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Research Article

Real Time PCR Detection of *Acanthamoeba* Species in the Egyptian Aquatic Environment

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ABSTRACT

Members of *Acanthamoeba* cause three main types of illness involving the brain and spinal cord (Granulomatous encephalitis), the eye (*Acanthamoeba* keratitis), and infections that can spread throughout the entire body (disseminated infection). A total of 96 water samples (Nile, ground, tap and swimming pool) were collected for detection of *Acanthamoeba* species that were cultivated on non-nutrient agar at 30ºC. The isolated strains of *Acanthamoeba* spp. were identified based on the morphologic criteria of trophozoites and cyst stages. Molecular characterization of the isolated strains of amoebae was performed by using real time polymerase chain reaction (PCR). The results of the present study *Acanthamoeba* spp. was detected in 26 % of 96 different water samples by culture and in 22.9% by real time PCR. Percentages were 41.7 %, 29.2 %, 12.5 % and 8.3 % in Nile water, swimming pool water, ground water and tap water, respectively by real time PCR. Statistically, type of water had a strong significant effect on the detection rate of *Acanthamoeba* spp which prevailed in warmer months in different water types. However, seasonal association of *Acanthamoeba* spp. with water types was not statistically significant. In conclusion, due to the prevalence of *Acanthamoeba* species in many diverse environmental settings, more public awareness is needed about general hygiene procedures to prevent disease.

Keywords: *Acanthamoeba* – Real time PCR – Aquatic environment – Egypt.

INTRODUCTION

Genus *Acanthamoeba* is free-living amoebae distributed ubiquitously in various ecological environments[1,2]. *Acanthamoeba* spp. are found worldwide in intestines of animals, vegetative material, dust, soil, marine water, fresh water, sediments, air, compost, sewage, tap water, and bottled water[3]. High numbers of *Acanthamoeba* spp. are found in surface layers of fresh-water lakes and sediments, corresponding to high-density of bacterial populations. Members of genus *Acanthamoeba* colonize chemical showers, hot tubs, drinking water fountains, eyewash fountains, dialysis units, dental units, air conditioning systems, swimming pools, hot-water systems and humidifiers[4-9]. The quantitative real-time PCR is a highly sensitive and specific method that is also simple, rapid, less labor-intensive, and less time-consuming. Real-time PCR has the capacity to efficiently detect and quantify pathogens, even microorganisms that are not detected or easily identified after conventional cultivation[10]. *Acanthamoeba* spp. has been reported as causative agents of serious and even fatal diseases in humans and animals. *Acanthamoeba* spp. cause diseases, such as *Acanthamoeba* keratitis, dermatitis, granulomatous amoebic encephalitis, sinusitis, chronic granulomatous lesions, pneumonitis and disseminated tissue diseases[11]. *A. griffini, A. rhysodes*, *A. lugdunensis, A. culbertsoni, A. quina, A. hatchetti, A. polyphaga,* and *A. castellanii* are the most common species infecting humans[12,13]. In addition to their own pathogenicity, *Acanthamoeba* spp. are natural hosts of many bacterial (*Legionella* spp., *Burkholderia cepacia*, *Vibrio cholerae*, *Escherichia coli* O157 and *Listeria monocytogenes*) and viral pathogens (coxsackie viruses and adeno viruses)[14-16]. Few studies were available concerning the prevalence of *Acanthamoeba* spp. in Egypt. In Egyptian aquatic environment, *Acanthamoeba* were isolated from Nile water, drinking water and swimming pool[7-9]. Because of these threats and their potential impact on human health, it is crucial to assess the prevalence of *Acanthamoeba* spp. in the Egyptian aquatic environment.

MATERIAL AND METHODS

A total of 96 water samples (Nile, ground, tap and swimming pool waters) were collected from Cairo and Giza governorates during one year. Nile water (24 samples) and tap water (24 samples) samples were collected from localities in the vicinity of drinking water treatment plant. Swimming pool water samples (24 samples) were collected from a private and sport club in Cairo, while ground water (24 samples) was collected from Al Ayat district in Giza. Two water samples (1liter 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

