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Prevalence of Free-Living Amoebae in Tap Water and Biofilm, Egypt.

Gazaa H Morsy[1], Ahmad Z Al-Herrawy[2], Waled M Elsenousy[3], and Mohamed A Marouf[2]*.

[1]Zoology Department, College of Science, Benha University, Benha, Egypt.
[2]Parasitology Laboratory, Water Pollution Research Department, National Research Centre, Giza, Egypt.
[3]Virology Laboratory, Water Pollution Research Department, National Research Centre, Giza, Egypt.

ABSTRACT

Free-living amoebae are protozoa found in soil and water. Among them, some are pathogenic and many have been described as potential reservoirs of pathogenic bacteria. In the present work, a total of 48 tap water samples were collected from Giza governorate, Egypt. Samples were processed for detection of free-living amoebae using non-nutrient agar (NNA) and were incubated at 30^{o}C. The isolates of free-living amoebae were identified morphologically and molecularly to the genus level. Members of genus *Acanthamoeba* were identified to species level based only on the morphologic criteria. The obtained results showed that free-living amoebae were found in 60.4% of the examined tap water samples. Concerning seasonal variation, the highest occurrence percentage of free-living amoebae in water samples was observed in summer season (75%), and then it decreased to be 66.7, 58.3 and 41.7% in autumn, spring and winter, respectively. Morphological identification and molecular confirmation of the isolated free-living amoebae revealed the presence of 4 genera namely *Acanthamoeba* spp., *Naegleria* spp, *Vermamoeba* (*Hartmannella*) and *Vannella* spp. The formed biofilm inside the sampled drinking water pipe lines contained only *Acanthamoeba* and *Vannella* spp. In conclusion, the appearance of free-living amoebae especially potentially pathogenic amoebae in tap water might represent a significant threat to public health.

Keywords: Free-living amoebae, tap water, biofilm, Egypt.

Corresponding author

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INTRODUCTION

There is a global concern that all the world population should have access to safe drinking water. Even in the 21st century, there are many people without access to appropriate water, in quantity and/or quality, for the basic needs [1]. One of the important issues in water contamination is the presence of pathogenic free-living amoebae in tap or drinking water supply sources [2].

Free-living amoebae (FLAs) are ubiquitous in aquatic environments and are aerobic, mitochondriate, eukaryotic protists [3]. They are often referred to as amphizoic amoebae due to their ability to live freely without a host in addition to having the capability to invade a host and live as parasites [3,4]. Humans are continually exposed to these amoebae due to their ubiquitous occurrence in the environment. Free-living amoebae have been shown to colonize upon the internal pipe line surfaces of drinking water by adhering and secreting metabolic substances, thereby creating a biofilm [5]. They are mobile and feed on bacteria, algae, fungi, protozoa, or other organic particles [6,7]. They can be found in two morphological stages: trophozoites and cysts. The trophozoite, or vegetative form, corresponds to the period of metabolic activity of the amoeba with division, feeding, and motility, whereas the cyst form corresponds to the dormant phase of amoeba that can resist hostile environmental conditions [8,9]. Some species, for example *Naegleria fowleri*, may exist in a temporary third stage, flagellate form, under specific environmental conditions [3]. *Acanthamoeba* and *Hartmannella* are the genera most frequently isolated from the environment and water networks, but others such as *Naegleria*, *Vahlkampfia*, *Balamuthia* or *Nuclearia* may also be found [3,10].

In particular, *Naegleria fowleri* is responsible for primary amoebic meningoencephalitis (PAM), while some species of *Acanthamoeba* and *Balamuthia mandrillaris* can induce granulomatous amoebic encephalitis (GAE), mainly in immunocompromised patients [11]. Also, *Acanthamoeba* species may give rise to a severe corneal infections designated as amoebic keratitis (AK) [12,13]. Some species within these genera are able to harbor other micro-organisms, and can influence the growth of these agents or afford them protection [6,14,15]. Specifically, they establish symbiotic relationships with other microbes including bacteria of the genera *Legionella*, *Mycobacterium*, *Campylobacter* and *Listeria*, which, apart from being a nutritional source, are able to survive amoeba digestion and multiply within them [6,16]. *Hartmannella vermiformis*, now known as *Vermamoeba vermiformis*, although rarely responsible for human disease, also serves as a host for pathogenic yeasts or bacteria and may thus have indirect public health significance [14,15]. Also, free-living amoebae share in creating a biofilm by colonization on surfaces through adhering and secreting metabolic substances [5].

