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Detection of *Naegleria* Isolates from the Egyptian Aquatic Environment

Wafaa M. Hikal[1, 2, *], Ahmad Z. Al-Herrawy[2], Mahmoud M. Bahgat[3], Abd-Elhafez H. Mohammed[4], Ameen A. Ashour[4]

[1]Department of Biology, Faculty of Science, University of Tabuk, Tabuk, Saudi Arabia
[2]Parasitology Lab, Water Pollution Research Department, National Research Centre, Dokki, Giza, Egypt
[3]Therapeutic Chemistry Department, National Research Centre, Dokki, Giza, Egypt
[4]Zoology Department, Faculty of Science, Ain Shams University, Cairo, Egypt

Abstract

Free-living amoebae of the genus *Naegleria* have been recognized as etiologic agents of amoebic encephalitis, keratitis, otitis, lung lesions and other skin infections mainly in immuno-compromised individuals. *Naegleria fowleri* is the causative agent of primary amoebic meningo-encephalitis (PAM), a rapidly fatal disease of the central nervous system. The disease is generally acquired while swimming and diving in freshwater. In the present study samples from swimming pools water in Egypt were examined for *Naegleria* using a polymerase chain reaction (PCR) method. Members of genus *Naegleria* were detected in 27.5% of the examined swimming pool water samples. Based on the morphological attributes of trophozoites and cysts, flagellation test, all the isolates were classified to the genus *Naegleria.* Molecular identification of the amoebae isolated from water samples confirmed their affinity to *Naegleria* genus. The isolated species of *Naegleria* could provoke variable degrees of infections to the swimmers. Thus there is a need for further investigation to establish *Naegleria* genotype.

Keywords

Free-Living Amoebae, *Naegleria*, Flagellation Test, PCR, Swimming Pools

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1. Introduction

Naegleria is a free-living amoeba that is ubiquitously distributed in the environment worldwide in fresh water as well as in marine water cooling towers (Barbaree et al., 1986),. Moreover, they have been recovered from various domestic water systems such as drinking tap water (Michel et al., 1998).Only *Naegleria fowleri* has been shown to cause human disease, that result in primary amoebic meningo-encephalitis (PAM), a rapid fatal infection of the central nervous system (CNS) that occurs generally in previously healthy children and young adults with a history of exposure to contaminated recreational, domestic, or environmental water sources (Ithoi et al 2011).

More than 200 cases of PAM, predominantly in children and young adults, were reported worldwide as of July 2002 (Visvesvara, 2003).

Naegleria fowleri (*N. fowleri*) feed on red blood cells, white blood cells, and brain tissue. PAM is almost fatal, usually killing its victims within 3–7 days after the onset of symptoms (CDC, 2010; Cabanes et al, 2001; John, 1982).Pathogenic FLA are not dependent upon a host for transmission and spread, nor does host-to-host transmission of these amoebic diseases occur. They feed by phagocytosis, mainly on bacteria, fungi and algae (Bass and Bischoff, 2001).They have the ability to multiply and grow well in tropical climate and in water body with high temperatures of 40-45ºC (De Jonckheere, 2006).Molecular methods such as PCR offer an attractive

* Corresponding author
E-mail address: wafaahikal@gmail.com (W. M. Hikal)

alternative to microscopy and culture, since they can be performed by personnel without a high level of expertise in recognizing diagnostic morphological features of amoebae. Furthermore, molecular methods are very sensitive and may allow the detection of fewer microorganisms per volume of sample analyzed than morphological methods would. The aim of this study was to determine the presence of *Naegleria* spp. in different aquatic environment of Egypt using morphological and molecular characterization methods which can be a risk factor for people especially contact lens wearers and immuno-compromised patients.

2. Materials and Methods

2.1. Samples and Sampling Sites

Water samples (1 liter volume each) were collected monthly from ten different swimming pools in Cairo, Egypt for one year period. Samples were collected in clean, dry autoclavable polypropylene containers and sent to the laboratory of parasitology, water pollution Research Department, National Research Centre, in icebox and processed at the same day of collection.

