Distribution of *Naegleria* in water resources in Egypt.

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Abstract

Members of genus Naegleria are free-living amoebae inhabiting various aquatic environments. Some of heat-tolerant Naegleria species can provoke fatal infections in humans, and the most important source of infection is through enforced inhalation of water containing these species of Naegleria. Methods: A total of 192 water samples were collected from the river Nile, tap water, ground water and swimming pools. Samples were examined for detection of Naegleria species by cultivation on non-nutrient agar at 30oC and 45oC. Naegleria were identified based on trophozoite and cyst morphology, flagellation test and heat tolerance test. Results: Members of genus Naegleria were detected in 38.0% and 18.8 % of 192 cultured water samples at 30°C and 45°C, respectively. The highest occurrence of Naegleria grown at 30 °C was recorded in Nile water (70.8%), followed by 31.3% in ground water and 25% in each of tap water and swimming pool samples. On the other hand, the highest occurrence of Naegleria grown at 45 °C was recorded in Nile water (41.7%), followed by 16.7, 10.4 and 6.3% in ground water, swimming pools and tap water, respectively. Concerning seasonal variations, the greatest percentage of recovery of Naegleria (cultured at 45°C) occurred during summer in tap water and swimming pools, during autumn in ground water, during spring and summer in Nile river. The greatest percentage of recovery of Naegleria (cultured at 30°C) occurred during autumn and winter in Nile river, during autumn in tap water, during autumn, winter and summer in ground water, during the summer in swimming pools. Conclusion: The presence of heat-tolerant Naegleria species in Egyptian aquatic environment should be considered a potential health threat.

Keywords: *Naegleria* – Egyptian aquatic environment

Introduction

Naegleria are free-living amoebo flagellate amphizoic protozoa that are ubiquitous in nature. They have been found in soil, fresh water lakes and rivers (Hsu *et al.*, 2009; Niyyati *et al.*, 2012; Yousuf *et al.*, 2013), swimming pools (Heggie, 2010), therapeutic pools (Visvesvara and Stehr-Green, 1990), tap water (Edagawa *et al.*, 2009), natural thermal water(Rivera *et al.*, 1990), reservoirs (Garcia *et al.*, 2013), air samples (Rivera *et al.*, 1987;Lawande, 1983) and the gills of fresh-water fish (Shin and Im, 2004).

Naegleria amoebae belong to the family Vahlkampfiidae which contains 13 genera. Forty four species of *Naegleria* have been recognized based upon sequencing data (De Jonckheere,

2006). Most of identified *Naegleria* spp. until now have a high maximum growth temperature, usually between 37 and 45ºC (De Jonckheere, 2002).

Naegleria usually have three stages of life development. The first stage (amoeba or trophozoite stage) is morphologically cylindrical, with a size range of 10-30 um and a contracting area in the rear. It is uninucleated with its cytoplasm allowing locomotion by means of eruptive, hyaline, hemispherical bulges. When *Naegleria* tophozoites are diluted naturally by rainwater or any other means, they rapidly develop into their temporary second stage (flagellate stage), and during unfavorable conditions the third stage (cyst stage) is formed (Page, 1976).

Materials and Methods

A total of 192 water samples (tap, ground, swimming pool and Nile waters) were collected from Cairo and Giza governorates during the period from September 2011 to August 2012. Nile water (48 samples) and tap water (48 samples) samples were collected from localities in the vicinity of 4 drinking water treatment plants. Swimming pool water samples (48 samples) were collected from a social and sport club in Cairo, while ground water (48 samples) was collected from some villages in Giza. Two water samples (2 liters for each) were collected every two weeks from each type of water. Samples were collected in clean, dry autoclavable polypropylene containers and sent to the laboratory where they were processed at the same day of collection. One liter of each water sample was filtered through a nitrocellulose membrane filters (0.45 μm pore size and 47 mm in diameter) (Whatman, WCN type, Cat No. 7141-104) (APHA, 2005).After filtration the membrane was inverted face to face on the surface of a non-nutrient agar (NNA) plate seeded with *E-coli*. The plate was wrapped with parafilm and incubated at 30ºC. These steps were repeated for the second liter of the same sample, except that the incubation temperature was 45 ℃ for isolation of heat-tolerant strains of *Naegleria* (De Jonckheere, 2002).Daily microscopic examination for the presence of any amoebic growth was carried out for one week (De Jonckheere*et al.,* 1974). Flagellation test was carried out using distilled water to differentiate *Naegleria* spp. from other Vahlkampfiidae (Page, 1976). Identification of the obtained *Naegleria* spp. were achieved according to the morphological characteristics of trophic, temporary flagellate and cyst stages (Barnett *et al.,* 1996; Rose *et al.,* 2001; Visvesvara *et al.,* 2007; Bennett, 2008).

