ORIGINAL ARTICLE

Recognition of Pathogenic Free-Living Amoebae in the Surface Water in Shiraz, Iran

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Received: 15 July 2014
Revised: 10 August 2014
Accepted: 6 September 2014

Abstract

Background: There are many genera of free-living amoeba in the environment, but members of only four genera (*Naegleria*, *Acanthamoeba*, *Balamuthia* and *Sappinia*) have an association with human infection. Water, soil and air are main sources of infective types of these pathogenic organisms for human.

Methods: Totally, 30 samples were collected from the surface water sources of Shiraz city, the capital of Fars province, during July and August 2009. The samples were filtered and their sediments were cultured on non-nutrient agar medium and seeded with non-pathogen *Escherichia coli*. Then, they were incubated at three different temperatures, 22˚C, 37˚C, and 44˚C. The media were checked with invert microscopy and amoebae were recognized by phase–contrast microscopy and observed by light microscopy after Trichrome staining. Polymerase chain reaction (PCR) was performed for molecular detection.

Results: Of the 30 samples, 29 were recognized morphologically as *Acanthamoeba,* the characteristics of 20 of which were confirmed by PCR. The growth rate of amoeba in 22˚C was more than 37˚C. Eight of the samples grew at 44˚C, but flagellate forming test and PCR were negative for *Naegleria fowleri*. Two of them were identified morphologically as *Balamuthia* and *Sappinia*.

Conclusion: Since Fars province is located in the subtropical region where there are a lot of parks and green areas with surface water, the potential risk of diseases caused by free-living amoebae should be considered. Further investigations about various aspects of these important opportunistic protozoa are recommended especially for establishment of appropriate prevention tools.

Please cite this article as: Mohammadi-Ghalehbin B, Hatam GR, Mohammad-Pour I, Ghobakhloo N, Foroughi-Parvar F. Recognition of Pathogenic Free-Living Amoebae in the Surface Water in Shiraz, Iran. J Health Sci Surveillance Sys. 2014;2(4):164-167.

Keywords: Surface water, *Acanthamoeba*, Shiraz

Introduction

Free-living amoebae (FLA) are ubiquitous protozoa that are presented in various environments. These opportunistic parasites have been proved to cause serious health problems such as granulomatous amoebic encephalitis, amoebic keratitis, cutaneous lesions, and primary amoebic meningoencephalitis in humans and other mammals.[1-3] Among several genera of FLA, only four genera have been reported to act as pathogen for human; they are *Acanthamoeba* spp, *Balamuthia*, *Naegleria,* and *Sappinia*.[4]

Recently, infections caused by these amoebae have been increased in many countries. Because of their wide scattering in nature, awareness about the prevalence of these protozoa in various environments could be very effective for control of infections by FLA. Many studies have shown that aquatic environments can provide an important potential source for human infection.[5-7]

Until now, a few investigations have been

conducted to identify FLA in the soil, water and hot springs in Iran.[8-10] There are many parks with superficial waters and pools in Shiraz and people especially children are exposed to these pathogens. Therefore, due to the rate of amoebic keratitis, FLA contaminated superficial water sources and the potential risk of FLA transmission, this study aimed to identify the genera of FLA in superficial waters in Shiraz by using cultivation methods, morphological keys and confirmation by molecular methods.

Materials and Methods

Sampling

During July and August of 2009, 30 water samples were collected from various locations as fountains and pools in specified parks and green spaces in Shiraz city. The samples were transferred to the Basic Sciences Infection Research center, Shiraz University of Medical Sciences.

Isolation and Cultivation

Water samples in 500-1000 ml volume were passed through a cellulose nitrate membrane filter (Millipore, pore size 0.45 µm), using a vacuum pump. The filter membrane from each sample was divided into three pieces. Each piece of filter membrane was cultivated on three non-nutrient agar media that were seeded with non-pathogen *Escherichia coli* ATCC 25922. The plates were sealed with Parafilm and incubated at three different temperatures: 22ºC (room temperature), 37ºC and 44ºC. Each sample was examined daily by the inverted microscopy to detect trophozoites or cysts of amoebae. Wet mount slides were prepared from positive cultures and observed by light microscopy to follow active and motile trophozoites. The positive samples were stained by Trichrome method in order to identify morphological structures. *Acanthamoeba* species were recognized based on double wall cysts with creased or star-shape endocyst. Trophozoite forms were characterized by typical pseudopodia. Double nucleus amoebae were considered as *Sappinia spp.* while *Balamuthia* spp. was identified by trophozoite special morphology.

Enflagellation test for Naegleria Fowleri

Enflagellation test was done for detection of *Naegleria fowleri* to confirm eight samples that were grown in 44˚C. For this purpose, the trophozoites were suspended in sterile water and placed in 37˚C for 20 min to see flagellum creation.

