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Research Article

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# *Isolation and identification of free living amoebae from water sources with respect to Acanthamoeba, Naegleria in Jeddah city, Saudi Arabia*

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**ABSTRACT**

*Free-living amoeba (FLA) are ubiquitous protozoa that have been isolated from a wide range of environments particularly water and soil. Only Acanthamoeba, Naegleria, Balamuthia and Sappinia genera are responsible for opportunistic and non-opportunistic infections in humans and other animals. Also, FLA serve as reservoirs for several bacteria, virus and protozoa. The present study has been conducted to characterize the distribution of both Acanthamoeba and Naegleria species in water sources and swimming pools in Jeddah city, in addition to determination of the potential risk of human health through bacterial acquisition by these amoebae.*
*A total of 64 water samples; houses (32), mosques (16) and swimming pools (16) were collected. Water samples were prepared and sterile cotton swaps were cultured on plate of non-nutrient agar supplemented with E. coli for up to 14 days, in addition, negative controls were carried out, each inoculated with sterile distilled water. And respectively, the total positive samples were 32.8%; houses were 31.25%, mosques were 31.25% and swimming pool achieved 37.5%. Acanthamoeba accounted for 80.95% from the total positives while Naegleria was 19.05%. On examining some positive samples of FLA with electron microscope there were many specimens which showed intracellular micro-organisms which are needed to be further identified by polymerase chain reaction or through specific probes.*
**Keywords**: *Isolation and identification of free living amoebae Acanthamoeba, Naegleria*

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## INTRODUCTION

Free-living amoeba (FLA) are ubiquitous protozoa that have been isolated from a wide range of environments, such as water sources, soil, dust, air conditioning vents, sewage, contact lenses, and dialysis units, but they are particularly abundant in water and soil [1,2,3,4].

Out of the many FLA that are found in nature, only four genera, namely Acanthamoeba, Naegleria, Balamuthia and Sappinia are responsible for opportunistic and non-opportunistic infections in humans and other animals [5,2,6].

The genus Acanthamoeba has been currently classified into 20 genotypes [7,8,9]. Only a few species of Acanthamoeba are human pathogens, with the potential to cause granulomatous amoebic encephalitis (GAE) in immunosuppressed patients [10,11]. Acanthamoeba keratitis has been investigated in several countries. Human infection with Acanthamoeba, causing keratitis, occurs through contamination when contact lenses are rinsed in nonsterile water or when lenses are worn while bathing or swimming in a no disinfected aquatic environment

[1,12,11]. In addition, skin lesions and systemic infections of Acanthamoeba were reported among patients with immunodeficiency as well as healthy individuals [13]. Amoeba-associated bacteria as agent of ventilator-associated pneumonia were isolated from intensive care units [14,15].

More than 40 species of the genus Naegleria (N.) have been classified by molecular techniques, among them, only N. fowleri infections is the most virulent FLA [16,17].

N. fowleri is the causative agent of primary amoebic meningo-encephalitis (PAM) [2,18]. The infection begins when contaminated water passes through the nose, usually during swimming of diving, where amoebae reach the brain along the olfactory nerve fibers, and through the perforated plate. PAM is almost always fatal and the victims die within 3-7 days after the onset of symptoms and was most frequently reported in healthy young persons with a recent history of aquatic activities [19].

In addition to their pathogenicity, FLA serve as reservoirs for several bacteria such as Pseudomons aeruginosa, Legionella pneumophila, Helicobacter pylori, Mycobacterium avium, Chlamydia and Vibrio cholera, Klebsiella spp. and Aspergillus spp. [20,2,21,22,23] and for viruses such as Mimivirus [14], enterovirus [24], and Adenoviruses [25].

Furthermore, it has been shown that the growth of bacteria in FLA increases bacterial virulence, consequently representing an important reservoir of human pathogens [26].

The aim of this work is (I) to characterize the distribution and genera richness of free-living amoebae especially Naegleria and Acanthamoeba through the morphological identification (II) to determine bacterial endosymbiosis in the recovered Acanthamoeba isolates as a potential risk to human health in Jeddah city, Saudi Arabia.

## MATERIAL AND METHODS

### Sampling sites

Tap water samples (as all water tanks were underground) taken from houses (H) (6), mosques (M) (6) and 6 swimming pools (SP) from the following districts: Alnahdhah, Alnaem, Alsalamah and Alrawdahah of the region of Jeddah city, western margin Saudi Arabia.

