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Original Article

A Case-Control Study of Detection of the Potentially Pathogenic *Acanthamoeba* Strains From the Oral Cavity of Diabetic Patients, Cancer Patients, and Healthy Individuals in Iran

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Abstract

Background and aims: *Acanthamoeba* is a genus of free-living amoebae (FLA) that can cause granulomatous amoebic encephalitis and amoebic keratitis. This study aimed to identify *Acanthamoeba* strains among diabetic and cancer patients and healthy individuals.

Methods: In this cross-sectional study, 324 sterile cotton swab samples were collected from the oral cavity of 108 diabetic patients, 106 cancer patients undergoing chemotherapy, and 110 healthy individuals from June to October 2018 in Shahrekord, Iran. Samples were cultured onto 1.5% non-nutrient agar, and the *Acanthamoeba* spp. were investigated with morphological, molecular (polymerase chain reaction targeting 18SrRNA), and pathogenicity assays.

Results: FLA was found in 130/324 (40.1%) oral cavity samples of individuals. Microscopic results using Giemsa staining revealed that 14/37 (37.8%) and 31/93 (33.3%) FLA isolated from healthy individuals and immunocompromised patients were identified as *Acanthamoeba* spp. Moreover, the PCR confirmed the existence of *Acanthamoeba* spp. in 4.7% of patients and 3.6% of controls. In addition, the PCR demonstrated that *Acanthamoeba* spp. were isolated in 6.6%, 8.2%, and 6.3% of cancer patients, diabetic patients, and healthy individuals, respectively. According to the sequence analysis of PCR products of 18S rRNA, the T4 (10 isolates) and T5 (3 isolates) genotypes of *Acanthamoeba* were identified in two groups. Finally, nine isolates genotyped as T4 were positive for potential pathogenic assays.

Conclusion: The presence of the potentially pathogenic *Acanthamoeba* T4 genotype in both immunocompromised patients and healthy individuals indicated that it may pose a risk factor for immunocompromised individuals.

Keywords: *Acanthamoeba* spp., Genotype, Immunocompromised patients, Healthy individuals, Oral mucosa, Iran

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Introduction

Free-living amoebas (FLA) are amphizoic microorganisms that are widely distributed in aquatic and arid environments and even biological specimens, causing opportunistic and pathogenic as well as non-opportunistic infections (1, 2). Some species of these potentially pathogenic protozoa (e.g., *Acanthamoeba* spp., *Naegleria fowleri*, *Sappinia pedata*, *Balamuthia mandrillaris*, and *Vermamoeba vermiformis*) cause complications and occasionally fatal diseases in humans and mammalian animals worldwide (1, 3, 4). The life cycle of *Acanthamoeba* has trophozoite and cyst forms. The trophozoite is the active form of protozoa, and the cyst is formed in unfavorable environmental conditions as a dormant form. The cysts exhibit high resistance to many harsh environmental conditions, including different chemical and physical factors (e.g., drought, detergents, ultraviolet rays, osmotic pressure, chlorinating agents, and antiseptics); therefore, they can endure and survive for years (5). Humans can be infected through direct transmission of trophozoites and cysts of *Acanthamoeba* spp. into the body in various ways. These trophozoites and cysts can enter the eye by swimming in contaminated water, failing to observe hygiene when using contact lenses, being exposed to sewage, working with contaminated soil, rubbing the eyes with contaminated hands, and experiencing trauma to the eyes. Similarly, they may enter the nasal passages and the lower respiratory tract by the inhalation of contaminated dust (5). Furthermore, trophozoites and cysts can enter the body from ulcerated or broken skin by contact with wounds with contaminated soil and equipment, resulting in hematogenous dissemination to the body. The mucosa

