Original Article

Acanthamoeba species in Swimming Pools of Cairo, Egypt

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Abstract

Background: The free-living amoebae Acanthamoeba spp. have been recognized as etiologic agents of amoebic encephalitis, keratitis, otitis, lung lesions and other skin infections mainly in immuno-compromised individuals. The purpose of this study is to detect the presence of Acanthamoeba in swimming pools in Egypt using a polymerase chain reaction (PCR) method.

Methods: Water samples were collected from 10 different swimming pools in Cairo, Egypt. Samples were cultured on non-nutrient agar for the detection of Acanthamoeba isolates that were confirmed by PCR amplification using genus specific primers. The molecularly confirmed Acanthamoeba isolates were morphologically identified to the species level.

Results: Members of genus Acanthamoeba were detected in 49.2% of the examined swimming-pool water samples. Morphologically, six Acanthamoeba species were isolated from the examined swimming pool water namely A. polyphaga, A. castellanii, A. rhyzodes, A. mauritaniensis, A. royreba and A. triangularis. All the identified species of Acanthamoeba were molecularly confirmed to be related to the genus Acanthamoeba.

Conclusion: The isolated species of Acanthamoeba could provoke variable degrees of infections to the swimmers. The culture method is cheaper and easier than PCR techniques that are faster for the detection of free-living amoebae.

Keywords: Acanthamoeba, Cyst morphology, PCR, Swimming pools, Egypt

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Introduction

Most species of genus Acanthamoeba are free-living protozoan potential pathogens that have gained increasing attention during the last few decades due to their ability to produce serious, as well as fatal, human and animal infections (1). These infections are documented as skin, nasal passages, lung, and brain lesions (2-5). "In ad-
dition to its natural distribution, *Acanthamoeba* can be opportunistically pathogenic, being identified as the causative agent of a painful and sight-threatening infection of the cornea, *Acanthamoeba keratitis* (AK)” (6). “Variations in the pathogenicity of different *Acanthamoeba* strains have been recognized in laboratory studies, but the relevance of these results to human disease is unclear ”(6).

*Acanthamoeba* species are presented worldwide in fresh water as well as in marine water. Moreover, they have been recovered from various domestic water systems such as drinking tap water (7), cooling towers (8), swimming pools (9), hydrotherapy baths (10) and hospital water networks (11). Waterborne transmission, acquired through forceful inhalation of surface waters or poorly maintained swimming pools, is uncommon (12).

"Traditional taxonomy of *Acanthamoeba* has used morphological features, such as cyst morphology and trophozoite size and shape, as classification characters "(6). Species of *Acanthamoeba* are categorized into three morphological groups based largely on exocyst and endocyst criteria as well as number and shape of cyst pores. Detection of *Acanthamoeba* can be improved by means of a molecular detection of the organisms by polymerase chain reaction (PCR). This technique can detect the presence of DNA specific to *Acanthamoeba* present even in small amounts which can be missed by culture techniques (13). A previous estimate of domestic tap water *Acanthamoeba* colonization has been reported but as the culture method on non-nutrient agar was used the level may have been underestimated (14). Data collected over the last decade now allow us to quickly analyze environmental samples using molecular methods to determine and classify the *Acanthamoeba* genotype (15, 16).

In the present study, we examined *Acanthamoeba* isolates obtained from swimming pools of Cairo, Egypt. Isolates of free-living amoebae having finger-like pseudopodia were examined by PCR using genus-specific primers for *Acanthamoeba*. PCR-confirmed isolates of *Acanthamoeba* were identified morphologically to the species level.

Materials and Methods

Samples and sampling sites

Water samples (1 liter volume each) were collected monthly from ten different swimming pools in Cairo, Egypt for one year period. Samples were collected in clean, dry autoclavable polypropylene containers and sent to the laboratory of parasitology, water pollution Research Department, National Research Center, in icebox and processed at the same day of collection.

Isolation of *Acanthamoeba* spp. from water samples

Collected swimming pool-water samples were separately concentrated by using the membrane filtration technique. One liter of each water sample was filtered through a nitrocellulose membrane filters (0.45μm pore size and 47mm in diameter) (Whatman, WCN type, Cat No. 7141-104) (17). After filtration the membranes were separately inverted face to face on the surface of a non-nutrient (NN) agar plates previously seeded with 100μl *Escherichia coli* suspension. All the inoculated plates were incubated at 40°C for one week with daily microscopic examination for the presence of any amoebic growth (18). Identification of the obtained *Acanthamoeba* spp. were achieved according to the morphological characteristics of both trophozoite (presence of finger-like tapering pseudopodia) and cyst(inner wall often polygonal or stellate and outer wall often rippled or wrinkled) stages and resulted in the classification of the isolates as *Acanthamoeba* species (19).

