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Acanthamoeba genotypes and *Vermamoeba vermiformis* in drinking water treatment facilities

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ABSTRACT

Free-living amoebae species, such as *Acanthamoeba* and *Vermamoeba vermiformis* are found worldwide and cause severe infections in humans. In the present study, inlet and outlet water samples (n = 96) were collected from conventional and compact drinking water treatment facilities (DWTF), concentrated through nitrocellulose membrane filters (0.45µm pore size) and cultured on non-nutrient agar covered with dead *Escherichia coli*. The morphologically positive *Acanthamoeba* and *Vermamoeba* isolates were subjected to molecular identification and 19 morphologically different *Acanthamoeba* and two *Vermamoeba* isolates were genotyped for further confirmation. The results revealed that the predominance free-living amoebae species in surface water samples were belonging to *Acanthamoeba* (68.8%). All morphologically positive samples for *Acanthamoeba* proved to be positive by PCR. The conventional DWTF showed better results (66.7%) for removing *Acanthamoeba* and *Vermamoeba vermiformis* than the compact DWTF (50%). At the sequence level, three *Acanthamoeba* genotypes (T3, T4, and T15) and one *Vermamoeba* species (i.e., *Vermamoeba vermiformis*) were obtained. In conclusion, conventional DWTF was more effective than compact DWTF in removing *Acanthamoeba*. The presence of viable *Acanthamoeba* strains especially the pathogenic types (e.g., *Acanthamoeba* T4) in outlet water could cause health hazards to consumers.

INTRODUCTION

Safe and clean drinking water is considered one of the human rights essentials **(United Nations, 2015)**. Biological agents like protozoa, viruses, parasitic helminths, and pathogenic bacteria are the most common and widespread health risks associated with waterborne disease **(Gorche and Ozolins, 2011)**. Protozoa represent an extremely diverse group of unicellular parasites; some of them are considered problems for the water industry **(APHA, 2017)**. Moreover, protozoa are resistant to inactivation by chemical disinfectants used in drinking water **(Gorchev and Ozolins, 2011; APHA,**

2017). Protozoa generally cause diarrhea or gastroenteritis of varying severity, although more serious consequences including death can occur **(APHA, 2017)**.

Free-living amoebae (FLA) are unicellular protozoans distributed in diverse environments worldwide **(Chan *et al.*, 2011; Al-Herrawy *et al.*, 2016; Gad and Al-Herrawy, 2016; Morsy *et al.*, 2016)**. For example, free-living amoebae occur abundantly not only in natural habitats (e.g., water and soil), but also in man-made environments such as swimming pools and air conditioning systems **(Angelici *et al.*, 2021)**. The FLA trophozoites feed on bacteria, fungi, algae, cyanobacteria, and even smaller protozoa. Due to the fact that FLA feed on bacteria, they have an impact on the bacterial communities and increase the return of nutrients to the biosphere **(Archibald *et al.*, 2017)**. Then, amoebophagous fungus and other microorganisms feed on FLA as a part of the microbial food web **(Scheid, 2014)**. Due to the wide distribution of these organisms, it is suggested that people are likely to come in contact with FLA species in their daily lives **(Moreira and Brochier-Armanet, 2008; Mungroo *et al.*, 2021)**. Free-living amoebae are known to interact not only with viruses but also with bacteria and fungi **(Siddiqui and Khan, 2012; Balczun and Scheid, 2017)**. They act as an important reservoir or host of pathogenic microorganisms and protecting them from adverse conditions such as disinfectants and therapeutic agents. Additionally, these endosymbionts enhance the pathogenicity of the *Acanthamoeba* that hosts them **(Sun *et al.*, 2020)**. *Acanthamoeba* (Discosea, Amoebozoa) and *Vermamoeba* (Tubulinea, Amoebozoa) have been used as model organisms for isolating giant viruses from environmental samples **(Suzan-Monti *et al.*, 2007; Boyer *et al.*, 2009; Arslan *et al.*, 2011; Reteno *et al.*, 2015).** *Acanthamoeba* causes a central nervous system, eye, and cutaneous infections **(Marciano-Cabral and Cabral, 2003; Visvesvara *et al.*, 2007).** Additionally, *Vermamoeba vermiformis* has been reported as a causative agent behind a painful ulcer adjacent to the eye and a case of amoebic keratitis **(Abedkhojasteh *et al.*, 2013; Scheid, 2019)**.

