

Isolation and Genotyping of Acanthamoeba from Soil Samples in Markazi Province, Iran

Mehri Meighani¹, Zahra Eslamirad^{2*}, Reza Hajihosseini³, Azam Ahmadi⁴, Sassan Saki⁵

¹Department of Biology, School of Basic Sciences, Islamic Azad University, Arak Branch, Arak, Iran; ²Molecular and Medicine Research Center, Department of Medical Parasitology and Mycology, School of Medicine, Arak University of Medical Sciences, Arak, Iran; ³Department of Medical Parasitology and Mycology, School of Medicine, Arak University of Medical Sciences, Arak, Iran; ⁴Infectious Disease Research Center (IDRC), Arak University of Medical Sciences, Arak, Iran; ⁵Department of Medical Laboratory Sciences, Faculty of Medical Sciences, Islamic Azad University, Arak Branch, Arak, Iran

Abstract

Citation: Meighani M, Eslamirad Z, Hajihosseini R, Ahmadi A, Saki S. Isolation and Genotyping of Acanthamoeba from Soil Samples in Markazi Province, Iran. Open Access Maced J Med Sci. <https://doi.org/10.3889/oamjms.2018.454>

Keywords: Acanthamoeba; Soil; PCR; Molecular method; Genotype

***Correspondence:** Zahra Eslamirad. Molecular and Medicine Research Center, Department of Medical Parasitology and Mycology, School of Medicine, Arak University of Medical Sciences, Arak, Iran. E-mail: z.eslami64@gmail.com

Received: 28-Aug-2018; **Revised:** 25-Oct-2018; **Accepted:** 27-Oct-2018; **Online first:** 16-Dec-2018

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Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

AIM: A previous study confirmed the contamination of water sources with this parasite in Arak, Markazi Province, Iran. The current study investigated soil sources and determined the predominant genotype of Acanthamoeba in this region of Iran.

MATERIAL AND METHODS: Forty-eight soil samples, collected from different regions of Arak, Markazi province, Iran, were evaluated in this study. The samples were processed and identified by culturing on a specific medium, performing PCR assay, and sequencing the PCR products. Finally, using the NCBI database, the genotypes were determined.

RESULTS: Of 48 soil samples, 33.3% and 31.25% were contaminated with Acanthamoeba according to the culture and molecular assays, respectively. The majority of these isolates belonged to the T4, T5 and T6 genotypes of Acanthamoeba.

CONCLUSION: The genotypes of most isolates from soil samples in Arak similar to other regions of Iran belong to T4 genotype of this parasite. New sequence accession numbers include MG066681 and MG298785-MG298794.

Introduction

Acanthamoeba is described as a free-living amoeba with a widespread distribution in the water and soil of various regions [1]. Host infection occurs through the entry of parasite cysts into the nose and eyes. Depending on the host conditions, this can lead to various diseases, such as granulomatous amoebic encephalitis (GAE) or keratitis [2]. Water and soil are important reservoirs of this parasite, and to date, parasites have been isolated from different environmental sources in Iran [3]. For instance,

46.25% of environmental samples collected from Tehran were contaminated with this parasite; also, all soil samples were contaminated [4]. Also, 71.6% and 26% of water and soil samples from south of Iran were contaminated with Acanthamoeba, respectively [5].

A limited number of studies have examined water sources in different regions of Iran. In Isfahan, 45.16% of water sources were contaminated with this parasite, and a higher prevalence was found in environmental water sources in comparison with tap water [6]. Similar results have been reported from

West Azerbaijan Province (Northwest of Iran) [7] and Shiraz [8]. Moreover, 70.3% of surface water samples were contaminated with *Acanthamoeba* in North of Iran (Gilan Province) [9].

Morphological and molecular studies have revealed that the T4 strain is the predominant *Acanthamoeba* genotype in the environmental sources of Iran [3]. Considering the high prevalence of pathogenic *Acanthamoeba* strains in the environment, it is recognised as a dangerous organism. Also, it seems that fine airborne dust plays a role in the dissemination and transmission of this parasite [10]. The contamination of water sources with *Acanthamoeba* was confirmed by a previous study conducted in Markazi Province [11]; however, there is no information regarding the prevalence and isolates of this parasite in the soil of Markazi Province.

