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Co-occurrence of Free-living Amoebae and Amoebae-resisting Bacteria in Hospital Drinking Water System: Evaluation and Comparative Analysis

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Authors' contributions

This work was carried out in collaboration between all authors. Authors LG, OEB, MHMAR and HYB designed the study, collected the samples and wrote the protocol. Authors OEB, MHMAR and EM managed the experimental part. The author OEB did the statistical analysis. Authors MHMAR and HYB wrote the first draft of the manuscript and managed the literature searches. Analysis of study and final draft was done by author LG. The final draft was revised by author HYB. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: Conventional routine water analysis does not search for free-living amoebae (FLA) and Amoebae-Resisting Bacteria (ARB) in spite of their morbid impact on human health especially towards patients in health care services. In Egypt, very limited data are available in FLA, existing in the treated drinking water and the ARB naturally inhabits them. Therefore, this study was to elucidate the obscure side of this problem trying to find their actual co-occurrence and strength of such relations in nature.

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Study Design: Cross-sectional study.
Place and Duration of Study: This study was conducted between June and December 2014 at six of Assiut University Hospitals (AUH) buildings.
Methodology: A total of 54 samples (27 water and 27 biofilms) were collected from the drinking water system (DWS) of AUH. The samples were filtered and processed to Fluorescent *In-situ* Hybridization (FISH) using specific oligonucleotide probes for *Legionella* spp., *Legionella pneumophila, Parachlamydiaceae,* and eukaryote-specific probes for *Hartmannella* spp. and *Naegleria* spp.
Results: Forty-four out of 54 samples (81.4%) were positive for FLA, 45% *Hartmannella* species, 27.27% *Naegleria* spp. and 27.27% (co-occurrence of both amoebae). *Legionella* spp. was the most detected ARB within their FLA host 84% (37/44) in contrast to 54% (24/44) *Parachlamydiaceae.* Co-existence of *Legionella* spp. and *Parachlamydiaceae* in both amoebae within the same sample was observed. The number of *Hartmannella* harboring *Legionella* spp. was higher than *Naegleria* spp., but the number of FLA decreased when both amoebae coexisted and harboring *Parachlamydiaceae*.
Conclusion: It is worth mentioning it could be the first comparative study in Egypt pointing to the relation between FLA and ARB in their natural environment and not from in vitro culture using FISH. New strategies are to be implemented for efficient infection prevention and control to restrict the spread of nosocomial infections through hospital water systems.

Keywords: Free-living amoeba; Naegleria; Hartmannella; amoeba resisting bacteria; hospitals; drinking water system; Parachlamydiaceae; Legionella spp.

1. INTRODUCTION

Treated drinking water is still responsible for a number of water based diseases, of which pathogens, grow naturally in water systems [1]. Mortality of water-associated diseases exceeds 5 million people per year in developing countries [2]. Water-based pathogens are FLA, reservoirs for Amoebae-Resisting Bacteria (ARB), and are widely spread in the environment [3,4]. Internal water systems like dental treatment units, eyewash solutions, contact lenses, dialysis units are their site of predilection [5]. They are including *Acanthamoeba* species (spp.), *Hartmannella* spp., and *Naegleria* spp. and are hosts for the ARB such as *Legionella* spp.(an established agent of pneumonia) and *Parachlamydiaceae* (emerging human pathogen) [6–8]. The ARBs are able to challenge the microbicidal effector mechanisms of amoebae [7] and use the amoebae as "Trojan horses" to fight macrophages destruction [9]. FLA also afford ARB shield from extreme environmental conditions like temperatures [3,10,11]. desiccation and exposure to ultraviolet radiation [10,12] as well as FLA cysts mostly, provide protection from a range of chemicals used in water disinfection [13]. Moreover, FLA can increase the virulence of some of the ARB including *Legionella* spp., *Parachlamydiaceae*, *Legionella pneumophila* and other amoeba-resistant microorganisms [14,15]. Water-based pathogens have the capability to develop to high concentrations within biofilms on pipe walls, particularly during periods of water stagnation and warmer conditions [1]. The presence of FLA in treated drinking water system, therefore, represents a health risk to both immunocompromised and immunocompetent individuals [15]. These pathogens appear to cause a higher health burden via hospitalization than waterborne enteric pathogens [16]. Patients in the healthcare settings are more vulnerable to contamination either by their contact with treated potable water used for many purposes other than drinking like showering or through dealings with soiled health utensils washed with tap water or when they have invasive devices, open wounds exposed to the hands of healthcare workers washed using tap water [17,18].

