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Isolation and identification of free-living amoeba from contact lenses: Thermal and osmotic tolerance in relation to their pathogenicity.

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ABSTRACT

Free-living amoeba (FLA) such as *Acanthamoeba* spp., *Naegleria fowleri*, *Balamuthia mandrillaris*, and *Sappinia diploidea* are widely distributed natural and human-made environments which may cause human diseases. For example, *N. fowleri* causes fatal encephalitis, *Acanthamoeba* and *Balamuthia* cause chronic granulomatous encephalitis and *Acanthamoeba* also can cause cutaneous lesions and Amoebic Keratitis (AK) that is associated with the use of contact lens. In the present work, FLA were isolated from new and used lenses and lenses preservative solutions and identified by morphological characteristics then their tolerance to high temperature and osmosis were studied. The results showed that *Acanthamoeba* spp. is the main amoeba isolated from lenses and lens solution, and *A. polyphaga* had higher pathogenicity than the rest of the genera due to its ability to grow at all temperatures and osmosis tested. While both *N. fowleri* did not grow at any of the tested osmotic levels but grew at the tested temperatures so they were considered as non-pathogenic.

INTRODUCTION

Free-living amoebae (FLA) are universal and opportunistic protozoa, they showed variation in distribution among natural environments such as soil dust, air, seawater, drinking water, swimming pools, sewage, eyewash solutions, contact lenses, dialysis units, and dental treatment units. Among these FLA, only four genera such as *Acanthamoeba*, *Naegleria*, *Balamuthia*, and *Sappinia* are responsible for opportunistic and non- opportunistic infections in humans and other animals. *Acanthamoeba* sp. causes amoebic keratitis in immunocompetent persons and granulomatous amoebic encephalitis

(GAE) and cutaneous lesions in immunocompromised patients. *Balamuthia mandrillaris*, causes skin, lung infections, and a fatal GAE, mostly in immunocompetent children. *Naegleria fowleri* (*N. fowleri*) causes primarily amoebic meningoencephalitis (PAM) in healthy children and young adults. *Sappinia* sp. has been reported, only once, from a brain infection in a healthy man [1, 2].

Acanthamoeba spp. feed on organic molecules and reproduce by binary fission under optimal conditions, and, potentially, on contaminated cleaning solutions of contact lenses. It can also grow in contact lenses that are cleaned with contaminated tap water [3, 4]. The trophozoites of *Acanthamoeba* species are active, more sensitive, and can change into a cyst by altering its phenotype in strict environmental conditions [5]. The cyst form is highly resistant and has a double wall of cellulose and other polysaccharides with a fibrous laminar exocyst and a granular smooth endocyst that make the wall tougher for protection [6, 7]. The ability *of Acanthamoeba* to tolerate a wide range of temperatures, osmosis, pH, and other factors indirectly contributes to its pathogenicity [8].

N. fowleri has been isolated from various aquatic habitats and thermally polluted streams and rivers. Also, it has been isolated from the nasal mucosa of healthy asymptomatic children and nasal passages of patients with PAM [9]. *N. fowleri* amoebae can exist in three morphological stages, a trophozoite, a flagellate, or a cyst [10]. *N. fowleri* is thermophilic and able to grow at different degrees of temperature and can proliferate during warmer months of the year when the temperature is likely to be high [11].

Sappinia diploidea (*S. diploidea*) normally lives in soils contaminated with faeces of elk, bison, and cattle, and was identified as causing encephalitis in a healthy young man [12]. *S. diploidea* has two stages during its life cycle, a monopodial locomotory trophozoite (50 – 60 μm by 20–30 μm), with a large hyaloplasm in the anterior part of the cell, and a smaller bi-nucleated cyst, 18 – 25 μm in diameter [13,14].

Depending on the foregoing literature we planned to isolate the free-living amoeba from contact lenses and preservative solution under normal conditions and study the ability of isolated and morphologically identified genus to grow under abnormal temperature and osmosis.

MATERIALS AND METHODS

Collection and cultivation of samples.

From April to August 2019, one hundred samples of lenses and contact lenses solutions, new cosmetic lenses (NL, n= 50), used lenses (UL, n=30), and opened contact lenses solutions (LS, n= 20), were collected from cosmetic shops in Assiut Governorate, Egypt and contact lenses users. Specimens were applied directly to the centers of the

surface of a non-nutrient agar (NNA) plate seeded with live *Escherichia coli*, then incubated at 30° C under standard atmospheric conditions and were observed daily for 7 days for trophozoites and 14 days for cysts [15, 16].

Direct unstained and temporary staining by iodine preparations:

Two drops of clean sediment were dropped on a glass slide, covered with a coverslip, examined, and photographed for permanent record. The wet mount staining was prepared by spreading out two drops (50 µl) of amoeba suspension on a glass slide, a drop of 0.2 % iodine was added, and finally put a coverslip to enclose the material. Stained slides were examined under a light microscope and photographed for the permanent record [17, 18].

Pathogenicity tests *in vitro*:

The physiological behavior of the isolates under different temperatures and osmolarity conditions were evaluated with the bacteria co-cultures in non-nutrient agar. The positive growth media were washed with sterile phosphate buffered saline (pH 7.2) then gently scraped the surface of the agar and the content was centrifuged for the collection of *Acanthamoeba* trophozoites or cysts. Pathogenicity is expressed as test scores, and high pathogenicity is considered if the results obtained in both thermal and osmotic assays are positive. A positive result in either test was considered as low pathogenic and negative results in both tests were considered non-pathogenic [19, 20].

A. Thermotolerance assays:

Trophozoites of *Acanthamoeba* (10^3/plate) were transferred to the center of freshly prepared 1.5% NNA plates seeded with *E. coli*, then incubated at various temperatures 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. The plates were observed under a microscope (100× magnification) and the number of trophozoites or cysts were counted in the middle region of the cultured plates. Results of pathogenicity were scored based on the number of counts, zero count (–, non-pathogenic), 1–15 (+), 16–30 (++), and >30 (+++).

