

Investigating the Frequency of Free-living Amoeba in Water Resources of Bandar Abbas City, Hormozgan Province, Iran 2019-2020

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Research note

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Abstract

Objective

These amoebas can cause dangerous illnesses when they accidentally enter the human body, so it is necessary to determine various forms of organisms in water resources to prevent the danger they can cause risks to human health. Currently, in Bandar Abbas, there is no sufficient information about the distribution of *Acanthamoeba*, and we intended to study its frequency.

Results

Out of 83 water samples collected from different resources in the city, 31 plates (37.3%) were found to be positive for free-living amoebae. Of these, five were identified (6%) by culture method and 8 (9.6%) by molecular method. Positive sample sequence analysis enables us to distinguish two genotypes of T4 and T15 in this study.

Introduction

Pathogenic and opportunistic free-living amoebae such as *Acanthamoeba* spp., *Balamuthia mandrillaris* and *Naegleria fowleri* are aerobic, mitochondriate, eukaryotic organisms that occur worldwide and can potentially cause infections in humans and other animals. They are ubiquitous in soil and water resources, aquatic environments, ponds, hot springs, swimming pools, domestic sewage, air, air conditioning chambers, sediments, stagnant water, artificial humanmade creatures, and can infect human and animals (1). These amoebae use bacteria, yeasts, and other organisms as food sources. Unlike real parasites, pathogenic free living amoebae can complete their life cycle without entering the human and animal host body in the environment. An increasing number of people with immunodeficiency diseases, including AIDS treating the patients with corticosteroids or people undergoing chemotherapy, are at increased risk of developing amoebae (2, 3).

In many parts of the world, the pathogenicity of these amoeba has been considered and many studies done on epidemiology and genotyping. Amoebic diseases are challenging to diagnose, which cause delayed diagnosis and cure, and results in a high rate of mortality. Considering those facts, there is an urgent necessity to pay more attention to this type of diseases.

Despite being a relatively rare disease in comparison with other forms of infectious diseases, *Acanthamoeba* has hygienic importance in developed and developing countries (4). In Turkey, environmental samples (100%), in USA 2454 tap water samples (51%), water supply in Osaka, Japan (19%), and in 40 water and sanitation samples in Tunisia Hospital (47.6%) *Acanthamoeba* was found. Also, 14 samples taken from swimming pools in Malaysia, were contaminated with *Acanthamoeba* (5-7).

In Iran, some studies carried out on water samples in different cities, including stagnant surface water of Qazvin city (43.8%), water resources of Bojnourd (68%), hot springs of Mazandaran province (40.9%), river waters of Tonekabon region (23%). Also, some investigations performed on the water samples of swimming pools and ponds in Sistan - Baluchistan squares (47.56%) and various results reported as mentioned here (8-11).

Acanthamoeba detection is usually carried out based on the structural characteristics of the cyst through direct microscopic diagnostic methods and culture techniques. This technique has its limitations due to the impacts of cultivation conditions. In recent years, the molecular method has largely solved the problem, and the molecular test is a useful confirmation tool for Acanthamoeba differentiation from other free-living amoebae.

Due to the lack of sufficient information about the epidemiological situation of Acanthamoeba in Bandar Abbas water resources, it was necessary to conduct research on various water samples of the city, including swimming pools, squares and fountains, nearby hot springs, stagnant waters, hospital tap water, and water consumption supplies in student dormitories. Some of these areas are recreational and hydrotherapy and are available to the public. That's why it's important to follow the health tips, especially in people who use swimming pools and hot springs.

Also, free-living amoeba is reservoirs for transmission of bacterial agents such as Legionella pneumophila, Helicobacter pylori, and Vibrio Cholera (12).

A review on the published papers and articles revealed that no such study had been conducted in Bandar Abbas Hormozgan, Iran, so far. Finally, the objective of this study was to determine the frequency of free-living amoeba in Bandar Abbas water resources by cultivation and polymerase chain reaction methods.

