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Original Article

Genotyping of *Acanthamoeba* Isolated from Hospital Environments and Thermal Water of Recreational Baths in Markazi Province, Iran

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Abstract

Background: Due to the opportunism character of *Acanthamoeba*, the presence of this parasite in the thermal water of recreational baths and hospital environments can be a risk to the health of staff, patients and others. The aim of this study was to investigate the distribution of potentially pathogenic *Acanthamoeba* genotypes isolated from the hospital environment and the thermal water of recreational baths in Markazi Province, central Iran.

Methods: Overall, 180 samples including thermal water from recreational baths in Mahallat City and dust, soil and water from different hospitals of Arak, Farahan and Komijan cities, central Iran were collected. The presence of *Acanthamoeba* was investigated using microscopic examination and molecular methods. The PCR and sequencing was performed based on a specific 18S fragment of ribosomal DNA.

Results: Based on the microscopic survey, totally 134 positive samples were detected including 35% in thermal water samples and 44.7% in hospital samples. In molecular analysis, 53.5% of the samples were identified as *Acanthamoeba* and 46.7% as *Protacanthamoeba bohemica*. The genotypes were detected as T4 (33.3%), T2 (10%), T11 (6.7%), and T5 (3.3%).

Conclusion: The T4 was the most common genotype found in hospitals sampling sites while the T2 genotype and *P. bohemica* were detected in thermal water sampling sites.

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Introduction

Free-living amoebae (FLAs) are cosmopolitan and are widely found in soil, freshwater, saltwater, various pH and high / low ambient temperature samples (1). *Acanthamoeba* is a member of the FLA group is seen as trophozoite and cyst forms. The size of trophozoites, depending on the isolates and different genotypes, is between 20 and 40 µm, and cysts, which are usually double-walled, are between 10 and 20 µm (2). *Acanthamoeba* is found in a variety of environments as microbial predators (3). Different species of *Acanthamoeba* can cause amoebic granulomatous encephalitis (GAE), amoebic keratitis (AK) and cutaneous acanthamoebiasis. GAE is commonly found in people with suppressed immune systems, including diabetics, people undergoing organ transplants, HIV-AIDS patients, drug abusers and chemotherapy for cancer. AK is an infection of the cornea of the eye that can lead to blindness and usually occurs in immune competent people, mainly due to the use of contact lenses. Cutaneous acanthamoebiasis is more commonly reported in HIV-positive patients. The presence of both CNS and skin lesions at the same time can indicate *Acanthamoeba* infection (4).

Acanthamoeba is a thermophilic organism that lives in temperatures between 37 and 45 °C and feeds on bacteria and debris, so it can be active in the hot springs of recreational and therapeutic baths (1). In fact, the high temperature of hot springs (37-45 °C) provides the basis for the growth and transmission of this parasite (6). On the other hand, the presence of *Acanthamoeba* in water, soil and dust in the environment of some hospitals has been proven to be a danger to the health of staff, patients and others (7).

Acanthomba has several species and genotypes. Knowing the parasite genotype can help us to know the exact cause of the disease and then control the parasite. According to worldwide research, 20 *Acanthamoeba* genotypes (T1-T20) have been reported so far, some of which are pathogenic and cause human infections. According to some studies, genotypes T2, T3, T4, T5, T6, T10, T11 and T15 are associated with AK disease and genotypes T1, T2, T4, T5, T10, T12 and T18 are associated with GAE. Among these, genotype T4 is the most prevalent type in environmental and clinical cases worldwide and has the highest pathogenicity among *Acanthamoeba* genotypes (8).

Markazi Province is located in the center of Iran (34.0954°N 49.6909°E). Arak is the largest City and center of the province. Relatively mild summers and cold to relatively cold winters are the characteristics of the climate of Markazi Province. This Province is known as the industrial capital of Iran, covers an area of 29,530 km^2 and has a population of about 1.5 million. Markazi Province has 12 cities including Arak, Mahallat, Farahan and Komijan. Mahallat is known as a recreational-therapeutic area due to having hot springs. Local people and tourists enjoy the area's springs all year round (*https://en.wikipedia.org/wiki/Markazi_province*).

