Detection of Free Living Amoeba Infection in Patients with Suspected Central Nervous System and Keratitis Disease in Shiraz, Southern Iran

Mohammad Saleh Bahreini[1], PhD; Mohammad Hossein Motazedian[1], PhD; Shahram Bamdad[2], MD; Mohammad Javad Abbaszadeh Afshar[3], PhD; Qasem Asgari[1], PhD

[1]Department of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
[2]Department of Ophthalmology, Poostchi Eye Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[3]Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

Correspondence:
Qasem Asgari, PhD;
Department of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Science, Shiraz, Iran
Tel/Fax: +98 71 32305291
Email: asgarig@sums.ac.ir
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Abstract

Background: Free Living Amoebas, as opportunistic protozoa, can cause more problems such as meningoencephalitis, encephalitis and keratitis in human being. These protozoa have been isolated from many sources in Iran. This study was undertaken to determine the diseases due to these parasites in the south of Iran.

Methods: In this cross-sectional study, 200 cerebrospinal fluid (CSF) samples and 15 corneal scrapings were collected from patients admitted in clinics of Shiraz city. The samples were examined by light microscopy, cultivation and molecular methods. Phylogenetic relationship was also conducted among the sequences and various *Acanthamoeba spp.* based on nucleotide sequences in NCBI GenBank.

Results: No infection in CSF samples was seen, while one patient suspected with keratitis was positive to *Acanthamoeba sp.* infection only by PCR. Using Sequencing technique and Phylogenetic tree, the genotype of the parasite was demonstrated T4. This sample belonged to a 26 year old woman who used a contact lens.

Conclusion: Our results indicate that it is necessary to pay attention to the complexity of the free living amoeba infections, especially in soft contact lens wearers. Also, PCR as an appropriate method in diagnosis is recommended for the detection of free living amoebae. However, it is unavoidable to suspect these protozoa as an infectious agent in patients with central nervous system infection due to increased immunodeficiency disorders.

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Keywords: Free living amoeba, Cerebrospinal fluid, Keratitis, Iran

Introduction

Free living amoebas (FLA), as opportunistic parasites, can cause a variety of diseases in humans and animals.[1] These protozoans thrive in water, soil, air and even dental units, eyewash solutions, contact lenses, and dialysis units.[2] The amphizoic traits of these parasites help them to live in *a wide range of environmental conditions* and infect humans as pathogens.[3] In essence, sometimes the phagocyted pathogenic bacteria and fungi can been conserved in their cytoplasm and transmitted to human.[4] Four forefront genera of FLA are known as ubiquitous human pathogens: *Acanthamoeba, Balamuthia, Hartmannella* and *Naegleria*.[5]

Evidence showed that the FLA hasd a superior affinity to the nervous system, skin and eye. Central nervous system disorders such as granulomatous amoebic encephalitis (GAE) are usually related to *Acanthamoeba spp.* and *Balamuthia mandrillaris* infection, while *Naegleria fowleri* is considered as an etiological agent in primary amoebic meningoencephalitis (PAM). Moreover,

Acanthamoeba spp. can cause *Acanthamoeba* Keratitis (AK) and complexity in the skin and lungs involvement.[6] Several different genotypes in *Acanthamoeba spp.* cause an insidious and chronic disease, granulomatous amebic encephalitis (GAE), principally in persons infected with HIV/AIDS.[7] Amoebic keratitis, another manifestation of *Acanthamoeba spp.* Infection, has increased in soft contact lens wearers around the world.[8]

Considering the high prevalence of *Acanthamoeba spp.* and other FLA in different environmental water sources of Shiraz, Southern, Iran[9, 10] and *lack of data* about the epidemiological status of these etiological agents, this study aimed to find out the presence of these parasites in clinical samples of suspected individuals.