DNA Extraction and Aamplification

Organisms were grown in agar media and washed by PBS thoroughly; the suspended trophozoites and cysts were centrifuged at 1000g for 10 minutes. Sediments were used for DNA extraction by Phenol-chloroform technique.[11] Amplification of *Acanthamoeba Spp.* and *Naegleria fowleri* DNA was performed using *Acanthamoeba* 18S ribosomal fragment genus

Specific primer pair (5GGCCCAGATC GTTTACCGTGAA3, 5 TGACTCCCTAGCA \ GCTTGTGAGA 3) and *Naegleria fowleri* specific primer pair (5 GTGAAAACCTTTTTTCCATTTACA 3, and 5 AAATAAAAGATTGACCATTTGAAA 3).[12] Reaction conditions for all primer sets were done by the following protocol. Briefly, 25 µl PCR reaction contained 10ng of total genomic DNA, 10 pmole of each primer, 0.2 mM each dNTP, 2.0 mM $MgCl_2$ and 1U of Tag polymerase in the 2.5 µl PCR buffer. The cycling conditions were 95°C for 5 min, followed by 35 cycles of 94°C for 45s, 60°C for 30s, and 72°C for 45s. The PCR products were visualized on a 1.5% agarose gel containing ethidium bromide and a 100 bp DNA ladder (Sina gen, Iran).

Results

Twenty nine out of 30 superficial water samples were identified as *Acanthamoeba* genus after cultivating for 2 weeks and just according to morphological key (Figure 1). The growth speed rate of amoeba in 22°C was more than 37°C. Eight of 30 samples grew at 44°C, but flagellate forming test for them was negative. Two of the samples were identified morphologically as *Balamuthia spp.* and *Sappinia* (Figure 1).

Twenty of 29 specimens considered as *Acanthamoeba* by microscopy were reconfirmed as *Acanthamoeba* by molecular method (PCR). Also, PCR technique demonstrated that 8 samples grown at 44°C were not *Naegleria fowleri* (Figure 2).

Discussion

Finding *Acanthamoeba spp.* in superficial waters is very important, because they are one of the most available sources for people, so it increases the risk of keratitis and the other health problems caused by FLA in population.

In the present study, we have isolated *Acanthamoeba* species from 29 out of 30 (99.6%) superficial water samples by cultivation and morphologic assessment in different areas of Shiraz city; also, 20 samples were reconfirmed by molecular method as *Acanthamoeba*.

Studies in Iran revealed a significant increase in the incidence of keratitis in recent years.[13] In a study on surface waters of Gilan province, north of Iran, 19 out of 27 samples were morphologically positive for *Acanthamoeba* species, while 14 of 19 positive samples were verified by PCR.[10]