Producing drinking water free from waterborne/water-based pathogens is considered the main objective of water treatment providers. Because no single treatment process can be expected to remove all of the different types of pathogens found in water, multiple barriers (pre-chlorination, coagulation, sedimentation, filtration, and post-chlorination) are desirable **(Stanfield *et al.*, 2003).** In Egypt, conventional and compact DWTFs are the major drinking water treatment types. They comprise several treatment steps to combat waterborne and water-based pathogens. However, there is still scarce researches to evaluate the performance of different drinking water treatment facilities regarding protozoa removal **(Al-Herrawy and Gad, 2017; Al-Herrawy *et al.*, 2019)**. Moreover, few studies used sequencing technology to identify the *Acanthamoeba* isolates and confirm the morphological results. So, the present study aimed to assess the removal of *Acanthamoeba* species and *Vermamoeba* via two different drinking water treatment

facilities as well as the molecular characterization of *Acanthamoeba* isolates and *Vermamoeba*.

MATERIALS AND METHODS

Samples and sampling sites

Water samples were collected from two different drinking water treatment facilities (conventional DWTF and compact DWTF) in Giza governorate (Egypt). The daily production of conventional DWTF and compact DWTF were about 1.300.000 and 1200 m^3, respectively (Fig. 1). The treatment processes in the two types of water treatment facilities are the same, including pre-chlorination, flocculation, sedimentation, sand filtration, and post-chlorination. Two water samples were collected monthly from each stage (inlets and outlets) of the two drinking water treatment facilities for one year. Water samples (one liter from each sampling type) were separately collected in sterile polypropylene containers. Collected water samples were transported to Environmental Parasitology Laboratory (National Research Centre, Egypt) in an ice-box at the same day of collection **(Health Protection Agency, 2014)**.

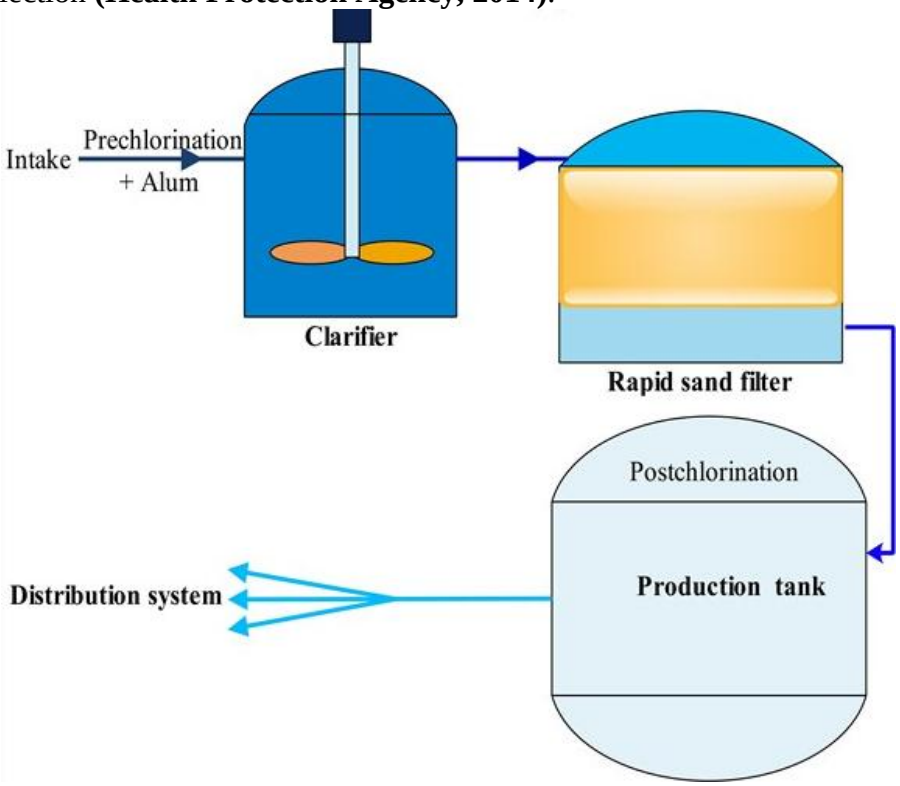

Figure 1. Schematic diagram showing the treatment stages in conventional and compact DWTFs

Concentration and culturing

One liter of each water sample was used for the detection and cultivation of free-living amoebae. Samples were separately filtered under a sterile condition through 0.45 µm nitrocellulose membranes (47 mm in diameter) by using stainless steel vacuum filter holder (Sartorius) and then the membrane was placed face to face on non-nutrient (NN)

agar plate covered with dead *Escherichia coli* and incubated at 37ºC for one week with a daily microscopic examination **(Health Protection Agency, 2014)**. FLA were morphologically characterized as mentioned according to **Page, 1976,** and **Pussard and Pons, 1977**. *Acanthamoeba* and *Vermamoeba* were the FLA types appeared in the current research, so we focused on them.

