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Isolation, Morphotyping, Molecular Characterization and Prevalence of Free-Living Amoebae from Different Water sources in Makkah city, Saudi Arabia

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ABSTRACT

Free-living amoebae (FLA) are protozoa that can be found in a wide range of habitats around the world. Some species have been found to be pathogenic to humans in addition to their normal distribution. There is no research on the prevalence of FLA in various water sources in Makkah city that we are aware of. The aim of this study was to investigate the prevalence of FLA from various water sources in a different part of the city. A total of 86 water samples were processed and cultured with *Escherichia coli* overlay on non-nutrient agar medium (NNA). Microscopic examination was used to examine the samples. Fifty (58.1%) of the 86 samples tested were positive for FLA. Amoebae identified by morphology belonging to the genus *Acanthamoeba* were 30 (34.9%) and that belonging to the genus *Naegleria*, were 20 (23.3%). Among the positive cases, 100% of ponds, 80% of air conditioner exhaust, 50% of water fountains and tape water filters, 40% of swimming pools and 33.3% of stored tanks. The absence of FLA in the examined wells was a striking feature. Polymerase chain reaction (PCR) on DNA showed a 229-bps fragment using Nelson primers and a 500-bps fragment using *Acanthamoeba* genus-specific primer pair JDP1/JDP2. In conclusion, *Acanthamoeba spp.* was the most predominant type of free-living amoeba and more prevalent in ponds water absent in wells as shown by culture, microscopy and PCR.

1. Introduction

There is widespread concern that everybody in the world should have access to safe drinking water. Even in the 21st century, many individuals lack adequate water for basic needs in terms of quantity and/or quality [1]. The emergence of free living amoebae (FLA) in various water sources is one of the most significant problems in water pollution [2]. FLA is widely distributed in water systems because of its resistance to temperature, pH, and various chemicals used in water disinfection [3, 4].

FLA are unicellular protozoa that can live and reproduce in the environment without a host. They are found on all continents. FLA has been found in a variety of areas, including soil, raw, brackish, and sea water; field-grown crops, trash, swimming pools, contact lens supplies, therapeutic pools, dental treatment facilities, dialysis equipment, and heating, ventilation, and air conditioning systems, to list some [3]. They are known as amphizoic amoebae because of their ability to survive without a host as well as their ability to enter a host and live as parasites [5]. FLA differentiates from trophozoites, the vegetative form, to cysts, the resting form, in response to adverse conditions or stresses [6]. FLA encystment occurs in a variety of situations, including nutrient deprivation, osmotic stress, and in response to bacterial toxins [7-10]. Cysts are especially resistant to treatment and, as a result, play an important role in FLA survival and spread. The amoeba's metabolic function, which includes division, feeding, and motility, is represented by the trophozoite, or vegetative form [11, 12].

FLA serve as reservoirs for bacteria such as *Pseudomonas aeruginosa, Legionella pneumophila, Helicobacter pylori, Mycobacterium avium, Chlamydia and Vibrio cholera, Klebsiella spp., and Aspergillus spp.*, in addition to their pathogenicity [4, 13-15] and for viruses such as *Mimi virus* [16], *enterovirus* [15], and *Adenoviruses* [17]. FLA has a number of interactions with bacteria. Despite the fact that they are effective predators of bacteria and fungi, some of them are resistant to FLA digestion [18]. These amoeba-resistant bacteria (ARB) can multiply, survive, and lyse their host cells before spreading in enormous numbers throughout the environment [19-23]. Furthermore, ARB can grow and maintain virulence traits such as antibiotic resistance within amoebae, as well as adapt to exist within human macrophages [18]. As a result, the presence of FLA in the atmosphere poses a serious threat to both immunocompromised and immunocompetent people by transmitting pathogenic bacteria in aquatic environments.

Acanthamoeba, *Naegleria*, *Balamuthia*, and *Sappinia* are the only four FLA genera known to cause infections in humans and animals [3]. *Acanthamoeba* and *Balamuthia mandrillaris* cause granulomatous amoebic encephalitis (GAE). *Naegleria fowleri* causes primary amoebic meningoencephalitis (PAM). GAE and PAM are also central nervous system diseases that are sometimes lethal. Amebic keratitis, a painful and sight-threatening infection of the cornea, may also be caused by *Acanthamoeba spp* [3, 24, 25].

