Original Article

Isolation and identification of free living amoeba (Naegleria and Acanthamoeba) in Shiraz water resources by morphological criteria

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Abstract

Introduction:
Free living amoebas are opportunistic pathogens that usually exist in different environmental conditions such as warm and polluted water, even in water supply networks and they could cause serious diseases in humans. So, due to their medical importance, identification of free living amoeba in water resources is necessary.

Materials and Methods:
Water samples were collected from 70 water wells, 30 water resources and 20 water supply networks in the first six months of 2010. Then, the samples were cultured on non-nutrient agar and the amoeba were collected and stained by Giemsa stain for morphological studies.

Results:
42 out of 120 samples (35%) contained free living amoeba. Out of them, 31 samples (73.81%) were polluted with one amoeba and 11 (26.19%) with one amoeba. The frequency of Acanthamoeba species (39 cases) was higher than that of Neagleria (14 cases) and the wells were more polluted than others (44.40%). Based on morphological characteristics, four pathogenic ameba were identified (Naegleria fowleri, Acanthamoeba polyphaga, A.castellani and A.astronyxis).

Conclusions:
The results showed that water resources contained free living amoeba and some important and pathogenic species of these amoebas were identified by morphological characteristics. Thus, it is necessary to employ new methods for disinfection and filtration of water resources so that the infection with free living amoeba and infectious agents is prevented.

Keywords: Amoeba, Acanthamoeba, Naegleria, Water Resources, Morphology

Introduction:

Free-living amebas are the opportunistic amebas which usually live freely in saline and fresh waters, seawaters, humid soils, on decaying plants, etc. (1). These amebas are abundantly found in open surface waters such as water resources, but most of them are found in nutrient-rich environments like biological treatment systems of sewage in which there are bacterial nutritional activities. Free-living protozoa are present in a broad scope of

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environmental conditions in marine environments from hot ground waters and contaminated hot waters to pipes of water supply system (2). Some of these amebas are harmful to health. In recent years, the reported cases have been increasing considering the progress of laboratory diagnostic methods for these amebas (3). Although these protozoa do not have any parasitic stage in their life at all, when they accidentally enter human or animal body, they will colonize and reproduce, which causes problems for humans and animals (1).

Naegleria and acanthamoeba are the most widely known opportunistic free-living pathogenic amebas. The infection caused by naegleria fowleri mostly results from water and can cause a rare but fatal brain infection called primary amoebic meningoencephalitis. Acanthamoeba species are usually found in water and soil and cause diseases in central nervous system or granulomatous amoebic encephalitis and an eye disease called amoebic keratitis (2). Cases of granulomatous amoebic encephalitis resulted from different species of acanthamoeba, especially acanthamoeba castellani and colbertessoni acanthamoeba, have been reported in people suffering from chronic diseases. Amoebic keratitis can result from different species of acanthamoeba, particularly acanthamoeba castellani and acanthamoeba palyphaga (1&4). These amebas can also carry and transmit important and pathogenic bacteria such as vibrio cholera, legionella, mycobacterium leprae, listeria monocytogenes, francisella tularensis, helicobacter pylori and escherichia coli serotype O157 and, as a result, these pathogenic agents may be transmitted by these amebas through water resources to humans (5).

Different studies have been conducted across the globe to identify free-living amebas. In Iran, contamination with acanthamoeba and naegleria has been reported by studying water and soil samples from rivers and Parishan Lake in Kazeroon (6), surface waters and squares of Tehran (7) and water tap samples of hospitals (8). Also, contamination with free-living amebas has been reported in water resources, tanks, tankers and different hospital wards in Portugal (9), well water and tap water in Nicaragua (10), domestic water sample in Florida (11), drinking water in Osaka, Japan (12) and drinking water treatment plants in Switzerland (13).

Reproduction and growth of free-living amebas in culture media have allowed for studying these protozoa. Selection of culture media depends on the goal of the study for isolating and maintaining strain or determining biochemical characteristics and antigenic properties of a special strain (14). Observation of amoeba cysts and trophozoite by optical and invert microscopes, trichrome staining, H&E and giemsa, isoenzyme analysis, indirect fluorescence antibody method using specific antiserum (14) and molecular methods such as polymerase chain reaction (PCR-RFLP) (15&16) and sequence determination (16-18) are among the methods for identifying free-living amebas.

