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Molecular characterization and phylogenetic analysis of FLA from different water sources in Egypt

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ABSTRACT

Free-living amoebae (FLA) are protozoa ubiquitously found in nature. In addition to their natural distribution, some species have been documented as pathogenic to humans. The main aim of the current study was the molecular identification, sequencing and phylogenetic analysis of morphologically detected FLA in water sources in El-Qalyubia, Egypt. A total of 96 water samples were collected from different water sources. Each water sample was filtrated and cultured on non-nutrient agar (NNA). Morphologically positive FLA were subjected to PCR, PCR products were sequenced and the obtained sequences were phylogenetically analysed. FLA were found in 41 water samples examined (42.7%). Nile water and groundwater were the sources with the highest prevalence rates (83.3 and 62.5%, respectively). *Naegleria italica* was first identified in Egypt from the waters of the Nile. In addition, *Vahlkampfia* spp. and *Hartmannella* spp. were also detected. However, other FLA species, including *Acanthamoeba* spp. and the pathogenic *Naegleria fowleri*, previously reported in Egypt, were not included in this study. The recent identification of these FLA in the Egyptian waters related to human populations indicates the need for more phylogenetic studies using larger sample sizes to investigate their potential threat to human health.

Key words: Egypt, free-living amoebae, PCR, sequence analysis, water sources

HIGHLIGHTS

- This study documented the presence of FLA in different water sources in El-Qalyubia, Egypt.
- *Naegleria italica* was identified for the first time in Egypt from the waters of the Nile.
- Besides, *Vahlkampfia* spp. and *Hartmannella* spp. were also detected.
- This study presents knowledge of the prior prevalence of FLA that should be considered by the clinicians and the environmental professionals in the region.

1. INTRODUCTION

Free-living amoebae (FLA) are protozoa that can survive and proliferate in the environment independently or found within a host hence the name amphizoic amoeba. FLA are found all over the world in various natural and man-made aquatic environments such as lakes, ponds, swimming pools and even treated water (Mahmoudi *et al.* 2021).

Humans are constantly exposed to these amoebae which can cause severe diseases due to infection (Fabros *et al.* 2021). The causative agents of the disease in humans belong to two super-groups: Amoebozoa, including the genera *Acanthamoeba* and *Hartmannella*, and Excavata, including Vahlkampfiidae family with the genera *Naegleria* and *Vahlkampfia* as members (Di Filippo *et al.* 2015; El-Badry *et al.* 2020).

FLA can induce a wide range of clinical complications. For instance, *Naegleria fowleri* causes an acute fatal central nervous system infection known as primary amoebic meningoencephalitis (PAM) in healthy young adults and children (Ithoi *et al.* 2011; El-Badry *et al.* 2020). *Acanthamoeba* species cause granulomatous amoebic encephalitis (GAE), and occasionally skin, pulmonary and kidney infections that may affect immunocompromised patients. Besides, amoebic keratitis (AK) can

be detected in immunocompetent individuals (Al-Herrawy *et al.* 2017). *Vahlkampfia* and *Hartmannella* species have been detected from the eye surface of humans (Al-Herrawy & Gad 2017).

In Egypt, there is a dearth of available data regarding the prevalence of the FLA in aquatic environments, with a shortage of phylogenetic studies.

The present study aims to identify FLA from water sources used by local populations for drinking, washing, cooking, agriculture and recreational activities in El-Qalyubia, Egypt. Moreover, it aims to characterize isolates at the species/genotypes level to better understand their distribution in the environment and to assess potential risks to human health.

