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Original Article

Detection and Molecular Characterization of Potentially Pathogenic Free-Living Amoebae from Recreational and Public Soils in Mazandaran, Northern Iran

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Abstract

Background: Free-living amoeba (FLA) belonging to *Acanthamoeba* spp., *Naegleria*, and *Balamuthia mandrillaris* are the soil-born protozoa. This study aimed to survey the occurrence of FLA, including *Acanthamoeba* spp., *B. mandrillaris*, *Vermamoeba* spp., and *Naegleria* spp., in soil samples collected from various districts of Mazandaran Province (Northern Iran) from July to December 2018.

Methods: Overall, 118 soil samples from the recreational and public places were surveyed for the existence of *Acanthamoeba* spp., *Vermamoeba*, *Naegleria*, and *B. mandrillaris* using both morphological key and molecular tools with genus-specific primers of JDP1, NA, ITS1, and Bal, respectively. To verify the taxonomic status of isolated amoeba, the phylogenetic tree was made based on sequences of 18S rRNA by MEGA (5.05) software with the maximum likelihood model.

Results: Overall, 61/118 samples (51.6%) were contaminated with FLA, and based on the sequencing data, 29 isolates were successfully sequenced. Among the samples, all isolated *Acanthamoeba* (52.4%) belonged to the T4 genotype with amplification of the DF3 region (18S rRNA gene). Internal transcribed spacer (ITS) sequencing revealed the presence of one strain of *Naegleria americana*. Twenty-eight *V. vermiformis* were also confirmed based on Nuclear SSU rDNA. Morphological survey and PCR assay did not show any positive samples for *B. mandrillaris*.

Conclusion: The present study indicates the occurrence of FLA in soil sources of the recreational and public places in Mazandaran province that it can be a severe risk to human health. Thus, more studies are expected to survey the infection source in patients with FLA-related diseases.

Introduction

Free-living amoebae (FLA) including several genera, *Acanthamoeba spp.*, *Balamuthia mandrillaris* (*B. mandrillaris*), and *Naegleria* spp. can be found in the environment (1). Potentially pathogenic FLA able to cause severe and fatal diseases with poor prognoses such as the central nervous system (CNS), amoebic keratitis (AK), and cutaneous ulcers (2, 3).

The prevalence of *Acanthamoeba* in different water sources such as tap water, freshwater, hot springs, and bottled mineral water is estimated to be 42.6% in Iran (1, 4). Only a few studies are surveying the contamination with FLA in soil sources of Iran (42.1%), and in most studies, *Acanthamoeba* has been identified according to the morphological characteristics, and the distribution of other FLA has not thoroughly evaluated.

There are several studies on AK caused by *Acanthamoeba* spp., *Vermamoebae*, and *Valkampfia* from Iran as well as a case report of primary amoebic meningoencephalitis (5). Moreover, *B. mandrillaris* has been reported in three studies from this country. For the first time, *B. mandrillaris* strain (ID-19) was isolated from dust particles of a hospital in Tehran (6); the second study reported *B. mandrillaris* in 5 of 55 soil samples collected from recreational areas in East Azerbaijan (7); and the third study showed that 2 of 66 water samples collected from hot-spring in northern Iran were positive for *B. mandrillaris* (8, 9).

Only some *Naegleria* species including *Naegleria americana* (*N. americana*), *N. australiensis*, *N. dobsoni*, *N. polaris*, *N. fultoni*, and *N. pagei* were isolated from hot-spring sources located in Iran. However, so far no report of *N. fowleri* has been found in the environmental sources of this country (9). Studies focusing on the farmlands soils are missing or empty in Iran.

On the other hand, the irrigation of agricultural lands and farmlands with untreated urban wastewater, which are potential sources for FLA colonization, is particularly the concern that agricultural and environmental officials and experts have been focusing on this problem for years as this is an essential and intensely vital issue for the national health insurance system (5).

This study aimed to survey the occurrence of FLA, including *Acanthamoeba* spp., *B. mandrillaris*, *Vermamoeba* spp., and *Naegleria* spp., in soil samples collected from various districts of Mazandaran province (Northern Iran) by using both morphological and molecular-based approaches. Besides, the phylogenetic tree was constructed for isolated stains.