There is no research on the prevalence of FLA in different water sources in Makkah, Saudi Arabia that we are aware of. The aim of this research is to investigate, isolate, and classify FLA from a variety of water sources in Makkah, Saudi Arabia.

2. Methods

2.1. Study Area

The study was conducted in Makkah city located at the western part of the Saudi Arabia.

2.2. Samples

Eighty-six water samples were collected from different water sources including Swimming pool (10), water fountain (4), Stored tanks (18), Tape water filter (8), air conditioner exhaust (30), ponds (10) and water wells (6) as shown in (table1).

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2.3. Culture

To eliminate debris and mud from each water sample, multiple folded sterile gauze filters were used. Each filtrate was centrifuged for 20 minutes at 250 × g. The sediments were dissolved in Page's Amoeba saline solution after the supernatant was discarded. (2.5 mM NaCl, 1 mM KH2PO4, 0.5 mM Na2HPO4, 40 mM CaCl2.6H2O e 20 mM MgSO4.7H2O) [26]. *Acanthamoeba* was identified based on the size and morphological characteristics of both trophozoites and cysts.

The mixed solution was inoculated into a Petri dish with 1.5 % non-nutrient agar (NNA) Difco agar and Gram-negative bacteria Escherichia coli (ATCC 25922) culture. All of the plates were firmly sealed with Parafilm and incubated for up to two weeks at 37°C. All the cultured plates were examined daily for up to 14 days by inverted and light microscopes before being discarded. Checking cultures was made first to the eye and the inverted phase contrast microscope at least 14 days. The specific morphological appearances of the trophozoites, cysts and flagellates were identified accordingly based on the reports by several workers [5, 27, 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.

2.4. Flagellation test

The amoebae were removed from the plates and placed in a 1 mL distilled water solution. 100 µL of material was transferred to slides and inspected under a light microscope for the existence of any free-swimming flagellates after 2 hours of incubation at 37°C. Observations for the motile flagellates were carried out every 30 minutes for up to 6h. For the morphological identification of isolates, we utilized the study of [28, 29].

2.5. DNA extraction and PCR amplification

The amoeba cells were centrifuged (1000 ×g) for 10 minutes at room temperature before being rinsed three times with phosphate-buffered saline (PBS) pH 7.2. Cell pellets were resuspended in QIAamp DNA Mini® kit Nuclei Acid Lysis Buffer (ATL buffer) and treated with 10 mg/ml proteinase K for 1 hour at 56 °C. The amoeba DNA was purified according to company manufacture and then was stored at −20 °C until used.

2.6. PCR AMPLIFICATION

Two primer pairs were utilized for molecular identification: an A*canthamoeba*-specific PCR and a JDP1-JDP2 primer combination targeting the ASA. *Acanthamoeba* 18S rDNA gene S1 sequence. Forward primer JDP1(5'-GGCCCAGATCGTTTACCGTGAA-3') and reverse primer JDP2 (5'-TCTCACAAGCTGCTAGGGAGTCA-3') were used to target conserved regions of Acanthamoeba 18S rDNA in this PCR [30]. Amplification procedures for all PCRs were carried out in a 25 µl mixture containing 50 ng DNA, 10 µl 5X AmpliTaq Gold buffer, 0.2 mM each of dATP, dGTP, dCTP, and dTTP, and 20 pmol of each primer, 2.5 units of AmpliTaq DNA polymerase (AmpliTaq Gold 360 master mix, Applied Biosystems, CA, USA).

Denaturation (94° C, 45 s), annealing (55° C, 45 s), and primer extension (72° C, 45 s) were used in the JDP1/JDP2 PCRs. After the last cycle, a primer extension was performed at 72° C for 10 minutes. All PCR amplification products were electrophoresed on a 1.5 % agarose gel for analysis. Nelson primers targeting the sequence on the Acanthamoeba 18S rDNA gene are another pair of primers for Acanthamoeba-specific PCR. Forward primer F (5'-GTTTGAGGCAATAACAGGT-3') and reverse primer R (5'-GAATTCCTCGTTGAAGAT-3') were used to target conserved regions of *Acanthamoeba* 18S rDNA in this PCR [31]. Denaturation (94° C, 45 s), annealing (55° C, 45 s), and primer extension (72° C, 30s) were all done in 45 cycles for the Nelsons. After the last cycle, a primer extension was performed at 72° C for 5 minutes. All PCR amplification products were electrophoresed on a 1.5 percent agarose gel for analysis.