Therefore, this study aimed to assess the contamination status and genotypes of this parasite in soil samples from this region.

Material and Methods

Forty-eight soil samples were collected from Arak, capital of Markazi Province, located on the crossroad of northern, eastern, southern, and western provinces of Iran (Arak, Iran; 34°00' N 49°40' E; Figure 1). Because of the presence of various industries, this city has many immigrants. In this study, the soil of parks and gardens were collected for analysis. For this purpose, approximately 50 g of soil was collected in sterile bags and transferred to the Parasitology and Mycology Laboratory, Arak University of Medical Sciences.



Figure 1: Geographic Location of Arak city in Iran

The soil samples were prepared using a 250- μ m sieve and then a 0.45- μ m nitrocellulose filter, as described in Figure 2. The nitrocellulose filter was transferred to a non-nutrient agar (NNA) plate, coated with *E. coli* (killed) at a temperature of 28°C. After monitoring the plates for four weeks, the surface of positive cultures was rinsed with sterile Page's saline. The parasites were centrifuged for 5 minutes at 1500 \times g after collecting and washing them with phosphate buffer. These samples were used for molecular analysis.

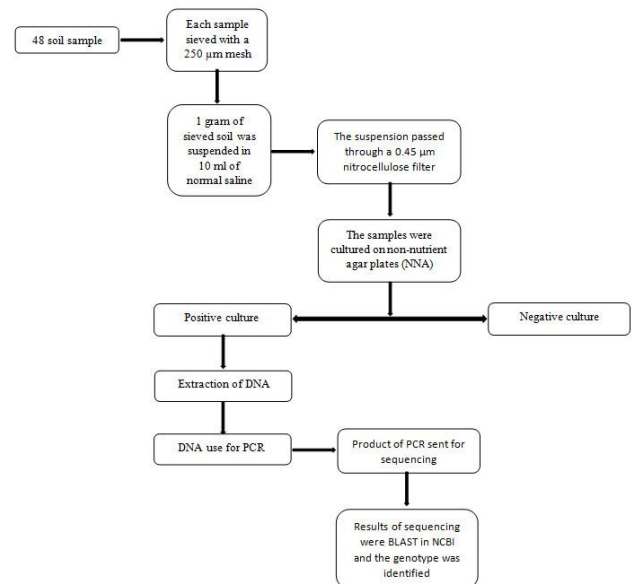


Figure 2: Preparation and testing steps on soil samples

According to the literature, the phenol-chloroform method was used for DNA extraction [12, 13]. For PCR amplification, genus-specific primers were used. To amplify a nearly 500-bp fragment of *Acanthamoeba*-specific 18S ribosomal DNA, reverse primer JDP2 (5'- TCTACAAGCTGCTAGGGGAG TCA-3') and forward primer JDP1 (5'- GGCCAGATCGTTTACCGTGAA-3') were used [14].

A Master Mix Kit (CinnaGen Co.) was used to optimise the PCR reactions. A final volume of 50 μ L was used for amplification in an Eppendorf thermocycler with incubation at 95°C for 3 minutes, followed by 35 cycles at 94°C for 45 seconds, at 55°C for 45 seconds, at 72°C for 45 seconds, and incubation at 72°C for 10 minutes. Using electrophoresis on 1.5% agarose gel, the products were assessed and then visualised under ultraviolet light. After sequencing the PCR products from positive samples, homology analysis was conducted through comparison of the sequences with *Acanthamoeba* DNA sequences available in the GenBank. The sequences generated in the current study were submitted to the GenBank database.

Results

Sixteen out of 48 soil samples contained *Acanthamoeba* isolates according to the culturing method (16 samples, 33.3%). Molecular evaluation of the positive samples confirmed 15 (31.25%) isolates.

The eleven sequences (11 sequences) reported in the current study were submitted to the GenBank (accession numbers, MG066681 and MG298785-MG298794). Using the BLAST tool,

homology of sequences was compared with the sequences available in the GenBank. The specificity of the detected isolates is presented in Table 1.