Materials and Methods

The geographical location of the study area Mazandaran Province is one of the most densely populated provinces in Iran and is divided into 20 counties. This province is located in the central-northern of Iran, along the southern coast of the Caspian Sea and the Central Alborz mountain range. It has various climates, including the mild and humid climate of the Caspian shoreline and the moderate and cold climate of mountainous regions with 700 mm rainfall annually (Fig. 1).

Samples Collection and Processing

Overall, 118 soil samples from the recreational and public places, including parks, hospitals, surrounding spaces of swimming pools, and school campuses, were collected from 14 cities in Mazandaran Province, Northern Iran from July to December 2018.

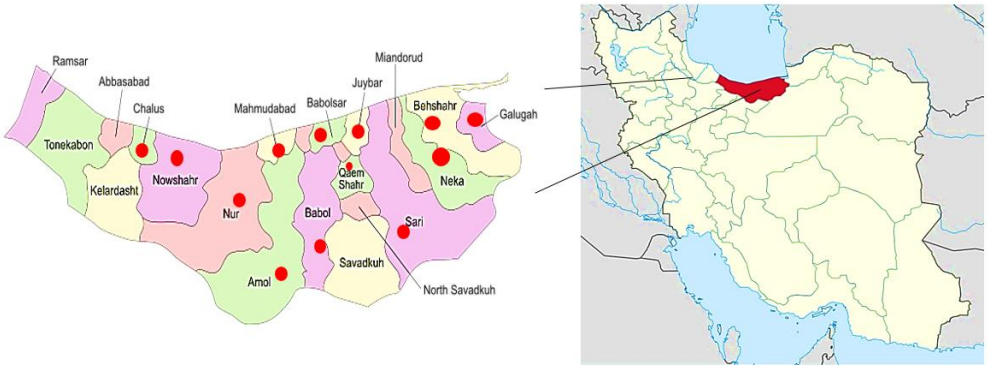

Fig. 1: Map showing the location of the study area in Iran (The map has been given from Wikipedia)

Briefly, 50 g of soil samples were placed in the sterile plastic bags and transferred immediately to the laboratory of the Department of Parasitology, Mazandaran University of Medical Sciences, Sari, Iran, for further processing. The soil samples were dissolved in 10 ml sterile distilled water; remained for about an hour, and the suspension of samples filtered using a pumping machine with a 0.45-µm cellulose nitrate membrane (Millipore, SA).

The filters were inoculated on 1.5% non-nutrient agar (NNA) plates covered with a layer of heat-killed *Escherichia coli*. Then, all the plates were incubated at 37 °C, and amoebae were morphologically identified to detect FLA plaques and the presence of cysts and trophozoites, daily for three weeks, according to taxonomic criteria using an inverted microscope (10, 11).

Positive samples were purified to harvest amoebae. Plates were stored for up to 2 months, and positive plates (plates exhibiting amoeba growth) were subjected to the following processes.

DNA Extraction, Polymerase Chain Reaction (PCR), and Phylogenetic Analysis

The genomic DNA from the positive samples was extracted using the phenol-chloroform method base on our previous study (12).

PCR assay was done using genus-specific primers, including JDP1 and 2 for *Acanthamoeba* spp. (12), NA1 and NA2 for *Vermamoeba vermiformis* (13), ITS1 and ITS2 for *Naegleria* spp. (14), and Bal1 and 2 for *B. mandrillaris* (15) (Table 1). The PCR reaction was carried out in a volume of 25µl, containing 12.5µl PCR Master Mix (Pishgam Co., Iran), 1µl forward primer, 1µl reverse primer, 8.5µl of distilled water, and 2µl DNA template.

The PCR cycles were set up using a thermocycler (BIO-RAD, Hercules, California, USA) with the programs listed for *Acanthamoeba*, *Vermamoeba*, *Naegleria*, and *Balamuthia*. Amplified DNA was detected on 1.5% agarose gel electrophoresis, and the DNA was visualized by the solution of safe stain under a UV gel documentation system.

PCR products were submitted for sequencing using the ABI 3130X automatic sequencer. For homology analysis, the sequences were assembled and analyzed with Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information homepage (NCBI) for comparing the identified sequences with other sequences available in the GenBank database.

The multiple sequence alignment was done using the SequencherTmv.4.1.4 software based on the ClustalW method version 7.2.5. To identify genetic associations and taxonomic status of isolated FLA from soil sources, phylogenetic trees were displayed using the MEGA 5 software and maximum likelihood algorithm by Kimura 2-parameter model.