Molecular characterization:

The surface of NN agar plates, cloned with amoebae trophozoites were washed with sterile phosphate buffer saline (PBS), and the washing solution was then centrifuged at 5000 rpm for 10 min. Extraction of DNA from amoebae trophozoites in the obtained sediment was performed using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Three freezing-thawing cycles, each cycle consisting of 1 min in liquid nitrogen followed by 1 min in boiling water were performed before the application of the manufacturer's protocol. The obtained DNA was stored at −20°C until used.

Amplification of *Acanthamoeba*- and *Vermamoeba*-DNA was performed using GoTaq G2 green master mix (Promega, USA) according to the manufacturer's manual. PCR reaction mixture per sample consisted of 12.5µl master mix, 3µl template DNA, 1µl of each primer (conc. 10pmol) for *Acanthamoeba;* JPD1 (5′-GGCCCAGATCGTTTACCGTGAA -3′) and JPD2 (5′-TCTCACAAGCTGCTAGGGAGTCA -3′) **(Schroeder *et al.*, 2001)** and for *Vermamoeba vermiformis;* Hv1227F (5′- TTACGAGGTCAGGACACTGT -3′) and Hv1728R (5′- GACCATCCGGAGTTCTCG -3′) **(Kuiper *et al.*, 2006)** and completed the final volume to 25µl by nuclease-free water. The thermal profile for DNA amplification was achieved with a pre-denaturation step at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension step was performed at 72°C for 10 minutes. The removal rate of the amoebae was determined according to the following equation:

$$Removal = \frac{Total\ positive\ samples\ in\ inlet - Total\ positive\ samples\ in\ outlet}{\text{Total positive samples in inlet}} \times 100$$

The PCR product was visualized using gel stained with ethidium bromide. The PCR products were subjected to purification using GeneJET PCR Purification Kit (Thermo Scientific, USA) according to the manufacturer's instruction. The purified DNAs served as templates for DNA sequencing using the ABI PRISM® automated DNA Sequencer to identify *Acanthamoeba* genotypes. Nucleotide sequences were analyzed and assembled using the Laser gene 6 Package® (DNASTAR) and BLAST analysis tools (http://www.ncbi.nlm.gov/BLAST). Gene sequences were prepared and aligned using Clustal W implemented in the Bio-Edit program (version 7.0.4.1). Phylogenetic tree was constructed by neighbor-joining analysis with the Tamura-Nei model implemented in the MEGA6© program **(Tamura *et al.*, 2013)**.

RESULTS

Based on the morphological characteristics, the identified genera of FLA were *Acanthamoeba* and *Vermamoeba*. Thus, we mainly presented these FLA types in our results. All morphologically positive samples for *Acanthamoeba* (Fig. 2A-D) and *Vermamoeba* were proved to be positive by PCR (Fig. 3A-B).