Materials and Methods

Ethical Statement

This study was apprved by the Ethics Committee of Shiraz University of Medical Sciences (Ethical code: IR.SUMS.REC.1394.S131). Written informed consent was obtained from all patients.

Study Area

Shiraz, capital of Fars province, is the sixth densely populated city in Islamic Republic of Iran. It has a moderate climate and an average height of 5200 ft. above the sea level (Figure 1).

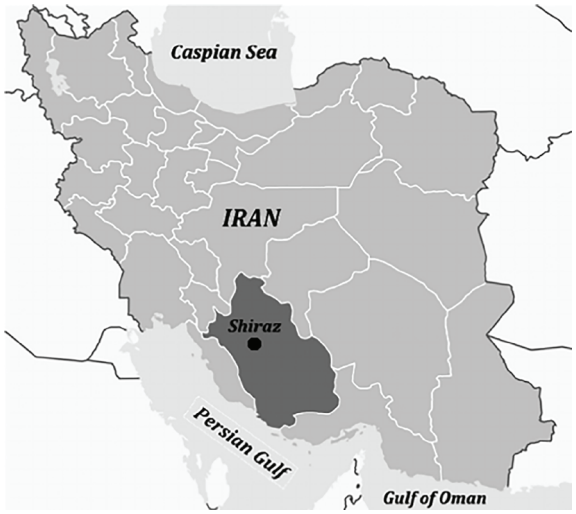

Figure 1: Map of Iran, showing Shiraz city in Fars province, Southern Iran

Sampling

From 2015 to 2017, two hundred CSF samples were collected from the patients suspected with encephalitis and meningoencephalitis from hospitals of Shiraz, southern Iran. The samples were taken by the physician and were poured into 500µL micro-tubes. 15 samples of the corneal scrapings were also taken from patients suspected with AK from Shiraz ophthalmology centers and were dispensed into micro-tubes containing normal saline. The samples were transferred to the department of Parasitology and Mycology of Shiraz University of Medical Sciences.

Direct Examination (Light Microscopy)

To increase the chance of detection of FLA, we centrifuged all the samples for 10 minutes at 1500 rpm. After centrifugation, the supernatant was discarded and the pellet was observed by light microscope.

Culture

After wet analysis, the pellets were placed centrally onto a 90 mm 1.5% non-nutrient (NN) agar plate covered with a lawn (100 µL) of a 24 h old culture of *Escherichia coli* (ATCC 25922). The plates were sealed with Parafilm, incubated at 25°C, 35°C and 45°C and screened daily for FLA by invert microscopy. The samples should be observed for up to 1 month to reliably prove a negative result.

Molecular Analysis

DNA was purified from all samples by DNA extraction kit (FAVORGEN Company, Taiwan).

PCR amplication was based on 18srRNA by JDP1: 5- GGCCCAGATCGTTTAC CGTGAA-3 and JDP2: 5- TCTCACAAGCTGCTAGGGAGTCA-3 primers which were specified for *Acanthamoeba spp.* That yielded 423-551 bp fragments.[9]

ITS1: 5'- GAACCTGCGTAGGGATCATTT-3' and ITS2: 5'-TTTCTTTTCCTCCC CTTATTA-3 primers were used for *Naegleria fowleri* which were specified for detection of all its species. This primer yielded 400-450 bp fragments.[11]

The PCR reactions for *Acanthamoeba spp.* were based on 25µL total volume which contained: mastermix 12.5µl, primer (F&R) 1 µL, DNA 1µL, and distilled water 8.5 µL. In addition, the PCR reactions for *N. fowleri* was performed at 25µL total volume which include mastermix12.5µl, primer (F&R) 1 µL, DNA1 µL, and distilled water 8.5 µL.

Thermo-cycler program for JDP and ITS was a primary incubation of 94°C for 3 minutes and 33 frequency cycles at 94°C for 45s, annealing at 56°C for 35s, and extension at 72°C for 1 min with final extension 0f 72°C for 5 min. PCR products were detected under UV in 1.5 % agarose gel using gel red staining.