So, the aim of this study is to establish the prevalence of free-living amoebae in tap water and consequently formed boifilm.

MATERIALS AND METHODS

Water and biofilm samples

A total of 48 tap water samples were collected from newly changed drinking water pipe and tap (that were used for the first time) in Giza governorate during the period from November 2012 to December 2013. A water sample (1 liter volume each) was collected every week. Water samples were separately collected in a clean, dry and autoclavable polypropylene containers then sent to the laboratory where they were processed at the same day of collection. Samples were transported at ambient temperature [17,18].

After one year period, previously sampled tap water pipes were externally disinfected by 70% ethyl alcohol and then by autoclaved distilled water. A part of the pipe line was released and rinsed in 100ml autoclaved distilled water. The internal surface of the pipe was carefully brushed to detach the biofilm in the rinsing water. The released debris and biofilm were vortexed in rinsing solution and then treated the same as a water sample for the detection of free-living amoebae concentrated by using the membrane filtration technique.

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Cultivation and morphologic identification of FLAs

Water samples (either tap water or biofilm rinsing water) were separately filtered through a nitrocellulose membrane filter (0.45 µm pore size and 47 mm in diameter) using a stainless steel filter holder connected with a suction pump. Filtration was stopped just before drying of the membrane [17,18].

After filtration process, the membrane was inverted face to face on the surface of a non-nutrient agar (NNA) plate seeded with heat-killed *Escherichia coli*. The plate was wrapped with parafilm and incubated at 30^{o}C to permit growth and multiplication of free-living amoebae existing in water samples [18,19]. Incubated plates were daily examined by inverted microscope (Olympus CXK 41, Japan) for 7 days for the presence of any amoebic growth. All cloned amoebae were identified morphologically to the genus level according to the key described by Page [20]. Isolated members belonging to *Acanthamoeba* were morphologically identified to the species level according to the key of Pussard and Pons [21].

Molecular confirmation of detected FLAs to genus level

Cloned plates were washed with sterile PBS buffer. The amoebae were then collected from the washing solution by centrifugation at 250xg for 5-10 min. Amoebic DNA was extracted using phenol-chloroform method as described by Winnepenninckx *et al*. [22] and modified by Abdel-Hamid et al. [23].

Genus-specific primers and their fragments of target genes used for detecting *Acanthamoeba*, *Naegleria*, *Hartmannella* and *Vannella* were displayed in Table 1. The PCR reaction was performed using Maxima™ Hot Start Green PCR Master Mix (Thermo Fisher Scientific Inc, Waltham, MA, USA) according to the manufacturer manual. PCR products were electrophoresed using 2% agarose gel stained with a solution of ethidium bromide and visualized under UV light.

Table 1: Primer set used for confirmation detected genera of FLAs

Organism	Sequence	Fragment length	Reference
Acanthamoeba	**ttt gaa ttc gct cca ata gcg tat att aa ttt gaa ttc aga aag agc tat caa tct gt**	**910 to 1170 bp**	**Kilic et al. [24]**
Hartmannella	**gct cca ata gcg tat att aa aga aag agc tat caa tct gt**	**600 to 650 bp**	**Nazar et al. [25]**
Naegleria	**gaa cct gcg tag gat cat tt ttt ctt ttc ctc ccc tta tta**	**409 bp**	**Pelandakis et al. [26]**
Vannella	**gct cca ata gcg tat att aa aga aag agc tat caa tct gt**	**700-800 bp**	**Nazar et al. [25]**