However, most of these transient associations of bacteria and amoebae have been observed under laboratory conditions. The actual occurrence and strength of such relations in nature and their impact on the ecology of both bacteria and amoebae are unknown [4]. In Egypt, according to the available literature, very limited information is available concerning ARB from nosocomial and environmental samples [19] and no data are available on nosocomial *Legionella*, *Parachlamydiacae*, or amoebal infection rates in Assuit University Hospitals so far. Only a few studies were done to survey the biodiversity of FLA within various water sources [20–24]. According to Thomas and Ashbolt [25], very restricted documents on which FLA act as hosts in treated drinking water is available.

Conventional routine water analysis does not search for ARB &FLA in spite of their morbid impact on human health. Therefore comes up the role of this study to elucidate the obscure side of this problem to prevent waterborne parasites transmission. The health effects of water contamination are vital to national public health due to the fact that access to safe drinking water is an essential cornerstone of public health.

2. MATERIALS AND METHODS

2.1 Sampling

Fifty-four samples (27 water and 27 biofilm samples) were collected between June and December 2014 from drinking water system (DWS) of six of Assiut University hospital (AUH) buildings: Main hospital, outpatient clinic, Children Hospital, Woman Health Hospital, Al-Rajhi Liver Hospital, and Urology & Neurology Hospital. All these buildings are supplied by the same source (waterworks).

Water and biofilm samples were taken from the main water tank and faucets at different sites in each building. A single biofilm swab and bulk water sample were collected from each sampling site. Samples were taken under complete aseptic precautions (wearing appropriate disposal, powderless gloves, changing gloves before each new step during sample collection and processing and avoiding hand contact with contaminating surfaces and with collected samples). Biofilm samples were collected by vigorously rubbing the interior surface of the water tanks and faucets with sterile swabs soaked in sterile distilled water. Swabs were then kept moist during transport in 3-5 ml of water from the same water tank or faucet [26]. Swabs were discarded after forceful stirring into the collection tube water. After the biofilm swab was collected, water was turned on to run for a few minutes until the water is warm but not hot, then 1 L of water from the faucet was collected into a sterile plastic bottle, leaving a 1-inch space at the top. Sodium thiosulfate solution (0.1N) was added to biofilm and water samples to neutralize residual disinfectants [27]. Samples were transported to the laboratory and kept at 4ºC for subsequent analysis.

Water samples were filtered using 0.22 µm pore size cellulose nitrate membranes (ADVANCED MICRODEVICES PVT. LTD, India) in order to arrest particles bigger than 0.22 µm. Intact membranes were aseptically removed and placed into sterile 50 ml Falcon tubes containing 10 ml sterile distilled water. Sonication for five min was done using Soniprep 150 ultrasonic disintegrator (MSE, UK) to dislodge the target organisms from the membranes [28].

2.2 Fluorescence *In situ* Hybridization Method (FISH)

The molecular fluorescence in situ hybridization (FISH) method was used to screen all samples via probe EUK516 complementary to a unique target site on the 18S rRNA of many eukaryotes and the bacterial probe EUB338 targeting most but not all bacteria. Then positive samples were further investigated for the numbers of FLA *(Naegleria* spp*. and Hartmannella* spp*.)* and the presence of different phylogenetic bacterial groups (*Legionella* spp*., L. pneumophila and Parachlamydiaceae)* living naturally inside them. At the end of sample collection, all samples were centrifuged and a fixative (3 methanol: 1 glacial acetic acid) was applied on the sediments.

The hybridization was performed according to Grimm et al. [29]. FLA cells retained in the sediment were hybridized using *Hartmannella* and *Naegleria* fluorescein-labelled eukaryote-specific probes (HART498, and NAE1041 respectively) and *Legionella* spp*., L. pneumophila,* and *Parachlamydiaceae* -specific fluorescence-labelled oligonucleotide probes (LEG705, LEGPNE1, and Bn9-658 respectively). Oligonucleotide probes sequences and their fluorescent dyes (Eurofins Genomics, Germany) are shown in Table 1. The probes were suspended in the hybridization buffer consisting of formamide [whose concentration depended on the probe sequence as shown in Table 1 (in volumes per volume)], NaCl 0.9 M, sodium dodecyl sulfate 0.01 % and Tris/HCl (20 mM), pH 7.6. The probe diluted 1: 9 hybridization buffers for prokaryotic and eukaryotic organisms. The sediments were dropped on positive charge slides. Probes were applied in a hybridization chamber (denaturation at 75 c for 2 min and hybridization at 37ºC (overnight program). After that, in order to remove the unbound probes, the slides were placed for 15min in the washing buffer having the temperature of 48°C (the composition of the buffer, 20 mM Tris/HCl, pH 7.6;0.01% sodium dodecyl sulfate; 5 mM Ethylene Diamine Tri-acetic acid (EDTA)-160 mM NaCl for all probes, rinsed with the distilled water and dried. Subsequently, the slides were covered with coverslips; Fluorescence was detected using automated motorized fluorescent