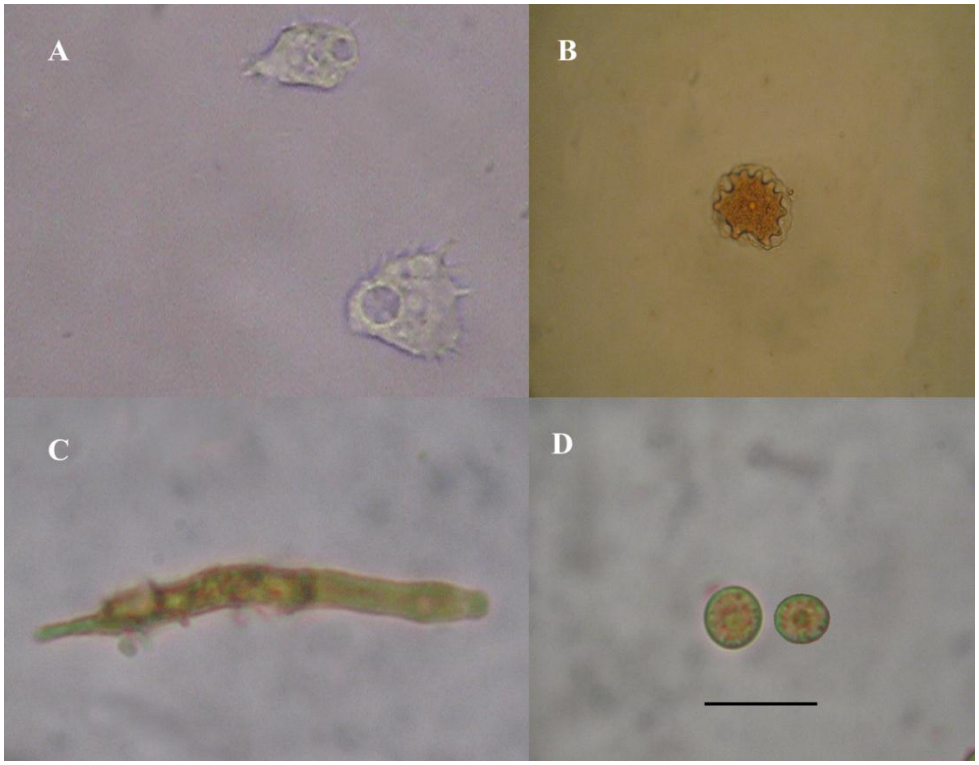

Figure 2. Photomicrograph for (A) *Acanthamoeba* trophozoites, (B) *Acanthamoeba* cyst., (C) *Vermamoeba vermiformis* trophozoite, (D) *Vermamoeba vermiformis* cysts. Bar = 10µm.

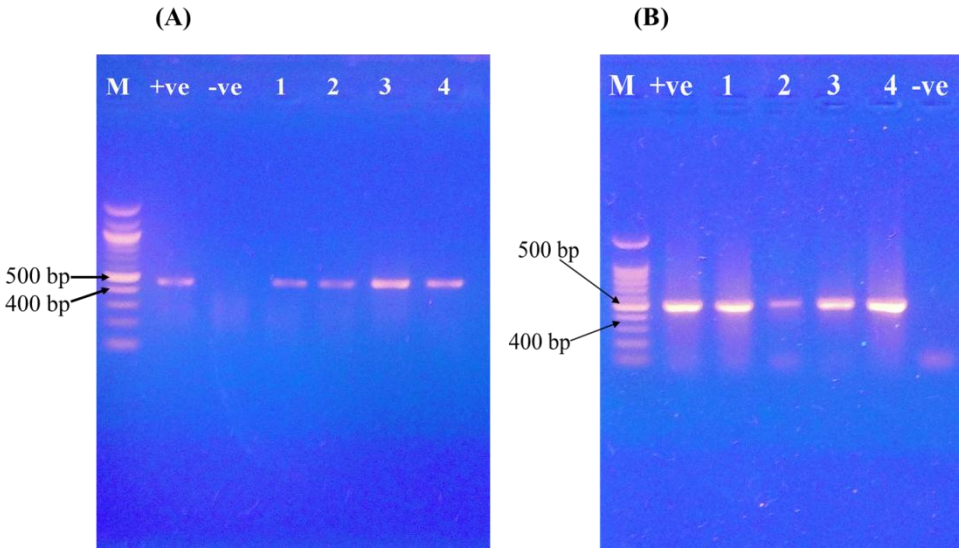

Figure 3. Agarose gel electrophoresis for the PCR amplified product of (A) *Acanthamoeba* isolates DNA by using the genus-specific primers, (B) *Vermamoeba vermiformis* isolates using species-specific primer. M: 100 plus DNA Ladder, +ve: positive control, -ve: negative control. Wells no. 1 to 4 are positive samples.

The prevalence of *Acanthamoeba* was 62.5% and 20.8% in conventional DWTF inlet and outlet samples, respectively. While the prevalence of *Vermamoeba vermiformis* was 12.5% and 4.2% in conventional DWTF inlet and outlet samples, respectively. In Compact DWTF, the prevalence of *Acanthamoeba* in the inlet (75%) and outlet (37.5%) was higher than that of *Vermamoeba vermiformis* (*i.e.*, 16.7% for inlet and 8.3% for outlet). The removal rate of *Acanthamoeba* via conventional DWTF and compact DWTF reached 66.7% (n = 10/15) and 50% (n = 9/18), respectively. A similar removal rate for *Vermamoeba vermiformis* was observed for conventional DWTF (66.7%; n = 2/3) and compact DWTF (50%; n = 2/4). The removal of *Acanthamoeba* via conventional DWTF and compact DWTF was significant (Wilcoxon test; $P < 0.05$). In contrast, the removal of *Vermamoeba vermiformis* was not significant (Wilcoxon test; $P > 0.05$) (Table 1, Fig. 4 and 5).