So far, there is no data on the isolation and determination of *Acanthamoeba* genotypes from the hospital and hot springs in Markazi Province. The main objective of this study was to identify *Acanthamoeba* genus in dust, soil and water samples of hospital environments of three cities (Arak, Mahallat, Farahan and Komijan) of Markazi Provine (Iran) as well as in samples from the hot springs of Mahallat City, using microscopic and molecular approaches.

Materials and Methods

Sampling

This study was a cross-sectional study and a total of 180 samples were collected from hospital sources, including 7 hospitals (Amiralmomenin, Taleghni, Amirkabir, Khansari, Valiasr, Ghods, Taminejtemaei) in Arak

City (115 samples), one hospital in Farahan City (10 samples), and one hospital in Komijan City (9 samples), as well as from hot springs of the Mahallat City (46 samples) during August and September 2018.

The hospital samples were collected from dust, soil and water of different clinical unit of the hospital comprising: general wards (admission, triage area, injection room, laboratory, drug store, hemodialysis, radiology, hematology); specialized wards (departments of psychiatry, orthopedic, heart & lung, children, infants, midwifery, intensive coronary care unit (ICCU), cardiac care unit (CCU), ear, nose & throat (ENT), surgery, ophthalmology, mammography); and service departments (kitchen, laundry, corporate social responsibility (CSR), equipment and emergency wards).

Sterile swabs were used to sample dust from the surfaces and the swabs were placed inside sterile tubes. Soil samples were collected from the hospital yard, a weight of 100 g in sterile jar, then 500 ml sterile distilled water was added to it. Samples were taken from medical instruments, windows, floors and air conditioners in patients' rooms of different wards.

Fixed biofilms were sampled from swimming pools, hot springs, small pools and hot tubs. The floating biofilms sampling was collected from the indoor swimming pool for men and women, hot tubs and tap water. Temperature, pH and free chlorine concentration of water were measured (10).

This study was approved by the Ethics Committee of the Tarbiat Modares University with code number: IR.MODARES.REC.1397.074.

Filtration, cultivation, and cloning of Acanthamoeba

The Xenic Cultivation was used for growth of *Acanthamoeba* including: non-nutritive medium (15 g bacto-agar, Page Amoeba Saline solution of MgSO4,7 H2O (4mg), CaCl2,2H2O (4mg), Na2HPO4(142 mg), KH2PO4 (136mg) and NaCl (120 mg) in 1 lit) seeded with 0.1 ml of a heat inactivated 48-hour culture of *Escherichia coli* (2, 3). *E. coli* was cultivated on EMB or MacConkey agar plates and was incubated at 37° C for 24 hours (11). Water samples were filtered using sterile membrane filters (pore size 2.5µm) by a vacuum pump. Non nutrient agar medium (NNA) (1.5%) was prepared into 6 cm plates using Page's saline and 1ml autoclave inactivated suspension of *E.coli* was added as a food source for amoebae outgrowth. Then each filter was placed face to face on the surface of non-nutrient (NN) agar medium. To prevent the plates from drying out, parafilm tape was used for sealing (9).

Swab samples were placed in tubes containing 50 ml of sterile distilled water. The swabs were gently shaken, squeezed and discarded. The collected material was then centrifuged (250 g, 10 minutes) and the precipitate was resuspended in 0.5 ml of Page's saline. 100 µl of the suspension was injected directly onto the plate (NNA) directly, and the plates were sealed with parafilm tape (1).

DNA extraction

After adding 5 ml of page saline (PSA or PBS) to the medium, the surface of the positive plates was scratched with a sterile swab. *Acanthamoeba* (trophozoites and cysts) were harvested from positive plates by gentle scraping in a tube, centrifuging (2000 G/5 min) (4) and in order to eliminate the bacteria incubating them in 1ml HCl 3% overnight (5), then washed three times with page saline. The pellets were resuspended in 200 µl PAS and kept on -20°C for DNA extraction. DNA was extracted using the phenol-chloroform method or by the tissue protocol of the DNGTM- Plus kit (Cinnagen, Tehran, Iran) (4). The genomic DNA was stored at –20 ˚ C until use. Control DNA was also extracted from an *Acanthamoeba* sp. as a positive control, and from sterile water as a negative control (6).