RESULTS AND DISCUSSION

In the present investigation, free-living amoebae were detected in 29 (60.4%) out of 48 examined tap water samples (Table 2). Other workers in Egypt recorded free-living amoebae in tap water in a lower occurrence reaching 4% [27] and 23.6% [28]**.** In other countries like USA, Brazil and Turkey, free-living amoebae were recorded in 9.3% [29], 22.79% [30] and 29.4% [31] of their tap water, respectively. On the other hand, other workers in the United Kingdom and USA recorded higher occurrence of free-living amoebae in tap water representing 89% [32] and 79% [29], respectively. The conflicts of occurrence of FLAs in tap water from different countries might be attributed to factors known to affect the presence of FLAs, such as water source, water treatment method, geographic location, and differences in water temperature [29]**.**

Concerning seasonal variation, the highest occurrence percentage of free-living amoebae in tap water samples was observed in summer season (75%) and then it decreased to be 66.7, 58.3 and 41.7% in autumn, spring and winter, respectively (Table 2). Other workers in Egypt found that free-living amoebae predominated in tap water of Greater Cairo in winter (41.7%), followed by summer (25%) while they were evenly distributed in both spring and autumn (16.7% for each) [33]. In another study in Egypt, the highest occurrence of free-living amoebae in distribution systems in fayuom governorate was recorded in autumn

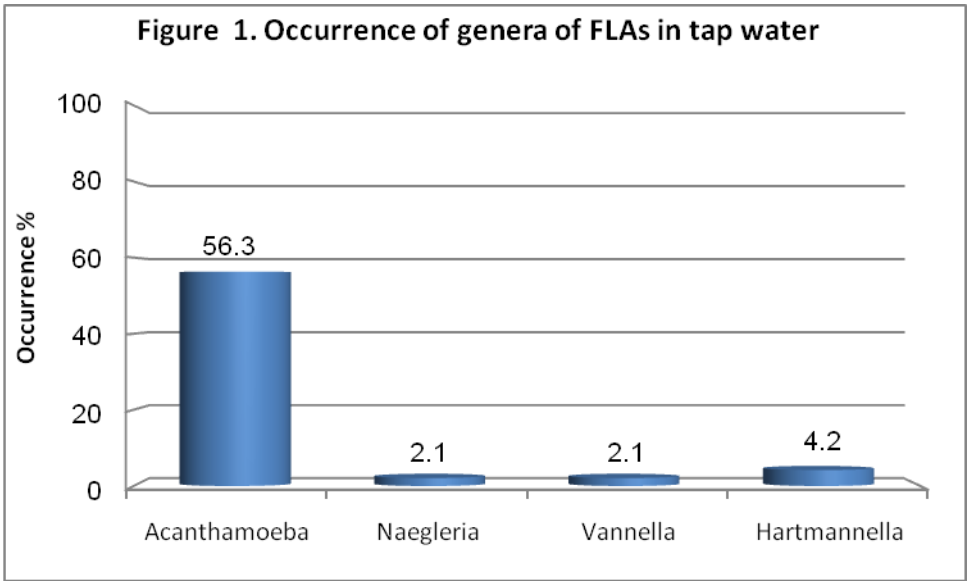
(41.7%), followed by winter, spring and summer in percentages of 25, 16.7 and 8.3%, respectively [34]. Intervention between results might be attributed to differences in water quality based on the differences in drinking water treatment plants processes and in the distribution system from which samples were collected [29]. There was a trend of increased amoeba detections in summer months in USA. For the months of January through May, detections across all households averaged 65% (range, 61–76%); this proportion increased in June through October to average 91% of households positive for amoebae [29].