Concerning seasonal variations, *Acanthamoeba* showed the highest prevalence in summer (100%), followed by 50% in each of autumn, winter, and spring in inlet samples of conventional DWTF (Fig. 4 A-B). A similar pattern was observed for *Acanthamoeba* in outlets of conventional DWTF and compact DWTF, with a noticed peak in summer. The temporal peak of *Vermamoeba vermiformis* was observed in summer for the inlet and outlet of conventional DWTF. However, the same microorganism peaked in the spring for the inlet of compact DWTF (Fig. 5 A-B).

Table 1. Prevalence of *Acanthamoeba* and *Vermamoeba* in DWTFs.

Seasons	Collected samples no.*	*Acanthamoeba*-positive samples								*Vermamoeba vermiformis*-positive samples							
		Conventional DWTF				Compact DWTF				Conventional DWTF				Compact DWTF			
		Inlet		outlet		inlet		Outlet		inlet		Outlet		inlet		Outlet	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Summer	6	6	100	3	50	6	100	3	50	2	33.3	1	16.7	1	16.7	1	16.7
Autumn	6	3	50	2	33.3	4	66.7	4	66.7	0	0	0	0	1	16.7	0	0
Winter	6	3	50	0	0	2	33.3	0	0	0	0	0	0	0	0	0	0
Spring	6	3	50	0	0	6	100	2	33.3	1	16.7	0	0	2	33.3	1	16.7
Total	24	15	62.5	5	20.8	18	75	9	37.5	3	12.5	1	4.2	4	16.7	2	8.3

*Number of collected samples per each stage in each drinking water treatment facility

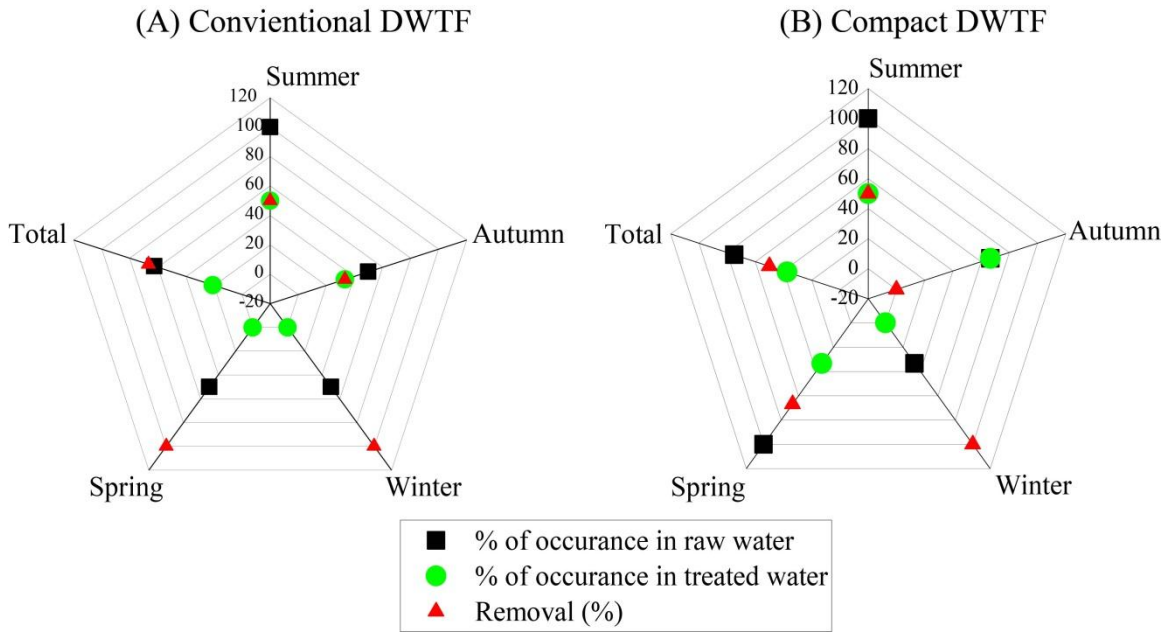

Figure 4. Occurrence and removal of *Acanthamoeba* spp. in conventional and compact DWTFs.

