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## Original Article

# Genotyping of Environmental Isolates of *Acanthamoeba* in Hamadan, West of Iran

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***Abstract***

**Background:** Different genotypes of *Acanthamoeba* have been abundantly isolated in environmental samples such as water, soil, and dust, as well as in different hospital departments and eyewash stations. This protozoan is a potential hazard for immunocompromised patients and contact lens wearers. The aim of the present study was isolation and genotyping of environmental and corneal isolates of *Acanthamoeba* in Hamadan, west of Iran.

**Methods:** During 2018-2020, a total of 104 environmental samples including, water, soil, and dust and 16 corneal scraping samples were collected and investigated for the presence of *Acanthamoeba* using morphological and molecular identification tools. Genotypes were determined using sequence analysis of the diagnostic fragment 3 (DF3) from *Acanthamoeba*-specific amplimer S1 (ASA.S1) gene. Phylogenetic tree was constructed with the MEGA7 software using Neighbor-Joining method.

**Results:** The presence of *Acanthamoeba* spp. was determined in 87.5% of water, 53.1% of soil, and 25% of dust samples. From 30 dust samples collected from eight wards of three hospitals, 7 (23.3%) were contaminated with *Acanthamoeba*. Sequencing analysis of environmental samples revealed that the T4 genotype was the most prevalent (92.6%) one. Genotypes T2 (1.9%), T2/T6 (1.9%), and mixed T4 and T2/T6 (3.7%) were also identified in environmental samples. *Acanthamoeba* was seen in none of the examined corneal scraping samples from patients with suspected keratitis.

**Conclusion:** The widespread occurrence of this potentially pathogenic amoeba in most hospital wards and environmental resources and areas of the region highlights a strong need to increase awareness regarding this ubiquitous amoeba among susceptible individuals, such as immunocompromised patients and contact lens wearers.

## Introduction

*Acanthamoeba* is an opportunistic amoeba that is widely distributed in numerous habitats such as water, soil, air, sewage, ventilation ducts, and so on (1). In addition, this protozoan has been detected in different units of hospitals and eyewash equipment and can cause amoebic keratitis in people with corneal damage, as well as granulomatous amoebic encephalitis in immunocompromised patients (2). Pool water, soil and dust are major risk factors for keratitis, especially in contact lens wearers (1, 2).

So far, 23 *Acanthamoeba* genotypes (T1-T23) have been identified based on 18s rRNA gene sequence variation, among which T4 is the most prevalent and pathogenic genotype in clinical and environmental specimens (3). T5 genotype is the second and T3 is the third most common genotype in the environment. However, the clinical prevalence of T3 genotype and its frequency in cases of amoebic keratitis is higher than T5 genotype (4, 5). The main cause of amoebic keratitis in Iran is *Acanthamoeba*, and T4 is the most common genotype causing the disease (6). The frequency of this disease is higher among people who wear contact lenses (7).

Considering the increase in diseases caused by *Acanthamoeba* in recent years and the lack of information in Hamadan about the distribution of *Acanthamoeba* genotypes in soil, dust, and corneal specimens, this study was carried out with the aim of molecular identification and genotyping of environmental and corneal isolates of *Acanthamoeba* in Hamadan, west of Iran.

## Materials and Methods

### *Sampling*

Overall, 120 (104 environmental and 16 corneal scraping) samples were investigated during 2018-2020. Environmental samples consisted of 32 water, 32 soil, and 40 dust samples. Samples of water were randomly collected from park ponds, square ponds, rivers, pools and springs, soil samples from parks, squares, flower pots and gardens, dust samples from different laboratories and different wards of Farshchian, Shahid Beheshti, and Besat hospitals of Hamadan. Corneal scraping samples of suspected keratitis patients were also prepared by ophthalmologists in Farshchian Hospital.