Zeiss microscope and the appropriate filter. The slides were analyzed at ×40 magnification to detect hybridized eukaryotic cells, and then positive slides were further analyzed at × 63 (oil immersion) magnifications to detect hybridized intracellular prokaryotic cells. Thirty fields were counted for each sample in DAPI and FITC and Texas red filters settings.

2.3 Statistical Analysis

Statistical package for social sciences (SPSS), version 16 was used for data analysis. Mann-Whitney Test and ANOVA were used for calculation of differences in mean numbers of free-living amoebae harboring bacteria. *P*-value is significant when ≤ 0.05.

3. RESULTS

A total of 54 samples (27 water and 27 biofilms) were tested by FISH using specific oligonucleotide probes (Table 1). About 81.5% (44/54) of the samples were found contaminated with FLA: *Hartmannella* spp. was the most prevalent amoeba 20/44 (45.4%) followed by *Naegleria* spp. 12/44 (27.3%) and the last 12/44 (27.3%) represents the co-occurrence of both amoebae. ARB (*Legionella* spp. and *Parachlamydiaceae*) were also detected living in the FLA.

When either *Naegleria* spp. or *Hartmannella* spp. were present solely in samples, it was found that *Legionella* spp. were invading 95% (19/20) of *Hartmannella* spp. (Fig. 1) and 83% (10/12) of *Naegleria* spp. positive samples. *Parachlamydiaceae* were found in 12/20 (60%) of *Hartmannella* spp. and 50% (6/12) of *Naegleria* spp. positive samples (Fig. 2). Fifty-five percent (11/20) of *Hartmannella* spp. and 33.3% (4/12) of *Naegleria* spp. positive samples have affirmed *Legionella* spp. / *Parachlamydiaceae* co-existence in the same sample. *Naegleria* spp. and *Hartmannella* spp. were co-occurring in the same sample in 12/44 (27.3%) harbored by ARB *Legionella* spp. 66.6% (8/12) & *Parachlamydiaceae* 50% (6/12) (Table 2).

Legionella spp. was the most detected ARB within their FLA host 84% (37/44) in contrast to 54% (24/44) *Parachlamydiaceae. Legionella pneumophila* was the dominating spp. among *Legionella*-infected *Naegleria* samples 67% (12/18) in comparison to non-*pneumophila Legionella* spp. 33% (6/18).Whereas non-*pneumophila Legionella* spp. was the dominating species of among *Legionella*-infected *Hartmannella* 59% (16/27) (Table 2).

The highest mean number of amoebae was recorded among *Hartmannella* spp. but with no significant differences between the mean numbers of both free-living amoebae, regardless of their harbored bacteria. Meanwhile, it was noticeable that the mean number of *Hartmannella* spp. harboring non-*pneumophila Legionella* had markedly decreased from 23 $x10^6$ to 13 $x10^6$ (mean number/L) when both amoebae co-occurred together (Table 3). No significant discrepancy was observed between water and biofilm samples in the mean number of tested free-living amoebae harboring bacteria. But, an obvious increase in mean number of FLA was observed in the biofilm .When both ameabe were found in the same sample *Hartmannella* associated with *Legionella* spp. had almost tripled its intensity from $6x10^6$ in water to $17x10^6$ in the biofilm (Table 4).

