Original Article

Identification of *Acanthamoeba* genotype T4 in three drinking water treatment plants in Menofia Governorate

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ABSTRACT

Background: Water contamination with variable microbial contaminants is a major problem worldwide. *Acanthamoeba* spp. are able to survive in different environments including various water sources, thus posing important public health hazards.
Objective: To investigate the presence of *Acanthamoeba* spp. in three drinking water treatment plants (DWTPs) in Menofia governorate.
Material and Methods: Overall, 72 water samples were collected from three DWTP located in Shebin El-Kom, Tala, and El-Shohadaa cities. Water samples were collected monthly from the inlet and the outlet of each DWTP during the period from September 2021 to August 2022. Samples were filtered and processed for detection of *Acanthamoeba* spp. using direct microscopic examination, Gimenez stain, cultivation on non-nutrient agar (NNA), as well as molecular and phylogenetic analyses. *Acanthamoeba* isolates were microscopically identified to the genus level based on morphological criteria while molecular confirmation was accomplished by sequencing of the positive-PCR products, and phylogenetic analysis.
Results: *Acanthamoeba* was detected in 22 water samples (30.6%) by cultivation on NNA, out of which 17 were from raw water, and 5 from treated water samples. The highest detection rate of *Acanthamoeba* spp. was in Tala DWTP (11/22) followed by El-Shohadaa DWTPs (6/22), and Shebin El-kom DWTP (5/22). Seasonal distribution of *Acanthamoeba* isolates was highest in summer (40.8%) while the lowest was in winter (13.7%). Morphological identification revealed six *Acanthamoeba* spp. namely, *A. triangularis*, *A. polyphaga*, *A. astronyxis*, *A. castellanii*, *A. royreba* and *A. quina*. Utilizing phylogenetic analysis, only 9 samples (12.5%) were identified, all of which belonged to genotype T4.
Conclusion: Identification of *Acanthamoeba* spp. (genotype T4) in municipal water represents a potential health risk which should alert the authorities to adjust the procedures used to control this waterborne pathogen.
Keywords: *Acanthamoeba*; culture; drinking water; Menofia governorate; NNA; PCR; seasonal variation; sequencing.

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INTRODUCTION

Acanthamoeba spp. are free-living amoeba that have the ability to survive in variable environments, and have been isolated from soil, different water sources, and even air. They are also isolated from hospitals, dialysis units, eyewash solutions, and many clinical samples including human lung tissues, nasal cavities, corneal biopsies, pharyngeal swabs, skin lesions, brain tissue and cerebrospinal fluid[1]. *Acanthamoeba* trophozoites are the active feeding reproducing motile forms with characteristic acanthopodia. Its inactive resistant cysts are protected from harsh environmental conditions, desiccation and disinfecting agents by the presence of a double-cellulose wall[2,3].

Although not all *Acanthamoeba* spp. are pathogenic to humans, few species cause serious infections such as granulomatous amoebic encephalitis (GAE), and the painful sight threatening *Acanthamoeba* keratitis (AK)[4]. The clinical significance of *Acanthamoeba* is not limited to its direct pathogenesis. In nature, *Acanthamoeba* isolates include a variety of microbial endosymbionts. Therefore, *Acanthamoeba* spp. could potentially act as carriers for a number of human pathogens such as *Legionella pneumophila, Pseudomonas* spp., *Listeria monocytogenes, H. pylori. Mycobacterium avium, Aeromonas* spp., *Chlamydia* spp. and adenovirus leading to disease transmission[5]. The co-existence of these pathogens within water systems can be seriously detrimental to human health. Moreover, this endosymbiosis can promote drug resistance and decrease the efficacy of the commonly used disinfection procedures[6].

Acanthamoeba trophozoites invade the body through nasal mucosa and/or any skin lesions and then disseminates via the olfactory nerve or by hematogenous spread. Later, they can have access to brain tissue leading to GAE. Investigating the presence of these amoebae in various environmental and water

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sources is important since they hold a great risk to human health[7]. The identification of *Acanthamoeba* spp. at the genus level is made by discriminating the morphological features, especially the shape of the cyst double wall. *Acanthamoeba* was initially classified into three morphological groups (I, II and III). However, this morphological classification was insufficient for discriminating different species. After the successful implementation of molecular techniques, taxonomy and classification of *Acanthamoeba* has been re-evaluated. Genotype discrimination was based on the diversity of the *Acanthamoeba* genus-specific amplicon S1 (ASA.S1) of the small subunit (18S) of ribosomal RNA (rRNA). The ASA.S1 includes a region designated the diagnostic fragment 3 (DF3) which encodes a highly variable sequence in different genotypes. The strains in which the differences in this region were below 5% were categorized under a single genotype[8,9].