Results

● Prevalence of *Naegleria* at 30°C and 45°C in different water samples.

Members of genus *Naegleria* were detected in 38.0% and 18.8 % of the cultured water samples at 30°C and 45 °C, respectively. The highest occurrence of *Naegleria* grown at 30 °C was recorded in Nile water (70.8%), followed by 31.3% in ground water and 25% in each of tap water and swimming pool samples. On the other hand, the highest occurrence of *Naegleria* grown at 45 °C was recorded in Nile water (41.7%), followed by 16.7, 10.4 and 6.3% in ground water, swimming pools and tap water, respectively. In general, prevalence of members of genus *Naegleria* was higher in cultures grown at 30°C than at 45°C (Table 1 and figure 1, 2 and 3).

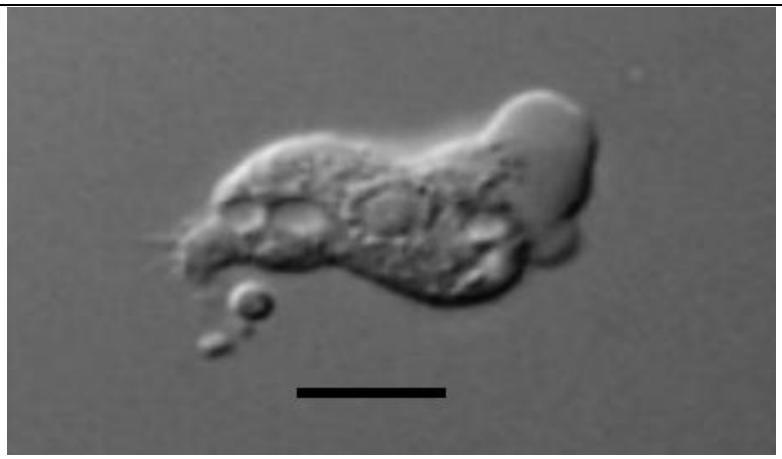


Figure (1): Unstained trophozoite form of *Naegleria.* Bar = 10 µm. 100X magnification.

