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Isolation and molecular characterization of *Acanthamoeba* genotypes isolated from soil sources of public and recreational areas in Iran

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Abstract

Pathogenic strains of *Acanthamoeba* are causative agents of a sight threating infection of the cornea known as *Acanthamoeba keratitis*. AK cases have been reported in Iran recently due to inappropriate usage of contact lens maintenance and most patients report a contact with contaminated sources such as dust, water or soil. Sixty soil samples were collected from public and recreational areas in the province of East Azerbaijan, Iran and checked for the presence of *Acanthamoeba* spp. Samples were cultured on non-nutrient agar plates seeded with heat killed *Escherichia coli*. PCR and sequencing of the DF3 region were carried out in order to genotype the isolated strains of *Acanthamoeba*. Thermotolerance and osmotolerance assays were performed in order to investigate the pathogenic potential of isolated *Acanthamoeba* strains. *Acanthamoeba* spp. was isolated from 41.6% of soil samples and genotyping of the strains resulted in the identification of genotypes T3, T4, T5 and T11. Most of the isolates belonging to genotypes T3 and T4 showed high pathogenic potential, indicating that they might present a potential health hazard for humans and other animals in this region. To the best of our knowledge, this is the first report on the identification of genotypes T3 and T11 from soil sources in the country.

Keywords

Acanthamoeba spp., Iran, soil

Introduction

Free-living amoebae (FLA) are extensively protozoan parasites that colonize soil, water, dust, biofilms and water-air interface (Visvesvara *et al*. 1990, 2007; Khan 2009; Niyyati *et al.* 2015c; Lasjerdi *et al.* 2015). In this regard some FLA are of medical importance and these include *Acanthamoeba* spp., *Balamuthia*, *Naegleria* and *Vermamoeba* (Khan 2006; Martinez *et al*. 1997; Visvesvara *et al.* 2007). Some genera including *Acanthamoeba* could resist harsh environmental sources due to their resistant cyst stage and thus they could lead to *Acanthamoeba* related disease including blindness keratitis and fatal encephalitis in high risk people such as contact lens wearers and immunosuppressed patients (Lorenzo-Morales *et al.* 2015; Marciano-Cabral and Cabral 2003). However, infections caused by the free living amoebae are not usually reported in Iran with the exception of *Acanthamoeba keratitis* (AK) (Niyyati *et al*. 2009; Niyyati *et al*. 2014). It is important to mention AK cases have been raised in Iran during past years due to inappropriate usage of contact lenses and most patients report a contact with contaminated sources such as dust, water, biofim or soil (Niyyati *et al.* 2009; Niyyati and Rezaeian 2015c; Lasjerdi *et al.* 2015). In the USA amoebic keratitis is reported in over 1 to 2 cases per million contact lens wearers each year (Thomas *et al*. 2008; Diaz 2010). In addition, *Acanthamoeba* spp. and *Balamuthia mandrillaris* are capable of causing granulomatous amebic encephalitis (GAE), whereas *Naegleria* induces primary amebic meningoencephalitis (PAM) (Visvesvara *et al*. 2007). To date, there is a single report of PAM in a six month boy in Iran (Movahedi *et al*. 2012). Among 20 genotypes, *Acanthamoeba* belonging to T4 genotype, are the most cause of amoebic keratitis (AK) worldwide (Gast 2001; Evyapan *et al*. 2014; Lorenzo-Morales *et al*. 2015; Nuprasert *et al*. 2010; Qvarnstrom *et al.* 2013). Genotypes belonging to T2, T3, T4, T5, T6 and T11 were recognised for severe AK in Iran and in the world so far (Maghsood *et al*. 2005; Niyyati *et al*. 2009). Unfortunately, there is limited report regarding successful treatment of AK

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in the region and most patient showed poor prognosis. In addition, FLA could act as hosts for many pathogenic bacteria and viruses (Niyyati *et al*. 2015b; Żbikowska *et al*. 2014, Scheid 2014). Interestingly, FLA can increase virulence of these bacteria and viruses (Greub *et al*. 2004). Considering the role of these amoebas in environmental sources, controlling polluted resources through their identification and therefore prevent potential diseases caused by them is a high priority.