of the oral cavity and the intestine are the other sites of infection (1, 5). *Acanthamoeba* spp. are more attracted to the cornea and conjunctiva tissues of the eye, nervous system, and skin of hosts that are the causative agents of blindness due to amoebic keratitis in immunocompetent persons (6). Moreover, these protozoa can cause fatal granulomatous amoebic encephalitis (GAE), skin ulcers, sinusitis, nasopharyngeal, lung, and kidney infections, and disseminated acanthamoebiasis in individuals with a compromised immune system as life-threatening infections (4, 7). Despite the low occurrence of acanthamoebiasis in humans, the mortality rate of acanthamoebiasis is extremely high, especially in immunosuppressed hosts (8). Certain individuals are at a higher risk of *Acanthamoeba* infection due to compromised immune function or other predisposing factors, including people living with HIV, patients with hematologic malignancies, organ transplant recipients, and those undergoing immunosuppressive treatments (e.g., steroid therapy). Additionally, systemic lupus erythematosus, diabetes mellitus, chronic antibiotic overuse, alcoholism, malnutrition, and pregnancy can increase susceptibility. Other risk factors involve liver cirrhosis, major surgical procedures, severe burns, open wounds, and radiotherapy, which may all lead to a vulnerability throughout their lives to *Acanthamoeba*-related disorders (4). *Acanthamoeba* spp. can act as a carrier or reservoir for various prokaryotic microorganisms, such as many pathogenic bacteria, yeast, and viruses, called harbor endosymbionts (Trojan horses), thereby leading to dangerous outcomes (e.g., tuberculosis, anthrax, legionellosis, listeriosis, chlamydiosis, cryptococcosis, histoplasmosis, and cholera) for humans (9, 10). So far, 23 different genotypes of *Acanthamoeba* (T1–T23) have been identified by sequencing the diagnostic fragment 3 (DF3) of the ASA.S1 region of the *18S rRNA* gene (11). Although some previous studies indicated that the pathogenicity of *Acanthamoeba* is related to its genotype, this relationship has repeatedly been debated by some researchers. However, the T4 genotype is considered the most abundant species in diverse environments and the most pathogenic genotype that has been associated with neurological and pulmonary acanthamoebiasis (5, 11, 12). In addition, recent studies have reported the pathogenicity of other genotypes, such as T1, T2, T3, T5, T6, T10, T11, T12, T13, T15, and T18, which may cause GAE, *Acanthamoeba* keratitis, and pneumonia in humans (4, 12, 13). According to previous research, the FLA, especially the *Acanthamoeba* spp., were isolated from various sources, such as water and soil of Chaharmahal and Bakhtiari province (14). Hence, the present study aims to determine the frequency of *Acanthamoeba* spp., according to morphological and molecular characteristics in diabetic patients, cancer patients undergoing chemotherapy, and healthy individuals in Shahrekord, the capital of Chaharmahal and Bakhtiari province.

Materials and Methods

Sampling, Cultivation, and Microscopic Identification

In this cross-sectional study, 324 oral cavity samples (including the pharynx and mouth) were collected from 108 diabetic patients, 106 cancer patients undergoing chemotherapy, and 110 healthy individuals using sterile cotton swabs in Hajar Hospital in Shahrekord, in the southwest of Iran, from June to October 2018. First, the aim of this study was explained to individuals who agreed to participate in the investigation, and written informed consent was obtained from them. Furthermore, their demographic data were collected through questionnaires and face-to-face interviews. Then, the samples were taken from individuals using sterile swabs, and one centimeter above the cotton part was cut off and placed in 1.5 mL microtubes containing 1 mL of the sterile phosphate-buffered saline (PBS) solution (pH = 7.3). Next, the samples were sent to the parasitology laboratory for parasitological and molecular tests at the Department of Parasitology and Mycology and the Cellular and Molecular Research Center, Shahrekord University of Medical Sciences. Subsequently, they were centrifuged at 2,000 rpm for 10 minutes, and the sediment was cultivated on plates containing 1.5% non-nutrient agar medium (NNA) that was prepared with amoeba Page's saline (1 mM KH_2PO_4, 2.5 mM NaCl, 0.5 mM Na_2HPO_4, 40 µm $CaCl_2$-$6H_2O$, and 20 µm $MgSO_4$. $7H_2O$ (Merck, Germany), which was added to 1.5% agar (Merck, Germany) coated with a layer of heat-killed *Escherichia coli* (ATCC 25922) and incubated at 30˚C for up to one month (15). In addition, sterile PBS was cultivated on NNA as a negative control to evaluate the contamination of the culture method. The growth of trophozoites and cysts of FLA in the plates was evaluated daily by observing under an optical microscope (Olympus CX52) with low power (the magnifications of 100x and 400x). Further, the smears were prepared from amoebae-positive samples and stained by Giemsa dye (Labtron Company, Tehran, Iran) in order to accurately identify the structure of the trophozoite and cyst of amoebae according to morphological characteristics (e.g., size and shape of cysts by 1000 × magnification) based on the diagnostic keys of Pussard and Pons and Page (16, 17). Moreover, the slow-growing strains of *Acanthamoeba* spp. were cultivated in plates containing 500 µL of the peptone-yeast extract-glucose medium [glucose 1.5% (w/v), Merck, Germany], proteose peptone 0.75% (w/v) [QUELAB, UK], and yeast extract 0.75% (w/v) [QUELAB, UK] (18). It should be noted that FLA were continuously subcultivated into fresh NNA plates to reduce fungal and bacterial contamination by cutting out a piece of agar containing the amoebic growth using a sterile scalpel and then inoculated it into a fresh NNA plate that was coated with heat-killed *E. coli*.