The obtained DNA was dissolved in a 50 µl buffer and 1 mM TE, then visualized with 1% agarose, and the concentration and purity of the DNA product was estimated by a NanoDrop (15).

Primers

The primers are ASA.S1 based on the amplification of a specific gene portion of the 18S rRNA genotype for the detection of genotype-specific gene segment for *Acanthamoeba* (423–550 bp) (15) that includes the hyper variable diagnostic fragment3 (DF3) (8). The PCR assay was performed using a forward primer JPD1(GGCCCAGATCGTTTACCGTGAA) and a reverse primer JPD2(TCTCACAAGCTGCTAGGGGAGTCA)(15, 16).

PCR reaction set up

PCR was done with positive control (*A. castellanii*) and annealing temperature was calculated 58 °C. In each sample, PCR reaction mix was performed in a volume of 15 µl. The PCR cycle is set as follows: initial denaturation at 94° C for 5 minutes followed by 35 cycles (denaturation at 94 °C for 30 seconds, annealing at 58° C for 30 seconds, expansion at 72 °C for 30 minutes (s) and a final extension for 10 minutes at 72 °C in a thermocycler (Bio Rad, USA) (20). Distilled water was added instead of DNA as a negative control. A ladder of 100 to 3,000 bp was used as a DNA size indicator (Smobio, Taiwan) (16). PCR product was successfully observed on 1.5% agarose gel under UV gel documentation system (10).

Sequencing

The PCR reaction was performed on about 32 positive samples with a volume of 25 µl and was sent to Niagen Company and were sequenced in two directions (reverse and forward) using ABI 3730XL automatic sequencer after clean up based on standard Sanger dideoxynucleotide method. Sequence data processing inclusive of a DNA chromatograms survey of AB1 file, comparison of forward and reverse sequences to one another, editing of the sequences was performed and assembled into a single contig applying Sequencher software version5.4.6 and each contig was used as a query for analyzing with reference sequences using the basic local alignment search tool (BLAST) analysis system (http://www.ncbi.nlm.nih.gov/BLAST).

Phylogenetic analyses

The CLUSTAL W alignment program implemented in MEGA7 was used to align the sequences. The phylogenetic was generated using the neighbour-joining method (K2+G model) (10).

Statistical analysis

Data were analyzed by chi-square test using SPSS software version 18 (Chicago, IL, USA) to determine the correlation between sampling location, sample type and *Acanthamoaba* contamination. *P* value less than 0.05 was considered significant.

Results

Microscopy

Out of 134 of hospital samples, 112 (83.5%) were dust, 9 (6.7%) soil, and 11 (8.2%) tank & tap water. Out of 46 of thermal water samples, 20(43.5%) were swimming pool, 9 (19.5%) fixed biofilms (swab), 8 (17.4%) tank and tap water, 7 (15.2%) bathtub and small pond and 2 (4.3%) mineral spring.

Overall, *Acanthamoeba* were detected in 125 samples out of 180 (69.4%) by microscopic examination. Of which 31/46 (67.4%) of thermal water, and 94/134 (70.1%) of hospital samples consist of: Amir Kabir Hospital 76.2%, Ghods Hospital 83.3%, Khansari Hospital 42.1%, Taleghani Hospital 90%, Taminejtemaei Hospital 77.8%, Valiasr Hospital 45.4%, Amiralmomenin Hospital 88.9%, Farahan Hospital 80%, and Komijan Hospital 66.6%. The sources and number of positive samples of *Acanthamoeba* identified by microscopic and PCR techniques are shown in Table 1.

