Parasitol Res (2012) 110:473–477
DOI 10.1007/s00436-011-2530-1

SHORT COMMUNICATION

Isolation of *Acanthamoeba* species in surface waters of Gilan province-north of Iran

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Received: 28 March 2011 / Accepted: 24 June 2011 / Published online: 12 July 2011

Abstract We analyzed water samples to determine the prevalence of free-living *Acanthamoeba* in water sources from Gilan, greater area, Iran. A total of 27 surface water samples were collected from environmental sources, including natural (rivers, lakes, springs, and lagoon) and freshwater source. The samples were filtrated and transferred to non-nutrient agar plates seeded with *Escherichia coli* and incubated for 2 to 7 days at 30°C or 42°C. The plates were examined by microscopy to morphologically identify *Acanthamoeba* species. Following DNA extraction, PCR was used to confirm the microscopically identification. A total of 19 out of 27 samples (70.3%) were positive for *Acanthamoeba* species based on the morphological criteria, and 14 (73.7%) were confirmed by PCR method. The high frequency of *Acanthamoeba* spp. in different environmental water sources of Gilan is an alert for the public health related to water sources in Iran.

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Introduction

Acanthamoeba spp. are free-living opportunistic protozoan parasites that pervade the entire environment, and they can be found in tap, fresh, coastal and bottled mineral water, sewage, soil, dust and air, heating or air-conditioning units and contact lens solutions, eyewash stations, and gastrointestinal washings (Mergeryan 1991; Marciano-Cabral et al. 2000; Khan 2003). These amoebae are the causative agents of multifocal encephalitis called granulomatous amebic encephalitis, a chronic central nervous system disease that usually occurs in immunocompromised hosts, amoebic keratitis (AK) and pneumonitis. AK is a corneal infection mainly associated with contact lenses use (Visvesvara et al. 2007). Most episodes of keratitis occur after water exposure or a history of swimming in lakes and ponds while wearing contact lenses and the infection is also linked to non-sterile home-made saline solutions for contact lenses (Radford et al. 1998; Khan 2003). In addition to their own pathogenicitiy, *Acanthamoeba* are known to be a reservoir of bacterial pathogens such as *Legionella pneumophila*, *Mycobacterium avium*, and other "amoeba-resistant micro-organisms" (Greub and Raoult 2004). *Acanthamoeba* feed on bacteria by phagocytosis, but some bacteria are able to survive and sometimes multiply in the host, resulting in new properties of the bacteria (Sandström et al. 2011).

Recently, the ability of free-living amoebae (FLA) to serve as vectors of *Cryptosporidia* has been reported for the first time (Scheid and Schwarzenberger 2011). FLA appear able to act as carriers or vectors of the oocysts and thus may play a certain role in the transmission of *Cryptosporidium parvum* oocysts.

As a co-investigation of our current research for *Giardia* and *Cryptosporidium* distribution in water supplies in Iran, we carried out a survey to determine the prevalence of *Acanthamoeba* spp. in drinking water sources of Iran.

Materials and methods

General information on geography

Gilan is one of the provinces of Iran, and it lies along the Caspian Sea. Gilan has a humid temperate climate with plenty of annual rainfall and is known for its moderate, mild, and Mediterranean-like climate. Large parts of the province are mountainous, green, and forested. Thousands of domestic and foreign tourists come to the seashore river for swimming and camping.

Sampling

During March to November 2009, 27 surface water samples were collected from environmental sources, including natural (rivers, lakes, springs, and lagoon) source from different parts of the Gilan province, north of Iran (Table 1; Fig. 1). From each sampling point, one to three water samples were collected in 500-ml sterile bottles and transported immediately to the laboratory for further processing. The samples were examined for the presence of *Acanthamoeba* in the laboratory of Protozoology Unit, Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Isolation of *Acanthamoeba* species and culture

For the isolation of *Acanthamoeba* species, approximately 500 ml of the collected water samples were filtered through a cellulose nitrate membrane with pore size 0.45 μ. Filter was transferred on non-nutrient agar plates seeded with Gram-negative bacteria (*E. coli*) as a food source. Plates were incubated at room temperature, and 3 days later, they were microscopically examined for the presence of *Acanthamoeba* trophozoites. However, in the absence of amoebae, plates were monitored for up to 14 days. *Acanthamoeba* were identified at the genus level, based on morphological characteristics of trophozoites and cysts using phase-contrast microscopy (Fig. 2).

