Detection of Free-Living Amoeba in a tertiary care hospital

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Summary

This study aims to investigate the presence of Free-Living Amoebae (FLA), particularly *Acanthamoeba spp*., in diverse water systems and body fluids within a hospital. Water and body fluid samples were collected from various locations. Cultivation of FLA was performed using Non-Nutrient Agar (NNA) culture with *Escherichia coli*, and microscopic observations after staining. Microscopic observations revealed the presence of *Acanthamoeba spp*. in pre-dialysis tank water and dental flush water samples. The identification of *Acanthamoeba* in specific water sources underscores the importance of continuous monitoring and preventive strategies to mitigate potential health risks.

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Introduction

Amoebae are a group of protists characterized by their unique mode of movement using pseudopods and their feeding mechanism through phagocytosis. These versatile organisms are prevalent in various environments, such as water, soil, and air. The multifaceted nature of amoebae is reflected in their role as essential contributors to ecosystem dynamics as well as their potential hazards to human well-being [28]. Amoebae can be categorized into two main groups based on their lifestyles: Free-Living Amoebae (FLA) and parasitic amoebae.

FLA live independently in various environments, such as soil, water, and decaying vegetation. Unlike parasitic amoeba, they do not require a host organism for survival. The majority of FLA are harmless and play roles in nutrient cycling and decomposition in various ecosystems [27]. However, some species can pose health risks, particularly to individuals with weakened immune systems or through specific modes of transmission.

The distinction between free-living and parasitic amoebae is not always clear-cut, as some amoebae can switch between free-living and parasitic lifestyles depending on environmental conditions. Additionally, certain amoebae may have commensal or mutualistic relationships with other organisms. FLA, notably *Naegleria fowleri*, *Acanthamoeba spp*., and *Balamuthia mandrillaris*, are identified as emerging organisms with considerable pathogenicity, particularly in their capacity to affect the Central Nervous System (CNS) [19]. These amoebas have two morphological stages called the active trophozoite form and the resistant cyst form [8], and they pose a significant health threat, as infections caused by them in the trophozoite form exhibit a strikingly high mortality rate, surpassing 95% [1].

In recent years, FLA have been identified not only in natural environments but also in human-made water systems, including hospital water networks [8,22], swimming pools [4], air-conditioning cooling towers [21], dental treatment units [14] and drinking water systems [25]. The discovery of pathogenic amoebae in these systems is of concern. For example, Acanthamoeba is generally harmless, but several species of Acanthamoeba (*A. culbertsoni, A. polyphaga, A. castellanii, A. hatchetti, A. healyi, A. astronyxis, A. divionensis and A. rhysodes*) have been identified as pathogenic and capable of infecting humans [17] leading to conditions such as keratitis, an eye infection that can result from improper contact lens hygiene, and Granulomatous Amoebic Encephalitis (GAE), especially in individuals with compromised immune systems or those who wear contact lenses. Acanthamoeba keratitis is a severe and vision-threatening infection of the eye, and although it is a significant risk to contact lens wearers, cases have been reported in those who do not wear contact lenses [10]. On the other hand, GAE is a progressive infection of the CNS also caused by *Acanthamoeba*.

This condition is particularly dangerous and often fatal, with a mortality rate exceeding 90%, especially among immunocompromised individuals, including those with HIV/AIDS, organ transplant recipients, diabetes patients, and others undergoing immunosuppressive treatments [6]. The pathogenesis of GAE involves complications such as cerebral edema, hemorrhagic areas, effusions, necrotizing arteritis, and the selective accumulation of *Acanthamoeba* trophozoites and cysts in the brain [27]. Acanthamoeba can enter the CNS through various routes, including the olfactory neuroepithelium pathway and/or the lower respiratory tract, with subsequent dissemination through the bloodstream [15,28]. Skin lesions can also serve as a direct entry point into the bloodstream, bypassing the lower respiratory tract [15,28]. In the case of the hematogenous route, the entry of amoebae into the CNS is likely to occur at specific sites of the blood-brain barrier [28]. Additionally, a study [33] found that under conditions of hypoacidity or altered normal flora, the intestinal tract became susceptible to amoebae invasion, serving as a potential new portal of entry for CNS infections. The understanding of these mechanisms of entry and the subsequent development of therapeutic interventions and preventative strategies are still in the preliminary stage. Hence, pathogenic Acanthamoeba in man-made water systems may be of concern.