B. Osmotolerance assays

Trophozoites of *Acanthamoeba* (10^3/plate) were transferred to the center of freshly prepared 1.5% NNA containing 0.5 M, 1 M concentrations of mannitol. *E. coli* suspension was over- layered to each plate and incubated at 30°C for a maximum of 2 weeks. On days 7–9, the plates were observed under a microscope (100× magnification) and the number of trophozoites or cysts were counted in the middle region of the cultured plates. Results of pathogenicity were scored based on the number of counts, zero count (–, non-pathogenic), 1–15 (+), 16–30 (++), and >30 (+++).

RESULTS

Isolated and detected FLA.

In the present study, the whole positive result percentage was 33% and that of isolated FLA in NL, UL, and LS was 18 (36%), 4 (13.3%), and 11 (55 %), respectively as shown in **figure (1A & B).**

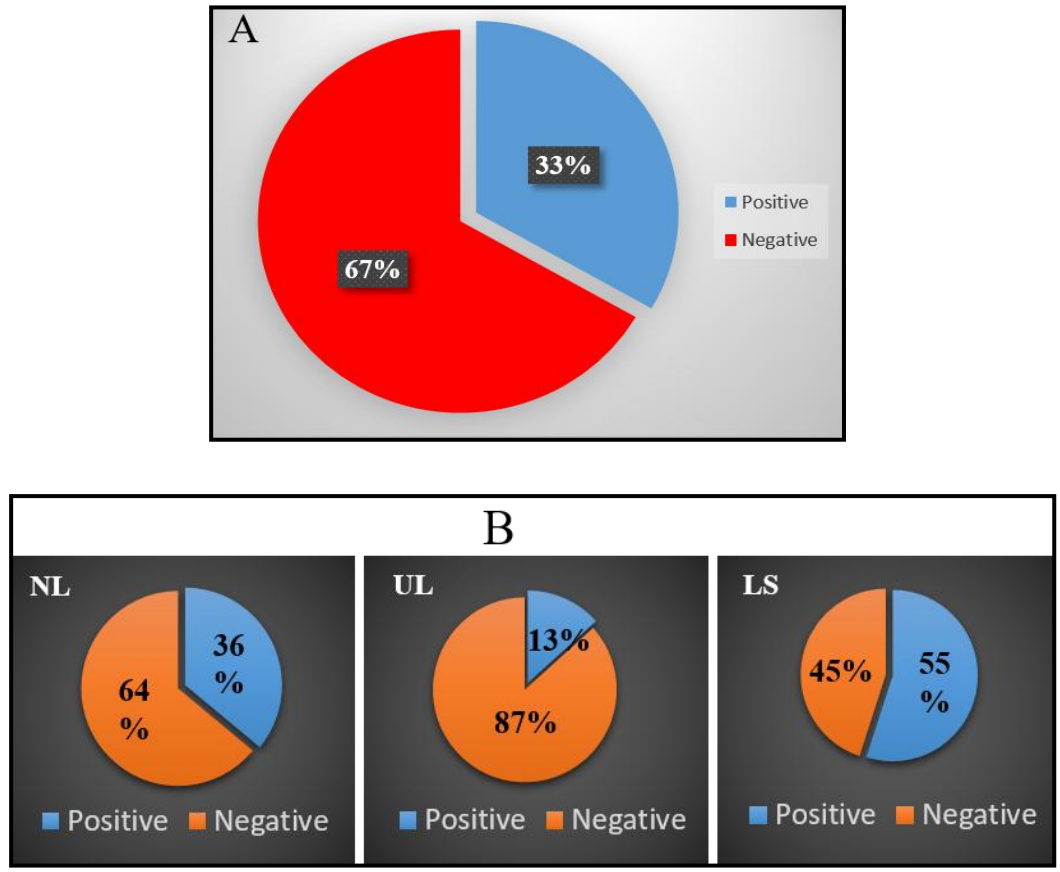

Figure (1): Percentage of FLA isolated from lenses and preservative solutions.

Morphological characterization of the isolated FLA:

A- Morphological characterization of *Acanthamoeba* sp.

Trophozoites of *Acanthamoeba* were easy to be identified under the light microscope by the presence of needle-like as the minute projections of the false feet on their surface known as acanthopodia. The average size of *Acanthamoeb*a trophozoites was in the range of 12- 45 microns and it had one nucleus. The endoplasm contained many food vacuoles and prominent contractile vacuoles that controls the water content of the cell **(Figure. 2)**.

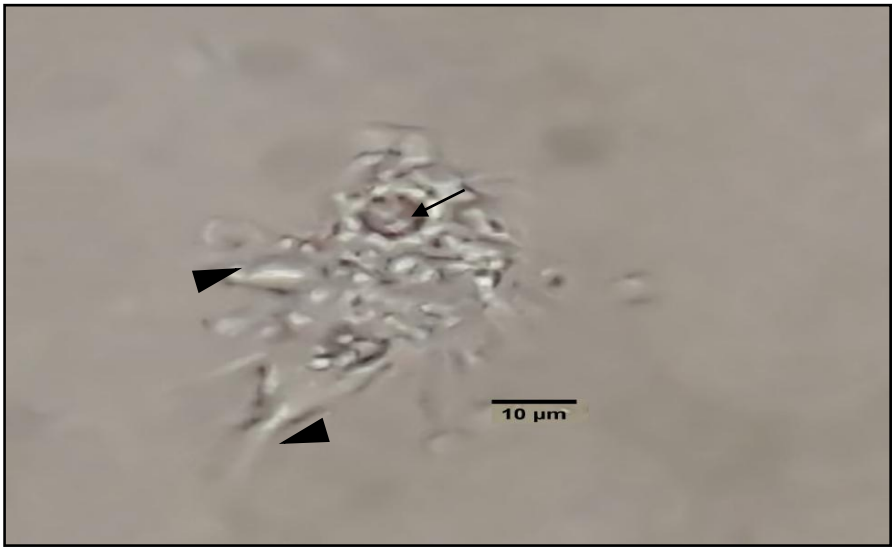

Figure (2): Photomicrograph of wet-mount *Acanthamoeba* trophozoites showing acanthopodia (arrowhead) and vacuole (arrow) (×400).

