Genotyping of *Acanthamoeba* isolates from clinical and environmental specimens in Iran

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**A R T I C L E   I N F O**

Article history:
Received 28 September 2008
Received in revised form 11 November 2008
Accepted 17 November 2008
Available online 25 November 2008

Keywords:
*Acanthamoeba*
Iran
Genotype
Keratitis
Environment

**A B S T R A C T**

In this study, 15 *Acanthamoeba* isolates from AK patients and 10 environmental samples (water, soil and animal-origin samples) were classified at the genotype level based on the sequence analysis of the Diagnostic Fragment 3 (DF3) of *Acanthamoeba* small subunit rRNA gene. The obtained results revealed that most of these *Acanthamoeba* strains belonged to genotype T4 both in clinical and environmental samples. The presence T11 genotype in clinical samples was also revealed after the genotyping analysis and to our knowledge this is the first report of T11 genotype in Iran. Moreover, the isolation of T4 genotype from cow faeces in this study highlights a possible transmission of *Acanthamoeba* through animal faeces in Iran.

Overall, the widespread distribution of pathogenic *Acanthamoeba* T4 across the environmental sources and the increasing number of contact lens wearers in Iran, demands more awareness within the public and health professionals as this pathogen is emerging as a risk for human health in Iran and worldwide.

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1. Introduction

Free-living amoebae belonging to *Acanthamoeba* genus are emerging as a serious problem in soft contact lens wearers (Khan, 2003; Visvesvara et al., 2007; Marciano-Cabral and Cabral, 2003). In fact, specific genotypes of these amoebae are causative agents of a sight-threatening corneal infection known as *Acanthamoeba* keratitis (AK). To date, isolates belonging to T4 genotype are globally known as the most common genotype related to AK cases and in the environment (Khan, 2006; Schuster and Visvesvara, 2004). However, other genotypes have been reported worldwide as causative agents of AK such as T2B, T3, T5, T6 and T11 (Ledee et al., 1996; Stothard et al., 1998; Walochnik et al., 2000; Booton et al., 2002; Maghsood et al., 2005; Spanakos et al., 2006).

AK cases have risen in the recent years in Iran, as it was revealed by a 10 year study by Rezaeian et al. in this region. This study reported that most of AK patients in Iran are between 15 and 25 years old and female individuals are mostly affected may be due to the higher rate of contact lens wearers within women compared to the male population. Regarding keratitis cases in Iran, this study also demonstrated that *Acanthamoebae* were the causative agents in a 34.5% of the keratitis cases in the region in the last 10 years (Rezaeian et al., 2007). It is noteworthy that poor hygiene and insufficient disinfection measures were the major identified risk factors among these cases (Rezaeian et al., 2007). In addition, previous studies in Iran revealed that there are three species of *Acanthamoeba* which are associated to AK in this region including: *Acanthamoeba castellanii* (T4), *Acanthamoeba griffini* (T3) and *Acanthamoeba palestinensis* (T2) and also reported the presence of T2 and T4 genotypes in water sources from Tehran and other cities in the north of Iran (Maghsood et al., 2005). No previous studies on the presence and distribution of *Acanthamoeba* genotypes in other environmental (soil) or animal sources have been previously reported in this region and thus, samples from these sources were included in this study.

As suggested before, the high isolation rate of T4 genotype related to AK cases worldwide may be due to their greater abundance in the environment, greater virulence or both (Khan, 2006). In order to clarify this and give further evidences of T4 as the predominant genotype associated with AK cases in Iran, 15 clinical isolates from AK cases presented during 2005–2008 at the School of Public Health, Tehran University of Medical Sciences, Iran, eight environmental isolates and two strains isolated from
animal faeces collected from various locations in Iran were genotyped by sequencing the Diagnostic Fragment 3 (DF3) of the Acanthamoeba small subunit rRNA gene.

2. Material and methods

2.1. Isolates

During 2005–2008, a total of 50 specimens from keratitis patients referred to the Parasitology Laboratory, School of Public Health, Tehran Medical University, Iran were tested for the presence of Acanthamoeba. Clinical specimens were identified based on detection of cysts in corneal smears and/or positive PCR of corneal scrapes and report of painful keratitis in contact lens wearers who were responding to a treatment with 0.1% brolene and neomycin® (0.025 mg of neomycin, 1.75 mg of polymyxin B and 10,000 units of gramicidin/ml). Regarding the clinical specimens, most patients were diagnosed AK within 20 days and the most common symptoms among these patients were photophobia, severe pain and eye watering.