In Iran most of the efforts were to identify the FLA isolation from various water sources including hot spring and recreational water sources of parks and swimming pools. These researches lead to isolation of various potentially pathogenic *Acanthamoeba* including T2, T3, T4, T5 and T11 (Niyyati *et al*. 2012, Niyyati and Rezaeian 2015c, Rezaeian *et al.* 2008, Solgi *et al*. 2012). Currently there were limited survey on the isolation of *Acanthamoeba* in soil sources in Iran and thus the present research was done to characterize *Acanthamoeba* genotypes in soil samples in two touristic region of East Azerbaijan province using culturing, microscopic investigation based on page key and sequencing analysis. Pathogenic potential of the isolates were also determined using thermo and osmotolerance assays.

Materials and Methods

Sample sites, filtration and cultivation

Sixty soil samples were collected across the recreational regions and public places in the East Azerbaijan Province about 130 km of the Tabriz (Fig. 1). The sampling area was public places including hospital area, swimming pool area, parks and school campus. One hundred gram (100 g) of soil samples were dissolved in sterile distilled water, remained for about an hour and were filtered using cellulose nitrate membrane (Millipore, SA) with a pore size of 0.4 µm. The filters paper were cut and placed upside down onto a 1.5% Non-nutrient agar (NNA) medium along with *Escherichia coli* and incubated aerobically at room temperature according to our previous studies (Lorenzo-Morales *et al.* 2005; Niyyati *et al*. 2015a). The cultures were check out after 72 h, for up to one months and were examined in the laboratory of Protozoology Unit, Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Iran.

Purification and Identification of the cloned amoebae

Positive samples (recognized under the inverted microscope) transferred to the new culture plates. To this end a single amoeba was taken from NNA medium which contained less fungi and bacteria were placed on fresh NNA medium. All plates were sealed, incubated at room temperature and monitored daily for 2 weeks to obtain pure cultures.

PCR analysis and Sequencing of DF3 region

DNA extraction was done using Phenol chloroform method and Instagene matrix kit (Chelex; Biorad) base on our previous studies (Lasjerdi *et al*. 2011). All of the cloned amoebae were submitted to PCR targeting the highly variable region of

