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# Real Time PCR Detection of *Naegleria* Species in Various Water Types.

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**ABSTRACT**

Members of genus *Naegleria* are known to cause infections of the central nervous system (CNS) of humans and other animals. A total of 72 water samples (24 Nile, 24 tap and 24 ground water) were collected from Giza governorate, Egypt. The collected samples were filtered through nitrocellulose membranes, then placed on non-nutrient agar seeded with *Escherichia coli* for cultivation of free-living amoebae. The obtained amoebae were molecularly identified to genus level for *Naegleria* species by real time PCR. The real time PCR results revealed that the highest occurrence of *Naegleria* species (54.2 %) was recorded in Nile water, followed by 25 % in each of tap and ground water samples. Statistically, various water types had a significant effect on the prevalence of *Naegleria* species by using one-way ANOVA. In contrary, conventional statistical criteria showed that the prevalence of *Naegleria* species in various water types was not significantly affected by the seasons. In conclusion, *Naegleria* species may be considered a potential health threat if pathogenic species exist in various water types. Therefore, long-term investigations on the prevalence of *Naegleria* species may be necessary, to prevent further infestations of water types.

**Keywords:** *Naegleria,* Real time PCR, Various water types, Egypt

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## INTRODUCTION

Waterborne diseases remain an important issue all over the world [1]. Because water is a human necessity, every human being is exposed to potential risk of diseases caused by waterborne and water-based pathogens. Waterborne diseases may originate from the contamination of water through human or animal feces infected with pathogenic viruses, bacteria, or protozoa [2]. Despite effective treatment of drinking water, outbreaks of waterborne disease are still attributed to exposure to pathogens in treated drinking water [3]. Although more than 880 such outbreaks were documented in the US between 1971 to 2008; numerous occurred that were either not reported or not recognized [4].

Members of genus *Naegleria* were isolated from organs of freshwater fishes (5), dental treatment units [6], soil [7], tap water and rivers [8,9], well water and reservoirs [10], natural hot springs [11], thermally polluted run-off from industrial zones [12] and saline bath [13]. More recently, the United States Environmental Protection Agency (USEPA) added *Naegleria fowleri* to their contaminant candidate list 3 (CCL3). The CCL is established by a process of identifying the waterborne infectious agents of concern in source waters by evaluating significant health effects and occurrence of unregulated contaminants [14].

The genus *Naegleria* attracted much more attention, especially from the biomedical world, when it was found that some *Naegleria* isolates cause a fatal brain infection named primary amoebic meningoencephalitis (PAM) in humans. The infection almost invariably results in death [15].

In Egypt, *Naegleria* spp. were detected in surface water, ground water, swimming pool samples, and tap water [16]. The aim of this work to apply rapid and accurate technique (real time PCR) to detect and identify *Naegleria* species in various type of water.

## MATERIALS AND METHODS

A total of 72 water samples (Nile, ground and tap water) were collected from Giza governorate during one year. Nile water (24 samples), tap water (24 samples) and Ground water (24 samples) were collected from Al Ayat district, Giza - Egypt. Two water samples (1 liter volume each) were collected every two weeks from each type of water. Samples were collected in clean, dry autoclavable polypropylene containers and sent to parasitological laboratory, National Research Centre where they were processed at the same day of collection. Samples were transported at ambient temperature [17, 18]. Each sample was filtered through a nitrocellulose membrane filter (0.45 µm pore size and 47 mm diameter) using a stainless steel filter holder connected with a suction pump. After filtration process, the membrane was inverted face to face on the surface of a non-nutrient agar (NNA) plate seeded with heat-killed *Escherichia coli*. The plate was wrapped with parafilm and incubated at 30$^{o}$C for the cultivation of *Naegleria* amoebae. Incubated plates were daily examined by inverted microscope (Olympus CXK 41, Japan) for 7 days for the presence of any amoebic growth [17, 18]. The morphologically positive samples for amoebae were processed by real time PCR (rt PCR) for identification of *Naegleria* species using genus specific primer.