### *Processing of the samples and culture method*

An approximate volume of 100 mL each water sample was passed through three filters with pore sizes of 8, 2, and 0.45 micrometers. The soil samples were passed through a sieve and 5 g of the samples were dissolved in 12 mL sterile distilled water and stagnated for 30 mins. Then, the supernatant was centrifuged at 1500 rpm for 5 min. To collect dust samples, the swabs were moistened with sterile distilled water and swept over surfaces such as beds edges, niches, walls corners, floors, dialysis machines, and cabinet surfaces. Then the swabs were placed in a tube containing 5 mL of sterile distilled water, vortexed and centrifuged at 1500 rpm for 5 min. Sediments of dust and soil samples and the filters of water samples were inoculated onto non-nutrient agar (NNA) medium seeded with *Escherichia coli*. The corneal scraping samples of suspected keratitis patients were prepared by ophthalmologists and inoculated onto NNA media along with *E. coli*. The culture media were incubated at room temperature (~30 °C) and checked daily for the presence of *Acanthamoeba* for up to 14 days. In environmental samples, various organisms were present, including other protozoa, various bacteria, fungi, and nematodes. Therefore, the positive cultures were passaged to a fresh plate as soon as possible.

### *DNA extraction*

Chelex 100 Sodium (SIGMA, USA) was used to extract DNA (6) as follows. One hundred µL of 10% Chelex solution was trans-

ferred into a 0.5-mL microtube, and about 100 trophozoites were gently picked up from the plate culture surface and mixed with Chelex solution in the microtube. The microtube was incubated in a heater block at 56 °C for 10 min. The microtube was then vortexed gently for 10 seconds and placed back in the water bath for 10 min. The microtube was removed from the heater block and vortexed for 10 seconds and then incubated at 100 °C. After 10 mins, it was severely vortexed for 10 seconds and centrifuged at 12,000 rpm for 3 mins. Then the supernatant (containing DNA) was transferred to a new microtube. DNA concentration of the specimens was measured by NanoDrop spectrophotometer (Thermo Scientific, USA) device and stored at -20 °C for PCR.

### PCR

The *Acanthamoeba*-specific amplimer S1 (ASA.S1) gene with approximately 423-551 bp length, located in the genomic region of the 18S rRNA gene of *Acanthamoeba* was amplified using JDP1: 5'-GGC CCA GAT CGT TTA CCG TGA A-3' and JDP2: 5'-TCT CAC AAG CTG CTA GGG GAG TCA-3' primers (8). PCR was performed in a volume of 50 µL containing 25 µL Mastermix (Ampliqon, Denmark), 15.2 µL dd water, seven µL DNA, and 1.4 µM of each primer. The PCR condition was carried out at 95 °C for 5 min for an initial denaturation step and 45 cycles (94 °C for 45 s, 61 °C for 45 s, and 72 °C for 45 s) with a final extension step for 5 min at 72 °C.

### Sequencing and genotyping of strains

The PCR product of *Acanthamoeba* isolates was directly sequenced by primer 892c: GTC AGA GGT GAA ATT CTT GG. This primer amplifies diagnostic fragment 3 from ASA.S1 genomic area of *Acanthamoeba*, which is highly variable and used to identify different genotypes of *Acanthamoeba* (8). Genotypes were identified using the Basic Local Alignment Search Tool (BLAST) and by comparison with available *Acanthamoeba* DNA sequences in GenBank.

### Phylogenetic analyses

Sequence alignments were performed using the Multalin website (http://multalin.toulouse.inra.fr/multalin), and phylogenetic tree was constructed with the MEGA7 software using a Neighbor-Joining method, with a Maximum Composite Likelihood substitution model.

### Gene sequence data

Sequence information obtained in this study was deposited in the GenBank database under the accession numbers OP443874-OP443890.

### Statistics

All statistical analysis was performed using SPSS V.16 (Chicago, IL, USA). Variables were analyzed by chi-squared test. The statistical level of significance was set at $P<0.05$.

## Results

From 104 environmental samples, 55 samples (52.9%) were contaminated with *Acanthamoeba* based on morphological characteristics of cysts and trophozoites (Fig. 1) and molecular methods.