Table 1 Surface water samples collected from various location in Gilan, Iran and investigated for *Acanthamoeba*

Samples	Name of the source	Number of samples in culture pos/exam	Number of samples after PCR pos/exam
1	Sefid roud River	3/3	3/3
2	Bandar-e Anzali Lagoon	1/1	1/1
3	Amlash River	1/1	1/1
4	Chamkhaleh Lagoon	2/2	2/2
5	Chamkhaleh Seawater	1/1	1/1
6	Chaboksar Seawater	1/1	1/1
7	Emam-Zadeh Hashem Dam	0/1	–
8	Emam-Zadeh Hashem River*	1/1	0/1
9	Kiashahr Lagoon	1/1	1/1
10	Kiashahr Seawater	0/1	–
11	Lahijan Cascade	1/1	1/1
12	Langroud River*	2/3	1/2
13	Lounak Cascade	0/1	–
14	Manjil Cascade*	1/1	0/1
15	Pole-Koumle River	0/1	–
16	zarjoob River*	2/3	1/2
17	Roudbar River	0/1	–
18	Roudsar River	0/1	–
19	Sangar Dam	1/1	1/1
20	Siyahkal River*	1/1	0/1
Total number of samples investigated		19 (70.3%)/27 (100%)	14 (73.7%)/19 (100%)

DNA extraction and PCR

Amoeba cells were harvested from culture plates, concentrated by centrifugation, and then lysed by treatment with lysozyme (100 mg/ml) and freeze-thawing (using liquid nitrogen and heating to 96°C). The samples were then treated with 2–5 μl proteinase K (18.9 mg/ml), and DNA extraction was performed by phenol–chloroform method. PCR assay: the *Acanthamoeba*-specific primer pairs JDP1 (5′-GGCCCAGATCGTT TACCGTGAA) and JDP2 (5′-TCTCACAAGCTGCTAGG GAGTCA) as described by Schroeder et al. (2001) were used for the amplification of the 500 bp of 18S rDNA gene (Edagawa et al. 2009).

Standard PCRs were performed in 50-μl volumes, containing 5 μl of 10× PCR buffer, 20 pM of each of the primers, 4 mM $MgCl_2$, 0.2 mM dNTP, 1.25 U Taq polymerase (Cinnagen), and 1–10 ng of template DNA. Thermal cycling conditions were 94°C for 5 min; 32 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 40 s; followed by a final extension at 72°C for 5 min.

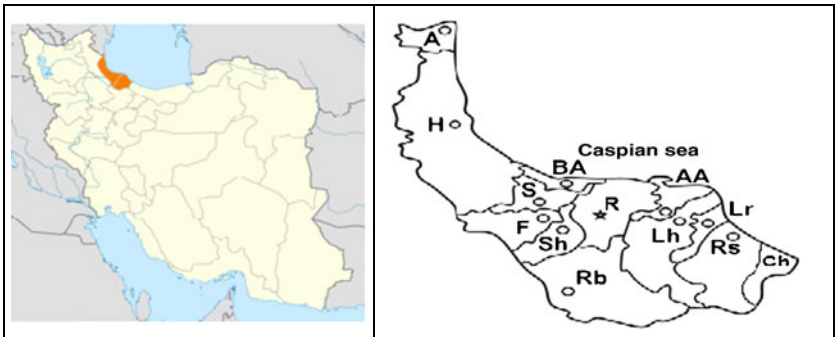

Fig. 1 Map of the investigated Gilan greater area (*right*) and its location in Iran (*left*)

Abbreviation in Gilan map	County
AA	Astaneh Ashrafiyeh
BA	Bandar-e Anzali
Lh	Lahijan
Lr	Langerood
R	Rasht
Rs	Roudsar
Ch	Chaboksar
Rb	Rudbar

Results and discussion

Culture Based on the morphological criteria, 19 out of 27 (70.3%) surface waters contained *Acanthamoeba* spp. (Table 1). Some of the samples contain other free-living organisms. *Acanthamoeba* can be identified at the genus level based on distinctive features of trophozoites and cysts, especially the double-walled cyst shape that is unique to the genus. *Acanthamoeba* species have been classified into three distinct morphological groups (I, II, and III). However, this division is inconsistent (Stothard et al. 1998; Alves et al. 2000). The taxonomy and classification of these parasites are currently under revision, following the successful application of molecular techniques (Booton et al. 2002; Kong et al. 2002; Khan 2003).

PCR Further detection and identification of *Acanthamoeba* species from the culture plates has been approved by the PCR (Fig. 3). The primers used in this study are specific for *Acanthamoeba* spp.. From 19 microscopically positive culture samples, 14 (73.7%) were also positive by PCR (Tables 1 and 2). Five negative PCR amplifications may include other FLA, like *Thecamoebidae* family (Table 2). According to Khan and Paget (2002) DNA sequencing is probably the most detailed and accurate technique for *Acanthamoeba* species differentiation.