### Sampling collection

From each site, 2000 ml of tap water of each house and mosques, while 1000 ml from the area within the middle of each swimming pool (SP) wall and (6) samples were collected by scrapping with sterile cotton wool of the SP margin.

### Preparation of samples for cultivation and isolation

Cotton wool scrapped samples were directly streaked in the middle of the (NNA-E. coli) plates and labeled accordingly. For the water samples, they were processed prior to cultivation. The bottle containing the water sample was shaken vigorously to mix the contents, then filtered through a 1.2 um pore size cellulose nitrate membrane (Millipore) by mild suction. Filtration was stopped when 3-5 ml of the water sample was left above the membrane and the trapped debris was carefully flushed in situ with 6.0 mL of sterile distilled water followed by spreading 1.0 mL evenly onto each of 6 NNA-E coli plates. The culture plates were sealed with parafilm and were incubated at room temperature (28±2°C), 37 and 45˚C respectively, for up to 14 days. A group of 2 plates from each of the sampling and sub-sampling sites were then incubated at room temperature (28±2°C), 37°C and 45°C respectively. Three sets of 2 types of negative controls were carried out, each inoculated with sterile distilled water and sterile cotton wool respectively, onto NNA-E. coil plates and incubated as for the test samples [27].

### Detection of FLA

All the cultured plates were examined daily for up to 14 days by inverted and light microscopes before being discarded. The presence of FLA could be seen by the clear tracks on the E. coli lawn, produced by the feeding

trophozoites of Acanthamoeba and Naegleria, which were readily apparent after 48-72 hours (h) of incubation. The specific morphological appearances of the trophozoites, cysts and flagellates were identified accordingly based on the reports by several workers [28], the images of the selected organisms were photographed using light microscope (Olympus BX51) which was attached to a photo adapter and a computer installed with imaging software.

**Enflagellation test for Naegleria species**

Culture plates that were seen to contain Naegleriatrophozoites were added with 3ml of PAS solution or sterile distilled water and placed onto a shaker at 50rpm observations for the highly motile flagellates were carried out every 30 minutes for up to 6h with light microscope followed by photography.

**Sub-cultivation and isolation of FLA**

Subculture was carried out for all the positive plates with growth of Acanthamoeba or Naegleria or both.

**Ultrastructure study**

The ultrastructure and the intracellular niche of the bacterial symbionts within their amoeba host cells were further investigated by transmission electron microscopy (TEM). For this analysis one representative of each was selected. Amoebae were harvested from axenic cultures and directly fixed with 2% glutaraldehyde in lml paged amoebic saline (PAS) for 1h at room temperature, followed by fixation with 2% osmium tetroxide for 1h at room temperature and dehydration in an ascending series of acetone. Subsequently, samples were embedded in spur resin (Sigma-Aldrich) with polymerization at 60 c for 8 to 12 h . Ultrathin sections were stained with 1% uranyl acetate for 4 min and 0.3% lead citrate for 2 min and examined with a Zeiss CEM 902 transmission electron microscope.

## RESULTS

**Isolation of FLA**

All culture plates were examined daily up to 14 days using a light microscope before being discarded, detection was done depending on the specific morphological appearance of trophozoite, cysts of Acanthamoeba and trophozoite, flagellates and cysts of Naegleria were identified based on the reports by several documents (Figure 1).

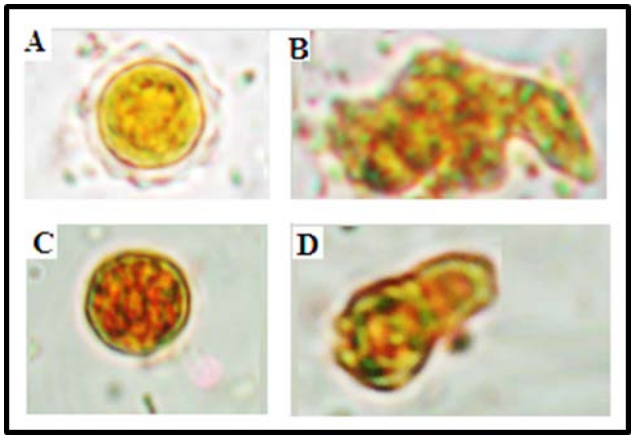


Figure (1): Cultured free living amoebae from different sources and stained with iodine. A) Acanthameoba cyst; B) Acanthameobatrophozoite; C) Naegleria cyst and D) Naegleriatrophozoites.

