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Original Article

Isolation and Identification of *Acanthamoeba* Strains From the Oral Cavity of Patients Undergoing Hemodialysis in Shahrekord County, the Southwest of Iran in 2018

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Abstract

Background and aims: *Acanthamoeba* spp. as opportunistic microorganisms are widespread in a wide range of environmental sources in the world and may cause severe diseases in immune-deficient patients. The aim of this study was to determine the prevalence and genotypes of *Acanthamoeba* spp. in hemodialysis patients in Shahrekord county of Chaharmahal and Bakhtiari province.

Methods: In this cross-sectional study, 79 oral swabs were collected from the mouth of patients with chronic renal disorder undergoing hemodialysis from June to October 2018. The samples were then cultured on non-nutrient agar and examined by Giemsa staining, polymerase chain reaction (PCR), sequencing, and tolerance testing methods.

Results: Morphological investigations indicated that 30.4% (24/79) of the subjects were infected with some free-living amoebae (FLA), and the PCR showed that only 3 samples contained *Acanthamoeba* spp. The sequencing of the latter samples demonstrated that these isolates belonged to T2 and T4 genotypes. After performing the assay tolerance test, 2 of these 3 isolates were related to T4 genotypes representing a high pathogenic potance.

Conclusion: The infection of hemodialysis patients with some of *Acanthamoeba* spp. particularly, the T4 genotype should be considered important for these patients. Therefore, it is recommended that dialysis machines and dialysis units in hospitals be checked and disinfected periodically.

Keywords: *Acanthamoeba*, Hemodialysis patients, Genotypes, Iran

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Introduction

Acanthamoeba is a genus of free-living amoebae (FLA) that is widely distributed in different environments including water resources, soil, swimming pools, dust, vegetables, air conditioning systems, and the like. This amoeba has been also isolated from the clinical samples of humans and animals, including the throat and nasal mucosa of healthy individuals throughout the world.[1-3] As an opportunistic pathogen amoeba, some species of *Acanthamoeba* may cause severe and even fatal diseases such as amoebic keratitis (AK), cutaneous acanthamoebiasis, and fatal granulomatous amoebic encephalitis (GAE) in individuals with intact or weakened immune systems.[4,5] In addition, the FLA can act as a 'Trojan horse' for a diverse group of microorganisms called 'endosymbionts', including amoeba-resisting bacteria. The mentioned bacteria included *Chlamydophila*, *Legionella* spp., *Burkholderia cepacia*, *Mycobacterium*, *Vibrio cholera*, *Listeria monocytogenes*, *Cryptococcus neoformans*, and *Escherichia coli* O157 causing serious diseases for infected patients.[6]

The life cycle of *Acanthamoeba* spp. consists of trophozoite and cystic stages. When unfavorable environmental conditions (e.g., food shortages, dryness, and high temperatures) occur, trophozoites transform into cysts which can survive for a long period of time (even years), and trophozoites are formed under favorable conditions. The cysts of *Acanthamoeba* spp. are highly resistant to hard environmental conditions such as high temperature, chlorination, and antibiotics.[7] Keratitis caused by some species of *Acanthamoeba* is known as a dangerous eye infection. It is less dependent on immunodeficiency compared to other *Acanthamoeba*-related diseases and is often observed in healthy individuals. AK occurs due to corneal ulcers caused by the contamination of contact lenses by some species of *Acanthamoeba* and may lead to vision loss and even blindness if not treated promptly.[8] GAE is a chronic central nervous system infection caused by certain species of *Acanthamoeba*. The disease mostly occurs in persons who are immunocompromised due to different reasons, including HIV patients, organ

transplant recipients, diabetic and renal failure patients, and those undergoing cancer treatment.[9] Moreover, other GAE-associated risk factors include prolonged and excessive use of antibiotics, chronic alcoholism, liver cirrhosis, malnutrition, pregnancy, surgical trauma, burns, wounds, and radiation therapy.[10] Some investigations on the oral and nasopharyngeal cavity of immunosuppressed patients (e.g., diabetic, hemodialysis, and AIDS patients and those undergoing therapies for malignancies, transplants, or lymphoproliferative disorders and steroids or other immunosuppressive therapy) indicated the presence of FLA, especially *Acanthamoeba* spp.[11-13] Currently, *Acanthamoeba* spp. have been classified into 21 different genotypes (T1 to T21), some of which may cause human infections.[10] This classification is based on the sequencing of the rRNA 18s DF3 region.[14] In the past, only T2, T3, and T4 genotypes of this amoeba have been introduced as infectious genotypes while recent investigations have shown that some other genotypes of this amoeba (e.g., T1, T2, T5, T10, T11, T12, T13, T15, and T18) may cause infections including amoebic keratitis and GAE.[10] According to available reports on both clinical and environmental sources, T4 is the dominant genotype of *Acanthamoeba*.[15] Patients with chronic renal failure undergoing hemodialysis are considered as an immunocompromised group and *Acanthamoeba* spp. may be found in their nasal and oral mucosa, as well as hemodialysis units and hospital environments. Accordingly, this study was conducted to isolate and characterize *Acanthamoeba* strains from the oral cavity of the subjects.

