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ORIGINAL PAPER

Genotypic, physiological, and biochemical characterization of potentially pathogenic *Acanthamoeba* isolated from the environment in Cairo, Egypt

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Abstract *Acanthamoebae* are the most common opportunistic amphizoic protozoa that cause life-threatening granulomatous amoebic encephalitis in immunocompromised individuals and sight-threatening amoebic keratitis (AK) in contact lens wearers. The present work aimed to determine the presence of *Acanthamoeba* isolates in different environmental sources: water, soil, and dust in Cairo, Egypt and to characterize the pathogenic potential of the isolated *Acanthamoeba* using physiological and biochemical assays as well as determination of the genotypes in an attempt to correlate pathogenicity with certain genotypes. The study included the collection of 22 corneal scrapings from patients complaining of symptoms and signs indicative of acanthamoeba keratitis (AK) and 75 environmental samples followed by cultivation on non-nutrient agar plates preseeded with E. coli. Positive samples for *Acanthamoeba* were subjected to osmo- and thermo-tolerance assays and zymography analysis. Potentially pathogenic isolates were subjected to PCR amplification using genus-specific primer pair. Isolates were classified at the genotype level based on the sequence analysis of *Acanthamoeba* 18S rRNA gene (diagnostic fragment 3). The total detection rate for *Acanthamoeba* in environmental samples was 33.3 %, 31.4 % in water, 40 % in soil, and 20 % in dust samples. Three and two *Acanthamoeba* isolates from water and soil sources, respectively, had the potential for pathogenicity as they exhibited full range of pathogenic traits. Other 12 isolates were designated as weak potential pathogens. Only ten of the environmental isolates were positive in PCR and were classified by genotype analysis into T4 genotype (70 %), T3 (10 %) and T5 (20 %). Potential pathogens belonged to genotypes T4 (from water) and T5 (from soil) while weak potential pathogens belonged to genotypes T3 (from water) and T4 (from water and soil). Additionally, T7 genotype was isolated from keratitis patients. There is a considerable variation in the response of *Acanthamoeba* members of the same genotype to pathogenicity indicator assays making correlation of pathogenicity with certain genotypes difficult. Presence of potentially pathogenic *Acanthamoeba* isolates in habitats related directly to human populations represent a risk for human health. Isolation of *Acanthamoeba* genotype T7 from AK cases, which is commonly considered as nonpathogenic, might draw the attention to other Acanthamoeba genotypes considered as non pathogenic and re-evaluate their role in production of human infections. To our knowledge, this is the first study on the presence and distribution of *Acanthamoeba* genotypes in the environment, Cairo, Egypt.

Keywords *Acanthamoeba* · Genotypes · Granulomatous amoebic encephalitis · Egypt · Environmental · Physiological · Biochemical · Characterization · Pathogenic

Introduction

Acanthamoeba spp. is the most common amphizoic protozoa commonly found in soil and aquatic environments worldwide. These amoebae have been isolated from very diverse habitats, including water from the Antarctic, bottled water, swimming pools, dental units, eye wash stations, and even from dust in the atmosphere (Goldschmidt et al. 2012).

Several species of the genus *Acanthamoeba* have been known to cause life-threatening granulomatous amoebic encephalitis (GAE) in immunocompromised individuals and sight-threatening amoebic keratitis (AK) in contact lens

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wearers. The incidence of AK infection has increased exponentially during the last 30 years (Hassan et al. 2012). In addition, these amoebae can act as vehicles for many pathogenic microorganisms with high virulence and resistance to antibiotics. It is suggested that such interactions may help transmit microbial endosymbionts to the susceptible hosts and/or endosymbionts may contribute to the pathogenicity of *Acanthamoeba* (Siddiqui and Khan 2012). The wide environmental distribution of *Acanthamoeba* is a key predisposing factor in *Acanthamoeba* infections (Kilic et al. 2004).

The genus *Acanthamoeba* consists of both pathogenic and non-pathogenic isolates and their differentiation is important for clinical diagnosis. *Acanthamoeba* pathogenesis is related to several factors. Among these factors, proteases are directly involved in host cell and tissue invasion and damage (Khan 2006). The differentiation of pathogenic and non-pathogenic *Acanthamoeba* has been shown by assaying physiological and biochemical characteristics and cytopathic effects (Khan 2003).

Over the last two decades, advances in molecular techniques have led to the development of methods for genotyping of *Acanthamoeba* isolates. A fast and reliable identification method based on the nuclear 18S small subunit ribosomal RNA gene is now used by investigators worldwide to identify *Acanthamoeba* isolates (Maciver et al. 2012). Three highly informative regions of the Rns could produce phylogenetic trees that are as robust as those based on the entire gene. These were designated diagnostic fragments 1, 2, and 3 (DF1, DF2, and DF3) of which the single highly variable and highly informative region DF3 could be used to identify genotypes rapidly (Kong 2009). Molecular characterization and phylogenetic analysis have led to the identification of 19 different genotypes (T1–T19); however, attempts to correlate pathogenicity with certain genotypes are under investigation in many laboratories (Armand et al. 2015, Todd et al. 2015, Magnet et al. 2014).