Until recently, more than 25 *Acanthamoeba* spp. were assigned to the National Center for Biotechnology Information (NCBI) taxonomy database according to the sequencing of the ASA.S1. It was found that the most predominant isolated genotype from environmental and clinical samples belonged to genotype T4. The increasing number of cases of *Acanthamoeba*-related infections have been documented worldwide, posing significant health concerns[5,10-12].

Water treatment primarily focuses on disinfection of pathogens in the water source, reducing them to acceptable national or international levels with minimal changes of water quality. This can be achieved through multiple chemical or physical methods including chlorination and removal of particulate or organic materials[13]. Accordingly, natural freshwater sources are essential for agricultural, household, and recreational purposes; however, they can act as a route for disease transmission to humans and animals. Producing safe, secure and pathogen-free drinking water is considered the main objective of any water treatment plant. In Menofia Governorate, there is insufficient data regarding detection rate (or identification) of *Acanthamoeba* spp. in the drinking water system. Therefore, we investigated the presence, the seasonal variation, and the genotypic characterization of the isolated *Acanthamoeba* spp. in three DWTPs.

MATERIAL AND METHODS

This descriptive analytical study was conducted at the Medical Parasitology Department, Faculty of Medicine, Menofia university during the period from September 2021 to August 2022.

Study design: Water samples were collected from three DWTPs located in Shebin El-Kom, Tala and El-Shohadaa cities, Menofia Governorate, Egypt. Samples were subjected to direct microscopic examination, permanent staining, and cultivation on NNA. Molecular identification of the samples with PCR followed by sequencing of the PCR-positive samples was performed.

Water sample collection and processing: A total of 72 samples were collected from three DWTPs in Shebin El-Kom, Tala and El-Shohadaa cities, Menofia Governorate, Egypt. Water samples (one-liter volume each) were taken monthly from the inlet (raw water 5-10 cm below the surface) and the outlet (completely treated water) of each DWTP. Samples were collected separately in sterile polypropylene bottles then concentrated at the corresponding DWTP Microbiology Laboratory using nitrocellulose membrane filters (0.45 µm pore size and 47 mm in diameter) according to the membrane filtration technique[14]. Membrane filters were kept in 10 ml phosphate-buffered saline (PBS) in sterile containers and transferred inside iceboxes to the laboratory where they were further processed on the same day.

Direct microscopic examination and staining techniques: After centrifugation of the PBS (from each container) at 250 x g for 20 min, the supernatant was discarded, and the deposit was resuspended in 1 ml PBS. Two drops of PBS were directly examined by wet mount preparation and then after Gimenez staining[15,16] using a light microscope (X100) for detection of *Acanthamoeba* cysts and trophozoites. Based on the morphological characteristics, *Acanthamoeba* were identified as previously described[17,18].

Cultivation on NNA: The membrane filter of each sample was inverted face to face on the surface of 1.5% NNA medium (Agar No. 1, Oxoid, Thermoscintefic, UK) seeded with living *E. coli* (kindly provided from Microbiology Department, Faculty of Medicine, Menofia University) and incubated at 30°C for two weeks, with daily microscopic examination to check for the presence of any amoebic growth. The plates were considered negative after 14 d of incubation and were discarded. Positive culture plates were sub-cultured on new NNA plates for further morphological and molecular analysis[19].

DNA extraction and molecular identification: Positive culture plates were washed with sterile PBS then centrifuged at 250 X g for 5-10 min. Prior to DNA extraction, destruction of *Acanthamoeba* cyst wall was performed by application of three successive freeze-thaw cycles (freezing in liquid nitrogen for few seconds followed by10 min incubation in a water bath at 100°C)[20]. *Acanthamoeba* DNA was extracted using QIAamp DNA Minikit tissue protocol (QIAGEN, Hilden, Germany) following the manufacturer's protocol[21].

The amplification was performed using the genus specific JDP primers set, JDP1 (5′-GGCCCAGATCGTTTACCGT GAA-3′) and JDP2

(5′-TCTCACAAGCTGCTAG-GGAGTCA-3′). The targeted amplicon was ASA.S1 gene including the DF3 region. The PCR procedure was performed using a PerkinElmer thermocycler (PerkinElmer Cestus, Norwalk, CT, USA) and the reaction mixture of final 25 µl volume consisted of 12.5 µl Maxima Hot Start Green PCR Master Mix (ThermoFisher Scientific Inc, Waltham, MA, USA), 5 µl template DNA, 1 µl of each primer, and 5.5 µl distilled water. The PCR products were visualized on 1.5% agarose gel electrophoresis using ethidium bromide under UV light[22].

