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Isolation and genotyping of *Acanthamoeba* strains from water sources of Kermanshah, Iran

Mitra Salehi[1], Hamid Reza Niazkar[2], Amirreza Nasirzadeh[2]

[1]Department of Medical Parasitology, Gonabad University of Medical Sciences, Gonabad, Iran
[2]Student Research Committee, Gonabad University of Medical Sciences, Gonabad, Iran

Corresponding Author: Amirreza Nasirzadeh; e-mail: nasirzadeharnz@gmail.com

ABSTRACT. *Acanthamoeba* spp. are free-living amoeba commonly found in environmental sources such as soil, water, and dust. This ubiquitous amoeba is the causative agent of amoebic keratitis (AK) and encephalitis. The present study aimed to investigate the presence of *Acanthamoeba* spp. in the water sources of Kermanshah city, Iran. Sixty water samples were taken from different localities of Kermanshah including agricultural canals, rivers, and swimming pools. Filtration and cultivation were carried out on non-nutrient agar medium (NNA). The axenic cultivation was performed for all of the positive isolates. PCR analysis was performed on positive samples. Sequencing was done for 12 PCR products. Genotypes were identified by blast search and homology analysis. The obtained data were analyzed using Statistical Package for the Social Sciences (SPSS 16) software. *Acanthamoeba* spp. was found in 46 (76.66%) water samples and amoebae were grown in the TYI-S-33 medium. Sequencing of 12 samples proved that *Acanthamoeba* belonged to T4 (75%), T2 (8.34%), T5 (8.33%) and T11 (8.33%) genotypes. In this study, *Acanthamoeba* T4 (75%), T2 (8.34%), T5 (8.33%) and T11 (8.33%) genotypes were isolated from the water of Kermanshah city. Thus, hygiene consideration is recommended to prevent the contamination.

Keywords: *Acanthamoeba*, water samples, genotyping

Introduction

Acanthamoeba spp. are amphizoic protozoa, which can be found in different environmental sources such as water, soil, sewage, tap water, swimming pool, contact lens solution, animal feces, human tissues and cavities [1–5]. This free-living amoeba has two forms in its life cycle including active trophozoite and resistant cyst. The double-walled cyst enables it to survive in the presence of disinfectants, such as chlorine compounds and some drug (antibiotics) [5]. The pathogenic *Acanthamoeba* spp. strains have shown more thermal tolerance than the nonpathogenic ones [6]. Amoebas with pathogenic properties can grow and develop at 42°C and above, which is most likely due to their high levels of heat shock proteins (HSP60 and HSP70) [7]. This protozoan can enter the human body in the form of cyst or trophozoite through contaminated water, soil or air [8]. In essence, the presence of *Acanthamoeba* spp.in surface waters is significant because they are sources of recreational and potable water and it increases the hazard of keratitis and other diseases caused by *Acanthamoeba* genus [9]. The taxonomy and classification of these amphizoic protozoa are still under revision due to the improvement of molecular methods [2,6,10,11]. Based on the rRNA gene sequence, this amoeba has been classified into 17 genotypes (T1-T17), while T4 genotype is the most prevalent one responsible for diseases in humans [12,13].

Acanthamoeba spp. are an agent of nasopharyngeal and skin infections. Moreover, several strains can cause granulomatous amoebic encephalitis (GAE). *Acanthamoeba* spp. are also isolated from the upper respiratory tract as natural flora in apparently healthy individuals [5]. Additionally, some strains of *Acanthamoeba* have the potential to cause a corneal infection known as *Acanthamoeba* keratitis (AK) [3,5,6,14], which leads to uvea wounds, severe eye pains, photophobia and blindness [15]. AK infection can

occur due to the application of contaminated contact lenses with non-sterile water or through swimming in contaminant water [4,5]. Recently, the prevalence of AK is increasing in Iran and the world [6,13].