Molecular Detection

Deoxyribonucleic Acid Extraction, Polymerase Chain Reaction, and Sequencing

The growing amoebae (cysts and trophozoites) were

removed from the surface of the plate by gently scraping and rinsing with sterile PBS solution, collected in 1.5 mL microtubes, and centrifuged at 2,000 rpm for 10 minutes. The sediment at the bottom of the tube was washed 3 times using PBS. Additionally, DNA extraction was performed using a nucleic acid extraction kit (DNG plus, Sinagene, Tehran, Iran) according to the manufacturer's instructions. Next, the obtained DNA was quantitatively and qualitatively analyzed using NanoDrop 2000™ (Thermo Scientific, Waltham, MA, USA). Then, PCR was performed using specific *Acanthamoeba* primers JDP1:5'-GGCCCAGATCGTTTACCGTGAA-3 and JDP2: 5'-TCTCACAAGCTGCTAGGGAGTCA-3', which targeted the DF3 region of the 18S rRNA (19). In addition, PCR amplification was conducted in a 25 µL volume containing 12.5 µL of amplicon buffer (Taq DNA Polymerase Master Mix RED, Denmark), 5 µL template DNA (20–50 ng), 1 µL forward and reverse primers (10 pmol), and 5.5 µL RNA-free water. Furthermore, DNA from the *Acanthamoeba* T4 strain detected in the previous research (14) and sterilized distilled water were used as positive and negative controls, respectively. The temperature and time conditions of the amplification reaction by ASTEC Thermal Cyclers Gene Atlas (Japan) included the first stage at 94°C for 4 minutes, the second stage in 30 cycles, including 94°C for 35 seconds, 56.5°C for 45 seconds (the annealing step), and 72°C for 1 minute, and the third step, including 72°C for 5 minutes (the final expansion step). Then, PCR products were loaded on 1.5% agarose (SERVA, Heidelberg, Germany) gel containing ethidium bromide solution (Sigma-Aldrich, USA, CAS No. 1239-45-8), and electrophoresis was observed using a BioDoc-It and VisiDoc-It Gel Documentation System (Cambridge, UK). The positive samples in terms of *Acanthamoeba* showed bands of 423 bp to 550 bp (19) and were sent to Genomin Company (Tehran, Iran) for sequencing. Moreover, the nucleotide sequences of isolated *Acanthamoeba* spp. were analyzed through a multi-step verification process. Additionally, initial sequence validation was conducted by manual editing and quality assessment using the Chromas program (version 2.6.6, http://www.technelysium.com.au/ChromasPro.html). Subsequent genotypic identification was performed by comparing the sequences against all known *Acanthamoeba* genotypes in the National Centre for Biotechnology Information database (NCBI) using Basic Local Alignment Search Tool analysis (http://www.ncbi.nlm.nih.gov), with matches determined based on the highest similarity scores. For phylogenetic analysis, a dataset comprising fourteen representative sequences from the study, along with reference sequences from GenBank, was used to construct a maximum-likelihood tree by MEGA software (version 6.0; Molecular Evolutionary Genetics Analysis; Pennsylvania State University, PA, USA; https://www.megasoftware.net). The representative sequences included MW866552.1, MW866553.1, MW866558.1, MW866561.1, PP527029, MW866563.1, PP527092, MW866542.1, MW866546.1, MW866548.1, MW866534.1, MW866535.1, MW866538.1, and MW866539.1. In addition, the evolutionary relationships were reconstructed through neighbor-joining and maximum composite likelihood methods, employing the Tamura-3 parameter substitution model. It should be noted that the gene sequence of *Hartmannella* sp. (GenBank accession: KF697197.1) served as the outgroup, and the bootstrap consensus tree was inferred from 1,000 replicates.

Pathogenicity Assay

The potential pathogenicity of different strains of *Acanthamoeba* was assessed by osmotolerance and thermotolerance assays. In the osmotolerance test, each *Acanthamoeba* isolate was cultured in the NNA medium containing concentrations of 0.5 M and 1 M d-mannitol (Merck, Germany). Further, in thermotolerance assays, each isolate of *Acanthamoeba* was cultured in NNA containing killed *E. coli* and was incubated at 37°C and 42°C (20). In two experiments, the samples were examined for *Acanthamoeba* growth after 24 hours, 48 hours, and 72 hours to 14 days. Then, pathogenicity was evaluated and scored based on the number of counts: zero counts (non-pathogenic), 1-15 (+), 16-30 (++), and > 30 (+++) (21).

Statistical Analysis

Pearson's chi-squared and Fisher's exact tests were used to determine the statistical significance of differences between categorical variables. Statistical significance was set at $P < 0.05$, and all analyses were performed using IBM SPSS software, version 26.