2. MATERIALS AND METHOD

2.1. Study location, water sample collection and filtration

A total of 96 water samples were collected from various districts in El-Qalyubia governorate as follows: 24 from the Nile River (collected from the inlet of Benha Drinking Water Treatment Plants (DWTP)), 24 samples from household potable water (distribution systems), 24 samples from untreated groundwater (pre-chlorinated water from a 128 m deep well of Kaha DWTP) and 24 from treated groundwater (chlorinated water of Kaha DWTP) (Figure 1). The Nile River samples were collected from the inlet of Benha DWTP with a depth of approximately 30 cm, making sure that there are no floating films or organic material, while the untreated groundwater samples were collected from a tape connected to the well pipes. The well had been purged sufficiently (the water is pumped for 5–10 min until the water temperature was stabilized) to ensure that the sample is representative of the groundwater (ISO/FDIS 2006; APHA 2017).

One litre volume of water samples was collected in dry, clean autoclavable polypropylene containers and transferred in iceboxes to the laboratory where they were immediately processed. The collected water samples were concentrated using the membrane filtration technique as per Di Filippo *et al.* (2015). Each separately collected water sample was concentrated and filtered through cellulose nitrate membranes (0.45 mm pore size and 47 mm in diameter) using a stainless-steel holder attached to a suction pump. When the suction was detached, the holder was separated and the membrane was removed before it dried.

2.2. FLA cultivation, sub-cultivation and morphological identification

Cultivation and sub-cultivation of FLA were performed according to Abd El Wahab *et al.* (2018). Briefly, filter paper for each sample was placed face-down on the surface of plates of a 1.5% non-nutrient agar (NNA) made with Page's amoebae saline, covered with a thin layer of *Escherichia coli*. NNA plates were incubated at 37 °C and observed daily for 14 days using an inverted microscope for the presence of any amoebic growth. When the amoebic growth was detected, a piece of agar enclosing the amoebic growth was cut out using a sterile scalpel and placed into a fresh NNA-*E. coli* plate. The sub-cultured plates were incubated in the same way described above and then plates were examined for amoebae growth. Morphological characteristics of trophozoites and cyst stages of FLA were performed according to Page's classification key. The flagellation test was done by incubating the amoebae in a test tube containing 2 ml of distilled water for 1–2 h to differentiate *Naegleria* spp. from other Vahlkampfiidae (Stockman *et al.* 2011).

2.3. DNA extraction, amplification and sequencing

The growing amoebae were harvested from all positive culture plates, placed in Eppendorf tubes and washed two times with saline buffer prior to the molecular procedure. DNA extraction was performed using G-spin™ Total DNA Extraction Mini Kit following the manufacturer's specifications (iNtRON Bio, South Korea).

For identification of FLA, we used two sets of *Naegleria* primer pairs, genus-specific primers Naeg1 (forward: 5′GAACCTGCGTAGGGATCATTT3′) and Naeg 2 (reverse: 5′TTTCTTTTCCTCCCC TTATTA3′) and *N. fowleri* species-specific (forward: 5′ GTGAAAACCTTTTTTCCATTTACA 3′) and (reverse: 5′ AAATAAAAGATTGACCAT TTGAAA 3′) targeting the internal transcribed spacer regions (ITS1 and 2) that contain the 5.8S rDNA gene. PCR reaction conditions and mixtures were performed as previously described (Pélandakis *et al.* 2000).

For identification of the genus *Acanthamoeba*, a PCR was carried out to amplify an 18S rDNA region defined as ASA.S1 (*Acanthamoeba*-specific amplimer) that includes the diagnostic fragment 3 (DF3), using the genus-specific primers JDP1 (forward: 5′GGCCCAGATCGTTTAC CGTGAA3′) and JDP2 (forward: 5′ TCTCACAAGCTGCTAGGGAGTCA3′). PCR reaction conditions and mixtures were performed as per Schroeder *et al.* (2001).