The morphologically positive isolates of *Naegleria* were subjected to molecular characterization. *Naegleria* Cyst walls were perforated by three consecutive applications of freezing and thawing in liquid nitrogen, followed by incubation in a water bath at 100°C for 10 minutes. *Naegleria* DNA was then extracted using Ez-10 spin column fungal genomic DNA mini-preps kit (BIO BASIC CANDA INC.). Real-time PCR reactions were performed in 20µl containing 4µl 5x HOT FIREPol® EvaGreenqPCR Mix, 2µl DNA (1–50.0 ng/µl), 2µl (4 mM $MgCl_{2)}$, 1µl forward primer (5´- gaacctgcgtagggatcattt - 3´) in concentration10 pmol/µl, 1µl reverse primer (5´- tttcttttcctcccctta tta -3´) in concentration 10 pmol/µl and 10µl PCR deionized water [19]. Amplifications for *Naegleria* spp. programmed as follows: 1 pre-PCR heat cycle at 95°C for 10 min; 40 cycles at 95°C for 30 sec, 60°C for 45 sec. Finally a melting temperature ramp was from 65 to 95 °C at 0.3 °C/s according to the manual instruction. By increasing number of cycles during amplification, fluorescence values from the examined DNA increase, demonstrating the presence of the target organism. Real time PCR *Naegleria*-positive isolates were processed by conventional PCR to confirm that the PCR products of these isolates were at 409 bp.

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**Statistical analysis**

The obtained data were statistically analyzed using one-way ANOVA through GarphPad Prism statistical program. A p value <0.05 was considered statistically significant [20].

## RESULTS

The highest occurrence of free living amoebae (100 %) was recorded in Nile water, followed by 58.3 % and 50 % in ground water and tap water, respectively. It was found that 34.7 % out of 72 examined water samples belonged to genus *Naegleria* by real time PCR. By real time PCR, the highest occurrence of *Naegleria* spp. (54.2 %) was recorded in Nile water, followed by 25 % in each of tap and ground water samples (Table 1, figure 1, 2 & 3). P. Value = 0.024 (i.e. less than 0.05) so various water types had a significant effect on the prevalence of *Naegleria* spp. by using one-way ANOVA (Table 2). The PCR products (obtained from conventional PCR) for rt PCR-*Naegleria* positive isolates were confirmed at 409 bp.

Concerning seasonal variations, the highest occurrence of *Naegleria* spp. in Nile water was recorded in autumn (75 %), followed by 66.7% in spring and 33.3 % in each of winter and summer. The highest occurrence of *Naegleria* spp. in tap water was recorded in both winter and spring (33.3 %), while the lowest was recorded in each of summer and autumn (16.7 %). In ground water, the highest occurrence of *Naegleria* spp. was recorded in both winter and autumn (33.3 %), while the lowest was recorded in both summer and spring (16.7 %) (Table 3). By conventional statistical criteria, the prevalence of *Naegleria* spp. in Nile water was not significantly affected by the seasons using one way ANOVA (P>0.05) (Table 4). Seasonal variation had no significant effect on the prevalence of *Naegleria* spp. in tap water by one way ANOVA (P>0.05) (Table 5). P. Value = 0.802 (i.e. more than 0.05) so that seasonal variations had no significant effect on the prevalence of *Naegleria* spp. in examined water types by one way ANOVA (Table 6).

**Table 1: Real time PCR detection of *Naegleria* spp. in different water types.**

| Water type | Number of examined samples | positive amoebic culture | | rt PCR-*Naegleria* +ve samples | |
|---|---|---|---|---|---|
| | | No. | % | No. | % |
| Nile water | 24 | 24 | 100 | 13 | 54.2 |
| Tap water | 24 | 12 | 50 | 6 | 25 |
| Ground water | 24 | 14 | 58.3 | 6 | 25 |
| Total | 72 | 50 | 69.4 | 25 | 34.7 |

**Table 2: One-way ANOVA for prevalence of *Naegleria* spp. in various water types**

| | SS | DF | MS | F | P value |
|---|---|---|---|---|---|
| variability among the water types (between columns) | 2.722 | 2 | 1.361 | 4.115 | 0.0254 |
| Variability within the water types (within columns) | 10.92 | 33 | 0.3308 | | |
| Total | 13.64 | 35 | | | |

**DF:** Degree of freedom **SS:** Sum of squares **MS:** Mean square
**F:** F-distribution variable **P:** Significance

**Table 3: Seasonal variation of *Naegleria* spp. in different water types.**

| Seasons | Number of examined samples for each water type | rt PCR-*Naegleria* positive samples | | | | | |
|---|---|---|---|---|---|---|---|
| | | Nile water | | Tap water | | Ground water | |
| | | No. | % | No. | % | No. | % |
| Winter | 6 | 2 | 33.3 | 2 | 33.3 | 2 | 33.3 |
| Spring | 6 | 4 | 66.7 | 2 | 33.3 | 1 | 16.7 |
| Summer | 6 | 2 | 33.3 | 1 | 16.7 | 1 | 16.7 |
| Autumn | 6 | 5 | 83.3 | 1 | 16.7 | 2 | 33.3 |