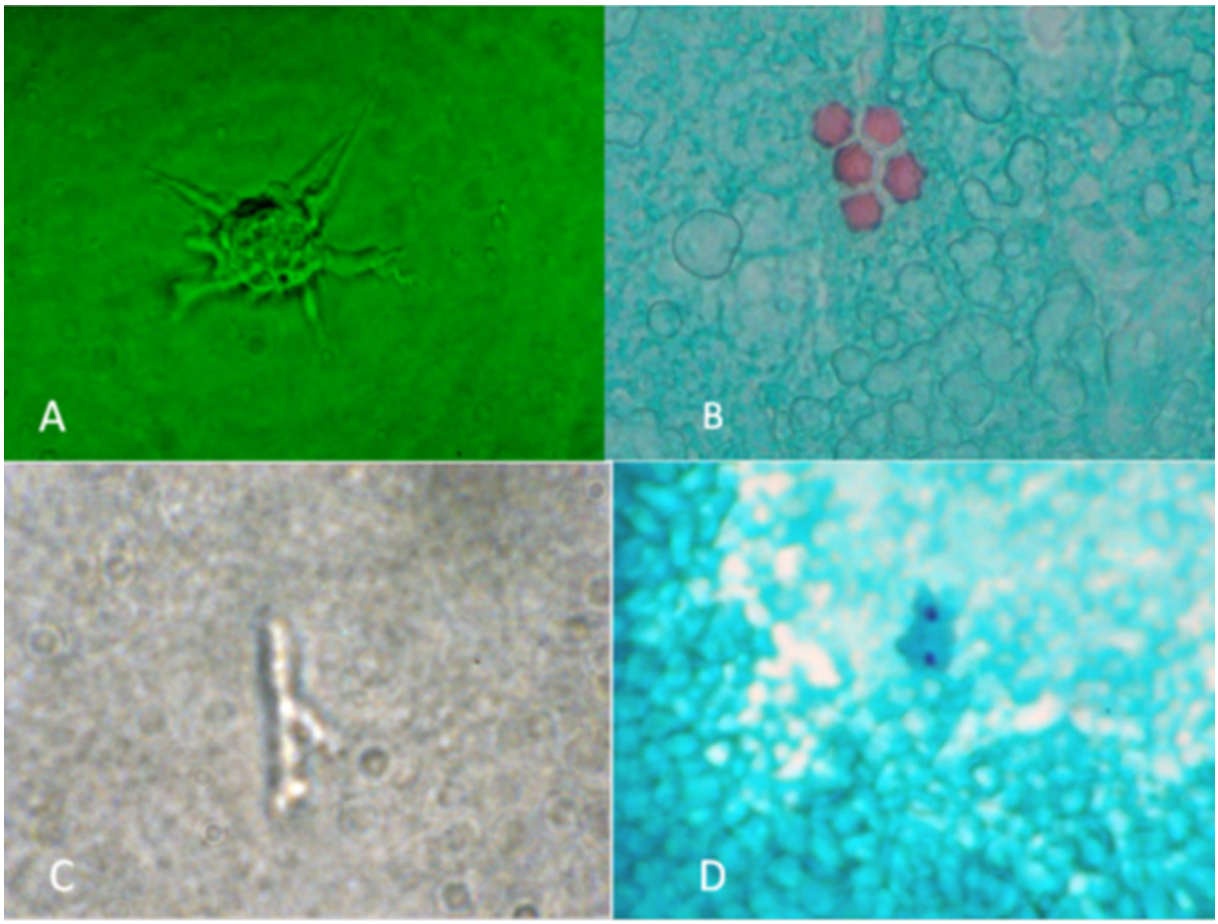

Figure 1: A: *Acanthamoeba spp.* trophozoite. (The distinctive feature is the presence of multiple finger-like acanthopodia projecting from the surface of the amoeba. (Phase contrast microscopy)) B: Cysts of *Acanthamoeba spp.* (Trichrome staining) C: *Balamuthia spp.* trophozoite. (Phase contrast microscopy) D: *Sappinia diploidea* trophozoite. (Two nuclei can be seen in this image. (Trichrome staining))

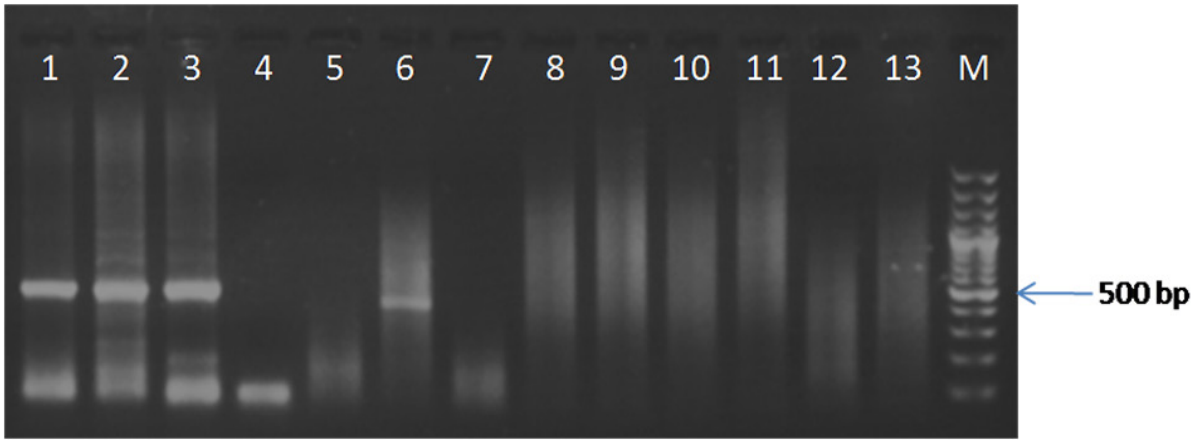

Figure 2: PCR products on a 1.5% agarose gel. Lane1: *Acanthamoeba* positive control was confirmed by morphologic and staining procedures, Lanes 2, 3, 6: *Acanthamoeba* positive isolates, Lane 4: *Acanthamoeba* negative control, Lanes 5, 7-13: *Acanthamoeba* negative isolates, Lane M: molecular marker.

In an attempt by Maghsood and colleagues, *Acanthamoeba* genotype T2 was the most frequent genotype in 12 environmental samples.[7] Rezaeian and colleagues reported that 46.25% of the samples from different habitats, such as tap water, soil, dust, cow faeces and medical instrument, were positive for *Acanthamoeba spp.*[14] Badirzadeh and colleagues claimed that 42.9% of water samples from hot springs were positive for FLA which contained *Acanthamoeba* and *Vahlkampfidae*.[8] 27.3% of the major rivers in southern suburbs of Tehran (associated with human activity) were identified as FLA, 80% of which were reported *Acanthamoeba* genus by Niyati.[15] Other sources like dust samples showed *Acanthamoeba* too and the genotypes were detected T4, T5 and T11.[16] These results are in agreement with those

of the current study. Also, investigations in other regions in the world showed T4 genotype as the most pathogenetic parasite.[17-19]

As mentioned above, based on the present and previous studies *Acanthamoeba* is distributed in various environments considerably. In addition, in two samples which were positive for *Acanthamoeba,* other FLA such as *Balamuthia* and *Sappinia* were also detected. This increases the importance of such studies on these protozoa in this area. Fars province is located in a subtropical region where there are a lot of parks and green areas with superficial waters. Therefore the potential risk of diseases caused by free-living amoebae should be considered in this area. Further investigations about various aspects of these important opportunistic protozoa are recommended especially for establishment of appropriate prevention tools.

Acknowledgments

The authors thank Mrs. P. Habibi, for technical assistance. The study was financially supported by the office of vice-chancellor for research of Shiraz University of Medical Sciences (Grant No: 88-4709).

Conflict of Interest: None declared.

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