Additionally, *Naegleria fowleri*, found in water systems, has gained attention due to its ability to cause a rare but severe non-opportunistic brain infection called Primary Amebic Meningoencephalitis (PAM) [3]. PAM is usually associated with activities such as swimming in warm freshwater, where the amoeba can enter the nasal passages and reach the brain. The presence of *N. fowleri* has been confirmed in various water sources in India, including swimming pools, pond water, and sewage canals [11]. It poses a particularly alarming threat as it can infect human brains. Despite its rarity, the global reporting of PAM has surpassed 400 cases [30], as documented in 2020. The most reported cases have been documented in the USA, while the rest are scattered across the globe. More specifically, the USA has reported nearly 143 cases from 1968 to 2019, with a strikingly high fatality rate [30]. The disease is almost always fatal, with a mere 5% of patients surviving globally [20]. There is relatively limited information about the incidence of infection due to *Naegleria* species in many parts of Asia, with the notable exception of Pakistan. In spite of that, more than 150 confirmed cases of PAM have been reported from Asia alone, and the majority of these cases are concentrated in southern Asia, including countries such as Pakistan, India, and Thailand [23]. According to a paper [11], India alone has reported only 16 cases of PAM, with a survival rate of only 4 patients out of this total. Another analysis [24], includes 25 cases of *N. fowleri* PAM in India and over half of these cases had a history of exposure to freshwater. This finding was also backed by another paper [29], as they reviewed eight reports of PAM in India and found a history of contact with water was present in four of those cases, and additionally, trophozoites were identified in all eight cases of the series. However, it is believed that neurological infections by these free-living amoebas are both misdiagnosed and underreported in India, potentially due to insufficient information about their pathology or the country's very low autopsy rate. In our paper we attempt to investigate the possibility of the presence of free-living amoeba in water systems and suspected patients to further the limited understanding on the pathogenicity of these organisms and their associated diseases by culturing water samples.

The cultivation of FLA involves several methods tailored to mimic their natural habitat and nutritional requirements. One common approach is the use of Non-Nutrient Agar (NNA) culture, a simple medium that supports bacteria without providing additional nutrients for amoebae and encourages their feeding behavior. In a paper [18], the authors advocate for the widespread adoption of NNA cultures with *E. coli* for diagnosing acanthamoeba infections. The use of NNA to isolate FLA from fluid samples has since been observed in several research papers in India [1,2,7]. Additionally, studies [9] have evaluated various cultivation media, such as grass-seed infusion, grass-seed agar, and NNA, with the highest recovery rates of species at 79%, 67%, and 58%, respectively, suggesting that grass-seed infusions are also highly efficient for exploring the diversity and ecology of naked amoebae in freshwater samples.

Monoxenic cultures involve amoebae cultivated with a single bacterial species, often *Escherichia coli*, as seen in a certain work [13] studying FLA in sewage treatment plants in Southern Brazil. Axenic culture, on the other hand, entails growing amoebae in the absence of other microorganisms, often requiring more complex nutrient-rich media. A study [19] investigated potential pathogenic FLA in water samples from various regions in Turkey by using monoxenic culture and PCR to confirm the presence of amoebae and identify species, respectively. They further subjected the positive samples to axenic culture to ensure single-species growth, and their research successfully identified *Acanthamoeba*, *Sappinia*, and *Balamuthia mandrillaris,* establishing the technique as an effective way to diagnose pathogenic FLA in a given sample.

Polymerase Chain Reaction (PCR) techniques are employed for molecular detection of FLA DNA directly from environmental samples, bypassing the need for cultivation. The choice of method depends on the specific goals of the study and the type of amoebae being investigated, with simpler techniques like NNA culture commonly used for routine observation and more sophisticated methods like axenic culture and PCR employed for research and diagnostic purposes.

Materials and Methods

For sample collection, 50 mL of water samples and 5 mL of body fluid samples were collected aseptically from various sites in sterile 100 mL plastic pods. A total of 14 samples were obtained from the following locations: pre-dialysis tank water from two separate tanks, running tap water from three different laboratories, kitchen water, dental flush water, stagnant pond water, one ascitic fluid sample, one pleural fluid, and two CSF samples from different patients, stagnant water from earthen pots and water from air cooling towers. All the pods were labeled from 1-14, and all samples except the body fluids were stored at room temperature. CSF, pleural, and ascitic fluids were stored in 2-8º C. Storage time ranged from 48-50 hours, during which any particulate matter in water was allowed to settle.