The extent to which *Acanthamoeba* isolates are present in water sources were previously studied in specific regions in Egypt (Lorenzo-Morales et al. 2006 and Al-Herrawy et al. 2015). However, no previous studies on the presence and distribution of *Acanthamoeba* genotypes have been previously reported in Cairo governorate. Therefore, the present study aimed to determine the presence of *Acanthamoeba* isolates in different environmental sources; water, soil, and dust from different districts in Cairo Governorate; and to characterize the pathogenic potential of the isolated strains by physiological (osmo- and thermo-tolerance) and biochemical (proteolytic activity) assays in correlation to *Acanthamoeba* isolated from keratitis patients in order to verify the existence of pathogenic potential in environmental isolates. It also aimed at determining the genotypes of the potentially pathogenic environmental isolates in an attempt to correlate pathogenicity with certain genotypes.

Subjects, materials, and methods

The present study is a cross-sectional study conducted in the Diagnostic and Research Unit at the Parasitology Department, Faculty of Medicine, Ain Shams University over the period from September 2011 to August 2014. All chemicals were purchased from Sigma Chemicals Co., USA, unless otherwise mentioned.

Sampling and processing of samples

1. Sampling from patients: Twenty-two corneal scrapings were obtained from patients attending the Corneal Outpatient Clinic, Research Institute of Ophthalmology, Giza by a specialized ophthalmologist. All patients were contact lens wearers, complaining of symptoms and clinical signs indicative of AK. Bacterial and fungal keratitis was excluded. Patients were 18 females and 4 males ranging in age from 18 to 40 years. The samples were kept in 5-ml screw-capped vials containing Page′s amoeba saline (PAS), each sample was centrifuged for 10 min at 2000 rpm then the sediment was re-suspended in about 0.5 ml of PAS (Init et al. 2010).
2. Sampling from environment: Seventy-five environmental samples were collected from Nasr city, Madinat al Salam, Al Nozha, Heliopolis, Kobri al Kobba, al Abassia, Shobra, Maadi, Al Rehab, and Ataba. They included 35 tap water, 30 dry soil, and 10 dust samples. Soil and water samples were collected in sterilized polypropylene containers while dust samples were collected using sterile swabs by swabbing windows and tiles. Tap water samples (about 100 ml) were centrifuged for 10 min at 2000 rpm and the sediment was re-suspended in about 0.5 ml of PAS (Booton et al. 2004). About 1 g of the soil sample was mixed with enough PAS (0.5 to 1 ml) using sterile Pasteur pipettes to make thick slurry (Rezaeian et al. 2008). The swabs containing dust samples were kept tightly closed until cultivated (Costa et al. 2010).

Cultivation of samples (Init et al. 2010)

Each sample were inoculated onto the center of nonnutrient agar (NNA) plate previously seeded with 100 μl *Escherichia coli* bacterial suspension and incubated at 25–28 °C, with daily microscopic examination using the inverted microscope for up to 7 days. Scraping of the agar surface was examined after adding few drops of Eosin stain (0.5 %) at ×1000 magnification under a light microscope. The grown *Acanthamoeba* trophozoites were characterized from other free-living amoebae by their pointed pseudopodia (acanthopodia), while the cyst forms were easily identified by their double cyst wall and conventionally stellate shape.

Plates positive for *Acanthamoeba* were selected for subculture every week on NNA-E. coli plates.

Determination of the potential pathogenicity of samples from AK and environment

For the Osmotolerance assay (Khan 2001): Positive samples for *Acanthamoeba* were sub-cultured on 1 M mannitol NNA-E. coli plates and incubated at 28 °C for up to 48 h.

For the Thermotolerance assay (Khan 2001): Positive samples for *Acanthamoeba* were sub-cultured on NNA-E-coli plates and incubated at 37 °C for up to 48 h.

For the biochemical assay, Zymography analysis was carried out at VACSERA (Alfieri et al. 2000): *Acanthamoeba* trophozoite lysate was prepared; trophozoites were subcultured on NNA plates pre-seeded with heat killed *E.coli* and harvested in sterile PAS, from the surface of the plates. After washing in PAS, addition of gentamycin (100 μg/ml) and centrifugation at 3000 rpm for 10 min, the pellet was resuspended in PAS and mixed well. The trophozoites were subjected to two cycles of rapid freezing and thawing, followed by sonication for six times, each of 30-s duration to be disrupted. The suspension was centrifuged at 15,000 rpm for 15 min. The supernatant of each sample was collected by a sterile Pasteur pipette, and stored at −20 °C. The procedure was repeated three times for each sample to reach a protein content of 500 μg/ml (Al Herrawy et al. 2013). Zymography on SDS-polyacrylamide gels copolymerized with gelatin (1 %) were used. Briefly, the prepared sample buffer was added to the *Acanthamoeba* trophozoite lysate to keep a final volume of 30–40 μl (equivalent to 30-μg protein) of *Acanthamoeba* and then applied to the gels. After electrophoresis, gels were soaked in 2.5 % Triton X-100 (*w*/*v*) solution for 60 min, incubated in a developing buffer (50 mM Tris–HCl, pH 7.5, containing 10 mM CaCl2) at 37 °C overnight, rinsed and stained with Coomassie brilliant blue. Areas of gelatin digestion were visualized as non-staining regions in the gel. The de-stained gel was stored in storage solution to stop de-staining. Molecular weights of proteins were determined using a gel documenting system software. The same experiment was repeated using the trophozoite lysates from all isolates pretreated with phenylmethylsulfonyl fluoride (PMSF, an inhibitor of serine protease), for 30 min prior to electrophoresis. Potentially pathogenic environmental *Acanthamoeba* isolates, as determined by osmo- and thermotolerance assays as well as zymography analysis, were subjected to the molecular characterization compared to AK isolates.

