Research Article

Isolation and identification of pathogenic *Acanthamoeba* species from different water sources in Minia Governorate, Egypt

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Abstract

Acanthamoeba species (spp.) are opportunistic pathogens and they are a member of free-living amoebae (FLA). They cause granulomatous amoebic encephalitis (GAE), a chronic disease of immunocompromised hosts such as acquired immune-deficiency syndrome (AIDS) patients and transplant recipients. The present study was conducted to isolate and to identify *Acanthamoeba* spp. from various water sources. During the study period, 150 water samples were collected from various sources such as streams and ponds, tap water, tanks, swimming pools and Nile River water. All samples were processed and cultured on non-nutrient agar medium (NNA) with *Escherichia coli* overlay for the isolation of *Acanthamoeba* spp. Organism identifying was based on the microscopic morphology of cyst and trophozoites forms. Confirmation PCR was done to positive culture samples. The pathogenicity of *Acanthamoeba* spp. was analyzed by thermo-tolerance assay. *Acanthamoeba* spp. were detected in 84 (56%) out of the 150 examined water samples. The highest percentage of *Acanthamoeba* presence was observed in streams and ponds water (70.73%), followed by swimming pools water samples (60%) and Nile River water samples (66.67%) and the lowest was in tap water samples (37.5%). Out of 84 water samples positive by NNA culture, 72 (85.71%) samples were positive by PCR using specific primers for *Acanthamoeba* spp. Based on pathogenicity test assays, among 84 positive cultures, 39 (46.43%) were thermo-tolerant. This study was showing the distribution of *Acanthamoeba* spp. in various water sources in the environment. Also, this study confirmed that the high presence of pathogenic strains in recreational water could threat contact lens wearers.
Key words: *Acanthamoeba*, water, culture, PCR, thermo-tolerance.

Introduction

Acanthamoeba spp. are opportunistic pathogens and they are a member of free-living amoebae (FLA). they cause granulomatous amoebic encephalitis (GAE), a chronic disease of immunocompromised hosts such as acquired immune-deficiency syndrome (AIDS) patients and transplant recipients. Also, they can cause infections of the lungs, sinuses and skin (Visvesvara et al., 2007). The presence of *Acanthamoeba* in water samples was considered as a double danger since some of these species are pathogenic and they could harbor pathogenic strains of *Helicobacter*, *Pseudomonas*, and *Legionella* (Balczun and Scheid 2017).

Acanthamoeba spp. are primarily diagnosed by isolating the characteristic trophozoite or the double-walled polygonal cyst after culturing

(Coşkun et al., 2013). Other methods include isoenzyme analysis, indirect fluorescence antibody using specific antiserum (Schuster, 2002), polymerase chain reaction (PCR) (Boost et al., 2008) and sequence analysis (Magliano et al., 2009). Molecular methods are very sensitive and may allow the detection of microorganisms which are difficult to identify. Thus, these methods are alternative to microscopy and culture. PCR diagnostic methods are useful for the diagnosis of both clinical and environmental specimens (Maďarová et al., 2010). This study is needed in order to assess the human risk from a variety of illnesses ranging from keratitis and infections of the lungs and skin to more serious illnesses such as granulomatous amoebic encephalitis caused by *Acanthamoeba* (Visvesvara et al., 2007).

Materials and Methods

Study type, Samples and sampling sites:

A cross sectional study was performed during the period from January 2018 to December 2018, where 150 water samples were collected from different water sources including streams and ponds, tap water, tanks, swimming pools and Nile River in Minia Governorate, Egypt. Each sample (500 ml water) were collected in sterile tubes and labeled with sampling date, time, and place of collection. Samples were examined in the Parasitology Department, Faculty of Medicine, Minia University, Egypt.

Isolation and identification of *Acanthamoeba*:

Each water sample was filtered using multiple folded sterile gauze to remove dirt and mud. Each filtrate was centrifuged at $250 \times$ g for 20 min. The supernatant discarded and sediments were dissolved in Page's Amoeba saline solution (PAS) (Caumo et al., 2009). The mixed suspension was inoculated into Petri dish containing 1.5% non-nutrient agar (NNA) over layered with *Escherichia coli* culture. All the plates were sealed tightly and incubated at 30°C for the duration of up to 2 weeks. All the processed culture plates were observed under the microscope on daily basis to check for the growth of trophozoites and cysts. *Acanthamoeba* spp. were identified based on the size and morphological characteristics of both trophozoites and cysts. The presence of finger-like tapering pseudopodia was observed in trophozoites, cysts would appear as an inner wall of polygonal and wrinkled outer wall (Caumo et al., 2009).