NNA plates (1.8-2%) were prepared by adding 1.5 to 2 g of agar and 150 mg of sodium chloride to 100 mL of water. The mixture was plated in a Petri dish and allowed to cool. The Agar plates were lawn cultured with the ATCC strain of *E. coli*, divided into two sections using a sterile blade, and 50 µL of water sample was pipetted onto one side of each plate (care was taken to pipette only the supernatant part of the water samples). Petri plates were covered with their lids and incubated at room temperature, sealed with paraffin wax tapes after 24 hours to avoid contamination, and observed daily for changes or haziness.

When bacterial growth was observed in any plate, a slide-smearing step was performed. A loopful of normal saline was placed on a clean slide using a sterilized inoculation loop. The loop was then sterilized over a burner, allowed to cool, and gently scraped on the agar. Microbes obtained in the scrapings were placed in normal saline and smeared on the slide. After drying, Leishman staining was performed, and the slide was observed under a 40X microscope (Figure 1).

Results

Microscopic observations were conducted on all 14 smears prepared from various fluid samples collected during the study. The first sample revealed the presence of suspected *Acanthamoeba* spp., with an estimated count of 1-5 amoeba per oil immersion microscopic field. In the second sample, fungus contamination was noted, so a smear was obtained, avoiding that area. Acanthamoeba was confirmed in this sample and characterized by pointed projections ranging from 0 to 3 organisms per oil immersion field. The third sample, aimed at detecting amoeba suspected when observed

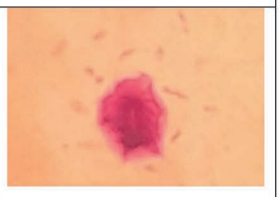
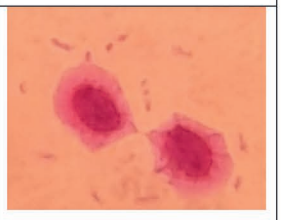
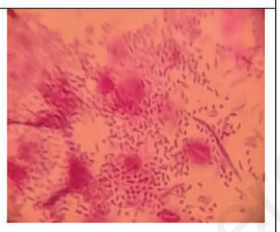
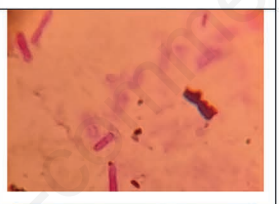
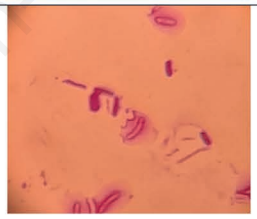
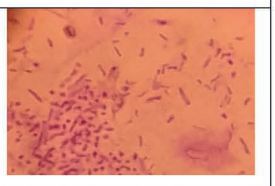
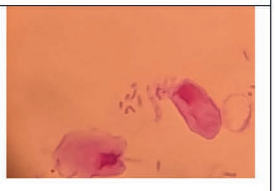
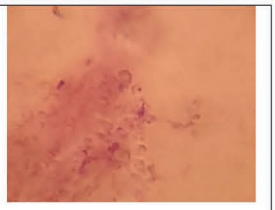
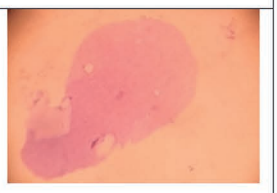
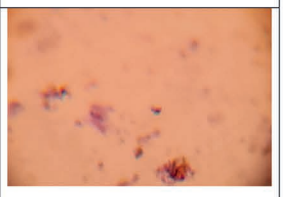
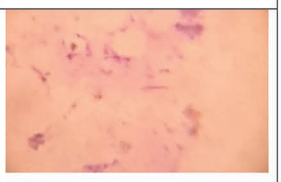
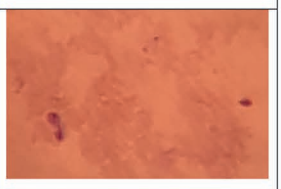
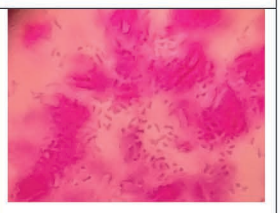
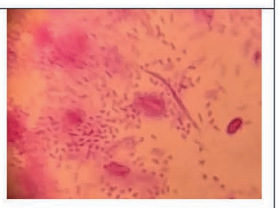
Sample number	Water source	Microscope 400X observation, followed by 1000x observation showing representing picture
Sample 1	Pre-dialysis tank 1 water	
Sample 2	Pre-dialysis tank 2 water	
Sample 3	Laboratory tap 1 water	
Sample 4	Laboratory tap 2 water	
Sample 5	Laboratory tap 3 water	
Sample 6	Kitchen water	
Sample 7	Dental flush water	
Sample 8	Pond water	
Sample 9	Ascitic fluid	
Sample 10	Cerebrospinal Fluid 1	
Sample 11	Cerebrospinal Fluid 2	
Sample 12	Pleural fluid	
Sample 13	Flower vase water	
Sample 14	Air coolant tower water	