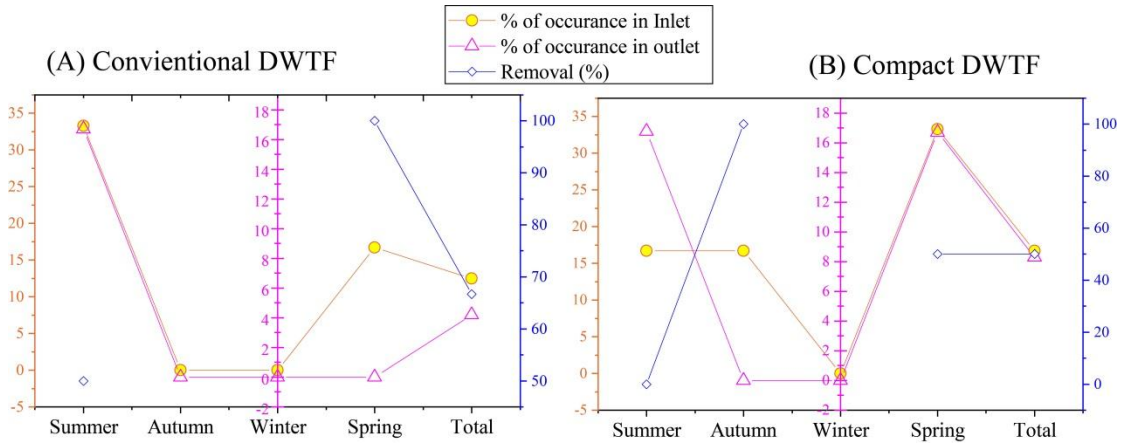

Figure 5. Occurrence and removal of *Vermamoeba vermiformis* in conventional and compact DWTFs. The colors of the legends correspond to the axes' colors.

The 19 *Acanthamoeba*-morphologically different isolates were subjected to sequence analysis. The sequence results showed that 5 (26.32%) out of 19 *Acanthamoeba* isolates could be typed, while 73.86% were not. Two *Acanthamoeba* isolates, isolate (1) and isolate (4) were found in outlet samples of conventional DWTF. Isolate (1) showed 99.35% similarity with *Acanthamoeba astronyxis* (Accession number: MN239988), while isolate (4) revealed 100% similarity to *Acanthamoeba* genotype T4 (Ref. strain: Accession no. MK297910). On the other hand, three isolates of *Acanthamoeba* (isolates 2, 3, and 5) were identified in the outlet samples of compact DWTP. Isolate (2) belonged to *Acanthamoeba* genotype T4 (Ref. strain: Accession no. MT292607) with a similarity of 100%. Two isolates (3) and (5) were 100% similar to *Acanthamoeba lenticulata* and *Acanthamoeba* genotype T15 (Ref. strain: Accession no. MK217509), respectively. Two strains of *Vermamoeba vermiformis* have been identified in the inlet and outlet of conventional DWTF (Fig. 6). The DNA sequences in the current study were deposited on the GenBank under accession numbers; OM021886 (*Acanthamoeba astronyxis* isolate 1), OM003587 (*Acanthamoeba* genotype T4 isolate 2), OM003585 (*Acanthamoeba lenticulata* isolate 3), OM003586 (*Acanthamoeba* genotype T3 isolate 4), OM003588 (*Acanthamoeba* genotype T15 isolate 5), OM003579 (*Vermamoeba vermiformis* isolate 6), and OM003590 (*Vermamoeba vermiformis* isolate 7).