Table 2: Seasonal variation of FLAs in tap water

Season	FLAs in tap water samples		
	Examined	+ve	%
Winter	12	5	41.7
Spring	12	7	58.3
Summer	12	9	75
Autumn	12	8	66.7

Table 3: Seasonal variation of identified genera of FLAs in tap water

Seasons	Detected genera of FLAs			
	Acanthamoeba	***Naegleria***	***Vannella***	***Hartmannella***
Winter	**5/12 (41.7%)**	**0/12 (0%)**	**1/12 (8.3%)**	**0/12 (0%)**
Spring	**6/12 (50%)**	**0/12 (0%)**	**0/12 (0%)**	**1/12 (8.3%)**
Summer	**9/12 (75%)**	**0/12 (0%)**	**0/12 (0%)**	**0/12 (0%)**
Autumn	**7/12 (58.3%)**	**1/12 (8.3%)**	**0/12 (0%)**	**1/12 (8.3%)**



The present investigation showed that the isolated FLAs from tap water in Egypt belonged to 4 genera (*Acanthamoeba, Naegleria, Vannella, Hartmannella*). The morphological features and seasonal variations as well as Molecular confirmation of detected genera of free-living amoebae from tap water in the present work were illustrated in tables 3, 4 & figures 1, 2 and 3. To the best of our knowledge, scarce previous studies concerning this subject in Egypt were published [33,35-37].

The trophozoites of *Acanthamoeba* varied in length from 20 to 45µm and measured 15 to 30µm in width with a single vesicular nucleus and large centrally located nucleolus. The cyst form of *Acanthamoeba* spp. measured 12 to 25µm in diameter and had a double cyst wall (ectocyst and endocyst) consisting of a smooth or wrinkled outer wall (ectocyst) and a stellate, polygonal, star–like or even fairly inner wall

(endocyst). Members of the genus *Acanthamoeba* were considered the most prevalent free-living amoebae presented in 56.3% of tap water samples in the present work. The highest occurrence of *Acanthamoeba* was recorded in summer (75%), followed by autumn, spring and winter in percentages 58.3, 50 and 41.7%, respectively. It has been established previously that *Acanthamoeba* species were the most common and opportunistic amphizoic protozoa [20,38].