Results

Parasitology and Molecular Evaluations

In this study, 324 samples of the oral cavity from 164 men and 160 women with an age range of 5–100 years (56.5 ± 17.8) were examined by microscopic and molecular methods. Moreover, other socio-demographic characteristics of participants were assessed that may be associated with the contamination of FLA (Table 1). The results of examinations demonstrated that 130/324 (40.1%) FLA were isolated from the oral cavity samples of individuals using the culture method in the NNA medium. Furthermore, Giemsa staining indicated that 45/130 (34.6%) of isolated FLA were morphologically identified as *Acanthamoeba* spp. based on flattened trophozoites with slender acanthopodia and double-wall cysts with different shapes (Figures 1 and 2), including 14/110 isolates (12.7%) in healthy individuals and 31/214 isolates (14.5%) in immunocompromised patients. The molecular tests revealed that 14/130 isolates (10.8%) of FLA were identified as *Acanthamoeba* spp. Based on PCR analysis, FLA were isolated from the oral cavities of 4 (3.6%) healthy individuals, and 10 (4.7%) immunocompromised patients were confirmed as *Acanthamoeba* spp. (Figure 3, Table 2). Furthermore, the results of this study showed

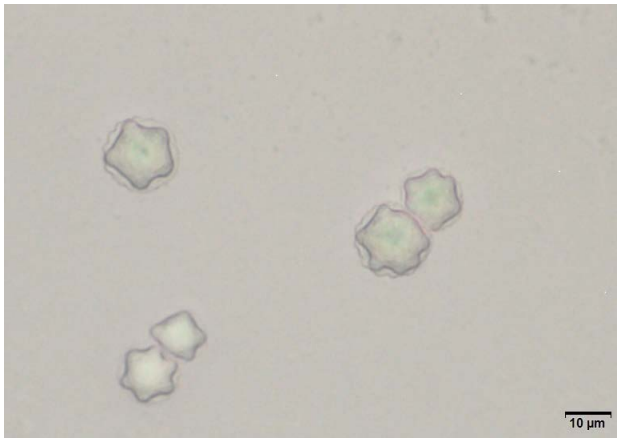

Figure 1. Star-Shaped Cysts of *Acanthamoeba* spp. in the NNA Medium Isolated From the Oral Cavity of Diabetic Patients, Shahrekord, Iran (400X)
Note. NNA medium: non-nutrient agar medium

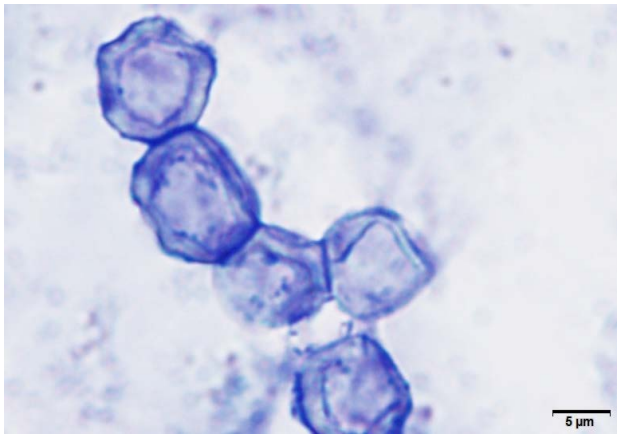

Figure 2. The Polygonal Shape Cysts of *Acanthamoeba* spp. With a Double-Layer Cell Wall Isolated From the Oral Cavity of Healthy Individuals, Shahrekord, Iran (Giemsa Staining at 1000X)

Table 1. Demographic Characteristics of Participants in This Study

Characteristics	Cancer n (%)	Diabetic n (%)	Healthy Individuals n (%)
Gender			
Male	41 (37.8)	58 (53.7)	65 (59.1)
Female	65 (61.3)	50 (46.3)	45 (40.9)
Age group (year)			
<15	10 (9.4)	0 (0)	16 (14.5)
16-30	9 (8.5)	14 (13)	18 (16.5)
31-45	13 (12.3)	21 (19.4)	22 (20)
46-60	21 (19.8)	24 (22.2)	16 (14.5)
61-75	28 (26.4)	23 (21.3)	17 (15.5)
>76	25 (23.6)	26 (24.1)	21 (19)
Job			
Employee	(8.5)	11 (10.2)	28 (25.5)
Manual worker	6 (5.7)	6 (5.6)	10 (9.1)
Retired	22 (20.8)	27 (25)	7 (6.4)
Jobless	69 (65.1)	64 (59.3)	65 (59.1)
Education level			
Illiterate	31 (29.2)	41 (38)	4 (3.6)
Primary school	43 (40.6)	34 (31.5)	19 (17.3)
High school	18 (17)	18 (16.7)	23 (20.9)
Academic	14 (13.2)	15 (13.9)	64 (58.2)
Living location			
Urban	77 (72.6)	70 (64.8)	75 (68.2)
Rural	29 (27.4)	38 (35.2)	35 (31.8)