Sequencing, Alignment, and Phylogenetic Analysis

For confirmation of the PCR product of positive sample, we used the sequencing technique through the sequencing service of Macrogen Genomics Laboratories (Macrogen), similarity of the obtained

Table 1: The population of patients suspected with encephalitis, meningoencephalitis and AK

Sex	Patients suspected to encephalitis or meningoencephalitis	Patients suspected to AK
Male	123	4
Female	77	11
Total	200	15

Table 2: Frequency of contact lens in patients suspected with AK

Sex	Use contact lens	Don't use contact lens
Male	2	2
Female	9	2
Total	11	4

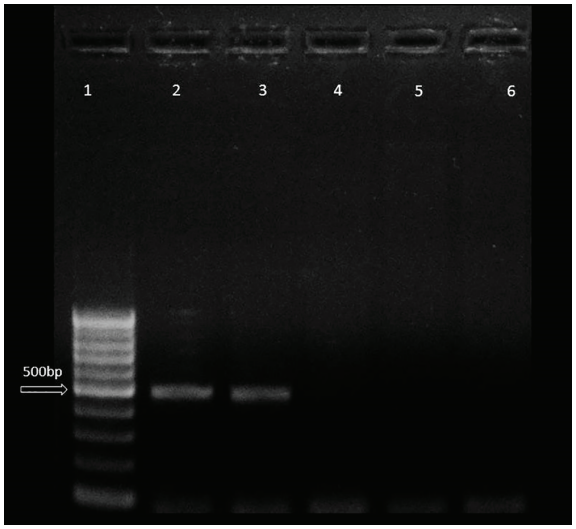

Figure 2: Gel electrophoresis of PCR-based products of *Acanthamoeba spp* using JDP primers. Samples prepared from the corneal scraping samples (Lanes 3–6), positive control (500 bp) (Lane 2) and the bands corresponding to the molecular weight marker (Lane 1)

sequence with sequences of the GenBank was performed by using BLAST option from the NCBI (National Center for Biotechnology Information) site. The strain of positive sample was recognized based on the highest homology and coverage.

The sequence was edited and aligned with the software BioEdit, version 7.2.5.[12] Alignment was done with data related to *Acanthamoeba* species from Iran and other countries in GenBank. Gaps were excluded from distance calculations using the pair-wise deletion option in the phylogenetic analysis computer program MEGA7. Phylogenetic tree was also constructed via the Kimura 2-parameter method of the neighbor-joining mode in MEGA7.[13]

Results

Of the 200 CSF samples of the patients suspected with encephalitis and meningoencephlitis, 123 were male (61%) and 77 were female (39%). 15 samples were gathered from individuals suspected with AK, of whom 11 were female (73%) and 4 were male (27%) (Table 1). Out of this number, 2 males and 9 females had used contact lens (Table 2).

Wet analysis of CSF and coronal scraping samples were negative for presence of FLA cyst and trophozoite. Also, the result of culture at different temperatures showed that FLA growth were absent for all samples.

The PCR did not detect *Naegleria fowleri* and *Acanthamoeba spp*. DNAs in the CSF samples, while it demonstrated the existence of *Acanthamoeba spp.* in a corneal scraping sample (Figure 2).

This sample belonged to a 26 year old woman with a positive history of using contact lens. The sequencing results and comparing them with Genbank showed most similarity to T4 genotype and 100% identity to *Acanthamoeba* species isolated from keratitis patients, Iran (accession no. MF576064). Nucleotide sequence was deposited in the GenBank database with MK 356382 accession numbers. Phylogenetic relationship was also conducted among the sequences and various *Acanthamoeba spp* based on nucleotide sequences in GenBank. The sequences of AK patients clustered with previous T4 genotype isolates in a common clade, most of which were obtained from AK patients. Also, the *Acanthamoeba spp.* isolated from tap water in Shiraz, Iran attached to it as sister clade (Figure 3).