Table 1: Comparison of Acanthamoeba spp. isolates from Arak soil samples with available strains found in the GenBank database

Isolate sequence	Isolate Accession number	Name of Isolate	Genotype	Name of similar isolates BLAST	Accession number of similar sequence (Genotype)	Region of origin	Reference
AGGTGAATTTCTGGATTTATG AAAGATTAACTTCTGCGAAGCC ATTTGCCAGAGGATTTTGATT AATCAAGAACGAAAGTTAGGG GATCGAAGACGATCAGATACC GTCGTAGTCTTAACCATAAACG ATGCCGACGAGGATTTGAGG ACGTTGAATCAAAACACACCC ATGATAATTCAGTATATGGCC AGTCAAATGGTTGTA TATTGTTTGTACATG	MG298785	Acs2	ND	Arc-T01 Arc-B08 Arc-NB03 BYB 2017-2 Arc-V13 Arc-E08 Arc-S07 RG-HD186	MF470298.1 (ND) MF470259.1 (ND) MF470254.1 (ND) MF113385.1 (T2) MF470280.1 (ND) MF470243.1 (ND) MF470242.1 (ND) LC184519.1 (ND)	United Kingdom United Kingdom United Kingdom USA - - -	Asif, Unpublished Asif, Unpublished Asif, Unpublished Martin-Perez et al.2017[15] Asif, Unpublished Asif, Unpublished Asif, Unpublished Rastri et al.2016, Unpublished
GGCCGAGATCGTTTACCCTGGA AAAAATTAGAGTGTTCAAAGCC GGGAGATTCAATTTCTGCCAC CGAATACATTAGCATGGGATAA TGGAAATAGGACCTGTCTCCTC ATCTTCAGTTGGTTAAGCTTGA GGGATCAAGGCTAATGATTAAT AGGGATAGTTGGGGGCAT	MG066681	AcAS4	T4	SSH40	KU885380.1 (T4)	Spain	Reyes-Balle et al. 2016[20]
CTGGGGCCGAGATCGTTTACC GTGAAAAATTAGAGTGTTCAA AGGAGCGAGATTCATTTGTTG CCACCGAATACATTAGCATGGG ATAATGAAATAGGACCTGTCTC TCCTTTTTCAGTTGGTTAATGA CAGAGGATCAAGGTAATGA TTAATAGGATAGTTG	MG298786	Acs5	T6	CRIB-25	EU273827.1 (T6)	France	Thomas (2008) [21]
GGGGTTGGCCGAGATCGTTTAC CCGTGAAAAATTAGAGTGTTC AAAGCAGGACGATCCAATTTTC TGCCACCGAATACATTAGCATG GGATAATGAAATAGGACCTGTCT CCTCCTATTTTCAGTTGGTTTGG GCACCGGAGGACTAGGGTAA TGATTAATAGGATAGTTGGGG GCATTAATA	MG298787	Acs7	ND	EGM3	EF050490.1 (ND)	India	Anand et al. Unpublished
AAAATTAGAGTGTTCAAAGCAG GCAGATTCAATTTCTGCCACC GAATACATTAGCATGGGATAA GGAATAGGACCTGTCTCCTCCT CTTTGGAGTTGGTTAATTAAGCT GTGAGGATCAAGGTAATGATTA ATAGGGATAGTTGGGGGCATT A	MG298788	Acs9	ND	Arc-SK07 Arc-NB06 T2/6C T5-1 250GILLE	MF470308.1 (ND) MF470264.1 (ND) J066966.1 (ND) EF378672.1 (T5) GQ087290.1 (T2)	- - USA - France	Asif, Unpublished Asif, Unpublished Crary, Unpublished Wildschutte et al. 2007 [23] Year et al. 2007 [24]