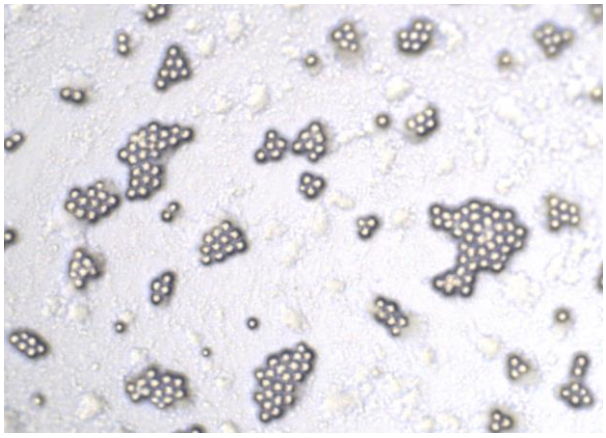

**Fig. 1:** Cysts of *Acanthamoeba* sp. in non-nutrient agar medium

The highest level of contamination was related to water samples with 87.5% (Table 1). All the samples collected from rivers, springs, and pools were positive, while 80% of the samples collected from the square pounds were contaminated with *Acanthamoeba* (*P*=0.7).

**Table 1:** The frequency of *Acanthamoeba* contamination in water samples

| ***Source*** | ***Sample no*** | ***Positive cases No (%)*** |
|---|---|---|
| Park | 8 | 7 (87.5) |
| Square | 10 | 8 (80) |
| River | 7 | 7 (100) |
| Pool | 1 | 1 (100) |
| Spring | 1 | 1 (100) |
| Others* | 5 | 4 (80) |
| Total | 32 | 28 (87.5) |

* Including pit of water, tap water and water stream

Of the ten dust samples of laboratories, three samples (30%) and of 30 samples of dust from different wards of the hospitals of Farshchian, Shahid Beheshti and Besat, seven samples (23.3%) were contaminated with *Acanthamoeba* (Table 2). The highest rate of contamination was observed in dialysis and transplantation wards at 60% and 50%, respectively (*P*=0.3). There were no cases of contamination in the operating rooms.

Of 32 soil samples collected from gardens, pots, parks, and squares, 17 samples (53.1%) were positive. The frequency of *Acanthamoeba* contamination in different soil samples is shown in Table 3. There was no significant relationship between *Acanthamoeba* frequency and soil sampling site (*P*=0.3).

**Table 2:** The frequency of *Acanthamoeba* isolates in dust samples of hospital wards

| ***Location*** | ***Sample no*** | ***Positive cases No (%)*** |
|---|---|---|
| Dialysis device | 2 | 0 (0) |
| Dialysis section | 5 | 3 (60) |
| Operation room | 7 | 0 (0) |
| Transplantation section | 2 | 1 (50) |
| Ophthalmology | 8 | 2 (25) |
| section oncology | 3 | 1 (33.3) |
| Hematology pediatrics | 3 | 0 (0) |
| Laboratory | 10 | 3 (30) |
| Total | 40 | 10 (25) |

**Table 3:** The frequency of *Acanthamoeba* contamination in soil of different places

| ***Location*** | ***Sample no*** | ***Positive cases No (%)*** |
|---|---|---|
| Park | 11 | 3 (27.3) |
| Square | 7 | 5 (71.4) |
| Garden | 6 | 3 (50) |
| Pot | 2 | 2 (100) |
| Garden | 4 | 3 (75) |
| Others* | 2 | 1 (50) |
| Total | 32 | 17 (53.1) |

* Including mountain soil and residential project

Molecular analysis revealed that the environmental isolates belonged to T4, T2 and T2/T6 genotypes, and the predominant one in water, soil and dust samples was T4 genotype with frequencies of 92.6%, 94.1% and 90%, respectively (Table 4). Also, in two samples of water, the contamination with both T4 and T2/T6 genotypes was detected. There was no significant relationship between the genotype and the type of examined samples ($P$=0.3). The phylogenetic tree showed correlation between *Acanthamoeba* isolates of this study and three reference *Acanthamoeba* strains (T2, T4 and T2/T6 genotypes) from GenBank (Fig. 2).