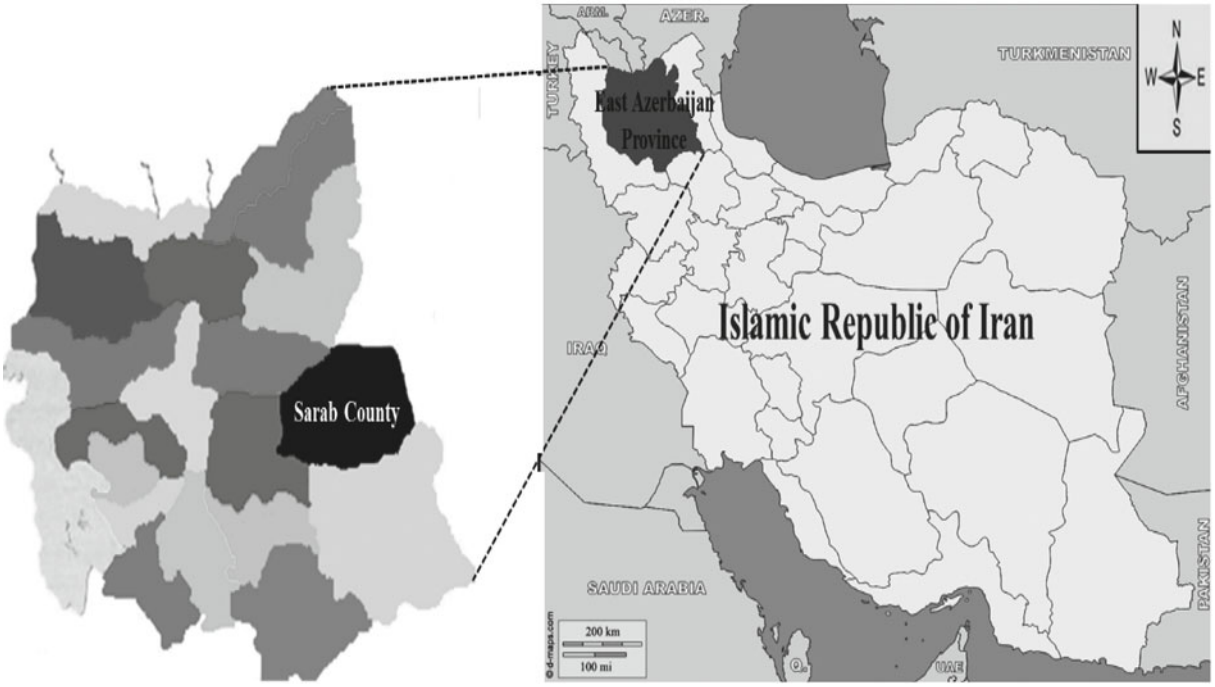

Fig. 1. Map of the East Azarbaijan province and Sarab county, Iran

Diagnostic Fragment 3 (stem 29-1 of 18S rRNA gene) within *Acanthamoeba*-specific amplimer. Molecular identification were done for *Acanthamoeba* positive sample using primers JDP1-2 (JDP1 5'- GGCCCAGATCGTTTACCGTGAA-3' and JDP2 5'-TCTCACAAGCTGCTAGGGAGTCA- 3') (Schroeder *et al*. 2001).

PCR was done using in 15 µl Ampliqone (Taq DNA Polymerase Master Mix RED, Denmark) as a ready-made mixture. 7.5 µl of Ampliqone were used with 2 µl template DNA, 0.8 µl of primer and 4.7 µl distilled water. The thermal cycling conditions were an initial denaturing step of 94°C for 1 min and 35 repetitions at 94°C for 35 s, annealing step were 56°C for 45 s, and extension were 72°C for 1 min. PCR products were electrophoresed on a 2% agarose gel stained with a solution of ethidium bromide and detected under UV light. PCR products were purified and resolved using the ABI 3130X automatic sequencer in Takapozist company and a homology analysis using the Basic Local Alignment Search Tool (BLASTn) was performed to search for the most similar reference sequences. Sequencing analysis were done using BLASTn in the genbank database.

Nucleotide sequence accession numbers

The DNA sequences for the new strains have been submitted in the genetic sequence database at the National Center for Biotechnical Information (NCBI) using the Sequin program (version 10.3) under accession numbers: KT985962-78.

Pathogenic assays of the positive strains

Pathogenic assay was performed using thermo tolerance and osmotolerance assay (Todd *et al.* 2015). Approximately 10^3 trophozoites were inoculated onto fresh non nutrient agar medium and each plate incubated at 30, 37 and 40°C. For osmotolerance assay 10^3 trophozoites were inoculated in non-nutrient agar plates containing mannitol 0.5 and 1 M. Positive and negative control were also applied. All of the plates were tested for the outgrowth of *Acanthamoeba* spp. after 24, 48 and 72 h.