Whereas, the isolated cysts of *Acanthamoeba* showed doubled-walled, circular oval or polyhedral inner wall (endocyst) and wrinkled or smoothly fibrous outer wall (exocyst). The outer cyst is separated well from the inner cyst. The size of *Acanthamoeba* cysts ranged from 7 - 25 microns in diameter. Cysts contain only one nucleus with a large karyosome. A wall pore with an operculum was observed **(Figure 3 A- F).**

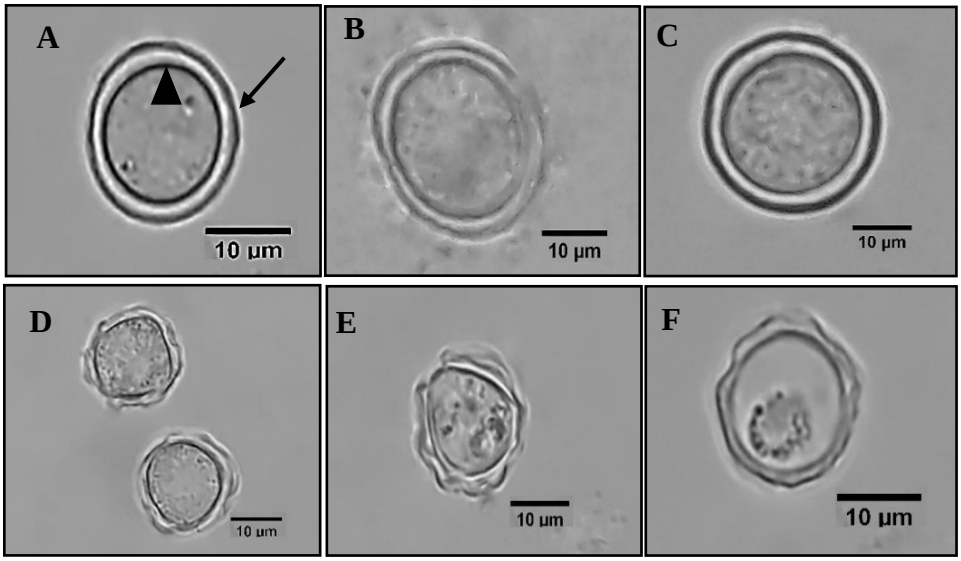

Figure (3): Photomicrograph of direct wet mount *Acanthamoeba* spp. cysts showing smooth ectocyst in A, B, C and shrinking ectocyst in D, E, F (arrowhead) and endocyst (arrow) (×1000).

Some stages of excystation and asexual division of *Acanthamoeba* were seen as in **figure (4A-J).**

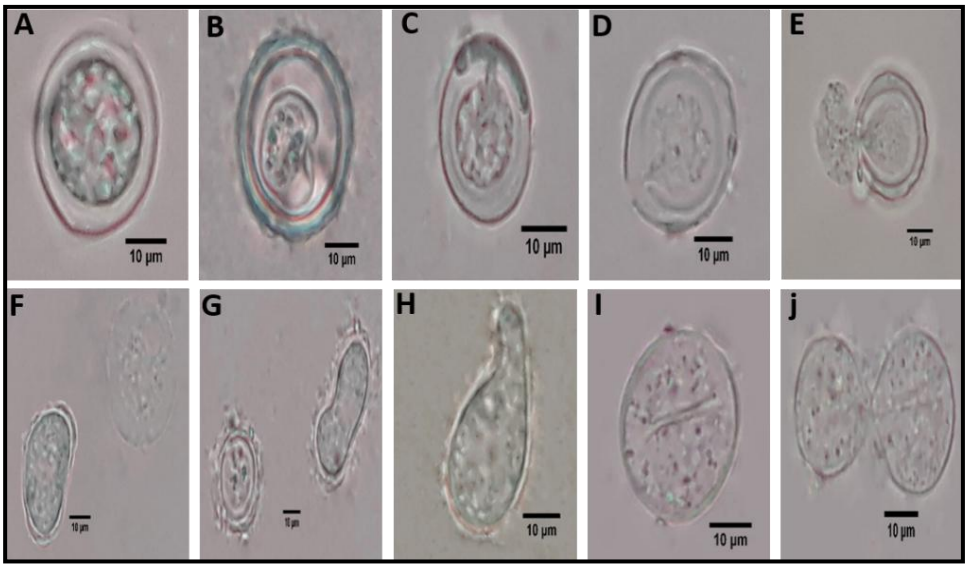

Figure (4): Photomicrograph showing some stages of *Acanthamoeba* spp. some stages showing growing cyst to trophozoite (excystation) (A-H) and asexual division (I-J) during the life cycle (×1000).

Wet preparation staining with Lugol's iodine stain showed that the cyst and trophozoites stages appeared yellowish-brown and had good differentiation as shown in **figure (5A& B).**

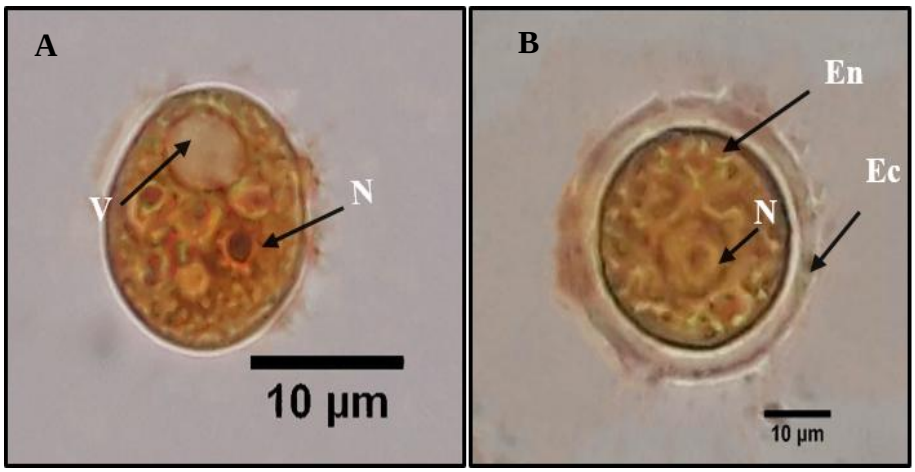

Figure (5). Photomicrograph showing *Acanthamoeba* in iodine wet mount stain. (A) Trophozoite appeared yellowish-brown with the dark nucleus; (B) Cysts appeared yellowish–brown with well- defined ectocyst (Ec), endocyst (En), and central nucleus (N) (×1000).

