

# DETECTION OF *ACANTHAMOEBA* SPP. FROM DUST PHENOMENON IN ILAM PROVINCE, WEST IRAN

REZA SABERI<sup>1,2</sup>, AZAR NAJAFI<sup>3,4</sup> and RAZI NASERIFAR<sup>3\*</sup>

<sup>1</sup>Department of Parasitology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

<sup>2</sup>Student Research Committee, Mazandaran University of Medical Science, Sari, Iran

<sup>3</sup>Department of Parasitology, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran

<sup>4</sup>Student Research Committee, Ilam University of Medical Sciences, Ilam, Iran

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In recent years, increasing dust phenomenon in the west of Iran has led to social, economic, and health concerns. This study aimed to represent the existence and genotyping of *Acanthamoeba* spp. in dust phenomenon in Ilam Province, Iran. In this study, 55 dust samples were collected and by targeting the diagnostic fragment 3 region of the 18S rRNA gene, the genotypes were determined. Utilizing the tolerance ability test, the pathogenic potential of all positive isolates was also recognized. Eighteen samples of *Acanthamoeba* (32.7%) were detected in the sampling areas. According to sequencing analysis, the isolates related to T4 (77.7%) and T2 (22.3%) genotypes were reported. It was revealed by thermo- and osmotolerance tests in which six strains are extremely pathogenic. To our knowledge, the pathogenic *Acanthamoeba* was potentially isolated initially from dust phenomenon in Ilam Province. Thus, these strains are probably highly virulent, and dusts are possible sources of *Acanthamoeba* infection in humans.

**Keywords:** *Acanthamoeba*, genotyping, dust, Ilam, Iran

## Introduction

*Acanthamoeba* spp. are free-living amoebae dispersed in different ecological environments and isolated from soil, air, dust, recreational and mineral water, and sewage samples [1]. *Acanthamoeba* spp. include two steps of the trophozoite and the cyst in their life cycle [2]. Trophozoites are slender, with spine-like processes known as acanthopodia, with the diameter of 10–50 µm and they are

\*Corresponding author; E-mail: [razinaserifar@yahoo.com](mailto:razinaserifar@yahoo.com)

divided by binary fission. Cysts are wrinkled, double-walled, star-shaped, hexagonal, polygonal, or spherical with the diameter of about 20–25  $\mu\text{m}$  [2, 3].

Several strains of *Acanthamoeba* are the causative agents of *Acanthamoeba* keratitis (AK), a painful sight-threatening disease of the eyes [4]. They are also a causative agent of granulomatous amoebic encephalitis, which is a severe infection of the central nervous system in immunocompromised people [5]. In addition, these amoebae can act as carrier of pathogenic microorganisms such as bacteria and viruses. Bacteria could remain in amoebae and develop the high virulence and resistance to antibiotic. The results of the previous studies reported a high-level resistance of *Legionella pneumophila* within *Acanthamoeba polyphaga* to disinfection and antimicrobials [6, 7].

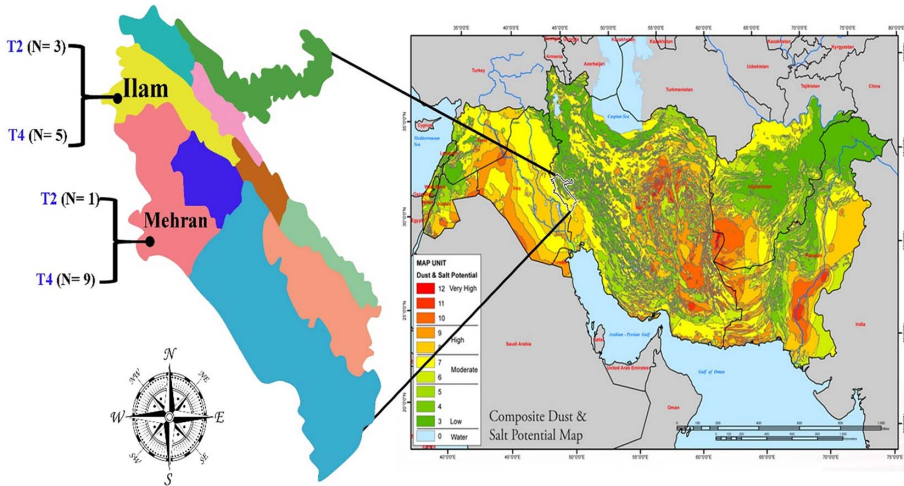
To date, molecular classification of *Acanthamoeba* genus based on the 18S ribosomal RNA sequence has described 21 genotypes (T1–T21) [8]. Numerous *Acanthamoeba* genotypes T4 have been reported in literature as common genotype in the environmental and clinical samples and the causative agent of different diseases [9]. Other pathogenic genotypes including T2, T3, T5, T6, T10, T11, T12, T15, and T18 were identified [10]. Khan et al. provided a simple plating assay to differentiate pathogenic isolates from the non-pathogenic. In the mentioned study, amoeba with ability to grow at high temperature and osmolarity seems to be pathogenic amoeba [11].