Table 1: Primers and amplification conditions for the identification of free-living amoebae in the present study

Free-living amoebae	*Primer name*	*Primers*	*Amplicon size (bp)*	*Target gene*	*Amplification conditions*	*Ref.*
Acanthamoeba spp.	JDP1 JDP2	5-GGC CCA GAT CGT TTA CCG TGAA-3 5-TCT CAC AAG CTG CTA GGG AGT CA-3	~ 500	Nuclear SSU rDNA	95°C (1 min) followed by 45 cycles of 95 °C (15 sec), 60 °C (1 min) and 72 °C (40 sec)	(4, 16)
V. vermiformis	NA1 NA2	5-GCT CCA ATA GCG TAT ATT AA-3 5-AGA AAG AGC TAT CAA TCT GT-3	~ 700	Nuclear SSU rDNA	95 °C (3 min) followed by 45 cycles of 95 °C (20 sec), 58 °C (30 s) and 72 °C (40 sec)	(17)
Naegleria spp.	ITS1 ITS2	5-GAA CCT GCG TAG GGA TCA TTT-3 5-TTT CTT TTC CTC CCC TTA TTA-3	400	Internal Transcribed Spacer (ITS)	94 °C (1 min) followed by 35 cycles of 94 °C (45 sec), 56 °C (45 sec) and 72 °C (1 min)	(15)
B. mandrillaris	Bal1 Bal2	5-CGC ATG TAT GAA GAA GAC CA-3 5-TTA CCT ATA TAA TTG TCG ATA CCA -3	~ 1000	Mitochondrial SSU rDNA	94 °C (1 min) followed by 40 cycles of 94 °C (45 sec), 48 °C (45 sec) and 72 °C (3 min)	(18)

Results

Out of 118 soil samples in thirteen cities of Mazandaran Province, 61 (51.6%) were positive for FLA in cultivation based on morphological criteria of Page (10). Approximately 500 bp bands were identified as *Acanthamoeba* spp., using JDP1 and JDP2 primers based on the PCR technique.

Overall, 32 out of 61 (52.4%) soil samples were positive for *Acanthamoeba*. Besides, using the PCR method, approximately 750 bp bands using specific primers were considered as *V. vermiformis*, and 28 from 61 (45.9%) soil samples were positive for *V. vermiformis*.

In soil samples of all 13 cities surveyed in the present study, were found *Acanthamoeba* and *V. vermiformis*. Moreover, about 400 bp bands with ITS1 and ITS2 primers were identified as *Naegleria* spp., just observed in 1 out of 61 (1.7%) samples in Galougah city. Whereas, morphological survey and PCR assay using *B. mandrillaris* specific primers failed to report any positive results, but four samples had a mixed infection (*Acanthamoeba* and *vermamoebae*) (Table 2).

Table 2: Data regarding the source and the isolated genus of free-living amoebae from recreational and public places in Mazandaran, Northern Iran

City	*Total No. of samples*	*No. Acanthamoeba positive samples*	*No. Vermamoeba positive samples*	*No. Naegleria positive samples*	*No. Mix positive samples*
Galougah	9	3	1	1	-
Behshar	12	2	2	-	-
Neka	10	4	1	-	1 (*Achanthamoeba+ Vermamoeba)*
Sari	16	5	5	-	3 (*Achanthamoeba+ Vermamoeba*)
Qaemshahr	12	1	1	-	-
Babol	6	2	1	-	-
Amol	8	4	3	-	-
Mahmudabad	4	2	1	-	-
Babolsar	11	3	3	-	-
Chaloos	6	1	4	-	-
Noshahr	10	2	3	-	-
Feredonkenar	4	1	1	-	-
Noor	6	1	1	-	-
Joybar	4	1	1	-	-
Total	118	32	28	1	4

All sequenced *Vermamoeba* isolates (n=20) belonged to *V. vermiformis,* and all sequenced *Acanthamoeba* isolates (n=8) were identified as T4 genotype. ITS sequencing showed that one strain belonged to *N. americana* (Table 3). Phylogenetic analysis confirmed that all sequenced isolates had high homology (95%-100%) with reference isolates from Gene Bank (Figs. 2 and 3). Data sequences of 29 isolated strains were deposited in the GenBank under accession numbers present in Table 3.

