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An investigation on potentially pathogenic *Acanthamoeba* species from a wastewater treatment plant in Alexandria, Egypt

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ABSTRACT

From the Wardian wastewater treatment plant in Alexandria, 48 water samples were collected and filtered through nitrocellulose membranes and placed on non-nutrient agar with *Escherichia coli* to cultivate free-living amoebae (FLAs). Results revealed that the occurrence of FLAs reached 75.0% and 62.5% in raw and treated wastewater samples, respectively. Morphological identification of FLAs from raw and treated wastewater samples showed that 45.8% and 37.5%, respectively (from the positive samples) belong to the genus *Acanthamoeba*. Whereas, other FLAs belong to the other genera in raw and treated water samples that reached 29.2% and 25.0, respectively. The treated water process of the plant removed only 12.5 % of FLAs. Statistically, seasons recorded no significant effect on the prevalence of FLAs in the wastewater samples. 85.0 % from the morphologically identified *Acanthamoeba* spp. were confirmed, when molecularly tested using a simple PCR technique. In conclusion, the presence of FLAs in treated wastewater leads to direct and indirect public health hazards as they may harbor other pathogenic microorganisms that can escape from the wastewater treatment processes and reach the end-user.

INTRODUCTION

Wastewater treatment plants are usually designed to efficiently remove solids and dissolved pollutants (organic matter and nutrients), but are seldom specifically planned to remove pathogenic microorganisms from wastewater. The removal efficiency of pathogenic microorganisms during wastewater treatment is characterized by high variations, and depends on several factors such as type of treatment process, hydraulic retention time, retention of solids, dissolved oxygen concentration, pH, temperature and the efficacy in removing suspended solids **(Tyagi *et al*., 2011)**.

Due to the increase in demand for water, the reuse of treated urban wastewater has been extended for agricultural, industrial, environmental and municipal uses over the last few decades. One of the main concerns for the reuse of treated urban wastewater is human exposure to biological pollution, not only bacterial pathogens but also helminth eggs, protozoa and enteric viruses, and the possibility of diseases being spread, since biological pollution is not commonly eliminated by conventional secondary wastewater treatment **(Castro-Hermida *et al*., 2008)**.

Several FLAs have been known to be pathogenic to humans **(Schuster & Visvesvara, 2007; Rocha-Azevedo, 2009).** Those pathogenic amoebae have been recovered from municipal sewage sludge, farm soils fertilized with sewage wastes, freshwater streams receiving discharged effluent, and from sewage lagoons **(Sawyer, 1989)**. It is now widely accepted that foul or polluted environment is the principal source of potentially pathogenic FLAs. They have been isolated from processing water systems such as cooling towers, hospital water networks, drinking and wastewater treatment plants **(Thomas, 2006; Corsaro, 2009; Garcia, 2013)**.

Potentially pathogenic *Acanthamoeba* species are the causative agent of amoebic keratitis (AK) and granulomatous amoebic encephalitis (GAE), while *Naegleria fowleri* and *Balamuthia mandrillaris* have been associated with amoebic meningoencephalitis (PAM) and GAE, respectively **(Visvesvara *et al*., 2007)**. Moreover, FLAs can also act as reservoirs for pathogenic bacteria such as methicillin-resistant *Staphylococcus aureus*, *Vibrio cholerae*, *Legionella pneumophila*, and *Mycobacterium* spp. **(Goni *et al*., 2014)**.

There has been an increase in water pollution in the recent years mainly due to population growth and industrial development, which have made water treatment necessary in many communities and in various industries. Wastewater offers a good example of a simple and an inexpensive form for a community, especially in many developing countries (**Rivera *et al*., 1985**). Thus, the present investigation was organized to evaluate the occurrence of potentially pathogenic FLAs in raw and treated wastewater from a plant in Alexandria and assess the efficacy of conventional wastewater treatment steps in removing the FLAs.

MATERIALS AND METHODS

Wastewater treatment plant

Wardian wastewater treatment plant in the West of Alexandria is located in a commercial, industrial and residential area. The approximate serviced area is 4,200 acres, the area of the treatment plant is 136,000 m^2 and serves a population of about 1.3 million people. The primary treatment process consisted of six stages: mechanical filtration, primary sedimentation basins, microscopic filtration, chlorine units, gravity condensing units, and sludge lifting station.

Samples collection

A yearlong study, from January to December 2018, was conducted to examine a total of 48 (Raw "Inlet" and treated "outlet") wastewater samples collected every two weeks from the plant in one-liter volume autoclavable polypropylene Stoppard containers. Samples were transported in an ice box to Environmental Parasitology Laboratory, Water Pollution Research Department, National Research Center, Dokki, Giza, where they were processed at the same day of collection.

