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Genotyping of *Acanthamoeba* spp. Isolated from the Caspian Sea in Iran †

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Abstract: *Acanthamoeba* spp. are widely distributed in the environment and have been reported to be causative agents of lethal encephalitis and keratitis. In this study, thirty water samples from the Caspian Sea were collected during 2018. Water samples were filtrated and the filtrate used for culture. The positive samples were subjected to Polymerase Chain Reaction (PCR) and nucleotide sequencing. Free-living amoebae were identified in 50% (15/30) of the seawater samples. DNA sequencing revealed the presence of T2 and T4 genotypes. The results of the present study confirmed the presence of potentially pathogenic strains in seawater in this area. This awareness should be raised among environmental and public health professionals.

Keywords: *Acanthamoeba*; seawater; prevalence; Caspian Sea; Iran

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1. Introduction

Acanthamoeba, a free-living amoeba, can be present in different environmental sources, including air, soil, dust, drinking water, seawater, and recreational water [1–3]. *Acanthamoeba* is the causative agent of granulomatous amoebic encephalitis (GAE) and amoebic keratitis (AK). AK mostly reported in persons after swimming while wearing contact lenses or inadequate contact lens care [4]. *Acanthamoeba* could harbor some pathogenic organisms such as *Helicobacter*, *Pseudomonas*, and *Legionella* [5].

The presence of *Acanthamoeba* was investigated in many water samples, which are frequently exposed to humans [4,6]. Tourists frequently visit the coastal area of the Caspian Sea in Guilan province, especially during the summer. Even though *Acanthamoeba* was investigated in some surface water in Guilan province [7–9], there is still a lack of knowledge about the prevalence and pathogenicity of *Acanthamoeba* in seawater in this area.

Exposure to *Acanthamoeba* occurs frequently through water contact and knowledge of the presence of the organisms in water sources is important in understanding transmission dynamics.

In the present study, the distribution *Acanthamoeba* was investigated in water samples collected from the coastal area of Caspian Sea in various cities in Guilan.

2. Material and Methods

2.1. Sampling Area

This study was carried out in Guilan Province, in Northern Iran, May–June 2018. Guilan province (37.2809° N, 49.5924° E) lies along the Caspian Sea. It has a humid subtropical climate with, by a large margin, the heaviest rainfall in Iran, reaching as high as 1900 millimeters (75 in) in the southwestern coast and generally, around 1400 millimeters (55 in).

In total, 30 samples were collected from the Caspian Sea in various cities of Guilan. Water samples were collected randomly from the Caspian Sea, including, Chamkhaleh, Kiyahshahr, and Bandaranzali.

These water sources were near residential areas or in public places. Water samples were taken ~30 to 60 cm from the shore and ~10 to 20 cm in depth. At least a volume of 500 mL for each water sample was collected in each place at 1–2 month intervals.

2.2. Filtration and Culture Method

About 500 mL of each sample were filtered through a cellulose nitrate filter (Millipore Corporation, Bedford, Madison, WI, USA), pore size 0.45 μm, using a vacuum pump. Then, the filters were inverted onto 1.5% non-nutrient agar plates coated with heat-killed *Escherichia coli*. The plates were incubated at 37 °C and the presence of cysts and trophozoites were controlled daily for 14 days using an inverted microscope. Microscopy detection was performed according to the Pussard and Pons criteria [10]. In addition, the pathogenicity of each isolate as a human pathogen was evaluated using a thermo-tolerance test by culturing in 42 °C.

2.3. DNA Extraction

Material from positive plates was gently scraped and washed with PBS (pH 7.4) and centrifuge at 2000 rpm for 5 min. Finally, DNA was extracted using the tissue DNA extraction kit according to the manufacturer's instructions with some modifications. Briefly, the suspension including amoeba cysts or trophozoites were passed 10 times of freeze and thaw at −196 °C for 5 min, followed by 5 min in boiling water.