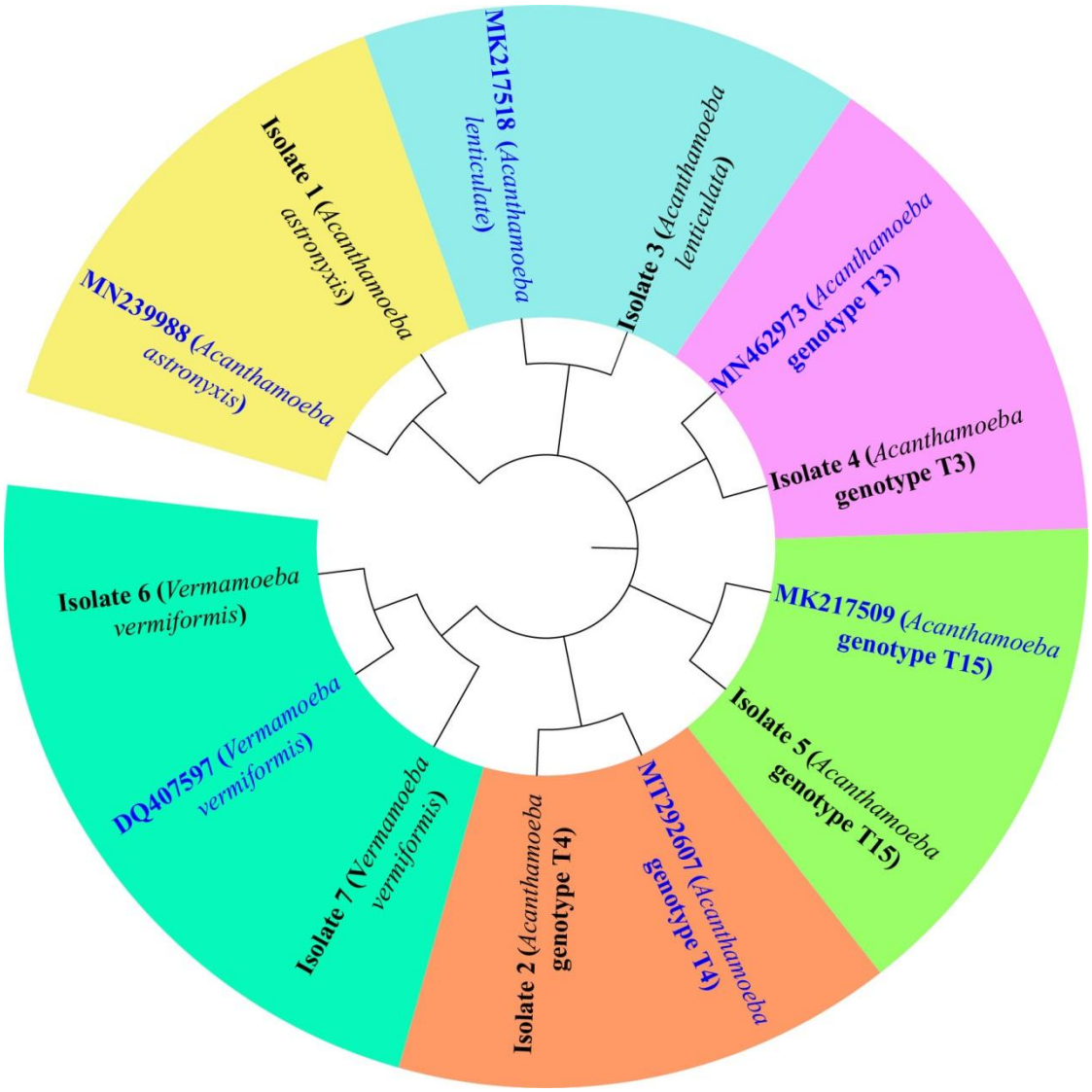

Figure 6. Neighbor-joining tree depicting the relationships between our environmental isolates (n = 7) and reference strains representing *Vermamoeba vermiformis* and *Acanthamoeba* genotypes.

DISCUSSION

An overall management strategy must be implemented in which multiple barriers, including source water protection and appropriate treatment processes, as well as protection during storage and distribution, are used in conjunction with disinfection to prevent or remove microbial contamination of drinking water **(Gorchev and Ozolins, 2011)**. Ensuring the parasitological safety of drinking water is of paramount importance. The safety of the drinking water starts from monitoring the water quality of drinking water treatment facilities. Thus, this research focused on the occurrence, molecular

characterization and removal of the two abundant protozoa (i.e., *Acanthamoeba* and *Vermamoeba*) in drinking water treatment facilities.

The presence of free-living amoebae (i.e., *Acanthamoeba* and *Vermamoeba*) in the outlet water (completely treated drinking water) may be due to the cyst formation. These cysts are hard and persistent to harsh environmental conditions and can escape from drinking water treatment processes. Also, the ability of FLA to reproduce in the environment without the need for a host enables these organisms to increase their numbers when favorable environmental conditions are available **(Aksozek *et al.*, 2002)**. Another reason is that the standard chlorine doses (2-5 mg/L) used for disinfection of the produced water in the drinking water treatment facilities are not enough to inactivate whether *Acanthamoeba* or *Vermamoeba*. For instance, *Acanthamoeba* cysts can survive at 50 ppm and 100 ppm chlorine for 18 hours and 30 minutes, respectively **(Kilvington *et al.*, 2004; Storey *et al.*, 2004)**. The aforementioned reasons could be responsible for the appearance of these microorganisms in the outlets of the studied DWTFs.