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processed at the same day of collection. Samples were transported at ambient temperature[3,17]. 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 30ºC for the cultivation of *Acanthamoeba* amoebae. Incubated plates were daily examined by inverted microscope (Olympus CXK 41, Japan) for 7 days for the presence of any amoebic growth[3,17]. The grown *Acanthamoeba* trophozoites were characterized from other free-living amoebae by their acanthopodia described as pointed pseudopodia, while the cyst forms were easily identified by their double cyst wall and conventionally stellate shape[18]. The morphologically positive isolates of *Acanthamoeba* were subjected to molecular characterization*Acanthamoeba* 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. *Acanthamoeba* 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´-** tttgaattcgctccaatagcgtatattaa - **3´**) in concentration10 pmol/µl, 1µl reverse primer (**5´-** tttgaattcagaaagagctatcaatctgt **-3´**) in concentration 10 pmol/µl and 10µl PCR deionized water[19]. Amplifications for *Acanthamoeba* spp. programmed as follows: 1 pre-PCR heat cycle at 95°C for 10 min; 40 cycles at 95°C for 60 sec, 60°C for 60 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.

Statistical analysis

The obtained data were statistically analyzed using one-way ANOVA through Minitab statistical program (Minitab Inc., Pennsylvania – USA). A p value <0.05 was considered statistically significant[20].

RESULTS

Acanthamoeba spp. was detected in 26.0 % out of 96 cultured water samples. By culturing method, the highest occurrence of *Acanthamoeba* spp. was recorded in Nile water (50 %), followed by 33.3 %, 12.5 % and 8.3 % in swimming pool, ground water and tap water, respectively. Statistically, type of water (Nile, Ground, Tap and swimming pools) had a strong significantly affect (P-value = 0.001) on the prevalence of *Acanthamoeba* spp. *Acanthamoeba* spp. was detected by real time PCR in 41.7 %, 29.2 %, 12.5 % and 8.3 % in Nile water, swimming pool water, ground water and tap water, respectively (Table 1, figure 1, 2, 3 and 4). Concerning seasonal variations, the highest occurrence of *Acanthamoeba* spp. in Nile water was recorded in summer (83.3 %), followed by 33.3 % both in autumn and spring, and 16.7 % in winter. Statistically, the difference in seasons was not significant (P-value = 0.119) on the prevalence of *Acanthamoeba* spp. in Nile water. On the other hand, the occurrence of *Acanthamoeba* spp. in ground water was similar (16.7 %) in summer, spring and winter. While no *Acanthamoeba* spp. was detected in autumn. In contrary, by statistical examinations, seasons had no significant effect (p-value = 0.802) on the prevalence of *Acanthamoeba* spp. in ground water. The prevalence of *Acanthamoeba* spp. in tap water was similar (16.7 %) in both summer and winter. While no *Acanthamoeba* spp. was found in spring and autumn. Moreover, seasons differences was not significant (P-value = 0.596) on the prevalence of *Acanthamoeba* spp. in tap water by using one-way ANOVA. On the other hand, the highest prevalence of *Acanthamoeba* spp. in swimming pool water was recorded in summer (50 %), followed by 33.3% in autumn, while the lowest was recorded in both winter and spring (16.7 %). By statistical analysis, the prevalence of *Acanthamoeba* spp. in swimming pool water was not significantly affected by the seasons using one-way ANOVA (Table 2, figure 5).

DISCUSSION

The occurrence of waterborne protozoan pathogens needs to be monitored in order to assess the human risk from a variety of illnesses ranging from gastroenteritis, keratitis, and infections of the lungs and skin to more serious illnesses such as granulomatous amoebic encephalitis (caused by *Acanthamoeba*), and primary amoebic meningoencephalitis (caused by *N. fowleri*)[21]. The present study is dealt with the natural distribution of *Acanthamoeba* app. by using real time PCR in the Egyptian aquatic environment. To the best of our knowledge, no previous studies concerning this subject in Egypt were published. In this investigation, it was found that *Acanthamoeba* species prevailed in Nile water more than other different water types. It has been established previously that *Acanthamoeba* spp. was the most common and opportunistic amphizoic protozoa[22]. In Egypt, *Acanthamoeba* spp. was isolated from 97.1 % of 24 inlet of the drinking water treatment plant (A branch from Nile river)[8]. Other researchers in Egypt, detected 56.0 % of *Acanthamoeba* spp. in of Nile water samples[7]. In Egypt, Lorenzo-Morales *et al.*,[23] detected a slightly higher occurrence of *Acanthamoeba* (43.3%) in freshwater samples using a genus-specific primer. Other study recorded the percentage of *Acanthamoeba* spp. was 26.4 % in the river water[24]. The difference in detection rates of amoebae in different countries and localities may be influenced by geographical conditions and water sources[10,25]. In the present study, *Acanthamoeba* spp. was detected by real time PCR in 29.2% of the tested swimming pool water samples. Other researchers in Egypt recorded a higher occurrence (49.2%) of *Acanthamoeba* species detected by culturing method in water samples collected from 10 swimming pools in Cairo[7]. The differences in the results between the two studies might be due to the using of different techniques. Other researchers in Brazil recorded a lower occurrence (20 %) of