The corneal scraping samples from 16 suspected keratitis patients referred to Farshchian Hospital were cultured, but none of the samples were positive for free-living amoeba.

**Table 4:** Frequency of *Acanthamoeba* genotypes isolated from environmental samples

| Sample | Genotype | | | | Total |
|---|---|---|---|---|---|
| | T4 No (%) | T2 No (%) | T2/T6 No (%) | T4 & T2/T6 No (%) | |
| Water | 25 (92.6) | - | - | 2 (7.4) | 27 |
| Soil | 16 (94.1) | 1 (5.9) | - | - | 17 |
| Dust | 9 (90) | - | 1 (10) | - | 10 |
| Total | 50 (92.6) | 1 (1.9) | 1 (1.9) | 2 (3.7) | 54 |

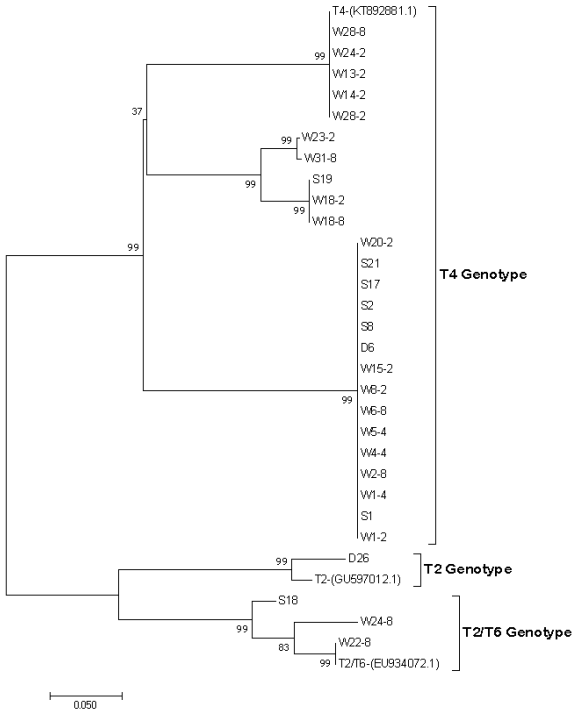


**Fig. 2:** Phylogenetic tree derived from 18S rRNA gene sequences corresponding to the DF3 from specific amplimer ASA.S1 showing the relationships between 29 representative *Acanthamoeba* isolates of this study and three reference strains of genotypes T2, T4 and T2/T6 (with their accession numbers in parentheses) from GenBank. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

## Discussion

In this study, 120 samples were collected and examined, of which 104 were environmental samples, including water, dust, and soil, and 16 were clinical samples from suspected keratitis patients referred to Farshchian Hospital, Hamadan. Accordingly, 100% of the samples collected from rivers, springs and pools, and 80% of the water collected from square ponds were contaminated with *Acanthamoeba*. Pollution in samples collected from fountains and recreational water of parks was 87.5%. Because the water is exposed to contaminated air and dust, it is one of the main sources of contamination to *Acanthamoeba* (9). There are several reports of surface water pollution in Iran and around the world, including recreational waters, hot springs, wetlands and swimming pools, in which the contamination rate was reported to be between 23% and 89% (10). In Tehran on 55 water samples, 27.3% of the samples were contaminated with free-living amoebae, of which 80% were *Acanthamoeba* (11). In a study conducted on 54 water samples in Arak, 61.11% of the samples were positive for *Acanthamoeba* cyst (12). In south of Iran and in the center of Turkey, the rate of *Acanthamoeba* contamination in environmental sources was 71.6% and 4.4%, respectively (13, 14).

One of the reasons for the high level of surface water contamination with *Acanthamoeba* in the present study may be due to the use of three different sizes of filters, which would reduce the risk of losing *Acanthamoeba*. While in most studies, only one filter (usually 0.45 µm) is used, which causes the filter pores to close early with suspended particles in surface water and thus lose its efficiency, or may even be ruptured under vacuum. However, if several filters are used in successive stages, the coarser particles are captured by the first (8-µm) and second (2-µm) filters and do not block the main filter (0.45 µm) pores. Perhaps another reason for the higher level of surface water contamination in this study is the windy area. Hamadan is one of the windy regions of Iran, and the wind can spread various pollutants, including *Acanthamoeba* cysts from one place to another and contaminate surface water. Considering that surface water sources are exposed to various types of environmental pollution, educating people, especially immunocompromised patients, people undergoing eye surgery or trauma, and those who use contact lenses, regarding the use of these types of water is essential.