4. DISCUSSION

The present study had chosen to investigate the presence of *Naegleria* spp. and *Hartmannella* spp. in DWS of the AUH facilities, driven by the highly pathogenic impact of the *Naegleria* parasite on human's health. In addition *Hartmannella* spp. seemed to increase the pathogenicity of their hosting bacteria [35], threatening the life of vulnerable persons in hospitals. Free-living amoebae and their endosymbiotic ARB were identified by FISH technique. FISH allows simultaneous molecular identification of different microorganisms at different taxonomic levels, by using multiple fluorescent dyes, without the need for their individual isolation [29]. Endosymbiotic microorganisms are frequently very difficult to obtain in pure culture. FISH is, therefore, an important tool for the identification within their host [36,33]. The technique also reveals the location of these microorganisms in the host [37]. Unlike PCR, FISH is aimed to distinguish the metabolically active cells and consequently, offers more relevant information about the infective threat [38]. The present study was not targeting specific species of FLA (*Hartmannella* spp. and *Naegleria* spp.) as many of them might be not pathogenic to man, but they still carry pathogenic bacteria which could invade the human body by many routes.

Table 1. Oligonucleotide probes used for fish detection and the targeted organisms

Probe name	Sequences	Targeted organisms	Fluorescent dyes	Formamide %	References
EUB338	5'- GCT GCC TCC CGT AGG AGT -3'	Most bacteria	Texas-red	0-50	[30]
LEG705	5'- CTG GTG TTC CTT CCG ATC -3'	*Legionellacea*	Texas-red	20	[31]
LEGPNE1	5'- ATC TGA CCG TCC CAG GTT -3'	*Legionella pneumophila*	Alexa fluor 350	25	[32]
HART498	5'-TCG CGG AGAG GGT GTC GGT-3'	*Hartmannella sp.*	CY3	40	[29]
EUK516	5'- ACC AGA CTT GCC CTC C -3	Many eukaryotes	FITC	49	[30]
NAE1041	5'- TGC ACT ACC CAC CAC ACA -3'	*Naegleria sp.*	FITC	30	[33]
Bn9-658	5'- TCC GTT TTC TCC GCC TAC-3'	Subgroup of the *Parachlamydiaceae*	Texas-red	10	[34]

Table 2. Comparison between the mean numbers of free-living amoebae harboring bacteria detected in samples AUH water distribution systems

Bacteria/Amebae		*Hartmannella* spp. [a]	*Naegleria* spp. [a]	Coexistence of both amoebae		*P*-value [b]
				Hartmannella spp. [a]	*Naegleria* spp. [a]	
Legionella spp.	All spp.	20×10^6	16×10^6	16×10^6	14×10^6	0.4
	L. pneumophila	17×10^6	14×10^6	20×10^6	15×10^6	0.8
	non *pneumophila Legionella*	23×10^6	19×10^6	13×10^6	12×10^6	0.3
Parachlamydiaceae.		20×10^6	20×10^6	11×10^6	14×10^6	0.3

[a] *mean number/L; One way ANOVA- 1*

[b] *P-value comparing amoebae (Hartmannella spp. vs. Naegleria spp. vs. Coexistence of both amoebae) harboring bacteria accordingly*
Significant P-value ≤ 0.05

Table 3. Mean numbers of free-living amoebae harboring bacteria detected in water and biofilm samples of Assuit University Hospitals' water distribution systems

Parasite	Endosymbiotic bacteria	Water[a]	Biofilm[a]	*P*-value[b]
Hartmannella spp (n=20)	*Legionella* spp.	$19x10^6$	$23x10^6$	0.7
	Parachlamydiaceae	$17x10^6$	$24x10^6$	0.3
Naeglaria spp (n=12)	*Legionella* spp.	$14x10^6$	$18x10^6$	0.6
	Parachlamydiaceae	$17x10^6$	$27x10^6$	0.5
Coexistence of both amoebae (n=12)	*Hartmannella /Legionella* spp.	$6x10^6$	$17x10^6$	0.5
	Hartmannella /Parachlamydiaceae	$10x10^6$	$12x10^6$	0.8
	Naeglaria /Legionella spp.	$13x10^6$	$14x10^6$	1
	Naeglaria/ Parachlamydiaceae	$10x10^6$	$16x10^6$	0.3

[a] *mean number/L; Mann-Whitney Test*

[b] *P-value comparing amoebae (either Hartmannella spp., Naegleria spp. or coexistence of both amoebae) harboring bacteria accordingly in water vs. biofilm samples. Significant P-value ≤ 0.05*