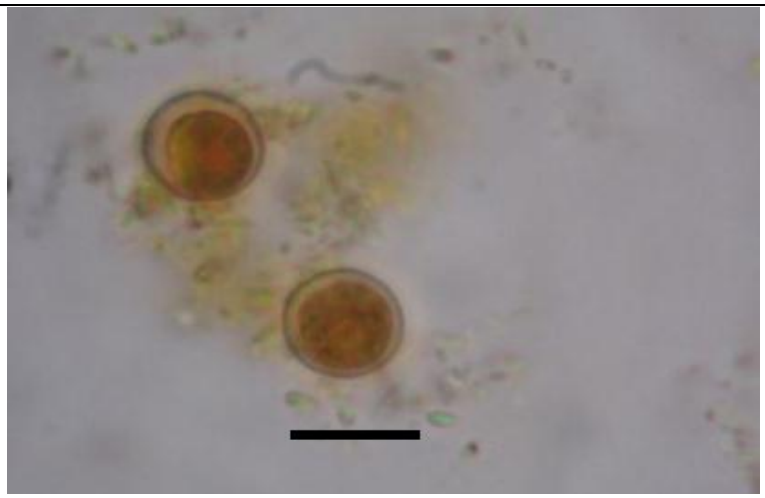
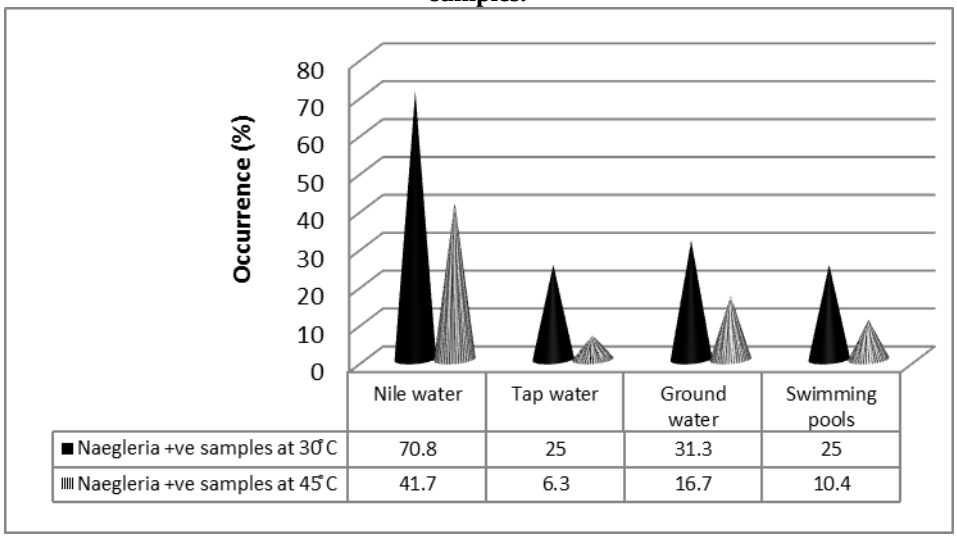


Figure (2): Cyst form of *Naegleria* stained with Lugol's iodine. Bar = 10µm.100X magnification.

Table (1): Prevalence of *Naegleria* at 30°C and 45°C in different water samples.

Water type	Number of examined samples	*Naegleria* +ve culture at 30°C		*Naegleria* +ve culture at 45°C	
		No.	%	No.	%
Nile water	48	34	70.8	20	41.7
Tap water	48	12	25	3	6.3
Ground water	48	15	31.3	8	16.7
Swimming pools	48	12	25	5	10.4
Total	192	73	38	36	18.8

Figure (3): Comparison between *Naegleria* +ve samples grown at 30°C and 45°C in different water samples.



● Seasonal variations of *Naegleria* in different water samples cultured at 30°C and 45°C:

Concerning the seasonal variations, the highest occurrence of *Naegleria* in Nile water (cultured at 30°C) was recorded in both winter and autumn (75%), while the lowest was recorded in both spring and summer (66.7%). On the other hand, the highest occurrence of *Naegleria* in Nile water (cultured at 45°C) was recorded in spring and summer (50%), while the lowest was recorded in both winter and autumn (33.3%).Concerning tap water, the highest occurrence of *Naegleria* (cultured at 30°C) was recorded in winter (41.7%), followed by 25% in summer and 16.7% in both autumn and spring. On the other hand the highest occurrence of *Naegleria* in tap water (cultured at 45°C) was recorded in summer (16.7%), followed by 8.3% in spring, while no *Naegleria* was detected in autumn and winter. Regarding ground water,

the highest occurrence of *Naegleria* (cultured at 30°C) was recorded in summer, autumn and winter (33.3%), while the lowest was in spring (25%). The highest occurrence of *Naegleria* in ground water (cultured at 45°C) was recorded in autumn (25%), followed by 16.7% in both summer and winter, while the lowest was in spring (8.3%).The highest occurrence of *Naegleria* (cultured at 30°C) was recorded in swimming pool samples collected in summer (58.3%), followed by 25 and 16.7 % in spring and winter, respectively, while no *Naegleria* was detected in autumn. On the other hand, the highest occurrence of *Naegleria* in swimming pools (cultured at 45°C) was recorded in summer (33.3%), followed by spring (8.3%), while no *Naegleria* was detected in autumn and winter (Table 2 and figure 4,5,6 and 7).