Current situation in Iran: A 10-year study in Iran reveals that there is a considerable increase in the incidence of amoebic keratitis during the recent years (Rezeaian et al. 2007). Bagheri et al. (2010) showed that about half of the investigated samples collected from cold and warm tap

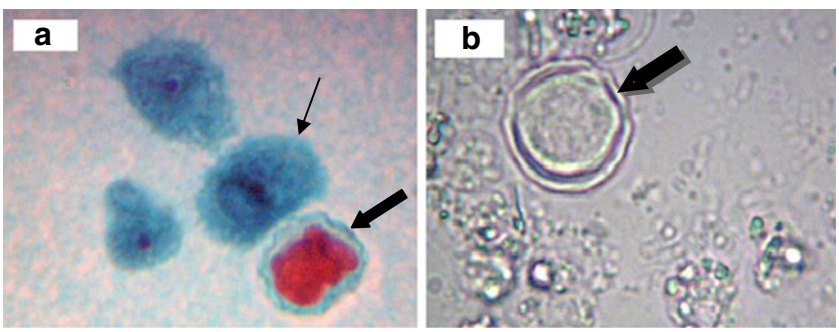

Fig. 2 *Acanthamoeba* species after isolation from water samples and cultivation. **a** Trophozoites (*thin arrow*) and a cyst (*big arrow*) after trichrome stain; ×400; **b** cyst form ×400

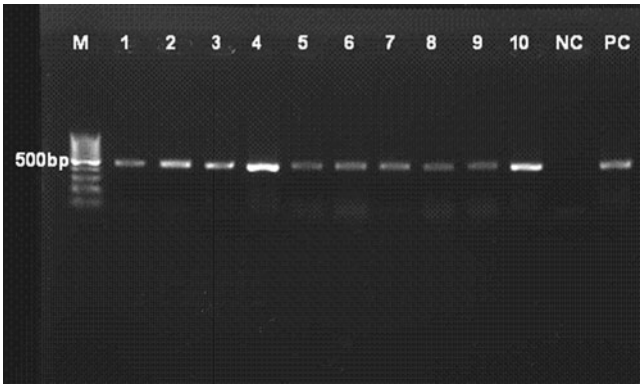

Fig. 3 PCR products on a 1.5% agarose gel. *Lanes 1–10* *Acanthamoeba* positive isolates with JDP primers. *Lane NC* *Acanthamoeba* negative control, *lane PC* positive control *Acanthamoeba* isolate NHE (Ac) 14-IR (Accession no.AB525824.1), *lane M* 100-bp marker

water in Iran were infected with *Acanthamoeba* species. Maghsood et al. (2005) examined 12 water samples in Iran by molecular methods and revealed that T2 genotype was the most frequent genotype in their samples and also isolated, T2 genotype from three keratitis patients although to date, only isolates belonging to the T3, T4, T6, and T11 genotypes have been associated with *Acanthamoeba* keratitis (Maghsood et al. 2005). Also Rezaeian et al. (2008) recovered *Acanthamoeba* spp. from 58% collected samples from a variety of ecological habitats such as tap water, soil, and dust. However, systematic information on the occurrence and distribution of potentially pathogenic species of FLA in environmental waters in different parts of Iran remain unclear.

According to Karanis et al. (2007), *Acanthamoeba* spp. was the causative agent of an outbreak associated with a contaminated municipal water supply in the USA and FLA has been commonly found in various environmental water sources throughout the world. Edagawa et al. (2009) reported the prevalence of FLA in tap water sources from rivers and water treatment plants located in Osaka Prefecture, Japan. A total of 257 of 374 samples (68.7%) were positive for FLA by microscopy. Tsvetkova et al. (2004) in Bulgaria environmental sources, determine FLA from 171 (61.1%) of 280 samples. There are also many cases of amoebic keratitis, which occurs after swimming in contaminated pools, usage of homemade saline for washing contact lenses, and exposure to dirt and dust (Khan 2006).

Table 2 Comparative results on the identification of *Acanthamoeba* species in surface water samples using culture and PCR

	Positive	Negative	Total
Culture	19 (70.3%)	8 (29.7%)	27 (100%)
PCR	14 (73.7%)	5 (26.3%)	19 (100%)

In conclusion, we found that nearly 70% of the investigated water samples in Gilan greater area in Iran were contaminated by potentially pathogenic *Acanthamoeba* species. The investigated surface waters, including natural sources (rivers, lakes, springs, lagoons), are used for agriculture and swimming activities, and in case of Emamzadeh Hashem Dam water, using for drinking water source after treatment. We suggest that the real risk of *Acanthamoeba* pathogenesis, especially immunocompromised patients, should be considered, and more attention must be paid on the distribution of *Acanthamoeba* species in surface waters to this organism in Iran. The presented work can serve as a platform for future investigations.

Acknowledgments The authors would like to appreciate Dr. Bahram Kazemi for his kind cooperation and assistance. DNA of Isolate NHE (Ac) 14-IR (Accession no.AB525824.1) used as a positive control obtained from Parasitology Department of Medical School of Shahid Beheshti Medical University. This work was supported by Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences.

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