Acanthamoeba genotype T4 from the UK and Iran and isolation of the T2 genotype from clinical isolates

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The majority of the keratitis-causing *Acanthamoeba* isolates are genotype T4. In an attempt to determine whether predominance of T4 isolates in *Acanthamoeba* keratitis is due to greater virulence or greater prevalence of this genotype, *Acanthamoeba* genotypes were determined for 13 keratitis isolates and 12 environmental isolates from Iran. Among 13 clinical isolates, eight (61·5 %) belonged to T4, two (15·3 %) belonged to T3 and three (23 %) belonged to the T2 genotype. In contrast, the majority of 12 environmental isolates tested in the present study belonged to T2 (7/12, 58·3 %), followed by 4/12 T4 isolates (33·3 %). In addition, the genotypes of six new *Acanthamoeba* isolates from UK keratitis cases were determined. Of these, five (83·3 %) belonged to T4 and one was T3 (16·6 %), supporting the expected high frequency of T4 in *Acanthamoeba* keratitis. In total, the genotypes of 24 *Acanthamoeba* keratitis isolates from the UK and Iran were determined. Of these, 17 belonged to T4 (70·8 %), three belonged to T2 (12·5 %), three belonged to T3 (12·5 %) and one belonged to T11 (4·1 %), confirming that T4 is the predominant genotype ($\chi^2 = 4{\cdot}167$; $P = 0{\cdot}0412$) in *Acanthamoeba* keratitis.

INTRODUCTION

Acanthamoeba are ubiquitous organisms found in a variety of environments including soil, water, air, food items, humidifiers, dialysis units and healthy individuals (reviewed by Khan, 2003; Marciano-Cabral & Cabral, 2003; Schuster & Visvesvara, 2004). Based on their ecological distribution, there is circulation of *Acanthamoeba* strains between humans and the environment. *Acanthamoeba* are opportunistic causative agents of nasopharyngeal and cutaneous infections, painful blinding keratitis and fatal *Acanthamoeba* granulomatous encephalitis (Khan, 2003; Marciano-Cabral & Cabral, 2003; Schuster & Visvesvara, 2004). The difficulty in assigning an explicit role of *Acanthamoeba* in infections is because *Acanthamoeba* are heterogeneous and only certain subgroups may be pathogenic. Recent advances in the taxonomy of *Acanthamoeba* have led to the identification of the pathogenic subgroups. The genus *Acanthamoeba* has been classified into 15 different genotypes (T1–T15) based on rRNA gene sequencing (Gast, 2001; Stothard *et al.*, 1998; Schuster & Visvesvara, 2004). To date, only isolates belonging to the T3, T4, T6 and T11 genotypes have been associated with *Acanthamoeba* keratitis (Booton *et al.*, 2002; De Jonckheere, 2003; Khan *et al.*, 2002; Ledee *et al.*, 1996; Stothard *et al.*, 1998; Walochnik *et al.*, 2000), and a large number of studies have identified T4 as the predominant keratitis-causing genotype. Indeed more than 90 % of *Acanthamoeba* keratitis cases in literature reports have been caused by T4 isolates. As suggested before, the frequent involvement of T4 isolates in *Acanthamoeba* keratitis may be due to their greater abundance in the environment and thus they are most likely to be picked up by the susceptible hosts, or because T4 isolates possess properties that enable them to be more virulent, or both. In an attempt to clarify this, we determined the environmental and clinical distribution of *Acanthamoeba* in Iran. We tested 13 *Acanthamoeba* keratitis cases presented during 1998–2003 at the Tehran University of Medical Sciences, Iran. In addition, *Acanthamoeba* from environmental sources from widespread geographic locations were isolated and identified at the genotype level using rRNA gene sequencing.

Abbreviation: HCEC, human corneal epithelial cell.

METHODS

Clinical isolates. During 1998–2003, specimens from keratitis patients were tested for the presence of *Acanthamoeba* at the Department

of Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. A total of 52 keratitis cases was tested. To determine the presence of *Acanthamoeba*, specimens (from contact lenses or corneal biopsies) were inoculated on to non-nutrient agar (Oxoid) plates seeded with *Enterobacter aerogenes* (formerly known as *Klebsiella aerogenes*). Plates were incubated at 30 °C and observed daily for the presence of amoebae as previously described (Khan & Paget, 2002).