ATTTTGGCCGAGATCGTTTACC GTGAAAAATTAGAGTGTTCAA AGCGGGCAGATTTTCTCCG CACCGAATACATTAGCATGGGA TAATGGAATAGGACCTGTCTCCT CCTATTTTCAGTTGGTTTGG ACAGCGAGGTTATATCAGGGTA ATGATTAATAGGATAGTTGGG GGCATTAAAT	MG298789	Acs10	T5	FL1	J0418506.1 (T5)	Brazil	Ota et al. (2012) [25]
GGCCGAGATCGTTTACCCTGGA AAAAATTAGAGTGTTCAAAGCGG GCAGATTTTCTGCCACCG AATACATTAGCATGGGATAA GAATAGGACCTGTCTCCTCCTAT TTTCAGTTGGTTTGGTTACAGC GAGGTTATCAGGTAATGAT TAATAGGATAGTTGGGGCA TTA	MG298790	Acs11	ND	AG-2012 clone AR551	J0678613.1 (ND)	Spain	Garcia et al. (2013) [26]
TGAGATGGCCGAGATCGTTTACC CGTGAAAAAATTAGAGTGTTC AAGCAGGACGATCCAATTTCTG GCCACCGAATACATTAGCATGG GATAATGGAATAGGACCTGTCTC CTCCTATTTTCAGTTGGTTTGG GCACCGGAGGACTAGGGTAA TGATTAATAGGATAGTTGGGG GCATTAAT	MG298791	Acs12	T4	JSS-2 JWS-37 GATAATGGAATAGGACCTGTCTC CTCCTATTTTCAGTTGGTTTGG GCACCGGAGGACTAGGGTAA TGATTAATAGGATAGTTGGGG GCATTAAT	KM189416.1 (T4) KM189412.1 (T4) KM189408.1 (T4)	Jamaica Jamaica Jamaica	Todd CD. (2015) [27] Todd CD. (2015) [27] Todd CD. (2015) [27]
TTTTTGGCCGAGATCGTTTACC GTGAAAAATTAGAGTGTTCAA AGCAGGACGATCCAATTTCTG CCACCGAATACATTAGCATGGG ATAATGGAATAGGACCTGTCTC TCCTATTTTCAGTTGGTTTGG CAGCGGAGGACTAGGGTAA GATTAATAGGATAGTTGGGG GCATTAATA	MG298792	Acs13	T4	A29	KT934544.1 (T4)	Venezuela	Wagner (2015) [28]
ATCGTTTACCCTGAAAAAATTA GAGTGTTCAAAGCGGACAGAA ACTTTTCTGCCACCGAATAC ATTAGCATGGGATAATGGA GACCTGACCTCCTATTTTGA GTTGGTTTGGTTTACAGCGAGG TTTCATCAGGTAATGATTAATA GGGATAGTTGGGGGCATTAA	MG298793	Acs14	T5	P7 JWS-26	J0268238.1 (T5) KM189414.1(T5)	Brazil Jamaica	Alves Dde S. (2012) [29] Todd CD. (2015) [27]
TGAAAAAATTAGAGGTTTCAA AGCAGGACGATTCATTTCTG CCACCGAATACATTAGCATGG ATAATGGAATAGGACCTGTCTC TCCTATTTTCAGTTGGTTTGG CAGCGGAGGACTAGGGTAA GATTAATAGGATAGTTGGGG GCA	MG298794	Acs16	T4	SSH40	KU885380.1 (T4)	Spain	Reyes-Balle M. (2016) [20]