In Iran, genotypes T2, T2/T6, T3, T4, T5, T6, T7, T13 and T15 have been observed in surface waters, genotypes T4, T5 and T11 in dust and genotype T3, T4, T5 and T11 in soil (6, 15-21). In the present study, three genotypes T4, T2 and T2/T6 were found, which T4 genotype was the most common one in water (92.6%), soil (94.1%) and dust (90%) samples. In the study of Maghsood et al. (6) on the surface waters of different regions of Iran, 12 isolates of *Acanthamoeba* were separated, of which seven isolates (58.3%) belonged to T2 genotype, and four (33.3%) were T4 that is not consistent with those of our study. In addition, Niyyati et al. (18) and Nazar et al. (15) reported T4 genotype as the dominant genotype in the study of water samples. In another study, Nayyati et al. (19) found T4 (84.6%), T5 (7.6%) and T11 (7.6%) genotypes in environmental dust. In the present study, as in most other studies, T4 was the dominant genotype, and T4 genotype is the main cause of *Acanthamoeba* infections in Iran and the world (1, 6, 16, 22), the presence of T4 genotype in recreational water can be a risk factor for susceptible people, and the education of these people is important in preventing the infection.

In examining the dust samples of hospitals in Brazil in 2010, 23% of the samples were contaminated to *Acanthamoeba* belonging to T4 and T3 genotypes (23). According to the findings of the present study, the rate of *Acanthamoeba* contamination in dust samples was 25%, of which 75% was related to the dialysis, ophthalmology, oncology and transplant

wards, and 25% to the laboratory environment. So, the results indicate a potential health risk in predisposed individuals, including immunocompromised patients and individuals undergoing eye surgery. Therefore, to prevent these nosocomial infections, it is recommended to use suitable disinfectants such as 3% hydrogen peroxide in ophthalmology hospital units instead of chlorine disinfectants, which do not have a lethal effect on *Acanthamoeba* (24).

In this study, the results of cultivating clinical specimens from suspected keratitis patients showed that none of the samples were positive for *Acanthamoeba*. One probable reason for negative results may be due to empiric therapy with antibiotics such as gentamicin, chloramphenicol, and econazole prior to sampling. But it should be noted that none of the studied patients had a history of using contact lenses. A 2002 survey in the United Kingdom showed that 93% of *Acanthamoeba* keratitis were associated with contact lenses use (25). In the study of Rezaeian et al., of 142 patients with keratitis, 49 cases (34.5%) were diagnosed as amoebic keratitis, of which about 90% were wearing contact lenses (medical or cosmetic) (26). *Acanthamoeba* was the cause of 15 cases (30%) of 50 cases of keratitis and all cases were related to the use of contact lenses (16). In another study, 92.3% of patients with amoebic keratitis used contact lenses and only one case had a history of trauma (7). The vast majority of cases of amoebic keratitis occur in contact lens wearers (27), whereas none of the patients under this study had worn contact lenses.

## Conclusion

Considering the high level of contamination of the studied environmental sources with the potentially pathogenic *Acanthamoeba* genotype T4 and the exposure of the residents of the region to this opportunistic amoeba, necessary education is recommended for at-risk groups to avoid their contamination by water and soil resources.

Furthermore, the widespread occurrence of *Acanthamoeba* in the dust samples of the oncology, dialysis, and transplantation units indicates that hygiene measures in the studied hospitals are insufficient. Due to the fact that patients hospitalized in these wards are susceptible to opportunistic infections, this study highlights a strong need for greater efforts regarding improved disinfection procedures in the hospitals.

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## Conflict of interest

The authors declare that there is no conflict of interest.

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