Considering the risk and role of these amebas in expansion of pathogenic microorganisms in water resources, effective actions can be taken for removing or controlling contaminated resources through their identification and thus prevent potential diseases caused by them or the bacteria carried by them. This research was conducted to identify free-living amebas in water resources in Shiraz using method of culturing and microscopic investigation based on morphological characteristics.

Materials and Methods:

In this cross-sectional, descriptive study, samples were collected from 70 wells, 30 tanks and 20 water distribution networks in all regions of Shiraz using 500-ml sterile bottles between January 2010 and

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September 2010. Sampling date and place were printed on the bottles and they were transferred to the laboratory in appropriate sample carriers. In the sampling site, temperature of the remaining chlorine and water was measured. In addition, coliform tests were performed using most probable number method under laminar flow, turbidity was measured using nephelometry method, pH, total hardness, electric conduction, nitrite, nitrate and ammonia of waters were also measured according to 2005 reference standard method of ABFA Organization. Meanwhile, samples were tested for all physical, chemical and microbial parameters on the sampling day (19).

In order to culture the amebas, a non-nutrient agar medium was used (14&20). After preparing the culture medium, a colony of escherichia coli K12 strain bacteria which was previously cultured was dissolved in 0.5 ml of buffer and two to three drops of this suspension were inoculated into the culture medium in the plate (the bacteria were distributed as a food source of parasites on the surface of culture medium). Then, the sampling bottle was shaken to homogenize the sample and 250 to 500 ml of water sample was passed through three-layered sterile cloth in order to remove planktons. The water sample was filtered using a vacuum pump and nitrocellulose sterile paper filters with 0.45 micron pores. Then, the filter was placed face-down on the non-nutrient agar medium surface, covered by Escherichia coli strain K12, by sterile forceps beside a flame. The plate was sealed by parafilm and then the media were incubated at 25 to 30ºC. In order to identify ameba, the culture media were microscopically observed since the second day to identify positive ameba sample (transparent points on culture medium indicate the presence of ameba). After 4 days, many cysts and trophozoites are usually found inside the positive plates. Other samples were also studied using an optical and invert microscope until one month after starting the culture on a daily basis and they were photographed under the microscope. Finally, the culture was reported negative after 1 month in case of observing no ameba (20&21).

The plates with mobile forms or cyst-like forms of free-living amebas were selected for the following stages. Since mixed growth was found in some plates, pure culture was prepared to obtain the related ameba. To this end, a microscope was used to find one or more ameba cysts with fewer bacteria and fungi. Then, the surrounding culture medium was cut by a sterile scalpel and transferred to the new culture medium. The plates were checked every day for ameba growth until a pure plate from the desired ameba was obtained (20&21).

In order to collect amebas from the plate surface, first, 4-5 ml of sterile normal saliene was poured in the culture medium by a sterile Pasteur pipette; then, the surface of plate was well mixed with the buffer using a cell scraper to immerse trophozoites and cysts in the buffer. In the next stage, trophozoites and cysts along with the buffer were transferred to the microtube using Pasteur pipette and sufficient sediment of amebas was obtained using centrifuge at 4000 rpm for 5 min. In order to remove additional agar, this action was repeated to isolate the highest amount of additional matters from the ameba (14&20).

In order to identify amebas, the samples collected from culture media were studied with or without giemsa staining under optical microscope with magnification of ×10, ×40 and ×100 and all the samples were photographed. The amebas were indentified considering morphological characteristics of cysts and trophozoites (22&23).

The data obtained from the above tests were descriptively analyzed in SPSS software.

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Results:

In this research, 42 out of 120 collected samples (35%) were positive for free-living amebas (Table 1). Generally, all the water samples were in normal range in terms of physical and chemical parameters (Table 2). The results showed that 39 out of 42 positive samples for free living amebas (92.85%) lacked remaining chlorine and 3 samples (7.15%) contained the remaining chlorine.