2.4. PCR Assay

PCR was performed using JDP primer; (JDP1: 5′-GGCCCAGATCGTTTACCGTGAA-3′) and JDP2: 5′-TCTCACAAGCTGCTAGGGGAGTCA-3′) were used for the amplification of approximately 500 bp of the 18S rDNA gene called Diagnostic Fragment 3 (DF3) [11,12].

Thermal cycling conditions were 94 °C for 3 min; 35 cycles of 94 °C for 35 s, 56 °C for 45 s, 72 °C for 45 s; followed by a final extension at 72 °C for 5 min. *Acanthamoeba* strains previously identified at our laboratory were used as a positive control and DNA-free water was used as a negative control.

The PCR products electrophoresis was done on 1.5% gel agarose stained with ethidium bromide solution and visualized under UV light.

2.5. Nucleotide Sequencing

For genotype identification, partial 18S rRNA sequencing (DF3 region) was performed with amplification primers. Sequencing results were compared with reference species for each genotype.

3. Results

In the present study, free-living amoebae were detected in 50% (15/30) samples by culture in NNA media at room temperature. Ten samples were sequenced and five sequenced results give good quality and revealed the presence of T4 (1 sample) and T2 (4 samples) genotype (Table 1). The pathogenic potential using the thermo-tolerance test was shown by 10% of the *Acanthamoeba* strains isolated from seawater.

Potentially pathogenic *Acanthamoeba* was detected in all three sampling sites. Frequency of *Acanthamoeba* in water samples collected from seawater was 60%, 40%, and 50% in Chamkhale, Kiyashahr, and Bandar Anjali, respectively.

Table 1. Frequency of *Acanthamoeba* spp. in water samples collected from the Caspian Sea in Guilan, Iran.

Sampling Site	Number of Samples	Culture Result	Sequencing Results
Chamkhaleh	10	6	T4
Kiyahshahr	10	4	T2
Bandaranzali	10	5	T2
Total	30	15	

4. Discussion

In the present study, free-living amoebae were detected in 50% samples by culture in NNA media.

In a study of seawater in Jamaica, West Indies, *Acanthamoeba* was identified in 49.6% of the seawater samples. The pathogenic potential was shown by 40.4% of the seawater strains according to the morphologies of the cysts and trophozoites, and thermo-tolerance and osmo-tolerance assays [6]. In the study of Gregory (2004), *Acanthamoeba* strains were isolated from beach sand (n = 20) and nearly all beach isolates were genotype T4 [13]. In our present study, one sample belonged to the T4 genotype and T2 (four samples) genotype.

This is in concordance with the study of Maghsood et al., (2005), who reported *Acanthamoeba* T2 strains as the predominant genotype in water sources of Iran [14]. In opposition to our study, Mahmoudi et al., (2005) reported the T4 genotype as the most isolated strain in surface water in Guilan province [9]. The T4 genotype is the main cause of *Acanthamoeba*-related infection in Iran and worldwide [4,5,14]. The T2 genotype, which is scarcely reported in patients worldwide, was also identified in the present study.

In Guilan, some reports address free-living amoeba (FLA) isolation from surface water in the previous study [7–9]. However, there is a lack of knowledge about the prevalence and pathogenicity of *Acanthamoeba* in seawater in this area. Interestingly, in the present study, *Acanthamoeba* was isolated from coastal water with high salinity. Previous research revealed that *Acanthamoeba* strains which withstand extremes of osmolarities such as high salinity are more likely to be a pathogen of human and animals [15]. Moreover, the fact that *Acanthamoeba* spp. can act as natural vectors for pathogenic microorganisms has an impact on the public health [16]. Guilan receives millions of tourists annually; all water sources included in the present study were associated with human activity, mainly swimming. Therefore, for preventing contamination, it is necessary for public health authorities to inform the population and high-risk people, including contact lens wearers and immuno-compromised patients.

5. Conclusions

The existence of pathogenic *Acanthamoeba* spp. in the coasts of Guilan province has been confirmed. Guilan receives millions of tourists annually and all water sources included in the present study were associated with human activity, mainly swimming. It is necessary to inform the population and the high-risk people, including contact lens wearers and immuno-compromised patients.

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Conflicts of Interest: The authors declare that there is no conflict of interest.

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