ORIGINAL ARTICLE

Isolation of Acanthamoeba species from fresh water sources in southern regions of Saudi Arabia

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ABSTRACT

Objective: The present study aimed to investigate the occurrence of *Acanthamoeba* species and other FLAs in freshwater sources in southern regions of the Kingdom of Saudi Arabia and to estimate the prevalence of their existence in these areas.
Methods: Water samples from both fresh water course and hot springs (n = 15) were collected from five different zones in Aseer region, southern western parts of the kingdom. Isolation and identification of *Acanthamoeba* species and other FLAs were carried out from these water sources (fresh water course and hot spring). The methods included filtration, *in-vitro* culture and incubation for up to three weeks for the selected samples according to standard methods.
Results: Examination of water samples by direct microscopic methods showed no amoebic parasites among all of the 15 samples collected from five different sites. However, when the water samples were cultured on non-nutrient agar (NNA), four (26.7%) of the 15 samples showed growth of *Acanthamoeba* species. Principle component analysis (PCA) indicated that the water bodies/ sources that were positive for *Acanthamoeba* spp. shared a common natural characteristic that the water bodies were exposed to contamination.
Conclusion: The study demonstrated that *Acanthamoeba* spp. were prevalent in some sites (3 out 5 sites were positives; 60%), low in two sites (1 out 3 samples) while absent in two sites (0 out of six samples). More comprehensive research is needed to find out the true prevalence of these parasites and factors affecting their existence.

Keywords: Free Living Amoeba (FLAs), *Acanthamoeba* spp., non-nutrient agar (NNA)

INTRODUCTION

Acanthamoebae are free-living aerobic amoebae and has a world-wide distribution. *Acanthamoeba* species have cysts and trophozoite stages in its life-cycle without the flagellated stage. The trophozoites replicate by mitosis. The trophozoite is 20-50µm in size with rough exterior with several spines like projections (acanthopoda) while the cyst is spherical (15µm in diameter). Equally these two forms can be the source of infection.[1-4] *A. polyphaga, A. rhysodes, A. astronyxis*, and *A. divionensis*.

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Acanthamoeba species along with its closely related free-living amoebae *Balamuthia mandrillaris* are causal agents of fatal chronic disease called granulomatous amoebic encephalitis (GAE). This is particularly serious among the immunocompromised individuals. Furthermore, *Acanthamoeba* causes Acanthamoeba keratitis which is a vision-threatening disease. Both *Acanthamoeba species* and *Balamuthia mandrillaris* cause infections of the lungs and skin.[7, 8]

Acanthamoeba spp. are abundant in nature and have worldwide distribution. They have been isolated from soil, fresh and brackish waters, bottled mineral water and many other sources of human and animal use. *Acanthamoeba* species have are tolerant of a wide range of osmolarity, enabling them to survive in distilled water, tissue culture media, mammalian body fluids, and sea water.[5, 9]

METHODS

Study Location, Sampling, isolation, and Identification of Amoebae

A total of 5 liters of water sample were collected in a sterile plastic container from five different sites at Aseer region (Fig.1). Samples were transferred to the laboratory immediately. FLA was isolated from the samples as per recommended procedures.[6]

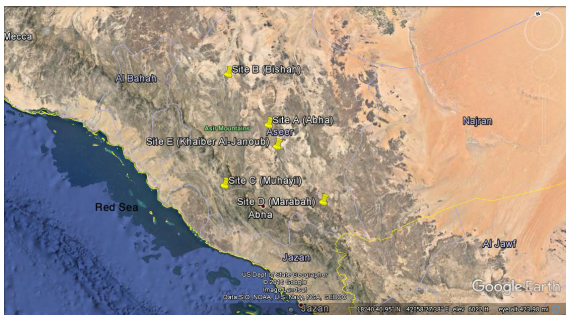


Figure 1: Map of Aseer region, KSA and cites of soil collection sites. Map was adapted from Google Earth (US State Department of Geography, 2016; http://earth.google.com/

Examination of water for parasite

Water sample was mixed thoroughly and 12 mL was transferred to a clean tube and centrifuged at 1500 rpm for 10 min. The supernatant was discarded and a drop of sediment was placed on the centre of glass slide. A cover glass was placed and the slide was examined under 100X and with 400X magnifications to look for the presence of amoeba parasites. For iodine preparation, a drop of sediment was placed on centre of glass slide and a drop of iodine was added. Then a cover glass was placed and the slide before examination.
Smears were also prepared from the sediment and Giemsa staining was performed on methanol fixed, dried slides. 10% Giemsa stain was added and slide was left for 25 minutes. Slide was then washed with distilled water, dried and examined under 1000X magnification