ND: not determined.

The genotyping study of these 11 positive specimens showed that 4 (36.4%), 2 (18.2%) and one (9.1%) sequence belonged to T4, T5 and T6

genotypes of Acanthamoeba, respectively. Phylogenetic tree with the neighbor-joining method was shown in Figure 3.

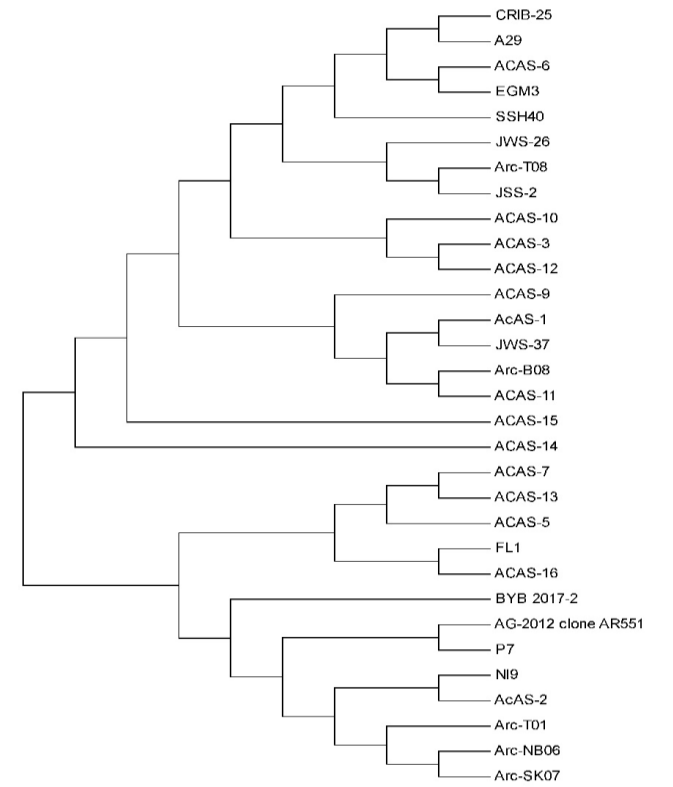


Figure 3: Phylogenetic tree constructed with the neighbour-joining tree using nucleotide sequences of DF3 region of the 18S rRNA gene by MEGA5 software

Discussion

Presence of Acanthamoeba spp. in natural resources of Iran has been confirmed in several studies. This organism is involved in dangerous infections of the nervous system and eyes [3]. The present study showed that 33.3% of soil samples from different parts of Arak were contaminated with free-living amoeba, and molecular examination confirmed that 31.25% of these contaminants were related to Acanthamoeba. Since previous studies have shown that water sources in Arak are contaminated with this parasite, the current results were not unexpected. However, the rate of soil contamination and the genotypes of the identified parasites should also be taken into consideration. In this study, seven out of eleven molecular-positive samples were contaminated with T4, T5 and T6 Acanthamoeba genotypes, while genotype of others cases was not determined.

The geographic location and climatic conditions of Markazi province have exposed it to the phenomenon of fine airborne dust. Airborne dust is a source of many microorganisms and has the potential to transfer Acanthamoeba. Therefore, identification of

pathogenic microorganisms that can be transmitted by soil and dust is important for proper planning and prevention of their spread. Strains of *Acanthamoeba* have been identified and reported in environmental sources from some regions in Iran. In the majority of these reports, the most prevalent *Acanthamoeba* genotype was T4 in water sources, comprising 62.96%, 91.7%, 83.3%, and 71.6% of water source samples from Shiraz [8], Tehran [30], Mazandaran [31], and Ahvaz [5], respectively. Also, the results of a systematic review showed that *Acanthamoeba* genotypes T4, T5, and T2 comprised 39%, 17%, and 16% of *Acanthamoeba* in water sources of Iran, respectively [32].

Some similar studies have examined soil and dust in Iran; T4 was the most common genotype of this parasite in soil and dust specimens in our country. Rezaeian et al., (2008) reported *Acanthamoeba* contamination in 100% of soil samples and 45.9% of dust samples; however, they did not investigate the parasite genotypes [4]. Niyiyati and colleagues (2009) identified the first case of pathogenic genotype *Acanthamoeba* in dust samples collected from hospital wards of Iran. The isolated strains were related to genotypes T4, T5, and T11 (84.6%, 7.6%, and 7.6%, respectively) [10]. In another study conducted in South of Iran, three genotypes of *Acanthamoeba*, T2, T5, and T4, were isolated from soil, and T4 (86.6%) was the predominant genotype [5]. Another study demonstrated that 17.3% of soil samples were molecularly positive for T4 genotype *Acanthamoeba* [33]. Moreover, the soil samples in East Azerbaijan were contaminated with T3, T4, T5, and T11 genotypes of *Acanthamoeba* [34].

Comparison of genotypes obtained in the current study with other studies from Iran indicates that the genotypes of most isolates from soil samples belong to the unclassified group in Arak. All of these genotypes have been reported in environmental samples from other parts of the world, whereas T4 genotype was dominant in other regions of Iran. Future research should determine the causes of genetic differences between the isolates in our study and other research in Iran.

The results of this study confirmed that soil from parks and gardens has the potential for transgenic transmission of *Acanthamoeba* to humans. Therefore, individuals, especially children and immunocompromised people, are more likely to develop parasitic infections.

Acknowledgement

This study was done as a MSPH thesis of Islamic Azad University (Mrs. Mehri Mighanee) and was approved by the Ethics Committee of Arak

University of Medical Sciences (IR.ARAKMU.REC.1395.178). The authors wish to thank the deputy of research at Arak University of Medical Sciences for their support.

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