Concentration, cultivation and isolation of FLAs

The collected water samples were separately concentrated, using cellulose nitrate membranes (0.45 µm pore size and 47 mm diameter). Then, the concentrated samples were placed on non-nutrient agar plates, spread with *Escherichia coli* bacteria and incubated at 37°C for one week, with a daily microscopic examination for the presence of amoebic growth according to the method of **Page (1974)**. Amoebic plaques on positive plates were separately sub-cultured for isolation and propagation of different amoebic isolates, following the method described by **Al-Herrawy (1992).**

Morphological identification of FLAs

Isolated wastewater FLAs (*Acanthamoeba* spp.) were identified on the bases of both trophozoite and cyst morphological characteristics according to **Pussard and Pons (1977), Page (1988)** and **Al-Herrawy (1992).**

Molecular identification of *Acanthamoeba* spp.

For molecular identification of morphologically identified *Acanthamoeba*, a simple 3- steps PCR was carried out; DNA extraction, amplification and agarose gel electrophoresis. DNA was extracted from the obtained FLAs using a QIAGEN®, DNA Mini-Prep Kit (Madison, USA). PCR was amplified using generic primers (JDP1 and JDP2) to identify *Acanthamoeba* spp. **(Table 1)**.

Table (1): **Sequence of a primer pair for detection of *Acanthamoeba* spp**.

Organism	Primer direction	Primer sequence (5`- 3`)	Reference
***Acanthamoeba* spp.**	**Forward**	**GGCCCAGATCGTTTACCGTGAA**	**Schroeder *et al.* (2001)**
	Reverse	**TCTCACAAGCTGCTAGGGAGTCA**	

Electrophoresis on agarose gel was done to separate DNA fragments as previously described (**Helling *et al.*, 1974)**. Each PCR reaction was carried out in a final volume of 25 µl, an initial denaturing step of 3 min at 95 ^{o}C, followed by 40 cycles of one min at 95 ^{o}C, one min at 55 ^{o}C, and 2 min at 72 ^{o}C. This was followed by a final extension of 10 min at 72 ^{o}C to generate amplification fragments from 423 – 551 bp **(Schroeder *et al.*, 2001)**. Then, PCR products were visualized on a 1% agarose gel and photographed using the documentation system.

Statistical analysis

The obtained data were statistically analyzed using Paired T-test through Minitab Statistical Program **(Mayer & David, 2004)**. The P value < 0.05 was considered statistically significant.

RESULTS

Occurrence of FLAs in the wastewater samples

Generally, the morphological examination of 48 wastewater samples collected from the plant along one-year period revealed that the highest percentage of FLAs reached 75.0% in raw wastewater samples, while the lowest percentage was 62.5% in treated water samples. The removal percentage of FLAs, after different treatment processes, was only 12.5% (Table 2).

Table (2): Occurrence of FLAs in wastewater samples.

	Samples no.	+ ve FLAs	Removal %
Raw water	24	18	12.5
Treated water	24	15	
Total	48	33	

Morphological identification of *Acanthamoeba* spp.

Examination of raw and treated wastewater samples showed that, the percentage of morphologically identified *Acanthamoeba* spp. was 11 (45.8%) and 9 (37.5%) in raw and treated water samples, respectively. Whereas, the other FLAs reached 7 (29.2%) and 6 (25%) in raw and treated water samples, respectively (Table 3 & Chart 1).

Table (3): Occurrence of morphologically identified *Acanthamoeba* spp. and the other FLAs in wastewater samples

<table>
<tr><th rowspan="2">Wastewater samples</th><th colspan="2">Raw</th><th colspan="2">Treated</th></tr>
<tr><th>N0. Examined</th><th>No. Positive</th><th>No. Examined</th><th>No. Positive</th></tr>
<tr><td>Morphologically identified Acanthamoeba spp.</td><td rowspan="2">24</td><td>11</td><td rowspan="2">24</td><td>9</td></tr>
<tr><td>The other FLAs</td><td>7</td><td>6</td></tr>
</table>