Materials and Methods

Sample Collection and Processing

In this cross-sectional descriptive study, a total of 79 patients were selected by the convenience sampling method. The samples were collected using sterile cotton swabs from the mouth of patients with chronic renal failure undergoing hemodialysis and hospitalized in Hajar Hospital in Shahrekord, in the southwest of Iran from June to October 2018. All patients consented to participate in the study after being informed about the study aim, and their socio-demographic characteristics including gender, occupation, age, living location, and level of education were collected by questionnaires. Then, the samples were processed with direct and indirect methods. In the direct method, the obtained samples were immediately cultivated on plates containing a non-nutrient agar medium (NNA) coated with a layer of heat-killed *E. coli* (ATCC 25922).[16] In the indirect method, after sampling, the cotton parts of swabs containing oral cavity samples were placed in micro-tubes containing 1 mL of sterile phosphate-buffered saline (PBS) solution (pH=7.3) and the contents of the swabs were well mixed with the buffer. Then, the plates and samples were transferred to the Department of Parasitology and Mycology, School of Medicine, Shahrekord University of Medical Sciences, Shahrekord, Iran. Subsequently, the samples were centrifuged at 2000 rpm for 10 minutes, and the sediment was cultivated on plates containing NNA coated with a layer of heat-killed *E. coli* and incubated at 30 ˚C for up to one month.

Morphological Identification

The plates were daily checked for the growth of trophozoites and cysts by observing under the 100x magnification of a light microscope for up to a month. Giemsa-stained smears were prepared from amoebae-positive samples و and the amoebae were microscopically detected at ×1000 magnification using the page detection key.[17] To provide plates without bacterial and fungal contamination and purification of amoebae (Axenification of isolates), a number of colonies were continuously transferred to new plates[18] containing a few drops of TYIS 33 for slow-growing strains.[11]

DNA Extraction, Polymerase Chain Reaction Analysis, Sequencing, and Phylogenetic Analysis

Amoebae were removed from the plate surface using sterile PBS (pH=7.3), and DNA was extracted by the DNG plus kit based on the manufacturer's instructions (Sinagene, Tehran, Iran). The PCR examination was conducted by targeting the DF3 region of the 18s rRNA[19] using the specific *Acanthamoeba* primers of JDP1: 5′-GGCCCAGATCGTTTACCGTGAA-3′ and JDP2: 5′-TCTCACAAGCTGCTAGGGAGTCA-3′[20]. The solution was prepared for the PCR in a 20 µL volume mixture of amplicon (Taq DNA Polymerase Master Mix RED, Denmark), 20-100 ng template DNA, 0.1 µM of each primer, and distilled water. The temperatures and required time for the amplification reaction in a thermocycler included three steps, namely, the first stage (denaturation) at 94˚C for 1 minute, the second stage with 35 cycles (including 94˚C for 35 seconds, 56.5˚C for 45 seconds (annealing), and 72˚C for 1 minute), and the final stage at 72˚C for 5 minutes.[11] The PCR products were mixed with power load and loading buffer and loaded on agarose gel, and then electrophoresed and detected by Gel Doc. The PCR product of positive samples for *Acanthamoeba* spp. was sent to Genomin Company (Tehran, Iran) for sequencing. The obtained sequences of the isolated *Acanthamoeba* spp. were manually edited by Chromas (version 2.6.6) and analyzed using the Basic Local Alignment Search Tool (BLASTn). Next, the identified nucleotide sequences were submitted to the genetic sequence database at the National Center for Biotechnical Information (NCBI) using the Bankit program (https://www.ncbi.nlm.nih.gov/WebSub/) under the accession numbers of MN900683, MN900688, and MN900689. The phylogenetic tree was drawn using the Neighbor-Joining method by the molecular evolutionary genetic analysis software, version 6.06. The bootstrap consensus tree was inferred from 1000 replicates.