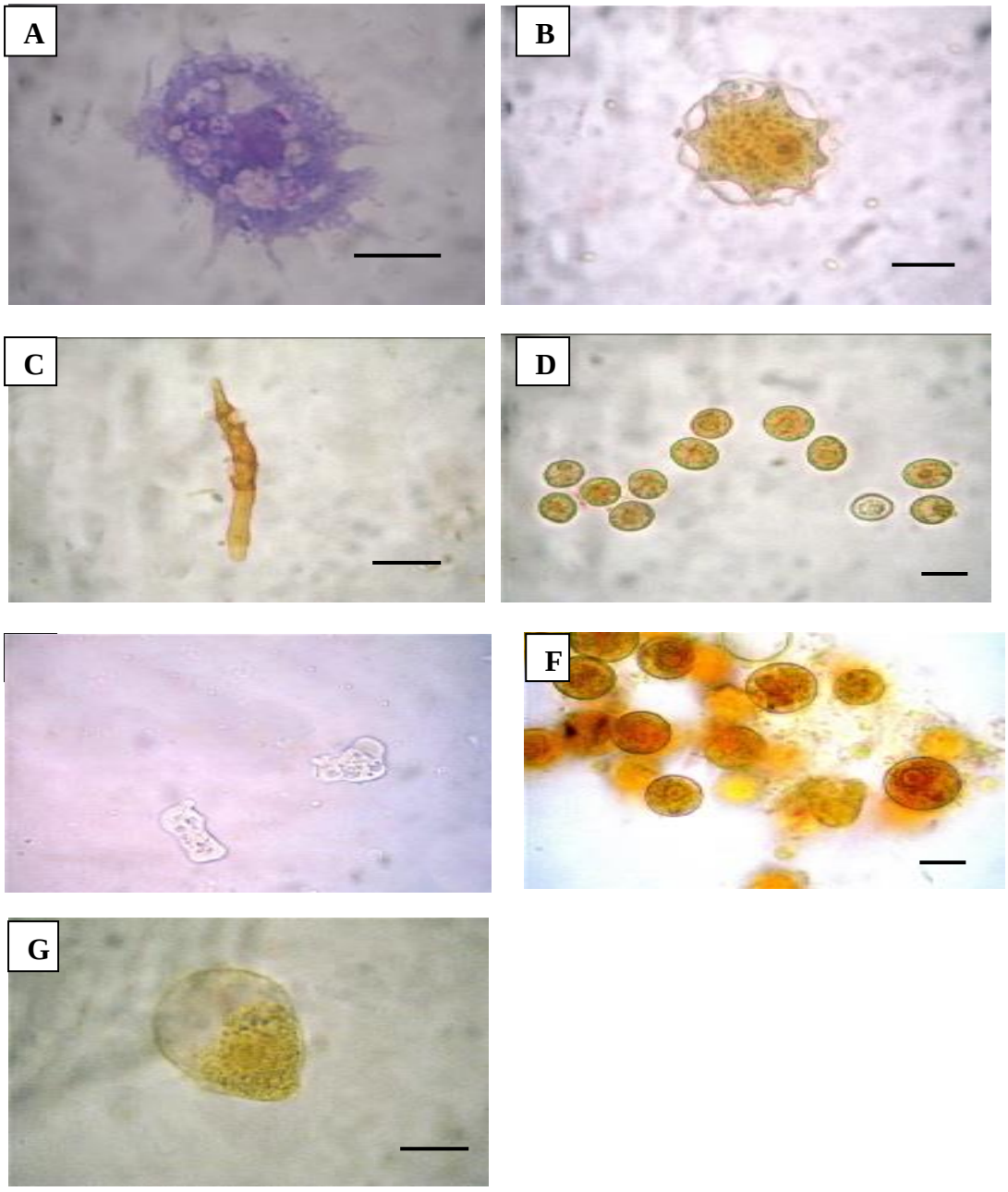


Figure 2: Photomicrographs for detected free-living amoebae. A) *Acanthamoeba* trophozoite; B*) Acanthamoeba* cyst; C) *Hartmannella* trophozoite; D) *Hartmannella* cyst; E) *Naegleria* trophozoite; F) *Naegleria* cyst; G) *Vannella* trophozoite. Bar= 10µm.

The frequency of occurrence of *Acanthamoeba* species in domestic tap water of Huntington, West Virginia, U.S.A reached 9.3% [39]. In general, the density and diversity of free-living amoebae at point of use were influenced by seasonal temperature variations [5,40] and also increased during the summer months [41].

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Table 4: Molecular confirmation of the morphologically identified genera of free-living amoebae isolates

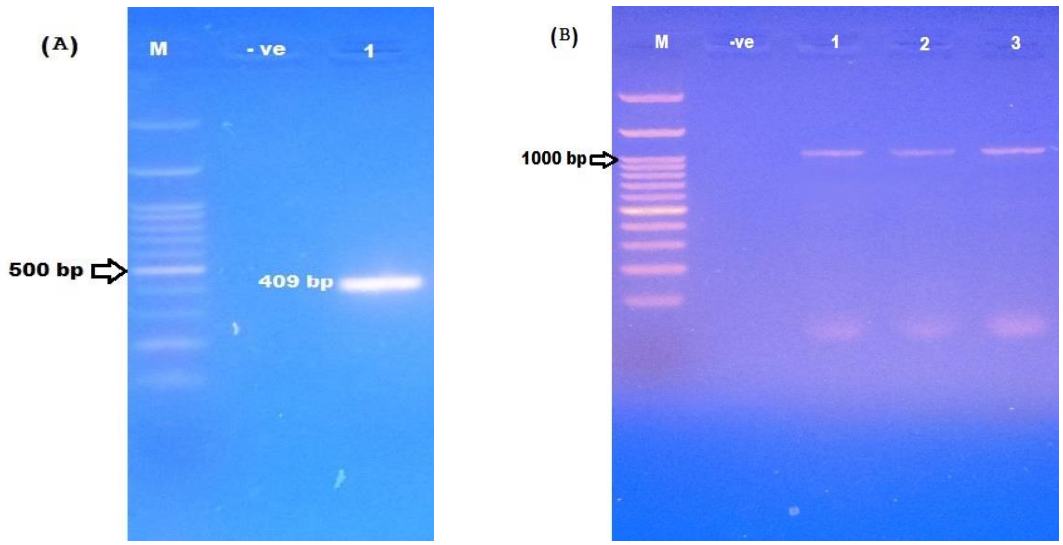
	Acanthamoeba		*Naegleria*		*Vannella*		*Hartmannella*	
	Tap water	Biofilm	Tap water	Biofilm	Tap water	Biofilm	Tap water	Biofilm
Morphologically +ve samples	23	1	1	0	1	1	2	0
PCR +ve samples	19	1	1	0	1	1	2	0
%	82.6	100	100	0	100	100	100	0