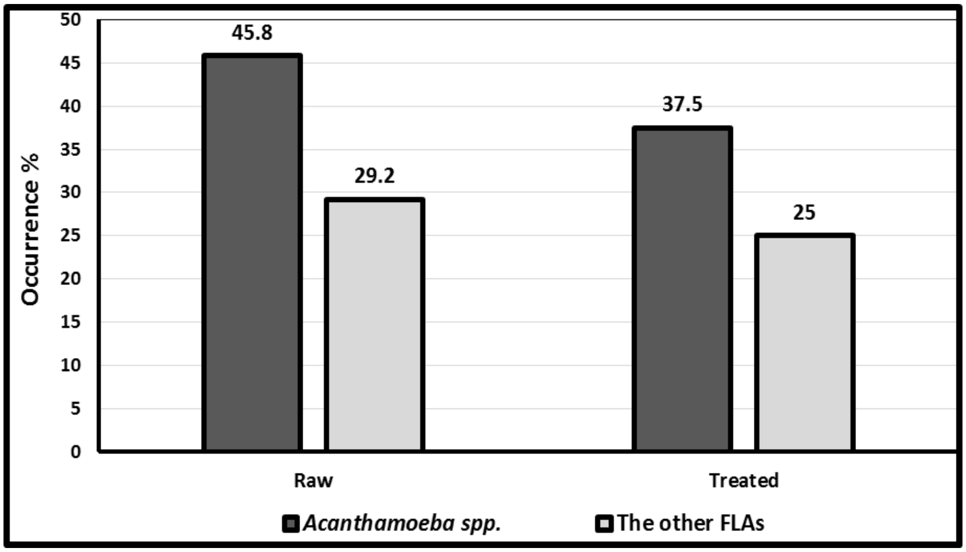


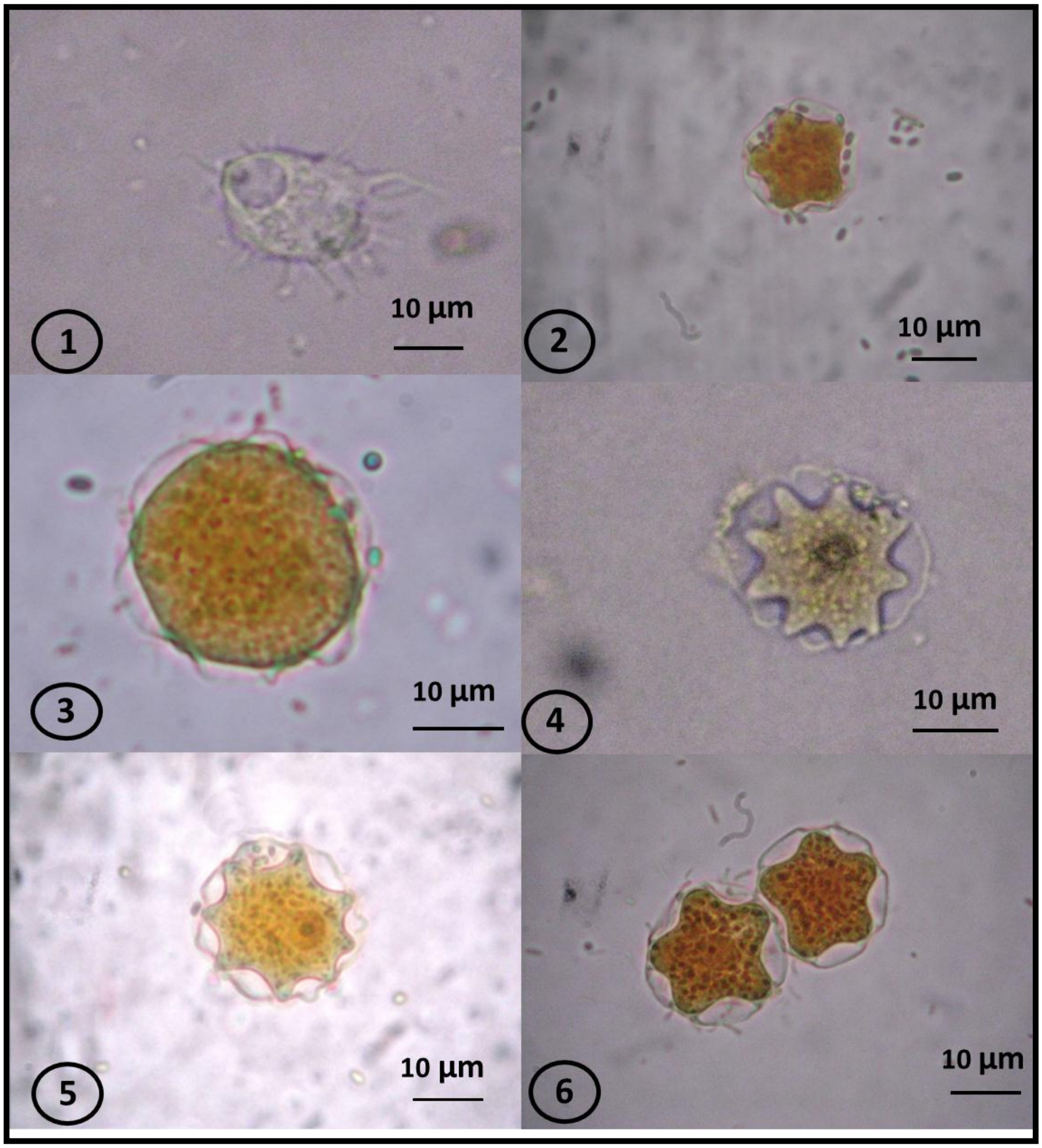
Chart (1): Showing the occurrence of morphologically identified *Acanthamoeba* spp. and the other FLAs in wastewater samples.