Pathogenicity Assays

The potential pathogenicity of *Acanthamoeba* spp. was determined using osmotolerance and thermotolerance assays. In the osmotolerance test, *Acanthamoeba* colonies (10^3/plate) were transferred and cultivated on NNA plates containing 0.5 M and 1 M concentrations of D-mannitol, respectively. Additionally, a plate with no mannitol was considered as control, and the plates were incubated at 30˚C for a maximum of 2 weeks and observed under a microscope (×100 magnification). The number of existing trophozoites or cysts in the middle region of the cultured plates was counted as well. Then, pathogenicity was evaluated based on the number of counts, zero counts (non-pathogenic), 1-15 (+), 16-30 (++), and >30 (+++) [21]. Similarly, in thermotolerance assay, the colonies were cultivated according to the same protocol followed by osmotolerance assay on the two sets of plates incubated at various temperatures of 30 ˚C (control), 37˚C, and 42˚C, respectively.[20] The plates were then checked at 24, 48, and 72 hours to 14 days. The growth of trophozoites or cysts was scored as "positive" while no growth was recorded as "negative".[3]

Data Collection and Analysis

The obtained data were analyzed by SPSS software, version 20.

Results

The age range of 79 hemodialysis patients in this survey varied from 26 to 96 years (mean 58.4±16.5), and most participants were males (59.5%) who lived in urban areas (64.6%). Likewise, the other socio-demographic characteristics of the patients are shown in Table 1. Out of 79 collected oral cavity samples, 24 (30.4%) cases were considered positive for FLAs in the culture method. After the Giemsa staining of these samples, 15 cases were similar to the trophozoite and cyst of *Acanthamoeba* spp. and thus were a candidate for PCR assay in order to confirm *Acanthamoeba* (Figure 1). The PCR examination of these samples amplified a fragment with a length of 423 to 460 bp of the 18S rRNA gene corresponding to the *Acanthamoeba* genus, indicating that 3 (3.8%) isolates were *Acanthamoeba* spp. (Figure 2). These isolates were morphologically considered as cysts. Moreover, the sequencing of PCR-positive isolates revealed that two of these isolates belonged to the T4 genotype (DH29 and DH39), and the other isolate was related to the T2 genotype (HD45) of *Acanthamoeba*. The demographic characteristics of infected hemodialysis patients with *Acanthamoeba* spp. are presented in Table 2. The other results of tolerance assays also indicated that DH29 and DH39 samples were highly pathogenic isolates (Table 3). Figure 3 illustrates a phylogenetic tree including the isolates.

Table 1. Socio-demographic Characteristics of Hemodialysis Patients Regarding *Acanthamoeba* spp.

Variable		Number	Percent
Gender	Male	47	59.5
	Female	32	40.5
Age group (y)	< 15	0	0
	16-30	3	3.8
	31-45	14	17.7
	46-60	10	12.7
	61-75	50	63.3
	> 76	2	2.5
Job	Employee	5	6.3
	Manual worker	5	6.3
	Retired	33	41.8
	Jobless	36	45.6
Level of education	Illiterate	33	41.8
	Primary school	36	45.6
	High school	8	10.1
	Academic	2	2.5
Living location	Urban	51	64.6
	Rural	28	35.4

Discussion

Due to weakened immune functions in immunodeficient patients, they are considered as a high-risk group for a wide range of infections including parasitic infections. Therefore, these patients should be periodically monitored for infection with these microorganisms.[22] The patients with renal failure undergoing hemodialysis are susceptible to septicemia, peritonitis, pneumonia, and GAE as a major cause of morbidity in these patients. Thus, these complications can be the second leading cause of death in this population.[11,23,24] The *Acanthamoeba* spp. are the most prevalent opportunistic protozoan parasites in nature and have been isolated from a wide variety of environments including the soil, air, sewage, seawater, chlorinated swimming pools, domestic tap water, bottled water, dental treatment and hemodialysis units, hospitals, air-conditioning units, and contact lens cases in the world. They have also been isolated from human skin, the nasal cavities, and throats.[7,17,25,26] The existence of this opportunistic amoeba in the environment can be a risk factor for immunocompromised patients. Some epidemiological studies indicate that the prevalence of *Acanthamoeba* spp. is variable from 4.8% to 45% in immunocompromised patients.[11,13,27] Humans can be infected with the trophozoite and cystic of *Acanthamoeba* spp. through different routes including the inhalation of contaminated dry soil and dust, consumption of contaminated food (unwashed raw fruits and vegetables) and drinking water (municipal and mineral water), penetration from breaks in the skin and swimming in contaminated waters, penetration of trophozoites through nasal cavity, and use of contaminated contact lenses. Furthermore, using contaminated dental and hemodialysis units may be other infected routes for humans.[28] It has been estimated that approximately 600 million people