Molecular characterization

It was carried out at Ain Shams University Genetic Engineering Research Services (ASUGEN). Cysts from different isolates were harvested by sterile PAS, from the surface of NNA-*E.coli* plates. After washing in PAS, addition of gentamycin (100 μg/ml) and centrifugation at 3000 rpm for 10 min, the pellet was resuspended in PAS and the suspension was centrifuged at 15,000 rpm for 15 min (Gatti et al. 2010). The deposit was stored at −20 °C for DNA extraction using "EasyQuick DNA extraction kit supplied by Genomix". For the detection of *Acanthamoeba* spp., a specific primer pair, namely: JDP1: 5′-GGCCCAGATCGTTTACCGTGAA (the forward primer-22 mer) and JDP2: 5′-TCTCACAAGCTGCTAGGGGAGTCA (the reverse primer-24 mer) was used (Schroeder et al. 2001). Amplification of DNA by polymerase chain reaction was performed using 2X Super-Hot PCR Master Mix DNA polymerase (Bioron). The PCR reaction mixture used per sample consisted of: 25 μL PCR Master Mix (2X), 2 μL template DNA, 2 μL of each primer, and 19 μL sterile deionized water. The amplification was performed in the thermal cycler (Biometra, USA) with cycling conditions as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 96 °C for1 min., primer annealing at 60 °C for1 min., and extension at 72 °C for1 min. A final extension step was performed at 72 °C for 7 min. PCR products were then electrophoresed using 1.2 % agarose gel (Ultrapure) stained with ethidium bromide (10 mg/ml in deionized H2O) and visualized under UV illumination. A 250–10,000 base pair (bp) ladder, (Gene Ruler TM, Fermentas) was used as a DNA size marker.

Sequencing and genotype identification (Chen, 1994)

PCR products from environmental and two representative keratitis isolates were purified using the AxyPrep PCR Clean-up kit (AXYGEN Biosciences-USA) and sequenced using an automated fluorescence sequencing system (Applied Biosystems 3730XL genetic DNA analyzer). Phylogenetic analysis of the obtained sequences was carried out using maximum parsimony, neighbor joining and maximum likelihood optimality criteria, implemented in the phylogenetic software program "Molecular and Evolutionary Genetics Analysis" (MEGA) (version 6.0). Genotype identification was based on sequence analysis of DF3 region as previously described (Booton et al. 2002) by comparison to the available *Acanthamoeba* DNA sequences in Genbank.

Statistical analysis

Friedman's test was used to assess the statistical significance of the difference between two or more markers measured for the same study group. A significance level of $P < 0.05$ was used in all tests. All statistical procedures were carried out using SPSS version 15 for Windows (SPSS Inc, Chicago, IL, USA).

Phylogenetic analysis of the obtained sequences was carried out using maximum parsimony, neighbor joining, and maximum likelihood optimality criteria, implemented in Molecular and Evolutionary Genetics Analysis (MEGA) program (version 6.0).

Ethical consideration

An informed consent was taken from the patients after explaining the aim of the study to them. The study was started after being approved by the Ethical Committee of Scientific Research, Faculty of Medicine, Ain Shams University.

Results

Out of 22 corneal scrapings and 35 tap water, 30 soil, and 10 dust samples, 5 (22.7 %) and 11 (31.4 %), 12 (40 %) and 2 (20 %), respectively, were positive for *Acanthamoeba* trophozoites and cysts according to morphological criteria (Table 1). Identification of trophozoites was based on the flat shape, prominent nucleus, and fine tapering thorn-like structures of acanthopodia as well as lobopodia which are hyaline pseudopodia arising from the cytoplasm (Fig. 1a–b, d). Identification of cysts was based on the characteristic double wall with wrinkled ectocyst and a smooth endocyst that varied in shape, i.e., they were stellate, polygonal, or spherical (Fig. 1b–e). Due to extensive contamination of cultures by organisms such as *Strongyloides* spp. and unidentified ciliates that could not be eliminated by repeated subcultures, 3 of the positive water samples and 5 of the positive soil samples were excluded from the study (Fig. 2).

Assays for pathogenicity indicated that out of 8, 7, and 2 *Acanthamoeba* isolates from tap water, soil, and dust samples, 5 (62.5 %), 5 (71.4 %) and 2 (100 %), respectively, were resistant to 1 M osmolarity and 6 (75 %), 7 (100 %) and 1 (50 %), respectively, were resistant to 37 °C temperature. Meanwhile out of 5 five *Acanthamoeba* isolates from corneal scraping samples, 3 three (60 %) and 5 five (100 %) showed growth at high osmolarity and 37 °C temperature, respectively (Table 2).

Zymography without protease inhibitor revealed that bacterial control samples showed bands of MWs 114, 87, 57, 53, 34, 26, and 23 kDa while *Acanthamoeba* isolates from AK cases gave bands ranging in MWs from 100 to 43 kDa. Out of 8, 7, and 2 *Acanthamoeba* isolates from tap water, soil, and dust samples, 3, 2, and 0, respectively, shared bands with 5 *Acanthamoeba* isolates from AK cases at 100, 75, 70, and 47 kDa. Zymography with protease inhibitor showed that proteolytic activity detected at 100, 75, 60, and 47 kDa were inhibited after treatment with protease inhibitor (PMSF) indicating their serine protease nature (Fig. 3).

Five *Acanthamoeba* isolates (3 from water and 2 from soil): expressed proteolytic activity common with all *Acanthamoeba* isolates from AK cases and showed osmo- and thermo-tolerance were considered potential pathogens. The remaining 12 environmental isolates that did not exhibit the full range of pathogenic traits were considered weak potential pathogen (Fig. 3 and Table 2). Both potentially pathogenic and weak potentially pathogenic *Acanthamoeba* isolates (17 isolates) were selected for further identification at the genotype level together with AK isolates.