Figure 1. Sampling results.

macroscopically due to haziness at the inoculation site, yielded negative results. Samples 4 and 5 exhibited contaminations with *Bacillus spp*. while sample 6 showed no observable growth or contamination. Sample 7 tested positive for suspected Acanthamoeba, with an estimated count of 0-10 organisms per oil immersion field, indicating clusters in certain regions. Sample 8, potentially affected by pond chlorination, resulted in a negative outcome. Sample 9 displayed fungus contamination, and care was taken to avoid that region during the smearing step. Fatty matter resembling amoeboid structures without nuclei was obtained, and thus, sample 9 was also negative. Samples 10, 11, 12, 13, and 14 all returned negative results, indicating the absence of any FLA. These results provide insights into the microbial composition of the collected samples, highlighting instances of Acanthamoeba presence. Hence, amoebic growth was confirmed in the pre-dialysis tank waters and dental flush water samples.

After the original surveillance presented positive results, all water sources were methodically scrubbed, and/or sterilized, and decidedly chlorinated. After 15 days, a reprised culture of all samples showed negative results. This designates the importance of an essential monitoring system for FLA in dialysis units, dental practice regions, and all areas with immune-compromised patients.

Discussion

In recent years, FLA have become a focal point in research due to their pathogenic potential in humans and their association with the survival and proliferation of amoeba-resistant bacteria. This study specifically investigated the presence of FLA in diverse man-made water systems and body fluids, emphasizing their potential pathogenicity and the associated risks to human health, especially in immunocompromised individuals or through specific modes of transmission.

Reports of isolation of FLA from the hospital milieu, although inadequate, widely distributed between countries throughout the globe. The frequently reported positive samples are from ventilators, air conditioners, tap water, dialysis fluids, and dental irrigation waters. Their existence carries risk in all immune-compromised patients as infection with FLA is headed to 100% mortality in the present scenario. Khurana *et al*. in 2015 [14] detected their occurrence in tap water and swab samples from diverse areas of the hospital, which were also confirmed by PCR. In another investigation in Tehran, 52.9% of hospital samples from wards with immuno-compromised patients showed positive results for FLA [12]. In this study, morphologically positive reports were established in pre-dialysis fluids and dental irrigation water. Amoebae in pre-dialysis fluid resembled Acanthamoeba, and amoebae in dental irrigation water looked like Naegleria. However, we could not endorse the species as we did not perform PCR. Again, in a study in Brazil [34], 77.8% of samples collected from air conditioners showed positive results for FLA. In our study, we could not isolate FLA from the air conditioning system.

The negative results in samples highlight the variability in microbial composition across different environments and emphasize the importance of comprehensive surveillance. Additionally, the absence of any amoeba in stagnant pond water, which may have been chlorinated, suggests the effectiveness of chlorination in eliminating amoeba in water. This theory is backed by a paper [32] which states that chlorination alone was effective in reducing the number of viable *N. fowleri* trophozoites while the combined action of chlorination and UV light is an even more effective disinfection strategy for mitigating the presence of *N. fowleri* in water systems, especially in settings like large pools where the risk of infection can be significant. Cultivation techniques, including NNA cultures, proved valuable in detecting Acanthamoeba presence. The method's simplicity and effectiveness support its adoption for routine surveillance and diagnostic purposes. The study contributes to the limited knowledge of the pathogenicity of these organisms and their associated diseases, particularly in regions where neurological infections may be underreported.

Conclusions

Our findings revealed the presence of Acanthamoeba spp. in pre-dialysis tank water and dental flush water samples, indicating potential concerns regarding water safety in these settings. The amoebic growth observed suggests the need for heightened awareness and preventive measures to ensure the safety of water used in medical facilities.

In conclusion, the identification of Acanthamoeba in specific water sources highlights the need for continuous monitoring and preventive strategies to mitigate potential health risks. Further research and surveillance are essential to broaden our understanding of the distribution and impact of pathogenic FLA, enabling more effective public health measures.

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