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Original Article

Isolation and Identification of Free Living Amoeba from Patients and Contact Lens Users in Iran

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Abstract

Background: Free-living amoebae (FLA) such as *Acanthamoeba* spp., are considered as opportunistic and pathogenic protozoans. *Acanthamoeba* granulomatous encephalitis (AGE) is a serious threat for immunodeficient patients and *Acanthamoeba* keratitis (AK) for contact lens users. We aimed to identify the presence of free living amoebae in nasal swabs of patients and contact lens users in Qazvin, Iran.

Methods: During 2019, 251 nasal and oral swabs (including the pharynx and mouth) were collected from patients with diabetes, AIDS and those under periodic dialysis in Qazvin, Iran. In addition, 27 soft contact lenses were collected from the participants. Following DNA extraction, PCR and sequencing were conducted to identify the genotypes of the amoeba. Phylogenetic analysis of the identified sequences was performed using MEGA 7 software.

Results: A strain of *Acanthamoeba* belonging to the T3 genotype was isolated from hemodialysis patients. Two specimens of *Acanthamoeba* with T3 genotype were isolated from keratitis patients.

Conclusion: The clinicians should pay attention to the possible complication of this organism because this amoeba is potentially pathogenic for immunocompromised patients. Since the amoeba is present in environmental resources, the use of contact lenses should be accompanied by considering proper hygiene.

Introduction

Free-living amoebae (FLA) such as *Acanthamoeba* spp., considered as opportunistic and pathogenic protozoans (1, 2). Among the FLA, *Acanthamoeba* spp. with different genotypes, show a cosmopolitan trend of distribution and are isolated from various environmental and clinical sources (3, 4).

Human infections with amoebic keratitis, fatal granulomatose encephalitis, and cutaneous acanthamoebiasis are the consequences of *Acanthamoeba* infection (1, 3). The development of the disease begins with direct transmission of *Acanthamoeba* to hosts through the oral cavity, respiratory tract, and injured skin (1). The cases of AK often occur in contact lens users with poor hygiene of the lenses which is a major risk factor for AK; in such conditions, even the amoebas isolated from lens solution or the cysts are resistant to disinfection solutions (1, 5). While AK cases have been repeatedly reported from Iran, no cases of *Acanthamoeba* encephalitis have been reported, mainly due to neglecting *Acanthamoeba* as an agent of central nervous system infections. Moreover, AGE is a chronic disease with common symptoms that makes it difficult to be easily diagnosed (4, 6-9).

Several domestic studies found FLA in the oral and nasopharyngeal cavity of immunosuppressed patients, including hemodialysis, cancer, diabetes, lupus, hepatitis, and AIDS patients (10-13). Isolation of FLA from immunocompromised patients can remind the risk of amoebic encephalitis in the country. Up to now, several genotypes of *Acanthamoeba* have been identified based on the 18S rRNA gene (14, 15). The relationship between pathogenicity and genotype has repeatedly been debated, but the results of several studies indicate that the genotype T4 is more associated with amoebic pathogenicity (2, 4, 8). Therefore, studies to identify *Acanthamoeba* in patients´ mucosa and contact lens in close contact with patients, is valuable to prevent the possible disease.

In the present study, the oral and nasopharyngeal cavities of patients plus the contact lenses of those wearing such device were investigated for FLA species.

Materials and Methods

The participants of this study were consciously and voluntarily enter the project. Ethical approval of the study was obtained from the Medical Ethics Committee of Qazvin University of Medical Sciences (IR. QUMS. REC.1398.069).

In total, 251 nasal and oral swabs (including the pharynx and mouth) were collected from patients with diabetes (n=106), AIDS (n=18) and dialysis (n=127) patients. Data on demographic was collected by questionnaires through a face-to-face interview. The specimens were collected during 2019 from the patients referred to the training medical centers and health centers in Qazvin, Iran.

Swabs were obtained using sterile cotton swabs, which were immediately cultured and submitted to the Department of Parasitology and Mycology, School of Medicine, Qazvin University of Medical Sciences. Overall, 27 soft contact lenses were also collected from the participants. Nasal and oral samples were instantly cultured on 1.5% NNA seeded with *Escherichia coli* and transferred to the Department of Parasitology and Mycology at Qazvin Medical School. Contact lenses of the were also cultured on NNA with *E. coli*. Plates were incubated at room temperature and checked for the outgrowth of amoeba every 24 h for 15 to 30 days (8, 13).

