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Genotype diversity, phylogenetic analysis and seasonality of isolates of *Acanthamoeba* spp. in swimming pools in Kafrelsheikh, Egypt

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Abstract: Species of *Acanthamoeba* Volkonsky, 1931 are the commonest among free-living amoebae that are widespread in different water resources but with lacking phylogenetic data. This study aims at detecting molecular prevalence and genetic diversity of *Acanthamoeba* isolates in Kafrelsheikh Governorate, Egypt. Forty-eight water samples were collected from 12 swimming pools; four samples during each season over one year. Samples were filtered, cultivated on non-nutrient agar plates and examined microscopically. Polymerase chain reaction (PCR) and sequence analysis of positive samples targeting diagnostic fragment 3 (DF3) of the small subunit rRNA gene were done. Cultivation succeeded to detect 14 (29%) positive samples while PCR missed three positive samples. The obtained sequences were phylogenetically analysed. The phylogenetic tree was constructed for them with sequences of reference species from the NCBI database. The identified species were *Acanthamoeba castellanii* Douglas, 1930 (T4), *A. astronyxis* (Ray et Hayes, 1954) (T9) and *A. hatchetti* Sawyer, Visvesvara et Harke, 1977 (T11). The prevalence of species of *Acanthamoeba* was higher during summer and fall. Therefore, the control of the presence of *Acanthmoeba* spp. in swimming pools needs immediate, effective and practical measures to prevent and control infection with species of *Acanthamoeba*.

Key words: Acanthamoebidae, sequence analysis, phylogenetic analysis, seasonal variation

Species of the genus *Acanthamoeba* Volkonsky, 1931 are considered the most common free-living amoebe (FLA) isolated from natural water resources such as springs, lakes, rivers and artificial water systems like cooling waters, drinking water networks and insufficiently chlorinated swimming pools (Üstüntürk-Onan 2020).

During the last few decades, species of *Acanthamoeba* gained attention as most of them are potentially pathogenic, causing serious and fatal diseases in the skin, nasal passages, lungs and brain in both human and animals (Al-Herrawy et al. 2014). *Acanthamoeba* spp. are the causative agents of granulomatous amoebic encephalitis (GAE), sometimes skin infections that may affect immunocompromised patients. In addition, amoebic keratitis (AK) could be detected in immunocompetent individuals wearing hygienically deficient contact lenses (Al-Herrawy et al. 2017).

Conventional microscopical and culture methods are used for the identification of *Acanthamoeba* spp. up to the genus level only. Genetic analysis and interstrain variations can be achieved based on 18S ribosomal RNA gene sequence analysis (Prithiviraj et al. 2020). Till now, there are 22 genotypes (T1–T22) of *Acanthamoeba* spp. identified from different clinical and environmental samples (Esboei et al. 2020).

Many reports over the last decades focused on the study of the ecology of FLA. A wide range of environmental factors may be correlated to the prevalence and genotypic differences of *Acanthamoeba* spp. such as soil moisture, water type and seasonal changes (Kao et al. 2013).

In Kafrelsheikh Governorate, Egypt, the available data regarding the prevalence of *Acanthamoeba* spp. are lacking. The objectives of this study were to determine the occurrence and genotypic characterisation of *Acanthamoeba* spp. in swimming pools in Kafrelsheikh Governorate, Egypt, and to investigate the seasonal variations on its genotypic diversity.

MATERIALS AND METHODS

Study location, sample collection and processing

This is a cross-sectional study that was conducted along one-year period from January, 2019 to January, 2020.

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Table 1. List of NCBI 18S rDNA nucleotide sequence accession numbers for the five isolates of *Acanthamoeba* spp.