Figure (4): Seasonal variations of *Naegleria* in Nile water cultured at 30°C and 45°C

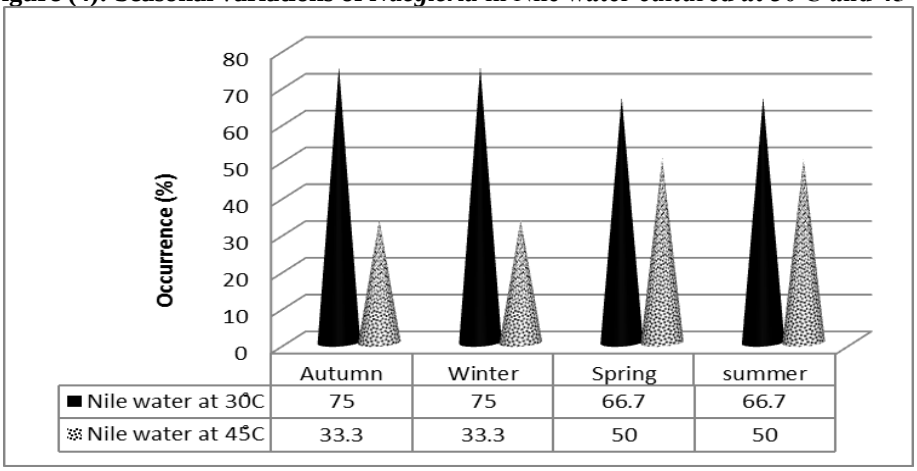


Figure (5): Seasonal variations of *Naegleria* in tap water cultured at 30°C and 45°C

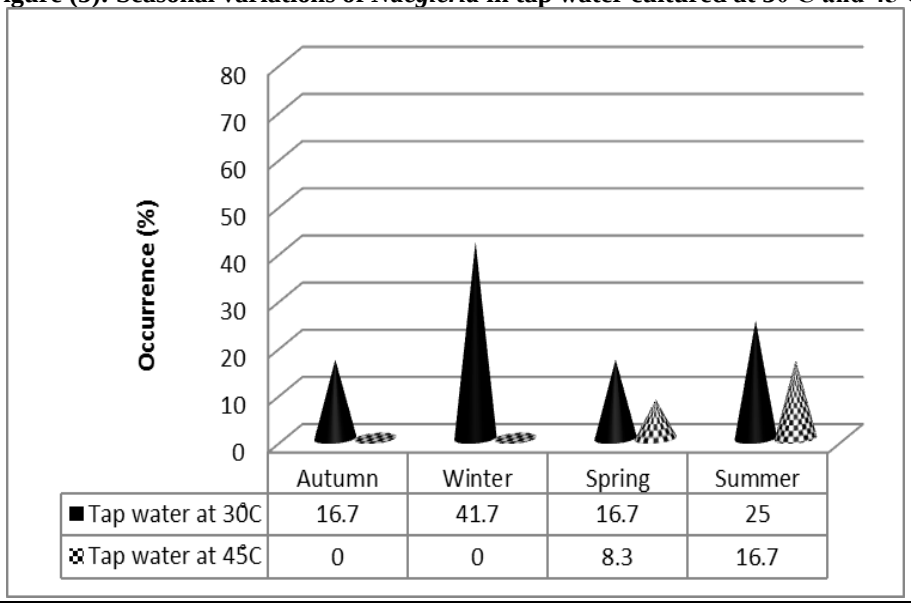


Figure (6): Seasonal variations of *Naegleria* in ground water cultured at 30°C and 45°C.