Remarkably, trophozoites of different potentially pathogenic *Acanthamoeba* species were nearly morphologically similar to each other. They had finger-like locomotive projections arising from the cytoplasm. However, those trophozoites varied in length that ranged from 20.0 to 45.0 µm, and width ranging from 15.0 to 30.0 µm. The outline of *Acanthamoeba* spp. trophozoites was often irregular, but was generally longer than the broad. The endoplasmic zone had a single vesicular nucleus in the anterior half. The nucleus measure was 4.0 – 8.0 µm in diameter and had a characteristically large central located dense nucleolus surrounded by a clear halo (Figure 1).

On the other hand, cysts of *Acanthamoeba* species were characterized by the presence of a double cyst wall. The outer cyst wall (ectocyst) was smooth or wrinkled, while the inner (endocyst) was a stellate or polygonal or even star-like measuring 12.0 to 25.0 µm in diameter. There were plugged pores scattered on the surface of the cyst wall and covered by opercula. *Acanthamoeba* spp. cysts had different shapes, which were species - specific (Figures 2 – 6).

Concerning the effect of seasonal variations on prevalence of *Acanthamoeba* spp in raw wastewater samples, it reached 80.% in winter, followed by 75.0% in summer and 40.0% in spring, while the lowest prevalence of *Acanthamoeba* spp. (33.3%) was recorded in autumn. Whereas, in treated water samples, *Acanthamoeba* spp. had the highest prevalence (75.0%) in both winter and spring, followed by autumn (50.0%), and the lowest prevalence (20.0%) was recorded in summer. The other FLAs in raw wastewater sampels recorded the highest prevalence (66.7%) in autumn, followed by a 60.0% in sping and 24.0% in summer, and the lowest prevalence (20.0%) was recorded in winter. Moreover, in treated water samples, the other FLAs had the highest prevalence

(80.0%) in summer, followed by autumn (50.0%) and the lowest prevalence (25.0%) was recorded in both winter and spring (Table 4) and (Chart 2).



Figures (1 - 6): Photomicrographs of morphologically identified *Acanthamoeba* spp., isolated from wastewater samples: Figure (1): Trophozoite. Figures (2–6): Cysts.

Table (4): The occurrence of morphologically identified *Acanthamoeba* spp. and the other FLAs in wastewater in different seasons.

	Raw wastewater samples				Treated wastewater samples			
	Samples No.	+ ve samples	Morphologically identified *Acanthamoeba*	The other FLAs	Samples No.	+ ve samples	Morphologically identified *Acanthamoeba*	The other FLAs
Winter	6	5	4	1	6	4	3	1
Spring	6	5	2	3	6	4	3	1
Summer	6	4	3	1	6	5	1	4
Autumn	6	3	1	2	6	2	1	1

Statistically, as the *P* value was 0.119 (more than 0.05), seasons had no significant effect on the prevalence of *Acanthamoeba* spp. and the other FLAs in raw and treated wastewater samples (Table 5).