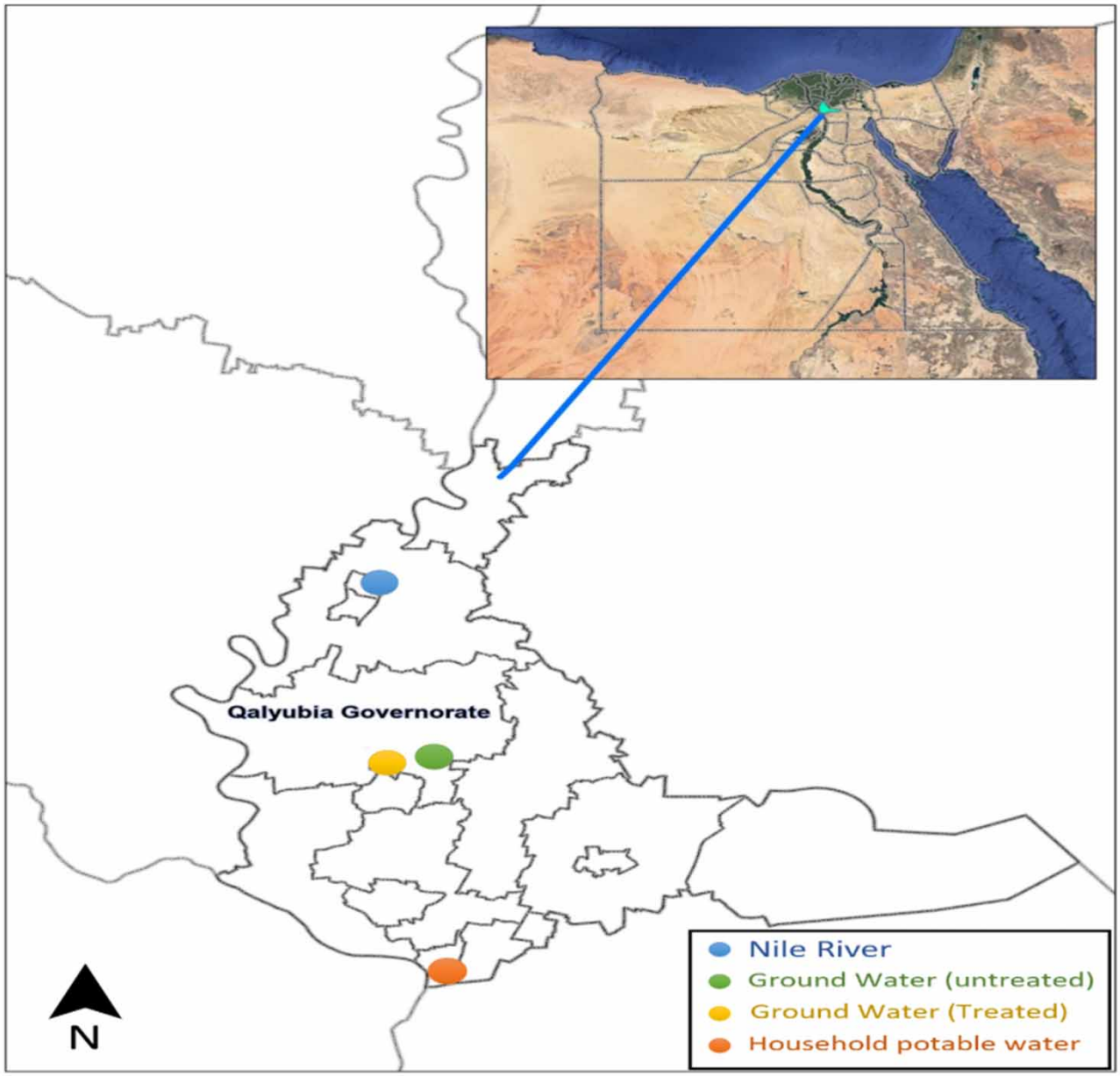

Figure 1 | Diagrammatic map for studied areas and water sampling sites (dots).

PCR products were purified using the QIAquick PCR & Gel Cleanup Kit (Qiagen, Germany) according to the manufacturer's instructions. Sequencing was done with the primer pair (Naeg1 and Naeg 2) and using Big-Dye™ Terminator v3.1 with the Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA) on the ABI Prism 310 genetic analyser (Applied Biosystems, USA) according to the manufacturer's instructions.