3. Results

Of the 86 samples examined, 50 (58.1%) were positive for FLA. Amoebae identified by morphology belonging to the genus Acanthamoeba were 30 (34.9%) and that belonging to the genus Naegleria, were 20 (23.3%). (table1) and (Fig.1)

Frequency of FLA in different water sources

Table1: Frequency of FLA in different water sources

Sample source		Number of Acanthamoeba positive samples	Number of Naegleria positive samples	Total Positive	%
Swimming pool	10	2	2	4	40
water fountain	4	0	2	2	50
Stored tanks	18	2	4	6	33.3
Tape water filter	8	0	4	4	50
Air conditioner exhaust	30	10	14	24	80
Pond	10	6	4	10	100
Water wells	6	0	0	0	0
Total	86	20	30	50	58.1

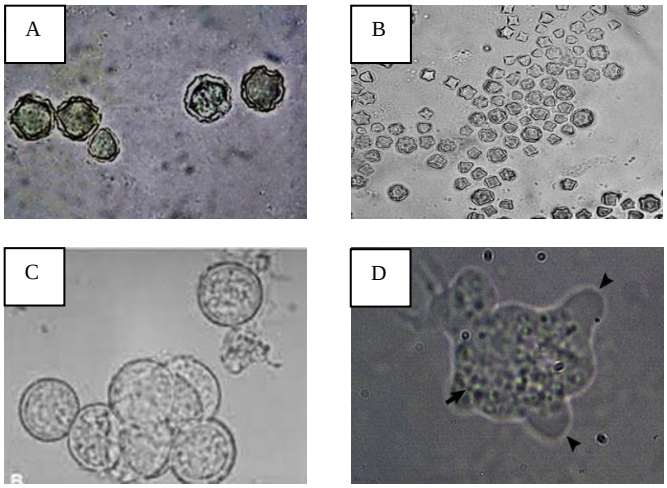

Figure 1: Trophozoites and cysts of identified FLA. Acanthamoeba cysts (A & B). Naegleria cyst (C). Naegleria trophozoite (D)

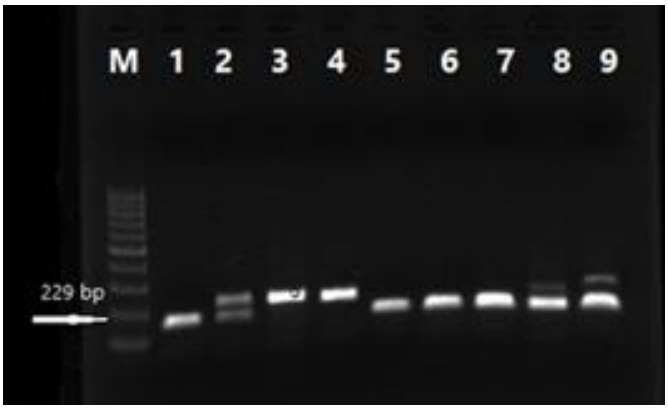

Figure 2: PCR reaction analysis using Nelson primers generated a 229-bps fragment. M indicates 100-bp marker, samples from 1-9 is the positive samples.

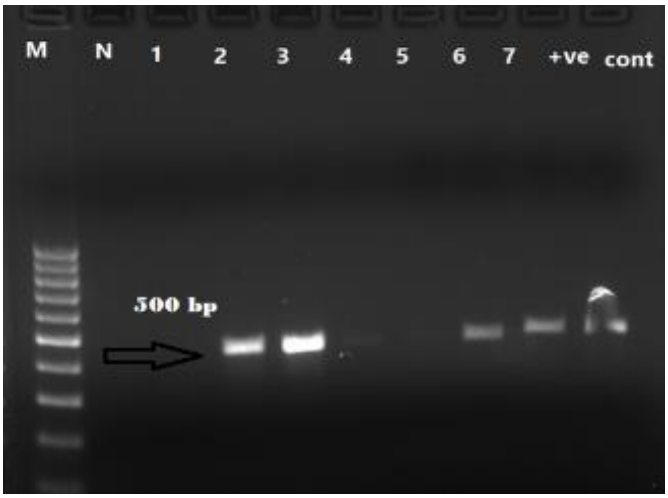

Figure 3: PCR amplification of ASA.S1 using *Acanthamoeba* genus-specific primer pair JDP1/JDP2. M: size markers (100-bp DNA ladder); N negative control (without DNA), sample 1 is negative, samples 2 and 3 are positive (500-bps), samples 4and 5 are negative, samples 6 and 7 are positive while the last is +ve control.