**Growth capability of both Acanthameoba and Naegleria species**

All these isolates showed growth rates 91.8%, 87.2% at room temperature 28±2C˚ and 37C˚ and 6.4, 0% at 45C˚ respectively.

Acanthamoeba and Naegleria occurrence (Table 1 and 2)

Table (1): Prevalence of Acanthamoeba and Naegleria species at the selected sites

| Sample site | No. of samples | No. of +Ve sites | % of +Ve |
|---|---|---|---|
| Houses (H) | 32 | 10 | 31.25% |
| Mosques (M) | 16 | 5 | 31.25% |
| Swimming pool (SP) | 16 | 6* | 37.5% |
| Total examined | 64 | 21 | 32.8% |

* +Ve SP, samples were mainly of those cotton wool scrapped (86.4%).

Table (2). Occurrence of Acanthamoeba/ Naegleria genera in the selected studied samples.

| Protozoan/ site | H | | M | | SP | | total | % |
|---|---|---|---|---|---|---|---|---|
| | No | % | No | % | No | % | | |
| Acanthamoebaspp | 9 | 52.5 | 4 | 23.5 | 4 | 66.6 | 17 | 80.95 |
| Naegleriaspp | 1 | 25 | 1 | 1.25 | 2 | 33.3 | 4 | 19.05 |
| Total | 10 | | 5 | | 6 | | 21 | 100 |

**Transmission Electron Microscopy (TEM)**

Through ultrastructural study, micro-organisms were observed inside both trophozoites and cysts (Figure 2).

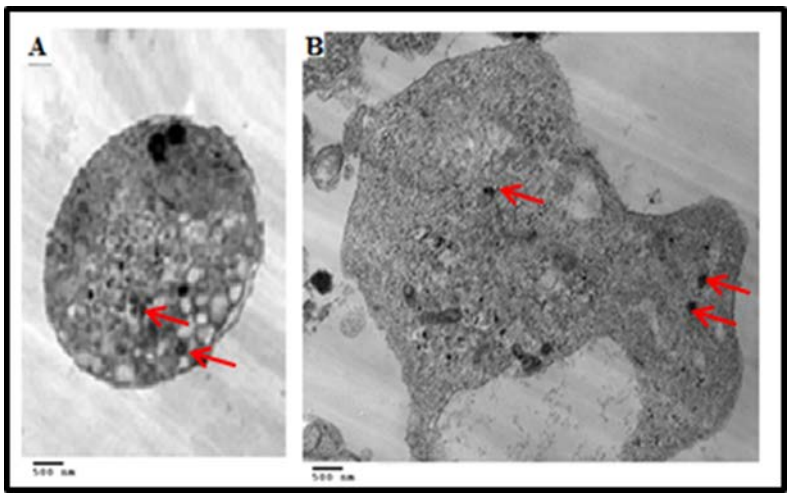


Figure (2) Transmission electron microscopy. A) Acanthameoba cyst; B) Acanthameobatrophozoite. Red arrows point to bacteria which are endosymbiosis.

## DISCUSSION

Free living amoebae are ubiquitous protozoa including many phylogenetically diverse genera as Acanthamoeba and Naegleria. Several species within these genera are recognized as potential human pathogens which are responsible for opportunistic and non-opportunistic infections in humans and other animals [5,6,11] .

They can infect the central nervous system resulting in granulomatous amoebic encephalitis (GAE), primary amoebic meningoencephalitis (PAM). PAM due to Naegleria [19] could be found in healthy people, while GAE is usually found in immunosuppressive patients [27]. In addition, FLA can also act as vectors and reservoirs for several viruses [14,25], and for pathogenic bacteria [20,21].

FLA have been isolated from freshwater lakes, thermally polluted waters, sediment, hot springs, swimming pools, soil, dust, air conditioning vents, sewage, the hospital water supply, contact lenses, and dialysis units [1,3,4,29].

The present study has been conducted to characterize the distribution of both Acanthamoeba and Naegleria species in water sources and swimming pools in Jeddah city, in addition to determination of the potential risk of human health through bacterial acquisition by these amoebae.