Molecular Identification and Genotyping

Out of 125 microscopic positive samples 42.4% (53/125) were found to be positive by PCR.

In total, 35.5% of thermal water, and 44.7% of hospital samples were found to be positive as shown in Table 1.

Table 1: The sources and numbers of positive samples of *Acanthamoeba* identified by microscopic and PCR techniques.

Source of samples	***Microscopic Examination***				***PCR-Acanthamoeba***			
	Positive		**Total**		**Positive**		**Total**	
	No.	%	No.	%	No.	%	No.	%
Mahallat (S)	31	67.4	46	25.5	11	35.5	31	24.8
Komijan (H)	6	66.6	9	5	2	33.3	6	4.8
Farahan (H)	8	80	10	5.5	1	12.5	8	6.4
Amiralmomenin (H)	16	88.9	18	10	10	62.5	16	12.8
Taleghni (H)	18	90	20	11.1	7	38.9	18	14.4
Amirkabir (H)	16	76.2	21	11.6	6	37.5	16	12.8
Khansari (H)	8	42.1	19	10.5	6	75	8	6.4
Valiasr (H)	10	45.4	22	12.2	5	50	10	8
Ghods (H)	5	83.3	6	3.3	2	40	5	4
Taminejtemaei (H)	7	77.8	9	5	3	42.8	7	5.6
Total	125	69.4	180	100	53	42	125	100

S: Spring pools; H: Hospital

In total, 40/83 (48.2%) of dust samples, and 2/8 (25%) of soil from 94 hospital samples were positive. There was a significant association between dust and positive cases (*P*=.01) (Table 2). In addition, according to clinical units, the most positive cases were found in general wards of the hospitals (20/38 or 52.6%) (Table 2).

Table 2: Frequency of positive samples of *Acanthamoeba* identified by PCR technique from dust, tap water and soil of different hospitals

Sample type		***PCR-Acanthamoeba***			
		Positive		**Total**	
		No.	%	No.	%
Dust from	General wards*	20	52.6	38	40.4
	Specialized wards**	15	45.4	33	35.1
	Service departments***	5	41.6	12	12.7
Soil		2	25	8	8.5
Tap water		0	0	3	3.2
Total		42	40	94	100

*__General wards:__ Admission, triage area, injection room, laboratory, drug store, hemodialysis, radiology, hematology. // **__Specialized wards:__ Department of psychiatry, orthopedic, heart & lung, children, infants, midwifery, ICCU, CCU, ENT, surgery, ophthalmology, mammography. ***__Service departments__: Kitchen, laundry, CSR, equipment and emergency wards

In Mahallat City, 43.7% of swimming pool (hot spring), 50% of fixed biofilms (swab) and 11.1% of the water of other sources were found (Table 3).

Table 3: Frequency of positive samples of *Acanthamoeba* identified by PCR technique from different water sampling source units of Mahallat City

Water source units		***PCR-Acanthamoeba***			
		Positive		**Total**	
		No.	%	No.	%
Floating Biofilms	Swimming pool (hot spring)	7	43.7	16	51.6
	Other Sources*	1	11.1	9	29
Fixed biofilms (swab)**		3	50	6	19.4
Total		11	35.5	31	100

*Other sources: Bathtub, tap water, small pond and tank.
** From swimming pools, hot springs, small pools and hot tubs

Sequencing and phylogenetic analysis

Thirty positive isolates were sent for sequencing. The sequences were performed by BLAST analysis showing at least 97% sequence identity with those accessible in GenBank.

According to molecular analysis, unambiguously identified 46.7% samples as *P. bohemica* and 53.5% as *Acanthamoeba* which related genotypes including 33.3% (T4), 10% (T2), 6.7% (T11), and 3.3% (T5).

The potential pathogenic *Acanthamoeba* T4 was the most common genotype distributed in hospitals sampling sites while in thermal water sampling sites were *Acanthamoeba* T2 genotypes, *P. bohemica*. The confirmed *Acanthamoeba* genotype T11 was isolated from dust in Ghods Hospital surgical ward and Taleghani hospital inpatient ward, whereas T5 were isolated from the Amirkabir Hospital Radiology Department.