Clinical specimens (contact lenses and cases, corneal scrapes or contact lens maintenance solution) were examined by both direct microscopic and culture methods as previously described (Rezaeian et al., 2007). Additionally, 10 isolates of Acanthamoeba which were obtained from soil (7), water (1) and cow faeces (2) were also included in this study. These samples were collected in a previous study and were identified as Acanthamoeba based on only morphological criteria (Rezaeian et al., 2008).

Water samples (500 ml approximately) were filtered through a 0.45 μm pore-size cellulose nitrate membranes (Rezaeian et al., 2008). Other samples including soil and animal faeces were dissolved in distilled sterile water and filtered as described above. Regarding the cow faeces, it is important to mention that these samples were collected from the interior of the faeces in order to avoid any contaminations.

After that, filters were placed on to 2% non-nutrient agar (NNA) plates overlaid with heat-killed Escherichia coli. Plates were incubated at 28 °C and monitored daily for the out-growth of Acanthamoeba using an inverted microscope. After 1–2 weeks, swabs were transferred to axenic culture by placing the amoeba in PYG medium and monitored daily for their elimination. Fungi and bacteria-free plates were then incubated for up to 2 weeks. In order to eliminate fungi contamination in environmental plates, fluorescein (1.25 μg/ml) was applied on the new plates and monitored daily for their elimination. Fungi and bacteria-free plates were then transferred to axenic culture by placing the amoeba in PYG medium as previously described for further morphological and molecular analyses (Lorenzo-Moraes et al., 2005, 2006).

It should be mentioned that in two plates (ICL-20 and IW-2), two different (morphologically) cyst forms of Acanthamoeba were detected and therefore cysts of these plates were cloned before the molecular analyses were performed.

2.2. DNA extraction and DF3 PCR amplification

Total genomic DNA was extracted using the phenol–chloroform method as previously described (Sambrook et al., 1989). DNA extraction of the cyst form was successfully done by prolonged incubation of samples with DNA lysis buffer (50 mM NaCl, 10 mM EDTA, 50 mM Tris–HCl, pH 8.0) and protease K (0.25 mg/ml) at 60 °C, overnight.

rRNA gene amplifications (DF3 region) were performed as previously described with minimal modifications (Booton et al., 2002) in a 30 μl volume containing 1.25 U Taq DNA polymerase (Ecogen), 30 ng DNA, 1.5 mM MgCl2, 200 μM dNTP and 0.2 μM each primer. Amplification products were fractioned by 2% agarose electrophoresis stained with a solution of 0.5 μg/ml of ethidium bromide and visualized under UV light using a Chemi-doc Image Analyzer (Bio-rad, Madrid, Spain).

2.3. Sequencing and genotyping of the isolates

PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced using a MEGABACE 1000 automatic sequencer (Healthcare Biosciences, Barcelona, Spain) in the University of La Laguna Sequencing Services (Servicio de Secuenciación SEGAL, University of La Laguna). The obtained sequences were aligned using Mega 3.0 software program (Kumar et al., 2004). 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 or with the Acanthamoeba DNA database (Department of Molecular Genetics, The Ohio State University, OH, USA). DF3 sequences for the new isolates are deposited in the GenBank data-base under the Accession Nos.: EU934046–EU934072.

3. Results

3.1. Isolation of Acanthamoeba from clinical and environmental specimens

Acanthamoeba was identified in 15 (30%) of 50 keratitis samples. Among these clinical isolates, 13 (86.7%) belonged to female patients and 2 (13.3%) were male patients. All positive specimens belonged to soft contact lens wearers excepting one of them which belonged to a hard contact lens wearer (Table 1). Regarding genotype identification, 13 (86.7%) of these isolates belonged to T4 genotype. However, it is important to mention the identification of two different genotypes in ICL-20 sample that presented Acanthamoeba DNA sequences in Genbank or with the Acanthamoeba DNA database (Department of Molecular Genetics, The Ohio State University, OH, USA).

Environmental isolates were collected in a previous study where identification of isolates was based only on morphological criteria (Rezaeian et al., 2008). From this study one pool water, two animal-origin isolates and seven soil-related samples (Table 2) were selected for further identification at the genotype level in the current study. All soil-related isolates belonged to genotype T4 in contrast to IW-2 sample (pool sample, see Table 2) that presented strains belonging to T2 and T6 genotypes. In the case of the animal-origin samples IA-1 and IA-2 (strains isolated from cow faeces) genotype T4 was identified in both. Sequence analysis of the DF3 region in these two strains showed high homology (>99%) between them and A. polyphaga strains from the Genbank database (AY026243.1).