The dust phenomenon is one of the risks in nature, i.e., climatic–atmospheric disasters, that has caused a major concern in recent years [12]. The dust phenomena exist highly in the arid and semi-arid areas. In recent decade, Ilam Province exposed to dust phenomenon throughout the year and this phenomenon has been in higher severity and length [13]. To our knowledge, this is the first study to assess the presence of *Acanthamoeba* spp. in dust phenomena collected in Ilam Province.

## Material and Methods

### *Study areas*

This study was conducted from June to September 2017 in Ilam Province (32° 03'–34° 02' north latitude and 45° 40'–48° 03' east longitude), located in the west of Iran, with the same borders with Khuzestan Province in the south, Lorestan Province in the east, Kermanshah Province in the north and with the same borders with Iraq in the west, with 425 km shared borders. It is a non-industrialized province that is affected by dust phenomenon. Sampling sites in this study were limited to two cities located in Ilam Province, Ilam and Mehran (Figure 1).



**Figure 1.** Sampling locations map in Ilam Province (map indicates composite dust and salt potential)

### *Sampling and Acanthamoeba culture*

Fifty-five dust samples were collected in these regions and were dissolved in distilled sterile water and filtered using nitrocellulose filters (pore size: 0.45  $\mu\text{m}$ ; Sigma-Aldrich, USA). The filters were put onto 2% non-nutrient agar (Difco, USA) covered with trypticase-yeast extract-maltose and incubated at room temperature [14]. The plates were monitored daily for the presence of *Acanthamoeba* for up to a month. The observed trophozoites or cysts of *Acanthamoeba* were subcultured through transferring small pieces of agar with amoebae to a fresh plate to reduce fungal and bacterial contamination and increases *Acanthamoeba* to molecular assays.

### *DNA extraction and PCR amplification assay*

DNA was extracted using a modified phenol–chloroform method [15]. To amplify the DF3 region of 18S rRNA (rDNA), specific primers JDP1 (forward: 5' GGC CCA GAT CGT TTA CCG TGA A 3') and JDP2 (reverse: 5' TCT CAC AAG CTG CTA GGG AGT CA 3') were used [16]. Amplification reactions were set for a total volume of 25  $\mu\text{l}$ , containing 12.5  $\mu\text{l}$  Ampliqon (Taq DNA Polymerase Master Mix RED, Denmark), 1  $\mu\text{l}$  forward and reverse primers (10 pmol), 3  $\mu\text{l}$  DNA templates, and 8.5  $\mu\text{l}$  double-distilled water. PCR amplification was performed with a primary denaturing step at 94  $^{\circ}\text{C}$  for 1 min, after 35 cycles at 94  $^{\circ}\text{C}$  for 35 s, the annealing step was 56  $^{\circ}\text{C}$  for 45 s, and 72  $^{\circ}\text{C}$  for 1 min. After this process, the final extension was performed at 72  $^{\circ}\text{C}$  for 5 min.

### *Gel electrophoresis*

Electrophoresis of PCR products was visualized using agarose gel electrophoresis on the 1/5% agarose gel (Invitrogen, Life Technologies GmbH, Germany) stained with safe stain.

### *Sequencing and genotyping of the isolates*

PCR amplicons were sequenced at both directions, with PCR primers by Pouya Gostar Gene Company ([www.pggene.com](http://www.pggene.com)) in a Genetic Analyzer ABI 3730 (Bioneer, Daejeon, South Korea). The sequences analysis was performed with Bioedit Sequence Alignment Editor 7.1.3.0. Using the basic local alignment search tool (BLAST) analysis (<http://blast.ncbi.nlm.nih.gov>), sequencing data were aligned with *Acanthamoeba* genotype sequences available in the GenBank database, to determine the genotypes. Nucleotide sequences were submitted to the GenBank using BankIt.