Acanthamoeba has been isolated from water, soil, dust, and cow feces in Iran [16,17]. Potentially pathogenic strains are detected in environmental samples worldwide [18–21]. In Turkey, Pakistan, Italy, and Egypt, the prevalence of *Acanthamoeba* in environmental sources was reported to be 40%, 70%, 39%, and 43%, respectively [22–25]. The overall prevalence rate of *Acanthamoeba* spp. among 1850 water and soil samples in Iran was estimated to be 42.7% using a random-effect model. Also, the genotyping results of the *Acanthamoeba* isolation indicated that the T4 (81.2%) genotype was the predominant strain in Iran. However, other genotypes, T2, T3, T4, T5, T6, T11, T13, T15, mixed T3/T4, and mixed T2/T6, were also detected in different environmental samples of Iran [9]. Also, researchers have been isolated *Acanthamoeba* from tap waters of the hospitals in Mashhad [26]. Another study has shown that the T4 genotype is the main etiological agent of *Acanthamoeba*-related infection such as GAE, AK in Iran and worldwide [17]. Previous studies have announced that T4, T3, and T2 were the causal agent of *Acanthamoeba* related infections; however, it was proved later that several genotypes could lead to AK such as T11, T13, T15 [4,5,27]. Contaminated water with *Acanthamoeba,* which is unsuitably used for washing and cleaning contact lenses, is usually due to a shortage of awareness. Therefore, the awareness about keeping contact lenses clean is very serious.

Acanthamoeba is detected using the culture method in the 1.5% non-nutrient agar medium with *E. coli* bacteria. However, in recent years, the PCR molecular method has been used for the final confirmation and to distinguish *Acanthamoeba* from other free-living amoebae [10,17]. The genetic typing is based on the18S rRNA gene and the sequencing of diagnostic fragment 3 (DF3) of the 18S rRNA gene [17]. Since there was no information regarding the distribution of *Acanthamoeba* in Kermanshah Province, Iran, the present research aimed to investigate the presence of *Acanthamoeba* genotypes in water sources of Kermanshah Province, Iran.

Materials and Methods

Sampling. This descriptive study was conducted in Kermanshah and its suburbs from April to July 2016. In this study, the total number of 60 water samples was obtained based on a previous study [17]. Water samples were collected (each sample 100–500 ml) from different areas of the province as follows: rivers (15 samples), agricultural canals (20 samples), tap water (11 samples), the park pools (9 samples) and swimming pools (5 samples) (Table 1).

Culture. Filtration of water samples was performed using nitrocellulose membranes (45-µm diameter) [13]. The filters were placed on a 1.5% Non-nutrient agar (NNA) medium, which was prepared with amoeba Page Saline. Amoeba Page Saline consists of 2.5 mM NaCl, 1 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 40 µm $CaCl_2$-$6H_2O$, and 20 µm $MgSO_2$. $7H_2O$. The final pH of this solution was adjusted to about 6.9 with KOH [17]. A small scrape was done on the plate so that when the amoeba penetrates the medium, it emerges on its surface through the scratch. Plates were incubated for one to two weeks at 28–30°C [3]. For this purpose, the part of the medium containing the highest amount of *Acanthamoeba* and the lowest fungi was identified and later placed in another non-nutrient medium, which continued until the fungi were removed from the medium. The *E. coli* bacteria cultured (as a food source of the Amoeba) were added to the medium. Then, the medium was incubated for one month at 30°C until the number of the above amoeba increased. The sterile PBS was used to wash both the bottom and upper parts of the medium, and the amoeba cysts were collected in a sterile tube, centrifuged and sediments were collected [3].

