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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. rhysodes, 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

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 sample 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 Winnepenninckx et al.

(20) and modified by Abdel-Hamid et al. (21), overlaid with 500 ml of phenol-chloroform-isoamylalcohol (PCI), and shaken gently for 5 hr. The suspension was centrifuged at 3000 x*g* 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 x*g* 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 (5ÕTTTGAATTCGCTCCAA-TAGCGTATATTAA-3Õ) and Reverse primer (5ÕTTTGAATTCAGA-AA-GAGCTATCAATCTGT-3Õ) Kilic et al. (23). All amplification reactions of PCR were performed in a 50 µl. PCR consisted of 1 min denaturation at 94°C, 1min annealing at 56°C and 1 min elongation at 72 °C for 35 cycles. After that, 10min of extension time at 72 °C was done. Finally, the PCR products were cheeked 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

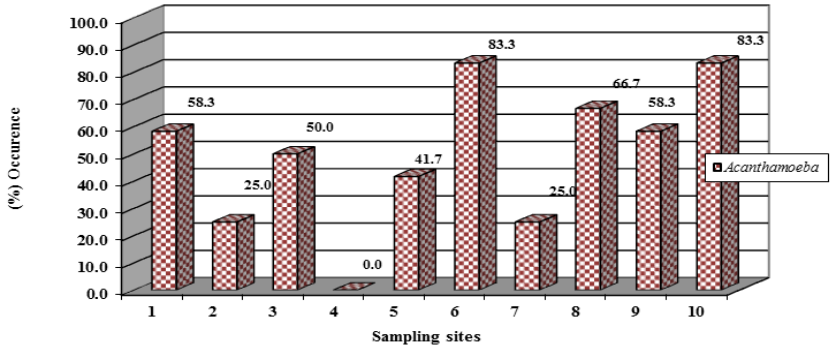

Fig. 1: Occurrence of *Acanthamoeba* spp. in swimming-pool samples

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. rhysodes, A. mauritaniensis, A. triangularis* and *A. royreba* (Fig. 3).

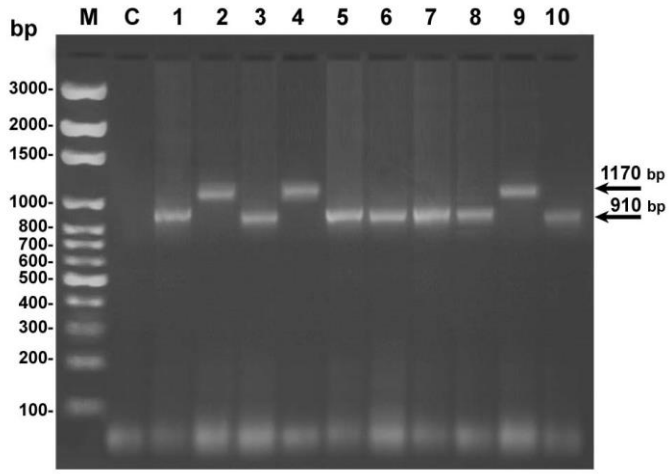

Fig. 2: Agarose gel electrophoresis showing amplification of 18S rDNA of different *Acanthamoeba* isolates were subjected to electrophoresis on 1.5% agarose gel parallel containing ethidium bromide to 100bp DNA. C: negative control bacteria; 1: *A. polyphaga*; 2: *A. mauritaniensis*;3: *A. castellanii*;4: *A. polyphaga*;5: *A. royreba*;6: *A. castellanii*;7: *A. triangularis*; 8: *A. rhysodes*;9: *A. castellanii*;10: *A. polyphaga*. M: 100bp DNA ladder

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.

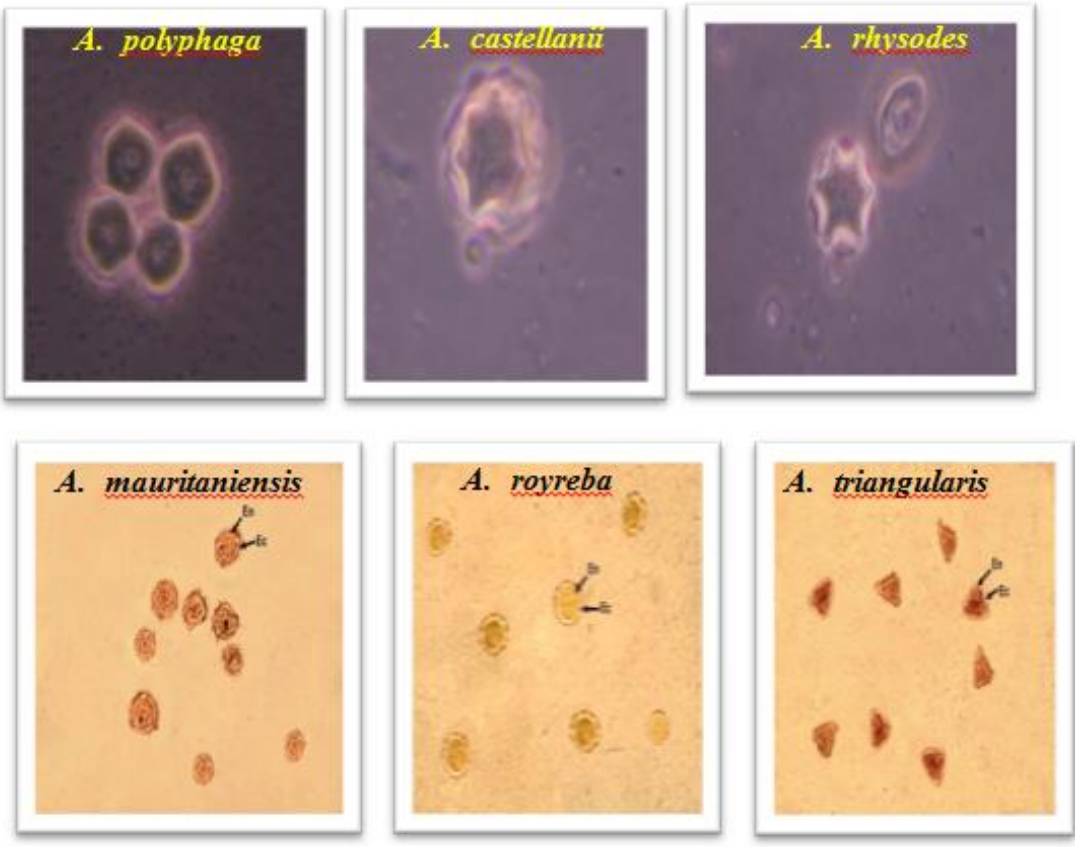

Fig. 3: Cyst stage of different *Acanthamoeba* isolates from swimming- pool water samples

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* keratitic 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.

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