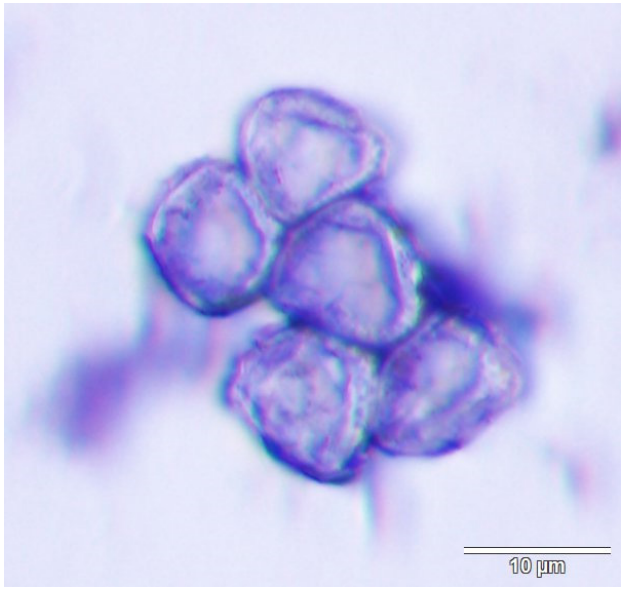

Figure 1. *Acanthamoeba* spp. Cysts Stained with Giemsa (×1000).

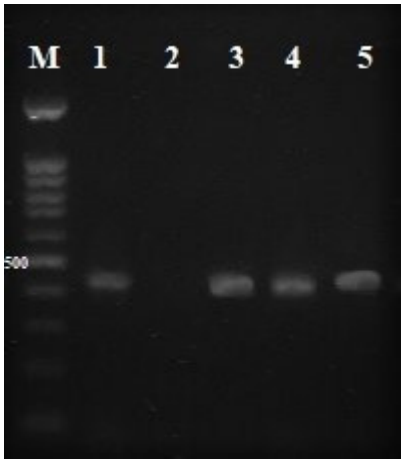

Figure 2. The Results of the Gel Electrophoresis of the Culture-positive Samples of *Acanthamoeba* spp.
Note. M: 100 bp marker; 1=: Positive control; 2: Negative control; 3, 4, 5: Positive samples for *Acanthamoeba*. This image shows fragments with lengths ranging from 423 to 460 bp that are related to the genus *Acanthamoeba*.