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**Table 4: One-way ANOVA for testing the seasonal variation of *Naegleria* spp. in Nile water**

| | SS | DF | MS | F | P value |
|---|---|---|---|---|---|
| Variability among seasons (between columns) | 2.25 | 3 | 0.75 | 2.250 | 0.1598 |
| Variability within seasons (within columns) | 2.667 | 8 | 0.3333 | | |
| Total | 4.917 | 11 | | | |

**DF:** Degree of freedom **SS:** Sum of squares **MS:** Mean square
**F:** F-distribution variable **P:** Significance

**Table 5: One-way ANOVA for testing the seasonal variation of *Naegleria* spp. in tap water**

| | SS | DF | MS | F | P value |
|---|---|---|---|---|---|
| Variability among seasons (between columns) | 0.333 | 3 | 0.1111 | 0.3333 | 0.8018 |
| Variability within seasons (within columns) | 2.667 | 8 | 0.3333 | | |
| Total | 3 | 11 | | | |

**DF:** Degree of freedom **SS:** Sum of squares **MS:** Mean square **F:** F-distribution variable **P:** Significance

**Table 6: One-way ANOVA for testing the seasonal variation of *Naegleria* spp. in ground water**

| | SS | DF | MS | F | P value |
|---|---|---|---|---|---|
| Variability among seasons (between columns) | 0.333 | 3 | 0.1111 | 0.3333 | 0.802 |
| Variability within seasons (within columns) | 2.667 | 8 | 0.3333 | | |
| Total | 3 | 11 | | | |