Discussion

Attention to FLA has increased due to increasing populations of immunocompromised patients and the increasing number of contact lens wearers.[14] Also, broad distribution of these parasites in medical instrumentation, air conditioners, windows, floors, sinks, water taps, bathrooms, and showers are other reasons which increase the risk of FLA infection.[15]

In our study, using light microscopy, culture and PCR methods, no *Naegleria fowleri and Acanthamoeba spp* infection was detected in 200 CSF samples from patients suspected with meningoencephalitis and encephalitis.

The first case of primary amoebic meningoencephalitis from Iran was reported in a 6-month-old boy with a history of washing his

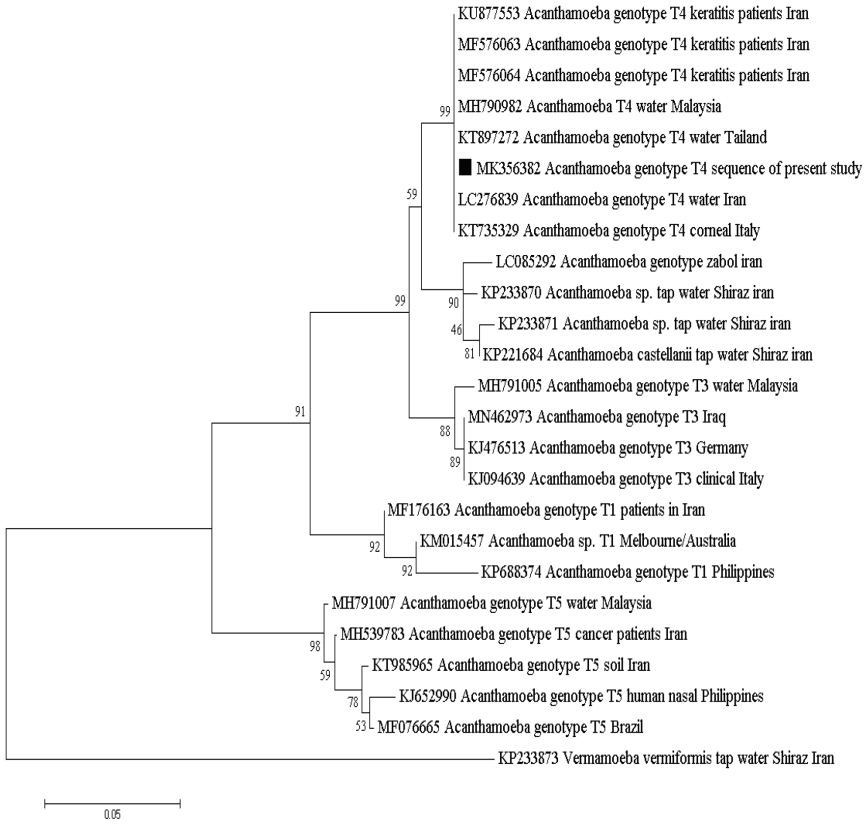

Figure 3: Phylogenetic relationship among various *Acanthamoeba* species to each other as inferred by neighbor-joining tree based on GenBank. Numbers on branches are percentage bootstrap values of 1,000 replicates. The evolutionary distances between the sequences were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The scale bar indicates an evolutionary distance of 0.05 nucleotides per position in the sequence. The reference sequences accession numbers are inserted. Evolutionary analyses were conducted in MEGA-7.

nasal passages with contaminated tap water without presence of bacteria and fungi on the culture and stain and imaging findings; infection to *Naegleria fowleri* was confirmed by wet analysis and culture in NNA media.[16]

Other studies in recent years have indicated that the presence of this disease in the world is rare and are case reported.[17, 18] However, *Shakoor et al.* in 2011 revealed that 13 cases of *Naegleria fowleri* infection were reported in Pakistan as the neighbor country.[19]