Table 1: Detection of *Acanthamoeba* spp. in different water types by culturing method and Real time PCR.

Water type	Total number of examined samples	Positive samples for *Acanthamoeba* by culturing method		Positive samples for *Acanthamoeba* by real time PCR	
		No.	%	No.	%
Nile	24	12	50	10	41.7
Ground	24	3	12.5	3	12.5
Tap	24	2	8.3	2	8.3
Swimming pool	24	8	33.3	7	29.2
Total	96	25	26.0	22	22.9

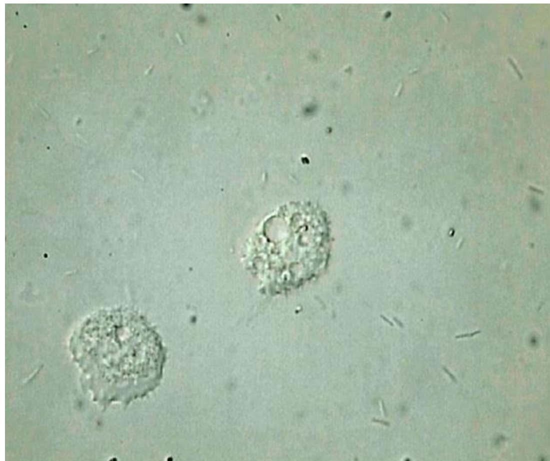

Figure 1: Photomicrograph for living unstained trophozoite form of *Acanthamoeba* spp.

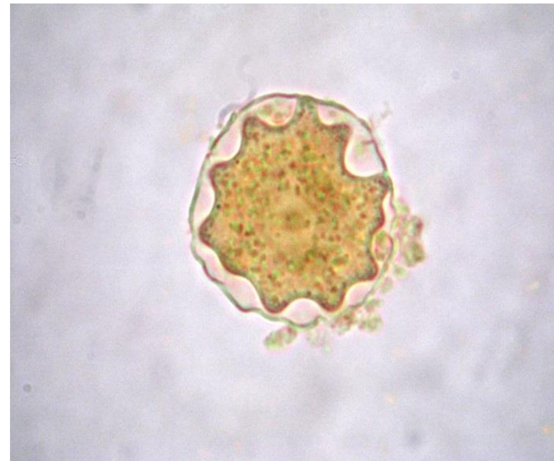

Figure 2: Photomicrograph for cystic form of *Acanthamoeba* spp. stained with Lugol's iodine.

Table 2: Seasonal variations of *Acanthamoeba* spp. in different water types.

Seasons	Total number of examined samples for each water type	Prevalence of *Acanthamoeba* spp. in different water types							
		Nile		Ground		Tap		Swimming pool	
		No.	%	No.	%	No.	%	No.	%
Winter	6	1	16.7	1	16.7	1	16.7	1	16.7
Spring	6	2	33.3	0	16.7	1	0	1	16.7
Summer	6	5	83.3	1	50	3	16.7	3	50
Autumn	6	2	33.3	0	33.3	2	0	2	33.3

