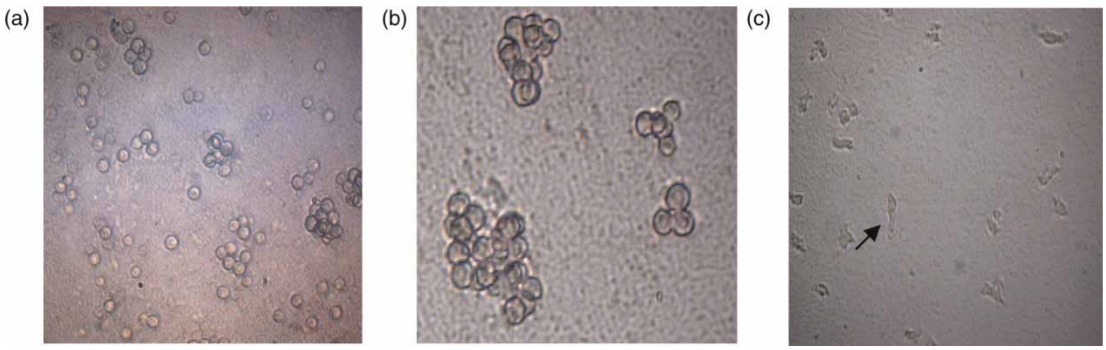

Figure 2 | Light micrograph of *Naegleria* cysts (a ×100, b ×400) and trophozoite (c) in non-nutrient agar.

homology with *N. clarki*, *N. pagei* and *N. fultoni*. *Naegleria* spp. were found in samples collected at water temperatures ranging from 30.5 to 33.4 °C.

DISCUSSION

This study is the first molecular-based survey of potentially pathogenic FLA in recreation areas of rivers in suburban Tehran. Only one report of the presence of FLA in rivers of southern Iran has been published, which indicated the presence of *Acanthamoeba* and *Naegleria*, based on morphology, and showed the presence of FLA in 3.7% of tested rivers (Rezaeian *et al.* 2004). The present study revealed 27.3% of samples positive for FLA, as opposed to those of Ettinger *et al.* (2003), who found a 43.3% rate of FLA contamination of James River near Richmond. Our results confirmed the T4 genotype as the predominant type as opposed to Huang & Hsu (2010a), who found T15, and Edagawa *et al.* (2009), who found T3 to predominate in waters surveyed. Our negative samples (72.7%) indicated that flow and circulation of waters, as well as the presence of other phagocytosing microorganisms, may make the situation unfavourable for a high presence of these amoebas, as suggested by Jamerson *et al.* (2009). Other probable causes for negative results may be due to water speed, biotic and abiotic factors. However, potentially pathogenic T4 *Acanthamoeba* in river waters, and especially in municipal tap-water sources (Table 1), could be a hazard for higher risk individuals. The T4 strains possess properties that render them more virulent with higher potential for transmission to humans (Maghsood *et al.* 2005; Khan 2006), and this can lead to greater involvement of the T4 genotype in human infections. The presence of T4 is therefore a risk factor for people engaged in water-related recreation. Moreover, isolation of genotype T15 (corresponding to *A. jacobsi*) for the first time in Iran indicates that further study is needed to identify genotype populations. Recently two cases of AK resulting from the T15 genotype have been reported in Italy (Di Cave *et al.* 2009). The T15 genotype is able to retain pathogenic bacteria for longer compared to other *Acanthamoeba* strains, and thus could act as an important Trojan horse for microbes (Flint *et al.* 2003).

We have isolated only two types of *Acanthamoeba* (T4 and T15), unlike Lorenzo *et al.* (2006), who showed the presence of several genotypes including T1, T2, T3, T4 and T7 in freshwater sources of Egypt. Environmental and climatic factors may contribute to distribution of different genotypes (Jamerson *et al.* 2009), and further study is needed to clarify this.

The incidence of AK showed an increased rate in summer in Iran than in cold weather, and most patients reported a history of recreational water contact before the onset of disease (unpublished data). Providing adequate knowledge and education to higher risk people such as contact lens wearers and posting of information at river recreation areas may decrease the development of severe disease.

An increase in *Acanthamoeba*, *Vannella* and *Naegleria* populations during summer has been found in Oklahoma, Virginia and South Carolina waters in the USA (Kyle & Noblet 1986; Stockman *et al.* 2011). High temperatures lead to favourable conditions for proliferation of amoebae. Our result showed that samples from rivers with higher water temperatures were more likely to contain vahlkampfiid amoebae.