2.2. Isolation and Morphologic Identification of *Naegleria* Spp from Water Samples

Collected swimming pool-water samples were separately concentrated by using the membrane filtration technique. One liter of each water sample was filtered through a nitrocellulose membrane filters (0.45µm pore size and 47mm in diameter) (Whatman, WCN type, Cat No. 7141-104) (Gradus et al., 1989; Hikal, 2010). After filtration the membranes were separately inverted face to face on the surface of a non-nutrient (NN) agar plates previously seeded with 100 µl *Escherichia coli* suspension. All the inoculated plates were incubated at 40°C for one week with daily microscopic examination for the presence of any amoebic growth (Hikal, 2015). Identification of the obtained *Naegleria* spp. were achieved according to the morphological characteristics of both trophic and cyst stages (Pussard and Pons, 1977, Hikal, 2010, Al Herrawy et al., 2013).

2.3. Flagellation Test

The obtained amoebic trophozoites were gently scraped from the surface of agar plates with a bacteriological loop and suspended in a test tube containing 5 ml distilled water and incubated at 37°C for 30 minutes. Every 10 minutes one drop from the content of the tube was suspended in the concavity of a clean glass hanging drop slide and examined under the microscope for the formation of temporary flagella (Behets et al., 2003).

2.4. Molecular Characterization of Isolated Freshwater Amoebae Using Polymerase Chain Reaction (PCR)

2.4.1. DNA Extraction

The amoebae pellet was resuspended in lysis buffer containing 2% CTAB as described by Winnepenninckx et al.(1993) and modified by Abdel-Hamid et al. (1999), overlaid with 500 ml of phenol-chloroform-isoamylalcohol (PCI), and shaken gently for 5 hr. The suspension was centrifuged at 3000 x*g* for 10 min, and the upper, aqueous phase was transferred to a new tube. PCI extraction was repeated two times for 10 min each time. DNA was precipitated at -80°C overnight, pelleted at 12000 x*g* for 30 min at 4°C, washed in 70% ethanol, air dried, and re-suspended in 30 ml of sterile double-distilled water (Walochnik et al., 2000).

2.4.2. Polymerase Chain REACTION (PCR)

For molecular identification, the genus specific primers were used. Forward primer sequence (5 TTTGAATTCGCTCC-AATAGCGTATATTAA-3**)** and Reverse primer (5- TTTCTT-TTCCTCCCCTTATTA-3) (Pelandakis *et al*. 2000). All amplification reactions of PCR were performed in a 50 µl. PCR consisted of 1 min denaturation at 94°C, 1min annealing at 47°C and 1 min elongation at 72 °C for 35 cycles.After that, 10min of extension time at 72 °C was done. Finally, the PCR products were cheeked by electrophoresis in a 1.5 % agarose gel (Helling et al., 1974).

3. Results

3.1. Prevalence of *Naegleria* in the Examined Swimming Pools

Naegleria species were detected in 33 (27.5%) water samples collected from 10 swimming pools in Cairo (Table 1).

Table 1. Prevalence of *Naegleria* spp. in swimming pool samples

Swimming pools	Examined samples (n)	*Naegleria* spp.	
		Number	%
1	12	2	16.7
2	12	-	-
3	12	4	33.3
4	12	-	-
5	12	7	58.3
6	12	8	66.7
7	12	1	8.3
8	12	6	50.0
9	12	3	25.0
10	12	2	16.7
Total	120	33	27.5