Acanthamoeba spp. in 65 swimming pools water samples. Interestingly there is no single case of *Acanthamoeba* granulomatous encephalitis or *Acanthamoeba* keratitis reported in Egypt, despite the prevalence of *Acanthamoeba* in the Egyptian aquatic environment. This is probably due to lack of awareness and the difficulty in diagnosis. The present investigation showed that *Acanthamoeba* spp. was detected in 8.3 % out of 24 tap water samples. In Egypt, *Acanthamoeba* spp. was detected in 37.5 % of the examined 48 tap water samples[8]. Other researchers in Holland isolated *Acanthamoeba* spp. from 30 % of 27 tap water samples. In Korea, ten percent of water samples were contaminated by *Acanthamoeba.* Jeong and Yu[26] reported 6% of *Acanthamoeba* spp. in domestic tap water in Busan Korea. The differences in occurrence percentages of *Acanthamoeba* spp. in the sources of drinking water treatment plants leads to differences in contamination percentages of drinking water by these organisms. The current study showed that *Acanthamoeba* spp. was detected in 12.5 % of the 24 examined ground water samples. Other researchers in Mexico recorded a higher percentage (67.3 %) of *Acanthamoeba.* in ground water samples[27]. The authors concluded that the prevalence of *Acanthamoeba* in groundwater due to at least in part to the structure of the cyst wall. The cyst wall contains cellulose and confers a high degree of protection allowing them to tolerate a wide range of environmental conditions. The present investigation showed that *Acanthamoeba* spp. prevailed in summer months. The increase in detections from spring to summer months was seen in all genera except for *Naegleria* in which case the percentage of households positive was lower than it was in the spring and fall. There was no appreciable difference in detections across the years of the study[2]. Kao *et al.*[10] found that the greatest percentage of *Acanthamoeba* species is detected during summer (32.4%), followed by winter (8.8%), spring (2.9%) and autumn (2.9%). The authors discussed that rain fall in Taiwan mainly occur in summer, with occasional

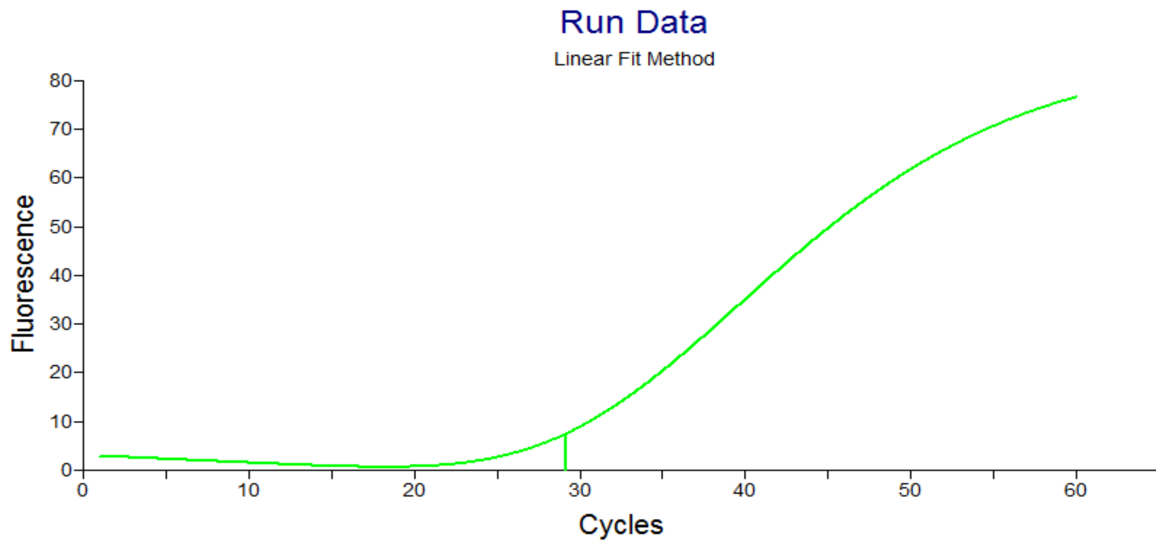

Figure 3: Analysis of real-time PCR, with increasing number of cycles during amplification, the values of fluorescence from genus *Acanthamoeba*-DNA increase, demonstrating the presence of genus *Acanthamoeba*.

