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Original Article

Morphological and Molecular Survey of *Naegleria* spp. in Water Bodies Used for Recreational Purposes in Rasht city, Northern Iran

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Abstract

Background: *Naegleria* spp. is a free-living amoeba of which some species including *N. fowleri* and *N. australeinsis* are highly pathogenic in human and animals. These widespread amoebae could be found in different environmental sources particularly in aquatic resources of tropical and subtropical regions. The most important source of infection is via recreational water contact. Due to the lack of thorough research regarding species of *Naegleria* spp. in aquatic sources, the present study was conducted.

Methods: In the present study, 60 samples were collected from recreational water resources of Rasht city, Guilan province, north of Iran. After filtering and culturing the samples, plates were examined by microscopic method and according to the page criteria. DNA of vahlkampfiid-positive samples were then extracted using phenol-chlorophorm method. Amoebae genus was identified by targeting the ITS-region and sequencing based-approaches.

Results: Nine (15%) samples out of a 60 total samples were positive for *Naegleria* spp. of which seven belonged to potentially pathogenic *N. australiensis*. Two other strains were belonged to non-pathogenic *N. pagei*.

Conclusion: The present research was the first report of occurrence of *N. australiensis* and *N. pagei* in Rasht city, north Iran. This study reflects the occurrence of *Naegleria* spp. in water sources of Guilan Province, Iran.

Introduction

Free-living amoeba (FLA) such as *Naegleria*, *Valhkampfia*, *Acanthamoeba* and *Vermamoeba* genera are widespread protozoa that can be isolated from different natural sources particularly from water resources (1). Among these FLAs, some genera such as *Naegleria fowleri, Acanthamoeba* spp., *and Balamuthia mandrillaris* are important pathogens affecting human and animals (1-3).These genera of protozoan parasites is especially important in at risk groups such contact lens wearers and also immunecompromised patients most notably cancer patients, so numerous studies were carried out on epidemiology of these parasites in these groups (4, 5).

Naegleria genus, as well as, *Vahlkampfia* and *Paravahlkampfia* belong to *Vahlkampfidae* family and the mentioned genera could be pathogenic for human, but, only pathogenic *Naegleria* species called *N. fowleri* and *N. australiensis* are medically important (6-8). *Naegleria* genus consists of 47 species, of which some species (*N. fowleri* and *N. australiensis*) have been reported as pathogenic agents for human and animals (3). Primary Ameobic Meningitis (PAM) is a disease that can be caused by both species. PAM is an acute and rapidly fatal meningoencephalitis (3, 9, 10), occurs in both humans and animals (10). It is worthy to mention most of PAM cases have been reported in young adults with a history of thermal water contacts. In Iran, a single case study reported meningoencephalitis due to pathogenic *N. fowleri* in a six-month Iranian infant. The report suggests paying more attention on isolation and molecular characterization of *Naegleria* spp. in Iran (11). There is another report from Iran detecting a mixed infection of *Acanthamoeba* genotype T3 and *Vahlkampfia* in a cosmetic soft contact lens wearer (6).

Gilan Province is located in north of Iran by the Caspian Sea on the north and by the Alborz Mountains on the south, with humid subtropical climate and the heaviest rainfalls in Iran. Because of beaches and forest, the province attracts many tourists every year. Rasht is the capital city of Gilan Province and at the 2011 its population was 639951 (Wikipedia). Despite the studies suggesting *Acanthamoeba* occurrence in this region, the presence of *Naegleria* spp. in water bodies and other environmental sources has not been reported. However, in other regions of Iran, few reports exist on the presence of *Naegleria* spp. Two studies were conducted in hot springs of Ardebil province to explore the presence of *Acanthamoeba* and Vahlkampfiids (12, 13). While the prevalence of Vahlkampfidae, particularly *Naegleria* spp. has not been studied in Gilan Province, there are several studies about the prevalence of other FLAs, especially within *Acanthamoeba* genus (14, 15), which showed the predominance of pathogenic T4 in the tested samples (14).

Thus, the present study aimed to isolate *Naegleria* spp., from recreational water resources using both morphological and molecular-based approaches. To the best of our knowledge there were no previous research regarding *Naegleria* spp. in north Iran.