The sequences of the studied isolates were matched to the reference sequences registered in the Gene Bank database through BLAST-NCBI (https://blast.ncbi.nlm.nih.gov), after which all sequences were aligned using the BioEdit software, which depends on the ClustalW multiple alignment conditions (Hall 1999). Evolutionary analyses were conducted in MEGA X (Version 10.2.4) (Kumar *et al.* 2018).

3. RESULTS

Of the 96 water samples, 41 (42.7%) were positive for FLA after cultivation on NNA. Nile water and groundwater were the sources with the highest prevalence rates (83.3 and 62.5%, respectively) (Table 1). Microscopically, vahlkampfiids and

Table 1 | Distribution of positive FLA in different water samples with reference to results of sequencing according to sampling site

Water samples	Examined samples Total	Positive FLA samples *n* (%)	Results of sequencing		
			Vahlkampfiidae		
			Hartmannella	*Naegleria*	*Vahlkampfia*
Nile water	24	20 (83.3%)	1	2	1
Untreated ground water	24	15 (62.5%)	–	1	1
Household tap water	24	1 (4.2%)	–	1	–
Treated ground water	24	5 (20.8%)	–	1	–
Total	96	41 (42.7%)	1 (*N. italica*)	5	2

Hartmannellidae were identified based on morphological characteristics. Vahlkmapfiids had spherical double-wall cysts and temporarily branched trophozoites (Figure 2(a) and 2(b)). *Hartmannella* had a small spherical or ovoid cyst, while the trophozoite form was *Limax* containing a small nucleolus (Figure 2(c) and 2(d)).

All the 41 morphologically identified vahlkampfiids and Hartmannellidae were positive by using the Ng.spp_FW and Ng.spp_RV primers. About 500 and 800 bp PCR products were obtained from *vahlkampfiids* and *Hartmannella*, respectively (Figure 3). Sequence analysis was successfully performed for only eight PCR products (Figure 4), 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 generated with sequences of reference species from NCBI-BLAST. The sequencing results are summarized in Table 2 and Figure 4. Isolates 10, 11 and 12 showed 94–98% homological identities with *Vahlkampfia* Sp. unclassified (MT109103.1) with accession numbers MW843639–MW843641, while isolate 23 showed 96% homological identities with *Hartmannella* sp. unclassified (JX910447.1) with accession number MW843642 and isolate 29 showed 100% homological identities with *Hartmannella* sp. unclassified (HE617189.1)