Table 1- Frequency and percent of samples in terms of free-living amebas

Number Sampling place	Presence of ameba (%)	Absence of ameba (%)	Total (%)
Well	32(44.4)	38(56.6)	70(100)
Tank	8(26.6)	22(74.4)	30(100)
Water supply network	2(10)	18(90)	20(100)
Total	42(35)	78(65)	120(100)

Microbial investigation of 120 water samples showed that 57 samples (47.50%) were positive for total coliforms and 29 (24.16%) were positive for thermotolerant coliforms. After microbial investigation of 42 samples containing free-living amebas, it was found that 21 samples (50%) were positive for total coliform and 13 (31%) were positive for thermotolerant coliforms; however, no significant relationship was found between water contamination with free-living amebas and the presence of coliforms (P>0.05).

Table 2: Mean, minimum and maximum values of physical and chemical parameters of water samples

Index Parameter	Standard error ±mean	Minimum	Maximum
Turbidity (NTU)	0.743 ± 0.09	0.12	8.25
Remaining chlorine (ppm)	0.132 ± 0.026	0	1.5
Temperature (°C)	19.54±0.15	12.0	29.0
pH	7.54±0.190	6.98	7.96
Ammonia (mg/Liter)	0.103±0.028	0.01	3.0
Nitrite (mg/Liter)	0.010±0.001	0.01	0.08
Nitrate (mg/Liter)	21.20±1.62	3.0	67.0
Electric conduction (µs)	949.69±35.21	314.10	1858.00
Water hardness (mg/Liter)	468.32±52.01	160.32	5826.00

Investigation of culture media using optical microscope showed that 42 samples had trophozoite- or cyst-like free-living amebas. In some cases, mobile trophozoites of naegleria or double-walled cysts of acanthamoeba with wrinkled external wall and angular and polygonal inner wall were easily distinguishable (Figures 1 and 2). In the microscopic investigation of slides stained with giemsa, trophozoites and cyst forms of naegleria, and acanthamoeba were found (Figures 4, 5, 6). Study of naegleria forms showed that their amebic forms were 10-25 micron long and had large round pseudopod; also, cytoplasm had flat and fine grains and contained a clear and distinct nucleus and a dense central nucleolus which makes its halo or ring of light (Figures 1 and 3). Flagellate form also had a nucleus and large nucleolus and usually two anterior flagellates but sometimes 3 or 4 flagellates were also found (Figures 1 and 3). Studying the stained slides of

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acanthamoeba showed that some of these cysts had mean diameter of above 18 microns, star-shaped inner wall (endocyst) and their flat or wrinkled external wall (exocyst) and the number of arms was below 6. Therefore, these should be A. asteronyxis (23) (Figure 4). Also, some cysts had an average diameter of below 18 microns with polygonal, triangular, round, elliptical and asteroid inner wall and irregular external wall and species of acanthamoeba in this group of samples can be one of the species of A.castellani, A.polyphaga, A.rhysodes, A.hatchetti, A.quina, A.griffini, A.lugdunensis, A.triangularis, A.divionensis or A.paradivionensis (Figure 5).

The research results demonstrated that 31 out of 42 ameba contaminated sample (73.81%) were contaminated with one type of ameba, 11 (26.19%) were contaminated with two types of ameba and frequency of acanthamoeba species (39 cases) was higher than that of naegleria (14 cases).

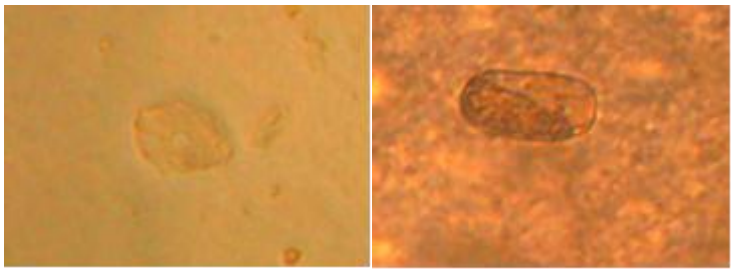

Figure 1: Flagellate and amebic form of naegleria (A- 40X) inside the culture medium (B- 10X)