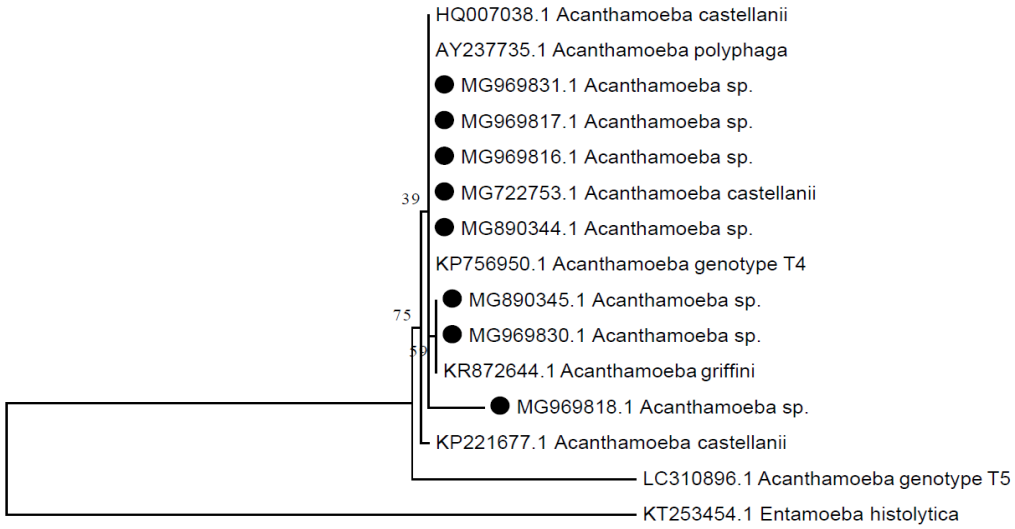

Fig. 2: Phylogenetic analysis of *Acanthamoeba* isolates. Neighbor-joining tree based on 18S rDNA sequences, with 1000 bootstrap replicates. The sequences from these isolates were aligned by MEGA 5 software using reference isolates from Gene Bank. The bar is an index of evolutionary distances (0.02) among the different sequences

Fig. 3: Phylogenetic analysis of *Vermamoeba* isolates. Neighbor-joining tree based on 18S rDNA sequences, with 1000 bootstrap replicates. The sequenced isolates were aligned by MEGA 5 software using reference isolates from Gene Bank. The bar is an index of evolutionary distances (0.05) among the different sequences

Table 3: Free-living amoebae genotypes in the present study

Code	*sequenced FLAs*	*Genotype/spicese*	*Gene Bank accession no.*
MGS961109	*Acanthamoeba*	T4	MG722753
MGS961107	*Acanthamoeba*	T4	MG890344
MGS961108	*Acanthamoeba*	T4	MG890345
Acn8F	*Acanthamoeba*	T4	MG969816
Acn5F	*Acanthamoeba*	T4	MG969817
Acn2F	*Acanthamoeba*	T4	MG969830
Acn9F	*Acanthamoeba*	T4	MG969818
Acn10F	*Acanthamoeba*	T4	MG969831
Hart1HviF	*Vermamoeba*	*vermiformis*	MG969819
Hart8HviR	*Vermamoeba*	*vermiformis*	MG969820
Hart16HviR	*Vermamoeba*	*vermiformis*	MG969823
Hart6HviR	*Vermamoeba*	*vermiformis*	MG969821
Hart15HviR	*Vermamoeba*	*vermiformis*	MG969826
Hart1HviF	*Vermamoeba*	*vermiformis*	MG969829
Hart8HviR	*Vermamoeba*	*vermiformis*	MG969833
Hart14HviR	*Vermamoeba*	*vermiformis*	MG969828
Hart11HviR	*Vermamoeba*	*vermiformis*	MG969825
Hart13HviR	*Vermamoeba*	*vermiformis*	MG969827
Hart5HviF	*Vermamoeba*	*vermiformis*	MG969835
Hart3HviF	*Vermamoeba*	*vermiformis*	MG969836
Hart17HviF	*Vermamoeba*	*vermiformis*	MG969838
Hart16HviF	*Vermamoeba*	*vermiformis*	MG969840
Hart4HviF	*Vermamoeba*	*vermiformis*	MG969837
Hart2HviF	*Vermamoeba*	*vermiformis*	MG969839
Hart9HviR	*Vermamoeba*	*vermiformis*	MG969824
Hart12HviR	*Vermamoeba*	*vermiformis*	MG969832
MGS961109	*Vermamoeba*	*vermiformis*	MG969863
Hart12HviR	*Vermamoeba*	*vermiformis*	MG969832
NEG1	*Naegleria*	*americana*	MH000323

Discussion

Among soil-born free-living amoebae, commonly *Acanthamoeba* spp., *Vahlkampfiids,* and *B. mandrillaris* cause severe amoebic keratitis and encephalitis. The current research showed that 51.6% of recreational and public places soil sources are contaminated with FLA. The present study was the first to survey the recreational and public soils in northern Iran.