Materials and Methods

Sampling area, Filtration and Cultivation

Rasht is the largest Iranian city on Caspian Sea coast. It is a major trade center between Caucasia, Russia and Iran using the port of Bandar-e Anzali. Rasht is also a major tourist center with the resort of Masouleh in the adjacent mountains and the beaches of Caspian as some of the major attractions (Wikipedia). A total of 60 water samples (500-1500 ml) were collected from Rasht ponds waters (20 samples), pools (20 samples) and streams (20 samples). All of the samples were taken within summer and the temperature of the water

sources at the time of sampling were 8-20 ^{0}C. The samples were transferred to protozoology laboratory, Shahid Beheshti University of Medical Sciences, Tehran, Iran within one day and stored at room temperature. Approximately 250 ml of each sample were filtered through cellulose nitrate membrane with 1.6µm pore size. After that, middle of each membrane was cut out and cultured in plates of 1.5% non-nutrient agar medium along with heat inactivated *Escherishia coli* (16). Then plates were incubated at room temperature up to two months.

Morphological identification and cloning

Investigation of Vahlkampfiids was started after one week and continued up to two months. Light microscope with magnification of x100 and page's morphological criteria were used for identification of positive plates. Positive plates were then cloned to eliminate bacteria and fungi contamination and achieving pure plates for DNA extraction. Briefly, cloning was performed by transfer of single Vahlkampfiid- amoebae to new plates according to our previous studies (4).

DNA extraction

After adding sterile normal saline, pH 7, plates were scraped to harvest the amoebae. Samples were then centrifuged three times at 500g for 5 minutes and the sediments were used for DNA extraction. DNA samples were suspended in lysis buffer and incubated at 56 °C overnight. Proteinase K was also added to samples and finally the mixture was put in boiling temperature for 30 minutes. Finally, the isolated DNA of the samples was purified using phenol-chloroform method (17).

PCR analysis and Sequencing

Internal transcribed spacer (ITS) primer was targeted for amplification of Vahlkampfiids DNA including *Naegleria* spp. the PCR assay primers were designed included forward 5'GAACCTGCGTAGGGATCATTT3' and reverse primer ITS2 5' TTTCTTTTCCTCC-CCTTATTA 3'.

The PCR reaction was accomplished in a 30 µl Ampliqone (Taq DNA Polymerase Master Mix Red, Denmark) as a readymade mixture. To achieve volume of 30-µl PCR reaction, 25 µl of master mix was combined with 5 ng DNA templates and 20 pmol primers. PCR products were separated on a 1.5% agarose gel. Products were visualized by ethidium bromide stain and were imaged under UV light.

The entire PCR product was then submitted to sequencing. DNA chromatograms were tested using chromas software (version 1.45) and the sequences were then aligned. Homology analyses were done using BLAST software and each sequence was blasted against all available eukaryotic sequences in the GenBank database.

Results

Out of 60 collected water samples from park ponds, pools and streams, 9 (15%) samples were found to contain Vahlkampfiids. The trophozoite form was detected after the third days of cultivation and the cysts were observed after one week of culture. Cloning of all Vahlkamfiids was done successfully. The most contamination source was found in ponds water sources (Table 1).

Table 1: Isolated *Naegleria* species in water samples of Rasht city according to the source of contamination

Sampling sources	No. of samples	Positive samples (%)
Ponds	20	7 (35)
Pools	20	1 (5)
Streams	20	1 (5)
Total	60	9 (15)

Amoebae isolated from pools only once. Vahlkampfiids were detected by their elongated shape of trophozoites, vesicular nucleus

measuring between15-20 micron. The cysts were presented with smooth round wall measuring between 10-15 microns (Fig.1, 2).