The amoebas were collected from the culture plates using sterile PBS, at a pH of 7.2. DNA extraction was done using High Pure polymerase chain reaction (PCR) Template Preparation Kit (Roche, Mannheim, Germany)

according to the manufacturer's protocol. PCR was performed, targeting the region approximately 500 bp in 18S rRNA gene of *Acanthamoeba*, using the primers JDP1 and JDP2. Primers of *Vermamoeba* spp. were NA1, NA2, which amplified partial 18srRNA gene (7, 8). Standard PCR was performed in a 30 µl volume containing a ready-made mixture of Amplicon (Taq DNA Polymerase Master Mix RED, Denmark), template DNA, 0.1 µM of each primer and distilled water. TECHNE thermal cycler (UK) program was performed as follows: 94 °C for 1 min (initial denaturing) then 35 cycles at 94 °C for 35 s, annealing step at 56 °C for *Acanthamoeba* and 50 °C for *Vermamoeba*, 72 °C for 1 min, and 10 min at 72 °C as final extension (7). Sequencing of PCR products was conducted using an automatic ABI3130 sequencer machine (Applied Biosystems, USA). The sequences analyzed against all eukaryotic nucleotide sequences available in the GenBank database. The sequences obtained in our experiments were deposited in the GenBank database under the Accession numbers, MW547527, MZ701918 and MZ701919**.** The phylogenetic tree was constructed using the MEGA7 software; the maximum-likelihood algorithm with Tamura-3 parameter substitution model was applied. Bootstrap analysis was performed based on 1,000 replications. As an outgroup, the sequence of *Balamuthia mandrillaris* was used in the dendrogram (Fig. 1). Osmo-tolerance (0.5 and 1.5 M mannitol) and thermo-tolerance (37 and 40 °C) assay were performed to investigate of the pathogenicity of the isolates. The plates were monitored daily for a week (10, 16).

Data are expressed frequency and percent. Pearson chi-square or Fisher's exact test was used to determine significant differences between proportions. The values $P < 0.05$ were considered statistically significant. Statistical analysis was done using SPSS version 16.0 statisticsal software (SPSS Inc., Chicago, IL, USA).

Results

Out of 251 patients, 18 (7.2%) had AIDS, 106 (42.2%) had diabetes and 127 (50.6%) had renal failure with the history of hemodialysis. The mean age of patients was 58.8 ± 13.9 with a range of 17 to 89 years. Women were the most common. Six patients (2.4%) were addicted to drugs. The most comorbidity disease among patients was hypertension and the lowest was tuberculosis (Table 1). No significant differences observed in infection status and demographic characteristics. The mean duration of the disease among the subjects was 6.9 ± 4.7 with a range of 3 to 30 years.

Table 1: Demographic characteristics of patients in Iran

Variable	***Freq. (%)***
Gender	
Female	137 (54.6)
Male	114 (45.4)
Job	
Self-employment	45 (17.9)
Retired	53 (21.1)
Employed	40 (15.9)
Housekeeper	113 (45.0)
Comorbidity	
Blood Pressure	110 (43.8)
Heart	42 (16.7)
Lipid	75 (29.9)
Eye	29 (11.6)
Hepatitis	8 (3.2)
TB	2 (0.8)

FLA species were identified by morphological features among the patients. The result of sequencing clarified one isolate as *Acanthamoeba* (0.8%) among renal failure patients with 2 years history of hemodialysis treatment, no contamination with *Vermamoeba* was observed by molecular approaches. *Acanthamoeba* belonging to the T3 genotype (labeled as 257D strain) was recovered from a male patient,

aged 65 and the patient had diabetes. Two (7.4%) specimens (labeled as L19 and L25) with *Acanthamoeba* were isolated from keratitis patients. The T3 genotype of *Acanthamoeba* was identified by molecular surveys in both specimens. The patients with amoebic keratitis were two females with soft contact lenses aged between 20-30y. The patient's complaints included eye pain, photophobia, foreign body sensation, redness and tearing. The pathogenicity of the strains was classified as low for all isolates (257D, L19 and L25). The phylogenetic analysis confirmed T3 genotype of the *Acanthamoeba* (Fig. 1).

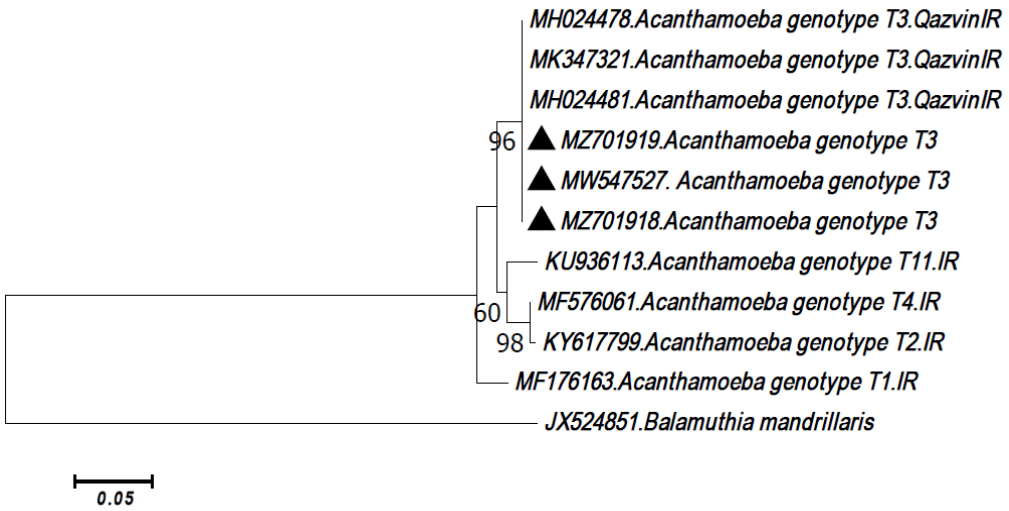

Fig. 1: Phylogenetic relationships among the genotypes of *Acanthamoeba* taxa inferred by ML methods. The tree was designed by the Tamura 3-parameter model as implemented in the MEGA7 software. The *Acanthamoeba* spp., clarified in the present study (▲). Close intra-specific proximity was observed among the isolates of *Acanthamoeba* obtained from the present study, comparable with those previously found in Qazvin province