The present study showed that conventional DWTF could eliminate 66.7% of *Acanthamoeba* present in the raw untreated water (Fig. 4). While removing FLA in drinking water treatment facilities using the same treatment technologies in Behara and Fayium governorates, Egypt reached 75% and 71.4%, respectively **(Al-Herrawy *et al.*, 2015; Al-Herrawy and Gad, 2017)**. A lower removal percentage (50%) of *Acanthamoeba* spp. was recorded by compact DWTF (Fig. 5). Higher removal of *Acanthamoeba* (69.23%) by compact DWTF was recorded before (Al-Herrawy et al., 2019). The removal of *Vermamoeba vermiforms* was 66.7% and 50% in the conventional and compact DWTFs, respectively (Fig. 5). In previous research, no *Vermamoeba* was detected in the conventional drinking water treatment facility, which used the rapid sand filter technology **(Al-Herrawy and Gad, 2017)**. Additionally, the compact unit failed to remove the *Vermamoeba* from the only positive sample in the previous study **(Al-Herrawy *et al.*, 2019).**

In the present study, three different genotypes of *Acanthamoeba* (T3, T4, and T15) were isolated from the outlets of DWTFs (Fig. 6). Several findings in previous reports revealed that genotype T4 was the most prevalent type found in the environment and also in most of the clinical isolates, especially from patients suffering from Acanthamoeba keratitis (AK) **(Booton *et al.*, 2005; Maciver *et al.*, 2013; Martín-Pérez *et al.*, 2017)**. Other *Acanthamoeba* genotypes T3, T4, T5 and T15, like that in our study were isolated from clinical and environmental samples **(Basher *et al.*, 2018)**. Also, *Acanthamoeba* genotypes T3, T4, T5 and T11 were detected in tap water from Southern Iran **(Niyyati *et al.*, 2015)**. Clinical studies showed that several distinct genotypes including T2, T3, T5, T6, T10, T11, T13, T15, and T16 have been associated with AK **(Alves *et al.*, 2000; Maghsood *et al.*, 2005; Booton *et al.*, 2005; Corsaro *et al.*, 2010)**, whereas few genotypes (T1, T10, T12) seem to be rare and associated with GAE **(Stothard *et al.*, 1998; Booton *et al.*, 2005)**. Based on sequence analysis, *Acanthamoeba astronyxis* was detected in one outlet water sample of the conventional DWTF (Fig. 6). *Acanthamoeba astronyxis* and *Acanthamoeba comandoni* were also isolated from the patients suffering from AK **(Hajialilo *et al.*, 2016; Tawfeek *et al.*, 2016).** *Acanthamoeba* have been involved in infections of eye, central nervous system, skin, and nose **(Visvesvara *et al.*, 2007).** Awareness on the pathogenicity of *Acanthamoeba* infections is important in health control programs in both humans and animals. Most pathogenic FLA, especially

Acanthamoeba and *Vermamoeba* are known to facilitate intracellular multiplication of *Vibrio cholerae*, *Mycobacterium tuberculosis*, *Bacillus anthracis*, and *Legionella pneumophila* which are responsible for cholera, tuberculosis, anthrax, and legionellosis, respectively **(Dey *et al.*, 2012; Scheid, 2014; Sun *et al.*, 2020)**. Large-scale environmental studies from diverse habitats are needed for future studies to identify different free-living amoebae and *Acanthamoeba* genotypes.

CONCLUSION

The presence of potentially pathogenic *Acanthamoeba* genotypes and *Vermamoeba vermiformis* in finally treated drinking water is considered a potential health threat. *Acanthamoeba* spp. more abundant than *Vermamoeba vermiformis* in the two DWTFs. Three *Acanthamoeba* genotypes (T3, T4 and T15) and two *Acanthamoeba* species (*A. astronyxis* and *A. lenticulata*) were detected. The sequence analysis confirms the PCR results for *Vermamoeba vermiformis*. The conventional DWTF was more efficient than compact DWTF for the removal of *Acanthamoeba* and *Vermamoeba vermiformis*. Management strategies and better surveillance are required to evaluate the risk of waterborne/water-based pathogens.

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