4. Discussion

The hypothesis that FLA would be found in many water sources serving people for diverse activities in Makkah motivated this research. The spread of FLA in natural and man-made habitats, as well as the possible damage that FLA causes to humans who come into contact with such amoebae, is of critical medical interest. The isolates capable of proliferating at temperatures of 37°C and higher, as well as the environments chosen by these FLA, were of particular interest. Despite the fact that amoebae from the genera *Acanthamoeba*, *Naegleria*, and *Hartmannella* are free-living organisms, scientists and medical experts are aware of the first two's potential to cause life-threatening CNS and of *Acanthamoeba* to cause abscesses in the cornea, skin lesions and other disorders [30].

This is the first time a preliminary search for free-living amoebae in an aquatic environment has been carried out in Makkah city. Water was obtained from various water sources (Swimming pools, fountains, stored tanks, tape water filters, air conditioner exhaust, ponds and wells). The overall prevalence in our study (58.1%) positive for FLA. Comparing our results with other publications, there is a wide range of variability in the prevalence of FLA worldwide ranging from 4.4% in turkey [33,34] up to 73.5% in Iran [35] Acanthamoeba spp. were found in 25 (73.53 %) out of 34 environmental water samples and 17 (28.8 %) out of 59 tap waters. Generally, Acanthamoeba spp. were found in 42 (45.16 %) of the samples in different regions of Isfahan, Iran. Despite using the same separation methods, we were unable to find out the underlying reasons for such variation in our research; it could be related to the characteristics of the water source and/or geographic regions. Amoebae belonging to the genus Acanthamoeba accounted for 34.9 % of the total, while those belonging to the genus Naegleria accounted for 23.3 %. FLA is recovered from all samples obtained from ponds (100%), which could be explained by the abundance of organic matter and bacteria in ponds. Air conditioner exhaust was the second most common site where FLA were found (80%), this may be explained by the presence of high concentrations of coliform bacteria with a secondary importance attributed to higher concentrations of oxygen, a finding that should increase our concern for further research into the pathogenicity of these isolates, as most Makkah residents use air conditions. The absence of FLA from the examined wells was a striking feature, which could be due to the superficial collection technique used for wells.

The nucleotide sequences of the PCR amplifying ASA.S1 region were used to identify the Acanthamoeba genus. A 423 to 551 bp Acanthamoeba-specific amplimer ASA, as previously demonstrated by [36]. S1, produced using primers JDP1 and JDP2, includes significant inter-strain sequence variation, allowing differentiation between numerous 18S rDNA genotype groups (genotypes T1-T15). The A. polyphaga reference strain has a band size of 450 pb. This band was also found in our samples. It could potentially be the DNA amplification of other harmful species (A. castellanii or A. hatchetti), which are capable of infecting humans [37]. In the majority of samples retrieved from the environment [30], discovered the same band 450 bp. They tested multiple stocks of Acanthamoeba spp. reference species and discovered band widths of 420 bp (genotype T5), 450 bp (genotype T4 and T11), 500 bp (genotype T9), and 550 bp (genotype T9) (genotype T7). The species with a 550-bp band in our study must correspond to 18S rDNA genotype T7, and this Acanthamoeba species is not harmful. The presence of these two bands (450 bp, 550 bp) in four samples could correspond to an association of two different 18S Acanthamoeba spp.

5. Conclusion

We found FLA species that are known as potential eye pathogens on the one hand, and as hosts and carriers for many pathogenic bacteria including eye pathogens on the other hand, in all water sources in Makkah, particularly tap water utilized by contact lens wearers. To our knowledge, this is the first time Acanthamoeba has been molecularly detected in Makkah water sources. The findings of this study may serve to enhance awareness of these facultative pathogens among clinicians and contact lens wearers, and so help to reduce the risk of FLA infections in the future.

We recommend for assessing the risks posed by FLA to Makkah residents, additional investigations are needed to fully determine the pathogenicity of these isolates.

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