Molecular characterization of isolated freshwater amoebae using polymerase chain reaction (PCR)

DNA extraction: The amoebae pellet was resuspended in lysis buffer containing 2% CTAB as described by Winnepenninx et al.
(20) and modified by Abdel-Hamid et al. (21), overlaid with 500 ml of phenol-chloroform-isooamylalcohol (PCI), and shaken gently for 5 hr. The suspension was centrifuged at 3000 xg for 10 min, and the upper, aqueous phase was transferred to a new tube. PCI extraction was repeated two times for 10 min each time. DNA was precipitated at -80°C overnight, pelleted at 12000 xg for 30 min at 4°C, washed in 70% ethanol, air dried, and re-suspended in 30 ml of sterile double-distilled water (22).

**Polymerase Chain Reaction (PCR):** For molecular identification, the genus specific primers were used. Forward primer sequence *(5'\TTTGAATTGC\TTAAT\GGTA\TAGA\TT\TATATTAA\TT\TTTT\TT\T3')* and Reverse primer *(5'\TTTGAATTCAGA\AA-GAGCTATCA\ATCTGT\TT\TT\TT\TT\T3)* Kilic et al. (23). All amplification reactions of PCR were performed in a 50 μl. PCR consisted of 1 min denaturation at 94°C, 1 min annealing at 56°C and 1 min elongation at 72 °C for 35 cycles. After that, 10 min of extension time at 72 °C was done. Finally, the PCR products were checked by electrophoresis in a 1.5 % agarose gel (24).

**Morphological identification of the PCR-confirmed isolates of Acanthamoeba**

The confirmed isolates of *Acanthamoeba* by PCR technique were morphologically identified to the species level using the method of Pussard and Pons (17) based on assessment of the size and shape of the endo- and ectocysts and the mean number of opercula (17, 25-27).

**Results**

**Prevalence of Acanthamoeba in the examined swimming pools**

*Acanthamoeba* species were detected in 59(49.2%) water samples collected from 10 swimming pools in Cairo (Table 1).

**Table 1:** Prevalence of *Acanthamoeba* spp. in swimming pool samples

| Swimming pools | Examined samples (n) | *Acanthamoeba* spp. |
|----------------|----------------------|---------------------|
|                |                      | No. | %     |
| 1              | 12                   | 7   | 58.3  |
| 2              | 12                   | 3   | 25.0  |
| 3              | 12                   | 6   | 50.0  |
| 4              | 12                   | -   | -     |
| 5              | 12                   | 5   | 41.7  |
| 6              | 12                   | 10  | 83.3  |
| 7              | 12                   | 3   | 25.0  |
| 8              | 12                   | 8   | 66.7  |
| 9              | 12                   | 7   | 58.3  |
| 10             | 12                   | 10  | 83.3  |
| Total          | 120                  | 59  | 49.2  |

**Fig. 1:** Occurrence of *Acanthamoeba* spp. in swimming-pool samples
Water samples collected from swimming pools number 6 and 10 showed the highest incidence of heat-tolerant *Acanthamoeba* species (83.3%). The heat-tolerant *Acanthamoeba* species were not recorded in water samples collected from swimming pool number 4. In addition, swimming pool number 2 recorded the least incidence of heat-tolerant *Acanthamoeba* species (25.0%) (Table 1, Fig. 1).

**PCR product of genus Acanthamoeba**

94.9% of microscopically *Acanthamoeba* +ve swimming pool samples were also +ve by using PCR technique.

Microscopically *Acanthamoeba* +ve swimming pool samples collected from site 1 (n=7), 3 (n=6), 5 (n=5), 6 (n=10), 8 (n=8) and 10 (n=10) were all +ve by PCR. 85.7, 66.7 and 66.7% of microscopically *Acanthamoeba* +ve swimming pool samples collected from sites 9, 2 and 7, respectively, proved to be +ve by PCR. Electrophoresis of amplification products from 18S rDNA of different *Acanthamoeba* species were subjected to electrophoresis on 1.5% agarose gel parallel containing ethidium bromide to 100 bp DNA ladder and products from control negative bacteria, where 910-1170 bp specific amplification products were visualized in most of environmental samples tested that were not evidenced in the negative control (Fig. 2).

