Distribution of potentially pathogenic Acanthamoeba isolates in the environment of Helwan University, Egypt

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ABSTRACT
Acanthamoeba species are free-living amoebae having worldwide distribution. These amoebae can cause granulomatous amoebic encephalitis and amoebic keratitis in humans. They can produce proteases that are considered virulence factors. Acanthamoeba can also harbor pathogenic bacteria, fungi, and viruses.

The objective of this study is to evaluate the presence of Acanthamoeba in the environment of Helwan University, Egypt. Six types of samples (tap water, irrigation water, wastewater, swabs from surfaces, soil, and air) were collected, processed, and cultured on non-nutrient agar medium. Positive plates for Acanthamoeba were subcultured, purified and amoebae were identified morphologically and confirmed by PCR using Acanthamoeba genus-specific primers. Obtained results declared that members of genus Acanthamoeba were detected in 91.7, 83.3, 54.2, 45.8, 12.5 and 12.5% of irrigation water, soil, swabs, wastewater, tap water, and air samples, respectively. The morphologically identified Acanthamoeba species proved to be related to genus Acanthamoeba when tested by PCR. Statistically, the sampling source had a strong significant correlation with the prevalence of Acanthamoeba. The highest appearance of Acanthamoeba was recorded in the spring season for samples from irrigation water, soil, and swabs from surfaces.

In conclusion, the high prevalence of Acanthamoeba species in irrigation water and soil exert public health hazards to students and workers in Helwan University.

INTRODUCTION

Acanthamoeba was first isolated in 1913 by Puschkarew as amoeba from the dust and named Amoeba polyphagus. Later in 1930, Castellani isolated an amoeba that occurred as a contaminant in a culture of the fungus Cryptococcus pararoseus (Castellani, 1930). From that time until now, Acanthamoeba species show up their ability to survive in diverse environments. Consequently, they have been isolated from these environments...
and even from the atmosphere. In addition, *Acanthamoeba* have been recovered from hospitals, dialysis units, eye wash stations, corneal biopsies, skin lesions, human nasal cavities, pharyngeal swabs, lungs tissues, cerebrospinal fluid (CSF) and brain necropsies (Khan, 2003; Marciano-Cabral and Cabral, 2003; Schuster and Visvesvara, 2004).

*Acanthamoeba* trophozoite possesses a large number of mitochondria (Burger et al., 1995). *Acanthamoeba* trophozoite moves a relatively fast, with a locomotion rate of approximately 0.8 μm/second. The movement involves the formation of a hyaline pseudopodium called acanthopodium (Preston et al., 2001).

Under harsh conditions, the trophozoites differentiate into a non-dividing, double-walled resistant cyst form. Cyst walls contain cellulose (not present in the trophozoite stage) that accounts for 10% of the total dry weight of the cyst although cyst wall composition varies between isolates belonging to different species and genotypes (Derda et al., 2009; Dudley et al., 2009). The most abundant *A. castellanii* cyst wall proteins are three sets of lectins, which have carbohydrate-binding modules (Magistrado-Coxen et al., 2019).

*Acanthamoeba*, a free-living amoeba, is an opportunistic pathogen of humans and other animals including gorillas, monkeys, dogs, ovines, horses and kangaroos, as well as birds, reptiles, amphibians, and fishes (Martinez and Visvesvara, 1997; Dykova et al., 1999). *Acanthamoeba* is the most common cause of illness, usually infecting the eyes and sometimes causing a sight-threatening keratitis (Yoder et al., 2010). *Acanthamoeba* spp. can also cause a highly fatal CNS infection known as granulomatous amoebic encephalitis (GAE), in addition to infections of the lungs and skin (Visvesvara et al., 2007; Visvesvara, 2010).

*Acanthamoeba* cysts can withstand desiccation for more than 20 years. It is therefore necessary to continuously monitor isolates of *Acanthamoeba* for their resistance to environmental pollutions (Sriram et al., 2008). So, the aim of the present work is to remind the decision-makers about the presence of potentially pathogenic *Acanthamoeba* species in the environment of Helwan University and announcing their hazards on the students.

**MATERIALS AND METHODS**

**Samples and sampling sites**

A total of 144 samples were collected from Helwan University environment during one year period from March 2017 to February 2018. Different types of environmental samples were collected (Tap water, irrigation water, wastewater, soil, swabs from surfaces and air samples). Samples were regularly collected two times per month during the study period. Collection of samples was performed following to Health Protection Agency (2004) and American Public Health Association (2017) as follows:

- Water samples (from tap, irrigation and wastewaters) were separately collected (1L volume each) in clean, dry and autoclavable polypropylene containers.
Distribution of potentially pathogenic *Acanthamoeba*

- Soil samples (about 100g each) were separately collected from the gardens of Helwan University in sterile autoclavable polypropylene plastic beakers that were then wrapped with parafilm.
- Swabs were separately collected from bench surfaces of laboratory number 3 of Zoology and Entomology Department, Faculty of Science by sterile cotton swabs stored in 10ml sterilized Page's saline (*Page, 1988*).
- Air samples were collected by leaving uncovered non–nutrient (NN) agar plates, soaked with heat-killed *Escherichia coli* suspension, in direct contact with air at different areas outside the buildings. The plates were left open for 2hr then covered with their lid, sealed with parafilm and immediately transported to the laboratory for incubation.