Out of 64 water samples from four different districts of Jeddah city, 21 positive samples for Acanthamoeba, Naegleria or both were detected with 32.8%. The genus Acanthamoeba has reported as the most common FLA in the study (80.95%), while Naegleria (19.05%), this in accordance with [30,31], authors attributed these findings to that Acanthamoeba is often associated with contaminated, bacteria- rich water and that Naegleria are more sensitive to environmental conditions such as dry, PH and extremes of temperature.

Dealing with the occurrence of both Acanthamoeba and Naegleria in tap water and swimming pools in Saudi Arabia, to our knowledge, few studies have been conducted [32,33,34].

The current study recorded the prevalence of Acanthamoebaspp, among the positive cases were 52.5, 23.5 and 23.5% in tap water of houses, mosques and swimming pools water respectively. These findings are higher than that recorded by [33] and lower than those recorded by [34] in Saudi Arabia and in accordance with [35] from households of UK and [12] in Spain. These results demonstrate that domestic tap water is an important source of potentially pathogen protozoan runs in direct contact with human activities.

The difference in the prevalence of Acanthamoeba contamination in tap water in different countries might be due to the difference in the tap water hygiene in each country. FLA were more frequently isolated in community dwelling house types than in independent ones, community dwelling type houses and most independent ones in this survey area have water storage tanks, which are not often tightly covered. Therefore, environmental organisms can easily contaminate them [36].

These two genera showed better growth capability 91.8 and 87.2% at 28± 2 and 37 C˚ respectively, while it was 6.4, 0% at 45C˚. For the growth capability test, both of the FLA were found to be suitable and best grown at room temperature (28± 2 C˚), followed by 37°C incubation. The growth rate was slower at 37C˚ which might be due to the overgrowth of E. coli, which sometimes made the detection of FLA difficult. At 45 C˚ incubation, none of the Naegleria was able to grow. All of the Naegleria detected were from the non-pathogenic species due to its inability to survive at high tens temperature (45C˚). On the contrary, (64%) of the culture plates showed positive growth of Acanthamoeba at 45 C˚. [27,37] recorded that these genera grow better at higher water temperature specially those pathogenic species.

Out of the 16 sampled SP, Acanthamoeba were detected in 4 and Naegleria in 2 of them with 37.5% of all, Acanthemoeba was detected in higher number of positive culture plates. This could be explained by the fact that the thick double-walled cyst of Acanthamoeba resists chlorine disinfection at levels up to 50 parts per million (ppm) and this concentration is far in excess of those used in swimming pools (<40 ppm). Only 5 chlorine concentration higher than 1.5 mg/ml affectively destroys spore from of free-living amebae in swimming pools, but are being introduced over and over again with soil by people [24].

In Saudi Arabia, Acanthamoeba spp. was detected in 50% and 78% of swimming pools [33,34], in Egypt [38] recorded 49.2% and in Malaysia both Acanthamoeba and Naegleria were detected in the walls of the SP (76.2%). The current study recorded that the positive samples were mainly of those sterile cotton swaps scrapped walls of SP, this could be explained by the fact that the surface of the wall was always covered with algae which might enhance the growth of other organisms such as bacteria, other microbes, organisms, etc, thus providing sufficient requirement

for the FLA to attach and obtain food. The presence of the detritus, filamentous cyanobacteria and eubacteria may also provide food sources for the FLA [39].

Herein, many micro-organisms were detected by electron microscope in many isolated FLA. Free living amoebae have been reported to feed mainly on bacteria, fungi, and algae by phagocytosis. With digestion occurring within phagolysosomes some bacteria have evolved to become resistant to protists. They are thus able to survive, grow, and exit free living amoebae after internalization. Among the amoeba resistant bacteria, some are obligate intracellular bacteria, and others are considered endosymbionts, because a stable host-parasite ratio is maintained [40].

The study of parasites and symbionts of FLA is interesting. [24] summarized the role of FLA in transmission of amoeba-resistant micro-organisms through acting as a vehicle known as "Trojan horses" [41] or may vesicles filled with these micro-organisms [24].

The found intracellular bacteria and organisms are needed to be further identified by PCR chain reaction or specific probes. In the future, the study of free-living amoebae and their intracellular microbes will contribute to the growing field of study of emerging pathogens and will shed some light on the virulence mechanisms of environmental micro-organisms.

## CONCLUSION

This study indicated that tap water is an important source of FLA and further studies are recommended to investigate the mode of spread of this micro-organisms and factors affecting their pathogenesis, in addition to proper identification of the micro-organisms that FLA harbor.

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