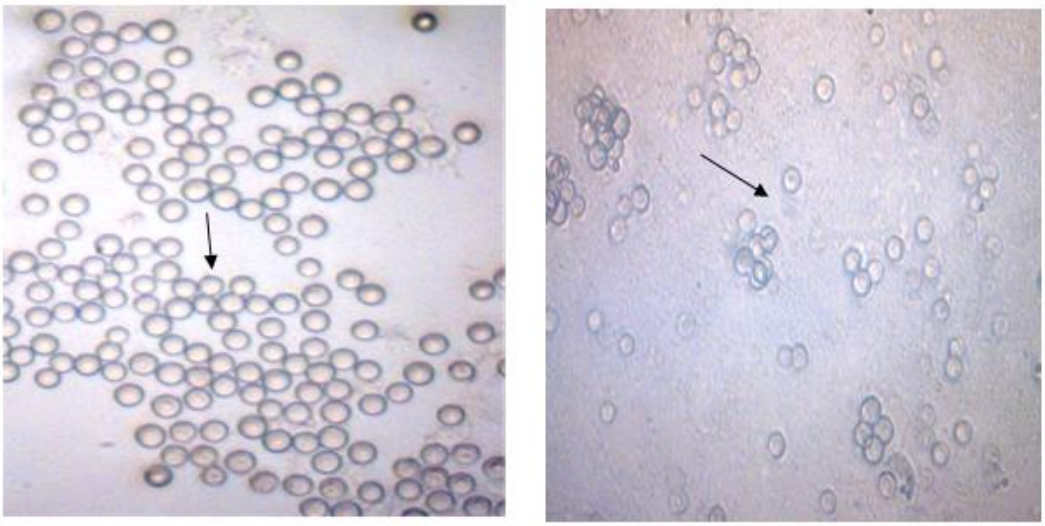

Fig.1: Cloned *Naegleria australiensis* cysts in non-nutrient culture medium (note the round ectocysts)

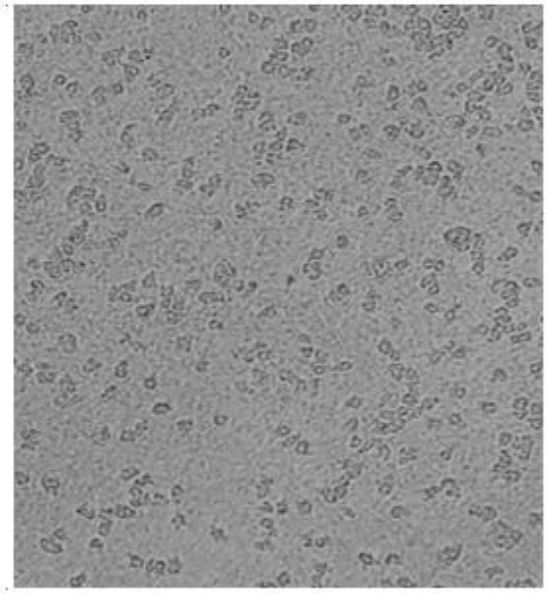

Fig. 2: Elongated shape of *Naegleria* trophozoites in non-nutrient culture medium

Eight of isolated amoebae showed growth on high temperatures (37-40 ^{0}C) and one strain did not growth in 40 ^{0}C. PCR analysis targeting ITS region revealed a 450 bp product in 9 isolates (15%) (Fig. 3).

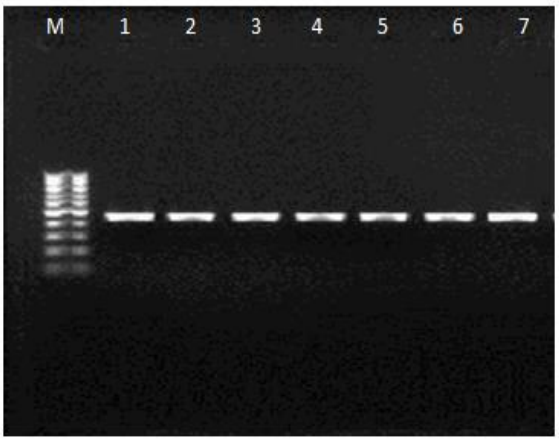

Fig.3: PCR products of some positive samples on a 1.5% agarose gel presenting 450 bp band (M= Marker, 1-7=samples).

Sequencing and homology analyses of the obtained sequences in Basic Local Alignment Search Tool (BLAST) showed that all strains belonged to the *Naegleria* genus. Seven strains isolated from pond 6 and stream 1 contained *N. australiensis*. Two other *Naegleria* belonged to *N. pegei* (Table 2).