The cysts size and angle number of *A. castellanii* and *A. polyphaga*.

Depending on the morphological characteristics of the cyst out of the 29 samples, 2 (6.9 %) culture plates were *A. castellanii*, 15 (51.7%) were *A. polyphaga*, and 12 samples (41.4%) were other *Acanthamoeba* species. The size of *Acanthamoeba* sp. cysts were different from one species to another as shown in (**Table. 1, Table. 2,** and **figure 6 A and B)** showed the number of *A. castellanii* and *A. polyphaga* cysts angles.

Table (1). Size of *A. castellanii* and *A. polyphaga* cysts.

***Acanthamoeba* sp.**	***A. castellanii***	***A. polyphaga***
Size average	10 - 22µm	7- 30µm
Mean	16	19
SD	6	12

Table (2): Number of cysts angles

***Acanthamoeba* sp.**	***A. castellanii***	***A. polyphaga***
Number of angles	3- 4	2-5
Mean	3.5	3.5
SD	0.5	1.5

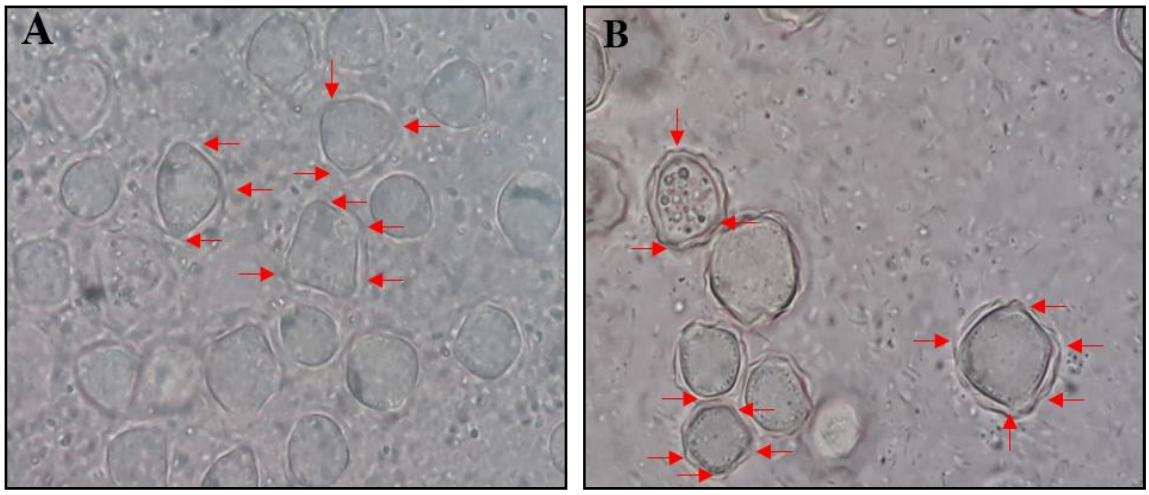

Figure (6): Photomicrograph of (A) *A. castellanii* and (B) *A. polyphaga* cysts showing the numbers of angles (×1000)**.**

B. Morphological characterization of *Naegleria* sp.

Naegleria sp. appeared in three stages (the trophozoite stage, the flagellate stage, and the cyst stage). The trophozoite stage appeared irregular in shape, measuring 15- 25 µm in diameter, it had a single nucleus with a prominent nucleolus. The endoplasm contained numerous food vacuoles and it moves by lobopodia. The flagellate stage was pear-shaped and had two flagella at the pointed end and had actively moved. Finally, the cyst stage was spherical with a double- walled poorly differentiated outer wall; it had a single inconspicuous nucleus and measured 8-12 µm in diameter. **Figure (7A, B & C).**

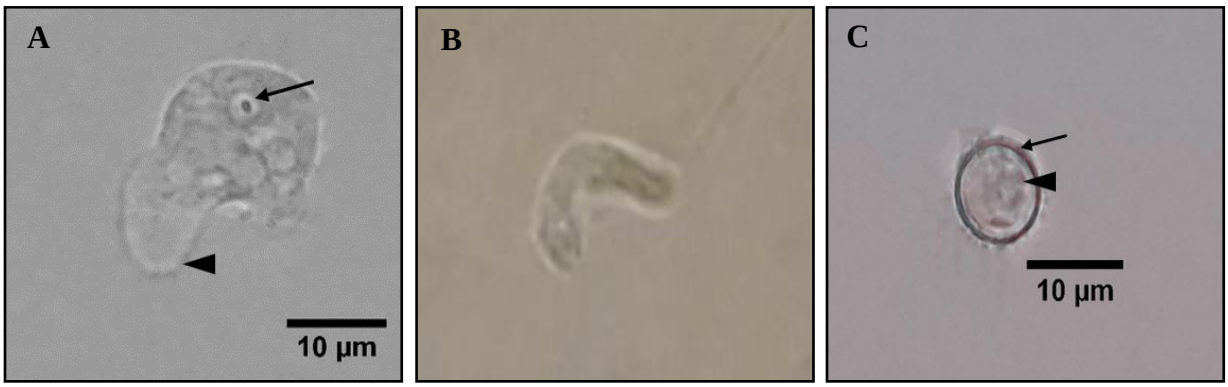

Figure (7): Light micrographs of *Naegleria* sp. showing (A) A prominent nucleus (arrow) and lobopodia (arrowhead) of *Naegleria* trophozoite; (B) Showing flagellate stage of *Naegleria* sp; (C) Cyst stage of *Naegleria sp* showing cyst wall (arrow) and a single nucleus (arrowhead) (×1000).