Out of 8, 7, and 2 *Acanthamoeba* isolates from tap water, soil, and 2 dust samples, 5, 5, and 0, respectively, as well as all isolates from AK samples showed expected PCR product of approximately 440–500 base pair (bp). The remaining 7 environmental isolates gave no amplification products. The PCR products from 10 environmental *Acanthamoeba* isolates as well as 2 representative isolates from AK cases were purified followed by sequencing (Figs 4 and 5).

The results of alignment proved that all the introduced isolates were belonging to the genus *Acanthamoeba* (Table 3). The 2 keratitis isolates were identified as genotype T7 (99-100 % homology with *Acanthamoeba* astronyxis). Out of 5 water isolates, 4 (80 %) were identified as genotype T4 and 1 (20 %) as genotype T3, while out of 5 soil isolates, 3 (60 %) were identified as genotype T4 and 2 (40 %) as genotype T5. The study showed that among the environmental samples studied T4 was the predominant genotype of *Acanthamoeba* (70 %) followed by T5 (20 %) and T3 (10 %) (Table 4).

Environmental isolates which were considered potential pathogens belonged to *Acanthamoeba* genotypes T4 and T5, while isolates which were considered weak potential pathogens belonged to genotypes T3 and T4. Phylogenetic relationships within the genus were examined with maximum parsimony, neighbor joining, and maximum likelihood optimality criteria. The tree topology was identical regardless of the method used. The partial 18S rRNA gene sequence was also compared with the reference species of each genotype. The GenBank accession numbers and the origins of the isolates used for comparison are: T3, *Acanthamoeba griffini* S-7 ATCC 30731 (GenBank accession number: U07412); T4 Neff, *Acanthamoeba castellanii*

Table 1 Frequency of *Acanthamoeba spp.* in environmental and corneal scraping samples

Sample source	Sample size	Number (%) of detection for *Acanthamoeba*	Locality of detection of *Acanthamoeba*
Keratitis	22	5 (22.7)	
Water	35	11 (31.4)	Nasr city, Madinat al Salam, Al Nozha, Kobri al Kobba, Shobra, Al Abassia, and Al Ataba
Soil	30	12 (40)	Nasr city, Heliopolis, Al Nozha, Kobri al Kobba, Maadi, Al Abassia, Al Ataba, and Al Rehab
Dust	10	2 (20)	Al Nozha and Al Abassia,
Total	75	25 (33.3)	

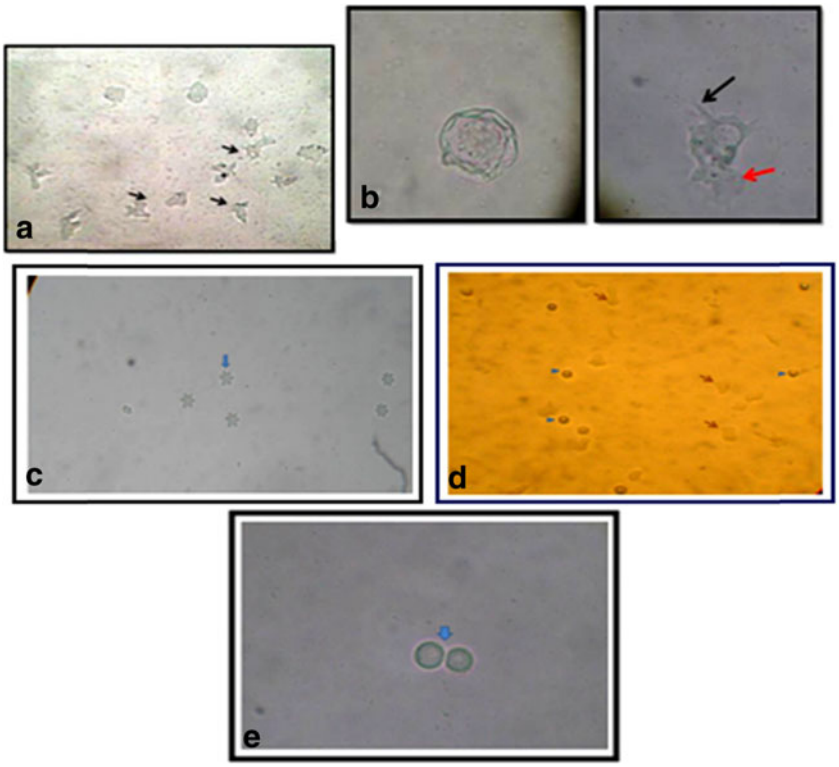

Fig. 1 *Acanthamoebae* trophozoites in culture (AK case, ×400) (**a**). *Acanthamoeba* cyst and trophozoite in culture (AK case, ×1000). *Left* Cyst showing stellate endocyst, *right* trophozoite showing lobopodia (*red arrow*) and acanthopodia (black arrow) (**b**), *Acanthamoeba* cysts in culture (water sample, ×400) (**c**), *Acanthamoeba* trophozoites (*arrows*) and cysts (*arrow heads*) in culture (soil sample, ×250) (**d**) and *Acanthamoeba* cysts in culture showing double wall and single nucleus (dust sample, ×1000) (**e**)

Neff ATCC 50373 (U07416); T5, *Acanthamoeba lenticulata* PD2S (U94741); T7, *Acanthamoeba astronyxis* CCAP 1534/1 (AF239293) (Fig. 6).