PCR confirmation:

A) DNA extraction:

Cysts from different isolates were harvested by sterile PAS, from the surface of NNA *E. coli* plates. After washing in PAS, centrifugation at 3000 rpm for 10 min, the pellet was resuspended in PAS and the suspension was centrifuged at 15,000 rpm for 15 min (Gatti et al., 2010). The deposit was stored at −20 °C for DNA extraction using the QIAamp DNA Mini Kit (Qiagen, Cairo, Egypt, 50 reactions).

B) PCR amplification assay for *Acanthamoeba* spp.:

Amplification of the partial 18 S rRNA gene from *Acanthamoeba* was performed by PCR, largely according to the method of Schroeder et al., (2001), using forward primer JDP1 (5′-GGCCCAGATCGTTTACCG TGAA) and reverse primer JDP2 (5′-TCTCACAAGCT GCTAGG GAGTCA) (Schroeder et al., 2001). Briefly, 5 μl of the extracted genomic DNA was used in a 50-μl reaction mixture containing 5 μl of 10× PCR buffer (100 mM KCL, 20 mM MgCl2, 20 mM Tris–HCl [pH 8.0]), 5 μl of 2.5 mM dNTP mixture, 0.5 μl of each 100 μM primer, and 0.25 μl of 5 U/μl Ex Taq DNA polymerase (Bioron). The thermal cycling conditions began with an initial incubation at 95°C for 7 min, followed by 45 cycles at 95°C for 1.5 min, then at 60°C for 1 min, and at 72°C for 2 min. Genomic DNA from *Acanthamoeba castellanii* was used as a positive control. PCR products were then electrophoresed using 1.2 % agarose gel (Ultrapure) stained with ethidium bromide (10 mg/ml in deionized H_2O) and visualized under UV illumination. A 250–10,000 base pair (bp) ladder, (Gene Ruler TM, Fermentas) was used as a DNA size marker.

Thermo-tolerance assay:

Trophozoites or cysts of *Acanthamoeba* (10^3/ plate) from culturing method were transferred to the center of freshly prepared 1.5% NNA and incubated at various tempera-tures: 30°C (control), 37°C and 42°C for 14 days. The results were recorded based on the growth at the end of the incubation period. Growth of trophozoites or cysts was scored as "positive" and no growth was recorded as "negative" as described by (Duarte et al., 2013).

Results

This study included 150 water samples that were collected from different localities of Minia Governorate, Egypt. Eighty-four (56%) out of the 150 water samples were positive as *Acanthamoeba* species by culturing method. Microscopic examination with iodine stain is used to visualize *Acanthamoeba* cysts and trophozoites are showed in Figure (1). The presence of *Acanthamoeba* species was the highest in streams and ponds (70.73%), followed by Nile River and swimming pools water samples (66.67% and 60% respectively) and the lowest was in tap water samples (37.5%). These data were statistically significant (Table 1).

Out of 84 water samples positive by NNA culture, 72 (85.71%) samples were positive by PCR using specific primers for *Acanthamoeba* species (Table 2). Agarose gel electrophoresis showed representative PCR product of the 18s rRNA gene of *Acanthamoeba* spp. (Figure 2). Based on thermo-tolerance test among 84 positive cultures, 39 (46.43%) were thermo-tolerant (Table 2).