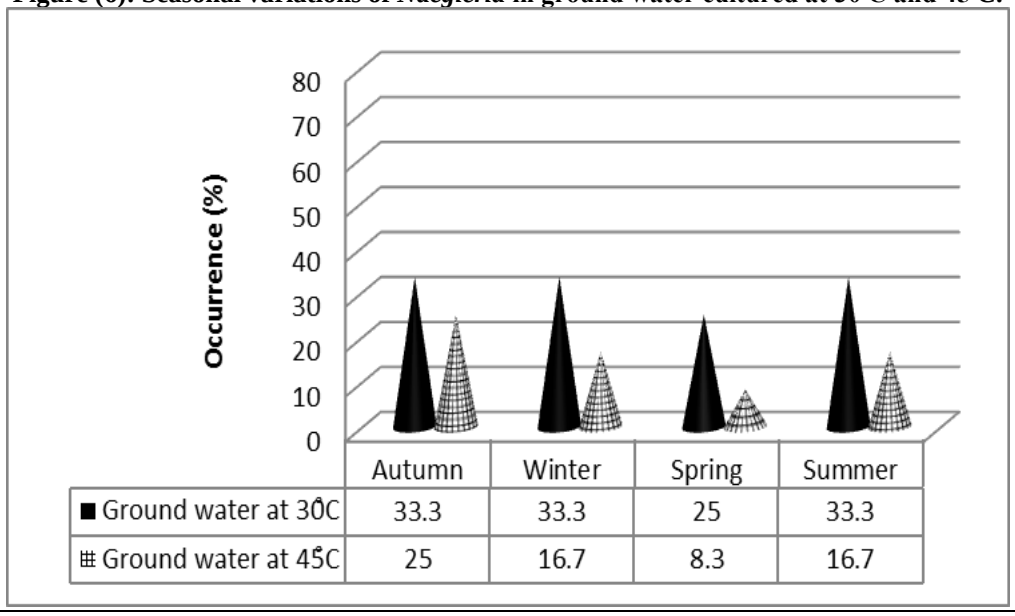


Figure (7): Seasonal variations of *Naegleria* in Swimming pools cultured at 30°C and 45°C

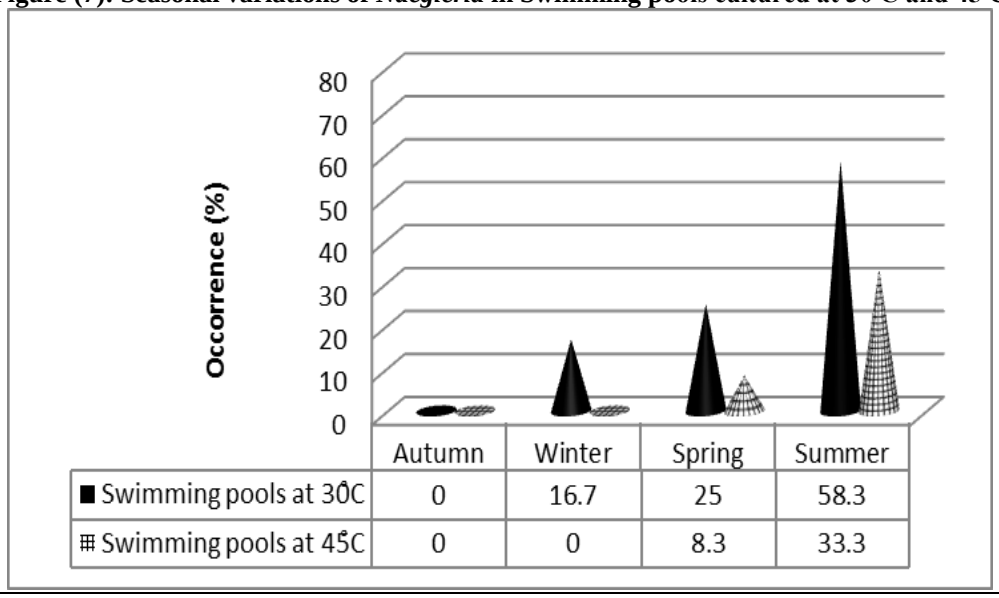


Table (2): Seasonal variations of *Naegleria* in different water samples cultured at 30°C and 45°C

Season	Number of cultured samples	Culture positive samples															
		Nile water				Tap water				Ground water				Swimming pools			
		At 30C		At 45C		At 30C		At 45C		At 30C		At 45C		At 30C		At 45C	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Autumn	12	9	75	4	33.3	2	16.7	0	0	4	33.3	3	25	0	0	0	0
Winter	12	9	75	4	33.3	5	41.7	0	0	4	33.3	2	16.7	2	16.7	0	0
Spring	12	8	66.7	6	50	2	16.7	1	8.3	3	25	1	8.3	3	25	1	8.3
Summer	12	8	66.7	6	50	3	25	2	16.7	4	33.3	2	16.7	7	58.3	4	33.3
Total	48	34	70.8	20	41.7	12	25	3	6.3	15	31.3	8	16.7	12	25	5	10.4

Discussion

Prevalence of *Naegleria* in different water samples:

In the present study, the highest occurrence of *Naegleria* grown at 30°C was recorded in Nile water (70.8%), followed by 31.3% in ground water and 25% in each of tap water and swimming pool samples. Other workers, in Egypt, examined different water types and reported that the greatest percentage of *Naegleria* (27.5%) was found in swimming pool samples (Hikal, 2010).