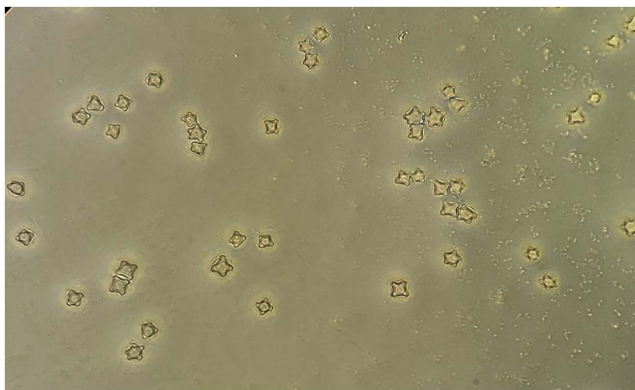
### *Thermotolerance and osmotolerance assays*

To predict the pathogenic potential of the positive isolates, thermal and osmotolerance tests are used currently. Osmotolerance and thermotolerance assays were developed as previously described [11]. Briefly, for the thermotolerance test, each positive strain was subcultured and incubated at two temperatures (37 and 44 °C) for 24, 48, and 72 h, and using D-Mannitol (Merck, Germany), each positive strain was subcultured in two plates at different molarities (0.5 and 1 M) for the osmotolerance assay. For both pathogenic tests, outgrowths of amoebae were checked by inverted microscopy [11, 17].

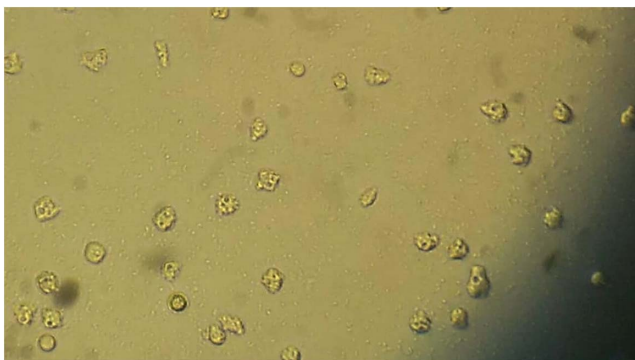
## **Results**

### *Identification of Acanthamoeba isolates based on the morphological characteristics*

Based on the morphological characteristics of amoebae, among the 55 dust samples, 18 (32.7%) were positive for *Acanthamoeba* spp. Monoxenic culture was done by successive passages, such that only amoeba cysts and trophozoites remained in the cultures. Cysts are double-walled measuring 20–25 µm (Figure 2), and trophozoites have flat shape and spine-like structures (Figure 3), which illustrate them as related to the genus *Acanthamoeba* [2, 18]. The incidence of



**Figure 2.** The image of *Acanthamoeba* cysts isolated from a dust sample in Ilam Province (magnification: 600×)



**Figure 3.** The image of *Acanthamoeba* trophozoites isolated from a dust sample in Ilam Province (magnification: 400×)

*Acanthamoeba* spp. was 26.6% (8 out of 30 samples) and 40% (10 out of 25 samples) in Ilam and Mehran cities, respectively. Eventually, 18 strains were submitted for molecular analysis.

#### *Molecular analysis of Acanthamoeba isolates based on 18S rRNA gene*

*Acanthamoeba* DNA was detected in all 18 morphological positive samples and was subjected to PCR amplification. A single approximately 460-bp product of the 18S rRNA gene was amplified in all the samples, which was consistent with the product size of *Acanthamoeba* genus. DNA sequence of the *Acanthamoeba*

**Table I.** Data related to the positive strains of *Acanthamoeba* genotypes isolated from dust phenomenon in Ilam Province, Iran

Sampling site	Culture/ PCR	Thermo- and osmotolerance		Genotype	Acc. no.
		(37 °C/44 °C)	0.5 M/1 M		
Kodak Park	+/+	+/-	+/-	T2	MK192784
Public place	+/+	+/+	+/+	T4	MK192785
Imam Khomeini Hospital	+/+	-/-	-/-	T4	MK192786
Residential houses	+/+	+/-	+/-	T2	MK192787
Chaghasabz Park	+/+	+/+	+/+	T4	MK192788
School of Medicine	+/+	-/-	-/-	T4	MK192789
Residential houses	+/+	+/-	+/-	T2	MK192790
Qhaem Hospital	+/+	+/+	+/+	T4	MK192791
Health centers	+/+	+/+	+/+	T4	MK192792
Public place	+/+	-/-	-/-	T4	MK192793
Residential houses	+/+	+/-	+/-	T4	MK192794
Public place	+/+	+/+	+/+	T4	MK192795
Parvin Etesami High School	+/+	-/-	-/-	T2	MK192796
Mehran Park	+/+	+/-	+/-	T4	MK192797
Residential houses	+/+	+/-	+/-	T4	MK192798
Residential houses	+/+	+/-	+/-	T4	MK192799
Public place	+/+	+/+	+/+	T4	MK192800
Health centers	+/+	+/-	+/-	T4	MK192801