Isolate ID	Accession No.	Genotype	Species	Reference strains No.
sp3	MZ504290	T4	*Acanthamoeba castellanii*	KX018021.1
sp8s	MZ504291	T9	*Acanthamoeba astronyxis*	MN239988.1
sp8a	MZ504292	T11	*Acanthamoeba hatchetti*	MN700300.1/ MN700304.1
sp11	MZ504293	T4	*Acanthamoeba castellanii*	KX018021.1
sp11s	MZ504294	T9	*Acanthamoeba astronyxis*	MN239988.1

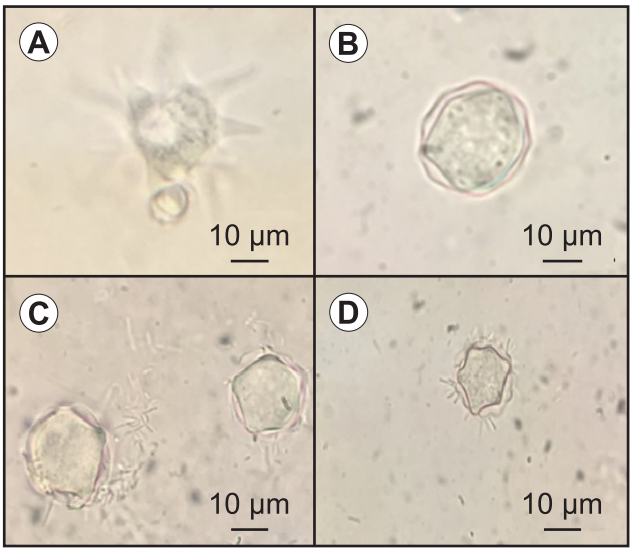

Fig. 1. Fresh unstained trophozoites (A) and cysts (B–D) of *Acanthamoeba* spp.

Forty eight water samples were collected from 12 outdoor swimming pools during the four different seasons (spring, summer, fall and winter) in Kafrelsheikh Governorate, Egypt. Water samples (1 litre volume each) were collected from the subsurface water of each swimming pool in sterile polypropylene bottles and transferred in iceboxes to the Laboratory of Parasitology, Theoder Bilharz Research Institute (TBRI), Giza, Egypt, where they were processed within 24 h.

Collected water samples were separately concentrated and filtred through nitrocellulose membrane (0.45 µm pore size and 47 mm in diameter) by using the membrane filtration technique (Di Filippo et al. 2015).

***Acanthamoeba* species cultivation and subcultivation**

After filtration, the membrane was placed face-down on the surface of non-nutrient agar (NNA) plates made with Page Amoebae Saline (PAS) and overlaid by a thin layer of *Escherichia coli*. All the cultured plates were incubated at 37°C with daily microscopic examination to detect trophozoites and cysts of *Acanthamoeba* spp. The plates were considered negative after 14 days of incubation and were discarded. When amoebic growth was observed, a piece of agar enclosing the amoebic growth was cut and placed into a fresh NNA-*E.coli* plate. Afterwards, the growing amoebae from the positive subculture plates were harvested by using a bacteriological loop. The surface of the non-nutrient agar was scraped and transferred to a sterile tube containing about 1 ml of PAS and centrifuged for 10 min at 6000 g. The pellet was resuspended in 100 µl of fresh saline buffer and stored in sterile Eppendorf at –20 °C for subsequent DNA extraction, molecular confirmation by conventional PCR technique using genus specific primers, and further sequencing.

DNA extraction and PCR assay

DNA extraction was performed by Quick-g DNA™ MiniPrep (Zymo Research, California, USA) according to manufacturer's instructions. For identification of species of the genus *Acanthamoeba*, a PCR was carried out to amplify 18S rDNA region defined as *Acanthamoeba* Specific Amplimer (ASA.S1) that includes the Diagnostic Fragment 3 (DF3), using the genus-specific primers JDP1 and JDP2. The *Acanthamoeba*-specific primer sequences were as follows: the forward primer JDP1(5'–GGCCCAGATCGTTTACCGTGA A-3') and the reverse primer JDP2(5'-TCTCACAAGCTGCTAGGGAGTCA-3') (El-Badry et al. 2020). Samples were subjected to initial denaturation at 94 °C for 3 min, then 35 cycles, each comprising denaturing at 94 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 30 s followed by a final extension at 72 °C for 7 min. Depending on the genotype, the primers amplified 423–551 bp of 18S rDNA between reference 936 bp and 1,402 bp (Schroeder et al. 2001).