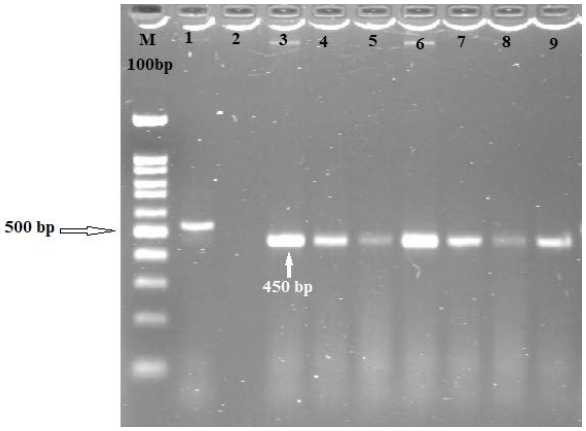

Figure 3. Gel Electrophoresis of PCR Products of *Acanthamoeba* Species Using JDP1 and JDP2 Primers, Isolated From the Oral Cavity of Diabetic Patients, Undergoing Treated Cancer Patients, and Healthy Individuals in Shahrekord, Iran
Note. PCR: Polymerase chain reaction. M: 100 bp marker; 1: Positive control (the *Acanthamoeba* T4 strain); 2: Negative control (sterilized distilled water); 3-9: Positive samples for *Acanthamoeba*. This image shows fragments with lengths of ~450 bp that are related to the genus *Acanthamoeba*

that the oral cavities of 7 (6.6%) cancer patients, 3 (2.8%) diabetic patients, and 4 (3.6%) healthy individuals were positive for *Acanthamoeba* spp. by the molecular method, respectively. Several variables, including gender, age, job, education level, and living location, which may be associated with FLA infections, were evaluated by statistical methods. The results indicated that gender, age group, job, and level of education were significantly related to the presence of *Acanthamoeba* spp. in the oral cavities of immunocompromised patients and healthy individuals. In this study, the partial *18S rRNA* gene sequences of 14 *Acanthamoeba* isolates were successfully amplified and manually curated using Chromas software (version 2.6.6) to eliminate ambiguous regions. Then, the cleaned sequences were analyzed via the Basic Local Alignment Search Tool for homology assessment. The confirmed nucleotide sequences have been submitted to the NCBI GenBank database using the Bankit submission portal (https://www.ncbi.nlm.nih.gov/WebSub/) and assigned accession numbers MW866552.1, MW866553.1, MW866558.1, MW866561.1, MW866563.1, MW866542.1, MW866546.1, MW866548.1, MW866534.1, MW866535.1, MW866538.1, MW866539.1, PP527029, and PP527092. Next, they were compared with sequences in the GenBank database with high genetic similarity identity of 97–100% to determine the genotype of the *Acanthamoeba* isolates (Table 3). The sequencing results of positive samples revealed that all *Acanthamoeba* spp. isolated from healthy and diabetic individuals were the T4 genotype. In addition, out of 7 positive samples isolated from cancer patients, 5 and 1 isolates were identified as T4

Table 2. Distribution of *Acanthamoeba* spp. in the Oral Cavity of Diabetic, Undergoing Treated Cancer Patients and Healthy Individuals by Giemsa Staining and PCR Methods According to Demographic Characteristics, Shahrekord, Iran

Characteristic	*Acanthamoeba* Positive in Cancer Patient's N (%)		*Acanthamoeba* Positive in Diabetic Patient's N (%)		*Acanthamoeba* Positive in Healthy Individual's N (%)		*P*-Value
	Giemsa	PCR	Giemsa	PCR	Giemsa	PCR	
Gender							
Male	8 (61.5)	4 (57.1)	11 (61.1)	2 (66.7)	8 (57.1)	3 (75)	0.009
Female	5 (38.5)	3 (42.9)	7 (38.9)	1 (33.3)	6 (42.9)	1 (25)	
Age group (year)							
<15	0 (0)	0 (0)	0 (0)	0 (0)	1 (7.1)	0 (0)	0.004
16-30	2 (15.4)	0 (0)	2 (11.1)	0 (0)	3 (21.4)	0 (0)	
31-45	2 (15.4)	1 (14.3)	4 (22.2)	0 (0)	2 (14.3)	0 (0)	
46-60	3 (23.1)	2 (28.6)	2 (11.1)	0 (0)	1 (7.1)	1 (25)	
61-75	2 (15.4)	1 (14.3)	4 (22.2)	1 (33.3)	2 (14.3)	1 (25)	
>76	4 (30.7	3 (42.8)	6 (33.4)	2 (66.4)	5 (35.8)	2 (50)	
Job							
Employee	4 (30.7)	1 (14.3)	3 (16.7)	0 (0)	2 (14.3)	0 (0)	0.001
Manual worker	1 (7.7)	2 (28.6)	6 (33.4)	2 (66.7)	6 (42.9)	3 (75)	
Retired	3 (23.1)	0 (0)	4 (22.2)	1 (33.3)	3 (21.4)	0 (0)	
Jobless	5 (38.5)	4 (57.1)	5 (27.7)	0 (0)	3 (21.4)	1 (25)	
Education level							
Illiterate	2 (15.4)	2 (28.6)	5 (27.7)	1 (33.3)	4 (28.6)	1 (25)	0.001
Primary school	4 (30.7)	2 (28.6)	6 (33.4)	2 (66.4)	5 (35.7)	2 (50)	
High school	3 (23.2)	1 (14.2)	3 (16.7)	0 (0)	3 (21.4)	0 (0)	
Academic	4 (30.7)	2 (28.6)	4 (22.2)	0 (0)	2 (14.3)	1 (25)	
Living location							
Urban	7 (53.8)	4 (57.1)	10 (55.6)	1 (33.3)	8 (57.1)	2 (50)	0.580
Rural	6 (46.2)	3 (42.9)	8 (44.4)	2 (66.7)	6 (429)	2 (50)	