**Species identification of molecularly confirmed Acanthamoeba isolates**

Identification of the different species of *Acanthamoeba* was performed according to the shape and size of cysts in addition to the number, shape, size and arrangement of the cyst pores.

Six species of *Acanthamoeba* could be morphologically recognized, namely *Acanthamoeba castellanii*, *A. polyphaga*, *A. rhyzodes*, *A. mauritanensis*, *A. triangularis* and *A. royreba* (Fig. 3).

**Discussion**

The present study deals with the natural distribution of members of the genus *Acanthamoeba* in the examined swimming-pool water of Cairo, Egypt. To the best of our knowledge, few studies were conducted reporting the detection and existence of *Acanthamoeba* in Egypt (25, 27-29).

**Prevalence of heat tolerant free-living amoebae in different types of water**

Free-living amoebae were isolated at 37°C from 73.3% of the examined swimming pool samples. In Egypt, a lower incidence of free-living amoebae (32%) in swimming pools (28). Other workers in Poland detected free-living amoebae in 59.7% of the examined swimming pool samples (30). In the present study, free-living amoebae were isolated from 60% of the swimming pool samples.
In Poland, Gronik and Kuzna-Grygiel, (30) recorded a lower incidence of free-living amoebae (37.2%) isolated at 42 °C from swimming pools.

**Morphological characterization of genus Acanthamoeba**

In the present study, it was shown that the trophozoites of species of *Acanthamoeba* were characterized by finger-like cytoplasmic projections (acanthopodia) used for locomotion. However, the cyst forms of *Acanthamoeba* were characterized by their clearly distinguishable double cyst walls that varied in shape according to species. *Acanthamoeba* possesses a distinctive large nucleolus and contractile vacuole, have slender acanthopodia, form cysts with wrinkled or ripple walls and moves in a slow slug-like fashion. Previous workers used the same criteria for the differentiation between *Acanthamoeba* species and other free-living amoebae (17, 20, 27, 31-33).

**Molecular Characterization of isolated free-living amoebae**

Swofford (34) stated that "in the past 20 years", molecular methods for characterizing pathogen strains have taken a center stage as modern approaches in diagnostic and epidemiological studies of infectious diseases. These techniques are more sensitive than the conventional morphological and biochemical methods, since DNA amplifications can be achieved from a single cell (35). Although rDNA sequencing provides detailed information, these methods are expensive and are not common in many laboratories. On the other hand, PCR-based restriction analyses are
more applicable in developing countries (35). In the present study the morphologically identified free-living amoebae belonging to the genera *Acanthamoeba* were confirmed by PCR using genus-specific primers.

Our result showed that 96.5% out of 141 morphologically *Acanthamoeba* +ve samples (i.e. 56.0% of the total examined) were also *Acanthamoeba* +ve by PCR. In Egypt, Lorenzo-Morales et al., (36) detected a lower incidence of *Acanthamoeba* (43.3%) in freshwater samples using a genus-specific primer. Other workers in Turkey and UK observed that 100% of freshwater samples exhibited *Acanthamoeba* by using genus-specific primers (35, 23).

By using PCR technique in the present study, the incidence of *Acanthamoeba* spp. in swimming pool samples reached 94.9% out of 59 morphologically *Acanthamoeba* +ve samples (i.e. 49.2% of the total examined) using genus-specific primers for *Acanthamoeba* species. In Iran Maghsood et al., (37) molecularly identified *Acanthamoeba* spp. in 11 (91.6%) environmental samples out of 12 mountain pool water samples and they also identified the same *Acanthamoeba* spp. from clinical samples of *Acanthamoeba* keratitis patients. Other workers in Taiwan identified *Acanthamoeba* spp. in a much lower incidence (16.4%) from swimming pool samples by using genus-specific primers (38).

**Conclusion**

The use of molecular methods to identify free-living amoebae of genus *Acanthamoeba* could provide a more rapid means to diagnose infections caused by those amoebae. The culture method is more reliable, easier and sensitive than direct DNA extraction and analysis for the detection of *Acanthamoeba* species.

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The authors declare that there is no conflict of interests.