Discussion

In the present work, frequency of *Acanthamoeba* spp. in studied keratitis cases was only 22.7 % (5 out of 22), although clinical manifestations were highly suggestive. This could be attributed to the need of a deep stromal scrape for the retrieval of *Acanthamoeba* trophozoites and/or cysts, which might not be possible as the depth of the scrape may be hazardous (Leck 2009). A quick overview on the presence of *Acanthamoeba* in the environment showed that out of the 75 environmental samples, 25 (33.3 %) were positive for *Acanthamoeba*. *Acanthamoeba* was isolated from 31.4 % of studied tap water samples. In other studies on tap water in Egypt, Al-Herrawy et al. (2013) recorded a higher occurrence of *Acanthamoeba* (58.6 %), while Al-Herrawy et al. (2015) and Hamadto et al. (1993) detected *Acanthamoeba* in a lower incidence 16.7 and 4 %, respectively. Sadaka et al. (1994) reported that no *Acanthamoeba*e were encountered in the drinking water in Alexandria Governorate. In Spain, the prevalence of *Acanthamoeba* in tap water was found to be 59.5 % (Lorenzo-Morales et al. 2005). Lower prevalence of 26.9 and 5.8 % in tap water was recorded in UK and Korea, respectively (Kilvington et al. 2004 and Jeong and Yu 2005). The difference in detection rates of *Acanthamoeba*e in different countries and localities may be influenced by raw water sources either fed by rooftop tanks or directly from water pipes. Stagnation of water leads to biofilm formation which augments the number of free-living amoebae (Hassan et al. 2012).

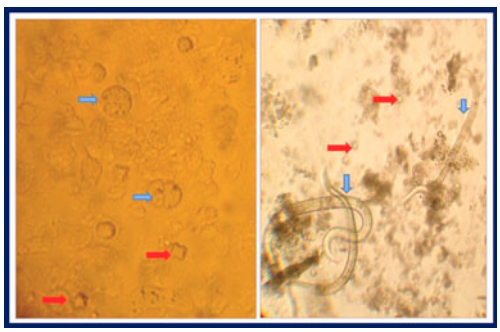

Fig. 2 *Left* unidentified ciliates and *right*: *Strongyloides spp.* contaminating a soil sample positive for *Acanthamoeba. Blue arrows* contaminants, *red arrows Acanthamoeba* cysts

In the present study, the frequency of *Acanthamoeba* among studied soil samples was 40 %, which almost matches the results of Booton et al. (2004) presenting occurrence of 38 % among soil samples in South Florida. A higher frequency of detection 88 % in Asssiut, Egypt and 100 % in Tehran, Iran was recorded by Abed et al. (2013) and Rezaeian et al. (2008), respectively. The difference in the results obtained may be due to diversity of environments tested as if it is dry or wet soil as well as seasonal variation as mentioned by Kao et al. (2013). The frequency of *Acanthamoeba* among studied dust samples was 20 % which is

Table 2 Characteristics of *Acanthamoeba* isolates from water, dust, and soil samples in Cairo districts

Sample Source	Locality	Growth at 37 °C	Growth at 1 M mannitol	Proteolytic activity
Water	Nasr city	+	+	√
	Al salam	+	+	√
	Al Nozha	−	−	√
	Al Nozha	+	+	√√
	Kobri Al kobba	+	+	√√
	Al Abassia	−	−	√
	Shobra	+	+	√√
	Shobra	+	−	√
Dust	Al Nozha	+	+	−
	Al Abassia	−	+	√
Soil	Nasr city	+	+	√√
	Heliopolis	+	+	√
	Kobri Al kobba	+	−	√
	Al Abassia	+	+	√
	Al Maadi	+	-	−
	Al Maadi	+	+	√√
	Attaba	+	+	√

Potential pathogens

Weak potential pathogens

+ represent growth of *Acanthamoeba,* − represent no growth of *Acanthamoeba,*√√ proteolytic profile sharing all bands common with all *Acanthamoeba* isolates of AK cases,√ proteolytic profile sharing some bands common with all *Acanthamoeba* isolates of AK cases

lower than other studies 45.9 % in Tehran, Iran and 100 % in Brazil by Rezaeian et al. (2008) and Costa et al. (2010), respectively. The difference in results may be attributed to the difference in numbers of samples examined.

In the current work, all *Acanthamoeba* isolates from keratitis cases were osmo-and thermo-tolerant except two out of five cases (40 %) were not osmotolerant. This finding could be explained by the assumption that scrapings from infected eyes have often been exposed to drugs which may result in alteration of physiological properties (Ledee et al. 1996). It was found that 70.5 % of all *Acanthamoeba* isolates obtained from the environment were osmotolerant. About 62.5, 71.4, and 100 % of *Acanthamoeba* isolated from tap water, soil, and dust, respectively, were osmotolerant. In terms of temperature tolerance, growth at 37 °C is a relevant indicator of pathogenicity since the temperature of the eye is around 34 °C (Booton et al. 2004). In this study, 76.5 % of *Acanthamoeba* isolates obtained from the environment showed growth at 37 °C. About 75, 85.7, and 50 % of