Table 2: contaminated Sources and *Naegleria* species isolated from recreational area in Rasht City, Guilan province

Code	Source	Species
MN1	Pool	*N. pagei*
MN2	Pond	*N. australiaensis*
MN3	Pond	*N. australiaensis*
MN4	Pond	*N. australiaensis*
MN5	Pond	*N. australiaensis*
MN6	Pond	*N. pagei*
MN7	Pond	*N. australiaensis*
MN8	Pond	*N. australiaensis*
MN9	Stream	*N. australiaensis*

Discussion

In the present study, *Naegleria* spp. were detected from 15% water samples of Rasht City, Gilan Province. Based on PCR products base pairs in the examined water bodies, the most pathogenic *Naegleria* spp., *N. fowleri*, was not identified. This may justifies lack of primary amoebic meningoencephalitis (PAM) report in the region. However, other studies reported *Naegleria* spp. in Iran. Except one study, other studies in Iran identified non-pathogenic *Naegleria* and they have been isolated from environmental samples. Recently there is just one case report of PAM from Qom Province, Iran (11). In addition, there is a report of mixed infection due to *Acanthamoeba* genotype T3 and *Vahlkampia* belonging to Vahlkampfiidea family in a cosmetic soft lens wearer in Iran with poor prognosis (6).

Despite the very low prevalence of PAM in Iran, because of carrier role of *Naegleria* for potentially pathogenic microorganisms, like other FLAs, study regarding *Naegleria* occurrence in environmental sources should not be neglected (18). However, more studies are needed to investigate water sources with more sample size. In addition, vast research studies need to be carried out on to examine the etiology of meningoencephalitis.

The present study is the first research on the occurrence of *Naegleria* spp. in environmental sources in Rasht City. Most studies were conducted on other FLAs occurrence, particularly *Acanthamoeba* genus (19). Behniafar et al. reported 40% occurrence of free living amoeba including *Acanthamoeba, Thecamoeba, Hartmannella, Vahlkampfiids and Vannella* in surface waters of Kaleybar and Khodaafarin counties (East-Azerbaijan province)using culture and microscopic method (20). Another study was conducted to estimate the presence of *Acanthamoeba* in Gilan Province with 50 samples of recreational water sources. Niyyati et al. reported 30% prevalence of *Acanthamoba* by cultivation and morphological detection and molecular analysis. They revealed that 13-isolated *Acanthamoeba* belong to T4 genotype (14). In other regions of Iran, researchers have studied prevalence of *Naegleria* spp. in water resources. While the results of the present study indicate no occurrence of *N. fowleri* in water samples, two studies report the occurrence of *Naegleria* in hot spring of Ardebil Province. Badirzadeh et al. found 11 samples out of 28 (39.3%) hot-spring water samples to be positive for Vahlkampfiids (12). However, their research was limited to morphological identification. Solgi et al. tested 30 samples collected from hot-springs of Ardebil Province that 8 samples (26.7%) were positive for Valhkampfiid and *Hartmannella* (13).This discrepancy could be explained by the fact that *Naegleria* spp. are thermotolerant so they are possibly more prevalent in hot springs.

The findings of the present study indicate the occurrence of Vahlkampfiids in 15% of the samples. This is in disagreement with other studies reporting a higher isolation of *Naegleria*. Wang et al. reported *Naegleria* occurrence of 92.9% in environmental water of Cangchun, Northeastern China using PCR method (21). Edagawa et al. found prevalence of 68.7% of FLAs from tap-water sources in Osaka, Japan (22). This difference may be attributed to the application of various isolation methods for FLA detection. It should be mention that although *N. pagei* is a non-pathogenic species but it can harbor pathogenic microorganisms.

Conclusion, the present study reflects the occurrence of *Naegleria* spp. in water sources of Rasht City that can be potential hazard for native people and tourists, thus posting of alarming signs in recreational places could be an option for decreasing the risk. However, we recommend more research investigations focusing on *N. fowleri* detection and their prevalence in the environmental sources.

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