Discussion

The result of the present study on nasal and oral cavities of the patients, based on morphological characteristics, showed the presence of *Acanthamoeba* and *Vermamoeba* among the specimens, however, the application of more accurate molecular survey only identified the existence of *Acanthamoeba* spp. It seems that the presence of candida yeast, a member of normal flora of mucosa, was the cause of confusion in identification of *Vermamoeba* cyst.

Acanthamoeba has been responsible for fatal diseases such as amoebic encephalitis (1), nevertheless, chronic nature of the disease, long incubation time of the amoeba, and manifestation of common symptoms of encephalitis, could cause the identification of amoeba to be neglected in different countries. Wide range of immune-suppressed patients, such as those with HIV infection, leukemia, organ transplant, periodic hemodialysis or excessive steroid users are considered as susceptible hosts (3).

In a study, disseminated infection of *Acanthamoeba* after hematopoietic stem cell transplant for acute lymphocytic leukemia, was reported (17). Since *Acanthamoeba* is isolated from various environmental niches, such as soil, water and even hydraulic system of a hemodialysis unit, it is quite possible that individuals and patients to easily come into contact with the amoeba (1, 18). Therefore, studies to identify *Acanthamoeba* in patients´ mucosa, hospital water supply, and various devices in close contact with patients, is valuable to prevent the possible disease. However, when the pathogenic protozoans such as *Acan-*

thamoeba and *Naegleria* are isolated from immunocompetent patients, they are considered as healthy carries of the amoeba, but finding the amoeba in immunosuppressed patients is highly important for both prevention and control programs of the disease (19). The result of the present study showed that the patient with chronic renal disease who required hemodialysis was contaminated with a strain of *Acanthamoeba* related to the T3 genotype. The patient had a two-year history of hemodialysis treatment with diabetes background. Molecular approaches clarified the presence of *Acanthamoeba* of T3 genotype in two specimens collected from keratitis patients.

The T3 genotype of *Acanthamoeba* is common in the water sources of Qazvin, so this genotype was highly expected to be found among the patients (20, 21). Also, the phylogenetic tree revealed a close intra-specific proximity among the isolates of *Acanthamoeba* identified in the present study compared to other *Acanthamoeba* genotype T3 isolates collected in Qazvin province. Therefore, they resided the same cluster (Fig. 1). Since contamination by amoeba occurs through environmental resources (1), the results are predictable. Memari et al found *Acanthamoeba* of T3, T4, and T5 genotypes with higher frequency of T4 genotype obtained from nasal swabs of cancer patients in which out of 80 samples, 36 (45 %) plates revealed *Acanthamoeba* trophozoites and double-walled cysts (10). Another study reported the identification of *Acanthamoeba* belonging to the T4 genotype from nasal and oral swabs of HIV patient (22). Also, *Acanthamoeba* of T1 and T4 genotypes, collected from oral cavity, was observed in hemodialysis patients in which 9 (4.8%) out of 187 specimens were positive for FLA (12). Furthermore, the nasal swabs obtained from the hospitalized patients in intensive care units and critical care units of a general hospital as well as the ICU and CCU of a heart hospital, both in Tehran, Iran, led to the identification of *Acanthamoeba* spp., *Vahlkampfiids*, and *Vermamoeba vermiformis* i.e. 4 nasal swap out of 34 specimens was positive for FLA (23).

Azabadi et al showed the existence of *Acanthamoeba* in nasal and oral discharges of HIV patients in the city of Arak, Iran. In the study, 11 out of 53 samples revealed positive results for the presence of *Acanthamoeba* (24). Finally, 63 (98.4%) out of 64 bronchoalveolar lavage (BAL) samples taken from immunocompromised patients in Arak were shown to be contaminated with *Acanthamoeba* (25).

We expected higher FLA contamination rate, but considering the presence of only one positive specimen in the patient was indicative of low incidence of *Acanthamoeba* among the patients in Qazvin. Since the pathogenicity of three isolates was low, it is assumed that FLA is not treated in patients in the study region. Neither the osmo-tolerance test nor the thermo-tolerance assay are considered as gold standard methods to investigate pathogenicity and more reliable methods should be used to test the pathogenicity of *Acanthamoeba*.

Conclusion

The result of our study shows low incidence of *Acanthamoeba* among the patients, but clinicians should be more aware of this lethal disease and consider the possible existence of this organism in their diagnostic approaches as the amoeba is potentially pathogenic for the patients. Contamination of contact lenses with *Acanthamoeba* highlights the need for health promotion in the society, specifically among the contact lens users and high risk people.

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Conflict of interest

The authors declare that there is no conflict of interest.

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