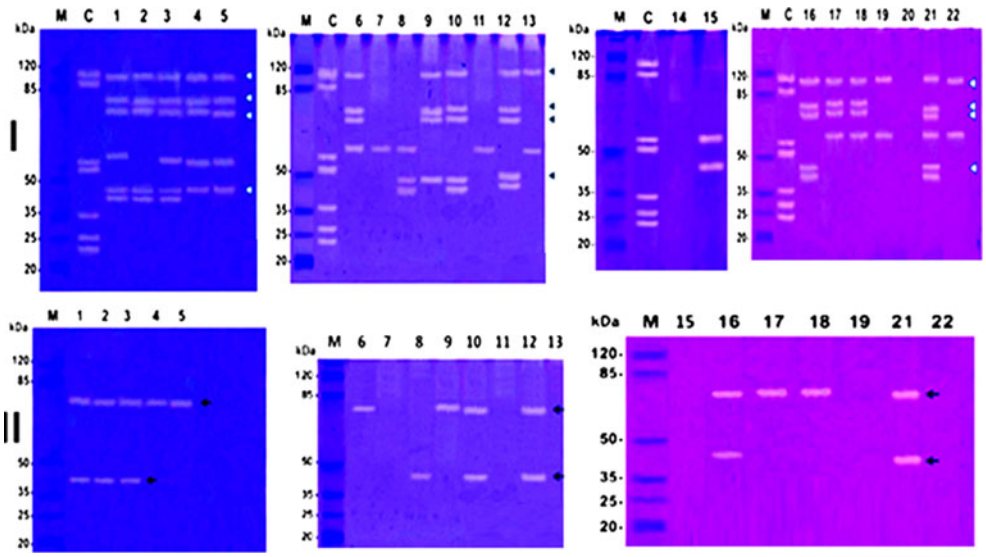

Fig. 3 I: Zymography analysis showing the proteolytic activity of the *Acanthamoeba* isolates. II: Serine protease activity of the *Acanthamoeba* isolates treated with PMSF prior to electrophoresis. M in I; molecular weight marker, *arrow heads* indicate proteolytic bands. M in II; molecular weight marker; *arrows* showing the unaffected gelatin digestion bands. Sample numbers: AK cases (1–5), water samples (6–13), dust samples (14–15), and soil samples (16–22). *C* bacterial control

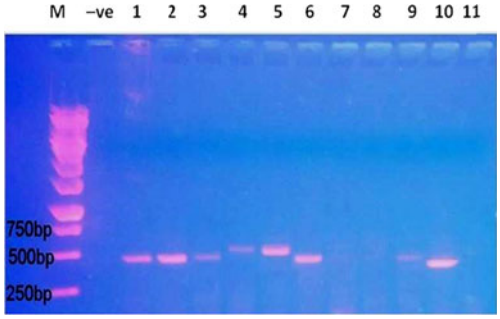

Fig. 4 PCR amplification products from *Acanthamoeba* isolates obtained from AK and water samples. *Lane M* DNA molecular weight standard, *−ve* negative control, *bp* base pair. *Lanes 1 to 5* AK *Acanthamoeba* isolates, *lanes 6 to 11* water *Acanthamoeba* isolates

Acanthamoeba isolated from water, soil, and dust, respectively, were thermotolerant. In Egypt, Al-Herrawy et al. (2013) showed that the percentage of *Acanthamoeba* spp. exhibiting osmo- and thermo-tolerance reached 22.2 and 50 % in tap water in Cairo and 15.2 and 58 % in the Delta region, respectively. In Ankara, Turkey, Kilic et al. (2004) reported that about 66 % of studied soil *Acanthamoeba* isolates was osmo- and thermo-tolerant. From soil samples, Booton and colleagues (2004) deduced that 100 % of isolated *Acanthamoeba* in South Florida, showed growth at 37 °C. The discrepancy in the results can be explained on the basis of different species of *Acanthamoeba* encountered in each study which may have different physiological properties.

In zymography analysis, the proteolytic profile of the prepared lysates from bacterial control differed from that of *Acanthamoeba* isolates. The proteolytic activity of environmental *Acanthamoeba* isolates showed that most of the isolates gave proteolytic activity ranging from 100 to 43 kDa. The bands mostly expressed in the *Acanthamoeba* isolates from tap water were of MWs 100, 75, 70, and 60 kDa and to a little extent MWs 47 and 43 kDa. In Cairo, Al-Herrawy et al. (2013) agreed with our study in two bands (75 and 70 kDa) produced by Acanthamoeba isolated from tap water. Magliano et al. (2009) agreed with only one proteolytic band at 47 kDa from a fresh water *Acanthamoeba* isolate in Brazil. The proteolytic

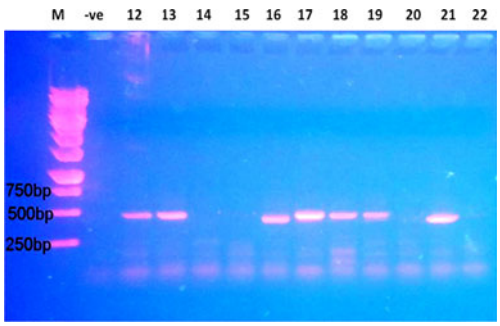

Fig. 5 PCR amplification products from *Acanthamoeba* isolates obtained from water and soil samples. *Lane M* DNA molecular weight standard; *−ve* negative control; *bp* base pair. *Lanes 12 and 13* water *Acanthamoeba* isolates; *lanes 14 and 15* dust *Acanthamoeba* isolates; *lanes 16 to 22* soil *Acanthamoeba* isolates

enzymes of trophozoite lysates that were inhibited by PMSF were those detected at 100, 75, 60, and 47 kDa. This result is compatible with the involvement of serine proteases in pathogenesis. This agrees with the results of Alfieri and colleagues (2000) who deduced through inhibition by PMSF that 100, 75, and 47 kDa bands, produced by *Acanthamoeba polyphaga* lysate originated from a human keratitis were of serine protease nature. Moreover, Omaña-Molina et al. (2013) found that the proteolytic activity of pathogenic *Acanthamoeba* strains at the region of MWs 60 and 47 kDa were of serine protease nature as they were inhibited by PMSF.