Table (1): Results of NNA culture in different water sources:

	Streams and ponds (N= 82)		Tap water (N= 40)		Tanks (N= 20)		Swimming pools (N= 5)		Nile River (N= 3)		Total (N= 150)		P value
	N	%	N	%	N	%	N	%	N	%	N	%	
NNA culture	58	70.73%	15	37.5%	6	30%	3	60%	2	66.67%	84	56%	0.001

Table (2): Results of PCR and thermo-tolerance assay in positive NNA cultures:

	Streams and ponds (N= 58)		Tap water (N= 15)		Tanks (N= 6)		Swimming pools (N= 3)		Nile River (N= 2)		Total (N= 84)		P value
	N	%	N	%	N	%	N	%	N	%	N	%	
PCR	50	86.21%	13	86.67%	5	83.33%	2	66.67%	2	100%	72	85.71%	0.006
Thermo-assay	24	41.38%	10	66.67%	4	66.67%	1	33.33%	0	0%	39	46.43%	0.1

Figure (1): Light microscopy showing different morphological forms of *Acanthamoeba* cysts (a, b, c, d) and trophozoites (e) by iodine wet mount stain (x 400).

Figure (2): Agarose gel electrophoresis showing representative PCR product of the 18s rRNA gene of *Acanthamoeba* spp. of positive samples (6, 7 and 8).

Discussion

Among opportunistic free living amoebae (FLA), species of the genera *Acanthamoeba* are the most frequently found in both natural and artificial places. They are the causative agents of granulomatous amoebic encephalitis (GAE) and *Acanthamoeba* keratitis (AK), which together comprise the largest number of reported FLA related infections (Schuster and Visvesvara, 2004). This study had demonstrated a wide distribution of FLA, including potentially pathogenic species, pathogenicity determined by thermo-assay.

In this work, the prevalence rate of *Acanthamoeba* spp. was 70.73% in streams and ponds raw water samples. These data matched with the data reported in Fayoum Governorate, Egypt by Al-Herrawy et al., (2015a) with prevalence rate of 91.7% in raw water samples. Besides, Al-Herrawy and others in Behera Governorate, Egypt found the prevalence rate of *Acanthamoeba* spp. in raw water samples was 100% (Al- Herrawy et al., 2015b). Also, in Bulgaria, Tsvetkova et al., (2004) recorded that freshwater *Acanthamoeba* spp. prevalence rate was 61.1%. On the other hand, some researchers recorded different result from our result. Ettinger and others collected samples from James River in Virginia, United States of America with 43.3% prevalence rate of *Acanthamoeba* spp. (Ettinger et al., 2002). This difference might be attributed to the lower atmospheric temperature in those countries. Also, FLA in this interface zone feed primarily on bacteria, although fungi, yeast, algae, and other protozoa may also serve as food sources. High levels of bacteria at the interface lead to an increase in the prevalence of amoebae and are probably one primary factor that stimulates excystment (Sadaka et al., 1994).

In this present study, the prevalence rate of *Acanthamoeba* spp. in tap water was 37.5%. This result was similar to the report obtained by Al-Herrawy et al., (2017), who found that 29.9% from tap water samples in Cairo, Egypt was positive for *Acanthamoeba* spp.. Similarly, a study by Kilvington and others in the United Kingdom reported closer occurrence of *Acanthamoeba* spp. in tap water (26.9%) (Kilvington et al., 2004). On the other hand, in Saudi Arabia and Brazil, researchers reported lower incidence rate of *Acanthamoeba* spp. was 9.5% and 10% respectively (Winck et al., 2011; Vijayakumar, 2018). The higher prevalence rate of *Acanthamoeba* spp. in tap water in this study could be explained by that water facility throughout different cities of Egypt is relatively poor especially in some rural areas. The prevalence rate of *Acanthamoeba* spp. in untreated raw water samples of streams and ponds was higher than that of treated tap water might attributed to the fact that tap water is often treated with chlorine or filtered (Gabriel et al., 2019).

In this study, it was found that 60% of the swimming pool samples were positive for *Acanthamoeba* spp.. This is comparable with other studies from Cairo, Egypt where Al-Herrawy et al., (2014) and (2016) reported that

the prevalence rates were 49.2% and 54.2% respectively. Additionally, in Poland Lass and other researchers detected close result with prevalence rate of *Acanthamoeba* spp. in swimming pools water samples was 59.7% (Lass et al., 2014). In contrast, other researchers in Thailand and Brazil detected lower occurrence of *Acanthamoeba* spp. (13% and 27.8% respectively) from swimming pools water samples (Lekkla et al., 2005; Fabres et al., 2016). The higher prevalence rate of *Acanthamoeba* spp. in swimming pools water samples in this work might be as a result of soil contamination introduced into water by humans. In addition, there is an improper maintenance and disinfection protocol in swimming pools. Moreover, *Acanthamoeba* resistance to chlorination may be considered as another reason for variation of prevalence (Vijayakumar, 2018).

