

Occurrence of *Acanthamoeba* species in the Damanhour Drinking Water Treatment Plant, Behera Governorate (Egypt)

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Abstract: Members of the genus *Acanthamoeba* are common free-living amoebae that may be pathogenic to humans. The aim of the present work was to evaluate the presence of *Acanthamoeba* species in the Damanhour Drinking Water Treatment Plant (DWTP) before and after conventional drinking water treatment. Water samples were filtered through nitrocellulose membranes of 0.45 µm pore size, cultured on nonnutrient agar plates seeded with *Escherichia coli*, and incubated at 37°C. The obtained amoebae, which proved to be morphologically consistent with *Acanthamoeba* spp., were confirmed by polymerase chain reaction (PCR). The confirmed isolates of *Acanthamoeba* were morphologically identified to the species level, based on assessment of the size and shape of the endo- and ectocysts and the mean number of pores per cyst. The obtained results showed that members of the genus *Acanthamoeba* were isolated from 33.3% and 16.7% of inlet and outlet samples, respectively. Six different species of *Acanthamoeba* were morphologically identified. Species morphologically identified as *Acanthamoeba castellanii*, *Acanthamoeba culbertsoni*, and *Acanthamoeba polyphaga* were isolated from the “finished” water of DWTP. Such species may be pathogenic to humans. In conclusion, the conventional drinking water treatment steps did not remove all the detected species of *Acanthamoeba* in the inlet water, and about half of them could escape to completely treated and finished water. These species may cause human infections.

Keywords: free-living, amoebae, identification, morphology, PCR

Introduction

Acanthamoeba spp. are free-living amoebae that are common in natural environments. They can cause serious human infections, such as granulomatous amebic encephalitis and amoebic keratitis. However, not all species or strains are pathogenic to humans.¹ *Acanthamoeba castellanii*, *Acanthamoeba culbertsoni*, *Acanthamoeba hatchetti*, *Acanthamoeba healyi*, *Acanthamoeba polyphaga*, *Acanthamoeba rhyodes*, *Acanthamoeba astronyxis*, and *Acanthamoeba divionensis* have all been implicated in human infections.² *Acanthamoeba* spp. can be found in soil, air, and water. They have the ability to survive in diverse aquatic environments and have been isolated from public water supplies, swimming pools, bottled water, seawater, pond water, stagnant water, freshwater lakes, salt water lakes, river water, distilled water bottles, ventilation ducts, the water–air interface, air-conditioning units, sewage, compost, sediments, soil, beaches, vegetables, air, surgical instruments, and contact lenses, indicating the ubiquitous nature of these organisms.^{2–8} In addition, *Acanthamoebae* have been recovered from hospitals, dialysis units, eye wash stations, human nasal cavities, pharyngeal swabs, lungs tissues, skin lesions, corneal biopsies, cerebrospinal fluid (CSF), and brain necropsies.⁹

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Acanthamoeba spp. have two stages during their life cycle: a vegetative or trophozoite stage (8–40 µm) and a dormant cyst stage (8–29 µm). Trophozoite morphology is characterized by the presence of a single nucleus and fine acanthopodia projecting outward from the surface of the body. The acanthopodia allow adhesion to surfaces, cellular movements, and capture of prey. Trophozoite forms feed, grow, and reproduce by binary fission.¹⁰ *Acanthamoeba* trophozoites cannot survive a long time in an adverse environmental condition (for example, lack of food, extremes in temperature or pH, or hypo- or hyperosmolarity) and differentiate into uninucleate cysts with a double-walled structure (endo- and ectocyst). The cyst forms are nonfeeding, nongrowing, and nonreproducing forms. The cyst form is a resistant form possessing pores, which are used to monitor environmental changes. The cyst form is protected from desiccation, starvation, and a variety of chemical and physical agents.^{11,12} Cysts have been known to survive in vitro for 20 years or more. With the return of optimal conditions for growth, especially food, cysts germinate to give rise to trophic forms.¹³ *Acanthamoeba* cysts have been shown to remain viable after treatment with 100 mg/L chlorine (free and combined) for 10 minutes; they were shown to survive as well at 80°C, implying that conventional hyperdisinfection may be insufficient for long-term control of *Acanthamoeba*.¹³