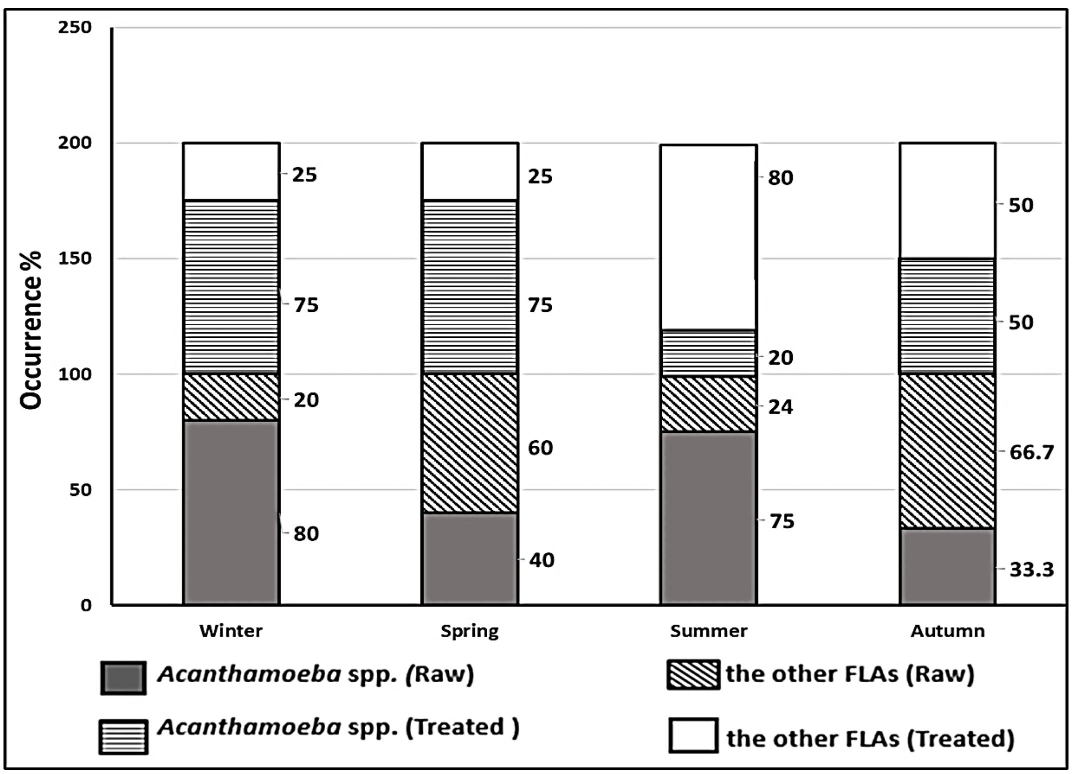


Chart (2): Showing the occurrence of morphologically identified *Acanthamoeba* spp. and the other FLAs in wastewater in different seasons.

Table (5): One-way ANOVA for testing response of *Acanthamoeba* spp. and the other FLAs from wastewater samples versus seasons.

Source	DF	SS	MS	F	*P*
Factor	**3**	**3.333**	**1.111**	**2.67**	**0.119**

DF:Degree of freedom; **SS**: Sum of squares; **MS**: Mean square; **F**: F-distribution variable; ***P*** Significance.

Molecular identification of morphologically identified *Acanthamoeba* spp.

85.0 % from the morphologically identified *Acanthamoeba* spp. were confirmed to belong to the same genus (*Acanthamoeba*), when tested by a simple PCR technique using a genus-specific primer pair (Figure 7).

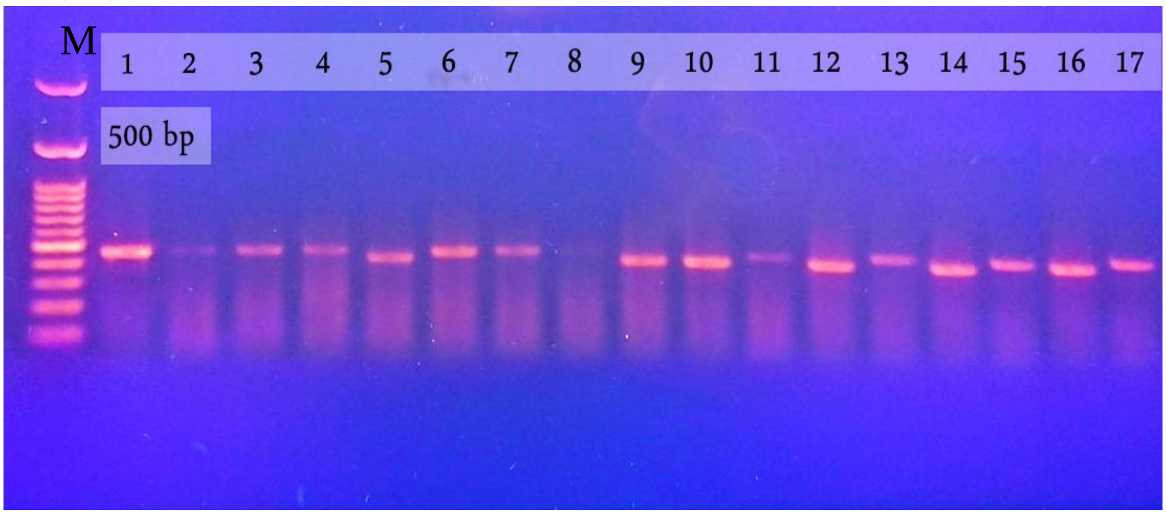


Figure (7): Agarose gel electrophoresis for PCR amplified product of DNA from *Acanthamoeba* spp. M: Marker; Lanes (1- 17) Positive samples.

DISCUSSION

Well-known concentration as membrane filtration and centrifugation methods were used to isolate and concentrate FLAs in environmental water samples **(Muchesa *et al.*, 2014)**. In the present study, using the concentration method, FLAs were not detected from all collected water samples from the wastewater treatment plant. However, **Muchesa *et al.* (2014)** found that, FLAs in all collected water samples from the wastewater treatment plant in Gauteng Province, South Africa, use the filteration method. The concentration method in recovering amoebae in seeded water samples was compared, results showed that samples that were filtered significantly recovered more amoebae as compared to samples that used centrifugation as a concentration method. The present findings are similar to those reported by Health Protection Agency **(HPA, 2004)** in membrane filtration method which was found to be more efficient in recovering amoebae, when compared to the centrifugation method. **Winiecka-Krusnell and Lind**

(**1998)** also concluded that, amoebae can be recovered more easily, when water samples are processed by filtration rather than by centrifugation, however, the concentration method was also used depending on some extent on the sample volumes. Studies using centrifugation method had concentrated relatively on the small sample volumes of 50 mL, compared to the filtration method, which was used to concentrate relatively on high sample volumes of 500 mL and 1000 mL **(Pagnier *et al.*, 2008; Ramirez *et al.*, 2008**).