In this study, it was found that 60% of the Nile River water samples were positive for *Acanthamoeba s*pp.. These data were supported by the data obtained by researches in Egypt. They recorded that the prevalence rate of *Acanthamoeba* spp. in Nile River was 40% from Nile delta region (Lorenzo-Morals et al., 2006). In Spain, Lorenzo-Morales et al., (2005) reported uncorrelated lower prevalence of *Acanthamoeba* spp. was 43.3% from sea water samples in Tenerife, Canary Islands, Spain. Also in Saudi Arabia researchers recorded a lower incidence of *Acanthamoeba* spp.. was 36.7 % in freshwater samples (Nacapunchai et al., 2001).

On the other hand, in USA, Ettinger et al., (2002) recorded that the prevalence rate of *Acanthamoeba* spp. from James River was 7% which was lower than that recorded in our result. The discrepancy in *Acanthamoebae* prevalence rate in different localities and countries might be due to geographic location, method of amoebae recovery, seasonal water temperatures or water treatment methods (Stockman et al., 2011).

Identification of some *Acanthamoeba* spp. can be accomplished by the morphological characteristics. In fact, more than 24 species of *Acanthamoeba* spp. have been identified based on morphological criteria (Caumo et al., 2009). Members of the genus *Acanthamoeba* spp. are

divided into three morphological groups according to the cyst size and other morphological features. Al Herrawy et al., (2014) concluded that the culture method was cheaper and easier than PCR techniques that were faster for the detection of FLA.

PCR has been found to be a more sensitive diagnostic test than culture (Pasricha et al., 2003). Xuan et al., (2017) found a poor relationship between *Acanthamoeba* spp. identification by cyst morphology and molecular studies. In addition, *Acanthamoeba* spp. morphology may change according to culturing conditions and different species in the same group can have similar morphology, rendering it difficult to be differentiated.

This work showed that 72 of 84 (85.71%) of morphologically identified *Acanthamoeba* spp. in all water samples, proved to be related to genus *Acanthamoeba* when they were tested by PCR technique. These data were close to other researchers in Egypt, who found that 94.9% of microscopically *Acanthamoeba* spp. positive swimming pool samples were also positive by using PCR technique (Al-Herrawy et al., 2014). In addition, in another work in Egypt, it was noticed that all water samples proved to be microscopically positive for *Acanthamoeba* spp. were also confirmed by PCR to be related to genus *Acanthamoeba* (AI-Herrawy et al., 2015a; El Wahab et al., 2018). Moreover, in Spain, researchers found that all positive samples with culture method were positive by PCR (Reyes-Batlle et al., 2014). However, in Malaysia, Gabriel, et al., (2019) showed that 64% of isolated FLA by culturing method were positive as *Acanthamoeba* spp. in PCR technique.

Tolerance to temperatures is one of the simplest assays to predict the pathogenic potential of *Acanthamoeba*, since the clinical isolates tend to be thermophilic (Walochnik et al. 2000; Khan et al. 2001). However, some non-pathogenic species can also tolerate temperatures 37°C (Schuster and Visvesvara 2004), making the criteria of thermo-tolerance by itself inconclusive to determine *Acanthamoeba* pathogenicity.

In this study, out of 150 water samples, 39 (46.43%) samples of *Acanthamoeba* spp. were

thermo-tolerant and show growth on NNA culture at 40° C. These data were supported by Al-Herrawy et al., (2013) who showed that the percentage of *Acanthamoeba* spp. exhibiting thermo-tolerance reached 50% in tap water in Cairo and 58 % in the Delta region. Moreover, in Saudi Arabia, Vijayakumar, (2018) reported that 40 % of *Acanthamoeba* spp. were thermo-tolerance. These data were very low in comparison to result obtained by Tawfeek et al., (2016). They found that 76.5% of *Acanthamoeba* spp. were thermotolerant. Furthermore, in Ankara, Turkey, Kilic et al., (2004) reported that about 66% of studied soil *Acanthamoeba* isolates were thermo-tolerant. Additionally, Booton et al., (2004) deduced that 100% of *Acanthamoeba* spp. isolates in South Florida were thermo-tolerant. The difference in the results could be explained on the basis of different species of *Acanthamoeba* spp. Encountered in each study which may have different physiological properties.