After collection, all samples were transported at ambient temperature in an ice box to Environmental Parasitology Laboratory, Water Pollution Research Department, National Research Centre, Dokki, Giza where they were processed at the same day of collection.

**Processing and cultivation of samples**

About 100g from every soil sample were separately added to 1L autoclaved Page’s saline with vigorous shaking for 10min and then left to settle for 5min. The supernatant was siphoned and treated as a water sample.

Water samples (whether tap water, irrigation water, wastewater and supernatant of soil samples) were separately filtered through a nitrocellulose membrane (0.45μm pore size and 47mm in diameter) using a stainless steel filter holder connected with a suction pump. Filtration was stopped just before drying of the membrane (*Health Protection Agency, 2004; American Public Health Association, 2017*). After filtration process, the membrane was inverted face to face on the surface of NN agar plate seeded with heat-killed *Escherichia coli*.

Swab samples in Page's saline were centrifuged at 1500xg for 10min. The last 1ml of centrifuged Page's saline of each swab sample was spread on the surface of NN agar plate seeded with heat-killed *E. coli* bacteria.

All the inoculated plates, in addition to air samples, were wrapped with parafilm and incubated at 30°C for one week (*Page, 1988; American Public Health Association, 2017*). Incubated plates were daily examined by the inverted microscope (Olympus CIX41, Japan) for the presence of any amoebic growth.

**Morphological identification of isolated FLAs**

The cloned amoebae (both trophozoites and cysts) on plates were morphologically examined for the presence of FLAs and identification of those belonging to *Acanthamoeba* according to the key described by Page (*Pussard and Pons, 1977; Page, 1988*). Amoebae, suspected to be *Acanthamoeba*, were sub-cultured to isolate and purify grown amoebae for further investigations (*Al-Herrawy, 1992*).
Molecular confirmation of the isolated *Acanthamoeba* by polymerase chain reaction (PCR) (Schroeder *et al.*, 2001).

A simple PCR technique was used, consisting of DNA extraction and amplification followed by agarose gel electrophoresis. *Acanthamoeba* DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) following the manufacturer instructions. PCR was done to amplify a restricted fragment of DNA through generic primers (JDP1 and JDP2) for identification of *Acanthamoeba* species (Table 1).

Each PCR reaction was carried out in a final volume of 50 μl (25μl master mix "Promega, USA", 3μl template DNA, 2μl forward and reverse primers and 20μl diethylpyrocarbonate "DEPC-treated water"). The amplification program included an initial denaturation at 95°C for 5min, followed by 35 cycles; each consisted of denaturation at 94°C for 30sec., annealing at 55°C for 40sec and extension at 72°C for 40sec. The program included a final extension step at 72°C for 10min to generate amplification fragments from 423-551bp (Schroeder *et al.*, 2001). The obtained PCR products were visualized and photographed using agarose gel electrophoresis and documentation system.

### Table 1. Sequence of a primer pair for detection of genus *Acanthamoeba*.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Primer direction</th>
<th>Primer sequence (5' - 3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acanthamoeba</em> spp.</td>
<td>Forward</td>
<td>GGCCCAGATCGTTTACCGTGAA</td>
<td>Schroeder <em>et al.</em> (2001)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>JTCTCACAAGCTGCTAGGGAGTCA</td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis

The obtained data were statistically analyzed using GraphPad Prism version 7.0 (USA) software. The critical *P*-value for the test was set at <0.05.

### RESULTS AND DISCUSSION

Members of genus *Acanthamoeba* exist in nature either as a trophic amoeba feeding on bacteria present in soil and water, or as a non-feeding dormant cyst. The trophic form of *Acanthamoeba* is characterized by the presence of thorn-like pseudopodia called acanthopodia and there is no flagellate form. The cyst form is characterized by a double-layered cyst wall having a varying number of pores (Pussard and Pons, 1977).

