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A REVIEW: ISOLATION OF ACANTHAMOEBA SPECIES IN SURFACE WATERS OF YASUJ DISTRICT SOUTH OF IRAN

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ABSTRACT

Background: *Acanthamoeba* spp. are opportunistic amphizoic protozoans, which are distributed in the natural and artificial environment water sources. This ubiquitous amoeba is the causative agent of Amoebic Keratitis(AK) and Granulomatous Amoebic Encephalitis(GAE). The main aim of the current study was to identify the presence of *Acanthamoeba* spp. In surface waters of Yasuj district south of Iran.
Methods: In this descriptive cross-sectional study, a total of 30 surface water samples were collected from environmental sources, including natural rivers, springs, waterfall and freshwater source in 2013. All samples were collected using 500 ml sterile plastic bottles during two month. After filtration through millipore nylon membrane, samples were cultured on non-nutrient bacto agar medium enriched with *Escherichia coli* and incubated for 3 to 14 days at room temperature. Identification of the *Acanthamoeba spp.* was based on morphological criteria of cysts and trophozoites. Following DNA extraction, PCR was used to confirm the microscopically identification.
Results: A total of 11 out of 30 samples (36.6%) were positive for *Acanthamoeba* species based on the morphological criteria. Five out of 11 positive samples (45.46%) were confirmed by PCR method. In total, 5 (16.6%) samples out of 30 samples were positive for *Acanthamoeba* species based on PCR method.
Conclusion: High frequency of *Acanthamoeba* spp. in different surface water sources specially promenades in that region is an alert for the public health and highlights the needs for more awareness of health professionals and for the related risks.

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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 (1,2,3). These amoebae are the causative agents of multifocal encephalitis called granulomatous amebic encephalitis, a chronic central nervous system disease that usually occurs in

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immunocompromised hosts, amoebic keratitis (AK) and pneumonitis(4). 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 (3,5).

The importance of Acanthamoeba more revealed according to recently studies in some parts of Iran. Positive rate of Acanthamoeba in drinking waters in several hospitals of Iran was 48% (6) and in hospital wards with immunodeficient patients in Tehran, was 52.9% (7) and in surface waters consist of rivers, lakes, springs, and lagoon of Gilan province-north of Iran was 70.3% (8), In Sarein, Ardebil province, north west of Iran, in hot springs were 42.9% (9) and in river recreation areas in Tehran province was 27.3% (10) and in environmental sources in ahvaz city, khuzestan province, southern Iran was 71.6% of samples of water and 26% soil samples (11)

Otherwise new reported cases of diseases show the importance role of Acanthamoeba in producing illness. In recent published paper the authors reported a 5-year-old Iranian immunocompetent girl who died of fulminant acanthamoeba meningoencephalitis. To the authors' knowledge, that was the first case of acanthamoeba meningoencephalitis in Iran(12)

Also, a case of young immunocompetent male adult with autopsy proven acanthamoeba meningoencephalitis was reported by Chandra and etal. From India. (13).

New studies in Iran revealed the genotype of Acanthamoeba spp. Isolated Acanthamoeba from water in recreational areas of Tehran, was belonged to the T4 and T5 genotypes(14). and in another study 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(10). In a study, Sequence analysis of the single isolate of Acanthamoeba revealed potentially pathogenic T(4) genotype corresponding to A. castellanii(9). In Ahvaz. Genotyping of positive samples proved that Acanthamoeba belonged to T4, T2, and T5 genotypes(11). In hospital research Acanthamoeba belonged to the T4 genotype was the most prevalent isolate. Presence of the T4 genotype on medical instruments, including an oxygen mask in an isolation room of an immunodeficiency pediatric ward, should be of concern for health authorities. Acanthamoeba T5 genotypes, Hartmannella vermiformis, and Vahlkampfia avara were also present(7). In therapeutic hot springs in Iran Acanthamoeba belonging to the potentially pathogenic T4 and T3 genotypes(15). Results of the first study for presenting the identification of pathogenic genotypes of Acanthamoeba in dust samples in Iran revealed the presence of T4, T5 and T11 genotypes within those samples(16).

In another study in Iran, The obtained results revealed that most of Acanthamoeba strains belonged to genotype T4 both in clinical and environmental(water, soil and animal-origin) samples and T11 genotype in clinical samples was also found after the genotyping analysis. Moreover, the isolation of T4 genotype from cow faeces in that study highlights a possible transmission of Acanthamoeba through animal faeces in Iran(17).

Furthermore, reports of new diseases due to Acanthamoeba is remarkable for researchers. Such as First report of a mixed infection due to Acanthamoeba genotype T3 and Vahlkampfia in a cosmetic soft contact lens wearer in Iran(18) and In a study authors results support the hypothesis that some parasitic microorganisms such as Acanthamoeba can involve and contribute toward the development of rheumatoid syndromes(19).

According to above evidences, this study was conducted to isolation of Acanthamoeba species in surface waters of Yasuj district south of Iran for use the results to identification areas with Acanthamoeba contamination.

2. Materials and methods

2.1. General information on geography

Yasuj is an industrial city in the Zagros Mountains of southwestern Iran, and is the capital of Kohgiluyeh and Boyer-Ahmad Province. Yasuj has an estimated population of 140,000 and famous to capital of nature of Iran.

The province is mostly mountainous in terrain, part of the Zagros range. The highest point is the Dena summit with a height of 4,409 meters.

The mountain range, which is located in Kohgiluyeh and Buyer-Ahmad province, is covered with oak forests. Natural springs, singing of the birds and fresh air fascinate all lovers of nature. The snow and rain falling is quite sizable in the city. Yasuj 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.