In the present study, *Acanthamoeba* isolates were classified according to a group of pathogenicity indicators. Costa et al. (2010) concluded that although a single parameter can provide an initial screening of the pathogenic potential, it must be associated with other parameters for more accuracy. This is in accordance with Lorenzo-Morales et al. (2006) who assigned grouping of *Acanthamoeba* isolates according to the expression of pathogenic traits. Based on these, 3 and 2 isolates from water and soil respectively were considered potential pathogens, while 5, 2, and 5 isolates from water, dust, and soil, respectively, were considered weak potential pathogens.

Acanthamoeba isolates from 7 out of 17 studied environmental samples showed no amplimers in PCR. Likewise, Schroeder et al. (2001) and Derda et al. (2014) obtained similar results as 4 environmental isolates out of 15 and 9, respectively, failed to produce amplimers using the same primers raising a question about the sensitivity of this primer pair as regards detection of environmental *Acanthamoeba* isolates. Moreover, antibiotics used in the wash of collected parasites from culture plates prior to PCR may act as PCR inhibitors as proposed by Farag et al. (2010). Another possible explanation may be that repeated passage of *Acanthamoeba*e in culture has led to mutations resulting in changes in gene structure as stated by Ebert 1998. PCR of *Acanthamoeba* isolates from keratitis cases and only 10 out of 17 samples from water and soil showed bands between 440 and 500 bp which agrees with the findings of Schroeder et al. (2001).

The present study revealed that most of the genotyped environmental *Acanthamoeba* isolates were T4 (70 %), 20 % of isolates were of T5 genotype while 10 % were of T3 genotype. This result is consistent with Booton et al. (2004) and Tanveer et al. (2013) who reported that T4 genotype is the most common *Acanthamoeba* genotype in the environment. Also, it is nearly in accordance with Booton et al. (2005) who found that the first, second, and fourth most common *Acanthamoeba* genotypes among environmental isolates were T4, T5, and T3, respectively. However, it is at odds with the results obtained by Kilic et al. (2004) and Edagawa et al. (2009) that the most abundant *Acanthamoeba* genotype in the environment was T2 and T3, respectively. The difference in distribution of *Acanthamoeba* genotypes between studies may be attributed to the seasonal variation at time of sample collection

Table 3 Genotypes of *Acanthamoeba* isolates from water and soil samples in Cairo districts

Source	Locality	*Acanthamoeba* species	Genotype	Accession number	Identity %
Water	Nasr city	*Acanthamoeba isolate AcaP 18*	T3	KJ094654	100
	Al Nozha	*Acanthamoeba genotype T4 voucher OSU08-014*	T4	JX423600	99
	Kobri Al kobba	*Acanthamoeba culbertsoni strain It-N*	T4	KF881887	93
	Shobra	*Acanthamoeba spp. AcaVNAK03*	T4	GQ905497	99
	Shobra	*Acanthamoeba spp. Ac_PCN20*	T4	GU808329	99
Soil	Nasr city	*Acanthamoeba lenticulata isolate 33195463*	T5	KC438381	100
	Heliopolis	*Acanthamoeba spp. UIC 1060 voucher OSU 06-033*	T4	EU168081	99
	Kobri Al kobba	*Acanthamoeba spp. MN/TRW14/IRN*	T4	JF317327	99
	Al Abassia	*Acanthamoeba spp. strain md-H*	T4	KF881880	98
	Al Maadi	*Acanthamoeba lenticulata clone CF1-249*	T5	KC164253	100
Keratitis		*Acanthamoeba astronyxis S1*	T7	HE653911	99
		Acanthamoeba astronyxis EFW	T7	DQ992178	100

Potential pathogens

Weak potential pathogens

that may play a role in the abundance of certain *Acanthamoeba* genotypes (Kao et al. 2013).

Currently, environmental *Acanthamoeba* isolates belonging to T5 genotype were potential pathogens. Members of the T5 genotype, designated as *Acanthamoeba lenticulata*, have been established cytopathogenic to human tissue culture cells and have been proved virulent in mice (De Jonckheere and Michel 1988 and Niszl et al. 1998). Ledee et al. (2009) have isolated T5 genotype from AK cases.

Three out of seven environmental *Acanthamoeba* isolates belonging to T4 genotype were potential pathogens. The inquiry most often raised is whether T4 isolates are the most ubiquitous genotype in the environment and thus are most likely to encounter humans to produce infections or they possess certain properties that make them more virulent. A possible explanation is that T4 are more capable of surviving different or harsher conditions that allow them to be found in a variety of locations. They may also be able to respond faster to changing conditions that allow them to have a competitive advantage in causing disease (Maciver et al. 2012). What is of particular interest in the current study is that four out of seven environmental *Acanthamoeba* isolates belonging to T4 were considered weak pathogens. A feasible explanation for this finding may be the high diversity observed among T4 isolates. T4 genotype can be divided into several different sub-clades, whether or not a particular subclade of T4 is more pathogenic than others is questionable. This has encouraged sub-classifying T4 on the basis of differences in the sequence (Booton et al. 2002). This later approach initially sub-classified T4 into one of ten groups, (T4/1 to T4/10), but this was expanded to 21 (Ledee et al. 2009), and then to 23 (Abe and Kimata 2010).