*Acanthamoeba* species were isolated from all the collected environmental samples from Helwan University. Morphologically, the trophozoites of different *Acanthamoeba* species were nearly similar. They have finger-like locomotive projections arising from the cytoplasm. However, these trophozoites varied in length from 20 to 45μm and ranged from 15 to 30μm in width. The outline of an amoeba was often irregular but it was generally longer than broad. A single vesiculate nucleus was seen in the anterior half of endoplasmic region. The nucleus measured 4 – 8μm in diameter and had a
characteristically large centrally located dense nucleolus surrounded by a clear halo and thin nuclear membrane (Figure 1A). The cyst form of *Acanthamoeba* species was characterized by the presence of a double cyst wall (ectocyst and endocyst). An *Acanthamoeba* cyst had a smooth or wrinkled outer wall (ectocyst) and a stellate, polygonal, star–like or even inner wall (endocyst) and measured 12 to 25 µm in diameter. There were plugged pores scattered on surface of the cyst wall; these pores were covered by opercula. Also, *Acanthamoeba* cysts had different shapes which were species specific (Figure 1B). All the morphologically detected *Acanthamoeba* proved to be belonging to genus *Acanthamoeba* when tested by PCR using a genus-specific primer pair (Figure 2). Other workers used riboprinting (RFLP analysis of the 18S small subunit ribosomal RNA (srRNA) gene) for the classification of *Acanthamoeba* species at the subgenus level (Chung et al, 1998; Kong and Chung, 2002).
Acanthamoeba species were isolated, in the present investigation, from all environmental samples of Helwan University. Examination of 144 environmental samples collected from Helwan University revealed that the highest percentage of Acanthamoeba (91.2%) was recorded from irrigation water samples, soil (83.3%), swabs samples from surfaces (54.2 %), domestic wastewater (50%), and lastly tap water and air samples with a similar occurrence (13%) for each (Table 2 and Figure 3).

In a previous study conducted on tap water from five governorates in Egypt, 26.6% out of 180 tap water samples were positive for Acanthamoeba species. They also found that Faiyum governorate was the highest site for occurrence of Acanthamoeba in tap water 36.1% (13/36), followed by Helwan 27.8% (10/36) and Cairo was the lowest site for occurrence of Acanthamoeba 19.4% (7/36) (Gad et al., 2019). Other several studies, conducted previously in Egypt, recorded that 80%, 58.6%, 56.3%, 31.4%, 67.7% and 29.2% of drinking water samples, collected from Beni-Suef governorate, Nile Delta governorates, Giza governorate, Cairo governorate and Faiyum governorate, respectively, were positive for Acanthamoeba species (Gad and Al-Herrawy, 2016; Morsy et al., 2016; Tawfeek et al., 2016; Sakran et al., 2017; Al-Herrawy et al., 2017; Abd El Wahab et al., 2018).

Globally, Acanthamoeba spp. have been documented in tap water in Korea (5.8%) Nicaragua (19%), Turkey (4.4% and 26.8%) and Philippines (9.1%) (Jeong and Yu, 2005; Leiva et al., 2008; Coşkun et al., 2013; Onichandran et al., 2014).

In our opinion, there are big differences in detection rates of Acanthamoeba in different sites and countries due to the difference in geographic areas, the quality of raw water sources or additional treatment technologies facilities in each country.

Statistical analysis of the obtained data revealed that the sampling source and types of samples had a strong significant correlation (P<0.0001 and R²=0.3784) with the prevalence of Acanthamoeba in the environment of Helwan University (Table 3).
Results of the present work declared that spring season recorded the highest appearance of *Acanthamoeba*. Irrigation water, soil and swabs from surface samples in spring season had the highest percentage of *Acanthamoeba* (100%). Also irrigation water samples recorded full appearance in summer season. The highest occurrence of *Acanthamoeba* in irrigation water samples was observed in spring and summer seasons (100%), and then it decreased to be 83% in winter and autumn. The highest occurrence of *Acanthamoeba* in
soil samples was observed in spring season (100%), and then it decreased to be 83% in winter, while it reached to the lowest occurrence 67% in autumn. The highest occurrence of *Acanthamoeba* in swabs from surfaces samples was observed in spring season (100%), and then it represented 50% in summer and autumn, while it reached the lowest occurrence (17%) in winter. The highest occurrence of *Acanthamoeba* in wastewater samples was observed in winter season (67%), and then it represented 50% in spring and autumn, while it reached the lowest occurrence (33%) in summer. On the other hand, the occurrence percentage of *Acanthamoeba* in tap water samples was the same in spring, autumn and winter(represented by 17% for each), while it was disappeared in summer. Concerning air samples, the highest occurrence of *Acanthamoeba* was recorded in summer season, while they disappeared in spring, autumn and winter (Figure 4).