Lugoĺs iodine stain Trophozoites and cysts of *Naegleria* sp appeared brown to yellowish-brown as shown in **figure (8A& B).**

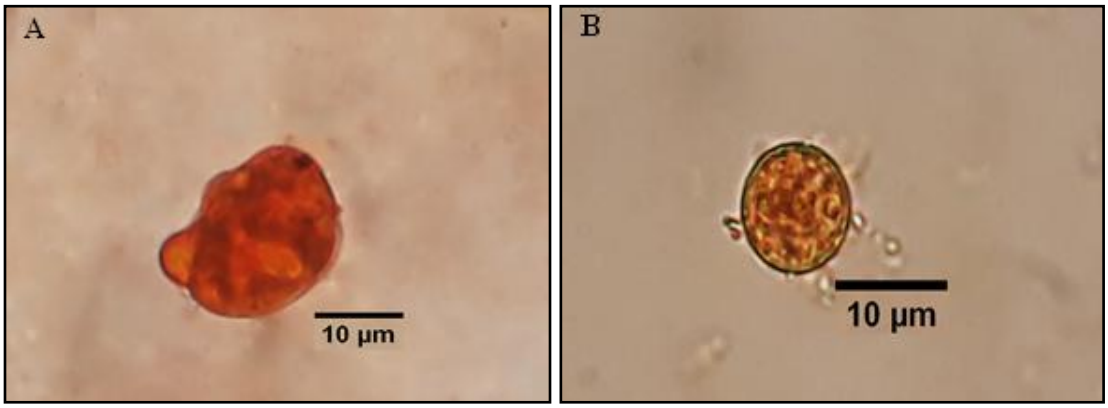

Figure (8): Light micrographs of *Naegleria* sp. showing trophozoite (A), and cyst (B) stained with iodine stain (×1000).

C: Morphological characterization of *Sappinia diploidea*

Sappinia diploidea isolated from the solution. It had two phases, trophozoite, and cyst as **in figure (9 A-D)**.

a. The trophozoite stage: had an irregular shape and moved by lobopodia. The size ranged from 20- 50µm.

b. The cyst stage: had a large size of 47-50 µm, had two divided nuclei, which sere sometimes appeared united.

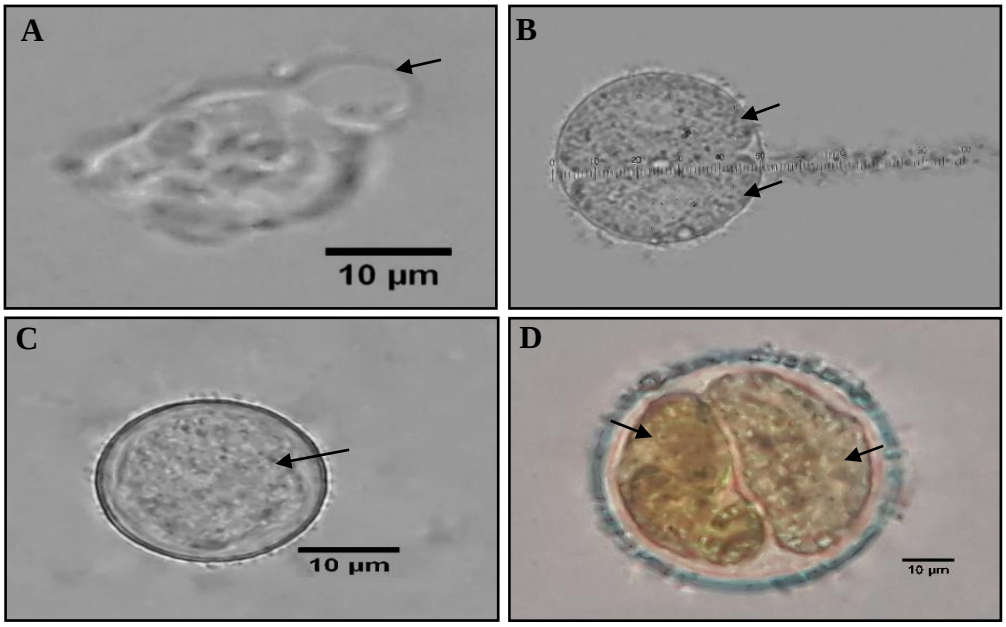

Figure (9). Light micrographs of *Sappinia diploidea* showing (A) Trophozoite stage with a hyaloplasm monopodial in the anterior part of the cell (arrow); (B) Binucleated cyst stage (arrow); (C) The two nuclei are united together (arrow); (D) A prominent binucleated cyst stained with iodine stain (arrow) (×1000).

Thermotolerance and osmotolerance assays for isolated FLA.

Table (3) showed thermotolerance assays and osmotolerance assays growth score (0 = none, + =1-15, + + = 16-30, + + + = >30). All species had a highly growing score of more than 30 at 30 ^{o}C, whereas other *Acanthamoeba* spp. showed moderate growing score (16- 30) at 37 ^{o}C, *Naegleria* sp. showed a growing score >30 at 37 ^{o}C. At 42 ^{o}C the growing score of *A. polyphaga* and *Naegleria* sp. was +. Osmotolerance assays showed *A. castellanii*, *A. polyphaga* at 0.5 M were more than 30. *A. castellanii* showed a growing score was + + at 1M, and *A. polyphaga* growing score was less (+). *S. diploidea* growth well at three temperature degrees, but osmotolerance assays growing score of *Naegleria* sp. and *Sappinia diploidea* was zero at 0.5 M and 1 M.

Table (3) Thermotolerance and osmotolerance assays.

Isolated species	No. of samples	Thermotolerance			Osmotolerance		Pathogenicity
		30 °C	37 °C	42 °C	0.5 M	1 M	
A. castellanii	2 (7 %)	+++	++	0	+++	++	Weak pathogenic
A. polyphaga	15 (52%)	+++	++	+	+++	+	Strong pathogenic
Other *Acanthamoeba* spp.	12 (41%)	+++	++	0	0	0	Non- pathogenic
***Naegleria* sp.**	3	+++	+++	+	0	0	Non- pathogenic
S. diploidea	1	++	++	+	0	0	Non- pathogenic

DISCUSSION

In the present study, FLA isolated from contact lenses and contact lenses solutions were matched with previous studies [21, 22] which reported that FLA is the widely distributed protozoa in the environment. Interestingly, the isolated FLA especially *Acanthamoeba* sp. in NL higher than that in UL, which may indicate poor storage of the lenses in stores, exposure to dust, dryness, poor manufacturing, and poor quality of some types. While the low insulation ratio in UL may indicate the health awareness of lens users in dealing with lenses in terms of using lens preservation solutions and avoiding contamination of lenses from hands or dust [23]. Furthermore, lenses and their solutions may be contaminated with *Acanthamoeba* from water and dust [24, 25]. In addition, we isolated FLA belonging to *Naegleria* sp. from three samples**.** Similarly**,** [26] mentioned that *Naegleria* sp. is commonly detected in the human environment worldwide.