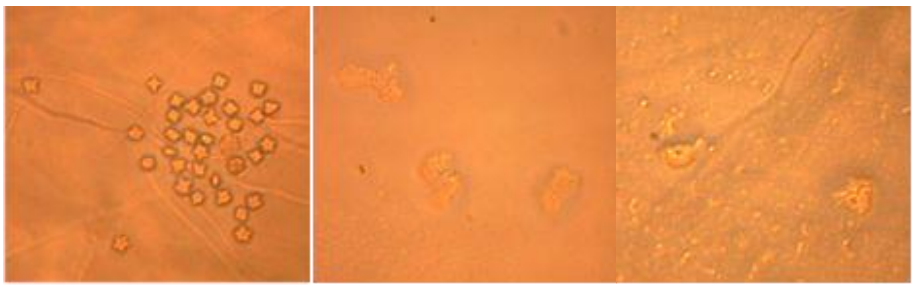

Figure 2: Trophozoite form and acanthamoeba cyst inside culture medium -10X

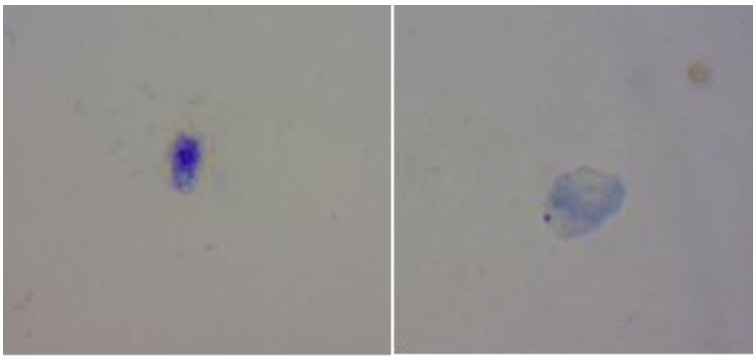

Figure 3: Flagellate and amebic forms of naegleria-giemsma staining -100X

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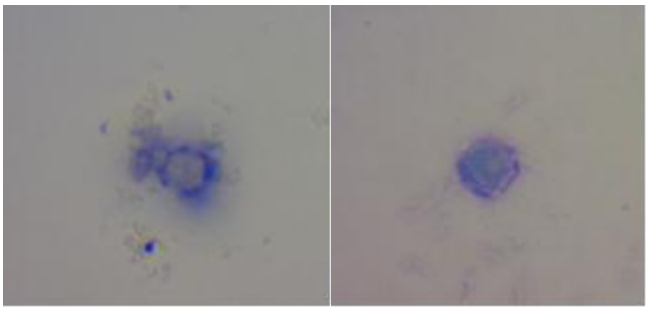

Figure 4: Cyst form of acanthamoeba.asteronyxis- giemsa staining -100X

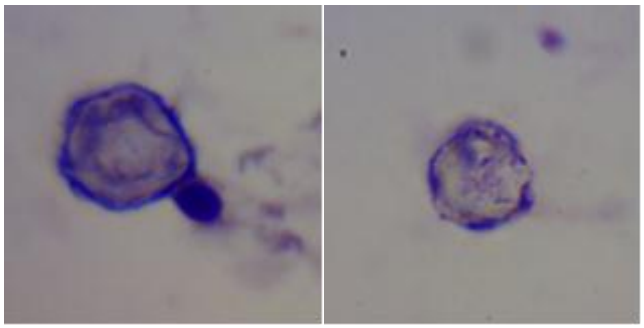

Figure 5- Cyst form of acanthamoeba- giemsa staining -100X

Discussion:

In the present research conducted to identify free-living amebas in water resources in Shiraz using culture method and microscopic investigation of morphological characteristics, 42 out of 120 water samples (35%) were positive. Thirty-one out of 42 ameba contaminated samples (73.81%) were contaminated with one type of ameba and 11 (26.19%) were contaminated with two types of ameba; also, frequency of acanthamoeba species (39 cases) was higher than that of naegleria (14 cases). The results of this research also showed that water resources of well, tank and water supply system might be contaminated with free-living amebas and wells were more contaminated than other resources (44.4%).