**DF:** Degree of freedom **SS:** Sum of squares **MS:** Mean square
**F:** F-distribution variable **P:** Significance

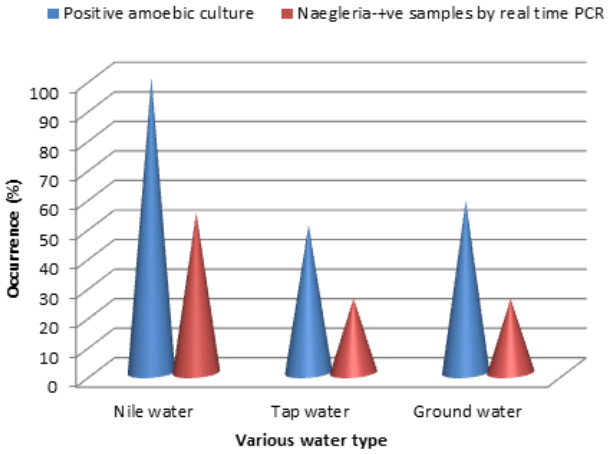


**Figure 1: Real time PCR detection of *Naegleria* spp. in different water types.**

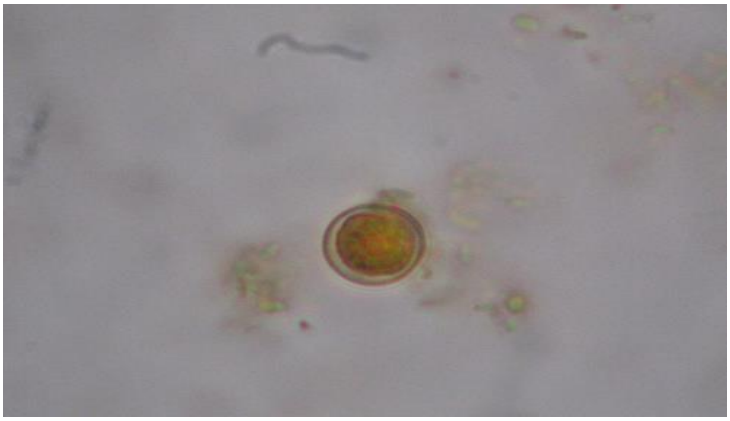

**Figure 2: Photomicrograph for cystic form of *Naegleria* spp. stained with Lugol's iodine.**

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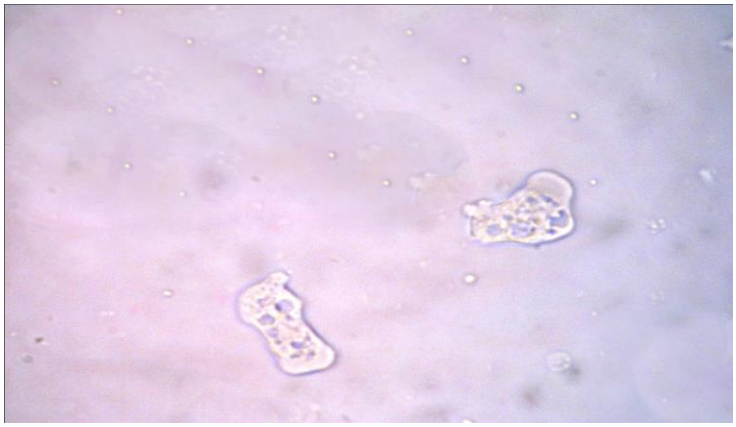

**Figure 3: Photomicrograph for living unstained trophozoite form of *Naegleria* spp.**

## DISCUSSION

Results of the present study revealed that members of genus *Naegleria* were detected by real time PCR in 54.2 % of Nile water samples. In Egypt, Al-Herrawy et al [16] found that a lower percentage of *Naegleria*-positive PCR samples reached 45.8 % in 48 Nile water samples. Other workers in Egypt detected *Naegleria* amoebae in surface waters from Lower Egypt and they mentioned that the isolated *Naegleria* amoebae were morphologically identified to the species level as *Naegleria fowleri* [21]. In other words, the identification of *Naegleria* amoebae to the species level could not be achieved morphologically only, but further approaches such as polymerase chain reaction (PCR) and deoxyribonucleic acid (DNA) sequencing should be applied [22,23]. Other workers, in Belgium, recorded members of genus *Naegleria* by PCR in 80 % of water samples collected from lakes and ponds [24]. In Malaysia, Ithoi *et al* [25] recorded a higher percentage of *Naegleria* spp. (100 %) in the 33 microscopically-positive isolates from recreational water. On the other hand, other workers recorded members of genus *Naegleria* by PCR in rivers in Belgium [26], Switzerland [27], Venezuela [28] and Germany [29] in percentages of 56.4, 27.7, 44.4, and 11.1 %, respectively. In Taiwan, Huang and Hsu [30] detected *Naegleria* spp. by PCR in 14.2 % recreational water samples. This difference in occurrence of *Naegleria* spp. might be due to the difference in the number of examined samples in different studies.

In the present study, the prevalence of *Naegleria* spp. by real time PCR reached 25 % in 24 tap water samples. Other researchers in Egypt detected *Naegleria* amoebae by PCR in a lower percentage (16.7 %) in 48 tap water samples [16]. Other workers in Spain, detected *Naegleria* amoebae in 31.7 % of total examined tap water samples [10]. In Australia, Dorsch *et al.* [31] established that the presence of *Naegleria* spp. was associated with the unusually high summer temperatures and inadequate chlorine residuals.

In the current study, the prevalence of *Naegleria*-positive samples by real time PCR reached 25 % in 24 ground water samples. Other researchers in Egypt recorded the same percentage of *Naegleria* in 48 groundwater samples [16]. In Thailand Lekkla *et al* [32] detected *Naegleria* spp. in 35.3 % of natural hot spring samples. The difference in detection rates of *Naegleria* spp. in different countries and localities may be influenced by geographical conditions and water sources [33, 34].

The present investigation showed that the winter season was the best one for the prevalence of *Naegleria* spp. in tap and ground water. Kyle and Noblet [35] concluded that the rainfall caused runoff of clay into Lake Issaqueena that led to the increased availability of particulate matter and subsequently higher concentrations of amoebae in surface waters. Other workers in Oklahoma, USA, also reported that high rainfall was associated with increased agitation of sediment within stock and golf-course ponds and a subsequent increase in the isolation of amoebae [36]. These results supported that of the current study because the winter in Egypt is the rain fall season.