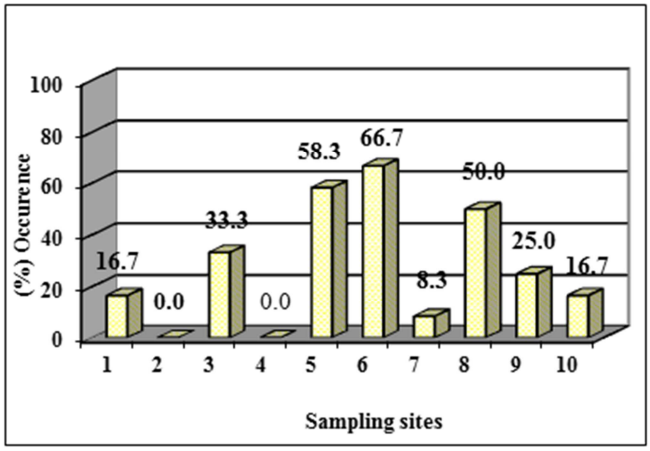
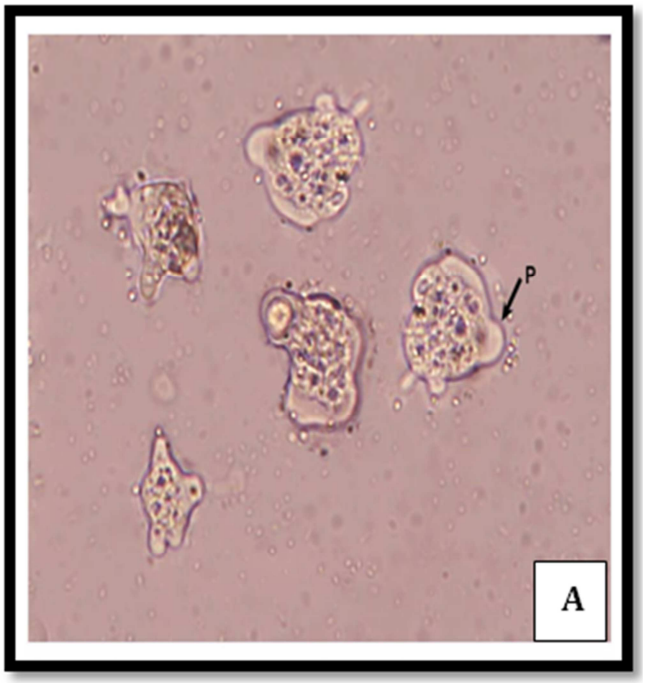


Figure 1. Occurrence of *Naegleria* spp. in swimming-pool samples

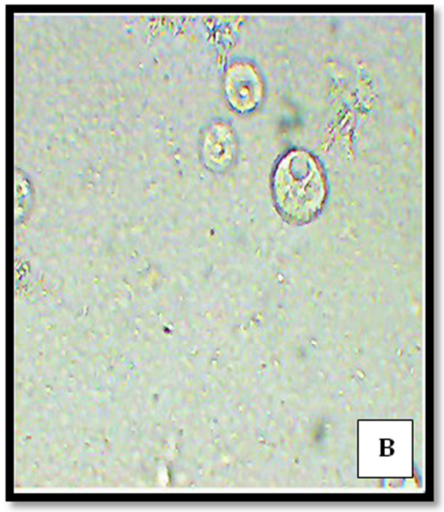
Swimming pool number 6 showed the highest incidence of heat-tolerant *Naegleria* species (66.7%).The heat-tolerant *Naegleria*species were not recorded in water samples collected from swimming pool number 4 and 2 (Table 1, Fig. 1).

3.2. Morphological Characterization of Genus *Naegleria*

Genus *Naegleria* represented the amoebo-flagellates whose members could transform from amoebae to flagellate forms. The life cycles included amoeboid and cystic stages and for most species a transient flagellate stage. Differentiation of *Naegleria* from other amoebae was based on their characteristic eruptive movement of the amoebic form, and their ability to transform to flagellates (Figure 2 [{A, B}).



Trophozoite form



Cyst form

Figure 2. Trophic and cystic form of *Naegleria*

3.3. PCR Product of Genus *Naegleria*

87.9% of microscopically *Naegleria* +ve swimming pool samples were also +ve by PCR technique. Microscopically *Naegleria* +ve swimming pool samples collected from site 1 (n=2), site 5 (n=7), site 8 (n=6) and site 9 (n=3) were all +ve by PCR. It was also observed that 87.5%, 75.0 and 50.0% of microscopically *Naegleria* +ve swimming pool samples collected from sites 6, 3 and 10, respectively proved to be +ve by PCR.Electrophoresis of amplification products from ITS primers of different *Naegleria* isolates were subjected to electrophoresis on 1.5% agarose gel parallel containing ethidium bromide to 100 bp DNA ladder where 409 bp specific amplification products were visualized in all environmental samples tested that were not evidenced in the negative control (Figure 3).