Note. PCR: Polymerase chain reaction.

Table 3. Sequencing Results and Pathogenicity Tests of *Acanthamoeba* Positive Samples

Code	Type of Examined Case	Genotype/Species	Thermotolerance (37°C/42°C)	Osmotolerance (0.5/1 M)	Shape of Cyst	Size of Cyst µm	Accession Number
49C	Cancer	T4/*Acanthamoeba* spp.	+/+	+/+	Wrinkled, Polygonal	10.6	MW866552.1
52C	Cancer	T5/*Acanthamoeba* spp.	-/-	-/-	Round, smooth	11.4	MW866553.1
53C	Cancer	T5/*Acanthamoeba* spp.	-/-	-/-	Round, smooth	16	MW866558.1
58C	Cancer	T4/*Acanthamoeba* spp.	+/+	+/+	Round, smooth	10.5	MW866561.1
72C	Cancer	T5/*Acanthamoeba* spp.	-/-	-/-	Wrinkled, Polygonal	14.2	PP527029
77C	Cancer	-/ *Acanthamoeba* spp.	-/-	-/-	Wrinkled, Polygonal	11.2	MW866563.1
90C	Cancer	T4/*Acanthamoeba* spp.	-/-	-/-	Wrinkled, Polygonal	12.4	PP527092
15Di	Diabetic	T4/*Acanthamoeba* spp.	+/++	+/++	Round, smooth	13.6	MW866542.1
44Di	Diabetic	T4/*Acanthamoeba* spp.	+/+	+/+	Wrinkled, Polygonal	10.4	MW866546.1
97Di	Diabetic	T4/*Acanthamoeba* spp.	+/+	+/+	Wrinkled, Polygonal	12.4	MW866548.1
5H	Healthy person	T4/*Acanthamoeba* spp.	+/+	+/+	Wrinkled, Polygonal	14.3	MW866534.1
21H	Healthy person	T4/*Acanthamoeba* spp.	+/+	+/+	Round, smooth	13.5	MW866535.1
48H	Healthy person	T4/*Acanthamoeba* spp.	+/+	++/+	Wrinkled, Polygonal	12.8	MW866538.1
87H	Healthy person	T4/*Acanthamoeba* spp.	+/+	+/+	Wrinkled, Polygonal	10.5	MW866539.1

and T5 genotypes, respectively. Further, the sequence of one sample did not relate to any of the sequences recorded on the NCBI site and was reported as *Acanthamoeba* spp. Moreover, the results of pathogenicity tests of different strains demonstrated that, unlike the T5 genotype, most specimens belonging to the T4 genotype grew in various temperatures and osmotic conditions, highlighting the high ability of this genotype to cause disease in humans

(Table 3). Additionally, the results showed no significant relationships between the prevalence of FLA, including *Acanthamoeba*, and variables such as age, gender, job, and level of education ($P > 0.05$). However, there was a correlation between the amoeba and the living location of individuals, so that the prevalence of FLA was significantly higher in urban people.

Phylogenetic Analysis

The taxonomic status of *Acanthamoeba* genotypes was determined by creating a maximum likelihood phylogenetic tree. Representative sequences of isolated *Acanthamoeba* in our study were supported by the distinct separation of species-specific clades obtained from phylogenetic analyses (Figure 4).