2.2. Sampling

During May to November 2013, 30 surface water samples were collected from environmental sources, including natural (rivers, lakes and springs) source from different parts of Yasuj, south of Iran. From each sampling point, one to three water samples were collected in 1000-ml sterile bottles and transported immediately to the laboratory of medicine school for further processing.

2.3. Isolation of Acanthamoeba species and culture

For the isolation of *Acanthamoeba* species, approximately 1000 ml of the collected water samples were filtered through a cellulose nitrate membrane with pore size 0.45 µ. Filter was transferred on bacto agar plates seeded with Gram-negative bacteria (*E. coli*) as a food source. Plates were incubated at room temperature, and after 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.

2.4. DNA extraction and PCR

Amoeba cells were harvested from culture plates, concentrated by centrifugation, and then lysed by treatment with lysozyme (100 mg/ml). 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′-GGCCCAGATCGTTTACCGTGAA) and JDP2 (5′-TCTCACAAGCTGCTAGGGAGTCA) as described by Schroeder et al. (*2001*) were used for the amplification of the 500 bp of 18S rDNA gene (20,21).

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.

3. Results

A total of 11 out of 30 samples (36.6%) were positive for *Acanthamoeba* species based on the morphological criteria. Five out of 11 positive samples (45.46%) were confirmed by PCR method. In total, 5 (16.6%) samples out of 30 samples were positive for *Acanthamoeba* species based on PCR method (fig1).

The name of five positive area are:

1- Tange Gange ei
2- Sadde Shah Ghasem (arrival river)
3- Beshar River (Ali Abad)
4- Sadde Shah Ghasem (going out river)
5- Tange Mehrian

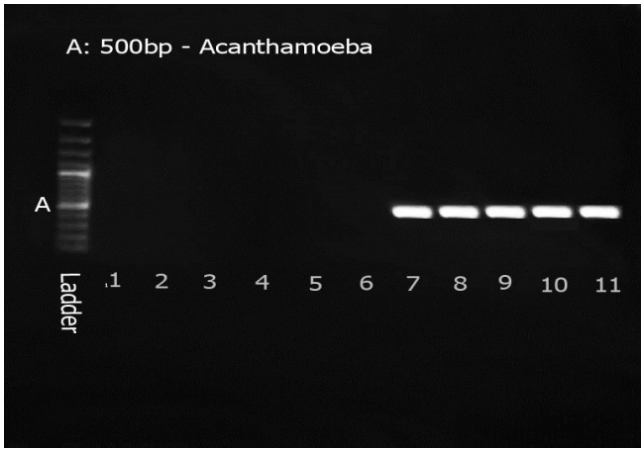

Fig1) Gel electrophoresis of PCR products
A: marker, 1-6: negative cases, 7-11: positive cases (500 bp)

4. Discussion

The present study highlight the presence of Acanthamoeba in surface waters of Yasuj city. All the waters included in the present research was in contact with human activity and thus this could be a hazard for high risk people including soft contact lens wearers and immunosuppressed patients especially those with frequent contact with such waters (22). On the other hand, there is a high risk for users, including children who have weak immune system, related to the high resistance of Acanthamoeba cysts to harsh environmental situation. There are various studies on surface waters and their contamination to Acanthamoeba in Iran and worldwide (23,24). In a study conducted on the surface waters by Rezaeian et al. there were a high occurrence of Acanthamoeba in environmental sources including water, dust, soil and animal feces (24). Their study showed that out of 80 samples of different environments in Tehran, 37 samples were contmanited with Acanthamoeba (46.25%).

The difference between Rezaeian et al study and the present research was the source of samples. The present study focused on only recreational water sources.

Another study conducted by Badirzadeh et al. showed the presence of free living amoeba including Vahlkampfiids (11) and Acanthameba (1) in hot prings of Sarein county, Ardebil province. The low occurrence of Acanthamoeba in hot spring samples in the mentioned research could be attributed to high temperature of waters in their study (9). Hoewever, despite

Badirzadeh et al research, Solgi et al. study in 2012 on all hot springs in Ardebil revealed that 20% of the hot springs were contaminated with Acanthamoeba (25). It should be mention that they also isolated other FLAs including Hartmannella and Naegleria (25).
The present study isolated FLA in recreational waters in order to find more detailed data about distribution of these amoebae in Yasuj city. It should be mention that no study has been performed on Acanthamoeba in this region and the present research was the first to investigate the presence of Amoebae in the region .
In Coşkun et al. research in 2013 in Turkey, 150 water samples were collected from six region of Sivas, Turkey The samples were cultured and of the 150 samples, 33 samples (22%) were positive for FLA. It should be mention that amoebae were detected using morphological keys, and most isolated amoebae were Acanthamoeba (26). Shoff et al. also conducted a study (2008) to examine the distribution of Acanthamoeba and other FLA in household waters. In their study, 283 water samples were collected from houses, and amoebae were detected using morphological criteria in the culture medium. Of 283 samples, 80 were positive for amoebae, and Acanthamoeba were detected in 8 samples (2.8%)(27).

One of the finding of the present research was the low number of PCR positive versus morphological identification. This is may be due to contamination of the plates to various microorganisms such as funji and bacteria. Another limitations of the present study was the impossibility of sequencing that could not be supplied due to financial supports, however it is hoped that we could achieve the goal in near future.
Overall, the present research reflect that warning signs should be implicated in recreational areas such as near water sources and education to high risk people are necessary for amoeba disease prevention.

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