Acanthamoeba spp., like other free-living amoebae, feed on microorganisms present on wet surfaces, in diverse environments,¹⁴ and even at the air–water interface.¹⁵ Based on morphological criteria, more than 24 species of *Acanthamoeba* have been identified, which can be assigned to three groups.^{16,17} The spiny structures or acanthopodia that arise from the surface of *Acanthamoeba* trophozoites may be used to capture food particles, which are usually bacteria,¹⁸ algae, and yeast.¹⁹

Acanthamoeba spp. can serve as hosts for a large number of pathogenic bacteria, including *Legionella* spp., *Vibrio cholerae*, *Burkholderia cepacia*, *Listeria monocytogenes*, *Escherichia coli* O157, *Mycobacterium bovis*, and *Mycobacterium avium*.^{20,21} Approximately, 20%–24% of clinical and environmental isolates of *Acanthamoeba* harbor intracellular bacteria.²² Moreover, *Acanthamoebae* can harbor echovirus.¹ Recently, a mimivirus was discovered in *A. polyphaga*.²³ In addition, it is known that coxsackie viruses and adenoviruses can infect *Acanthamoeba*.²⁴ The intracellular localizations of these microorganisms protect them from adverse conditions and allow the bacteria to evade host defenses, to resist antibiotic actions, and to increase their virulence.²⁵ Also, biofilms formed in aqueous environments (intravenous catheters,

contact lens, sclera buckles, dental units, etc) are known to be a reservoir for a large number of microorganisms. These play an important role in the pathogenesis of *Acanthamoeba*, particularly, in keratitis infections, and provide the parasite with an attractive niche and an abundant nutrient.¹⁴

In Egypt, scarce data are available concerning species of *Acanthamoeba* in freshwater and tap water. So, the aim of the present work was to identify *Acanthamoeba* spp. isolated from a drinking water treatment facility in Damanhour city, in the Behera Governorate, Egypt.

Materials and methods

Samples

Water samples (2 L volume, each) were collected from the Damanhour Drinking Water Treatment Plant (DWTP), Damanhour city, Behera Governorate, Egypt (Figure 1). The inlet of Damanhour DWTP receives its water from the Eastern Khandak Canal, which is a branch of the El-Rayah El-Behera Canal. The treatment system of this station begins with coarse sieving of the raw water, followed by prechlorination, clarification, slow sand filtration, and finally postchlorination of the treated water in storage tanks. Before pumping to the distribution system, another booster dose of chlorine is added to the drinking water in order to reach the consumer in a safe mode, with about 0.5 ppm residual chlorine. The Damanhour DWTP produces 1,200 cubic liters of drinking water per second and serves about 320,000 persons. For our study, two types of water samples were collected: raw surface water (intake of the Damanhour DWTP) and “finished” water (completely treated and chlorinated, from the outlet of the Damanhour DWTP). Two water samples were collected monthly – one from each water type – during the 1-year period from August 2010 to July 2011.

Collection of *Acanthamoebae*

The collected water samples were separately filtered through nitrocellulose membrane filters (0.45 µm pore size and 47 mm diameter) using a stainless steel vacuum filter holder (Sartorius SM, Göttingen, Germany). Then, each membrane was inverted face-to-face on the surface of a nonnutrient agar plate seeded with living *E. coli* bacteria and incubated at 37°C for 1 week, with daily microscopic examination using the inverted microscope.^{1,4} The grown *Acanthamoeba* trophozoites were characterized from other free-living amoebae by their pointed pseudopodia (acanthopodia), while the cyst forms were easily identified by their double cyst wall and conventionally stellate shape.²⁶

