Original Article

Reporting of T4 Genotype of *Acanthamoeba* Isolates in Recreational Water Sources of Gilan Province, Northern Iran

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Abstract

Background: *Acanthamoeba* spp. is the causative agent of blindness keratitis and fatal encephalaitis. Presence of *Acanthamoeba* spp. in a wide variety of niches such as different water types can lead to exposure of high risk people such as contact lens wearers. The main aim of the present study was to explore the occurrence of *Acanthamoeba* genotypes in the recreational water sources using both morphological and molecular approaches in Gilan province, Iran.

Materials and Methods: Overall, 50 samples were collected from recreational water sources including man-made and natural waters in Gilan province. Filtration and cultivation of samples was performed using non-nutrient agar. Cloning of *Acanthamoeba* spp. was done to eliminate bacterial and fungi contamination. PCR amplification and sequencing were performed using genus-specific primer pair. Genotype identification was based on homology analysis of 18S rRNA gene (DF3) of the obtained sequences with the available genes in the gene bank data base.

Results: Out of 50 water samples, 15 (30%) were positive for *Acanthamoeba* trophozoites and cysts according to morphological criteria. Cloning of 13 isolates (26%) was done successfully. Molecular analysis of 13 *Acanthamoeba* strain revealed that all isolates were belonged to potentially pathogenic T4 genotype.

Conclusion: T4 genotype is the main cause of *Acanthamoeba*-related infections. Presence of *Acanthamoeba* belonged to T4 genotype in recreational water sources is of concern for high risk people. Alarming sign and education to high risk people is of utmost importance to prevent such infections.

Keywords: *Acanthamoeba*, Sequencing, Recreational waters, Gilan province

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Introduction

There are free-living amoebae (FLA) includes various taxa such as; *Acanthamoeba spp*, *Naegleria fowleri* and *Balamuthia mandrillaris*. These FLA unicellular organisms can cause severe and fatal diseases including encephalitis, skin granulomatous and keratitis[1-3]. Among many genera of FLA, ubiquity of *Acanthamoeba* spp. leads to its worldwide habitat in many niches such as various water sources, soil, dust and air[4,5]. Up to now, *Acathamoeba* includes 17 identified genotypes (T1-T17) and is the most ubiquitous protozoan parasites which survives in many water types such as mineral water, fresh water, hot springs, hot tubs and sea water[6-9]. To this end, water can be a source of potentially pathogenic

Acanthamoeba spp. and thus, contaminated waters could be a hazard for high risk people such as contact lens wearers, patients with eye surgery, eye trauma and immune compromised patients[2,3]. On the other hand, *Acanthamoeba* spp. can act as Trojan horses for microbial world including pathogenic bacteria, viruses and fungi[2,10]. Therefore, even non-pathogenic *Acanthamoeba* such as T7 and T8 strains are also of clinical relevance[2].

The only reported FLA-related disease in Iran was Amoebic Keratitis (AK) due to *Acanthamoeba*[11, 12]. It is worthy to mention that infection with *Vahlakmpfia* has reported as a mix infection in this region, therefore the causality of *Vahlkampfia* as an agent of keratitis is still unclear[13]. Globally, the predominant *Acanthamoeba* spp. causing keratitis is T4 genotype. Interestingly, most of patients report water activity while wearing contact lenses before onset of disease[2]. On the other hand, the north of Iran such as Gilan province attract many tourists due to its Mediterranean climate and the presence of many recreational water sources such as coastal waters, pools and streams.

The extent to which that *Acanthamoeba* spp. is present in environmental sources such as dust, soil and water in Iran has been previously reported[11,12,14,15]. However, there is no report regarding distribution of *Acanthamoeba* genotypes in man-made and natural waters of Gilan province. The main aim of the present study was to address the occurrence of *Acanthamoeba* spp. in recreational water sources using both morphological and molecular approaches in Gilan province, Iran.

Methods

Sampling: Overall, 50 samples were collected during 2010-2011 from various recreational waters in Gilan Province, Northern Iran. All included water sources (natural and man-made) were used for recreational activity mainly swimming, washing and to some extent drinking. The water sources were sea water (n=10), pools (n=10), ponds (n=10), waterfalls (n=10) and streams (n=10). Briefly, 500 ml of water was placed into two sterile bottles and transported to the Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Processing of the samples and Culture method: Filtration was performed using cellulose membrane (pore size 1.2 µm). The filters were placed upside down onto 0.8% non- nutrient agar plates (Bacto-agar) overlaid with an autoclaved *Escherchia coli*. All plates were sealed and incubation was done in 30^{o}C for one month. Microscopic detection of cysts was performed according to page key by using light and inverted microscope[15]. Cloning of positive plates was done using serial passages in order to eliminate bacterial and fungal contaminations.

PCR and gel electrophoresis: Amoebae in plates were washed using Phosphate- buffered saline (pH 7). Extraction of DNA was performed using the Instagene matrix (Chelex; Biorad) according to manufactures instruction. Briefly, 10^5 cells were incubated with 50 µl Chelex. Incubation was performed at 56^{o}C for 20 minutes (min), followed by 10 min. incubation in boil water. After centrifuge at 10,000 g for 5 min. the supernatant was used as DNA template for PCR.

The PCR solution was obtained in a 30 µl Ampliqone (Taq DNA Polymerase Master Mix Red, Denmark) as a readymade mixture. Briefly, 25 µl of the kit with 5 ng DNA templates and 0.1 µM primers were mixed to achieve a total volume of 30 µl Primers JDP1 and JDP2[17] were used to amplify a fragment of 18S rRNA gene called Diagnostic Fragment 3 (DF3). The amplification was performed in the thermocycler with the following conditions: 94^{o}C for 1 min., followed by 35 repetition cycles at 94^{o}C for 35 second, annealing at 56^{o}C for 45 second and extension at 72^{o}C for 1 min. PCR products were then electrophoresed using 1.5% agarose gel, stained with ethidium bromide and visualized under UV illumination.