Trophozoites of *Naegleria* were long slender or oval in shape measuring from 12-25µm in length and 10-20µm in width. The nucleus had a distinct nuclear membrane and a centrally located prominent nucleolus. The cyst was spherical with a round, smooth double wall and measured 8-15 µm in diameter. At the level of ordinary microscopy, it was clear that cysts of different *Naegleria* species were too similar morphologically to be distinguished from each other [42]. Members of genus *Naegleria* were present in 2.1% of tap water samples with the highest occurrence (8.3%) in autumn season. They were not detected in the examined biofilm sample. Other workers in Egypt recorded a higher occurrence of *Naegleria* species (25%) in tap water samples [33].

In the current work, members of genus *Hartmanella* were present in 4.2% of the examined tap water samples and they were present only in spring and autumn, and absent from biofilm samples. Although they were uncommon in their distribution in the present investigation, other researchers in Turkey reported that *Hartmannella* species were the most common type in tap water samples [31].

In South Africa, Muchesa *et al.* [43] identified *Acanthamoeba* spp. and *Hartmanella* spp. morpholologically. These organisms have gained medical importance since some of them can produce infections in humans such as amebic encephalitis [44-46] and amebic keratitis, a sight threatening ulceration of the cornea [47,48].

In the present work, isolated trophozoites of *Vannella* species reached 10-25 µm in diameter with a single vesicular nucleus and had a hyaline, fan-like, flattened veil occupying anterior half of the cell. No cyst form was known. They were isolated from one tap water sample in winter only, but not in other seasons. They were also detected in the biofilm matrix. To date, the only species that is known to form cysts is *Vannella persistens* isolated from grassland soil [48]. The carrier property of *Vannella* species for intracellular microorganism such as microsporidia is of utmost health risk importance [49,50].



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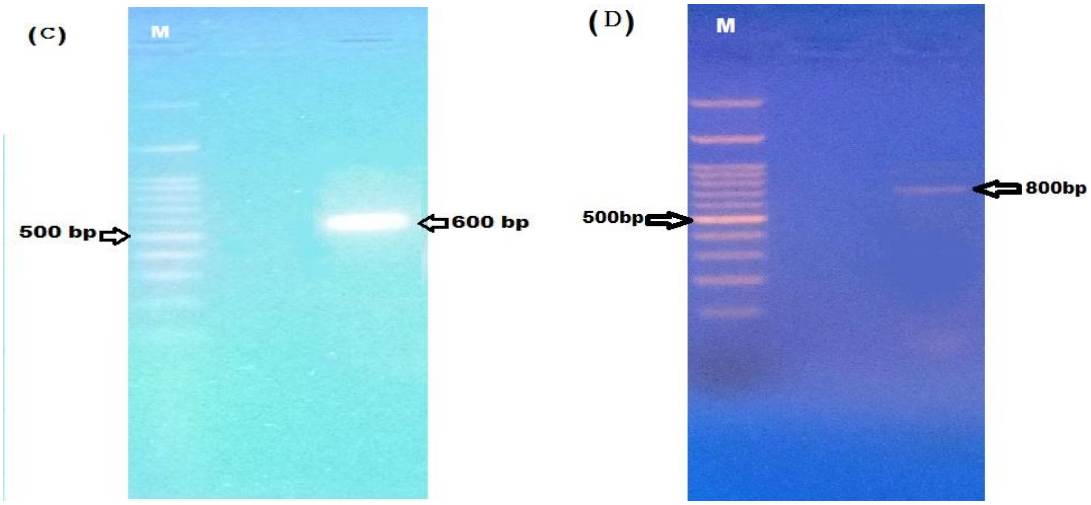