Observations of the present study demonstrated that the trophozoite of *Acanthamoeba* spp. was developed from the cyst on day 4 and appeared as described by [27] and [8] who reported that *Acanthamoeba* trophozoites have irregular, oval, round or pear-shaped acanthopodia that arise from the clear hyaline ectoplasm laterally and anteriorly, and different kinds of vacuoles. In addition, cysts of *Acanthamoeba* spp. appeared as described by [28] who found that ectocyst appears wrinkled and separated from the endocyst that was thin and smooth. Also, the ectocyst is wrinkled and the endocyst was polygonal and had a finely granular appearance [27].

Identification of *Acanthamoba* sp. showed that the cyst and the trophozoite morphology resembled the various species of group II and group III based on size, morphology, and number of opercula [29]. Cysts of group II were smaller than 18 μm in size and exhibited thick, wrinkled ectocysts and satellite, oval or polygonal endocysts. The cyst of group III diameter is < 19 um with globular or ovoid endocysts [30, 31& 32]. Species *A. castellanii* and *A. polyphaga* were classified under group II, and isolated from humans who have AK and GAE diseases [31].

The observed data of *Naegleria* sp**.** was matched with the observation of [33] and [34] who reported that the trophozoites of *Naegleria* sp. were ~10-20 μm in size and contain a single nucleus with a large karyosome; possessed lobopodia, and can transform to a flagellated form. However, cysts were single-walled, spherical, and 8 to 12 μm in diameter. Both trophozoite and cyst stages of *S. diploidea* are binucleate and can be cultivated on non-nutrient agar plate coated with bacteria [12].

In the present study trophozoite and cyst of *Acanthamoeba* sp. and *Naegleria* sp. were stained well with iodine. This agrees with the results of [17] and [35] who reported that Lugol's iodine stains the internal structures of trophozoites and cysts both in *Acanthamoeba* and *Naegleria* yellow to brown, exhibiting clear nuclei. The acanthopodia or lobopodia of trophozoites were stained brown to yellow-brownish and making the organisms clearly differentiated.

The distribution of amoeba species has faith in their ability to survive in front of a wide range of environmental factors, with the cysts being able to tolerate changes in salinity, desiccation, temperature, and osmotic pressure, as well as exposure to chemicals and prolonged starvation [36]**.** The ability of *Naegleria* and *Acanthamoeba* to grow at high temperatures appears to be indirect factors related to virulence, with non-virulent strains unable to grow at normal or elevated body temperatures [37]. So, the pathogenic potential of the isolated amoebae, thermotolerance, and osmotolerance was evaluated. As regards *A. castellanii* and other *Acanthamoeba* spp. growth at 30 ^{o}C, 37 ^{o}C only whereas, *A. polyphaga* growth at 30 ^{o}C, 37 ^{o}C and 42 ^{o}C. These results were in agreement with [15] who reported that the optimal cultivation temperatures of these FLA at 23 to 37 ^{o}C. Furthermore, [38] found that the growth of *A. castellanii* was good close to the surface temperature of the human cornea, while the higher body core temperature-induced encystment of the amoebae in both clinical and environmental strains of *A. castellanii*. Also, the ability of the amoeba to resist normal body temperature or even fever occurrences in the host is related to thermotolerance [39].

The corneal temperature is about 32-35°C. However, the corneal surface temperature changes with the ambient temperature, airflow, and humidity. In general, the surface temperature is measured to be 1– 4 °C, lower than the core body temperature and

the temperature of the corneal center is 0.5–1.0 °C, lower than at the limbus area; the human brain temperature is 37°C and strains that cannot grow at these temperatures most probably cannot cause disease. Therefore, colonization by the amoeba could be possible even when the organism is not able to grow at temperatures above 37°C. Moreover, the growth of amoebae at temperatures above 40°C is directly correlated to their capacity to produce cellular damage *in vitro.* However, different species of *Acanthamoeba* may be thermotolerant but non-pathogenic [40, 41]. Three *Acanthamoeba* isolated had low virulence which seems to be related to low water temperatures [42]. [43] concluded that T4 strains isolated from clinical and non-clinical isolates, prefer to grow at temperatures lower than 37°C. On the other hand, [20] reported that isolated *Acanthamoeba* strains were thermotolerant and grown at 42°C. The mechanisms by which pathogenic *Acanthamoeba* adapt to higher temperatures and maintain their metabolic activities remain entirely unknown [44]. The ability of *Acanthamoeba* cyst to tolerate the temperature due to the presence of high cellulose concentration in the inner wall of the cyst stages [45, 35].

This study also, showed that *A. castellanii* and *A. polyphaga* grow at 0.5 M and 1 M. Growth at high mannitol concentrations has been associated with the ability to resist high osmotic pressures, a situation that the amoebae could face when they act as parasites of the corneal epithelium [38].

The appropriate temperature for best growth for *Naegleria* spp. flagella stage is 35–46°C [34]. However, in the present study *Naegleria* spp. has the potential to grow at 30 °C, 37 °C, and 42°C and could not grow at 0.5 or 1.0 M mannitol. Similarly, *N. fowleri* has been detected in environmental water samples from 16°C to 47°C [46]. In addition, several amoeba species are also capable of growing at 40°C or higher, such as *N. fowleri* [47]. *Acanthamoeba* spp. are capable of surviving under conditions that are lethal for *Naegleria* spp. Although non-pathogenic *N. gruberi* appears to survive under normal temperatures far better than pathogenic *Naegleria* spp [48].