Nucleotide sequencing and phylogenetic analysis

PCR products were purified using Thermo Scientific Gene JET PCR Purification Kit according to manufacturer´s instructions. Partial 18S rDNA sequencing (DF3 region) was carried out for genotypic identification with primers' amplification according to Tamura and Nei (1993). Sequences of the studied isolates were matched with reference sequences registered in the GenBank database through BLAST-NCBI (https://blast.ncbi.nlm.nih.gov), all sequences were aligned using the BioEdit software which on the ClustalW multiple alignment conditions. The evolutionary history was inferred by the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei 1993). Evolutionary analyses were conducted in MEGA X (Version 10.2.4) (Kumar et al. 2018).

RESULTS

Prevalence of *Acanthamoeba* spp. in the examined swimming pools detected by microscopy

Cultivation of all collected water samples from swimming pool revealed that 14 out of a total 48 samples (29 %) were positive for *Acanthamoeba* spp. (Table 1). Examination of positive culture and subculture plates revealed both trophozoites of *Acanthamoeba* spp. (48–72 hours after culturing) and cysts (at least three days after culturing). Trophozoites of *Acanthamoeba* were similar morphologically with variable sizes ranging from 25 to 40 µm and the typical large karyosome within the nucleus. Acanthopodia, spin-like projections, arised from the cytoplasm and are used for locomotion Fig. 1A.

Table 2. Seasonal distribution and the prevalence of *Acanthamoeba* isolates, *Acanthamoeba*-DNA amplified by PCR and *Acanthamoeba* species.

		Winter	Spring	Summer	Fall	Total
Sample Size		12	12	12	12	48
Acanthamoeba isolates		0	6	6	2	14
Sequenced *Acanthamoeba* species	*A. castellanii* /T4	0	1	1	0	2
	A. astronyxis /T9	0	0	1	1	2
	A. hatchetti /T11	0	0	0	1	1

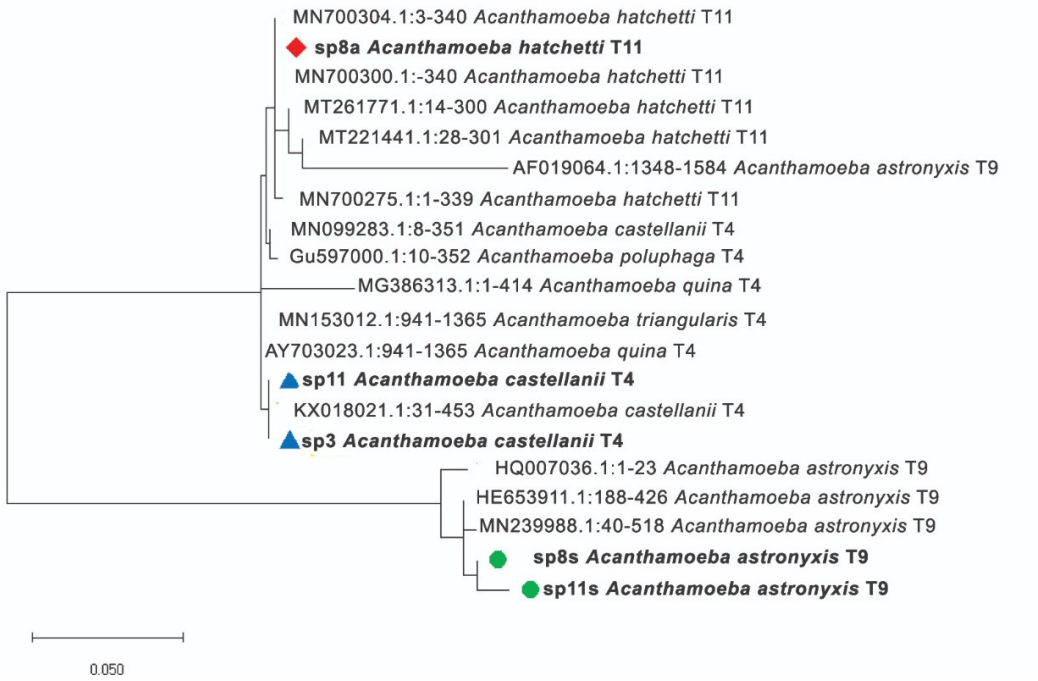

Fig. 2. The maximum-likelihood constructed phylogenetic tree of *Acanthamoeba* isolates inferred from the small subunit ribosomal RNA gene sequences from GenBank showing the phylogenetic position of three strains detected in the present study; T4 (blue), T9 (green) and T11 (red). The tree with the highest log likelihood (-1201.92) is shown.