Table 4 Frequency of *Acanthamoeba* genotypes among studied environmental isolates

Acanthamoeba genotypes	Environmental source		
	Water *N* (%)	Soil *N* (%)	Water and soil *N* (%)
T3	1 (20)	–	1 (10)
T4	4 (80)	3 (60)	7 (70)
T5	–	2 (40)	2 (20)

One out of ten environmental *Acanthamoeba* isolates (10 %) was belonging to T3 genotype and was considered a weak pathogen. T3 genotype has been found to be responsible for multiple cases of keratitis (Booton et al. 2005). The close genetic similarity relationship between T3 and T4 may lay the possibility of diversity also among T3 genotypes and may explain the observed weak pathogenicity of this isolate in the current study. Ledee et al. (2009) examined pathogenicity predictive factors of T3 genotype and found that T3 displayed high pathogenicity, although results were not always consistent between isolates.

Four out of five (80 %) *Acanthamoeba* isolates from tap water were belonging to T4 genotype and were classified as potential pathogens. This draws attention to the risk posed by continuous exposure to this pathogen in the everyday life, taking in consideration the high risk groups for development of AK as contact lens wearers and eye trauma patients and

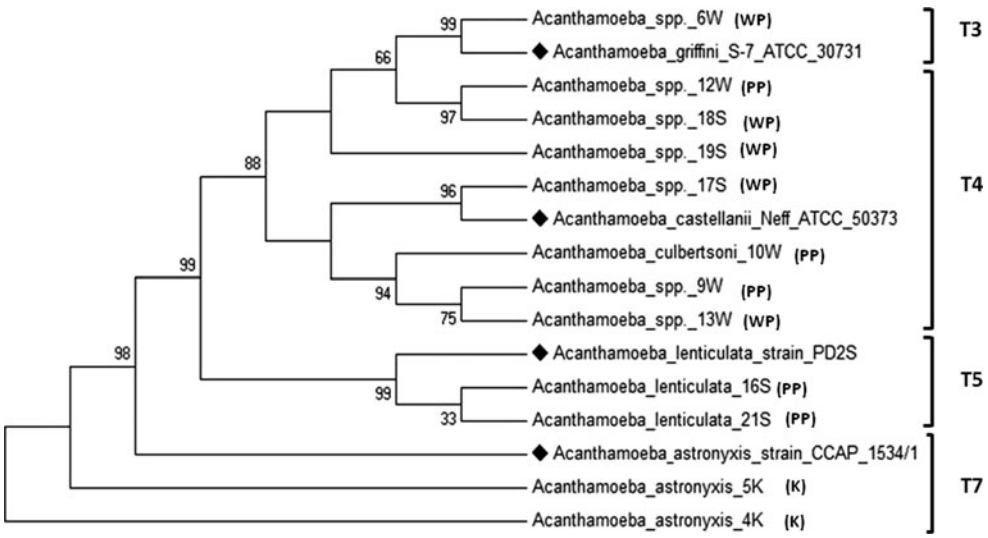

Fig. 6 Phylogenetic analysis based on partial 18S rRNA gene sequence, maximum parsimony tree. *PP* potential pathogen, *WP* weak potential pathogen, *K* AK case. *Black diamond* reference *Acanthamoeba* strains. *Numbers* represent bootstrap values based on 500 replicates

immunocompromised individuals. The presence of only limited variants of *Acanthamoeba* genotypes (T3, T4, and T5) in the current study is not surprising. Crary (2012) concluded that estimating the genotypic diversity of *Acanthamoeba* within an environmental sample is a major difficulty since the slow culturing of *Acanthamoeba* often result in ultimate overgrowth of a single clonal organism. This means that while numerous different *Acanthamoeba* may exist in a single sample, only the clonal lineage that is most successful under the culture conditions, either through faster excystment or a higher rate of division, will be isolated.

Though genotyping from AK cases was out of the scope of the study, two representative *Acanthamoeba* isolates from keratitis patients were subjected to genotyping as both isolates showed morphological characteristics suggestive of *Acanthamoeba astronyxis* which are designated as strictly nonpathogenic (Crary, 2012). Interestingly, both isolates were *Acanthamoeba* astronyxis (100 % identity) belonging to T7 genotype. *Acanthamoeba* astronyxis has been isolated from a corneal scrape of an AK case in Spain (Ortega-Rivas et al. 2005) as well as being reported earlier in human infection by Armstrong (2000). Although *Acanthamoeba* isolates belonging to T7 were not detected in the present environmental samples, they have been isolated before from fresh water for the first time in Egypt by Lorenzo-Morales et al. in 2006.

Taken together, the current findings serve as additional evidence for the presence of pathogenic *Acanthamoeba* isolates in habitats related directly to human populations which could represent a risk for human health. It has been shown that the environmental *Acanthamoeba* isolates obtained from Cairo districts belonged to T3, T4, and T5 genotypes. There is a considerable variation in the response of *Acanthamoeba* members of the same genotype to physiological and biochemical pathogenicity indicators making generalization difficult. More detailed analysis will determine the possibility of including sub-clades that might vary in the degree of virulence and pathogenicity. Studying the prevalence of *Acanthamoeba* requires examining a wider scale of different environmental samples. The association of T7 with human disease points out the urgent need to reevaluate the role of other *Acanthamoeba* genotypes that have been previously considered as non-pathogenic in production of AK and other non-keratitis infections.

Compliance with ethical standards An informed consent was taken from patients after explaining the aim of the study to them. The study was started after being approved by the Ethical Committee of Scientific Research, Faculty of Medicine, Ain Shams University.

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