Sequencing and phylogenetic analysis: Purification of the amplified product of DNA was performed using QIAquick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturers' instructions. Purified amplicons were loaded on agarose gel to determine the DNA yield and precision in gene excision. Sequencing was performed at SolGent Co. Ltd. (Solution for Genetic Technologies, South Korea) by ABI 3730XL DNA Analyzer and BigDye Terminator v3.1 cycle sequencing kit (Applied biosystems, Thermo-Fisher Scientific, USA)[23].

The identified sequences of PCR-positive water samples were trimmed, assembled, and aligned with reference *Acanthamoeba* genotypes (obtained by the basic local alignment search tool (BLAST) of GenBank). Using Clustal W, the phylogenetic tree was constructed in MEGA 11 software program (Molecular Evolutionary Genetic Analysis, version 11) applying the neighbor joining method with the bootstrap based on 1000 replicates[24].

Statistical analysis: The obtained data were collected, tabulated and statistically analyzed using an IBM compatible personal computer with Statistical Package for the Social Sciences (SPSS) version 26 (IBM SPSS statistics for windows, version 26.0, Armnok, NY: IBM Corp). Detection rates of *Acanthamoeba* in the examined DWTPs with different techniques was calculated using Pearson's Chi Square test (χ^2). A value of $P \leq 0.05$ was considered statistically significant.

Ethical consideration: Water samples were collected with approval from the official authorities at each DWTP. The ethical committee of Faculty of Medicine, Menoufia University, Egypt, approved this research protocol and procedures (IRP; PARA34).

RESULTS

Detection of *Acanthamoeba* spp. by microscopic examination: Results revealed detection of *Acanthamoeba* spp. in water samples taken from the three DWTPs (Table 1). *Acanthamoeba* spp. were detected in 19 out of a total 72 samples (26.4%), including 15 raw (38.9%) and 4 treated water samples (13.9%). Detection of *Acanthamoeba* spp. was mostly prevalent in Tala DWTP (10/19) followed by El-Shohadaa DWTP (5/19) then Shebin El-Kom DWTP (4/19). The total number of *Acanthamoeba* spp. in raw water samples was statically higher than treated water samples (P=0.016).

Detection of *Acanthamoeba* spp. by NNA: Cultivation of the collected water samples from the three DWTPs revealed that 22 samples (30.6%) were positive for *Acanthamoeba* spp., out of which 17 (47.2%) were from raw water and 5 (13.9%) from treated water. The highest detection rate was observed in Tala DWTP in 11 out of 22 samples constituting 50% of positive samples, followed by El-Shohadaa DWTPs (27.27%) then Shebin El-kom DWTP (22.73%) from both raw and treated water samples. The detected *Acanthamoeba* spp. from raw water were significantly higher than treated water in Tala DWTP (P=0.041). Both El-Shohadaa and Shebin El-kom DWTPs showed no statistical difference between raw and treated water samples (Table 1). Using culture as a gold standard method, the sensitivity and specificity of microscopic examination and PCR techniques was estimated (Fig. 1).

Elimination of *Acanthamoeba* by DWTPs treatment process: *Acanthamoeba*-positive samples from treated water were fewer than those from raw water,

Table 1. Detection rate of *Acanthamoeba* spp. in the examined DWTPs by microscopy and cultivation on NNA.

DWTP	Water source	Sample size	Microscopic examination			Culture		
			Positive No. (%)	Negative No. (%)	*P* value	Positive No. (%)	Negative No. (%)	*P* value
Elshohadaa	**Raw**	12	4 (33.3%)	8 (66.7%)	0.132	5 (41.7%)	7 (58.3%)	0.059
	Treated	12	1 (8.3%)	11 (91.7%)		1 (8.3%)	11 (91%)	
Tala	**Raw**	12	7 (58.3%)	5 (41.7%)	0.098	8 (66.7%)	4 (33.3%)	0.041*
	Treated	12	3 (25.0%)	9 (75.0%)		3 (25.0%)	9 (75.0%)	
Shebin El-Kom	**Raw**	12	3 (25.0%)	9 (75.0%)	0.273	4 (33.3%)	8 (66.7%)	0.132
	Treated	12	1 (8.3%)	11 (91%)		1 (8.3%)	11 (91.7%)	
Total	**Raw**	36	14 (38.9%)	22 (61.1%)	0.016*	17 (47.2%)	19 (52.8%)	0.002*
	Treated	36	5 (13.9%)	31 (86.1%)		5 (13.9%)	31 (86.1%)	
	Total	72	19 (26.4%)	53 (73.6%)		22 (30.6%)	50 (69.4%)	

*: Significant ($P \leq 0.05$).