Figure 3: Agarose gel electrophoresis for PCR amplified product of DNA from:

(A) *Naegleria* spp. Lane 1: Marker; Lane 2: Control negative; Lane 3: Positive samples.
(B) *Acanthamoeba* spp. Lane 1: Marker; Lane 2: Control negative; Lanes 3, 4 and 5: Positive samples.
(C) *Hartmannella* spp. Lane 1: Marker; Lane 2: Control negative; Lane 3: Positive sample.
(D) *Vannella* spp. Lane 1: Marker; Lane 2: Control negative; Lane 3: Positive sample.

CONCLUSION

Among the detected free-living amoebae, members of genus *Acanthamoeba* were the most prevalent in tap water. Presence of free-living amoebae in the formed biofilm on the inner surface of tap water pipes might facilitate their growth, multiplication, dissemination and persistent occurrence in drinking water distribution systems.

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REFERENCES

[1] WHO Guidelines for Drinking Water Quality, 4th ED 2011. World Health Organization.
[2] Yousuf FA, Siddiqui R, Subhani F, Khan NA. J Water Health 2013; 11:371–375.
[3] Visvesvara GS, Moura H, Schuster FL. FEMS Immunol Med Microbiol 2007; 50: 1-26.
[4] Page FC. Freshwater Biological Association 1976. Scientific publication no. 34.
[5] Hoffmann R, Michel R. Int J Hyg Environ Health 2001; 203: 215-219.
[6] Greub G, Raoult D. Clin Microbiol Rev 2004; 17: 413-433.
[7] Marciano-Cabral F, Cabral G. Clin Microbiol Rev 2003; 16: 273-307.
[8] Fouque E, Trouilhe MC, Thomas V, Hartemann P, Rodier MH, Hechard Y. Eukaryot Cell 2012; 11: 382-387.
[9] Dupuy M, Berne F, Herbelin P, Binet M, Berthelot N, Rodier MH, Soreau S, Héchard Y. Int J Hyg Environ Health 2014; 217: 335-339.
[10] Da Rocha-Azevedo B, Tanowitz HB, Marciano-Cabral F. Interdiscip Perspect Infect Dis 2009; 2009: 251406.
[11] Critchley M, Bentham R. J Appl Microbiol 2009; 106: 784-789.
[12] Smirnov AV, Chao E, Nassonova ES, Cavalier-Smith TA. Protist 2011; 162: 545–570.
[13] Trabelsi H, Dendana F, Sellami A, Sellami H, Cheikhrouhou F, Neji S, Makni F, Ayadi A. Pathol. Biologie 2012; 60: 1–7.
[14] Cateau E, Imbert C, Rodier MH. Lett ApplMicrobiol 2008; 47: 475-477.
[15] Vanessa B, Virginie M, Nathalie Q, Marie-He´le`ne R, Christine I. Water Res 2012; 46: 570-5714.