Results of the present study revealed that members of genus *Naegleria* were detected in 41.7% of the cultured Nile water samples at 45°C. Other workers in Egypt, (Hikal, 2010) detected lower a percentage of *Naegleria* (24.6%) in the examined freshwater samples cultured at 45°C. Also, Hamadto *et al.* (1993) recorded a higher percentage of *Naegleria* (60%) in 10 surface water & canals samples from different governorates in Egypt, but they did not mention the incubation temperature used. This difference in occurrence of *Naegleria* in the Nile river might be due to the difference in the number of examined samples in different studies. 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* (Mansour *et al.,* 1991). 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 (Init *et al.,* 2010).In Saudi Arabia, Al-Herrawy and Al-Rashied (1995) detected *Naegleria* in 47.22% of the examined freshwater samples from Wadi Hanifa, Ryadh region. In other countries like Venezuela, Cermeño *et al.* (2006) detected *Naegleria* spp. in44.4% (n=4) of a river water. The result of Cermeño *et al.* (2006) was nearly similar to our result. Other workers in USA detected *Naegleria* from James river in a percentage of 20% (Ettinger *et al.,* 2003). In Malaysia, Ithoi *et al.* (2011) did not detect *Naegleria* (cultured at 44°C) in recreational waters. The difference in detection rates of *Naegleria* in different countries and localities may be influenced by geographical conditions and water sources (De Jonckheere, 2011; Kao *et al.,* 2013a and b).

In the present work, it was found that the prevalence of *Naegleria* (grown at 45°C) reached 6.3% in the examined tap water samples. Other workers in Egypt detected *Naegleria* in 16.7%

(Hikal, 2010) and 9.7% (Hilali *et al.,* 1994) of the examined tap water samples. On the contrary, Ithoi *et al.* (2011) in Malaysia recorded *Naegleria* in 100% of drinking water tank samples grown at 37^{o}C, while no *Naegleria* grown at 44^{o}C was isolated at all. In Turkey, Ozçelik *et al.,* (2012) detected *Naegleria* in 10.0% of tap water samples.

Results of the present study showed that the occurrence of *Naegleria* reached 31.3% in ground water. To the best of our knowledge, there was no previous record concerning *Naegleria* in ground water in Egypt. Higher records were observed in 35.3% (24/68) of natural hot springs in Thailand (Lekkla *et al.,* 2005) and 43.3% of four examined thermal springs in Taiwan (Kao *et al.,* 2013a).

The current result showed that the prevalence of *Naegleria* in swimming pools reached 25%. Compared with our results and also in Egypt, Hikal (2010) detected *Naegleria* in a nearly similar percentage (27.5%), while Hamadto *et al.* (1993) recorded higher occurrence of *Naegleria* (75%) in swimming pool samples. This difference might be due to the difference in the number of examined samples and the way by which swimming pool waters were disinfected and treated (Dorsch, 1983). Other workers in New Zeland (Brown, 1983), Mexico (Rivera *et al.,* 1983) and Thailand (Lekkla *et al.,* 2005) recorded higher occurrence of *Naegleria* (60, 75.8 and 34.8%, respectively) in swimming pool samples. Init *et al.* (2010) in Malaysia detected *Naegleria* in 100% of the cultured swimming pools (n=14) at 37^{o}C, while no *Naegleria* species were detected from the same samples cultured at 44^{o}C.Ithoi *et al.* (2011) also in Malaysia recorded no *Naegleria* from swimming pool waters cultured at 44^{o}C.