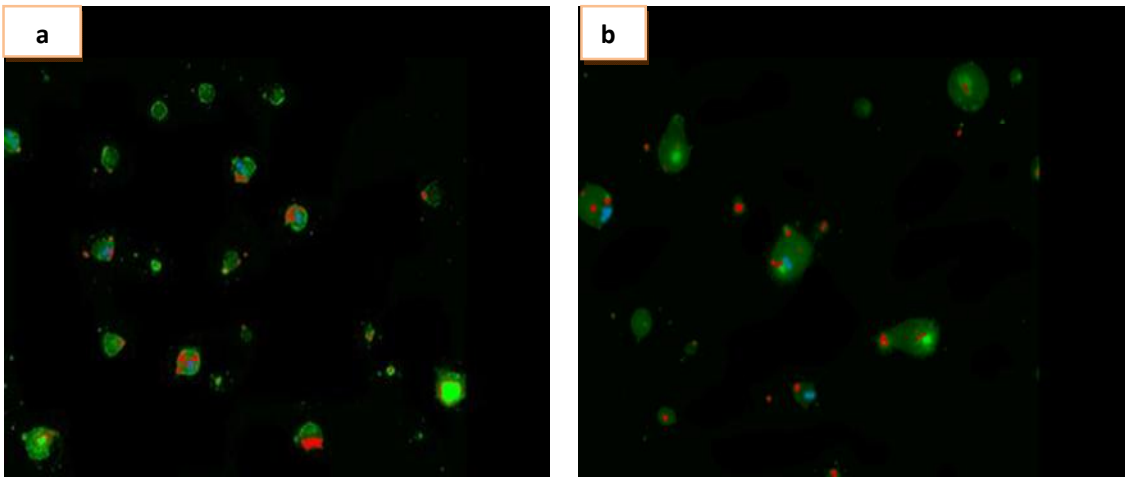

Fig. 1. *In situ* hybridization of *Hartmannella* spp. with CY3 labeled oligonucleotide probe HART498 (yellow green) showing endosymbiotic *Legionella* spp. hybridized by Texas red labeled probe LEG705 (red) and the *L. pneumophila* by Alexa fluor labeled probe LEGPNE1 (blue) x 400 (a) and x 630 (b) oil immersion

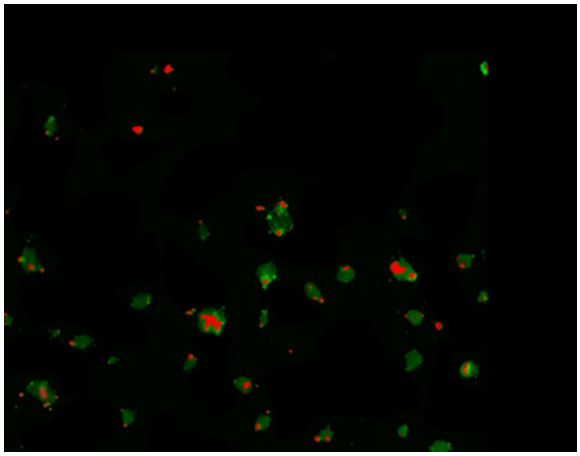

Fig. 2. *In situ* hybridization of *Naegleria* spp. with FITC labeled oligonucleotide probe NAE1041 (green) showing endosymbiotic *Parachlamydiaceae* hybridized by Texas red labeled probe Bn9-658 (red) x 400

Table 4. Total numbers and percentages of positive samples for some free-living amoebae harboring bacteria detected in AUH water distribution systems