Sequence data obtained from this study were deposited in the GenBank, with accession numbers *MZ318341-MZ318356* corresponding with *Acanthamoeba* genus and MZ314567-Z314580 corresponding with *P. bohemica* with separate accession number.

For phylogenetic correlations study, the maximum likelihood, maximum parsimony, and minimum methods were used (Fig. 1).

Discussion

So far, different species of FLA have been reported from different water sources, like drinking water, hot springs and recreational springs in Iran (11). Our results showed contamination in both thermal water sources and hospital samples with *Acanthamoeba* in Markazi Province of Iran. In general, the rate of contamination was 42.4%, which includes: 35.4% in thermal water spring samples and 44.7% in hospital samples. Molecular analysis showed that 46.7% of the samples contaminated with *Protacanthamoeba bohemica* and 53.3% with *Acanthamoeba*. Detected *Acanthamoeba* genotypes include: T4 (33.3%), T2 (10%), T11 (6.7%) and T5 (3.3%). *Acanthamoeba* T4 was the most common genotype distributed in hospital sampling sites, while *P. bohemica* and *Acanthamoeba* T2 genotype were more common in hot water sampling sites.

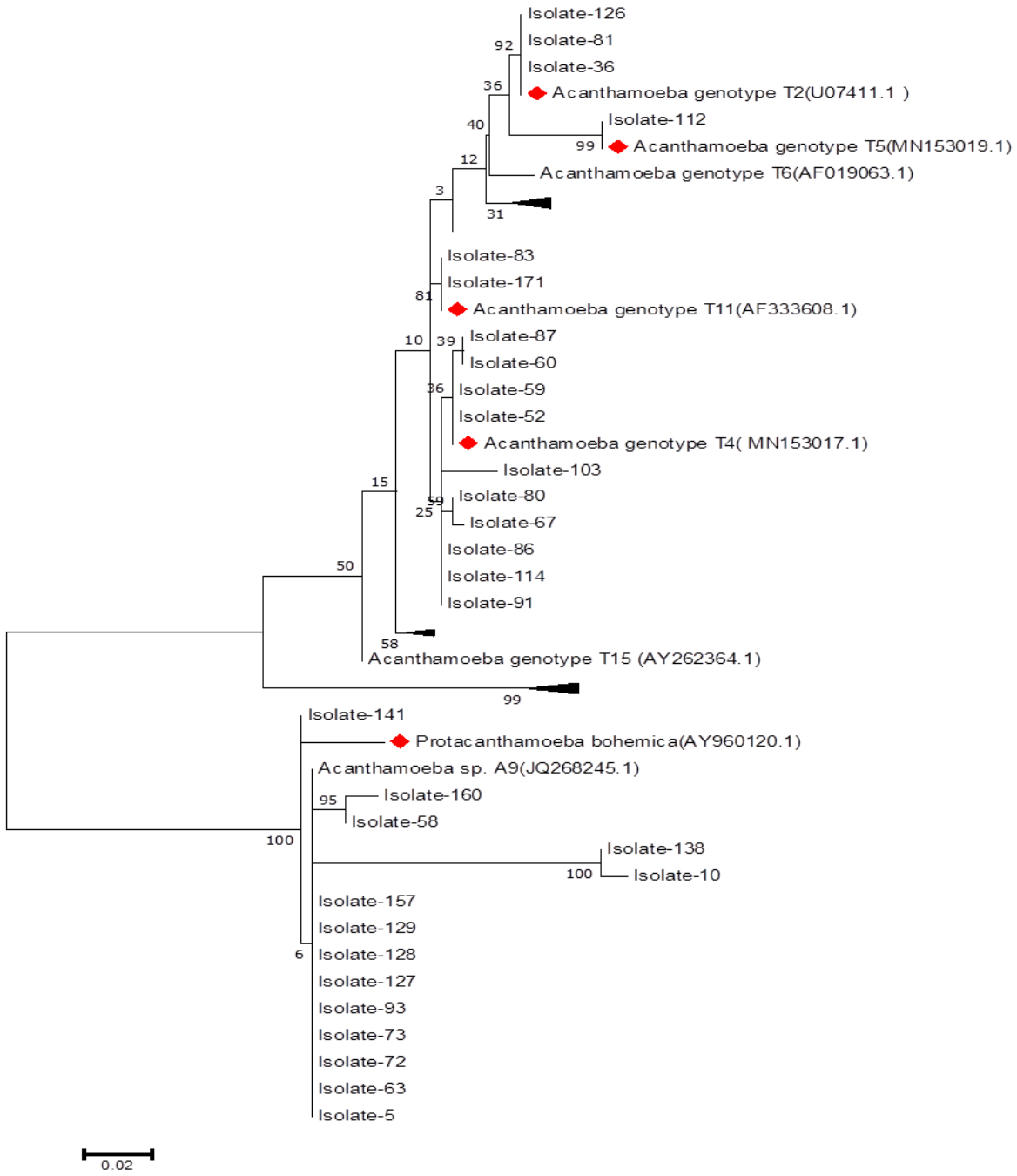

Fig. 1: Phylogenetic relationships of *Acanthamoeba* isolates in the present study and *Acanthamoeba* reference sequences registered in GenBank. The phylogenetic tree was constructed using maximum composite likelihood distance estimates with 500 bootstrap replications in MEGA7

In a similar study on soil samples in Markazi Province, the results indicated the genotypes of most isolates was belonged to T4 genotype. Of 48 soil samples, 33.3% and 31.25% were found to be contaminated with *Acanthamoeba* according to the culture and molecular assays, respectively. The majority of these isolates was belonged to the T4, T5 and T6 genotypes. (18)

In addition, in another survey in Sarein Hot Springs in Ardebil Province, *Acanthamoeba* was found in 3.6% of 28 samples (12). In Sari City, 55.8% of *Acanthamoeba* were isolated from 77 samples (4). In addition, 50% of the studied water samples in Zanjan Province were found positive (8). A study conducted in Gilan Province showed that 20% of the province's swimming pools are contaminated with *Acanthamoeba* (13). In Qazvin, 63.6% and 27.3% were *Acanthamoeba* and *P. bohemica* respectively have been reported from the water samples of public swimming pools (12).