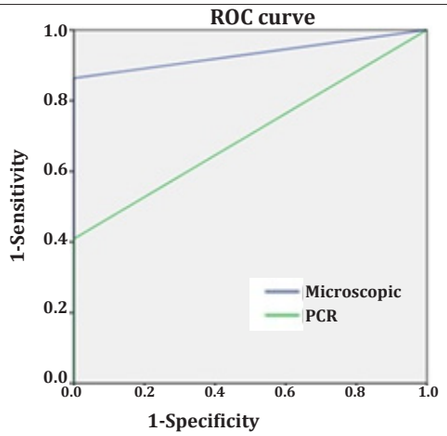

Fig. 1. Sensitivity and specificity of microscopic examination and PCR compared to culture.

indicating the efficiency of the drinking water treatment processes for elimination of *Acanthamoeba* spp. from the intake sources in the three DWTPs. The elimination percentage (negative treated samples/positive raw samples) reached 80% in El-Shohadaa DWTP followed by Shebin El-kom DWTP (75%) and the lowest was 62.5% in Tala DWTP (Table 2).

Seasonal distribution of *Acanthamoeba* isolates: The highest percentage of *Acanthamoeba* spp. was detected during summer (40.8%), followed by spring (27.3%) from all three DWTPs, then autumn (18.2%) from both El-Shohadaa and Tala DWTPs, and the lowest percentage was detected in winter (13.7%) interestingly from Tala DWTP alone. Shebin El-kom DWTP showed no *Acanthamoeba*-positive samples in autumn and winter. There was no statistical difference among the DWTPs regarding the season (Table 3).

Table 2. Elimination of *Acanthamoeba* spp. dy drinking water treatment process.

DWTP	*Acanthamoeba* culture-positive samples (No.)		*Acanthamoeba* spp. removal (%)
	Raw water	Treated water	
El-Shohadaa	5	1	80
Tala	8	3	62.5
Shebin El-kom	4	1	75

Morphological identification of the isolated *Acanthamoeba* spp.: *Acanthamoeba* isolates were morphologically identified based on trophozoite and cyst wall criteria. *Acanthamoeba* trophozoites had irregular shapes and variable sizes ranging from 25 to 40 µm. They had nuclei with large central karyosome and characteristic tapering thorn-like acanthopodia. The cysts had double walls (ectocyst and endocyst) and measured 15 to 25 µm in diameter. According to morphological criteria, six species were recognized in the examined samples, namely, *A. triangularis*, *A. polyphaga*, *A. astronyxis*, *A. castellanii*, *A. royreba* and *A. quina* (Fig. 2).

Table 3. Seasonal distribution of the culture-positive *Acanthamoeba* spp. in the examined DWTPs.

Water samples	Season (No./%)				Total	*P* value
	Autumn	Winter	Spring	Summer		
Sample size	18 (25.0%)	18 (25.0%)	18 (25.0%)	18 (25.0%)	72 (100.0%)	
Culture-positive samples	4 (18.2%)	3 (13.7%)	6 (27.3%)	9 (40.8%)	22 (30.6%)	0.139
El-Shohadaa DWTP	1 (16.6%)	0 (00.0%)	2 (33.4%)	3 (50.0%)	6 (8.33%)	0.217
Tala DWTP	3 (27.3%)	3 (27.3%)	1 (9.1%)	4 (36.3%)	11 (15.27%)	0.363
Shebin El-kom DWTP	0 (00.0%)	0 (00.0%)	3 (60.0%)	2 (40.0%)	5 (6.94%)	0.078