[16] Schmitz-Esser, S, Toenshoff ER., Haider S, Heinz E,Hoenninger VM, Wagner M, Horn M. Appl Environ Microbiol 2008; 74: 5822–5831.
[17] Health Protection Agancy. 2004;W17: issue 2.
[18] APHA 2 0 0 5 . 21th ED, APHA, WEF and AWWA, Washington, DC.
[19] De Jonckheere JF.Naegleria. Acta Protozool 2002; 41: 309-342.
[20] Page FC. Freshwater Biol. Ass.,Ambleside 1988; 3-170.
[21] Pussard M, Ponus R. Protistol 1977; TXIII: 557-598.
[22] Winnebenninckx B, Back Lijau T, De wachter R. Trends Gen 1993; 9: 407.
[23] Abdel-Hamid AZ, Molfetta JB, Fernandez V,Rodrigues V. Inst Med Trop 1999; 41: 291-295.
[24] kilic A,Tanyuksel M, Sissons J, Jayasekkera S, Khan N. Acta Parasitol 2004; 49: 246-252.
[25] Nazar m, Haghighi A, Taghipour N, Ortega-Rivas A, Tahvildar-Biderouni F, Mojarad EN, Eftekhar M. Parasitol Res 2012; 111: 835-839.
[26] Pelandakis M, Serre S, Pernin P. J Eukaryot Microbiol 2000; 47: 116-121.
[27] Hamadto HH, Aufy SM,El-Hayawan IA,Saleh MH, Nagaty IM. J Egypt Soc Parasitol 1993; 23:631-637.
[28] Hilali M, Ashmawy K, Samaha H, Draz AA, Abu El-Wafa SA, Salem A. J Egypt Vet Med Ass 1994; 54: 215-224.
[29] Stockman CJ, Wright GS, Visvesvara BS, Fields MJ. Parasitol Res 2011; 108: 621–627.
[30] Winck K, Caumo MB,Rott. MA.Curr Microbiol 2011; 63: 464–469.
[31] Ozçelik S,Coşkun KA,Yünlü O,Alim A, Malatyalı E. The Turkiye Parazitol Derg 2012; 36:198-203.
[32] KilvingtonS,GrayT,DartJ,MorletN,BeechingJR,FrazerDG,Matheson M. Invest Ophth Vis Sci 2004;45:165–169.
[33] Gad MA. Ph.D.thesis 2014; Fac. Sci., Ain Shams University.
[34] Al-Herrawy AZ, Mohamed SH, Mohamed AH, Zaghloul NM. Intern J Environ 2015; 4: 98-107.
[35] Al-Herrawy AZ. Ph.D.thesis1992; Fac.Vet. Med., Alexandria University.
[36] Al-Herrawy AZ, Bahgat M, Mohammed A, Ashour A, Hikal WM. Iranian J Parasitol 2013a; 8: 302-312.
[37] Al-Herrawy AZ, Lotfy WM, Hishmat MG, Abu Kabsha SH.Water and Sanitation in Africa and the Middle East 2013b;, held at the Bibliotheca Alexandrina, Alexandria, Egypt, during the period from October 28-29.
[38] Schuster FL , Visvesvara GS. Drug Resistance Updates 2004a; 7: 41–51.
[39] Trzyna WC, Mbugua MW, Rogerson A. Acta Protozool 2010; 49: 9–15.
[40] Marciano-Cabral F,Jamerson M, Kaneshiro ES. J Water Health 2010; 8: 71–82.
[41] Carlesso A,, Artuso G, Caumo K, Rott M. Curr Microbiol 2010; 60: 185–190.
[42] Schuster FL, Visvesvara GS. Int. J Parasitol 2004b; 34: 1001-1027.
[43] Muchesa P, Bamadr TG, Bartie C. South Afric J Sci 2015; 111: 1-3.
[44] Martinez A J Neurology 1980; 30: 567–574.
[45] Martinez A J. Rev Infect Dis 1991; 13:399–402.
[46] Marciano-Cabral F, Puffenbarger R, Cabral GA. J Eukaryot Microbiol 2000; 47: 29-36.
[47] Sharma S, Garg P, Rao GN. British J Ophthalmol 2000; 84: 1103-1108.
[48] Niederkorn JY, Alizadeh H, Leher H, McCulley J. Microb Infect 1999; 1: 437–443.
[49] Smirnov AV, Brown S. Protistol 2000; 1: 120-123.
[50] Hofmann R, Michel R, Schmid EN, Muller K-D. Parasitol Res 1998; 84: 164-166.
[51] Michel R, Schmid EN, Boker T, Hager DG, Muller KD, Hofmann R, Sertz HM. Parasitol Res 2000; 86: 514-520.