PAM, caused by *Naegleria fowleri,* is related with freshwater swimming and swimming pools and water activities.[20] Several cases of infection have also been reported in Muslim' people during ablution that involves taking water into the nostrils.[21] High temperature tolerance and life in warm water are the features of this parasite.[22]

Unfortunately, the prognosis of the disease is poor and its process is rapid; in this respect, it is important to consider amebic trophozoites in CSF wet mounting for early diagnosis and treatment.[23]

GAE is another central nervous system disease due to amoeba that is caused by *Acanthamoeba spp.* and *Balamuthia mandrillaris*.[24] There is no evidence of granulomatous amoebic encephalitis (GAE) in Iran yet;[25] however, by the use of wet analysis and culture, *Acanthamoeba* infection was reported in a five-year-old immunocompetent girl with meningoencephalitis manifestation. This study showed that *Acanthamoeba* not only primarily involves the brain, but also can *involve* the meninges.[26]

Studies have shown that the rate of the disease is directly related to the environment contamination.[27] Contact with soil is a major route to the infection. This indicates that contamination control plays the main role in the disease prevention.[28] Timely diagnosis can help the patients to be better treated and survive.[29]

In our study, using wet sample, culture in NNA and molecular methods, we detected *Acanthamoeba* DNA in a *corneal scraping sample* of 15 patients suspected with Amoebic Keratitis. The sample belonged to female patients who used contact lens.

Recent records *have* suggested a rising rate of AK seen especially in contact lenses wearer.[30] Among 142 patients referred to educational hospitals of Tehran University of Medical Sciences, Iran, 49 (34.5%) had Amoebic Keratitis that 44 patients (89.79%) were contact lens wearers.[31]

Similarly, a high incidence of the disease was reported in contact lens wearer in Egypt. In their

study, using contact lens was introduced as a risk factor in the disease transmission, and molecular method was presented as a sensitive method.[32]

In Hong Kong, in a 10 year period, fifteen eyes of 13 patients were studied for AK. 12 out of 13 patients (92.3%) were contact lens wearers. For diagnosis of AK, we used culture on to non-nutrient agar and also to confocal microscopy.[33]

Wet analysis, culture on NNA, and molecular methods are the routine and appropriate methods for AK diagnosis since the low number of parasites in some samples may decrease the chance of detection in direct method. Moreover, corneal scraping sample from patients treated by chemotherapy may reduce the likelihood of growth in NNA media. Studies showed that molecular methods were the most suitable and accurate approach in AK determination.

Based on the molecular analysis of small subunit of 18s rRNA gene, 17 Isotopes were identified within the *Acanthamoeba* genus.[7, 34] In our study, the AK *isolate* possessed the *most similar sequence to those* of T4 genotype.

T4 is the common genotype of *Acanthamoeba spp*. in AK.[35] Also, genotype T3,[36] genotype T6[37] and genotype T11[38] have been reported from keratitis patients. Studies have shown that T3, T4, and T5 are found in clinical samples and T4 is the highest contribution in both clinical and environmental samples.[36, 39] Other studies confirmed that T2, T4, T11 were identified to afford keratitis and genotypes T1, T4, T10, and T12 are in charge of GAE.[40] This study was conducted for the first time in patients suspected with FLA infection and admitted to clinical centers of Shiraz, Southern, Iran .We reported a case of AK infection in a woman that was contact lens wearer. However, there was no case of GAE and PAM in these patients. These results indicate the need for attention to the complexity of the free living amoeba infections and *Acanthamoeba* keratitis should be considered, especially in lenses wearers Moreover, molecular detection, along with other methods, can also be helpful as an appropriate method for the detection of free living amoebae. Also, due to an increase in the patients with immune deficiency, consideration of FLA as a potential factor should be put on the health programs.

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Conflict of Interest: None declared.

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