The cysts of *Acanthamoeba* spp. has a typical double cyst wall (ectocyst and endocyst). An *Acanthamoeba* cyst had a smooth or wrinkled outer wall (ectocyst) and a stellate, polygonal or star-like inner wall (endocyst) and measured 10–30 µm in diameter (Fig. 1B–D).

Sequencing and phylogenetic analysis of *Acanthamoeba* isolates

Five isolates were successfully sequenced while the rest of the sequences were non-interpretable possibly due to ineffective and/or insufficient amplified products. These sequences were phylogenetically analysed. The phylogenetic tree was constructed for them with sequences of reference species from NCBI-BLAST. The results of sequencing are summarised in Table 2. Isolates Sp3 and Sp11 showed 99.10–99.52% homology with *Acanthamoeba castellanii* (Pussard et Pons, 1977) (KX018021.1) ano 99.40–100% homological identities with genotype T4. While isolates sp8s and sp11s showed 96.20–98.74% homology with *Acanthamoeba astronyxis* (Pussard et Pons, 1977) (MN239988.1) and 95.86–98.52% homology with genotype T9. In addition, sp8a isolate showed 98.53–98.82% homology with *Acanthamoeba hatchetti* (Pussard et Pons, 1977) (MN700300.1) and (MN700304.1), with 99.42% homological identities with genotype T11. The sequences were submitted to GenBank with accession numbers MZ504290–MZ504294 (Table 1, Fig. 2).

Seasonal variation and genetic diversity of *Acanthamoeba* isolates

The greatest percentage of species of *Acanthamoeba* was detected during the summer (54.5%) followed by spring (27.3%), then fall (18.2%) and none was detected in winter (0%). Diversity of *Acanthamoeba* spp. related to seasonal temperature variations was detected. Site eight of swimming pool samples showed *A. astronyxis* (T9) in the summer season (Isolate sp8s) and *A. hatchetti* (T11) in fall season (Isolate sp8a). In site 11, *A. castellanii* (T4) in the spring season (Isolate sp11) and *A. astronyxis* (T9) in summer season (Isolate sp11s) (Tables 1, 2).

DISCUSSION

Swimming pools are continuously exposed to a wide range of contaminants either by swimmers or from environmental sources like rain or wind. Additionally, mi-

croorganisms, especially parasites including FLA, we reported as causative agents of most outbreaks associated with swimming pools (Al-Herrawy et al. 2017). During unfavourable conditions, trophozoites of *Acanthamoeba* spp. transform to the cyst, which is more resistant to dehydration, osmolarity, freezing, pH, irradiation, ultraviolet radiation and chemical disinfectant. Thus, investigating of contamination pools and necessary hygienic measures are vital to protect swimmers from different pathogens (Esboei et al. 2020).

The prevalence of *Acanthamoeba* spp. in swimming pools was addressed during the four different seasons in Kafrelsheikh Governorate, Egypt. *Acanthamoeba* spp. were found in 29% of the examined pools. Similar results were detected in another study in Alexandria that also recorded a prevalence of 29% (Al-Herrawy et al. 2017). The infection rate in the present study is not so high and this may be explained by the chlorine doses used in pools treatment in Kafrelsheikh Governorate.

Other studies in Egypt and Poland revealed a higher percentage of 37% and 60%, respectively (Al-Herrawy et al. 2014). Lower values were recorded by Mafi et al. (2017) who reported 24% prevalence in pools in Tehran (Mafi et al. 2017). The differences in the reported prevalence in different countries may be related to the sample size, type of water, geographical distribution, amoeba recovery methods, seasonal variation or water treatment protocols (Stockman et al. 2011).