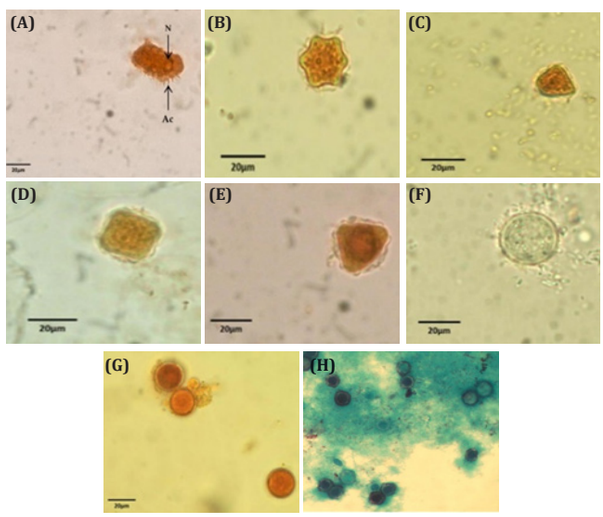

Fig. 2. Microscopic examination showing different stages of *Acanthamoeba*. **(A)** *Acanthamoeba* trophozoite with single nucleus (N), acanthopodia (Ac) and multiple contractile vacuoles stained with Lugol's iodine. **(B)** *A. astronyxis* cyst stained with Lugol's iodine. The cyst diameter was ~ 19-22 µm. Ectocyst was circular and very delicate. Endocyst was stellate with up to 6 rays ending with 4-6 pores. **(C)** *A. quina* cyst stained with Lugol's iodine. The cyst diameter was ~ 13-15 µm. The endocyst was globular, pentagonal or quadrangular and prominent. The ectocyst was wrinkled and thinner than the endocyst with 4-5 pores. **(D)** *A. polyphaga* cyst stained with Lugol's iodine. The average cyst diameter was 16 µm. The endocyst was polyhedral, quadrangular or pentagonal. The ectocyst was folded and wrinkled. **(E)** *A. triangularis* cyst stained with Lugol's iodine. The average diameter of the cyst was 13 µm. The endocyst was triangular in shape with broad rays. The ectocyst was thick, wrinkled, and corrugated. **(F)** *A. castellanii* unstained cyst. The cyst diameter ranged from 15-18 µm with mamillated or wrinkled ectocyst and stellate, irregular or nearly round endocyst. **(G)** *A. royreba* cyst stained with Lugol's iodine. The cyst diameter ranged from 14 to 16 µm and the endocyst was thick nearly rounded and the ectocyst was slightly wrinkled. **(H)** *A. castellanii* cyst colonies from culture stained with Gimenez stain (x100, scale bar 20 µm).

Detection of *Acanthamoeba* spp. by PCR: Nine samples, all from raw water, were successfully detected by PCR (12.5%), while the other 13 culture-positive samples could not be amplified by the PCR technique. *Acanthamoeba* spp. detected by the PCR was highly prevalent from El-Shohadaa DWTP (4/9) followed by Tala DWTP (3/9) and the least was from Shebin El-kom DWTP (2/9). All the *Acanthamoeba*-positive samples gave specific bands at ~500 bp. On the other hand, all treated water samples from the three DWTPs were negative by PCR (Table 4, and Fig. 3).

Sequencing and phylogenetic analysis of *Acanthamoeba* isolates: All of the PCR-positive isolates (9/72) were successfully sequenced and designated as strains 1w-WF to 10w-WF (GenBank accession numbers OR244182 to OR244190). These sequences were phylogenetically analyzed, and the phylogenetic tree was constructed by aligning them with similar sequences and reference species T1 to T23 sequences which were retrieved by NCBI-BLAST. Analysis of the isolates 1w-WF to 10w-WF showed homology with *Acanthamoeba* genotype T4 (Table 4 and Fig. 4).

Table 4. Detection rate of *Acanthamoeba* spp. in the examined DWTPs detected by PCR and their genotypes.

DWTP	Water source	Sample size	PCR		Isolate ID	GenBank accession number	Genotype	*P* value
			Positive No. (%)	Negative No. (%)				
Elshohadaa	**Raw**	12	4 (33.3%)	8 (66.7%)	2w-Wf	OR244183	**T4**	0.028
	Treated	12	0 (0.00%)	12 (100.0%)	6w-Wf	OR244186	**T4**	
					9w-Wf	OR244189	**T4**	
					10w-Wf	OR244190	**T4**	
Tala	**Raw**	12	3 (25.0%)	9 (75.0%)	1w-Wf	OR244182	**T4**	0.064
	Treated	12	0 (0.00%)	12 (100.0%)	4w-Wf	OR244184	**T4**	
					8w-Wf	OR244188	**T4**	
Shebin El-Kom	**Raw**	12	2 (16.7%)	10 (83.3%)	5w-Wf	OR244185	**T4**	0.140
	Treated	12	0 (0.00%)	12 (100.0%)	7w-Wf	OR244187	**T4**	
Total	**Raw**	36	9 (25.0%)	27 (75.0%)				0.001*
	Treated	36	0 (0.00%)	36 (100.0%)				
	Total	72	9 (12.5%)	63 (87.5%)				