Cultivation of *Acanthamoeba* spp

Water samples (approximately 5 liters were filtered through 0.45µm pore size cellulose nitrate membrane filter (47mm in diameter) under a vacuum. The membrane filter for each water sample was scraped and the collected materials were placed in 15 mL of sterile cover tubes containing 10 mL phosphate buffered saline (PBS). Tubes were incubated at room temperature overnight and then centrifuged for ten minutes at 1500 rpm to collect particles on filters. After centrifugation, the supernatant solution was discarded and the pellet was inoculated onto 1.5% non-nutrient agar (NNA) plates. A dense suspension of heat inactivated *Escherichia coli*, prepared in saline was seeded onto NNA plates to grow amoebae. After the inoculation of the samples, all plates were incubated at 30°C and examined daily for the presence of FLAs for up to 15 days using a light microscope (100x).

Morphological characterization of *Acanthamoeba* isolates

Amoeba cysts that were isolated from culture were stained with haematoxylin/eosin[10] and were directly examined under the microscope and identified. *Acanthamoeba* cysts (*Acanthamoeba* sp. I to III) were established as per as known scheme.[11]
Principle component analysis (PCA) was done to see whether water sources cluster with the presence of Acanthamoeba spp. PCA was done using PAST software (PAST Version 3.14; Øyvind Hammer, Natural History Museum, University of Oslo, 1999-2016).

RESULTS

In this study out of five sites in Aseer, three sites demonstrated the presence of *Acanthamoeba* spp., whereas 2 sites were negative with all of the three testes used. The overall prevalence of *Acanthamoeba* spp. in the five sites was estimated to be 26.7% (4/15*100) (Table -1).

Water collection regions	Sites	Description of water sources	Direct preparation for parasite	Iodine preparation for parasite	Smear from NNA (2 weeks)
Sites A: Abha	A1	Abha's dam	-	-	+
	A2	Well inside farms where export vegetables to the markets	-	-	+
	A3	swimming pool of famous gym (Alnakhil) in abha	-	-	-
Sites B: Bishah	B1	King Fahad Dam	-	-	-
	B2	Well inside farms where the animal drinks from it	-	-	-
	B3	swimming pool of famous gym	-	-	-
Sites C: Muhayil	C1	Muhayil valley	-	-	+
	C2	Well in village in Muhayil people use it for cooking	-	-	-
	C3	hot spring water	-	-	-
Sites D: Marabah	D1	Marabah's valley extend to Jazan	-	-	-
	D2	Still water which stray animals like dogs or cats can drink of it	-	-	-
	D3	Water tank beside mosque, people use it for ablution	-	-	-
Sites E: Khyber Al Janoub	E1	Almserg dam has benefit in groundwater recharge	-	-	+
	E2	Open well in farm	-	-	-
	E3	Swimming pool in house	-	-	-

Table 1. Detection of *Acanthamoeba* spp. in five sites in Aseer region, Saudi Arabia using microscopic and culture methods

Examination of water samples by direct microscopic methods showed no amoebic parasites among all of the 15 samples collected from five different sites (Table 1). When the samples cultured on non-nutrient agar (NNA), four (26.7%) of the 15 samples showed growth of *Acanthamoeba* species and were demonstrated microscopically (Fig. 2).

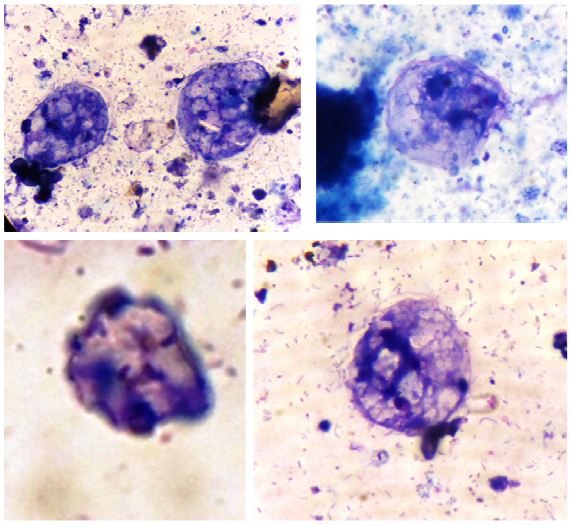


Figure 2. Different *Acanthamoeba* spp. shown on NNA incubated at room temperature for 2 weeks

PCA indicated that water sources that were positive for *Acanthamoeba* spp. shared a common natural characteristic. This included water sources exposed to contamination (Fig. 3).