Different contamination rates of swimming pools have been reported in different parts of the world. From the swimming thermal pool distributed throughout Turkey, 25.5% (26/102) of the samples were found to be contaminated by *Acanthamoeba* (7). Differences in the prevalence of *Acanthamoeba* contamination in swimming pool water may be due to differences in different sampling methods, sample number and size, examination methods and sampling season.

Pathogenic *Acanthamoeba* have the ability to grow at high temperatures. So, *Acanthamoeba* isolated from warmer waters such as natural hot springs may be potentially pathogenic. In our study, the average temperature of hot water was 36.4 °C. while in a study from northwestern Iran about natural hot springs, the mean temperature of the waters was 43.6 °C (11).

*Achanthamoeba c*ysts are usually resistant to changes in pH, dehydration, osmolarity, freezing, ultraviolet (UV) and chemical disinfectants (19). The standard free chlorine concentration in water is usually 1-3 ppm (9). In this study, mean concentration of free chlorine remaining in hot pools is less than the standard range (0.8 ppm). Thus, by increasing the amount of chlorine remaining in the water or swimming pool water, hygienic condition will be provided to protect swimmers from these infectious pathogens. Also, continuous assessment of pool contamination is crucial, and necessary measures must be taken to eliminate it (19).

To date, 20 genotypes (T1-T20) of *Acanthamoeba* have been reported in environmental and clinical specimens. T4 genotype has been known as the most common genotype and higher pathogenicity than other strains for human. On the other hand, several genotypes including T2, T3, T4, T5, T9, T11 and T13 have been reported from different regions of Iran (12). Sequencing studies have reported T4 and T3 as the most common *Acanthamoeba* genotypes in Iran (9, 20).