In the present study, isolated FLAs from both raw and treated water samples from Wardian plant in Alexandria, Egypt were 33 (68.75%) all over the year. **Al-Herrawy *et al*. (2020)** in Cairo, Egypt detected FLAs from 11 (45.8%) wastewater samples from Helwan University**.** However, **Muchesa *et al.*** (**2014)** in South Africa showed that the occurrence of FLAs in a wastewater treatment plant reached 87.2 % **(Muchesa *et al*., 2014)**. There is a big difference in the detection rates of FLAs in different sites and countries due to the difference in geographic areas and in the quality of raw water sources or additional treatment technological facilities in each country **(Al-Herrawy *et al.*, 2020)**.

The current investigation showed a highly occuecnce of *Acanthamoeba* spp. recording a value of 11 (45.8%) among isolated FLAs, in accordance with the study carried out by **Ramierz *et al*. (2005),** where 59% of the detected FLAs from the positive household wastewater samples belonged to the same genus (*Acanthamoeba*). It suggested that the presence of cellulose in the wall of *Acantamoeba* spp. cysts gave more protection against a wide variety of environmental factors and could therefore, account for its persistence and widespread disribution in the environment (**Page, 1988**).

The current research showed that the presence of *Acanthamoeba* spp. was more than other FLAs, belonging to the other genera. The aforementioned result is in accordance with the results obtained by **Marin *et al.* (2015)** in the Navarra Community of Spain, where *Acanthamoeba* spp. were found as the most common FLAs in the outlet water and sludge in wastewater treatment plants. This can be explained by the high resistance of *Acanthamoeba* spp. cysts in harsh conditions and also by the fact that the other FLAs are more fragile **(Ramirez *et al.*, 2014)**.

The present findings showed that, trophozoites of *Acanthamoeba* spp., were nearly morphologically similar to each other. On the other hand, cyst forms of the present *Acanthamoeba* spp. were clearly distinguished from each other by the presence of a double cyst wall that varied in shape and size according to the species. The description of both the trophozoites and the cysts of *Acanthamoeba* spp. coincides with that obtained by **Al-Herrawy (1992)**, **Ashmawy *et al*. (1993)** and **Schuster and Viseversa (2004)**. **Mattar and Byers (1971)** previously described cyst of *Acanthamoeba* spp. as spherical in shape, with a double wall; an exocyst, that was continuous over the entire surface of the cyst, and an endocyst, that was discontinuous in areas referred to as the ostioles.

Statistically, seasons had no significant effect on the prevalence of FLAs in wastewater samples, a result of which agrees with the that recorded by **Tsvetkova *et al.* (2004)** in Bulgaria, who reported that the production of highly resistant cysts by these protozoa may explain the lack of significant differences among the number of isolated amoebae during the different seasons of the year**.**

In the present work, 85% of the morphologically identified *Acanthamoeba* spp. were proved to belong to the same genus (*Acanthamoeba*), when tested using a simple PCR technique. In Hormozgan Province, Iran, **Shamseddin *et al.* (2020**) found that 6 % of the 83 samples were positive for *Acanthamoeba* spp. by the culture method, while the ratio of the presence of *Acancamoeba* spp. reached 9.6% using PCR technique. This indicates a high sensitivity of the molecular method in the diagnosis of *Acanthamoeba* spp. Considerably, several studies indicated that PCR can be a more sensitive and effective method in diagnosing of *Acanthamoeba* spp., and hence, can eliminate the need for skilled microscopist, however, no single method is suggested **(Rivière *et al*., 2006; Boggild *et al*., 2009; Lau *et al*., 2015).**

In conclusion, the presence of the potentially pathogenic FLAs, including *Acanthamoeba* spp. in water, exerts an indirect public health hazards that may harbor other pathogenic microorganisms that can escape from wastewater treatment processes to reach the end user.

REFERENCES

Al-Herrawy, A.Z. (1992): In *vitro* cultivation of agents of amoebic meningoencephalitis isolated from water and sewage. Ph.D. Thesis, Fac. Vet. Med., Alex. Univ.

Al-Herrawy, A.Z; Bahgat, M.; Mohammed, A.; Ashour, A.; Hikal, W.; (2013): Morphophysiological and biochemical criteria of *Acanthamoeba* spp. isolated from the Egyptian aquatic environment. Iran. J. Parasitol. 8: 302-312.

Al-Herrawy, A.Z.; Koteit, H. and Elowa, S.E. (2020): Distribution of potentially pathogenic *Acanthamoeba* isolates in the environment of Helwan University, Egypt. Egy. J. Aqu. Biol. Fish. 24: 61-73.