Axenic culture. The axenic cultures were

Table 1. Sampling point and percentage of positive cases

Sampling site	Total number	Positive number	Percent
Rivers	15	12	80
Agricultural canals	20	16	80
Tap water	11	6	54.54
park pools	9	8	88.88
Swimming pools	5	4	80
Total	60	46	66.76

Table 2. Samples and Accession number in Gene Bank

Sample ID	Genotypes	Accession number
Wat1	T4	KY587113
Wat2	T4	KY587114
Wat3	T4	KY587115
Wat4	T4	KY587116
Wat5	T4	KY587117
Wat6	T4	KY587118
Wat7	T5	KY617798
Wat8	T2	KY617799
Wat9	T4	KY617800
Wat10	T11	KY617801
Wat11	T4	KY617802
Wat12	T4	KY617803

performed on the *Acanthamoeba*-positive samples. The TYI-S-33 medium was used for axenic culture. To prepare this medium, 0.1 g of dibasic potassium phosphate (K2HPO4), monobasic potassium phosphate (KH2PO4), 0.2% sodium chloride, 0.2 g casein, 2 g yeast extract, 1 g glucose, 0.1 g cysteine hydrochloride, 0.1 g of ascorbic acid and 0.0023 ml of ferric ammonium citrate acid were dissolved in 100 ml of doubled-distilled water. Then, the amoebae were entered to axenic culture and penicillin and streptomycin antibiotics were added to the medium to prevent the bacterial growth [17]. The axenic culture was performed on the TYI-S-33 medium, which was successful in this study. A previous study proved that this medium is appropriate for the rapid growth of amoeba due to its nutrient nature. On the other hand, the rapid growth of the amoeba in this medium prevents the growth of fungi and bacteria.

DNA extraction and molecular analysis. DNA was extracted from the positive samples by using the phenol-chloroform method as previously described [17]. For extracting, DNA lysis buffer (50 mM NaCl, 10 mM EDTA, 50 mMTris–HCl, pH 8.0) and proteinase K (0.25 mg/ml) were used and incubated at 56°C for overnight. PCR analysis was performed using JDP primers including JDP1 forward 5′GGCCCAGATCGTTTACCGTGAA-3′ and JDP2 reverse 5′- TCTCACAACTGCTAG-GGGAGTCA-3′. These primers approximately amplified a 500 bp fragment. PCR reaction was performed in 30 µl Ampliqone (Taq DNA Polymerase Master Mix RED, Denmark). Twenty-five microliters of the Taq Master mix were used with 10 ng template DNA, 0.1 µM of each primer, and distilled water. Cycles of PCR were set up as following: pre-denaturation step at 94°C for 3 min and 33 cycles of denaturation at 95°C for 35 S, annealing at 56°C for 45 S and extension at 72°C for 1 min with an elongation step of 5 min at 72°C at the last cycle [17].

Gel electrophoresis. The PCR-products electrophoresis was done on 2% (w/v) agarose gel, stained with gel stain solution and visualized under UV light.

Sequencing and genotyping of the isolates. Due to the financial constraints, sequencing was performed on 12 samples. PCR products of 12 isolates were purified using the Column-based purification kit and sequenced using ABI 3730XL automatic sequencer by Takapozist Company. The obtained sequences were edited and aligned using the Chromas software program. Genotype identification was done by comparing with available *Acanthamoeba* DNA sequences in the GenBank based on sequence analysis of the DF3 region. Finally, data analysis was carried out using Statistical Package for the Social Sciences (SPSS 16) software.

Results

Of the 60 water samples cultured on the non-nutrient agar medium, *Acanthamoeba* spp. were found in 46 samples (76.66%) and culture of 46 positive samples was successful in the TYI-S-33 medium. PCR was applied to positive samples using JDP1 and JDP2 primers and confirmed by observing the 500 bp band on the agarose gel (Fig. 1). Sequences obtained in this study have been deposited in GenBank under the accession numbers KY587113-KY587118 and KY617798 -KY617803 (Table 2).