Rezaeian et al. studied water and soil of rivers and Parishan Lake in Kazeroon region studied 354 samples by culture and microscopic method and reported 10 cases of contamination with acanthamoeba and 3 cases of naegleria (6). The results of this research showed that percent of contamination with free-living amebas and frequency of each one of them were lower than those of the present study. Eftekhar et al. also studied 22 samples of surface waters and waters in squares of Tehran city in terms of contamination with free-living amebas through polymerase chain reaction method and found that 13 samples (59%) were contaminated, 6 of which were contaminated with acanthamoeba (7). In this research, the frequency of contamination with acanthamoeba was lower than that of the present study despite higher percent of contamination with free-living amebas. Based on the research results by Bagheri et al., acanthamoeba was found in 45 out of 94 (48%) samples of tap water in hospitals in different cities of Iran (8). Contamination percentage of drinking water with acanthamoeba in this research was lower than that in the present research (73.81%). Carloso et al. studied 135 samples of water resources, tanks, tankers and different wards of hospitals in

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Portugal and showed that 47 samples (35%) were contaminated with free-living amebas (9). Considering the sampling place, results of the recent studies were similar to results of the present research in terms of contamination with free-living amebas. Liva et al. studied 111 wells, 74 water taps and 21 water samples of tropical regions in Nicaragua in order to identify free-living amebas using molecular method and 43% of 201 studied samples were positive. The findings of this research showed that contamination with acanthamoeba was 21% and 2% and contamination with naegleria was 10% and 19% in urban regions and tropical regions, respectively (10). Results of this research compared with the present research indicate higher percentage of contamination with free-living amebas but percentage of contamination with each one of acanthamoeba and naegleria was lower than that of the present research. In the study by Shoff et al., 80 out of 283 domestic water samples were contaminated with free-living amebas and hartmannella and vahlkamfia were also found (11); however, percentage of contamination with free-living amebas and frequency of each were lower than those of the present study. Edagua et al. reported prevalence of free-living amebas with acanthamoeba and naegleria species as 68.7% in drinking water sources supplied by water of river and water treatment plant located in Osaka, Japan, and molecular study showed A. asteronexis species. Percent of contamination with free-living amebas was higher compared with the present study (12). Thomas et al. studied drinking water treatment plants in Switzerland and found that naegleria and acanthamoeba were available in the samples (13). Results of two recent researches were similar to the results of the present research in terms of contamination type and presence of naegleria and acanthamoeba.

Investigation of physical and chemical parameters of 120 water samples demonstrated that almost all the parameters (turbidity, remaining chlorine, temperature, pH, ammonia, nitrite. nitrate, electric conduction and water hardness) were in normal range considering standards of ABFA Organization. Also, temperature of water resources contaminated with free-living amebas was studied which showed that these amebas could grow and be present at temperate temperature ranging between 12 and 25.9 °C. Studying microbial parameters demonstrated that 21 out of 42 water sample containing free-living amebas (50%) were positive in terms of total coliform and 13 (31%) were positive in terms of thermotolerant coliform, but no significant relationship was found between water contamination with free-living amebas and presence of coliforms ($P>0.05$). Since coliforms are counted in water samples using MPN method, negative result of the water samples did not mean the absence of coliforms, but it indicated reduction in the number of coliforms and normality of water in terms of drinking and these amebas largely fed on bacteria. Investigation of the results from the remaining chlorine in water samples containing free-living amebas showed that 39 samples (92.85%) lacked remaining chlorine and 3 samples (7.15%) contained chlorine in terms of the free-living amebas, which indicated effect of chlorine on prevention of growth and reproduction of these amebas because the presence of a high amount of remaining chlorine can help prevent growth of these amebas.

Trophozoite and cyst forms of amebas vary in size, which makes their final diagnosis difficult using morphological method. As a result, this method alone is not able to make final diagnosis in terms of type and species of these amebas. Thus, it is suggested to use molecular methods such as PCR-RFLP (polymerase chain

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reaction - restriction fragment length polymorphism), real-time PCR and sequence determination in order to identify and determine species of free-living amebas.

Conclusion: Since it has been proved that some of these amebas such as naegleria fowleri, acanthamoeba palyphaga, A.castellani and A.astronyxis are pathogenic and it has been found that they can carry hazardous microbial and infectious agents, it is necessary to prevent potential contamination of water resources with free-living amebas and infectious agents using new disinfection and treatment methods.

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