***:** Significant ($P \leq 0.05$).

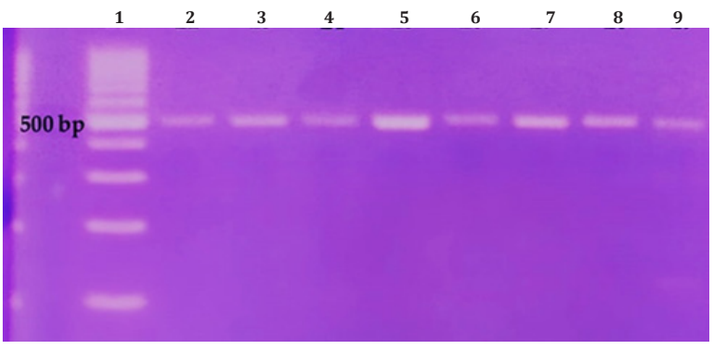

Fig. 3. Agarose gel electrophoresis of PCR amplified products of *18S rRNA* gene of different *Acanthamoeba* isolates. **Lane 1:** Marker ladder 100 bp; **lanes 2–9:** *Acanthamoeba* positive samples (≃ 500bp).

Fig. 4. Phylogenetic tree constructed by neighbor-joining model and 1000 bootstrap, representing sequence alignment of the nine detected *Acanthamoeba* isolates (T4) with their accession numbers (marked with blue circles) with sequences of *Acanthamoeba* strains T1–T123 retrieved from GenBank.

DISCUSSION

Acanthamoeba is one of the free-living amoeba (FLA) abundant in the environment. It can cause GAE and the sight-threatening AK. The high prevalence of *Acanthamoeba* spp. in water, soil and other environmental samples represents an important health risk especially for immunodeficient individuals and contact lens wearers[25]. Exposure to water sources while wearing contact lenses, through swimming, showering, rinsing the contact lenses with tap water and the lack of hand washing, were important risk factors for acquiring AK[26]. Safe drinking water production is a global public health concern, and adequate drinking water treatment processes in DWTPs are crucial in the reduction of microbial contamination of raw water sources[27].

In the present study, *Acanthamoeba* spp. were detected in three DWTPs in Menofia governorate. To our knowledge, it is the first report of detection and genotyping of *Acanthamoeba* spp. from water samples in Menofia governorate. The overall detection rate for *Acanthamoeba* spp. from both raw and treated water sources was 30.6% by culture. The highest number of *Acanthamoeba* spp. was isolated from Tala DWTP followed by El-Shohadaa DWTPs and the lowest was in Shebin El-Kom DWTP. This result was consistent with Tawfeek *et al*.[28] who detected *Acanthamoeba* ssp. in 31.4 % of water samples in Cairo, Egypt. Al-Herrawy *et al*.[1] isolated *Acanthamoeba* spp. from 33.3% of inlet water and from 16.7% of the outlet water samples with 25% overall detection rate from Damanhur DWTP, Behera Governorate. Moreover, *Acanthamoeba* spp. in tap water in Giza governorate, were detected in 29.9% of samples[29]. Furthermore, a study in Assiut City detected the presence of *Acanthamoeba* in 20.7% of samples from different water sources, and mixed *Acanthamoeba* and Vahlkampfiidae contamination in another source (5.3%)[30].

In Dakahlia governorate, *Acanthamoeba* was confirmed in 12.6% of samples from various water sources with detection rate of 35% in flowing water. Data from Dakahlia governorate in combination with surveys from other Egyptian governorates were analyzed and the results revealed that *Acanthamoeba* had the highest statistically significant presence in water with a mean prevalence of 43.03% throughout Egypt, with insignificant difference among various water sources. The mean prevalence of *Acanthamoeba* in raw water was 60.71%, whereas it was 22.62% in treated water[31].