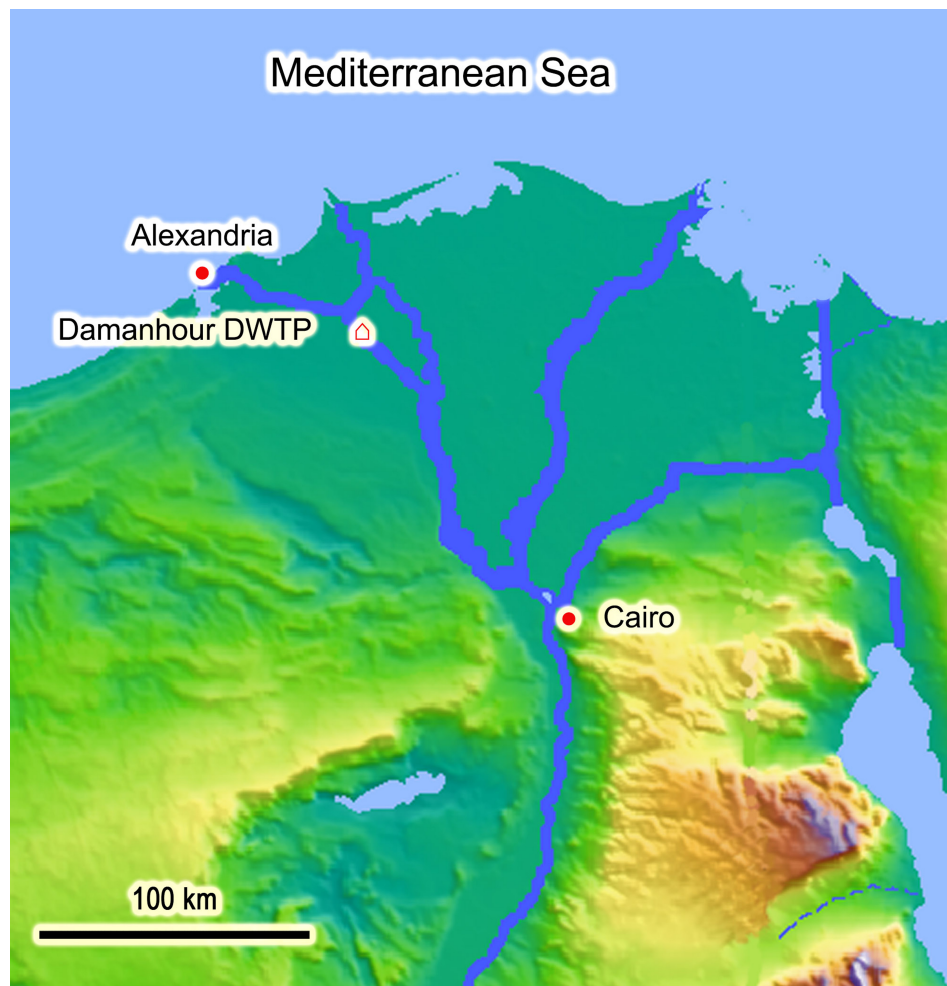


Figure 1 Location of the Damanhour DWTP.
Abbreviation: DWTP, Drinking Water Treatment Plant.

Molecular characterization

The morphologically suspected isolates of *Acanthamoeba* were subjected to molecular characterization. Cyst walls of these isolates 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. DNA was then extracted using phenol/chloroform/isoamyl alcohol, as described by Winneper-ninckx et al.²⁷ and modified by Abdel-Hamid et al.²⁸ For the detection of *Acanthamoeba* spp., a primer pair, namely AcantF900 (5'-CCC AGA TCG TTT ACC GTG AA-3') and AcantR1100 (5'-TAA ATA TTA ATG CCC CCA ACT ATC C-3'), were used to amplify a 18S ribosomal (r)RNA gene fragment of approximately 180 bp (differing by a few bases, depending on the species).²⁹ Amplification of DNA was performed using Maxima™ Hot Start Green PCR Master Mix (Thermo Fisher Scientific Inc, Waltham, MA, USA) according to the manufacturer manual. The polymerase

chain reaction (PCR) reaction mixture used per sample consisted of: 25 µL Maxima Hot Start Green PCR Master Mix (2×), 3 µL template DNA, 1 µL of each primer, and 25 µL diethylpyrocarbonate (DEPC)-treated water. A hot-start procedure for *Acanthamoeba* spp. was used, with an initial denaturation at 95°C for 4 minutes, followed by 35 cycles of denaturation at 94°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 DNA was visualized using ethidium bromide.