Methods

Bandar Abbas city is the capital of Hormozgan province. The city is located in the north of the Strait of Hormuz, which is located on the shores of the Persian Gulf, which is about 45 square kilometers, and its height above sea level is 10 meters. Hormozgan province is one of the hot and dry regions of Iran and its climate is affected by semi-desert and desert climate. The climate of the coastal strip is sweltering and humid in summers, and sometimes its temperature exceeds 52 degrees Celsius. The average annual temperature in this area is about 27 degrees Celsius. Hormozgan province's climate is characterized by a long warm season and a cold short season. The warm season, along with the sultry weather, lasts nine months (13).

Samples and sampling sites

Water samples were collected from different sources such as tap water, drinking water from Hospitals, university dormitories, public swimming pools, nearby hot water springs and recreational water in November 2019 to January 2020. A total of 83 water samples in a volume of 1000 ml were collected

from 42 sites and transferred to the laboratory of Bandar Abbas, Faculty of Health for 24 hours. Figure 1 shows a geographic information system (GIS) of sampling sites of study.

Isolation and identification of Acanthamoeba

water -samples were passed through a cellulose acetate filter paper, a diameter of 0.45, micrometers by a vacuum pump. The water samples, which contained visible particles, first passed through the gas and then was filtered by the device.

The filter paper was then cultured upside down in sterile conditions on a 1.5% non-nutrient agar culture medium enriched by a layer of Escherichia coli bacteria and incubated for two days at 37C. To prevent the culture medium from drying out, around the plates were sealed and tightly wrapped by parafilm during the culture or amoeba detection on microscope. After 48-72 hours, examination of plates by reverse-phase microscope continued for one month each day to detect growth and proliferation of amoebae.

DNA extraction

The PBS added to the surface of culture plates and washed thoroughly and gently to harvest the *Acanthamoeba* from the surface of the culture medium using a sterile scraper. The collected organism kept in microtubes containing PBS, pH 7.2. Sample centrifuged 5 min in 2000rpm to remove agar and excess materials.

The DNA extraction process was performed using the DynaBio™ Blood / Tissue DNA Extraction Mini Kit (Cat # K10015) by Takapouzist company (Tehran, Iran). The DNA yield assessed by Nanodrop to estimate concentration of extracted genome.

PCR analysis

The PCR reaction was performed using JDP1-JDP2 primer pair for *Acanthamoeba that can detect the genus of organism (genus-specific primer) that gives 500 bp amplicon. Also, it can identify the particular genotypes of this amoeba (T1-T17)*. Primer pair includes the forward primer JDP1 (5-GGCCCAGATCGTTTACCGTGAA) and the reverse primer JDP2 (5-TCTCACAAGCTGCTAGGGAGTCA).

Each reaction was carried out in a final volume of 25 µl containing 1x PCR buffer, 1U Taq polymerase, 1.5 mM MgCl₂, 200 µM of dNTP, 10 pmol of each primer (TAG; Copenhagen A/S, Denmark) and 6 ml of the extracted DNA. Amplification parameters were performed in a DNA thermal cycler (Bio-RadMyCyclerThermalCy-cycler). Electrophoresis of PCR products were done using 1.5% agarose gel stained with Gel Red, and amplicons were visualized under UV light. *Acanthamoeba* T4 genotype and distilled water implemented as positive and negative control, respectively.

Phylogenetic analysis

Each purified PCR products from the water samples and reference strains were delivered for sequencing in both directions (Bioneer Corporation, Daejeon, South Korea). The Neighbor-Joining method was performed using the phylogenetic program MEGA version 10 (14) and verified by the maximum likelihood method with 1,000 bootstrap replications (15). The phylogenetic tree was rooted using *Naegleria americana*.

Results

In this study, 83 water samples from different water resources were collected in Bandar Abbas city. Totally nine samples of hospital water pipes, eight samples of hospital water coolers (water dispensers) that located for referral people and patient companions, 13 samples of water pipes of student dormitories, five samples of water fountains and city squares, 42 samples of swimming pools, three samples of hot springs and three samples of stagnant water analyzed.

Water samples that collected from resources were immediately transferred to the laboratory. Physico-chemical properties of water were recorded. Using the culture method, 31 cases (37.3%) of free-living amoeba were detected. Five positive samples of *Acanthamoeba* was identified by culture methods (6%) and 8 cases (9.6%) by molecular methods (Figure 2).

Figure 3 shows the typical cysts of *Acanthamoeba* isolated on non-nutrient agar.