In a study that performed in Qazvin, *P. bohemica* (27.2%) and *Acanthamoeba sp.* (4.5%) were identified among the specimens (13) and 36.3%, 18.1% and 4.5% of *Acanthamoeba* specimens were detected as T3, T4 and T11 respectively. However, in our study on warm water, we found *P. bohemica* and T2 genotypes. *Protacanthamoeba* is classified as amoebae and a member of the family Acanthamoebaidae. This species has been reported in samples obtained from water sources but has not been reported in humans (12). The T2 genotype of *Acanthamoeba* was identified as the causative agent of GAE in a patient with immunodeficiency virus-negative tuberculosis (17) and also has caused ocular keratitis (14).

Acanthamoeba has been reported in drinking water, swimming pools and rivers in many studies. Water resources can play an actual important role in the spread of *Acanthamoeba* keratitis among individuals. Some believe that more than 80% of healthy individuals are positive for *Acanthamoeba* antibodies (15). In addition, isolation of T4 type of *Acanthamoeba* from ophthalmology wards of a hospital in Tehran have been reported previously (16). Our result revealed a rate of 44.7 % in hospital samples. No contamination was found in hospital water samples, ophthalmology and orthopedic departments, while the highest rates were found in the Hospital ward, laboratory, waiting room, radiology, and ICU-CCU wards. In a study conducted in Tehran, 47% of dust and biofilm samples were collected from different hospital

wards were found contaminated with *Acanthamoeba* (23). At a public hospital in Porto Alegre, Brazil, 23.4% of dust collected from five different hospital wards were found positive (24). In addition, in another study in Brazil, 135 samples from hospital wards and water storage tanks which 31 (23%) presented the *Acanthamoeba* with genotypes T5, T3 and T4 (17). In Kashan, 54.1% of the environmental samples were found positive (25). In addition, in two hemodialysis units in Mazandaran Province, 50% of the dust samples were positive.

Conclusion

The high rate of *Acanthamoeba* contamination in the thermal water and hospital samples, indicated that disinfection and hygiene measures are not enough to control this parasite in these places. Installing warning signs about the risk of disease around swimming pools and spas is highly recommended. In addition, raising public awareness of hospital staff, especially physicians, about the importance and risk of this parasite, especially for immunocompromised patients is essential.

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Conflict of Interest

The authors declare that there is no conflict of interest.

References

1. Landell M, Salton J, Caumo K, et al. Isolation and genotyping of free-living environmental isolates of *Acanthamoeba* spp. from bromeliads in Southern Brazil. Exp Parasitol. 2013;134 (3):290-4.
2. Orosz E, Posta K. Genotyping of *Acantamoeba* spp. from rhisophere in Hungary. Acta Microbiol Immunol Hung. 2019;67(3):171-175.
3. Corsaro D, Walochnik J, Köhsler M, et al. *Acanthamoeba* misidentification and multiple labels: redefining genotypes T16, T19, and T20 and proposal for *Acanthamoeba* micheli sp. nov.(genotype T19). Parasitol Res. 2015;114(7):2481-90.
4. da Rocha-Azevedo B, Tanowitz HB, Marciano-Cabral F. Diagnosis of infections caused by pathogenic free-living amoebae. Interdiscip Perspect Infect Dis. 2009; 2009: 251406.
5. Siddiqui R, Khan NA. Biology and pathogenesis of *Acanthamoeba*. Parasit Vectors. 2012;5:6.
6. Lekkla A, Sutthikornchai C, Bovornkitti S, et al. Free-living ameba contamination in natural hot springs in Thailand. Southeast Asian J Trop Med Public Health. 2005;36 Suppl 4:5-9.
7. Badirzadeh A, Niyyati M, Babaei Z, et al. Isolation of free-living amoebae from sarein hot springs in ardebil province, iran. Iran J Parasitol. 2011;6(2):1-8.
8. Haniloo A, Pezeshki A, Mahmmodzadeh A, et al. Genotyping of *Acanthamoeba* spp. from water sources from Northwestern Iran. Acta Parasitol. 2017;62(4):790-795.
9. Poor BM, Dalimi A, Ghafarifar F, et al. Contamination of swimming pools and hot tubs biofilms with *Acanthamoeba*. Acta Parasitol. 2018;63(1):147-153.
10. Sente C, Erume J, Naigaga I, et al. Xenic Cultivation and Genotyping of Pathogenic Free-Living Amoeba from Public Water Supply Sources in Uganda. New J Sci. 2016;2016:9.
11. Abdullah SW, Gul S, Farheen S, et al. Detection of *Escherichia coli* and total microbial population in River Siran water of Pakistan using Emb and Tpc agar. Afr J Microbiol Res. 2018;12:908-912.
12. Paknejad N, Hajialilo E, Saraei M, et al. Isolation and identification of *Acanthamoeba* genotypes and *Naegleria* spp. from the water samples of public swimming pools in