**References**

1. Visvesvara GS, Moura H, Schuster FL. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. FEBS Immunol Med Microbiol. 2007; 50: 1–26.
2. Marciano-Cabral F, Cabral GA. *Acanthamoeba* spp. as agents of disease in humans. Clin Microbiol Rev. 2003; 16:273–307.
3. Marciano-Cabral F, Puffenbarger R, Cabral GA. The increasing importance of *Acanthamoeba* infections. J Eukaryot Microbiol.2000; 47:29–36.
4. Martinez AJ, Visvesvara GS. Free-living amphyzoic and opportunistic amebas. Brain Pathol.1997; 7:583–598.
5. Moura H, Wallace S, Visvesvara GS. *Acanthamoeba* beadyn.sp.and the isozyme and immunoblot profiles of *Acanthamoeba* spp., groups 1 and 3. J Protozool.1992; 39:573–583.
6. Booiton GC, Visvesvara GS, Byers TJ, Kelly DJ, Fuerst PA. Identification and Distribution of *Acanthamoeba* Species Genotypes Associated with Non-keratitis Infections. J Clin Microbiol. 2005; 43: 1689–1693.
7. Michel R, Muller KD, Amann R, Schmid EN. Legionella-like slender rods multiplying within a strain of *Acanthamoeba* spp. isolated from drinking water. Parasitol Res. 1998; 84: 84-88.
8. Barbaree JM, Fields BS, Feeley JC, Gorman GW, Martin WT. Isolation of protozoa from water associated with a legionellosis outbreak and demonstration of intracellular multiplication of *Legionella pneumophila*. Appl Environ Microbiol. 1986; 51: 422-424.
9. Rivera F, Ramirez P, Vilaclosa G, Robles E, Medina F. A survey of pathogenic and free-living amoebae inhabiting swimming pool water in Mexico City. Environ Res.1983; 32: 205-211.
10. Scaglia M, Strosselli M, Grazzioli V, Gatti S, BernuzziAM,DeJonckheere JF. Isolation and identification of pathogenic *Naegleria australiensis* (Amoebida, Vahlkampfidae) from spa in northern Italy. Appl Environ Microbiol.1983; 46: 1282-1285.
11. Thomas V, Herrera-Rimann K, Blanc DS, Greub G. Biodiversity of amoebae and amoeba-resisting bacteria in a hospital water network. Appl Environ Microbiol.2006; 72: 2428-2438.

Available at: [http://ijpa.tums.ac.ir](http://ijpa.tums.ac.ir)
12. Karonis P, Kourenti C, Smith H. Waterborne transmission of protozoan parasites: A worldwide review of outbreaks and lessons learnt. J Wat Health. 2007; 5: 1-38.

13. Lehmann OJ, Green SM, Morlet N, Kilvington S, Keys MF, Matheson MM, Dart JK, McGill JI, Watt PJ. Polymerase chain reaction analysis of corneal epithelial and tear samples in the diagnosis of Acanthamoeba keratitis. Invest Ophthalmol Vis Sci. 1998; 39:1261–1265.

14. Houang E, Lam D, Fan D, Seal D. Microbial keratitis in Hong Kong: relationship to climate, environment and contact-lens disinfection. Trans R Soc Trop Med Hyg. 2001;95:361–367.

15. Booton GC, Kelly DJ, Chu YW, Seal DV, Houang E, Lam DSC, Byers TJ, Fuerst PA. 18S ribosomal DNA typing and tracking of Acanthamoeba species isolates from corneal scrape specimens, contact lenses, lens cases, and home water supplies of Acanthamoeba keratitis patients in Hong Kong. J Clin Microbiol. 2002; 40:1621–1625.

16. Booton GC, Rogerson A, Bonilla TD, Seal DV, Kelly DJ, Beattie TK, Tomlinson A, Lares-Villa F, Fuerst PA, Byers TJ. Molecular and physiological evaluation of subtropical environmental isolates of Acanthamoeba spp., causal agent of Acanthamoeba keratitis. J Eukaryot Microbiol. 2004; 51:192–200.

17. Pussard M, Pons R. Morphologie de la parakystique et taxonomie du genre Acanthamoeba (Protozoa, Amoebida). Protistol. 1977; TXIII: 557–598.

18. De Jonckheere J, Van Dijk P, De Voord H. Evaluation of the indirect fluorescent antibody technique for identification of Naegleria species. Appl Microbiol.1974; 28: 159–164.

19. Page FC. Nackete Rhizopoda, p. 3–145. In: D. Matthes (ed.), Protozoen fauna, Band 2. G. Fischer, Stuttgart, Germany.1991.