Sequencing and genotype identification

Homology analyses of the PCR products were done using BLAST (Basic Local Alignment Search Tool) software from the NCBI. We entered the genes into the Mega-x software environment and performed the alignment, then repeated the phylogenetic tree with the Neighbor-Joining method and the bootstrap test 1000 times and draw a distance of 0.1 (Figure S1).

In this study, after determining the sequence of nucleotides, two genotypes were presented as *Acanthamoeba*: T4 and T15.

Discussion

Current study focused on frequency of all types of free-living amoeba with emphasis on *Acanthamoeba* in water resources of Bandar Abbas. The free-living amoebae family consists of various genus and species with different potential pathogenic ability. Water is a suitable habitat for free-living amoebae; some of them can lead to serious complications. *Acanthamoeba* found in many environmental sources, including soil, dust, freshwater, sea, pools, Jacuzzis, and hot springs. Environmental resources play a potential role in transmitting accountants species to humans and other mammals (16, 17).

Our study is the first investigation that carried out in Bandar Abbas with culture and molecular methods. The results of our study showed that frequency of free-living amoebae in different water resources of Bandar Abbas city is considerable (37.3%). The study revealed that in culture method 6% of 83 samples were positive for *Acanthamoeba*, while PCR detected 9.6%, which indicates the higher sensitivity of the molecular method in the diagnosis of *Acanthamoeba*.

In a cross-sectional study by Inese Gavarāne et al. 2018, DNA extraction from amoebic isolates and genotyping of *Acanthamoeba* species from Latvian water sources were done. The results, type of study, and method of study are consistent with the present study. Their findings indicate that tap water in Latvia may be a source of *Acanthamoeba* (18). Wegdan M. Abd El Wahab in Egypt collected 80 samples of water supplies and proved that the dominant genotype of *Acanthamoeba* is T4.

Golestani in Kashan 2018, Pezeshki 2017 in Zanzan, Fraji 2017 in Lorestan showed that frequency of *Acanthamoeba* is between 30-80% in water samples, pools tap water, and hospital pipes (19-21). These studies are in agreement with our results, except in amount of chlorine in Lorestan study that has a significant relation with organism growth and multiplication, but in our chlorine did not affect *Acanthamoeba* viability.

In most studies, sensitivity of molecular methods in the detection of free-living amoebae were higher than culture method. We used culture and molecular methods to diagnose *Acanthamoeba* simultaneously, and high sensitivity of molecular method was confirmed.

There was a statistically significant difference between cultivation and molecular methods in identifying positive cases of *Acanthamoeba* ($P > 0.001$).

Polymerase chain reaction (PCR) method has been used since 1996 in identifying *Acanthamoeba*, and a recent study on its accuracy by Boggild et al. showed that it compared favorably with smear microscopy in terms of sensitivity. Still, DNA extraction should be done in very proper and precise manner, although specificity is slightly poorer (22).

There was no significant relationship between chlorine amount and frequency of *Acanthamoeba* by culture and molecular method ($P > 0.05$). Michael Storey showed that *Acanthamoeba* cysts could remain in 100 mg/l chlorine (free and combined) for 10 min (23). In our study, *Acanthamoeba* isolated from places with 2 mg/l free chlorine.

The most positive detected cases of *Acanthamoeba* have been related to swimming pools at the temperature range of 26-30 °C. Nielsen and Naveed Ahmed Khan showed that highest growth rate for six amoebic strains tested was close to 30-32 °C (24, 25). Result didn't show any positive samples from geothermal hot spring water of Bandar Abbas. Some studies showed that based on the morphological characteristics of amoebae, 42% of warm spring waters of southwest of Iran are positive for *Acanthamoeba*, and others revealed that genotype T2, T4, T15 could exist in such environments (26, 27).

No significant statistical differences were observed between pH variable and frequency of *Acanthamoeba* using culture and molecular methods (p-value = 0.014) and p-value (p = 0.116) respectively. In the range of pH 7-8.3, more positive cases of *Acanthamoeba* were identified.

Conclusion

Due to the importance of water sanitation on public health, in order to improve the quality of water resources, monitoring infectious agents like free-living amoeba, especially in swimming pools, squares and parks, water storage, and plumbing water is necessary. On the other hand, use of sensitive molecular detection along with culture method can increase the diagnosis efficacy.