However, higher detection rates were observed in other studies. Morsy *et al*.[32] found that 56.25% of tap water samples were contaminated with *Acanthamoeba* in Giza governorate. Gad and Al-Herrawy[33] also detected *Acanthamoeba* spp. in 50% of Nile water samples. In another study in Fayoum governorate, the overall detection rate for *Acanthamoeba* spp. was 72.7%, with detection of 93.3% from water tanks and 67.7% from tap water samples[27]. Moreover, in a study to investigate *Acanthamoeba* prevalence in household and hospital potable water in Beni-Suef governorate, the total detection rate for *Acanthamoeba* was 80%. Domestic water contamination was statistically higher than hospital water representing 90% and 70% respectively[34].

The difference in the detection rate of *Acanthamoeba* among different areas even within Egypt could be attributed to geographical conditions, environmental settings, different raw water sources, water distribution systems, accumulation of biofilms and drinking water treatment methods. Other influential factors include the source, number, volume, processing or filtration techniques of the collected samples[19,30].

In the current study, a statistically higher detection rate of *Acanthamoeba* was recorded in raw water samples compared to treated water samples. In contrast, the treatment processes in Damanhur DWTP, Behera governorate, could only remove 50% of *Acanthamoeba* present in the inlet water[1]. However, in Fayoum governorate, a removal efficiency of 72.7% was recorded in New Azab DWTP and 70% in Old Azab DWTP. However, both New Kohafa and Old Kohafa DWTPs recorded 57.1% removal efficiency[35]. In a further study in Fayoum governorate, the percentage of removal for FLA by different treatment processes was the highest (83%) in the DWTP using the slow sand filters, while by rapid sand filter DWTP it was 71.4%[19]. Using data analysis from multiple surveys in Egypt, the mean *Acanthamoeba* prevalence for raw water was 60.71%, whereas for finished water it was 22.62% with significant difference, while the estimated efficiency of water filtration for *Acanthamoeba* occurrence in different DWTPs in Egypt was 60.34%[31].

According to seasonal variation, the present study did not record statistical significance. This was similar to the results of several studies[1,29,30,33]. A slightly different result was found in Giza governorate, where the highest occurrence of *Acanthamoeba* in drinking water distribution systems was recorded in summer followed by autumn, spring and winter[32]. The lack of statistical difference in seasonal variation might be due to the presence of the highly resistant *Acanthamoeba* cysts that can withstand different weather factors and prevail all over the year[30].

According to morphological criteria, six species were recognized in the present work. Comparable results were reported in various studies. In Cairo, Al-Herrawy *et al*.[36] morphologically identified six *Acanthamoeba* spp. from samples of swimming pools. These were *A. castellanii*, *A. polyphaga*, *A. royreba*, *A. rhysodes*, *A. mauritaniensis* and *A. triangularis*. In Behera governorate, *Acanthamoeba* isolates were

identified, revealing the presence of six different species which were *A. astronyxis*, *A. royreba, A. culbertsoni*, *A. comandoni*, *A. quina*, and *A. polyphaga*[1]. Additionally, Al-Herrawy *et al*.[29] recognized eight species of *Acanthamoeba* from tap water in Giza governorate, Egypt, namely *A. triangularis*, *A. echinulata*, *A. astronyxis*, *A. comandoni, A. griffini, A. culbertsoni*, *A. quina*, and *A. lenticulata*. Seven species of *Acanthamoeba* were recognized, *A. triangularis*, *A. polyphaga*, *A. lenticulata,* and *A. culbertsoni, A. astronyxis, A. comandoni*, and *A. echinulate* from different water sources in Assiut[30]. The similarity in the results is probably due to the presence of these strains in the Nile River which is the main source of the water supply in Egypt.

In the present research, cultivation on NNA detected the highest number of *Acanthamoeba*-positive samples. Culture has the advantage of being a cheap and easy method, but it is time-consuming with limited ability in diagnosing different subtypes. *Acanthamoeba* could be morphologically identified to the genus level or to the species level but with much expertise. This observation conformed with a study that claimed three advantages of the culture method; being more sensitive, cheaper and easier than the PCR technique[36]. Scheid and Balczun[2] showed that culture followed by morphological identification is important, as the PCR methods might fail, thus combining several assays in the diagnosis of *Acanthamoeba* is recommended. Besides, Yera *et al*.[37] recorded that the sensitivity of PCR did not differ significantly from that of culture, and that the sensitivity could be increased by combining two or three assays. In contrast, several studies[2,9,38,39] considered the morphological classification of *Acanthamoeba* spp. not unreliable, as the morphology of *Acanthamoeba* cysts may change depending on different culture conditions. Furthermore, different species in the same morphological group can be closely similar thereby causing great difficulty in the identification of the species. Electron microscopy, immunostaining or molecular analysis may be needed to distinguish them.