Conclusions

The present study showed a high presence of *Acanthamoeba* spp. in different water source: streams and ponds, tap, tanks, swimming pools and Nile River water samples, representing a sanitary risk in aquatic sources. Also, this work identified a potentially pathogenic species of *Acanthamoeba*. Thus, genotyping analysis of *Acanthamoeba* isolated from environmental samples is needed.

References

1. Al-Herrawy, A., Bahgat, M., Mohammed, A., Ashour, A. and Hikal, W., 2013. Morpho-physiological and biochemical criteria of *Acanthamoeba* spp. isolated from the Egyptian aquatic environment. *Iranian journal of parasitology*,*8*(2), p.302.

2. Al-Herrawy, A.Z., Bahgat, M., Mohammed, A.E., Ashour, A. and Hikal, W., 2014. *Acanthamoeba* species in swimming pools of Cairo, Egypt. *Iranian Journal of Parasitology*, *9*(2), p.194.

3. Al-Herrawy, A.Z., Mohamed, S.H., Mohamed, A.H. and Zaghloul, N.M., 2015a. Surveillance of potentially pathogenic free-living amoebae through drinking water treatment processes in Fayoum Governorate, Egypt. *Intern Environ*, *4*, pp.98-107.

4. Al-Herrawy, A.Z., Heshmat, M.G., Kabsha, S.H.A., Gad, M.A. and Lotfy, W.M., 2015b. Occurrence of *Acanthamoeba* species in the Damanhour drinking water treatment plant, Behera Governorate (Egypt). *Reports in Parasitology*, *4*, p.15.

5. Al-Herrawy, A.Z., Gad, M.A., El-Aziz, A.A., Abou-El Nour, M.F., Shaldoum, F.M. and Salahuldeen, A., 2016. Morphological and Molecular Detection of Potentially Pathogenic Free-Living Amoebae in Swimming Pool Samples.*Egyptian Journal of Environmental Research EJER*, *5*.

6. Al-Herrawy, A.Z., Khalil, M.I., El-Sherif, S.S., Omar, F.A. and Lotfy, W.M., 2017. Surveillance and Molecular Identification of *Acanthamoeba* and *Naegleria* Species in Two Swimming Pools in Alexandria University, Egypt. *Iranian journal of parasitology*, *12*(2), p.196.

7. Balczun, C. and Scheid, P., 2017. Free-living amoebae as hosts for and vectors of intracellular microorganisms with public health significance. *Viruses*, *9*(4), p.65.

8. Boost, M., Cho, P., Lai, S. and Sun, W.M., 2008. Detection of *Acanthamoeba* in tap water and contact lens cases using polymerase chain reaction. *Optometry and Vision Science*, *85*(7), pp.526-530.

9. Booton, G.C., Rogerson, A., Bonilla, T.D., Seal, D.V., Kelly, D.J., BEATTIE, T.K., Tomlinson, A., LARES-VILLA, F.E.R.N.A.N.D.O., Fuerst, P.A. and Byers, T.J., 2004. Molecular and physiological evaluation of subtropical environmental isolates of Acanthamoeba spp., causal agent of *Acanthamoeba* keratitis. *Journal of Eukaryotic Microbiology*, *51*(2), pp.192-200.

10. Caumo, K., Frasson, A.P., Pens, C.J., Panatieri, L.F., Frazzon, A.P.G. and Rott, M.B., 2009. Potentially pathogenic *Acanthamoeba* in swimming pools: a survey in the southern Brazilian city of Porto Alegre. *Annals of Tropical Medicine & Parasitology*, 103(6), pp.477-485.

11. Coşkun, K.A., Özçelik, S., Tutar, L., Elaldı, N. and Tutar, Y., 2013. Isolation and identification of free-living amoebae from tap water in Sivas, Turkey. *BioMed research international*, *2013*.

12. Duarte, J.L., Furst, C., Klisiowicz, D.R., Klassen, G. and Costa, A.O., 2013.

Morphological, genotypic, and physiological characterization of *Acanthamoeba* isolates from keratitis patients and the domestic environment in Vitoria, Espírito Santo, Brazil. *Experimental parasitology*, *135*(1), pp.9-14.