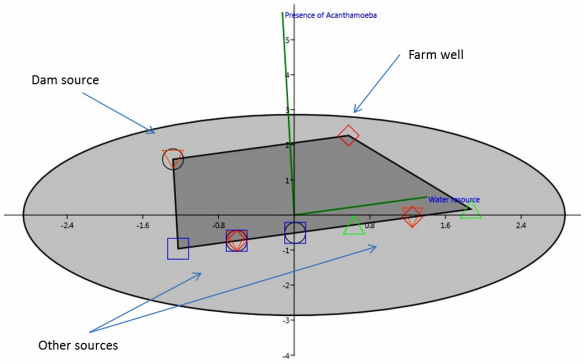


Figure 3. Plot of water bodies/ sources against results of *Acanthamoeba* spp. Principle component analysis (PCA) was done using PAST software (version 3.14). Note the clustering of sites with positive *Acanthamoeba* spp. spp. (A1, E1).

DISCUSSION

In the present study 26.7% prevalence of *Acanthamoeba* spp. was demonstrated microscopically from all 15 samples representing 5 geographical sites in Aseer region. NNA culture method has proven more sensitive than direct examination of the water deposits (Table 1). FLA**s** are distributed worldwide and the composition of these species at certain locations depends on the surroundings. FLA**s** are getting increasing attention as reservoirs and potential vectors for the transmission of pathogenic bacteria. [12, 13] Studies have shown that the food-borne and opportunistic pathogens are able to interact with species of *Acanthamoeba* and more so in *A. polyphaga*. These pathogens multiply intracellularly within the FLAs and in theory able to spread in the environment and to contribute to food contamination.[14]

In the environment, there exist many genera of pathogenic protozoa of both human and animal origin but only limited information is available regarding these organisms in developing countries. [15, 16] The spread of FLAs species depends on its tolerance to survive under adverse conditions. Therefore, the ecological importance of FLAs must be adequately studied to prevent occurrence of fatal human diseases. [17, 18] The presence of FLAs in tap

water may represent a health risk to both immunocompromised and immunocompetent individuals. [19] One of the earlier studies from this part of the kingdom has emphasized upon the hazards of water sources to human regarding the prevalence of *Acanthamoeba* spp. In their study, authors could isolate 33 strains of *Acanthamoeba* species from water sources at Wadi Hanifah, Saudi Arabia using non-nutrient agar with 1%NaCl added. The isolates were identified as *A. astronyxis, A. comandoni, A. culbertsoni* and *A. quina*. The optimum and maximum tolerated temperatures for the growth as well as the pathogenicity of each strain for mice were also recorded. [20] *Acanthamoeba* species are now becoming opportunists particularly in patients infected with (Human Immunodeficiency Virus (HIV) and a lack of effective therapeutic treatment represents a major problem and future emphasis must be placed on clinical trials dealing with the management of *Acanthamoeba* infections in patients with AIDS.[21]

The study concluded that *Acanthamoeba* spp. were common in some sites (66.7%) while absent or not detected in others or recorded low occurrence (33.3%). Further investigation covering larger area and sites is required to uncover the true prevalence and factors affecting the presence *Acanthamoeba* spp. in this part of the country.

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Conflict of interest

The authors declare that they have no conflicts of interest.