CONCLUSION

In conclusion, the current study showed that *Acanthamoeba* spp. were the most prevalent amoebae isolated from contact lenses and lenses solutions. *A. polyphaga* was the only amoebae that could survive in high osmosis and temperature that seem directly associated with virulence.

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REFERENCES

[1] H.Trabelsi, F. Dendana, A. Sellami, H. Sellami, F. Cheikhrouhou, S. Neji & A. Ayadi, Pathogenic free-living amoebae: epidemiology and clinical review. *Pathologie Biologie*, 60 (6), (2012) 399- 405.

[2] P. Scheid, Free-living amoebae as human parasites and hosts for pathogenic microorganisms. *In Multidisciplinary Digital Publishing Institute Proceedings*, Vol. 2, (2018) No. 11, p. 692).

[3] W. C. Lin, C. Y. Tsai, J. M. Huang, S. R. Wu, L. J. Chu & K. Y. Huang, Quantitative proteomic analysis and functional characterization of Acanthamoeba castellanii exosome-like vesicles. *Parasites & vectors*, 12 (1) (2019) 1-12.

[4] I. K. Susanto, S. Wahdini & I. P. Sari, Potential Transmission of *Acanthamoeba* spp. from Contact Lens Solution and Tap Water in Jakarta, Indonesia. *Open Access Macedonian Journal of Medical Sciences*, 8 (A) (2020) 333-337.

[5] A. Aghajani, M. Dabirzadeh, Y. Maroufi & H. Hooshyar, Identification of Acanthamoeba genotypes in pools and stagnant water in ponds in Sistan region in Southeast Iran. *Turkiye Parazitol Derg*, 40 (3) (2016) 132-136.

[6] F. Abjani, N. A. Khan, S. Y. Jung & R. Siddiqui, Status of the effectiveness of contact lens disinfectants in Malaysia against keratitis-causing pathogens. *Experimental parasitology*, 183 (2017)187-193.

[7] K. Moon, S. H. Lee & Y. H. Kim, Evaluation of reference genes for quantitative real-time PCR to investigate seasonal and labor-specific expression profiles of the honey bee abdomen. *Journal of Asia-Pacific Entomology*, 21(4) (2018) 1350-1358.

[8] A. G. de Lacerda & M. Lira, Acanthamoeba keratitis: A review of biology, pathophysiology and epidemiology. *Ophthalmic and Physiological Optics*, 41(1) (2021)116-135.

[9] R. A. Baquero, M. Reyes-Batlle, G. G. Nicola, C. M. Martin-Navarro, A. Lopez-Arencibia, J. Guillermo Esteban, J. Lorenzo-Morales, Presence of potentially pathogenic free-living amoebae strains from well water samples in Guinea-Bissau. *Pathogens and global health*, 108 (4) (2014) 206 -211.

[10] C. Lam, L. He & F. Marciano- Cabral, The effect of different environmental conditions on the viability of *Naegleria fowleri* amoebae. *Journal of Eukaryotic Microbiology*, 66(5) (2019) 752-756.

[11] A. Panda, S. Khalil, B. R. Mirdha, Y. Singh & S. Kaushik, Prevalence of *Naegleria fowleri* in environmental samples from northern part of India. *PloS one*, 10 (10) (2015) e0137736.

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

[13] J. Walochnik, C. Wylezich & R. Michel, The genus *Sappinia*: history, phylogeny and medical relevance. *Experimental parasitology*, 126 (1) (2010) 4-13.

[14] T. Tyml & I. Dyková, *Sappinia* sp.(Amoebozoa: Thecamoebida) and Rosculus sp.(SAR: Cercozoa) isolated from King Penguin guano collected in the Subantarctic (South Georgia, Salisbury Plain) and their coexistence in culture. *Journal of Eukaryotic Microbiology*, 65(4) (2018) 544-555.

[15] R. L.Penland & K. R. Wilhelmus, Comparison of axenic and monoxenic media for isolation of Acanthamoeba. *Journal of clinical microbiology*, 35(4) (1997) 915-922.

[16] F. Eroğlu, G. Evyapan & İ. S. Koltaş, The cultivation of Acanthamoeba using with different axenic and monoxenic media. Middle Black Sea Journal of Health Science, 1(3) (2015) 13-17.

[17] N. M. El-Sayed, & W. M. Hikal, Several staining techniques to enhance the visibility of *Acanthamoeba* cysts. *Parasitology research*, 114(3) (2015) 823-830.

[18] J. Lorenzo-Morales, N. A. Khan & J. Walochnik, An update on Acanthamoeba keratitis: diagnosis, pathogenesis and treatment. *Parasite*, 22 (2015).

[19] M. A. Abdul Majid, T. Mahboob, B. G. Mong, N. Jaturas, R. L. Richard, T. Tian-Chye, J. Chuah, Pathogenic waterborne free-living amoebae: An update from selected Southeast Asian countries. *PloS one*, 12 (2) (2017) e0169448.

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

[21] N. Lanocha, D. Kosik-Bogacka, A. Maciejewska, M. Sawczuk, A. Wilk & W.Kuzna-Grygiel, The occurrence Acanthamoeba (free living amoeba) in environmental and respiratory samples in Poland. *Acta Protozoologica*, 48(3) (2009) 271.

[22] B. Armand, M. H. Motazedian & Q. Asgari, Isolation and identification of pathogenic free-living amoeba from surface and tap water of Shiraz City using morphological and molecular methods. *Parasitology research*, 115(1) (2016) 63-68.

[23] N. Khoza, T. Moodley, S. Sokhulu, N. O. Sotyana, A. Suliman, R. Hansraj & D.van Staden, Knowledge, attitudes and practices of contact lens use in a South African adolescent population. *African Health Sciences*, 20 (2) (2020) 768-774.

[24] M. K. A. Ghani, S. A. Majid, N. S. Abdullah, A. Nordin, Y. Suboh, N. A. Rahim & N. Ahmad, Isolation of *Acanthamoeba* spp. from contact lens paraphernalia. *The Internal Medicine Journal*, 20 (2013) 66-68.