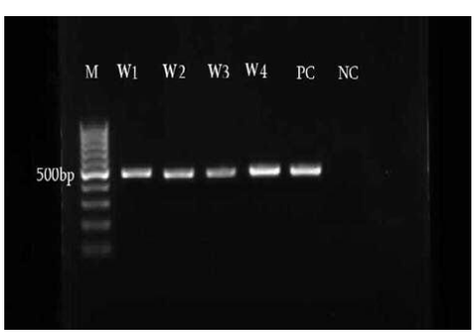

Fig 1. PCR-product of water samples. M=Molecular weight marker (100bp), W=Water, W=Water NC=Negative Control PC=Positive Control

Discussion

The present study aimed to investigate the presence of *Acanthamoeba* genotyping in Kermanshah province, Iran. In the present study, 76.66% of the samples of the river, agricultural canals, tap water, park and swimming pools were contaminated with *Acanthamoeba*. There are various studies on *Acanthamoeba* in Iran [1,17,28]. For example, the results of a study in Ahvaz showed that 71.66% of the water resources, such as agricultural canals, tap water, park, and swimming pools were polluted with *Acanthamoeba*, which is in concordance with the result of the present study [17]. Previous surveys in England and Korea have shown that *Acanthamoeba* is found in 15 to 30% of tap water samples, also, the rate of prevalence in the tap water of Chicago was reported to be 54% [29] . In other studies, rate of contamination of rivers was reported to be 72%, 88% and 23% in Bojnourd, Guilan, and Mazandaran, respectively [30]. The prevalence of *Acanthamoeba* was showed to be 88% in rivers of Iran [31], which is consistent with our study.

Acanthamoeba spp. has been seen in agricultural canals of North Khorasan and Ahvaz [30,32]. We also isolated this free-living amoeba from the agricultural canals. Similar to our study, *Acanthamoeba* spp. were seen in pools park of Ahvaz, Tehran, Bojnourd, and Mashhad [30,32,33]. It is also important for children, who play with the water in pool Park. Prevalence of *Acanthamoeba* spp. in pools swimming of Brazil and Egypt were reported to be 20% and 49%, respectively [34,35]. Also, this free-living amoeba has been detected in swimming pools of different regions in Iran [30,32]. This result is important for people, who are swimming in infected swimming pools. A different environmental and climatic factor can cause the distribution of various genotypes and further studies are required to clarify this [9]. The prevalence of T4 and T5 genotype was reported to be 79% and 16% in the waters of Iran [9]. Also, researchers have been isolated theT4 genotype of this free-living amoeba from the biofilm of hospitals in Tehran [36], which is very important, since the T4 genotype of this amoeba is a potential pathogen for immunocompromised patients. This amoeba can transmit bacteria such as *Vibrio cholera* and *Legionella*, therefore, it is considered as a threatening risk factor for the health of individuals. We also isolated the T4, T5, T2, and T11 genotypes from different water sources in Kermanshah province. Different genotypes have been isolated worldwide, T2, T4, T5, and T4 have been seen in Brazil, Japan and Egypt, respectively [18,37]. The dominant genotype in the environmental source was T4 in Bulgaria [38]. In India T2, T4, T5, and T11 have been detected which is similar to our findings (39). Previous studies indicated that the T11, T4, and T2 genotypes can cause AK, and also, T4 genotype is the dominant genotype in clinical and environmental samples [10,40,41]. In another study, the researchers isolated T2 genotype from agricultural canals, which is consistent with the result of the present study [17]. Also, the T4 and T11 genotypes were seen in environmental samples of Tehran (42). Also, in the present study, T2, T4, T5, and T11 genotypes were isolated from water sources. Besides, previous studies proved that these genotypes can cause AK [43]. The results of the present study showed that increasing knowledge of people, especially immunocompromised patients and individuals who clean their contact lenses with tap water in this region is important. In general, the contamination rate of water to *Acanthamoeba* spp. in Kermanshah is high. Therefore, people who live in this area should be careful.

In conclusion, since *Acanthamoeba* exists in environment and T4, T2, T5, and T11 genotypes were isolated from water samples in Kermanshah. Therefore, following *Acanthamoeba*-related hygiene is recommended for people in the area.

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