suffer from renal failure in the world. The prevalence rate of end-stage renal disease undergoing dialysis is estimated to be 380 per one million populations (30284 patients) per annum in Iran and it is expected to reach 95000 patients by 2023.[29,30] probably being infected with opportunistic microorganisms such as *Acanthamoeba* spp. In this study, 30.4% of hemodialysis patients were infected with FLAs. These results demonstrated that in addition to *Acanthamoeba*, other FLAs may cause infections in these patients. In this study, the prevalence of *Acanthamoeba* spp. was 3.8%, which is in line with the findings of Niyyati et al, indicating that 4.8% and 3.7% of hemodialysis patients in Tehran were infected with FLA and *Acanthamoeba* spp. Respectively.[11] Different frequencies of *Acanthamoeba* found in microscopic and molecular examinations were expected due to the high sensitivity and specificity of molecular tests in the detection of amoebae. Some studies focused on investigating the contamination of hemodialysis systems by morphological and molecular methods in Tunisia (39%), Egypt (42.9%), and Iran (17.5%) and reported the existence of the *Acanthamoeba* spp. in these systems. [17,26,31]. Furthermore, several studies showed the isolation of *Acanthamoeba* spp. from dust and biofilm samples in hospitals in different countries.[32-34] Therefore, the existence of *Acanthamoeba* spp. in the hemodialysis system and hospitals should be considered as a major risk for patients with renal failure who necessitate hemodialysis. In this study, the *Acanthamoeba* genotypes T2 and T4 were isolated from hemodialysis patients that could be due to the contamination of the plumbing water of the hospital and the formation of biofilms in the catheters of dialysate systems. Niyyati et al found that the genotypes of the isolated *Acanthamoeba* spp. from the oral cavity of hemodialysis patients in Iran belonged to T1 and T4 genotypes.[11] In other investigations performed in different parts of Iran, T3, T4, and T5 were among the *Acanthamoeba* genotypes that were isolated from hemodialysis systems.[31] In another study by Bagheri et al, the prevalence of *Acanthamoeba* in the water of some hospitals in Iran was reported at 48% and the abundance of *Acanthamoeba* in the cold and warm drinking waters of Shahrekord was 40%, which may have contaminated hemodialysis units.[35] Likewise, Khodabakhshi et al concluded that 22.1% of different water sources of Chaharmahal and Bakhtiari were contaminated with three genotypes of *Acanthamoeba* spp. (i.e., T2, T4, and T5) and the T4 genotype was the most prevalent one.[36] This indicates that in this region, similar to most regions of the world, the T4 genotype of *Acanthamoeba* is the most common genotype of *Acanthamoeba* spp. Although the T4 genotype of *Acanthamoeba* has been considered as the most frequent genotype in clinical and environmental sources, not all of them have pathogenesis potency for their hosts.[8,20,37] According to pathogenicity assays conducted in this study, most isolates of *Acanthamoeba* spp. had high potential pathogenicity. These results are consistent with those of many studies representing the pathogenicity of the T4 genotype isolated from clinical

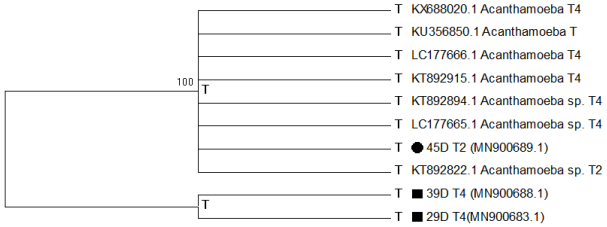

Figure 3. The Phylogenetic Tree of Identified *Acanthamoeba* Genotypes According to the Maximum-likelihood Algorithm Constructed Based on the Multiple Sequence Alignment of the 18S rRNA Gene
Note. Bootstrap values higher than 70% support the topology on each branch. The analyzed sequences in this study are marked by ■ and ●.

Table 2. The Characteristics of the Hemodialysis Patients Infected With *Acanthamoeba* in Shahrekord County

Number	Sample Code	Gender	Age (y)	Job	Location	Education
1	HD29	Male	80	Jobless	Urban	Illiterate
2	HD39	Male	37	Jobless	Rural	High school
3	HD45	Male	70	Retired	Urban	Primary school

Table 3. The Results of the Osmotolerance and Thermotolerance Tests of *Acanthamoeba* Genotypes

Number	Sample Code	Identity/Query Coverage (%)	Genotype	Thermotolerance (37/40 °C)	Osmotolerance (0.5/1 M)	Accession Number
1	HD29	97/93	T4	+/+	+/+	MN900683
2	HD39	99/96	T4	+/+	+/+	MN900688
3	HD45	97/95	T2	+/-	-/-	MN900689

samples such as amoebic keratitis specimens.[18,19,38] Finally, all *Acanthamoeba* positive samples were isolated from male patients in this study that may be due to their further contact with the environment.

Conclusion

The immunocompromised persons including patients with chronic renal failure undergoing hemodialysis may be at the risk of infection with different opportunistic pathogenic microorganisms such as *Acanthamoeba* spp., particularly the T4 genotype that may cause severe and fatal infections.

The contamination of the oral cavity of chronic renal failure patients undergoing hemodialysis with the T4 genotype of *Acanthamoeba* can cause some complications such as granulomatous amebic encephalitis as the fatal infection of the central nervous system and thus threaten patients' lives. Hemodialysis persons over the course of their life and the time of dialysis can be contaminated through various ways such as dialysis machines and dialysis units. Therefore, these patients are necessary to be periodically monitored to evaluate the infected with *Acanthamoeba* after dialysis. Eventually, it is recommended that the dialysis machines and dialysis units in hospitals be checked for contamination with FLA.

Ethics Approval

This survey was approved by the Ethics Committee of Shahrekord University of Medical Sciences (IR.SKUMS.REC.1397.68), and consent was received from all patients.

Conflict of Interest Disclosures

None.

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