[25] S. M. Lee, J. E. Lee, D. I. Lee & H. S. Yu, Adhesion of *Acanthamoeba* on cosmetic contact lenses. *Journal of Korean medical science*, 33(2018) (4).

[26] A. Edagawa, A. Kimura, T. Kawabuchi-Kurata, Y. Kusuhara & P. Karanis, Isolation and genotyping of potentially pathogenic *Acanthamoeba* and *Naegleria* species from tap-water sources in Osaka, Japan. *Parasitology research*, 105(4) (2009)1109-1117.

[27] A. M. Muslim & F. J. Azhar, Experimental Keratitis by Acanthamoeba polyphaga. *International Journal of Sciences*, 6(08) (2017) 62-66.

[28] T. Sampaotong, J. Roongruangchai & K. Roongruangchai, Viability and morphological changes of *Acanthamoeba* spp. cysts after treatment with effective microorganisms (EM). *Journal of Parasitic Diseases*, 40 (2) (2016) 369-373.

[29] J. K. Dart, V. P. Saw & S. Kilvington, Acanthamoeba keratitis: diagnosis and treatment update 2009. *American journal of ophthalmology*, 148(4) (2009) 487-499.

[30] Duarte, C. Furst, D. R. Klisiowicz, G. Klassen & A. O. Costa, Morphological, genotypic, and physiological characterization of Acanthamoeba isolates from keratitis patients and the domestic environment in Vitoria, Espírito Santo, Brazil. *Experimental parasitology*, 135(1) (2013) 9-14.

[31] Y. Qvarnstrom, T. A. Nerad & G. S. Visvesvara, Characterization of a new pathogenic *Acanthamoeba* species, *A. byersi n.* sp., isolated from a human with fatal amoebic encephalitis. *Journal of Eukaryotic Microbiology*, 60(6) (2013) 626-633.

[32] Y. Xuan, Y. Shen, Y. Ge, G.Yan & S. Zheng, Isolation and identification of *Acanthamoeba* strains from soil and tap water in Yanji, China. *Environmental health and preventive medicine*, 22 (1) (2017) 1-6.

[33] G. S. Visvesvara, J. F. De Jonckheere, R. Sriram & Daft, Isolation and molecular typing of Naegleria fowleri from the brain of a cow that died of primary amebic meningoencephalitis. *Journal of clinical microbiology*, 43(8) (2005) 4203-4204.

[34] M. Jahangeer, Z. Mahmood, N. Munir, U. E. A. Waraich, I. M. Tahir, M. Akram & R. Zainab, *Naegleria fowleri*: Sources of infection, pathophysiology, diagnosis, and management; a review. *Clinical and Experimental Pharmacology and Physiology*, 47(2) (2020) 199-212.

[35] h. e. Eldeek, , r. a. Attia, m. m.Nageeb & a. a. Sakla, Comparative evaluation of multiple staining techniques for identification of different developmental stages of Acanthamoeba and Naegleria. *journal of the egyptian society of parasitology*, 49(2) (2019) 409-422.

[36] S. Cervero- Aragó, S. Rodríguez- Martínez, O. Canals, H.Salvadó & R. M. Araujo, Effect of thermal treatment on free- living amoeba inactivation. *Journal of applied microbiology*, 116 (3) (2014) 728-736.

[37] N. A. Khan, Pathogenicity, morphology, and differentiation of *Acanthamoeba*. *Current Microbiology*, 43(6) (2001) 391-395.

[38] M. K. Nielsen, K. Nielsen, J. Hjortdal & U. B. S. Sørensen, Temperature limitation may explain the containment of the trophozoites in the cornea during *Acanthamoeba castellanii* keratitis. *Parasitology research*, 113(12) (2014) 4349-4353.
[39] E. Castro-Artavia, L. Retana-Moreira, J. Lorenzo-Morales & E. Abrahams-Sandí, Potentially pathogenic *Acanthamoeba* genotype T4 isolated from dental units and emergency combination showers. *Memórias do Instituto Oswaldo Cruz*, 112 (12) (2017) 817-821.
[40] C. Purslow & J. S. Wolffsohn, Ocular surface temperature: a review. *Eye & contact lens*, 31(3) (2005) 117-123.
[41] W. Pumidonming, M. Koehsler & J. Walochnik, *Acanthamoeba* strains show reduced temperature tolerance after long-term axenic culture. *Parasitology research*, 106 (3) (2010) 553-559.
[42] P. Bonilla-Lemus, A. S. C. Villegas, J. C. Jiménez & A. L. Vázquez, Occurrence of free-living amoebae in streams of the Mexico Basin. *Experimental parasitology*, 145(2014) S28-S33.
[43] G. C. Booton, A. Rogerson, T. D. Bonilla, D. V. Seal, D. J. Kelly, T. K. Beattie & T. J. Byers, Molecular and physiological evaluation of subtropical environmental isolates of *Acanthamoeba* spp., causal agent of Acanthamoeba keratitis. *Journal of Eukaryotic Microbiology*, 51(2) (2004) 192-200.
[44] N. A. Khan, *Acanthamoeba*: biology and increasing importance in human health. *FEMS microbiology reviews*, 30 (4) (2006) 564-595.
[45] N. A.Turner, A. D.Russell, J. R. Furr & D. Lloyd, Emergence of resistance to biocides during differentiation of *Acanthamoeba castellanii*. *Journal of Antimicrobial Chemotherapy*, 46(1) (2000) 27-34.
[46] L. M. Stahl & J. B. Olson, Environmental abiotic and biotic factors affecting the distribution and abundance of *Naegleria fowleri*. *FEMS Microbiology Ecology*, 97(1) (2021) fiaa238.
[47] J. F. De Jonckheere, Isolation and molecular identification of free-living amoebae of the genus *Naegleria* from Arctic and sub-Antarctic regions. *European Journal of Protistology*, 42(2) (2006) 115-123.
[48] C. J. Biddick, L. H. Rogers, & T. J. Brown, Viability of pathogenic and nonpathogenic free-living amoebae in long-term storage at a range of temperatures. *Applied and Environmental Microbiology*, 48(4) (1984) 859-860.