13. El Wahab, W.M.A., El-Badry, A.A. and Hamdy, D.A., 2018. Molecular characterization and phylogenetic analysis of *Acanthamoeba* isolates in tap water of Beni-Suef, Egypt. *Acta parasitologica*, *63*(4), pp.826-834.

14. Ettinger, M.R., Webb, S.R., Harris, S.A., McIninch, S.P., Garman, G.C. and Brown, B.L., 2002. Distribution of free-living amoebae in James River, Virginia, USA. *Parasitology research*, *89*(1), pp.6-15.

15. Fabres, L.F., dos Santos, S.P.R., Benitez, L.B. and Rott, M.B., 2016. Isolation and identification of *Acanthamoeba* spp. from thermal swimming pools and spas in Southern Brazil. *Acta parasitologica*, *61*(2), pp.221-227.

16. Gabriel, S., Khan, N.A. and Siddiqui, R., 2019. Occurrence of free-living amoebae (*Acanthamoeba*, *Balamuthia*, *Naegleria*) in water samples in Peninsular Malaysia. *Journal of water and health*, *17*(1), pp.160-171.

17. Gatti, S., Rama, P., Matuska, S., Berrilli, F., Cavallero, A., Carletti, S., Bruno, A., Maserati, R. and Di Cave, D., 2010. Isolation and genotyping of *Acanthamoeba* strains from corneal infections in Italy. *Journal of medical microbiology*, *59*(11), pp.1324-1330.

18. Khan, N.A., Jarroll, E.L. and Paget, T.A., 2001. *Acanthamoeba* can be differentiated by the polymerase chain reaction and simple plating assays. *Current Microbiology*, *43*(3), pp.204-208.

19. Kilic, A., Tanyuksel, M., Sissons, J., Jayasekera, S. and Khan, N.A., 2004. Isolation of *Acanthamoeba* isolates belonging to T2, T3, T4 and T7 genotypes from environmental samples in Ankara, Turkey. *Acta Parasitologica*, *49*(3), pp. 246-252.

20. Kilvington, S., Gray, T., Dart, J., Morlet, N., Beeching, J.R., Frazer, D.G. and Matheson, M., 2004. *Acanthamoeba* keratitis: the role of domestic tap water contamination in the United King-dom. *Investigative ophthalmology & visual science*, *45*(1), pp.165-169.

21. Lass, A., Szostakowska, B., Idzińska, A. and Chomicz, L., 2014. The first genotype determination of *Acanthamoeba* potential threat to human health, isolated from natural water reservoirs in Poland. *Parasitology research*, *113*(7), pp.2693-2699.

22. Lekkla, A., Sutthikornchai, C., Bovornkitti, S. and Sukthana, Y., 2005. Free-living ameba contamination in natural hot springs in Thailand. *Southeast Asian journal of tropical medicine and public health*, *36*, p.5.

23. Lorenzo-Morales, J., Ortega-Rivas, A., Foronda, P., Martínez, E. and Valladares, B., 2005. Isolation and identification of pathogenic *Acanthamoeba* strains in Tenerife, Canary Islands, Spain from water sources. *Parasitology research*, *95*(4), pp. 273-277.

24. Lorenzo-Morales, J., Ortega-Rivas, A., Martínez, E., Khoubbane, M., Artigas, P., Periago, M.V., Foronda, P., Abreu-Acosta, N., Valladares, B. and Mas-Coma, S., 2006. *Acanthamoeba* isolates belonging to T1, T2, T3, T4 and T7 genotypes from environmental freshwater samples in the Nile Delta region, Egypt. *Acta tropica*, *100*(1-2), pp.63-69.

25. Maďarová, L., Trnková, K., Feiková, S., Klement, C. and Obernauerová, M., 2010. A real-time PCR diagnostic method for detection of *Naegleria fowleri*. *Experimental parasitology*, *126*(1), pp.37-41.

26. Magliano, A.C., da Silva, F.M., Teixeira, M.M. and Alfieri, S.C., 2009. Genotyping, physiological features and proteolytic activities of a potentially pathogenic *Acanthamoeba* spp. isolated from tap water in Brazil. *Experimental Parasitology*, *123*(3), pp.231-235.