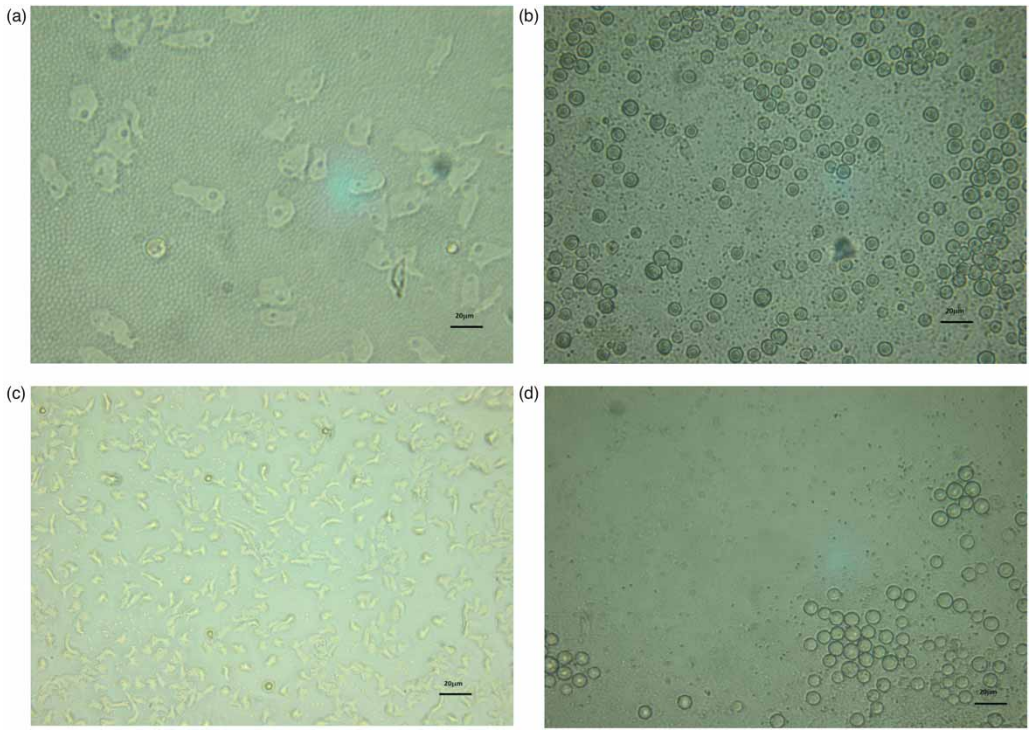

Figure 2 | Fresh unstained *Naegleria* trophozoite (a) and cysts (b) and fresh unstained *Hartmannella* trophozoite (c) and cysts (d).

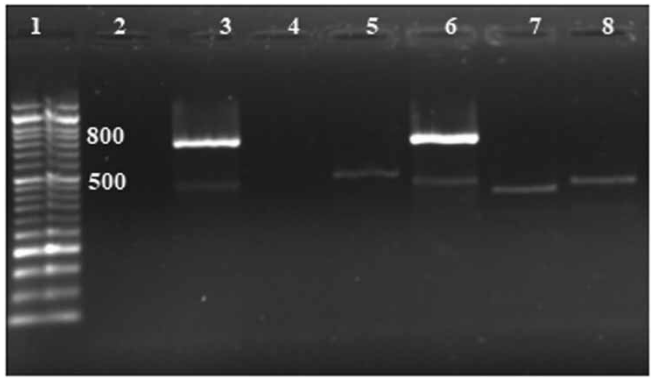

Figure 3 | PCR amplification of internal transcribed spacer (ITS) rDNA region. Lane 1: DNA marker of 50 bp molecular weight. Lane 2: negative control sample. Lanes 3 and 6: *Hartmannella*. Lanes 5, 7 and 8: Vahlkampfiidae samples. Lane 4: negative sample.