Morphological speciation

The isolates of *Acanthamoeba* confirmed by PCR technique were morphologically identified to the species level, based on assessment of the size and shape of the endo- and ectocysts, and the mean number of pores per cyst, using the method described by Pussard and Pons.¹⁶

Results

Prevalence of *Acanthamoeba* species in the Damanhour DWTP

Examination of 48 water samples collected from Damanhour DWTP revealed the presence of different *Acanthamoeba* species in 12 (25%) of them. About 33.3% of the examined inlet water samples were found to contain *Acanthamoeba* species, while only 16.7% of the examined outlet drinking water samples had *Acanthamoeba* species (Table 1).

Seasonal variation of *Acanthamoeba* species in the Damanhour DWTP

Concerning the seasonal variation of *Acanthamoeba* isolated from the Damanhour DWTP, it was observed that they were found in the inlet water samples in a same percentage (33.3%) in all seasons of the year. On the other hand, the occurrence of *Acanthamoeba* spp. in the outlet drinking water samples reached 33.3% in each of spring and summer seasons, while they were completely absent in the outlet samples collected in both winter and autumn (Table 2).

Elimination of *Acanthamoeba* species during the drinking water treatment process

It was found that the number of *Acanthamoeba*-positive samples in the finished (outlet) water was lower than that in the inlet samples, indicating that the efficiency of all treatment processes for the elimination of *Acanthamoeba* species from the inlet water in the Damanhour DWTP reached 50% (Table 3).

Confirmation of the morphologically identified isolates to the genus level by PCR

When the microscopically-positive water samples for *Acanthamoeba* spp. were subjected to DNA extraction, amplification, and agarose gel electrophoresis, genus-specific bands (for *Acanthamoeba* spp.) were visualized by ultraviolet

Table 2 Seasonal variation of *Acanthamoeba* in Damanhour DWTP

Season	<i>Acanthamoeba</i> spp.			
	Inlet samples		Outlet samples	
	+ve/examined	%	+ve/examined	%
Winter	2/6	33.3	0/6	0.0
Spring	2/6	33.3	2/6	33.3
Summer	2/6	33.3	2/6	33.3
Autumn	2/6	33.3	0/6	0.0

Abbreviations: +ve, positive; DWTP, Drinking Water Treatment Plant.

(UV) transilluminator. It was found that all the microscopically *Acanthamoeba*-positive samples gave specific bands at about 180 bp (Figure 2).

Morphological identification of *Acanthamoeba* spp. confirmed by PCR

The confirmed *Acanthamoeba* isolates were morphologically identified, revealing the presence of six different species on the basis of cyst morphology (Figure 3). The identified six *Acanthamoeba* spp. were *A. astronyxis*, *Acanthamoeba royreba*, *Acanthamoeba comandoni*, *A. culbertsoni*, *Acanthamoeba quina*, and *A. polyphaga*. Morphological criteria of the isolated six species of *Acanthamoeba* spp. are presented in Table 4.