20. Winnepenninckx B, Backeljau T, de Wachter R. Extraction of high molecular weight DNA from mollusca. Trends Gen. 1993; 9: 407.

21. Abdel-Hamid AZ, Molfetta JB, Fernandez V, Rodrigues V. Genetic variation between susceptible and non-susceptible snails to Schistosoma infection using random amplified polymorphic DNA analysis (RAPDs). Rev Inst Med Trop. 1999; 41: 291-295.

22. Walochnik J, Obwaller A, Aspock H. Correlation between morphological, molecular, biological and physiological characteristics in clinical and nonclinical isolates of Acanthamoeba spp. Appl Environ Microbiol. 2000; 66: 4408-4413.

23. Kilic A, Tanyuksel M, Sissons J, Jayasekera S, Khan N. Isolation of Acanthamoeba isolates belonging to T2, T3, T4, and T7 genotypes from environmental samples in Ankara, Turkey. Acta Parasitol. 2004; 49: 246–252.

24. Helling RB, Goodman HM Boyer HW. Analysis of R. EcoRI fragments of DNA from lambdoid bacteriophages and other viruses by agarose-gel electrophoresis. J Virol. 1974; 14: 1235–38.

25. Al-Herrawy AZ. In vitro cultivation of agents of amoebic meningo-encephalitis isolated from water and sewage. Ph.D. thesis, Fac. Vet. Med., Alexandria Univ., Egypt. 1992.

26. Page F. A New Key to Freshwater and Soil Gymnamoebae. Freshwater Biol. Ass: Ambleside, 1988.

27. Al-Herrawy AZ, Bahgat M, Mohammed A, Ashour A, Hikal W. Morpho-physiological and biochemical criteria of Acanthamoeba spp. isolated from the Egyptian aquatic environment. Iranian J Parasitol. 2013; 8: 302-312.

28. Hilali M, Ashmawy K, Samaha H, Draz A, Abu El-Wafa S, Salem A. Preliminary studies on amoebic pathogens isolated from water and sewage with respect to Naegleria and Acanthamoeba. J Egypt Vet Med Ass. 1994; 53: 215-224.

29. Ashmawy K, Hilali M, Abu El-Wafa SA, Samaha H, Draz AA, Salem A. In vitro identification of Naegleria and Acanthamoeba isolated from water and sewage. Assiat Vet Med J. 1993; 30: 87-100.

30. Gronik K, Kuzna-Grygiel W. Presence of virulent strains of amphihoic amoebae in swimming pools of the city of Szczecin. Ann Agric Environ Med. 2004; 11: 233-236.

31. Page FC. An Illustrated Key to Freshwater and Soil Amoebae. Freshwater Biol. Ass. 1976; ISSN 0367-18887.SBN 900386266.

32. Chang SL. Etiological, pathological, epidemiological and diagnostic considerations of primary amoebic meningo-encephalitis. Crit Rev Microbiol. 1974; 3: 135-159.

33. Schuster FL, Visvesvaras GS. Opportunistic amoebae: challenges in prophylaxis and treatment. Drug Resistance Updates.2004; 7:41-51.
Al-Herrawy et al.: Acanthamoeba species in Swimming pool...

34. Swofford D. Phylogenetic analysis using parsimony and other methods, PAUP*4.0b. Sunderland, MA, USA: Sinauer Associates.1998.
35. Khan NA, Paget TA. Molecular tools for speciation and epidemiological studies of Acanthamoeba. Curr Microbiol. 2002; 44: 444-449.
36. Lorenzo-Morals J, Ortiz-Rivas A, Martinez E, Khoubbane M, Artigas P, Periago MV, Foronda P, Abreu-Acosta N, Valladares B, Mas-Coma S. Acanthamoeba isolates belonging to T1, T2, T3, T4 and T7 genotypes from environmental freshwater samples in the Nile Delta region, Egypt. Acta Trop. 2006; 100: 63-69.
37. Maghsood A, Sissons J, Rezaian M, Nolder D, Warhurst D Khan NA. Acanthamoeba genotype T4 from the UK and Iran and isolation of the T2 genotype from clinical isolates. J Med Microbiol. 2005; 54: 755-759.
38. Hsu B, Ma P, Liou T, Chen J, Shine F. Identification of 18S ribosomal DNA genotype of Acanthamoeba from hot spring recreation areas in the central range, Taiwan. J Hydrobiol. 2009; 367: 249-254.

Available at: http://ijpa.tums.ac.ir