Limitations

Geothermal or hot spring waters are areas with extended regions, and we couldn't take enough samples, because obtaining representative samples of geothermal fluids requires specific sampling techniques.

Abbreviations

Not applicable

Declarations

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Authors' contributions

J S and H A conceived the project and designed the experiments.

J S, H A, and H T designed and collected samples.

J S, H A, A SM, A G, S S analyzed the data.

J S and H A supervised the collection of the samples.

J S wrote the manuscript. All authors reviewed and approved the manuscript

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Availability of data and materials

All data associated with this manuscript is inclusive in this paper.

Ethics approval and consent to participate

This work was approved (Code: IR.HUMS.REC.1397.295) by the research ethics committee of the Hormozgan University of Medical Sciences, Iran.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

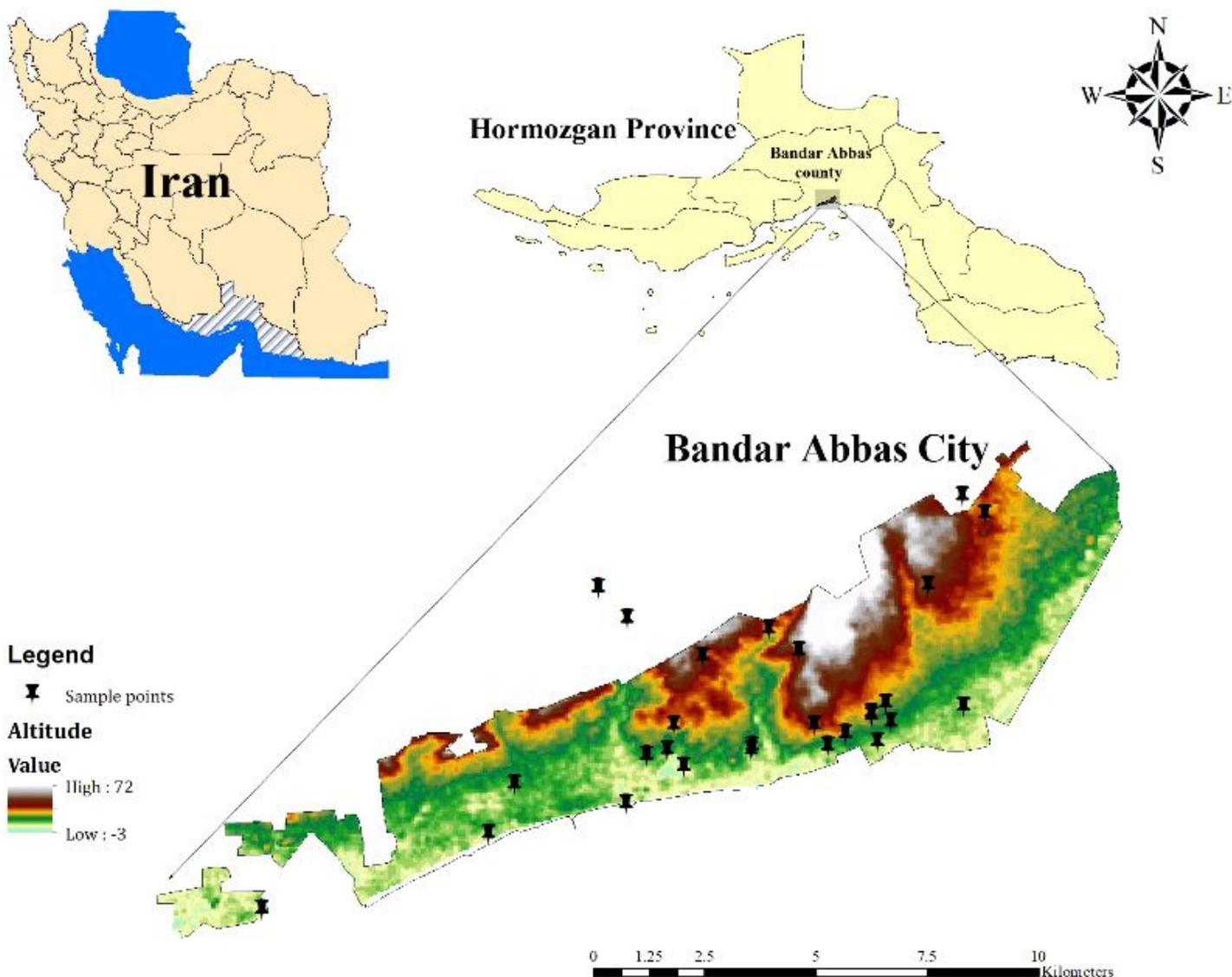


Figure 1

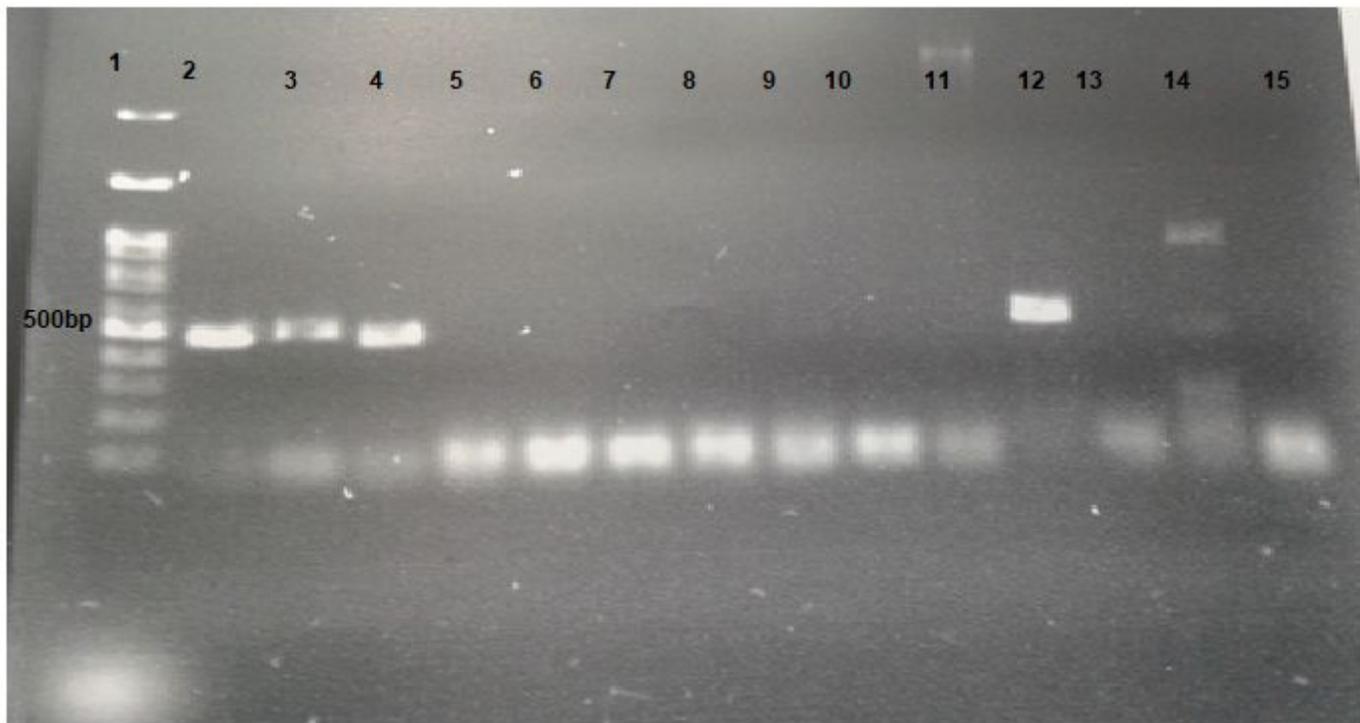


Figure 2

The PCR analysis of isolated amoeba recovered from water sample of Bandar Abbas. To confirm the presence of *Acanthamoeba*, DNA extracted from grown amoeba on NNA, and reaction done. As noted in material and method, the PCR produced 500bp amplicons. Lane1: marker: Molecular weight marker (100bp), Lane2: Positive Control, Lane3-14: Water sample, Lane15: Negative Control



Figure 3

Acanthamoeba cysts ($\times 400$) on non-nutrient agar plates when observed under inverted microscope

Supplementary Files

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