Hospitals building	Sample type	Total samples (n=54)													
		Hartmannella amoebae alone (n=20)[a]					Naeglaria amoebae alone (n=12)[b]						Coexistence of both amoebae (n=12)[c]		Negative (n=10)[d]
		Legionella alone (n=8)			Both (n=11)		Legionella alone (n=6)			Both (n=4)			Type of bacteria in		
		L. pneumophila (n=5)	*Non pneumophila (n=3)*	*Parachlamydiaceae (n=1)*	*L. pneumophila (n=4)*	*Non pneumophila Legionella* spp. (n=7)	*L. pneumophila (n=5)*	*Non pneumophila Legionella* spp.(n=1)	*Parachlamydiaceae (n=2)*	*L. pneumophila (n=1)*	*Non pneumophila Legionella* spp.(n=3)	*Number of +ve samples*	*Hartmannella*	*Naegleria*	
Main hospital building	Water (n=6)	-	-	-	1/5%	1/5%	-	-	-	-	-	1/8%	*Parachlamydiaceae*	*Parachlamydiaceae*	2/20%
												1/8%	*L. pneumophila* + *Parachlamydiaceae*	*L. pneumophila* + *Parachlamydiaceae*	
	Biofilm (n=6)	-	-	-	2/10%	2/10%	-	-	-	-	-	-	-	-	2/20%
Outpatient clinics building	Water (n=4)	1/5%	-	-	-	-	-	-	-	-	-	1/8%	*non pneumophila Legionella*	*non pneumophila Legionella*	2/20%
	Biofilm (n=4)	-	-	-	-	-	-	-	-	1/9%	-	1/8%	*L. pneumophila*	*L. pneumophila*	2/20%
Children hospital	Water (n=5)	-	-	1/5 %	-	-	1/9%	-	1/9%	-	-	1/8%	*non pneumophila Legionella*	*non pneumophila Legionella*	1/10%
	Biofilm (n=5)	-	1/5%	-	1/5%	-	1/9%	1/9%	1/9%	-	-	-	-	-	-
Women health hospital	Water (n=6)	1/5%	1/5%	-	-	1/5%	1/9%	-	-	-	1/9%	1/8%	*Parachlamydiaceae*	*Parachlamydiaceae*	-
	Biofilm (n=6)	1/5%	1/5%	-	-	1/5%	1/9%	-	-	-	1/9%	1/8%	*Parachlamydiaceae*	*Parachlamydiaceae*	-
Al-Rajhi liver hospital	Water (n=3)	1/5%	-	-	-	-	-	-	-	-	-	1/8%	*non pneumophila Legionella*	*L. pneumophila*	-
												1/8%	*non pneumophila Legionella*	*L. pneumophila*	
	Biofilm (n=3)	-	-	-	-	2/10%	-	-	-	-	-	-	-	-	1/10%
Uorology & neurology hospital	Water (n=3)	-	-	-	-	-	1/9%	-	-	-	-	1/8%	*non pneumophila Legionella*	*L. pneumophila*	-
												1/8%	*non pneumophila Legionella* + *Parachlamydiaceae*	*L. pneumophila* + *Parachlamydiaceae*	
	Biofilm (n=3)	1/5%	-	-	-	-	-	-	-	-	1/9%	1/8%	*Parachlamydiaceae*	*Parachlamydiaceae*	-
Total bacteria	*Legionella* *All spp.*			19					10				8	8	-
	Legionella *L. pneumophila*			9					6				2	6	-
	Legionella *non pneumophila Legionella*			10					4				6	2	-
	Parachlamydiaceae			12					6				6	6	-

n = number results are expressed as number/percentage, [a] Percentages calculated from the total number of samples +ve for Hartmannella alone, [b] Percentages calculated from the total number of samples +ve for Naegleria alone, [c]Percentages calculated from the total number of samples +ve for both Hartmannella & Naegleria, [d] Percentages calculated from the total number of negative samples

FLA was present in 44 (81%) out of 54 examined (water & biofilm) samples. Nearly similar results (79%) were recorded in the USA [39] and the UK 89% [40]. In other countries like Brazil, Turkey and Iran, free-living amoebae recorded 22.79% [41], 22% to 30% [42,43] and 38%,48% & 52% [44–46] of their tap water, respectively. On the other hand, lower prevalence had been recognized in studies done in Egypt in early 1990, where the prevalence of tap water contamination reached only 4% [47] and 23.6% [48] and the negative result was reached by Sadaka et al. [49]. Even so, with the advance of the technique of parasite diagnosis and retrieval, the prevalence has increased in Egyptian studies [21–24,50]. The discrepancy of the prevalence of FLAs in tap water from different countries might be due to differences in; water treatment method, geographic location, and differences in water temperature that disturbs the FLA existence [39], and lastly the methods by which the protozoa were recovered.