Discussion

During the present study, species of the genus *Acanthamoeba* were isolated from 33.3% of inlet water samples of the Damanhour DWTP. *Acanthamoeba* spp. were detected at higher percentages (66.7%, 40%, and 43.1%, respectively) in the examined Nile water of Egypt by Al-Herrawy,³⁰ Hamadto et al,³¹ and Hilali et al.³² Also, during the present work, *Acanthamoeba* spp. were isolated from 16.7% of the examined finished water samples of the Damanhour DWTP. In other studies in Egypt, Al-Herrawy et al recorded a higher occurrence (58.6%),³³ while Hamadto et al recorded a lower occurrence (4%).³¹ In the United Kingdom, *Acanthamoeba* spp. were isolated from 26.9% of tap water samples by Kilvington et al.³⁴ The difference in detection rates of free-living amoebae in different countries and localities may be influenced by geographical conditions and raw water sources.^{35–37}

Table 1 Prevalence of *Acanthamoeba* in Damanhour DWTP

Type of water	Total examined samples (N)	<i>Acanthamoeba</i> +ve samples	
		n	%
Inlet	24	8	33.3
Outlet	24	4	16.7
Total	48	12	25

Abbreviations: +ve, positive; DWTP, Drinking Water Treatment Plant.

Table 3 Elimination of *Acanthamoeba* during drinking water treatment process

Water type	Number of <i>Acanthamoeba</i> +ve samples
Inlet	8
Outlet	4
Elimination % (–ve outlet/+ve inlet)	50%

Abbreviations: –ve, negative; +ve, positive.

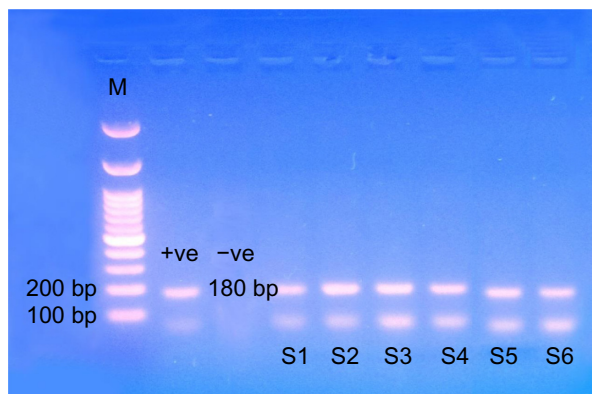


Figure 2 Ethidium bromide-stained 2% agarose gel showing PCR-amplified product of extracted DNA from *Acanthamoeba*.

Notes: Lane 1 shows the marker (100 plus DNA ladder); lane 2 shows the positive control; lane 3 shows the negative control; and lanes 4–9 show samples of the morphologically identified six species of *Acanthamoeba*, labeled S1–S6.

Abbreviations: –ve, negative; +ve, positive; M, marker; PCR, polymerase chain reaction.

The results of the present study showed that the percentage of *Acanthamoeba*-positive samples in the inlet water of the DWTP were the same in all seasons of the year (33.3%). However, in the outlet drinking water, the percentage of positive samples reached 33.3% in spring and summer, while they were 0% in both winter and autumn. Several workers in different countries reported seasonal variations in the distribution of *Acanthamoeba* in freshwater samples, with the highest percentage either in autumn^{38–40} or in summer.^{37,41,42}

During the present study, a simple PCR technique was applied, using genus-specific primers, to confirm the results obtained by morphological identification of the isolated *Acanthamoeba*, but PCR confirmation at the species level

is still needed. PCR has been found to be a more sensitive diagnostic test than culture.⁴³

Identification of some pronounced *Acanthamoeba* spp. can be accomplished by the morphological characteristics. In fact, more than 24 species of *Acanthamoeba* have been identified based on morphological criteria.^{16,17} Members of the genus *Acanthamoeba* are divided into three morphological groups according to the cyst size and other morphological features. Group 1 consists of *Acanthamoeba* spp. with relatively large cysts, distinctly stellate endocysts, and smooth spherical ectocysts. Group 2 and group 3 *Acanthamoeba* spp. have smaller cysts (less than 18 μ m in diameter). Species in group 2 have polygonal to stellate endocysts, with irregular or wrinkled ectocysts, while the cysts of group 3 species have rounded or slightly angular endocysts, with thinner and smooth or slightly wrinkled ectocysts.^{16,17} According to the results of the present work, six different species of *Acanthamoeba* were morphologically identified. Morphologically, two species (*A. astronyxis* and *A. comandoni*) of the isolated *Acanthamoeba* spp. belonged to group 1, two species (*A. polyphaga* and *A. quina*) belonged to group 2, and two species (*A. culbertsoni* and *A. royreba*) belonged to group 3.