Morphological characterization of *Naegleria* amoebae:

In the present study, it was found that trophozoites of *Naegleria* were long slender or oval, with a size range of 10-30 µm. *Naegleria* trophozoites were also characterized by a single vesicular nucleus and a single broad hemispherical hyaline eruptive lobose pseudopodium. These findings are in agreement with other workers (Page, 1974; Marciano-cabral, 1988; Al-Herrawy, 1992; Al-Herrawy and Al-Rashied, 1995; 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; Johan, 1982). The current result revealed that the cyst of *Naegleria* amoebae was spherical measuring 7-14 um in diameter and

composed of a double cell wall containing a thick endocyst and a thin ectocyst. Also cyst cell wall of *Naegleria* had hardly detectable 4-5 pores. These findings are in agreement with those of other workers (Barnett *et al.,* 1996; Rose *et al.,* 2001; Schuster and Visvesvara, 2004; Visvesvara *et al.,* 2007; Bennett, 2008). *Naegleria* species were too similar morphologically to be distinguished from each other at the level of ordinary microscope. This conclusion was also reached by De Jonckheere (1977) and Ashmawy *et al.* (1993).

Seasonal variations of genus *Naegleria*:

Concerning the seasonal variations in the present study, the highest occurrence of *Naegleria* in Nile water (cultured at 30°C) was recorded in winter and autumn (75% in each), while the lowest occurrence was recorded in spring and summer (66.7% in each). On the other hand, the highest occurrence of *Naegleria* cultured at 45°C was recorded in spring and summer (50% in each), while the lowest occurrence was recorded in winter and autumn (33.3% in each). In USA, Kyle and Noblet (1987b) found that *Naegleria* species were the most prevalent free-living amoebae in Lake Issaqueena especially during late summer. Kyle and Noblet (1987a) discussed 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 (John and Howard, 1995).

In the present work, the highest occurrence of *Naegleria* cultured at 45°C was recorded in Nile river water in spring and summer (50% in each), while the lowest occurrence was recorded in winter and autumn (33.3% in each).This result was in accordance with that of Ettinger *et al.* (2003) in Virginia, USA who recorded that *Naegleria* was mostly common in James river in spring and early to mid-summer. Moreover, John and Howard (1995) reported that the greatest percentage of recovery of pathogenic free-living amoeba (including *Naegleria*) occurred during the spring and autumn.

The current study revealed that the highest occurrence of *Naegleria* (cultured at 30°C) in tap water was recorded in winter (41.7%), followed by 25% in summer and 16.7% in each of autumn and spring. In north-central USA, *Naegleria* amoebae were detected in tap water in autumn, with *N. fowleri* being detected in summer (Marciano -Cabral *et al.*, 2010). In

Arizona, USA, it was founded that more positive tap water samples for *Naegleria* were identified during periods with higher average water temperatures (≥30.1°C) than during the period with a water temperature of 22.9°C (Sifuentes, 2012). *Naegleria fowleri* could be inactivated by using free chlorine concentration of 9-24 mg min/L (De Jonckheere and Van de Voorde, 1976).Other workers found that *Naegleria fowleri* could be inactivated by monochloramine with Ct values ranging from 44.5 to 51.6 mg min/L (Pringuez *et al.,* 2001). On the other hand, inactivation of *N. gruberi* could be achieved by the free chlorine dose 12.1 mg min/ L (Rubin *et al.,* 1983).

To date, heat-tolerant *Naegleria* species are identified namely *N. fowleri, N. australiensis* and *N. lovaniensis*. Concerning pathogenicity, *N. fowleri* is the cause of primary amebic meningo-encephalitis (PAM), a rapidly fatal infection involving the CNS (central nervous system). The PAM disease, which occurs naturally in humans, can be produced experimentally in mice. On the other hand, *N. australiensis* can cause a similar disease in experimentally infected mice, but it has not yet been isolated from humans. Nonetheless, because *N. australiensis* is pathogenic to mice, it should be considered a potential human pathogen (David *et al.,* 1998).

Recommendations: In high-risk areas, regular monitoring of recreational waters for potentially pathogenic *Naegleria* species (especially *N. fowleri*) should be considered and appropriate warnings should be posted.

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