Few studies have been dedicated to the investigation of the biodiversity and prevalence of FLA and ARB in drinking water production and distribution systems [13]. A strong association is present between waterborne pathogens and nosocomial infection and was largely ignored [18]. In health facilities, where the threat is more critical, studies are still insufficient .They were only conducted to detect FLA in hospital water systems and therapeutic pools [5,44,45,51,52] .Consequently, it is noteworthy to inspect the DWS of hospitals as a potential source of nosocomial outbreaks of FLA carrying bacteria causing diseases (Legionnaires' disease). We had intentionally conducted our study in the DWS of AUH buildings to record the magnitude of pathogens soiling the hospitals DWS. Yet, no literature in Egypt has been reported concerning the relation between ARB and their hosted FLA in hospital water systems. We recorded that, *Hartmannella* spp. (45%) has the highest prevalence compared to *Naegleria* spp. (27.7%). In the few Egyptian studies, the frequently detected FLA was *Acanthamoeba* and *Naegleria* spp. They were recovered from non-potable water (water canals or drains and swimming pools) [47,49]. Other studies have looked for single parasite [19,20,47]. In Morsy et al. [24] study, *Acanthamoeba* spp. was the most frequently detected FLA accounting for 56.3%, 2.1% *Naegleria* spp. and 4.2% *Hartmannella* spp. *Hartmannella* was present only in spring and autumn, and absent from biofilm samples . Their results are in discordance with ours, as *Hartmannella* was not only the most frequently detected FLA. *Hartmannella* was also the most abundant in number, whichever hosting *Legionella* spp. or *Parachlamydiaceae* and whether detected in biofilm or water samples. In accordance with our results, Coşkun et al. [43] had reported that *Hartmannella* spp. were the most abundant FLA to be identified in 72% of its tap water samples. However, no *Naegleria* spp. was recognized. They have attributed this high prevalence to high levels of active biomass and natural organic matter as was documented in an earlier study [53]. In fact, agriculture and animal farming is surrounding our hospital buildings and annexed water plant station. *Hartmannella* spp. presence in DWS is evidence that the parasite resists disinfection in treatment plants [54]. Some strains of *Hartmannella* spp. can tolerate up to 48°C [55]. Assiut Governorate, in which the work took place, is a very arid area in Egypt with extreme temperatures during the year [56]. Air temperatures have been reported from June 2014 to December 2014 with a Max to Min Temp (44 to 2°C) respectively, with a mean average Temp of 25°C according to "Weather underground report" https://www.wunderground.com/eg/asyut. It is worth mentioning that the identified FLA is tolerant to ecological conditions. The discrepancy in the FLA dispersion and FLA species composition at certain locations even within the same country seems to depend on the surroundings [43].

Grimm et al. [29] had recognized the simultaneous presence of *Legionella pneumophila* within the in vitro infected *Hartmannella vermiformis*. But, they failed to detect this simultaneous infection from environmental samples. Horn and Wagner [4] stated in their review that co-occurrence of phylogenetically diverse endosymbionts in a single amoeba isolate has never been witnessed, and significant differences regarding the host range have been shown for the different endosymbionts. The present study has endorsed the Grimm et al. [29] experiment as we had successfully found the concurrent infection of two genera of FLA (*Hartmannella* spp. *and Naegleria* spp.) with two genera of bacteria (*Legionella* spp. and *Parachlamydiaceae*) in treated drinking water in DWS of hospitals.The co-occurrence of the FLA (*Hartmannella* spp. and *Naegleria* spp.) and ARB (*Legionella pneumophila*, non-*pneumophila Legionella* and *Parachlamydiaceae*), was carried out by the alternate exposure of the sample to their

respective probe. Unfortunately, only three colors could be visualized as we were limited by the three color capacity of the Fluorescent microscope (DAPI blue, Red Texas and FITC greenish yellow). This disability was overcome by subdividing each sample into parts and interchanging probes to cover all tested FLA and ARB.

Legionella spp. happens to be the most detected ARB. They were present in 84% (37/44) of the FLA positive samples. *Legionella* spp. was found in 95% (19/20) of *Hartmannella* spp. positive samples, in contrast to 83% (10/12) of *Naegleria* spp. positive samples. *Legionella* spp. had a positive effect on *Hartmannella* spp. when amebae were found alone or in co-existence. The mean number of *Hartmannella* reached higher abundance than *Naegleria* and predominantly when they were loaded by *Legionella pneumophila* especially when both FLA (*Hartmannella* and *Naegleria* spp.) co-exist in the same sample. In addition, what our results reported and in accordance to many researchers' citations, *Hartmannella* spp. is the leading amoebae in man-made water systems among *Naegleria* and *Acanthamoeba* [57,58]. The mean number of any of the collected FLA in our study is incomparable to the several thousand of FLA/L retrieved from the domestic water system [40,59] or the few hundreds from DWS in other studies [53,54]. The higher FLA concentration was recovered from environmental samples and sediments of DWS [60] .