27. Nacapunchai, D., Kino, H., Ruangsittichai, C., Sriwichai, P., Ishih, A. and Terada, M., 2001. A brief survey of free-living amebae in Thailand and Hamamatsu District, Japan. *Southeast Asian journal of tropical medicine and public health*, *32*, pp.179-182.

28. Pasricha, G., Sharma, S., Garg, P. and Aggarwal, R.K., 2003. Use of 18S rRNA gene-based PCR assay for diagnosis of *Acanthamoeba* keratitis in non-contact lens

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wearers in India. *Journal of clinical microbiology*, *41*(7), pp.3206-3211.

29. Reyes-Batlle, M., Todd, C.D., Martín-Navarro, C.M., López-Arencibia, A., Cabello-Vilchez, A.M., González, A.C., Córdoba-Lanús, E., Lindo, J.F., Valladares, B., Piñero, J.E. and Lorenzo-Morales, J., 2014. Isolation and characterization of *Acanthamoeba* strains from soil samples in Gran Canaria, Canary Islands, Spain. *Parasitology research*, *113*(4), pp. 1383-1388.

30. Sadaka, H.A., El-Nassery, S.F. and Awadalla, H.N., 1994. Isolation and identification of free-living amoebae from some water sources in Alexandria. *Journal of the Egyptian Society of Parasitology*, *24*(2), pp.247-257.

31. Schroeder, J.M., Booton, G.C., Hay, J., Niszl, I.A., Seal, D.V., Markus, M.B., Fuerst, P.A. and Byers, T.J., 2001. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoebae* from humans with keratitis and from sewage sludge. *Journal of Clinical Microbiology*, *39*(5), pp.1903-1911.

32. Schuster, F.L., 2002. Cultivation of pathogenic and opportunistic free-living amebae. *Clinical Microbiology Reviews*, *15*(3), pp.342-354.

33. Schuster, F.L. and Visvesvara, G.S., 2004. Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. *International journal for parasitology*, *34*(9), pp.1001-1027.

34. Stockman, L.J., Wright, C.J., Visvesvara, G.S., Fields, B.S. and Beach, M.J., 2011. Prevalence of *Acanthamoeba* spp. and other free-living amoebae in household water, Ohio, USA—1990–1992. Parasitology research, 108(3), pp.621-627.

35. Tawfeek, G.M., Bishara, S.A.H., Sarhan, R.M., Taher, E.E. and Khayyal, A.E.,

2016. Genotypic, physiological, and biochemical characterization of potentially pathogenic *Acanthamoeba* isolated from the environment in Cairo, Egypt. *Parasitology research*, *115*(5), pp.1871-1881.

36. Tsvetkova, N., Schild, M., Panaiotov, S., Kurdova-Mintcheva, R., Gottstein, B., Walochnik, J., Aspöck, H., Lucas, M.S. and Müller, N., 2004. The identification of free-living environmental isolates of amoebae from Bulgaria. *Parasitology research*, *92*(5), pp.405-413.

37. Vijayakumar, R., 2018. Isolation, identification of pathogenic *Acanthamoeba* from drinking and recreational water sources in Saudi Arabia. *Journal of Advanced Veterinary and Animal Research*, *5*(4), pp.439-444.

38. Visvesvara, G.S., Moura, H. and Schuster, F.L., 2007. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. *FEMS Immunology & Medical Microbiology*, *50*(1), pp.1-26.

39. Walochnik, J., Obwaller, A. and Aspöck, H., 2000. Correlations between morphological, molecular biological, and physiological characteristics in clinical and nonclinical isolates of *Acanthamoeba* spp. *Appl. Environ. Microbiol.*, *66*(10), pp. 4408-4413.

40. Winck, M.A.T., Caumo, K. and Rott, M.B., 2011. Prevalence of *Acanthamoeba* from tap water in Rio Grande do Sul, Brazil. *Current microbiology*, *63*(5), p.464.

41. Xuan, Y., Shen, Y., Ge, Y., Yan, G. and Zheng, S., 2017. Isolation and identification of *Acanthamoeba* strains from soil and tap water in Yanji, China. *Environmental health and preventive medicine*, *22*(1), p.58.