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## REFERENCES


[1] APHA. Standard method for the examination of water and wastewater. 22th Ed 2012, APHA, WEF and AWWA, Washington, DC.
[2] Sifuentes LY. The occurrence of free living amoebae in water, Ph. D. thesis 2012; the university of Arizona, USA.
[3] Craun GF, Brunkard JM, Yoder JS, Roberts VA, Carpenter J, Wade T, Calderon RL, Roberts JM, Beach MJ, Roy SL. Clin Microbiol Rev 2010; 23: 507-528.
[4] Brunkard JM, Ailes E, Roberts VA, Hill V, Hilborn ED, Craun, GF, Rajasingham, A, Kahler A, Garrison L, Hicks L, Carpenter J, Wade TJ, Beach MJ, Msw JS. MMWR Surveill Summ 2011; 60: 38-68.
[5] Shin H and Im K. Korean J Parasitol 2004; 42: 93-119.
[6] Leduc A, Gravel S, Abikhzer, J, Roy S, Barbeau J. Can J. Microbiol 2012; 58: 884-886.
[7] Conza L, Pagani SC, Gaia V. PLOS One 2013; 8:e68244.
[8] Niyyati M, Lasjerdi Z, Nazar M, Haghighi A, Nazemalhosseini ME. Iran J Wat Health 2012; 10:140-146.
[9] Yousuf FA, Siddiqui R, Subhani F, Khan NA. J Wat Health 2013; 11:371-375.
[10] Garcia A, Goñi P, Cieloszyk J, Fernandez MT, Calvo-Beguería L, Rubio E, Fillat MF, Peleato ML, Clavel A. Environ Sci Technol 2013; 47: 3132-3140.
[11] Solgi R, Niyyati M, Haghighi A, Mojarad EN. Iran J Parasitol 2012; 7:47-52.
[12] De Jonckheere JF. Appl Environ Microbiol 1977; 33:751-757.
[13] Zbikowska E, Walczak M, Krawiec A. Parasitol Res 2013; 112:77-83.
[14] United States Environmental Protection Agency. Contaminant Candidate List - CCL3 2008.
[15] De Jonckheere JF. Acta Protozool 2002; 41:309-342.
[16] Al-Herrawy AZ, Mohamed SH, Ali M, Mohammed AH, Gad MA. EJER 2014; 2: 1-13.
[17] HPA. Isolation and identification of *Acanthamoeba* species. 2004; W 17, Issue 2. http://www.iremsaglik.com/HPA/w17. (Accessed 2015, July 14).
[18] APHA Standard method for the examination of water and wastewater. 2005; 21th Ed, APHA, WEF and AWWA, Washington, DC.
[19] Pélandakis MS, Pernin P. J Eukaryot Microbiol 2000; 47:116–121.
[20] Zar JH. Biostatistical Analysis 2010. Prentice Hall New Jersey USA. doi:10.1037/0012764
[21] Mansour NS Saoud AF, Nashed NN, Youssef FG. J Egypt Soc Parasitol 1991; 21:15-22.
[22] De Jonckheere, JF. Acta Protozool 2006; 45: 91-96.
[23] Init I, Lau YL, Arin Fadzlun A, Foead AI, Neilson RS, Nissapatorn V. Trop Biomed 2010; 27: 566-577.
[24] Declerck P, Behets J, Van Hoef V, Ollevier F. Water Res 2007; 41: 3159–3167.
[25] Ithoi I, Ahmad AF, Nissapatorn V, Lau YL, Mahmud R, Mak JW. PLoS One ; 6:e24327.
[26] Behets J, Declerck P, Delaedt Y, Verelst L, Ollevier F. Curr Microbiol 2006; 53: 506–509.
[27] Gianinazzi C, Schild M, Wüthrich F, Ben Nouir N, Füchslin HP, Schürch N, Gottstein B, Müller N. Res Microbiol ; 160: 367-374.
[28] Cermeño JR, Hernández I, El Yasin H, Tinedo R, Sánchez R, Pérez G, Gravano R, Ruiz A. Rev Soc Bras Med Trop 2006; 39: 264-268.
[29] Hoffmann R, Michel R. Int J Hyg Environ Health 2001; 203: 215-219.
[30] Huang SW, Hsu BM. Parasitol Res 2010; 106:1395-1402.
[31] Dorsch MM, Cameron AS, Robinson BS. Trans R Soc Trop Med Hyg 1983; 77: 372–377.
[32] Lekkla A, Sutthikornchai C, Bovornkitti S, Sukthana Y. Southeast Asian J. Trop. Med. Public Health 2005; 36:5-9.
[33] De Jonckheere JF. Infect Genet Evol 2011; 11:1520-1528.
[34] Kao PM, Tung MC, Hsu BM, Chou MY, Yang HW, She CY, Shen SM. Parasitol Res 2013; 112:1467-1474.
[35] Kyle DE, Noblet GP. J. Protozool 1987; 33: 422-434.
[36] John DT, Howard MJ. Parasitol Res 1995; 81:193-201.