Hartmannella, Naegleria, and *Acanthamoeba* spp. are known as avid bacteria feeders with some food preference. They mainly predate on *Legionella pneumophila* [29,61] but *Hartmannella sp* preference to *L. pneumophila* was twice as *Acanthamoeba* [62]. We have watched multiplication process of *Hartmannella* spp. to occur more successfully in the presence of *Legionella* spp. Moreover, in the co-occurrence of competing *Naegleria* spp., the number increased notably when both FLA fed on *L. pneumophila*, 20 x 10^6 and 15 x10^6 (mean number/liter) respectively , and particularly in the biofilm media.

Unlike *Legionella* spp. which are facultative, intracellular bacteria [63], *Parachlamydiaceae* comprise obligatory intracellular symbionts of free-living amoebae [8]. They are established agents of community and hospital-acquired pneumonia similar to *Legionella* spp. [6,7,64]. Fukumoto et al. [65] have coincidentally found *Parachlamydiaceae* and FLA in the hospital environment supporting that this potential human pathogen could spread through the hospital environment via FLA .Our study meets with Fukumoto et al. [65], as we have detected that *Parachlamydiaceae* in the DWS of our health facility together with the FLA genera hosting them. *Parachlamydiaceae* was additionally recognized in some samples co-existing with *Legionella* spp. (*L. pneumophila* or non-*pneumophila*),

We have demonstrated that *Parachlamydiaceae* are not only the endosymbionts of *Acanthamoeba* [8] but also *Hartmannella* spp. or *Naegleria* spp. In the co-existence of both FLA and *Parachlamydiaceae*, the competition of FLA for their prey, and the feedback effect was observed. The mean number of FLA either *Hartmannella* spp. or *Naegleria* spp. has reduced to 11 x 10^6 and 14 x10^6 (mean number/L) respectively .Unlike when *Hartmannella* spp. or *Naegleria* spp. were happened to occur alone associated with intracellular *Parachlamydiaceae*, their number was markedly abundant as 20 x10^6 (mean number/L). These relations need to be studied more to explain such difference in FLA multiplication in their natural environment.

It is important to mention that *Legionella* sp is more resilient to high temperature either inside or outside their host [63], unlike *Parachlamydiaceae* are endosymbiotic at 30°C but causing lysis their hosting FLA at 37°C [66]. This explains the more frequent presence of FLA hosting *legionella* sp than those hosting *Parachlamydiaceae* alone or co-existing with *legionella* spp.

In agreement with Lamoth and Greub [67], from our findings collectively we can say that the DWS is lined by multispecies biofilms, in which FLA (*Hartmannella* spp. and *Naegleria* spp) and ARB (*L. pneumophila*, non-*pneumophila* and *Parachlamydiaceae*) reside and grow naturally. Biofilms are to be the source of bulk water infection of the treated drinking water systems, despite an effective full treatment water chain [13,61]. Inside the biofilm, a multivariate media is growing, bacterial competition for food is high. The present proliferation and abundance of FLA in biofilm looks very alarming for all types of FLA in relation to ARB. Particularly when FLA co-exists, it had to be noticed that *Hartmannella* spp. hosted by *Legionella* spp. (17x106 mean number/L) had almost tripled in number in comparison to bulk water sample (6x106 mean number/L).Therefore biofilm is causing a great health concern.

The presence FLA filled with ARB represent a defy for whom are in charge for testing and inspection the quality and purity of surface waters, swimming pools, and drinking water systems, especially to those conveyed to heath care facilities [68]. Still, restricted data is available on which FLA act as hosts in treated drinking water, what may alter them or the ecological conditions that help ARB as potential human pathogens [25]. Even, if the full treatment water chain is effective, treated drinking water is still in charge for the spread of water-based pathogens (FLA and ARB).

5. CONCLUSION

The present study, so far, is the first report in our community to expose the biodiversity of FLA (*Hartmannella* spp. and *Naegleria* spp.) in DWS of hospitals, but also their co-existence relation to their hosting intracellular symbionts bacteria. The emerging *Parachlamydiaceae* was identified with the well-established (*L. pneumophila*) in their relation with their hosting FLA was studied from their natural environment and not from culture. Although the FLA may not be of the pathogenic species, but the hazard of infection is still present due to the hosted bacterial pathogens. It is to be considered in evolving new strategies for efficient infection prevention and control to restrict the spread of nosocomial infections through hospital water systems. Therefore, the ecological importance of FLA must be adequately studied to prevent fatal human diseases. The results of this investigation may change our view on the preferred disinfection strategies for drinking water systems in controlling exposure to health-threatening pathogens.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This study was approved by the local ethical committee of Faculty of Medicine, Assiut University.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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