Some *Acanthamoeba* spp. can cause human infections, such as *A. castellanii*, *A. culbertsoni*, *A. hatchetti*, *A. healyi*, *A. polyphaga*, *A. rhyodes*, *A. astronyxis*, and *A. divionensis*.^{1,2} During the present study, three species morphologically identified as *A. castellanii*, *A. culbertsoni*, and *A. polyphaga* were isolated from the finished water of the DWTP. Such species may be pathogenic to humans.

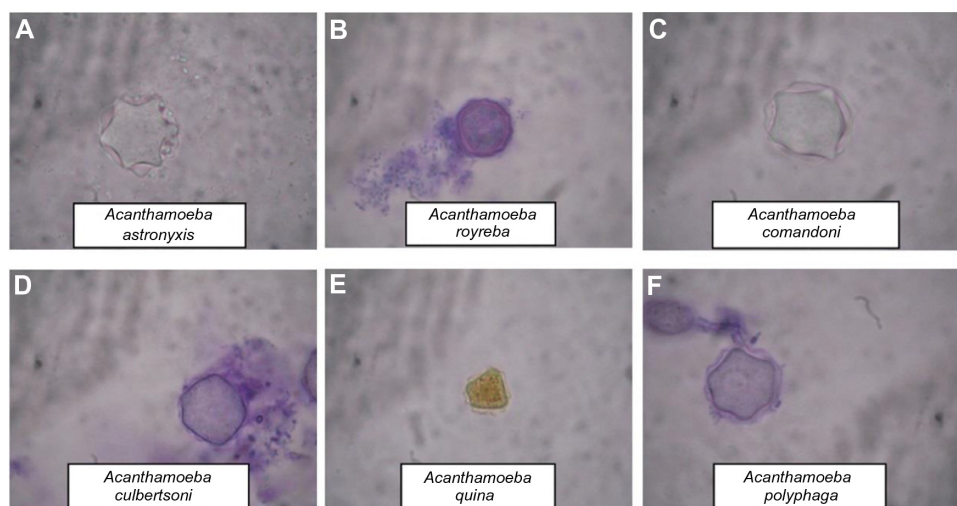


Figure 3 Cyst morphology of isolated *Acanthamoeba* spp.

Notes: (A and C) were unstained; (B), (D), and (F) were stained with Field's stain; and (E) was stained with Lugol's iodine stain.

Table 4 Morphological characters of isolated *Acanthamoeba* cysts of the different species

<i>Acanthamoeba</i> isolates	Cyst morphology			
	Cyst diameter (μm)	Endocyst	Number of pores	Ectocyst
<i>Acanthamoeba astronyxis</i>	19–22 μm	Stellate	5	Very delicate, not wrinkled
<i>Acanthamoeba royreba</i>	14–16 μm	Nearly round	12–14	Slightly wrinkled
<i>Acanthamoeba comandoni</i>	20–25 μm	Stellate	6–7	Delicate, not wrinkled
<i>Acanthamoeba culbertsoni</i>	13.5–15 μm	Not predominantly stellate, nearly rounded with slight angles	5–6	Little, slightly wrinkled
<i>Acanthamoeba quina</i>	12–13 μm	Firmly prominent	4–5	Noticeably wrinkled
<i>Acanthamoeba polyphaga</i>	12–15 μm	Nearly round with slight angles	5–6	Closely encircling endocyst

Conclusion

Acanthamoeba spp. were still present in the drinking water produced by the Damanhour DWTP, although they had been exposed to all steps of conventional drinking water treatment. This may denote a public health hazard to the users.

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Disclosure

The authors report no conflicts of interest in this work.

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