Environmental isolates. Water samples from different cities in Iran (Table 1) were collected and tested for the presence of *Acanthamoeba*. For each sample, approximately 100–500 ml water was filtered through a 0·45 μm pore-size filter. Filters were inoculated on to non-nutrient agar plates overlaid with *E. aerogenes* as described above. *Acanthamoeba* were identified at the genus level, based on morphological characteristics of trophozoites and cysts using phase-contrast microscopy. *Acanthamoeba* were axenically cultivated in PYG medium (0·75 %, w/v, proteose peptone; 0·75 %, w/v, yeast extract; and 1·5 %, w/v, glucose) at 30 °C as described previously (Khan & Paget, 2002).

Our previous studies have isolated *Acanthamoeba* from other geographic locations including the UK (Khan *et al.*, 2002) and these isolates were compared in the present study. In addition, *Acanthamoeba* spp. were isolated from six new keratitis patients in the UK (Table 1), identified at the genotype level using rRNA gene sequencing and used in comparative studies.

PCR analysis. To confirm the identity of *Acanthamoeba*, PCR reactions were performed using genus-specific primers as previously described (Khan *et al.*, 2001). Briefly, total DNA was extracted using the InstaGene matrix (Chelex; Bio-Rad) method as follows. Approximately 10^3 cells (counted using a haemocytometer) were incubated with 30 μl Chelex. Tubes were incubated at 56 °C for 20 min, followed by a 10 min incubation at 99·9 °C and finally centrifuged at 10 000 ***g*** for 5 min. The supernatants containing DNA were used as the template for PCR. Primer sequences were 5′-TTTGAATTCGCTCCAATAGCGTATATTAA-3′ and 5′-TTTGAATTCAGAAAGAGCTATCAATCTGT-3′ (Kong & Chung, 1996). PCR was performed in a volume of 50 μl containing 1·25 U *Taq* polymerase (Qiagen), 0·1–1·0 ng DNA, 200 μM dNTPs,

Table 1. *Acanthamoeba* isolates tested in the present study

No.	Patient no.	Source	Species	Genotype
(a) Environmental isolates (water samples)				
1	1-II	Fountain pool water, Shariati square, Hamedan, Iran	*Acanthamoeba palestinensis*	T2
2	3-I	Fountain pool water, Imam square, Hamedan, Iran	*A. palestinensis*	T2
3	6-V	Pond water, Estakhr-e Abas-abad, Hamedan, Iran	*Acanthamoeba castellanii*	T4
4	7-S	Pool water, Mellat Park, Hamedan, Iran	*A. palestinensis*	T2
5	8-II	Fountain pool water, Tehran, Tehran University, Iran	*A. castellanii*	T4
6	8-III	Fountain pool water, Tehran, Tehran University, Iran	ND*	ND*
7	9-S	Fountain pool water, Laleh Park, Tehran, Iran	*A. palestinensis*	T2
8	16-I	Fountain pool water, Ramsar, Shahed University, Iran	*A. palestinensis*	T2
9	19-I	Fountain pool water, Babataher square, Hamedan, Iran	*A. palestinensis*	T2
10	21-II	Fountain pool water, Tonkabon, Imam square, Iran	*A. castellanii*	T4
11	24-I	Fountain pool water, Ramsar, Shahrivar square, Iran	*A. castellanii*	T4
12	27-II	Waterfalls, Ganj-nameh, Hamedan, Iran	*A. palestinensis*	T2
(b) Clinical isolates (keratitis patients)				
13	30	Contact lens (*Acanthamoeba* keratitis patient), Iran	*A. castellanii*	T4
14	52	Contact lens (*Acanthamoeba* keratitis patient), Iran	*A. castellanii*	T4
15	53	Contact lens (*Acanthamoeba* keratitis patient), Iran	*Acanthamoeba griffini*	T3
16	54	Corneal tissue (*Acanthamoeba* keratitis patient), Iran	*A. palestinensis*	T2
17	55	Contact lens (*Acanthamoeba* keratitis patient), Iran	*A. castellanii*	T4
18	56	Contact lens (*Acanthamoeba* keratitis patient), Iran	*A. griffini*	T3
19	58	Contact lens (*Acanthamoeba* keratitis patient), Iran	*A. castellanii*	T4
20	59	Contact lens (*Acanthamoeba* keratitis patient), Iran	*A. castellanii*	T4
21	60	Contact lens (*Acanthamoeba* keratitis patient), Iran	*A. castellanii*	T4
22	61	Contact lens (*Acanthamoeba* keratitis patient), Iran	*A. castellanii*	T4
23	62	Contact lens (*Acanthamoeba* keratitis patient), Iran	*A. castellanii*	T4
24	64	Contact lens (corneal trauma by insect), Iran	*A. palestinensis*	T2
25	65	Contact lens (*Acanthamoeba* keratitis patient), Iran	*A. palestinensis*	T2
26	515	UK keratitis	*A. griffini*	T3
27	771	UK keratitis	*A. castellanii*	T4
28	984	UK keratitis	*A. castellanii*	T4
29	1117	UK keratitis	*A. castellanii*	T4
30	1230	UK keratitis	*A. castellanii*	T4
31	1311	UK keratitis	*A. castellanii*	T4