REFERENCES

1. Band RN Mohrlok S. The cell cycle and induced amitosis in Acanthamoeba. J Protozool; 1973; 20: 654-657.
2. Alves Dde S, Moraes AS, Alves LM, Gurgel-Goncalves R, Lino Junior Rde S, Cuba-Cuba CA, et al. Experimental infection of T4 Acanthamoeba genotype determines the pathogenic potential. Parasitol Res; 2016; 115: 3435-3440.
3. Cabello-Vilchez AM, Reyes-Batlle M, Montalban-Sandoval E, Martin-Navarro CM, Lopez-Arencibia A, Elias-Letts R, et al. The isolation of Balamuthia mandrillaris from environmental sources from Peru. Parasitol Res; 2014; 113: 2509-2513.
4. Wyburn-Mason R. The naeglerial causation of rheumatoid disease and many human cancers. A new concept in medicine. Med Hypotheses; 1979; 5: 1237-1249.
5. Visvesvara GS, Moura H Schuster FL. Pathogenic and opportunistic free-living amoebae: Acanthamoeba spp., Balamuthia mandrillaris, Naegleria fowleri, and Sappinia diploidea. FEMS Immunol Med Microbiol; 2007; 50: 1-26.
6. Visvesvara GS. Pathogenic and opportunistic amebae. In: Murray PR, Baron EJ, Jorgensen JH, Landry ML and Pfaller MA, (eds.). *Manual of Clinical Microbiology*. Washington, DC, USA: ASM Press, 2007, p. 2082–2091.
7. Satlin MJ, Graham JK, Visvesvara GS, Mena H, Marks KM, Saal SD, et al. Fulminant and fatal encephalitis caused by Acanthamoeba in a kidney transplant recipient: case report and literature review. Transpl Infect Dis; 2013; 15: 619-626.
8. Sison JP, Kemper CA, Loveless M, McShane D, Visvesvara GS Deresinski SC. Disseminated acanthamoeba infection in patients with AIDS: case reports and review. Clin Infect Dis; 1995; 20: 1207-1216.
9. Lorenzo-Morales J, Ortega-Rivas A, Foronda P, Martinez E Valladares B. Isolation and identification of pathogenic Acanthamoeba strains in Tenerife, Canary Islands, Spain from water sources. Parasitol Res; 2005; 95: 273-277.
10. Grossniklaus HE, Waring GOt, Akor C, Castellano-Sanchez AA Bennett K. Evaluation of hematoxylin and eosin and special stains for the detection of acanthamoeba keratitis in penetrating keratoplasties. Am J Ophthalmol; 2003; 136: 520-526.
11. Kong HH Chung DI. A riboprinting scheme for identification of unknown Acanthamoeba isolates at species level. Korean J Parasitol; 2002; 40: 25-31.
12. Walochnik J, Picher O, Aspock C, Ullmann M, Sommer R Aspock H. Interactions of "Limax amoebae" and gram-negative bacteria: experimental studies and review of current problems. Tokai J Exp Clin Med; 1998; 23: 273-278.
13. Scheid P Schwarzenberger R. Acanthamoeba spp. as vehicle and reservoir of adenoviruses. Parasitol Res; 2012; 111: 479-485.
14. Anacarso I, de Niederhausern S, Messi P, Guerrieri E, Iseppi R, Sabia C, et al. Acanthamoeba polyphaga, a potential environmental vector for the transmission of food-borne and opportunistic pathogens. J Basic Microbiol; 2012; 52: 261-268.
15. Bonilla-Lemus P, Caballero Villegas AS, Carmona Jimenez J Lugo Vazquez A. Occurrence of free-living amoebae in streams of the Mexico Basin. Exp Parasitol; 2014; 145 Suppl: S28-33.
16. Shanan S, Abd H, Bayoumi M, Saeed A Sandstrom G. Prevalence of protozoa species in drinking and

environmental water sources in Sudan. Biomed Res Int; 2015; 2015: 345619.
17. Adekambi T, Ben Salah S, Khlif M, Raoult D Drancourt M. Survival of environmental mycobacteria in Acanthamoeba polyphaga. Appl Environ Microbiol; 2006; 72: 5974-5981.
18. Winiecka-Krusnell J Linder E. Free-living amoebae protecting Legionella in water: the tip of an iceberg? Scand J Infect Dis; 1999; 31: 383-385.
19. Thomas V, McDonnell G, Denyer SP Maillard JY. Free-living amoebae and their intracellular pathogenic microorganisms: risks for water quality. FEMS Microbiol Rev; 2010; 34: 231-259.
20. Al-Herrawy AZ Al-Rasheid KA. Identification of Acanthamoeba strains isolated from a freshwater course in Saudi Arabia. J Egypt Public Health Assoc; 1998; 73: 621-633.
21. Bottone EJ. Free-living amebas of the genera Acanthamoeba and Naegleria: an overview and basic microbiologic correlates. Mt Sinai J Med; 1993; 60.