4. Discussion

This is the second study about the distribution of Acanthamoeba genotypes in clinical and environmental samples in Iran. The obtained results in our study give further evidence of genotype T4 as the most common one within AK-related specimens in Iran (86.7%, see Table 1) which is in accordance to other studies worldwide (Visvesvara et al., 2007; Marciano-Cabral and Cabral, 2003). Furthermore, T11 genotype was identified in clinical specimens from two patients and this is, to our knowledge, the first report of T11 genotype associated with AK cases in Iran. As it was mentioned before, the majority of AK cases worldwide are due to genotype T4 excepting occasional cases of AK due to T2B, T3, T6 and T11 (Khan and Paget, 2002; Ledee et al., 1996; Maghsood et al., 2005; Walochkin et al., 2000). Moreover, non-AK infections have been recently found to be associated with genotypes T3 or T11 (Booton et al., 2005). It is interesting to mention that in our case both pa-
patients that were positive for T11 were female soft contact lens wearers and one of them (ICL-20) suffered from a coinfection due to two genotypes of *Acanthamoeba*, being the other one a member of T4 genotype. Further studies are needed in order to establish whether T11 in Iran could be another AK-related genotype in contrast to the non-AK genotype as it was previously suggested for other areas.

The existence of *Acanthamoeba* in many different environmental sources worldwide, lead us to the analysis of different environmental sources in Iran. Regarding the environmental samples that were previously analyzed in Iran all of them were limited to pool and waterfall samples (Maghsood et al., 2005). Therefore, in our study other sources such as soil, animal-origin samples and one pool water were checked for the presence of this pathogen. In the previous study, the most abundant genotype (in a total of 12 water samples) was T2 (58.3%) followed by T4 (33.3%) (Maghsood et al., 2005). In our study, T4 was the only identified genotype in the analyzed soil sources whereas T2 and T6 were found in the only pool water sample included in this study. This is an important difference in comparison to the previous study and it may be due to the different sources that were tested in the present work. Therefore, it seems to be a high prevalence of T4 genotype in the soil sources of Iran and this may be important in the causation of AK in contact lens wearers. In the previous study by Maghsood et al. (2005) three water samples were collected from this city and one of them was genotyped as T4, another as T2 and one of them was not determined.

Additionally, two animal-origin samples were also analyzed for the presence of *Acanthamoeba* and the obtained isolates were classified as members of the T4 genotype. The extent to which *Acanthamoeba* are distributed in different animals is still unknown. There are reports on the presence of potentially pathogenic strains in wild squirrels from Morocco and the Canary Islands (Lorenzo-Morales et al., 2007) and also the isolation of a potentially pathogenic *Acanthamoeba hatchetti* strain (T11) was reported on a study about the presence of free-living amoebae in small rodents from Tunisia and Brittany (Simitzis-Le Flohic and Chastel, 1982). There are also reports of the isolation of *Acanthamoeba* strains in various animals such as tigers (González et al., 2007) and poikilothermic animals such as fish, amphibian and reptiles (Sesma and Ramos, 1989; Dykova et al., 1999; Walochnik et al., 1999) and captive burs-tards (Silvanose et al., 1998). Moreover, acanthamoebiasis cases has been also reported in horses (Kinde et al., 2007), a toucan, dogs, monkeys, a bull, a kangaroo and an Indian buffalo (Schuster and Visvesvara, 2004). *Acanthamoeba* cysts have been discovered in human faeces (Zaman et al., 1999) however, there has to date to our knowledge been no indication of the involvement of the amoeba in pathogenesis in the intestinal tract as there is of course with *Entamoeba*.

In conclusion, further studies about the distribution of *Acanthamoeba* genotypes in Iran should be carried out due to the increasing prevalence of AK in this country and worldwide. Moreover, more detailed studies should also be developed in this area due to the previous identification of T2B genotype in this region and also the new report of T11 genotype in AK cases in this country. These data together with the reports in Iran and worldwide about the high prevalence of T4 in clinical and environmental sources would give more detailed knowledge about the distribution of the different genotypes of *Acanthamoeba* worldwide.

**Acknowledgments**

Mrs. Maryam Niyyati was supported by an overseas fellowship in Spain from The Ministry of Health, Treatment and Medical Education of Iran. This project was funded by project # 85-02-27-3784 from Tehran University of Medical Sciences and also by the project RICET (project no. RD06/0021/0005 of the programme of Redes Temáticas de Investigación Cooperativa, FIS), Spanish Ministry of Health, Madrid, Spain. CM Martín-Navarro was funded by a PhD grant “Becas de Investigación para Postgraduados 2008”.

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Table 1
Clinical and laboratory data of 15 patients with amoebic keratitis.