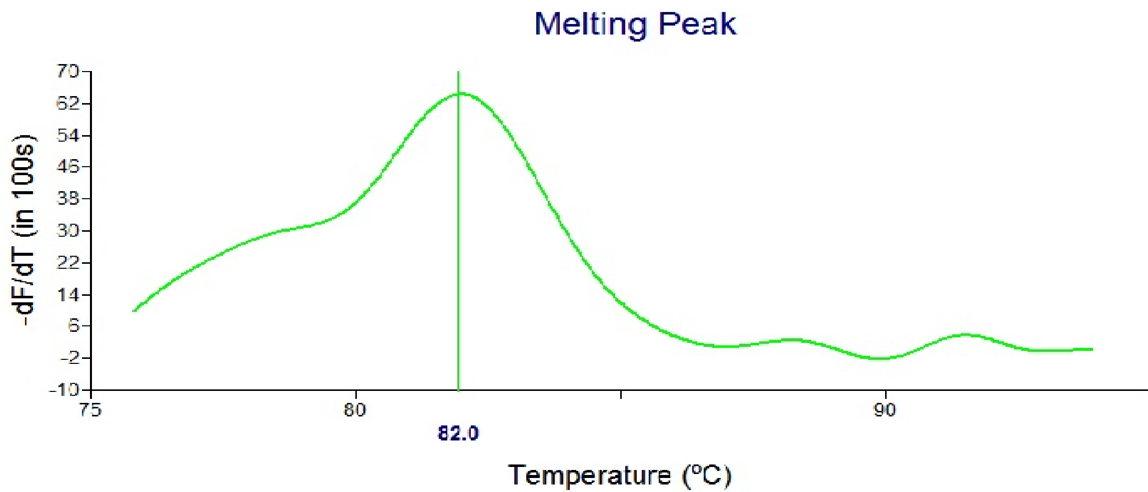

Figure 4: Melting curves of the DNA product from real time PCR amplification for genus *Acanthamoeba.*

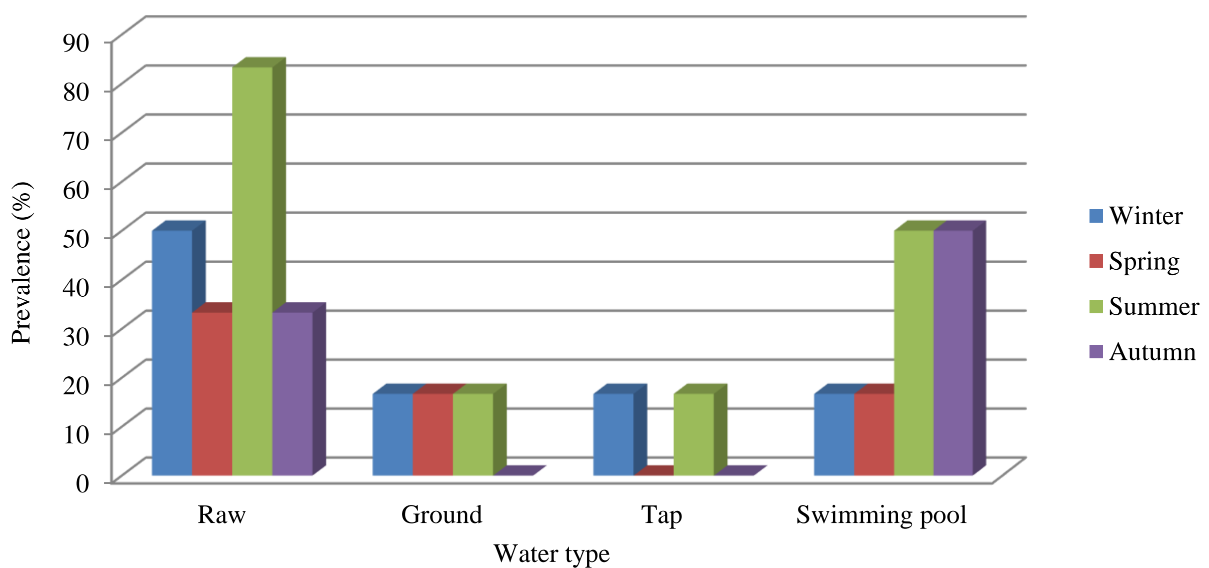

Figure 5: Prevalence of *Acanthamoeba* spp. in different water types.

typhoon and frequent thunderstorm. In addition, weather events were found to play a major role in the presence/absence of *Acanthamoeba* species in the river shed, with such changes probably due to resuspension of *Acanthamoeba* species from river side or river bed sediment by rain fall or wind action and input from the river shed via runoff[10]. These results were also supported by a previous report that *Acanthamoeba* species are most prevalent in summer in the aquatic environment**.**

RECOMMENDATIONS

Production of safe drinking water relies on the multiple barrier approaches to drinking water treatment. This begins with source water protection to prevent pollution, followed by appropriate treatment, and maintenance of water quality through proper storage and distribution to the consumer.

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