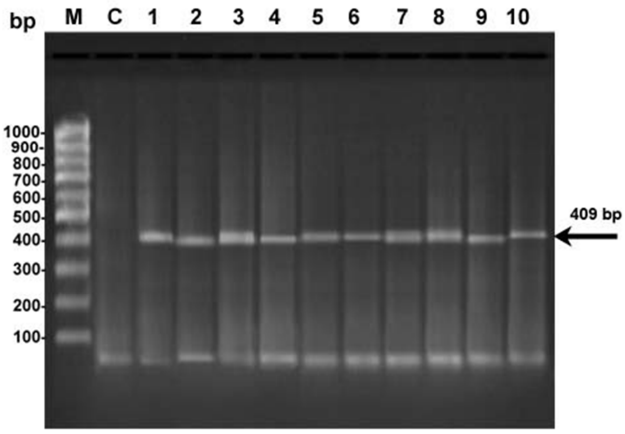


Figure 3. Electrophoresis of amplification products from DNA of different isolates of Naegleria (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10): N. spp., were subjected to electrophoresis on 1.5% agarose gel parallel containing ethidium bromide to M: 100 bp DNA ladder, where 409 bp specific amplification products were visualized in all samples. C: negative control bacteria

4. Discussion

The present study deals with the natural distribution of members of the genus *Naegleria*in the examined swimming pool water of Cairo, Egypt. To the best of our knowledge, few studies were conducted reporting the detection and existence of *Naegleria*in Egypt (Hikal, 2010;Al-Herrawy, 2014).

4.1. Prevalence of Heat Tolerant Free-Living Amoebae in Different Types of Water

Free-living amoebae were isolated at 37°C from 73.3% of the examined swimming pool samples. In Egypt, a lower incidence of free-living amoebae (32%) in swimming pools (Hamadto et al., 1993). Other workers in Poland detected free-living amoebae in 59.7% of the examined swimming pool samples (Gronik and Kuzna-Grygiel, 2004). Free-living amoebae grown at 40°C were isolated from 60% of the swimming pool samples. In Poland, Gronik and Kuzna-Grygiel, (2004) recorded a lower incidence of free-living amoebae (37.2%) isolated at 42°C from swimming pools.

4.2. Morphological Characterization of Genus *Naegleria*

In the present study, it was found that a trophozoite of *Naegleria* was long slender or oval, measuring 12-35µm in length and 10-30µm in width. *Naegleria* trophozoites were also characterized by a single vesicular nucleus having a large prominent centrally located nucleolus and a single broad hemispherical hyaline eruptive lobose pseudopodium. These findings are in agreement with those of most other workers (Page, 1974; Marciano-Cabral, 1988; Al-Herrawy, 1992; Ashmawy et al., 1993; Al-Herrawy and Al-Rashied, 1995; Schuster and Visvesvara, 2004; Shin and IM, 2004). In the present study, the flagellate stage of *Naegleria* amoebae usually had one pair of equal flagella arising from the pointed anterior end. Previous authors reported that the main process was the formation of one pair of flagella per cell (Page, 1967; Visvesvara, 1980; John, 1982; Ashmawy et al., 1993).

In the present study, the cyst form of *Naegleria* had a hardly detectable double wall with 4-5 shallow pores. These findings are in agreement with these of Schuster and Visvesvara (2004). *Naegleria* species were too similar morphologically to be distinguished from each other at the level of the ordinary light microscope. This conclusion was also reached by De-Jonckeree (1977) and Ashmawy et al. (1993).

4.3. Molecular Characterization of Genus *Naegleria*

In the present study the morphologically identified free-living amoebae belonging to the genus *Naegleria* were confirmed by PCR using genus-specific primers.

The incidence of *Naegleria* spp. were molecularly detected in 87.9% out of 33 morphologically *Naegleria* +ve samples (i.e. 27.5% of the total examined) collected from swimming pools. Based on the PCR amplification with a genus-specific primer pair in Taiwan Hsu et al. (2009) detected *Naegleria* spp. in 5.9% from swimming pool samples.

5. Conclusion

Swimming pools water may be the source of *Naegleria*invasion. The use of molecular methods to identify free-living amoebae of genus *Naegleria* could provide a more rapid means to diagnose infections caused by those amoebae.There is a need for further investigation to establish *Naegleria* genotype.

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