Although three vahlkampfiid amoebae were genetically confirmed to be *Naegleria*, none were positive in a flagellation test. This agrees with recent research in Japan (Edagawa *et al.* 2009), in which 50% of *Naegleria* amoebae failed to form flagellate forms. Thus sequence analysis of the rRNA gene is recommended for confirmation of presence of *Naegleria* (De Jonckheere 2006). We did not detect *N. fowleri*, a pathogenic vahlkampfiid amoeba, and the causal agent of PAM. This is in accordance with research conducted in Japan (Edagawa *et al.* 2009) and the USA (Jamerson *et al.* 2009), and our study provides further evidence that this may be due to lower distribution of this species in river water. However, three species of *Naegleria* (*N. fultoni*, *N. pagei and N. clarki*) were found, according to homology with isolates in the Genbank database (accession numbers: GU597046; AB332170; AJ243445, respectively). It is important to mention that three identified species of *Naegleria* are non-pathogenic, however a previous study revealed that *N. pagei* could coexist with pathogenic *Legionella pneumophila* (Huang & Hsu 2010b). Endosymbiosis relationship between *Naegleria* spp. and pathogenic

microbes can lead to their transmission via amoebae host (Huang *et al*. 2011). Thus, non-pathogenic FLA may have clinical relevance and they must be considered as a potential health hazard in recreation areas. This is the first report of molecular identification of *N. pagei*, *N. fultoni and N. clarki* in Iran (Figures 3 and 4).

In conclusion, although FLA-related disease is not as high as their environmental distribution, as the related disease has a poor prognosis, knowledge regarding FLA distribution in recreational areas is of utmost importance. The present study revealed that individuals may increase exposure to opportunistic pathogens such as potentially pathogenic *Acanthamoeba* and vahlkampfiid amoebae through water related activities. Therefore preventive measures and education regarding water-related activity to susceptible hosts, such as contact lens wearers, is critical for the prevention of FLA-related disease from water-related recreation.

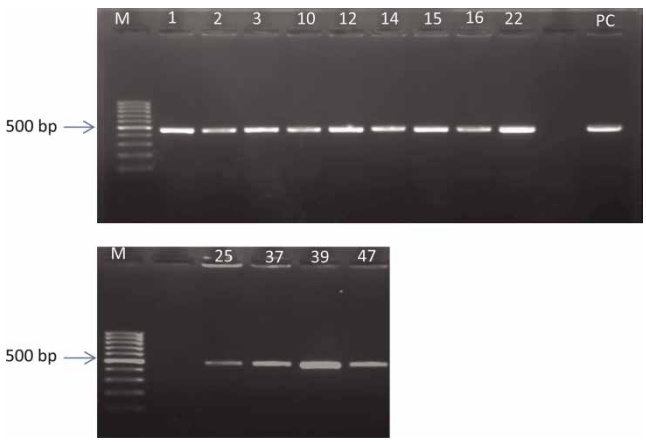

Figure 3 | PCR amplification of the isolated *Acanthamoeba* strains using JDP primers. Numbers: code of isolated amoebae. M: Marker, PC: Positive control.

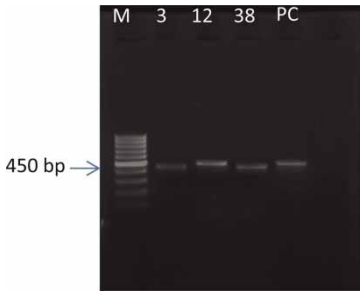

Figure 4 | PCR amplification of the isolated *Naegleria* strains using ITS primers. Numbers: code of isolated amoebae. M: Marker, PC: Positive control.

ACKNOWLEDGEMENTS

Dr Maryam Niyyati was supported by a grant from the National Elites Foundation for Distinguished Young Assistant Professors. The present research was funded by the project #88-01-91-6348 of the Shahid Beheshti University of Medical Sciences, Tehran. Thanks are due to Miss Niloofar Taghipour and Mr Mohammad Eftekhar for their kind help during this research and also the *Lucidus Consultancy*, for the English edition of the manuscript.

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First received 26 April 2011; accepted in revised form 23 October 2011. Available online 20 December 2012