Note: PCR: polymerase chain reaction.

isolates using BLASTn analysis revealed that 14 isolates (77.7%) belonged to the T4 genotype, and 4 isolates (22.3%) identified as T2 genotype under the accession numbers MK192784–MK192801 (Table I).

#### *Pathogenesis of Acanthamoeba isolates based on tolerance assays*

Six out of the 18 (38.8%) *Acanthamoeba* strains were considered as high-potential pathogenic *Acanthamoeba*. These isolates could grow at high temperatures (37 and 44 °C) and high osmolarity media (1 M). Eight isolates demonstrated growth at 37 °C and 0.5 M osmolarity; hence, they are categorized as low-potential pathogens (Table I).

## Discussion

Ilam Province due to arid and semi-arid climate having common borders with Iraq is one of the main centers of dust creation in Iran [19]. Dust is an

atmospheric phenomenon that the frequency and intensity of this phenomenon in Ilam Province has been an upward trend. In the study of Maghsudi et al. [20], the highest concentration of total suspended particles and respirable particles (PM10) has been recorded 22.5 and 1.3 mg/m<sup>3</sup> in Ilam city. In this study, we identified *Acanthamoeba* spp. in dust phenomenon of places that highly involved the human activities in dusty days.

Dust storms occur more in the summer and spring; therefore, during the summer, we collected 55 dust samples of dusty days. According to the results, 18 (32.7%) out of 55 samples were positive for *Acanthamoeba* spp., as Niyiyati et al. [15] also reported that the contamination of recreational and therapeutic geothermal water sources by *Acanthamoeba* in the Dehloran of Ilam Province was estimated to be 50%, whereas in Ahvaz City, Southern Iran, the contamination of soil samples was estimated to be 26% [21].

To date, the phylogenetic analyses showed 21 genotypes (T1–T21 genotypes *Acanthamoeba*). In Iran, genotype T4 has the predominant *Acanthamoeba* genotypes and other *Acanthamoeba* genotype related to T2, T3, and T5 (*Acanthamoeba lenticulata*), T6, T9, T11, T13, and T15 (*Acanthamoeba jacobsi*) [22]. In this study, the genotyping data based on DF3 sequences revealed T4 and T2 genotypes, which are in agreement with the results of other researchers [23, 24]. The findings of this study revealed that the most common *Acanthamoeba* genotype in dust samples is the T4 genotype. In the study conducted by Mirjalali et al. [25], pathogenicity of *Acanthamoeba* T4 genotype was assessed via *in vitro* and *in vivo* tests. In this work, pathogenic potential of T4 genotypes in Iran was studied. In addition, in this study, this pathogenicity test showed that all of isolates were considered as high-potential pathogenic *Acanthamoeba* belonged to the T4 genotype. According to the literature, T4 genotype was the most commonly isolated genotype on human infections. More than 90% of keratitis cases were related to this genotype [26]. Although the number of T4 Iranian keratitis isolates was higher than that of T2 isolates (61.5% vs. 23%), we determined T2 as the most genotype, followed by T4 [27]. Therefore, exposure to high concentrations of dust phenomenon in Ilam Province could be considered an important health hazard among the high-risk people. Eventually, public education is advisable to increase the consciousness among the high-risk individuals, such as contact lens users during a dusty day.

In conclusion, this study revealed *Acanthamoeba* related to T4 genotype as the most common strain dust phenomenon samples in Ilam Province. These strains could be a potential transmission for AK and high-risk people are exposed to them without being aware; therefore, considering health principles are suggested.

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RS designed all steps of the study and wrote the draft of the manuscript. RN made critical revisions and permitted the final version. RS and AN performed the experiments. All the authors reviewed and approved the final version of the manuscript.

## Conflict of Interest

The authors declare no conflict of interest.

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