Moreover, primary, recreational, and tourism counties such as Amol and Mahmudabad, showed a high pollution rate with FLA (50%), which is a significant threat to public health.

During the period between 1997 and 2000, overall 136 corneal scrapings of clinically-suspected *Acanthamoeba* ulcers were screened and tested for the existence of *Acanthamoeba*. Eleven out of 136 patients were positive for *Acanthamoeba* that all of these patients were agricultural workers and did not wear contact lenses (19).

Another study collected soil and tap water samples from several locations in Yanji, China. *Acanthamoeba* species identified in environmental samples belonged to T4, T5, and T16 genotypes (20). In another research, sixty soil samples were collected from public and recreational sides in East Azerbaijan, Iran, and examined the presence of *Acanthamoeba* spp. using morphological and PCR techniques. *Acanthamoeba* spp. was found in 41.6% of soil samples and led to the identification of four of the genotypic clades (T3, T4, T5, and T11) (21).

Moreover, 47.8% of hot spring samples in Mazandaran Province were contaminated with

Acanthamoeba, and the genotype of 100% isolates belonged to the T4 (12). The difference in the abundance of FLA in soil samples of different areas of the world could be due to the moisture rate, organic carbon and texture of the soil, and various climatic conditions (1).

In this study, the isolated *Acanthamoeba* strains belonged to the T4 genotype with the highest distribution compared to other FLAs. The results of the current study showed that most *Acanthamoeba* isolates are in soil samples. This finding may be due to their high resistance to harsh environmental conditions (2). There are very few reports about *Acanthamoeba* genotypes in soil sources in Iran and around the world. In a previous Iranian study, the prevalence rate of *Acanthamoeba* spp. (all belonging to T4 genotype) was 26.9% in soil sources of recreational parks in Tehran, Iran (22). Other research in Ahvaz city (southern Iran) also showed the low prevalence of *Acanthamoeba* (26%) from soil samples and the isolated genotypes belonged to T4 and T5 (23).

Besides, the results of the present research agreed with another findings, that presented T4 is the most frequent genotype isolated from environmental sources and patients with AK and granulomatous amoebic encephalitis in the USA (24). The *Acanthamoeba* belonging to genotype T4 was isolated from 8 clinical samples collected from 35 patients referred to the eye clinic (25). The researchers reported an increased prevalence of keratitis due to the *Acanthamoeba* T4 genotype (6, 26). To date, 90% of the infection-causing *Acanthamoeba* isolates belonged to the T4 genotype. The frequency of this genotype in human infections is probably due to their high virulence and properties that increase their transmissibility as well as decreased their susceptibility to drugs (27). Later, Iovieno et al. showed bacterial endosymbionts (*Legionella* spp. and *Neochlamydia* spp.) in the T4 genotype considering as a pathogenic genotype (28).

In the present study, non-pathogenic *Naegleria*, *N. americana,* was detected for the first time in soil samples of Iran. In the examined soil samples, pathogenic species of the *Naegleria, N. fowleri*, were not detected. However, other researchers reported *Naegleria* spp. in Iran. The presence of *Naegleria* spp. such as *N. americana*, *N. australiensis*, *N. dobsoni*, *N. pagei*, *N. fultoni*, and *N. polaris* were reported in therapeutic hot springs in Mazandaran, northern Iran (9).

Unfortunately, the isolation of *B. mandrillaris* from soil was not successful in this study. It may be because of favorable growth conditions needed for the cultivation of *Balamuthia* in non-nutrient agar. To date in Iran, three environmental studies have reported *Balamuthia* in environmental sources, including one from a soil sample from north-western (7), one from hot springs in Mazandaran (8), and one case from urban dust in Tehran (6).

Conclusion

The present study indicates the occurrence of FLA in soil sources of the recreational and public places in Mazandaran province that could be a severe risk for native people and tourists. Therefore, posting warning signs in recreational sites may be an option for decreasing the hazard of FLA-related diseases.

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Conflict of interest

The authors declare that there is no conflict of interests.

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