![Figure 4: Seasonal variation of Acanthamoeba Spp. in environmental samples from Helwan university](image)

Other workers in Egypt found that winter followed by autumn showed the peak for *Acanthamoeba* species in all inspected governorates. In Faiyum and Qalyubia governorates, winter was the highest season for occurrence of *Acanthamoeba* species (55.5 and 33.3%, respectively). Although *Acanthamoeba* species have been identified throughout the year, wet seasons showed the highest occurrence (Gad *et al.*, 2019).

*Acanthamoeba* species, the most common free-living amoebae, have been isolated from a wide range of environments particularly water. These amoebae have been reported to feed by phagocytosis on bacteria, fungi, and algae (Król-Turmińska and Olender, 2017; Chen *et al.*, 2018). According to the previous reports, *Acanthamoeba* might serve as an environmental reservoir for viruses living in the same environment, such as *Mimi* virus, *Coxsackie* virus and *Adenovirus* (Scheid and Schwarzenberger, 2012; Yousuf *et al.* 2019).
Other workers demonstrated that the environmental isolate *Acanthamoeba mauritaniensis* genotype T4D, which was previously characterized as a non-pathogenic amoeba by De Jockheere (1980), is able to produce and secrete serine proteases that can be involved in epithelial damage and in the alteration of TJ proteins (Coronado-Velázquez et al., 2020).

The seasonal variation of *Acanthamoeba* was noted, with a peak during summer months or warmer months either in clinical or water samples (Page and Mathers, 2013; Gad and Al-Herrawy, 2016). Other workers found that *Acanthamoeba* genotype T4 was the most predominant genotype in tap water in Egypt. Regardless of the disinfectant applied at a drinking water utility, cross-contamination can occur throughout the water distribution system due to cavitations; therefore, the use of secondary disinfectants in distribution systems is required (Gall et al., 2015). Recently, among the free-living amoebae (FLAs) microbiome, the highly pathogenic *Helicobacter pylori* bacteria were detected alive from the inside of these amoebae, pointing out that FLAs are carriers of these pathogens which can reach humans and cause a public health concern (Moreno-Mesonero et al., 2020).

**CONCLUSION**

The relatively high prevalence of *Acanthamoeba* species in tap water presents a public health hazards which reflect the importance of the presence of a regular monitoring plan for the water sources in Egypt. Generally, this work has underlined the need for additional deeper studies to investigate the actual genotypes of free-living amoebae and how they could be eliminated.

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**REFERENCES**


Distribution of potentially pathogenic *Acanthamoeba*


ARABIC SUMMARY

توزيع عزلات ال Acanthamoeba المسببة للأمراض في بيئة جامعة حلوان، مصر

1- قسم علم الحيوان والجراثيم - كلية العلوم - جامعة حلوان - مصر.
2- قسم بحوث تلوث المياه - المركز القومي للبحوث - جيزة - مصر.

تمثل أفراد جنس ال Acanthamoeba أكثر أجناس الأميبات حرة المعيشة تواجداً في البيئة في جميع أنحاء العالم. ومعظم أفراد هذا الجنس يمكن أن تسبب التهاب الدماغ الأمامي الحبيبي والتهاب القرنية الأمامي لدى البشر وذلك لقدرتها على إنتاج إنزيمات proteases التي تعتبر من أهم عوامل شرائها وضرواتها، إلى جانب قدرتها على إيواء البكتيريا المسببة للأمراض والفطريات والفطريات.

الهدف من هذه الدراسة هو تقييم وجود ال Acanthamoeba في بيئة جامعة حلوان، مصر. تم جمع ستة أنواع من العينات (ماء الصنبور، مياه الري، مياه الصرف الصحي، مسحات من الأسطح، التربة والهواء) وتتم معالجة هذه العينات وتركيزها واستزراعها على بيئة الأجار غير المعزلي. تم إعادة زرع العينات الإيجابية ل Acanthamoeba باستخدام البادئ الخاص بجنس ال PCR Acanthamoeba في PCR وتصور النتائج التي تم الحصول عليها. تم أنه تم الكشف عن جنس ال Acanthamoeba Acanthamoeba وأظهرت النتائج البنوكية عند استخدام تقنية ال PCR Acanthamoeba 12.5% من مياه الري، التربة، المسحات، مياه الصرف الصحي، عينات مياه الصنبور و Acanthamoeba المعرفة مورفولوجي أنها تتبع جنس Acanthamoeba Acanthamoeba عند اختبارها باستخدام تقنية ال PCR. كان لمصدر أخذ العينات ارتباط قوي وكبير على انتشار ال Acanthamoeba Acanthamoeba في موسم الري في بيئة مياه الري والتربة. ومسحات السطح Acanthamoeba ملاحظة أنه ارتفاع انتشار الأميبات التابعة لجنس ال Acanthamoeba في مياه الري والتربة يشكل خطرًا صحية عامة للطلاب والعاملين في جامعة حلوان.