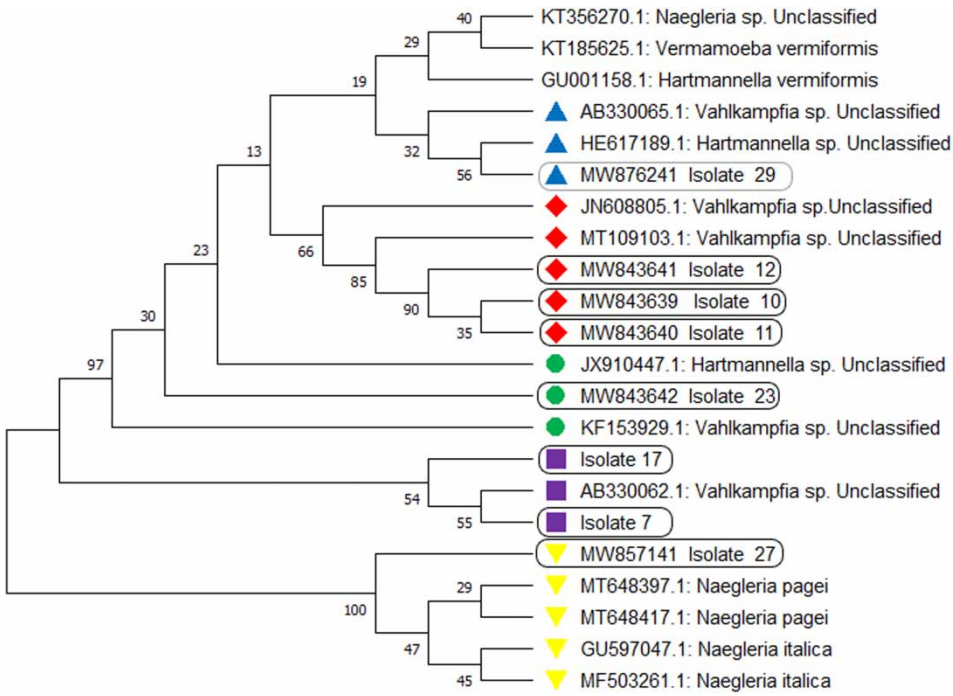

Figure 4 | Optimal tree of ITS1, 5.85, ITS2 and 28S rDNA sequences for the studied FLA samples. Neighbor-joining tree showing the evolutionary history of the studied FLA samples, inferred by the evolutionary distance analysis, was calculated using the maximum composite likelihood method. The percentage of replicate trees in which the associated taxa clustered together in a bootstrap test. The bootstrap value is 5,000 with the sum of the branch length = 0.5.

with accession number MW876241. Furthermore, isolate 27 showed 95% homological identities with *N. italica* (Gu597047.1) with accession number MW857141 (Table 2; Figure 4).

In our study, no representative of other pathogenic types of FLA (*Acanthamoeba* spp. and *N. fowleri*) was detected in any sample, neither by morphology nor by PCR.

4. DISCUSSION

Humans are constantly exposed to FLA due to their ubiquitous occurrence in the environment (Blair *et al.* 2008). According to Abdul Majid *et al.* (2017), the occurrence of these pathogens must be monitored due to their important role within ecosystems and their potential to cause serious infections in humans.

Table 2 | Sequences retrieved from the GenBank used for phylogenetic analysis

Organism	Source	Location	Accession number	References
Naegleria sp.	Raw water	Malaysia	KT356270	Richard *et al.* (2016)
Vermamoeba vermiformis	Culture media	USA	KT185625	Fučíková & Lahr (2016)
	Marine sediment sample	United Kingdom	GU001158	Glücksman *et al.* (2011)
Vahlkampfia sp.	Tap water	Japan	AB330062	Edagawa *et al.* (2009)
Unclassified	Hospital ward	Iran	JN608805	Unpublished (https://www.ncbi.nlm.nih.gov/nuccore/JN608805)
	Geothermal waters	Italy	MT109103	Unpublished (https://www.ncbi.nlm.nih.gov/nuccore/MT109103)
	Lake water	Pakistan	KF153929	Unpublished (https://www.ncbi.nlm.nih.gov/nuccore/KF153929)
Hartmannella sp.	Biofilm	Ghana	HE617189	De Jonckheere *et al.* (2012)
	Geothermal waters	France	JX910447	Moussa *et al.* (2013)
Naegleria pagei	Aquatic environment	Iran	MT648397	Unpublished (https://www.ncbi.nlm.nih.gov/nuccore/MT648397)
	Aquatic environment	Iran	MT648417	Unpublished (https://www.ncbi.nlm.nih.gov/nuccore/MT648417)
N. italica	Biofilm in hot spring water	Taiwan	GU597047	Unpublished (https://www.ncbi.nlm.nih.gov/nuccore/GU597047)
	Geothermal water	Italy	MF503261	Di Filippo *et al.* (2017)

In addition to their pathogenicity, FLA act efficient carriers for certain antibiotic-resistant bacteria and viruses rendering their detection a priority (Khurana *et al.* 2015).

In our research, FLA were detected in 41 (42.7%) out of 96 water samples examined in El-Qalyubia governorate, Egypt for identification of new species of *Naegleria* (*N. italica*), *Vahlkampfia* spp. and *Hartmannella* spp.

In the present study, FLA were isolated from 83.3% of the examined Nile water samples. Other studies in Egypt revealed a higher percentage of FLA in the examined Nile water samples with 100% in Minofeya governorate (Al-Herrawy *et al.* 2017), 91.7% in Fayuom governorate (Al-Herrawy *et al.* 2015) and 87.5% in Cairo (Hilali *et al.* 1994), while Hamadto *et al.* (2003) detected the presence of FLA in a lower percentage (20%) in surface water and canal water samples from different governorates in Egypt.