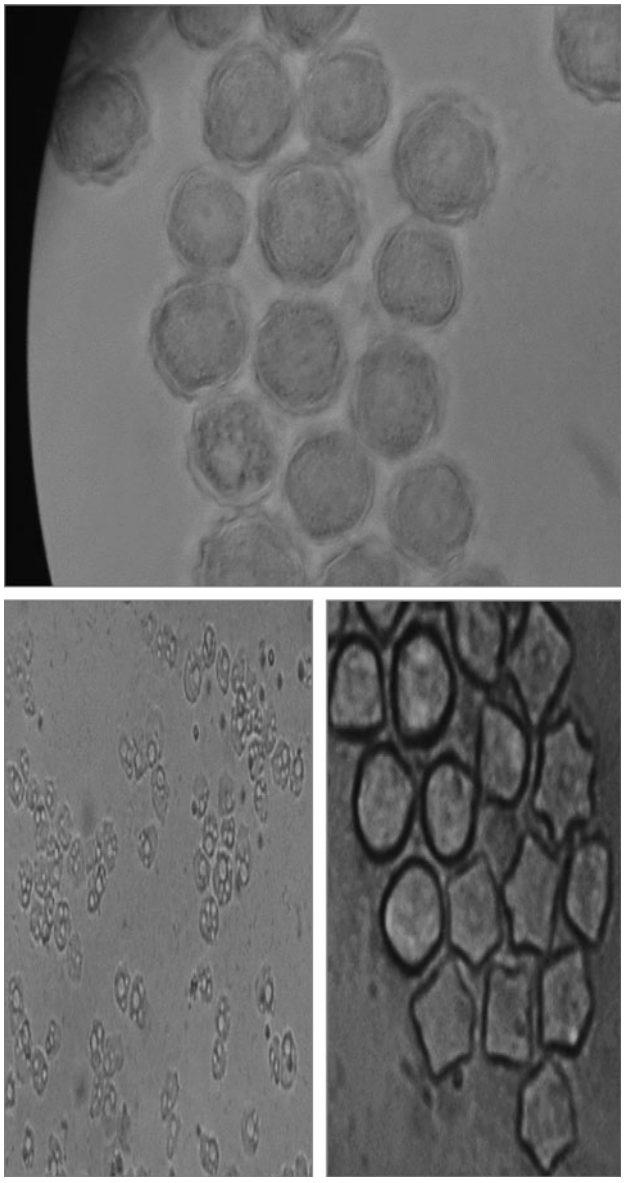

Fig. 2. Light microscopy photograph of cloned *Acanthamoeba* trophozoites (Left) and cysts (top and right) T4 genotype in non-nutrient agar (Magnification left × 400, top and right × 1000)

Results

In this study, 25 (41.6%) out of 60 collected samples were positive for *Acanthamoeba* based on morphological criteria. Contaminated sources were belonged to hospital environment, parks, school campus and swimming pool area (Table I). Cul-

Table I. Frequency of contaminated sources to *Acanthamoeba* genotypes

Source	Total No	No (%)/Positive	Genotypes
Play grounds	24	8 (33.3)	T3(1),T4(6),T5(1)
Campus	17	6 (35.2)	T3(1),T4(4),T11(1)
Pool area	17	2 (11.7)	T4(2)
Hospital	2	1 (50)	T4(1)
Total	60	17 (28.3)	T3(2),T4(13),T5(1),T11(1)

Table II. *Acanthamoeba* genotypes, sources and pathogenic potential in the present study

Sample code	Sampling area	Sources	NNA Culture/PCR	Temperature tolerance 37°C/40°C	Osmotolerance 1 M/0.5 M	Species	Genotype	Identity/ Query coverage	Genbank code
SK4	Hospital	Soil	+/+	-/-	-/-	*ND**	T4	99/97	KT985962
SK8	Pool area	Soil	+/+	+/+	+/+	*A.castellanii*	T4	99/98	KT985963
SK9	Park	Soil	+/+	-/-	-/-	*ND*	T4	99/98	KT985964
SK13	Park	Soil	+/+	-/-	-/-	*A.lenticulata*	T5	99/98	KT985965
SK18	Park	Soil	+/+	-/-	-/-	*ND*	T4	99/95	KT985966
SK19	Park	Soil	+/+	-/-	-/-	*ND*	T4	99/97	KT985967
SK25	Park	Soil	+/+	+/+	+/+	*A.griffini*	T3	100/100	KT985968
SK26	Park	Soil	+/+	+/+	+/+	*ND*	T4	99/97	KT985969
SK27	Campus	Soil	+/+	+/+	+/+	*ND*	T4	99/97	KT985970
SK28	Campus	Soil	+/+	-/-	-/-	*A.castellanii*	T4	99/97	KT985971
SK29	Park	Soil	+/+	+/+	+/+	*A.castellanii*	T4	99/97	KT985972
SK35	Pool area	Soil	+/+	+/+	+/+	*ND*	T4	99/97	KT985973
SK38	Campus	Soil	+/+	+/+	+/+	*ND*	T4	99/93	KT985974
SK41	Campus	Soil	+/+	+/+	+/+	*ND*	T4	99/98	KT985975
SK46	Campus	Soil	+/+	+/+	+/+	*A.griffini*	T3	100/100	KT985976
SK48	Park	Soil	+/+	-/-	-/-	*ND*	T4	99/98	KT985977
SK53	Campus	Soil	+/+	-/-	-/-	*A.hatchetti*	T11	99/71	KT985978

ture axenification were successful in 17 isolated strain after two month as there were high contamination to soil bacteria and fungi. Trophozoites were characterized by their pear-shape or irregularly renal-shape structures and some of them had fine pseudopodia-like extensions. Also double-walled cysts by wrinkled, angular, triangular and asteroid inner wall and round external wall called ectocysts of *Acanthamoeba* have been seen (Fig. 2).