In our study, PCR succeeded to detect only 79% of the previously morphologically diagnosed *Acanthamoeba* spp. Similarly, Al-Herrawy et al. (2014) documented that PCR detected only 96% of the morphologically positive *Acanthamoeba* spp. in swimming pools in Cairo, Egypt. In the present study, data obtained from *Acanthamoeba* spp. isolates were found to belong mainly to genotypes T4 *(A. castellanii*) and T9 (*A. astronyxis*) followed by T11 (*A. hatchetti*).

Genotype T4 was reported as the most prevalent and pathogenic genotype amongst all the known genotypes (Behera et al. 2016). Therefore, pools should be considered as an important source for infection transmission. This is in accordance with observations of Aghajani et al. (2016), Behera et al. (2016), and Abd El Wahab et al. (2018) who reported that T4 strain had the highest prevalence in the environment.

In contrast, Maghsood et al. (2005) reported T2 (58%) as the most prevalent genotype in Iran. Al-Herrawy et al. (2017) reported T3, T5, T11 and T15 genotypes in the swimming pools' samples. They also reported that genotypes T3 and T11 are closely related to T4 and this close genetic similarity relationship may explain why these three genotypes have all been observed in keratitis infections.

In Japan, T3 was recorded as the most prevalent genotype (Edagawa et al. 2009). Results of a study in Egypt revealed that the *Acanthamoeba* isolated strains belonged to T1, T2, T3, T4, and T7 (Lorenzo-Morales et al. 2006). T4 and T9 was recorded from western part of Turkey (Ertabaklar et al. 2007).

In the present study, seasonal detection rates were higher in summer and autumn (40% each), 20% in spring and nothing was detected in winter. This was in accordance with other studies in Taiwan and Egypt that reported that *Acanthamoeba* spp. is more prevalent in late summer (Al-Herrawy et al. 2017, Gad et al. 2019, Esboei et al. 2020). It is important to mention that the distribution of *Acanthamoeba* spp. and their impact on our public health remain questionable because of the sparse of study in our governorate.

T4, T9 and T11 are the prevalent genotypes the studied area and they are prevalent in the summer season. The high prevalence of *Acanthamoeba* spp. in swimming pools in Kafrelsheikh Governorate should be considered a major health problem that spot the light on the importance of gaining more efforts in sanitary principals, water resources management and training health authorities' personnel. There is a clear gap in the relationships between *Acanthamoeba* spp. and seasonal changes that need more studies.

Author contributions. All authors have contributed to the whole work, read and agreed to the published version of the manuscript.

REFERENCES

Abd El Wahab W.M., Ayman A., Doaa A.H. 2018: Molecular characterization and phylogenetic analysis of *Acanthamoeba* isolates in tap water of Beni-Suef, Egypt. Acta Parasitol. 63: 826–834.

Aghajani A., Dabirzadeh M., Maroufi Y., Hooshyar H. 2016: Identification of *Acanthamoeba* genotypes in pools and stagnant water in ponds in Sistan Region in Southeast Iran. Turkiye Parazitol. Derg. 40: 132–136.

Al-Herrawy A., Mahmoud B., Abd Elhafez M., Ameen A., Wafaa H. 2014: *Acanthamoeba* species in swimming pools of Cairo, Egypt. Iran. J. Parasitol. 9: 194–201.

Al-Herrawy A.Z., Khalil M.I., El-Sherif S.S., Omar F., Lotfy W.M. 2017: Surveillance and molecular identification of *Acanthamoeba* and *Naegleria* species in two swimming pools in Alexandria University, Egypt. Iran J. Parasitol. 12: 196–205.

Behera H. S., Anita P., Gita S., Pooja B., Murgesan V., Tushar A., Niranjan N., Radhika T. 2016: Genotyping of *Acanthamoeba* spp. and characterization of the prevalent T4 type along with T10 and unassigned genotypes from amoebic keratitis patients in India. J. Med. Microbiol. 65: 370–376.