<table>
<thead>
<tr>
<th>Code</th>
<th>Age (Y)</th>
<th>Sex</th>
<th>Contact lens type</th>
<th>Source</th>
<th>Direct smear of CS</th>
<th>NNA culture</th>
<th>PCR</th>
<th>Genotype</th>
<th>Accession No.</th>
</tr>
</thead>
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<tr>
<td>ICL-1</td>
<td>20</td>
<td>F</td>
<td>Soft</td>
<td>CL, CS</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>T4</td>
<td>EU934046</td>
</tr>
<tr>
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<td>20</td>
<td>F</td>
<td>Soft</td>
<td>CL,CS</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>T4</td>
<td>EU934047</td>
</tr>
<tr>
<td>ICL-3</td>
<td>25</td>
<td>F</td>
<td>Soft</td>
<td>CL</td>
<td>NA</td>
<td>*</td>
<td>*</td>
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<tr>
<td>ICL-4</td>
<td>18</td>
<td>F</td>
<td>Soft</td>
<td>CL,CS</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>T4</td>
<td>EU934049</td>
</tr>
<tr>
<td>ICL-5</td>
<td>31</td>
<td>F</td>
<td>Soft</td>
<td>CL,CS</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>T4</td>
<td>EU934050</td>
</tr>
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<td>ICL-6</td>
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<td>Soft</td>
<td>CL,CS</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>T4</td>
<td>EU934051</td>
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<td>ICL-7</td>
<td>27</td>
<td>M</td>
<td>Soft</td>
<td>CL,CS</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>T11/T4</td>
<td>EU934052</td>
</tr>
<tr>
<td>ICL-8</td>
<td>16</td>
<td>F</td>
<td>Soft</td>
<td>CL,CS</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>T4</td>
<td>EU934053</td>
</tr>
<tr>
<td>ICL-9</td>
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<td>Soft</td>
<td>CL</td>
<td>NA</td>
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<td>*</td>
<td>T4</td>
<td>EU934054</td>
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<td>Soft</td>
<td>CL,CS</td>
<td>+</td>
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<td>*</td>
<td>T4</td>
<td>EU934055</td>
</tr>
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<td>*</td>
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<td>*</td>
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<td>CS</td>
<td>+</td>
<td>-</td>
<td>*</td>
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<td>Soft</td>
<td>CL,CS</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>T4</td>
<td>EU934059</td>
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<td>33</td>
<td>F</td>
<td>Soft</td>
<td>CL,CS</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>T4</td>
<td>EU934060</td>
</tr>
<tr>
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<td>25</td>
<td>F</td>
<td>Soft</td>
<td>CL,CS</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>T4</td>
<td>EU934061</td>
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<td>F</td>
<td>Soft</td>
<td>CL,CS</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>T4</td>
<td>EU934062</td>
</tr>
<tr>
<td>ICL-18</td>
<td>33</td>
<td>F</td>
<td>Soft</td>
<td>CL,CS</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>T4</td>
<td>EU934063</td>
</tr>
<tr>
<td>ICL-19</td>
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<td>F</td>
<td>Soft</td>
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<td>+</td>
<td>*</td>
<td>*</td>
<td>T4</td>
<td>EU934064</td>
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<tr>
<td>ICL-20</td>
<td>27</td>
<td>F</td>
<td>Soft</td>
<td>CL,CS</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>T11/T4</td>
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<td>ICL-21</td>
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<td>Soft</td>
<td>CL,CS</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>T4</td>
<td>EU934066</td>
</tr>
</tbody>
</table>

* a ICL, indicates Iranian contact lens.
* b CL, contact lens.
* c CS, corneal scrapes.
* d NA, data not available.

Table 2
Data of environmental and animal-origin samples.

<table>
<thead>
<tr>
<th>Code</th>
<th>Source</th>
<th>Culture</th>
<th>PCR Genotype Accession No.</th>
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</thead>
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<td>IA-1</td>
<td>Cow faeces</td>
<td>+</td>
<td>T4 EU934062</td>
</tr>
<tr>
<td>IA-2</td>
<td>Cow faeces</td>
<td>+</td>
<td>T4 EU934063</td>
</tr>
<tr>
<td>IW-1</td>
<td>Pool water</td>
<td>+</td>
<td>T4 EU934071, EU934072</td>
</tr>
<tr>
<td>IS-1</td>
<td>Soil</td>
<td>+</td>
<td>T4 EU934067</td>
</tr>
<tr>
<td>IS-2</td>
<td>Soil</td>
<td>+</td>
<td>T4 EU934068</td>
</tr>
<tr>
<td>IS-3</td>
<td>Soil</td>
<td>+</td>
<td>T4 EU934069</td>
</tr>
<tr>
<td>IS-4</td>
<td>Soil</td>
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<td>Soil</td>
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</tr>
</tbody>
</table>

* a IA, indicates Iranian animal.
* b IW, indicates Iranian water.
* c IS, indicates Iranian soil.

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References