As expected sequence analysis of the DF3 region of *rRNA* genes of these isolates revealed *Acanthamoeba* belonging to T4 genotype as the most isolated type with homology analysis revealing 97–100% similarity (identity and query coverage) to available genes in the gene data bank (Table II). Three strains (SK8, SK28 and SK29) showed high homology to *A. castellanii*. However, genotypes belonging to T3 corresponding to *A. griffini* (SK25, SK46), T5 corresponding to *A. lenticulata* (SK13) and T11 (SK53) corresponded to *A. hatchetii* were also determined (Table II).

Pathogenic assay through thermo and osmotolerance explored the nine (52.9%) highly pathogenic strains (SK8, SK25, SK26, SK27, SK29, SK35, SK38, SK41, SK46) belonged to T3 and T4 genotype. Two *Acanthamoeba* strains (SK8 and SK35) belonged to T4 genotype isolated from swimming pool area were showed high pathogenic potential (Table I and II).

Discussion

Isolation of *Acanthamoeba* spp. in 41.6% of soil samples of recreational and public places in northwest Iran reflect that soil sources of the studied regions are suitable niches for outgrowth of amoebae. All of collected soils were in places with high human activity. There are only a few studies regarding genotypes of *Acanthamoeba* in soil sources in Iran and worldwide. In a previous Iranian study a 26.9% *Acanthamoeba* contamination rate was reported from soil sources (all belonging to T4 genotype) in recreational parks of Tehran, Iran (Niyyati *et al*. 2013). The lower frequency of *Acanthamoeba* spp. in their research may be due to seasons as Niyyati *et al*. have collected the soil samples in a dry and cold season (with average tem: 5–6^{O}C). Previous studies demonstrated that the abundance of free living amoebae could be affected by moister content, organic carbon and soil texture of the region (Rodríguez-Zaragoza 1994; Todd *et al*. 2015). These studies showed that wetter seasons are more suitable for occurrence of *Acanthamoeba* then dryer seasons. Other research in south Iran with a dry seasons also showed the lower frequency of *Acanthamoeba* from Soil samples (26%) and the isolated genotypes were T4 and T5 (Rahdar *et al.* 2012). However no pathogenic assays were performed on the isolates. Reyes-Batlle *et al*. studied soils of Gran Canaria, Canary Islands, Spain. In their study twenty-four soil samples were tested. Fifteen of the 24 samples (62.5%) were positive for *Acanthamoeba* based on morphological and molecular study. Genotypes belonging to T2, T5 and T4 were reported (Reyes-Batlle *et al*. 2014). Todd *et al*. also reported the 63.9% contamination of soil samples in Jamaica, West Indies and reported genotypes were T4, T5 and T11 (Todd *et al*. 2015). In the present study in addition to T4, T5 and T11 types, genotype T3 clustered to *A. griffinii* was also detected in two samples (SK25 and SK46) and both showed pathogenic potential using physical parameters. Interestingly this genotype showed high homology to the *Acanthamoeba* T3 isolated previously from keratitis patients in Iran (Ac-

cession number: EU934051) (Niyyati *et al.* 2009). All of the genotypes of the present research have been the casual agents of AK in Iran (Maghsood *et al*. 2005; Niyyati *et al*. 2009; Niyyati and Rezaeian 2015c). Most of isolated genotypes (T4 and T3) showed pathogenic potential using physical parameters. Earlier research explored that thermotolerance and osmotolerance assays are indicative of pathogenic potential. However more tests including invivo test and cell culture should confirm the pathogenic potential (Khan 2006, 2009).

Overall, high percentage of free living amoeba including *Acanthamoeba* spp. in soil and other environmental samples is a hygienic risk for public health mainly for individuals with immune deficiency and contact lens wearers. Therefore health experts must be conscious of FLA presence in such environments. Our results are a step toward considering contaminated sources as a leading risk factor for soil-borne diseases which reflect a potential hazard to human health.

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