Qazvin, Iran. J Water Health. 2020;18(2):244-51.
13. Shokri A, Sarvi S, Daryani A, et al. Isolation and Genotyping of *Acanthamoeba* spp. as Neglected Parasites in North of Iran. Korean J Parasitol. 2016;54(4):447-53.
14. Walochnik J, Haller-Schober EM, Kölli H, et al. Discrimination between Clinically Relevant and Nonrelevant *Acanthamoeba* Strains Isolated from Contact Lens- Wearing Keratitis Patients in Austria. J Clin Microbiol. 2000;38(11):3932-6.
15. Yera H, Ok V, Lee Koy Kuet F, et al. PCR and culture for diagnosis of *Acanthamoeba* keratitis. Br J Ophthalmol. 2021; 105(9):1302-1306.
16. Değerli S, Değerli N, Çamur D, et al. Genotyping by Sequencing of *Acanthamoeba* and *Naegleria* Isolates from the Thermal Pool Distributed Throughout Turkey. Acta Parasitol. 2020;65(1):174-186.
17. Taher EE, Méabed EMH, Abdallah I, et al. *Acanthamoeba* keratitis in noncompliant soft contact lenses users: Genotyping and risk factors, a study from Cairo, Egypt. J Infect Public Health. 2018;11(3):377-383.
18. Meighani M, Eslamirad Z, Hajihossein R, et al. Isolation and genotyping of *Acanthamoeba* from soil samples in Markazi Province, Iran. Open Access Maced J Med Sci. 2018; 6(12):2290-2294.
19. Mirahmadi H, Nia MM, Ebrahimzadeh A, et al. Genotyping determination of *Acanthamoeba* strains: an original study and a systematic review in Iran. J Water Health. 2019;17(5):717-727.
20. Saburi E, Rajaii T, Behdari A, et al. Free-living amoebae in the water resources of Iran: a systematic review. J Parasit Dis. 2017;41(4):919-928.
21. Bagheri H, Shafiei R, Shafiei F, et al. Isolation of *Acanthamoeba* spp. from drinking waters in several hospitals of Iran. Iran J Parasitol. 2010;5(2):19-25.
22. Niyyati M, Rezaeian M. Current Status of *Acanthamoeba* in Iran: A Narrative Review Article. Iran J Parasitol. 2015;10(2):157-163.
23. Lasjerdi Z, Niyyati M, Haghighi A, et al. Potentially pathogenic free-living amoebae isolated from hospital wards with immunodeficient patients in Tehran, Iran. Parasitol Res. 2011;109(3):575-80.
24. Carlesso AM, Artuso GL, Caumo K, et al. Potentially Pathogenic *Acanthamoeba* Isolated from a Hospital in Brazil. Curr Microbiol. 2010;60(3):185-90.
25. Saberi R, Fakhar M, Sedighi O, et al. First Molecular Evidences of *Acanthamoeba* T3, T4 and T5 Genotypes in Hemodialysis Units in Iran. Acta Parasitol. 2019;64(4):911-915.