In the current study, PCR detected 40.9% of the morphologically positive samples of *Acanthamoeba*, all of which belonged to genotype T4. Close results were obtained by Gad and Al-Herrawy[33], where *Acanthamoeba* spp. was detected in 41.7% of Nile water samples by real time PCR. In another study, Al-Herrawy *et al*.[40] declared that PCR confirmed the primary morphological identification of only 29.4% of the *Acanthamoeba* isolates. On the contrary, higher PCR detection rates were recorded in other studies. Al-Herrawy *et al*.[36] stated that 96.5% of microscopically *Acanthamoeba*-positive samples were also positive by the PCR technique. Other studies found that 98.5%, 82.6% or 79% of morphologically detected tap water samples were positive by PCR[23,32,35]. Several studies pointed out the performance limitations of some PCR assays in diagnosing *Acanthamoeba*. Besides, evaluation of sensitivity and specificity revealed that performance of a procedure might depend on the type or the preparation of the specimen[2,41].

Reports on the epidemiology and the environmental distribution of various genotypes of *Acanthamoeba* and other FLA in Egypt are still insufficient, with few studies addressing the distribution of various genotypes in AK patients. Researchers highlighted the importance of genotyping for the epidemiological study of *Acanthamoeba*, species separation, pathogenicity determination and distribution of different pathogenic species[9]. The T4 genotype is the most prevalent *Acanthamoeba* genotype in nature followed by T1, T10, T12, T5 and T2 genotypes respectively. Genotype T4 is also identified in the majority of human infections, especially AK and GAE. Most of the *Acanthamoeba* isolates from patients with severe infections are also from the T4 genotype followed by T3. However, T2, T5, T6, T8, T9, T10, T11, T12, T13, and T15 have also been identified in AK patients[39,42]. Moreover, *Acanthamoeba* T4 manifests a significantly higher binding ability and induces severe cytotoxicity on the host cells as compared to other genotypes. Thus, genotype T4 prevalent in any environmental sample poses a great health threat[40].

Similarly, Lorenzo-Morales *et al*.[25] identified 5 genotypes of *Acanthamoeba* from freshwater sources in the Nile Delta region. The isolates belonged to T1, T2, T3, T4 and T7 genotypes. In another study in Cairo, environmental isolates were classified by genotype analysis into T4, T3 and T5 genotypes[28]. Sequencing analysis of confirmed *Acanthamoeba* isolates from swimming pools in Alexandria revealed the presence of genotype T3, T4, T5, T11 and T15, and T4 was the most prevalent genotype in the examined samples[40]. In Beni-Suef governorate, Abd El Wahab *et al*.[34] detected T4 in 65%, and T2 in 35% of positive isolates of *Acanthamoeba* from domestic and hospital potable water sources. In addition, phylogenetic analysis of water samples from Assiut City, Egypt revealed genotypes T4 and T7[30]. Moreover, T4, T9 and T11 were the prevalent genotypes in a study conducted in Kafrelsheikh governorate[23]. In Dakahlia governorate, T3 and T4 were identified from multiple water samples[31]. There are various *Acanthamoeba* genotypes detected in Egypt and the highly pathogenic T4 was the most significantly identified genotype. Most of the T4 isolates from different water sources from Egypt were phylogenetically clustered in a common haplotype, suggesting the circulation of this haplotype in various water sources in Egypt. It was found that the same haplotype was isolated from AK patients in Assiut[43], verifying the potential role of water in the epidemiology of *Acanthamoeba* infections.

Our study had an important limitation; inability to examine other water sources and other DWTPs in the remaining districts in Menofia governorate. We

concluded that treated drinking water from the three DWTPs contains *Acanthamoeba* spp. The detected T4 genotype is the most encountered genotype in clinical infections. Owing to the significant role of FLA in ecosystems and the increasing number of human infections, future studies should be directed to identify their potential sources especially in water. Moreover, individuals, particularly those using CLs, should be aware of the potential risk of exposure to pathogenic strains of *Acanthamoeba* in warmer seasons. In addition, public authorities should make efforts to adjust water treatment regulations in order to achieve safe drinking water supply and eliminate any hazardous contaminations.

Authors contribution: Abokhalil NA initiated the research idea, designed the study, analyzed the data and wrote the manuscript. El Nahas NS, Afifi AF and Shalaan FH shared in study design and data analysis. Gaballah WG collected the samples and performed the parasitological examination. All authors revised the manuscript and approved the final version before publication.

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