Sequencing and genotype identification: PCR products have been submitted to ABI 3130X automatic sequencer in the Research Center for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran. The sequences were analyzed against all available nucleotide sequences in the GenBank database. 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) (GenBank ID: JN399011- JN399023).

Results

Out of 50 recreational water samples, 15 samples (30%) were positive for *Acanthamoeba* trophozoites and cysts according to morphological criteria[16]. Identification of *Acanthamoeba* spp. was based on characteristic double walled cysts with wrinkled endocyst and smooth or round ectocysts. Identification of trophozoites was based on flat shape, prominent nucleus and fine structures of acanthopodia. Culture of 13 strains were done successfully after 2-3 months, however 2 cultures showed very high fungi contaminations and therefore

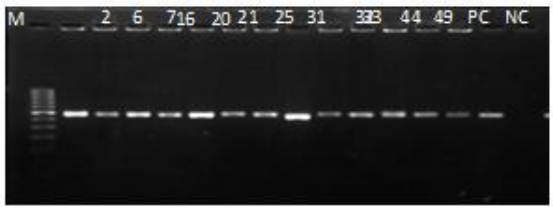

Figure 1. Gel electrophoresis of PCR products of 13 *Acanthamoeba* strains isolated from water sources of Gilan Province, Iran; M: Molecular size marker, Numbers: Code of each sample, PC: Positive Control, NC: Negative Control.

Table 1: Genotypes of *Acanthamoeba* isolates, obtained from recreational waters sources of Gilan Province, Iran.

Isolates code	City	source	Culture	PCR	Genotype	Accession number
***NW1**	Rasht	Pond	+	+	T4	JN399011
NW2	Rasht	Pond	+	+	T4	JN399012
NW6	Roodbar	Pond	+	+	T4	JN399013
NW7	Lahijan	Waterfall	+	+	T4	JN399014
NW16	Lahijan	Pool	+	+	T4	JN399015
NW20	Anzali	Stream	+	+	T4	JN399016
NW21	Anzali	Sea	+	+	T4	JN399017
NW25	Rasht	Sea	+	+	T4	JN399018
NW31	Astara	Sea	+	+	T4	JN399019
NW32	Astara	Sea	+	+	T4	JN399020
NW33	Astara	Pond	+	+	T4	JN399021
NW44	Rezvan shahr	Pond	+	+	T4	JN399022
NW49	Rezvan shahr	Stream	+	+	T4	JN399023
NW 38	Astara	Stream	+	**ND	---	-----
NW 50	Anzali	Pool	+	**ND	---	-----

* NW: North Water
** ND: Not Determined

Table 2: Number and percent of recreational water Contamination to *Acanthamoeba* spp.

Sample type	Total	Positive number (%)
Pools	10	2 (20)
Sea water	10	4 (40)
Streams	10	3 (30)
Ponds	10	5 (50)
Water falls	10	1 (10)
Total	**50**	**15 (30)**

the latter's were identified only at morphological level (Table 1).

PCR amplification using genus specific primers pairs revealed an approximately 450-500 base pair product in 13 isolates (26%) (Fig. 1). Sequencing and homology analysis of the obtained sequences in Basic Local Alignment Search Tool (BLAST) showed that all *Acanthamoeba* strains belonged to the potentially pathogenic T4 genotype (Identity 96-100%) (Table 1). Interestingly, four isolates (NW21, NW25, NW31 and NW32) have isolated from high salinity water (Caspian Sea water). It should be noted that Contamination of pond waters to *Acanthamoeba* spp. were higher than other sources (Table 2).

Discussion

This is the first study regarding the presence of *Acanthamoeba* spp. belonging to the potentially pathogenic T4 genotype in water sources of Gilan Province, Northern Iran. The present study showed that 30% of recreational water sources were contaminated with *Acanthamoeba* spp. and all isolated strains were belonged to the potentially pathogenic T4 genotype. This is in opposed to the study of Maghsood *et al* who reported *Acanthamoeba* T2 strains as the predominant genotype in water sources of Iran[11]. In concordance to our study Nazar *et al.* reported T4 genotype as the most isolated strains in waters of ponds and squires in Tehran Province[18]. This can be due to the difference of genotype distribution in various examined water. Researches have shown that T4 genotype is the main cause of *Acanthamoeba*-related infection in Iran and worldwide[1, 7, 11, 12]. Besides, previous researches in Iran revealed an increased rate of keratitis due to *Acanthamoeba* spp. and the predominant genotype in all studied patients was T4 genotype[11,12]. This is due to properties that make T4 strains more virulent as shown by higher binding and remarkable cytotoxicity on host cells[11]. On the other hand, researchers have reported that T4 genotype have more binding ability in comparison to T2, T3, T7 and T11 genotypes[19].

All water sources included in the present study were associated with human activity mainly swimming and washing. Interestingly, *Acanthamoeba* have been isolated from coastal water with high salinity (isolates: NW21, NW25, NW31 and NW32). Previous researches revealed that *Acanthamoeba* strains which withstand extremes of osmolarities such as high salinity are more likely to be a pathogen of human and animals[2]. Therefore, high risk people including contact lens wearers can be in exposure to potentially pathogenic *Acanthamoeba* by recreation in contaminated water sources.

Conclusion

Occurrence of potentially pathogenic *Acanthamoeba* T4 genotype in recreational waters could be a hazard for high risk people. Implication of alarming sign and education to high risk people such as contact lens wearers is of special importance in preventing people to recreation in such waters.

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