Discussion

Acanthamoeba spp. is an opportunistic protozoan that can infect individuals with impaired immunity, such as patients with AIDS, cancer, diabetes, and hepatitis, as well as those who undergo immunosuppressive therapy, which causes some disorders, such as GAE, as a life-threatening pathogen, ocular and pulmonary complications, skin lesions, and disseminated acanthamoebiasis (3, 5, 22). Similarly, *Acanthamoeba* causes a sight-threatening eye infection in immunocompetent people, mostly in contact lens wearers (3). Therefore, it can cause diseases with high mortality and considerable morbidity. It is also imperative to identify the patients at the earliest possible time and initiate appropriate treatments as soon as possible. This case-control study investigated the occurrence and characterization of potential pathogenic *Acanthamoeba* spp. from the oral cavity of 110 healthy individuals and

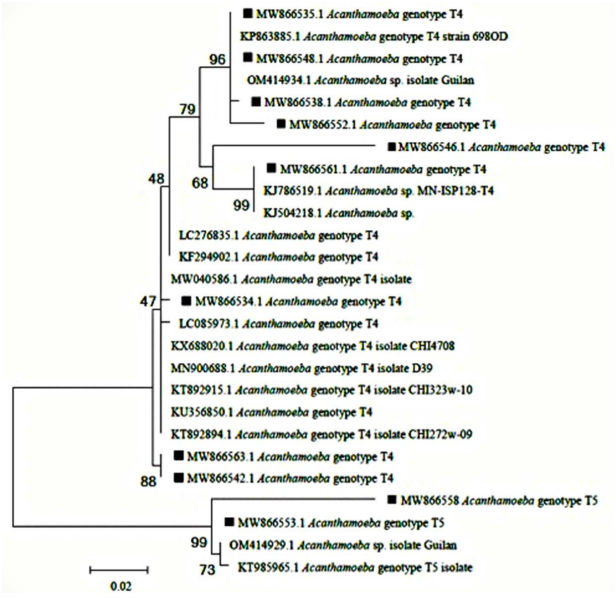

Figure 4. Phylogenetic Tree of the *18S rRNA* Gene of *Acanthamoeba* spp., Isolated From Diabetic Patients, Undergoing Treated Cancer Patients, and Healthy Individuals' Samples Together With Reference Sequences

Note. Based on the phylogenetic tree, all identified genotypes were clustered with the reference genotypes. The phylogenetic tree was drawn using the maximum-likelihood method and the Tamura 3-parameter model. The sequences generated in this study are marked by a square

214 patients with immunodeficiency in Shahrekord using morphological and molecular methods. The results showed that 40.1% of the total samples were positive for FLA using the culture method. The evaluation results of FLA isolated by staining and molecular methods indicated that 34.6% and 10.8% of them were detected as *Acanthamoeba* spp., respectively. These results are due to the great variety of other genera of FLA (e.g., *Vermamoeba*) in the living environment of people as a source of infection. In addition, the round or oval and smooth cysts of *Acanthamoeba* spp. with small sizes in the culture medium are similar to other FLA, such as *Vermamoeba* and *Vahlkampfiids*. Accordingly, the identification of *Acanthamoeba* spp. only based on morphological characteristics is unreliable, and molecular methods are more accurate and correct (23). Molecular techniques displayed more sensitivity and specificity in identifying *Acanthamoeba* spp. The epidemiological studies reported that the number of *Acanthamoeba* infections is increasing in recent years, especially *Acanthamoeba* keratitis (24). Based on previous studies, the prevalence of *Acanthamoeba* spp. in immunocompromised patients was from 3.08% to 100%, and T1, T3, T4, T5, T11, and T15 genotypes were identified in different clinical specimens, such as bronchoalveolar lavage (BAL) samples, as well as nasal and oral swabs (8, 25-28). The results of this study demonstrated that 6.6%, 8.2%, and 6.3% of the samples from cancer patients, diabetic patients, and healthy individuals were positive for *Acanthamoeba*, respectively, which, contrary to the expected frequency of *Acanthamoeba* in diabetic and cancer patients, was not much different from that of healthy individuals. Hence, in the current study, *Acanthamoeba* spp. were detected in all the studied groups, confirming the prevalence of *Acanthamoeba* spp. in different regions and resources. Based on previous studies regarding the isolation of *Acanthamoeba* spp. from hospital environments, air conditioning systems of hospitals, and medical devices (29), as well as dialysis systems (30) and dentistry units (31), these species are widely distributed in nature as a risk factor for the transmission of the protozoa to healthy and immunocompromised individuals. Moreover, in the present study, the identified genotypes in immunocompromised and healthy individuals were T4 and T5, with T4 being the most common genotype. The findings of a previous study performed by Khodabakhshi et al in Chaharmahal and Bakhtiari province, including Shahrekord, revealed that T2, T4, and T5 genotypes were isolated from different water sources of this province (14). Comparing these results with those of the present study confirmed that the genotypes isolated from the environment and oral samples of individuals in this province were highly similar to those of many studies that indicated the T4 genotype as the most common genotype isolated from individuals and different environments from many parts of the world (5, 32). The results of the frequency of *Acanthamoeba* in this study are consistent