Di Filippo M., Santoro M., Lovreglio P., Monno R., Capolongo C., Calia C., Fumarola L., D'Alfonso R., Berrilli F., Di Cave D. 2015: Isolation and molecular characterization of free-living amoebae from different water sources in Italy. Int. J. Environ. Res. Publ. Health. 12: 3417–3427.

Edagawa A., Kimura A., Kawabuchi-Kurata T., Kusuhara Y., Karanis P. 2009: Isolation and genotyping of potentially pathogenic *Acanthamoeba* and *Naegleria* species from tap-water sources in Osaka, Japan. Parasitol. Res. 105: 1109–1117.

El-Badry A.A., Sayeda M.A., Eman S.E., Enas M.R., Soheir S.M., Nahed Y.T. 2020: First identification of *Naegleria* species and *Vahlkampfia ciguana* in Nile water, Cairo, Egypt: seasonal morphology and phylogenetic analysis. J. Microbiol. Immunol. Infect. 53: 259–265.

Ertabaklar H., Meral T., Volkan D., Sema E., Julia W. 2007: *Acanthamoeba* keratitis due to *Acanthamoeba* genotype T4 in a non-contact-lens wearer in Turkey. Parasitol. Res. 241–246.

Esboei B.R., Mahdi F., Reza S., Mohammad B., Masoumeh M., Hadi H., Yousef D., Nahid J. 2020: Genotyping and phylogenic study of *Acanthamoeba* solates from human keratitis and swimming pool water amples in Iran. Parasite Epidemiol. Control 11: e00164.

Gad M.A., Allayeh A.K., Elmahdy E.M., Shaheen M.N.F., Rizk N.M., Al-Herrawy A.Z., Saleh F.R., Marouf M.A. 2019: Genotyping and interaction-reality of *Acanthamoeba*, enteric adenovirus and rotavirus in drinking water, Egypt. J. Aquat. Biol. Fish. 23: 65–79.

Kao P.M., Ming Y.C., Chi W.T., Wen C.H., Bing M.H., Shu M. S., Cheng W.F., Yi C.C. 2013: Diversity and seasonal impact of *Acanthamoeba* species in a subtropical rivershed. Biomed. Res. Int. 2013: 405794.

Kumar S., Stecher S., Li M., Knyaz C., Tamura K. 2018: MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol. Biol. Evol. 35: 1547–1549.

Lorenzo-Morales J., Antonio O., Enrique M., Messaoud K., Patricio A., Pilar F., Basilio V., Santiago M. 2006: *Acanthamoeba* isolates belonging to T1, T2, T3, T4 and T7 genotypes from environmental freshwater samples in the Nile Delta region, Egypt. Acta Trop. 100: 63–69.

Mafi M., Maryam N., Ali H., Zohreh L. 2017: Contamination of swimming pools and park ponds with free living amoebae in Tehran. Med. J. Tabriz. Uni. Med. Sci. Health Serv. 38: 60–67.

Maghsood A .H., James S., Mostafa R., Debbie N., David W., Naveed A. K. 2005: *Acanthamoeba* genotype T4 from the UK and Iran and isolation of the T2 genotype from clinical isolates. J. Med. Microbiol. 54: 755–59.

Prithiviraj S.R., Siva G.K.R., Hariharan G., Rameshkumar G., Ponlakshmi M., Sharma S., Lalitha P. 2020: Clinical presentations, genotypic diversity and phylogenetic analysis of *Acanthamoeba* species causing keratitis. J. Med. Microbiol. 69: 87–95.

Schroeder J.M., Booton G.C., Hay J., Niszl I.A., Seal D.V., Markus M.B., et al. 2001: Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of acanthamoebae from humans with keratitis and from sewage sludge. J. Clin. Microbiol. 39: 1903–1911.

Stockman L.J., Carolyn J.W., Michael J. B. 2011: Prevalence of *Acanthamoeba* spp. and other free-living amoebae in household water, Ohio , USA – 1990–1992. Parasitol. Res. 108: 621–627.

Tamura K., Nei N. 1993: Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10: 512–526.

Üstüntürk-Onan M. 2020: Isolation and identification of free-living amoebae isolated from well water in Istanbul. J. Water Health. 18: 1139–1145.

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