Ashmawy, K.; Hilali, M.; Abu-El wafa, S.A.; Samah, H.; Draz, A.A. and Salem, A. (1993): In vitro identification of *Naegleria* and *Acanthamoeba* isolated from water and sewage. Assuit Vet. Med. J. 30: 87-100.

Badirzadeh, A.; Niyyati, M.; Babaei, Z.; Amini, H.; Badirzadeh, H. and Rezaeian, M. (2011): Isolation of free-living amoebae from Sarein hot springs in Ardebil Province, Iran. Iran. J. Parasitol. 6: 1-8.

Barbeau, J. and Buhler, T. (2001): Biofilms augment the number of free-living amoebae in dental unit waterlines. J. Micrbiol. Res. 8: 753-760.

Behets, J.; Declerck, P.; Delaedt, Y.; Verelst, L.; Ollevier, F. (2007): Survey for the presence of specific free-living amoebae in cooling waters from Belgian power plants. Parasitol. Res. 100: 1249-1256.

Boggild, A.K.; Martin, D.S.; Lee, T.Y.; Yu, B. and Low, D.E. (2009): Laboratory diagnosis of amoebic keratitis: comparison of four diagnostic methods for different types of clinical specimens. J. Clin. Microbiol. 47:1314-1318.

Castro-Hermida, J. A.; Garcia-Presedo, I.; Almeida, A.; González- Warleta, M.; Correia Da Costa, J. M. and Mezo, M. (2008): Contribution of treated wastewater to the contamination of recreational river areas with *Cryptosporidium* spp. and *Giardia duodenalis.* W. Res. 42: 3528-3538.

Cavalier-Smith (1993): Kingdom Protozoa and its 18 phyla: Microbiol. Rev. 57: 953-994.

Cermeño, J.R.; Hernández, H.E. and Yasin, I. (2006): Meningoencephalitis by *Naegleria fowleri* epidemiological study in Anzoategui State, Venezuela Revista da Sociedade Brasileira de Medicina Tropical. 39: 264-268.

Corsaro, D.; Feroldi, V.; Saucedo, G.; Ribas, F.; Loret, J.F. and Greub, G. (2009): Novel Chlamydiales strains isolated from a water treatment plant. Environ. Microbiol. 11: 188-200.

Garcia, A.; Goni, P. and Cieloszyk, J. (2013): Identification of free-living amoebae and amoeba-associated bacteria from reservoirs and water treatment plants by molecular techniques. Environ. Sci. and Technol. 47: 3132-3140.

Goni, P.; Fern´andez, M. T. and Rubio, E. (2014): Identifying endosymbiont bacteria associated with free-living amoebae. Environ. Microbiol. 16: 339-349.

Health Protection Agancy (2004): Isolation and identification of *Acanthamoeba* species. W17, issue2.

Helling, R.B.; Goodman, H.M. Boyer, H.W. (1974): Analysis of R. Eco RI fragments of DNA from lambdoid bacteriophages and other viruses by agarose-gel electrophoresis. J. Virol. 14: 1235-1238.

Lau, R.; Cunanan, M.; Jackson, J.; Ali, I.K.M.; Chong-Kit, A. and Gasgas, J. (2015): Reevaluation of an *Acanthamoeba* molecular diagnostic algorithm following an atypical case of amoebic keratitis. J. Clin. Microbiol. 53: 3213-3218.

Marciano-Cabral, F. and Cabral, G. (2003): *Acanthamoeba* spp. as agents of disease in humans. Clin. Microbiol. Rev.16: 273-307.

Marin, I.; Goni, P.; Lasheras, A. and Ormad, M. (2015): Efficiency of a Spanish wastewater treatment plant for removal potentially pathogens: Characterization of bacteria and Protozoa along water and sludge treatment lines. Ecol. Eng. 74:28-32.
Maryland Soils. Amer. Soc. Microbiol. 55: 1074-1077.

Mattar, F.E. and Byers T.J. (1971): Morphological changes and the requirements for macromolecule synthesis during excystment of *Acanthamoeba castellanii*. J. Cell Biol. 49: 507-519.

Mayer, R.K. and David, D.K. (2004): A minitab guide to Statistics (3rd ed.). Upper Saddle River, NJ: Printce-Hall publishing. ISBN978-0-13-149272-1.

Morsy, G.; Al-Herrawy A.; Elsenousy, W. and Marouf, M. (2016): Prevalence of free-living amoebae in tap water and biofilm, Egypt. Res. J. Pharm. Biol. Chem. Sci., 7: 752-759.

Muchesa, P.; Mwamba, T.; Barnard, T.G. and Barite, C. (2014): Detection of free-living amoebae using amoebal enrichment in a wastewater treatment plant of Gauteng Province, South Africa. Bio. Med. Res. Int. Article ID 575297 10 pages.