with the findings of some previous studies. Cheshmpanam et al reported that the prevalence of *Acanthamoeba* infection in dialysis patients in Shahrekord was 3.8%. The sequencing results of this study showed T4 genotype strains (two isolates) and the T2 genotype strain (one isolate) in dialysis patients (33). In the study by Walochnik et al, the PCR analysis detected *Acanthamoeba* in the BAL sample of an HIV-negative patient, with genotyping confirming the strain as T2 (34), which is in line with the results of our study. Pezeshki et al found that 6% of patients with malignancy in Zanjan (Iran) were infected with *Acanthamoeba* spp. based on morphological methods (35). In another study conducted by Memari et al on immunocompromised individuals, including patients with diabetes, lupus, hepatitis, splenotomy, and steroid therapy in Tehran, the prevalence of *Acanthamoeba* was 13.4%, and the identified genotypes included T3, T4, and T11, with T11 as the most common genotype (8). However, in another study, Memari et al concluded that the prevalence of *Acanthamoeba* in the nasal swab samples of cancer patients was 45%, and T3, T4, and T5 genotypes were isolated from these patients (25). The prevalence of *Acanthamoeba* can vary in different regions depending on many factors, including the degree of environmental pollution. Eslamirad et al, investigating the BAL samples of patients with immunodeficiency in Arak, Markazi Province of Iran, reported that using culture, molecular, and direct smear methods, respectively, 100%, 98.4%, and 0% of patients were infected with *Acanthamoeba*, indicating the contamination of inhaled air with cysts and trophozoites of FLA as a risk factor for patients (28). However, Lanocha et al observed that the abundant contamination of lung samples of patients with immunodeficiency with symptoms of pulmonary complications in Poland was 3.3%, representing different levels of environmental pollution in diverse areas of various countries (26). Likewise, Niyyati et al evaluated nasal swab samples of patients with severe immunodeficiency hospitalized in special wards and environmental samples of these wards and found that 50% and 12% of environmental dust samples and swab samples of patients were infected with FLA, respectively. Therefore, they concluded that high-level contamination of the hospital environment can be a risk factor for immunocompromised patients who are referred to the hospital (36). On the other hand, another study of nasal swabs of healthy individuals in Peru showed that 28.4% of individuals were infected with *Acanthamoeba* spp., and T4 and T15 genotypes were isolated from them (37). The results of this investigation can be a reason for the effectiveness of contact with a contaminated environment as an agent for infection with FLA. The difference between Giemsa staining and PCR results, which were both used to identify *Acanthamoeba* in this study, indicates the high sensitivity and specificity of molecular methods in detecting organisms, including *Acanthamoeba*. Moslemzadeh et al investigated the prevalence and genotypes of *Acanthamoeba* spp. in the nasal mucosa of immunocompromised patients and healthy persons and reported that 15.6% and 4.6% of the patient and control groups were positive for the *Acanthamoeba* genus, respectively. Furthermore, the topology of the phylogenetic tree revealed that all the *Acanthamoeba* strains belonged to the T4 genotype (27). Compared to the outcomes observed in our study, these results demonstrated that the abundance of *Acanthamoeba* in immunocompromised patients was more than in our study, but it was lower in healthy individuals. Assessing the pathogenicity of *Acanthamoeb*a strains is an important aspect of the study of isolated and identified genotypes in most studies using osmotolerance and thermotolerance tests. In the present study, the results of pathogenicity tests confirmed that the T4 genotype has a high ability to cause disease, and this result has been proven in some studies (38, 39). The isolation of the T4 genotype from many clinical specimens (e.g., ocular keratitis, brain tissue, cerebrospinal fluid, lung and skin samples) in most parts of the world represents severe pathogenicity of this strain, especially in patients with immunodeficiency, mentioned as the most essential cause of amoebic granulomatous encephalitis (40).

Study Limitations

In this study, the lack of access to all types of immunocompromised patients in Chaharmahal and Bakhtiari province caused some problems in sampling and non-cooperation of the participants.

Conclusion

In general, our results revealed that healthy and immunocompromised individuals are infected with FLA, including *Acanthamoeba* spp., during their lifetime. In addition, the identified genotypes of *Acanthamoeba* in immunocompromised and healthy individuals were T4 and T5, which, according to the pathogenicity of T4, may cause severe complications in immunocompromised patients. With the increasing identification and introduction of new and powerful immunosuppressive agents, as well as the severe and occasionally fatal complications of *Acanthamoeba*, the medical community should be aware of the risks of this infection. In addition, they should design and implement programs to prevent and control the disease, especially among immunocompromised individuals with central nervous system lesions who are referred to medical centers. Additionally, it is highly recommended that the presence and coexistence of *Acanthamoeba* spp. with other pathogens in the environment of hospitals and health facilities (e.g., water and biofilm, dust, ventilation, and air conditioning systems) be evaluated as a reservoir for human pathogens.

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Competing Interests

The authors declare that there is no conflict of interests.

Ethical Approval

Permission was obtained from the Ethics Committee of Shahrekord University of Medical Sciences (IR.SKUMS.REC.1397.68). Moreover, consent was received from all patients and healthy individuals before the study.

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