Other studies in other countries detected FLA at high frequency like Japan (Edagawa *et al.* 2009) and the USA (Corsaro *et al.* 2009) found that the percentage of occurrence of FLA in surface water reached 94 and 100%, respectively. In contrast, other researchers recorded a lower incidence of FLA in freshwater samples. 28.7, 61.1 and 69% were recorded from Italy (Di Filippo *et al.* 2015), Bulgaria (Tsvetkova *et al.* 2004) and Central Mexico (Bonilla-Lemus *et al.* 2014), respectively.

In the present study, FLA were isolated from 62.5% of untreated groundwater samples. In Egypt, Al-Herrawy *et al.* (2017) reported a higher incidence (100%) of FLA in untreated groundwater samples from Minofeya governorate, while a lower incidence (58.3%) was documented by Gad & Al-Herrawy (2016).

In other countries such as Italy (Di Filippo *et al.* 2015) and Iran (Ghadar-Ghadr *et al.* 2012), FLA were found in 85.7 and 44.4% of groundwater samples, respectively.

In the present work, FLA were isolated from 4.2 and 20.8% of the examined household tap water and treated groundwater, respectively.

In Egypt, a nearly similar percentage (4%) of FLA was detected by Hamadto *et al.* (2003) in the examined tap water samples from different governorates. Other workers in Egypt recorded FLA in tap water in a higher percentage of 45.8% (Al-Herrawy *et al.* 2017), 41.7% (Al-Herrawy *et al.* 2015) and 50% (Gad & Al-Herrawy 2016) compared to the present work.

Other researchers in other countries such as Leońska-Duniec *et al.* (2015) in Poland, Shoff *et al.* (2008) in the UK and Jeong & Yu (2005) in Korea have reported FLA in tap water samples as 44, 46.9 and 48%, respectively.

Significant variation in the prevalence values of FLA was observed worldwide. According to De Jonckheere (2011), Stockman *et al.* (2011) and Di Filippo *et al.* (2015), the variation in the occurrence of FLA may be affected by many factors such as differences in geographical location, environmental conditions, water sources and different applied methodologies.

Concerning the phylogenetic analysis, this is the first phylogenetic study revealing the presence of *Naegleria italica*, *Vahlkampfia* spp. and *Hartmannella* spp. in Egypt. Nevertheless, other FLA species, including *Acanthamoeba* spp. and the pathogenic *N. fowleri*, previously reported in Egypt, were not investigated in this study.

In Egypt, there is a scarcity of phylogenetic studies. A study done by El-Badry *et al.* (2020) reported the presence of *Vahlkampfia ciguana* and the *Naegleria* species *N. australiensis*, *N. philippinensis* and *N. neojejuensis* in the Nile water. In agreement with our results, this study also documented the absence of the pathogenic *N. fowleri*.

Although *N. fowleri* is the only species of the genus *Naegleria* causing human pathology, *N. italica* detected in this study has previously proven to be pathogenic in experimental animals (De Jonckheere 2014).

The most common cause of AK is the genera *Acanthamoeba*, *Vahlkampfia* and *Hartmannella* species; nonetheless, it has been isolated from the cornea, alone (Alexandrakis *et al.* 1998; Al-Herrawy & Gad 2017) or in combination with *Acanthamoeba* (Arnalich-Montiel *et al.* 2013) as a cause of keratitis.

5. CONCLUSION

N. italica was first identified in Egypt from the water of the Nile River. In addition, *Vahlkampfia* spp. and *Hartmannella* spp. were also detected. These newly discovered FLA in Egyptian aquatic environments need further phylogenetic studies, using bigger sample sizes from a greater variety of water sources. These studies are needed in order to evaluate the potential pathogenicity of these species to humans.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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