Nagyova, V.; Nagy, A. and Timko, J. (2010): Morphological, physiological and molecular biological characterisation of isolates from first cases of *Acanthamoeba* keratitis in Slovakia. Parasitol. Res. 106: 861-872.

Page, F.C. (1974): A further study of taxonomic criteria for *limax* amoebae with limax amoebae with descriptions of new species and a key to genera. Arch. Protistenk. 116: 149-184.

Page, F.G. (1988): A new key to freshwater and soil Gymnamoebae, F. w. Biol. Asso. Cumbria. 5: 92-97.

Pagnier, I.; Raoult, D. and La Scola, B. (2008): Isolation and identification of amoeba-resisting bacteria from water in human environment by using an *Acanthamoeba polyphaga* co-culture procedure. Environ. Microbiol. 10: 1135- 1144.

Pussard, M. and Ponus, R. (1977): Morphologie de la pario Kystiqueet taxonomie du genre *Acanthamoeba* (Protozoa, Amoebida). Protistology TXIII: 557-598.

Ramirez, E.; Warren, A. and Rivera, F. (1993): An investigation of the pathogenic and non-pathogenic free-living amoebae in an activated sludge plant. Water, Air, and Soil Pollution. 691:135-139.

Ramirez, E.; Robles, E. and Martinez, B. (2014): Distribution of free-living amoebae in a treatment system of textile industrial wastewater. Exp. Parasitol.; 145 Suppl: 34-38.

Rivera, F.; Garcia, G.; Lugo, A.; Zierold, E.; Islas, J.; Ramirez, E. and Bonilia, P. (1985): Amoebae in a waste stabilization pond system in Mexico. Water, Air, and Soil Pollution. 28: 185-198.

Rivera, F.; Roy-Ocotla, G.; Rosas, I.; Ramirez, E.; Bonilla, P. and Lares, F. (1987): Amoebae isolated from the atmosphere of Mexico-City and environs. Enviro. Res. 42: 149-154.

Rivière, D.; Szczebara, F.M.; Berjeaud, J.M.; Frère, J. and Héchard, Y. (2006): Development of a real-time PCR assay for quantification of *Acanthamoeba* trophozoites and cysts. J. Microbiol. Meth. 64: 78-83.

Rocha-Azevedo, B. da Tanowitz, H. and Marciano-cabral, F. (2009): Diagnosis of infections caused by pathogenic free-living amoebae interdisciplinary perspectives on Infectious Diseases. Interdiscip. Perspect. Infect. Dis. doi: 10.1155/251406.

Sawyer, T.K. (1989): Free-Living pathogenic and nonpathogenic amoebae in Maryland soils. American Soc. Microbiol. 18:1074-1077.

Schroeder, J.M.; Booton, G.C.; Hay, J.; Niszl, I.A.; Seal, D.V.; Markus, M.B.; Fuerst, P.A. and Byers, T.J. (2001): Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoeba* from humans with keratitis and from sewage sludge. J. Clin. Microbiol. 39: 1903-1911.

Schuster, F.L. and Visvesvara, G.S. (2007): Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals," Int. J. Parasitol. 34: 1001-1027.

Shamseddin, J.; Attariani, H.; Turki, H.; Shoja, S.; Salahi-Moghaddam, A. and Ghanbarnejad, A. (2020): Investigating the frequency of free-living amoeba in water resources with emphasis on *Acanthamoeba* in Bandar Abbas City, Hormozgan Province, Iran in 2019–2020. BMC Res Notes. 13:420-427.

Thomas, V.; Herrera-Rimann, K. Blanc, D. S. and Greub, G. (2006): Biodiversity of amoebae and amoeba-resisting bacteria in a hospital water network," App. Environ. Microbiol. 72: 2428-2438.

Tsvetkova, N.; schild, M.; Panaiotov, S.; Kurdov-Mintcheva, R.; Gottstein, B.; Walochnik, J.; Aspock, H.; Lucas, M.S. and Muller, N. (2004): The identification of free-living isolates of amoebae from Bulgaria. Parasitol. Res., 29:405-413.

Tyagi, V. K.; Sahoo, B. K.; Khursheed, A.; Kazmi, A. A.; Ahmad, Z. and Chopra, A. K. (2011): Fate of coliforms and pathogenic parasite in four full-scale sewage treatment systems in India. Environ. Monit. Assess. 81: 123-135.

Visvesvara, G.S.; Moura, H. and Schuster, F.L. (2007): Pathogenic and opportunistic free-living amoebae *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri* and *Sappinia diploidea*. FEMS. Immunol. Med. Microbiol. 50: 1-26.

Winiecka-Krusnell, J. and Linder, E. (1998): *Acanthamoeba* keratitis: increased sensitivity of the detection of parasites by modified cultivation procedure, *Scandinavian* J. Inf. Dis. 30: 639-641.