**Acanthamoeba*, but species/genotype not determined. This sample was omitted from the statistical analysis of association with genotype, i.e. only 11 environmental samples were included.

4 mM $MgCl_2$ and 0·5 μM primer. PCR reactions were performed at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min for 30 cycles, with a final elongation step of 10 min at 72 °C. Amplified DNA was electrophoresed on a 2 % agarose gel, stained and visualized under UV illumination.

18S rRNA gene sequencing. For 18S rRNA gene sequencing, amplified DNA products were directly sequenced with an automated fluorescent sequencing system using a LI-COR 4200 DNA sequencer for diagnostic fragment 3 using primer 892C (5′-GTCAGAGGTGAA ATTCTTGG-3′) (Booton *et al.*, 2002) and identified at the genotype level by comparison with the available *Acanthamoeba* DNA sequences in GenBank or by comparison with the *Acanthamoeba* DNA sequence database (Department of Molecular Genetics, The Ohio State University, OH, USA). Diagnostic fragment 3 sequences for the new isolates are available upon request from N. A. Khan.

Cytotoxicity assay. For some *Acanthamoeba* isolates, cytotoxicity assays were performed as previously described (Khan & Tareen, 2003). Briefly, immortalized human corneal epithelial cells (HCECs) were grown to confluency in 24-well plates in supplementary hormonal epithelial medium (15 %, w/v, fetal bovine serum; 40 μg gentamicin ml^{-1}; 5 μg insulin ml^{-1}; 0·1 μg cholera toxin ml^{-1}; vitamins; 40 % Hanks F12; 40 % Dulbecco's modified Eagle's medium; 0·5 % DMSO; 10 ng epidermal growth factor ml^{-1}) (Invitrogen) at 37 °C in a 5 % CO_2 incubator as previously described (Araki-Sasaki *et al.*, 2000). *Acanthamoeba* isolates (5×10^5 amoebae per well) were incubated with cell monolayers in serum-free medium (RPMI 1640 containing 2 mM glutamine, 1 mM pyruvate and non-essential amino acids) at 37 °C in a 5 % CO_2 incubator for up to 24 h. At the end of this incubation period, supernatants were collected and cytotoxicity was determined by measuring lactate dehydrogenase release using a cytotoxicity detection kit (Roche Applied Science) as previously described (Khan & Tareen, 2003).

RESULTS AND DISCUSSION

Isolation of *Acanthamoeba* from the clinical specimens in Iran

Of 52 specimens taken from keratitis patients in Iran, 13 were found to contain *Acanthamoeba*, seven from women (53·9 %) and six from men (46·1 %); their ages ranged from 15 to 54 years (mean 22 years). Twelve patients (92·3 %) wore soft contact lenses and one patient (7·7 %) wore hard lenses. Patients were treated with topical application of propamidine isethionate (Brolene) plus Neosporin (neomycin, polymyxin B sulfates and bacitracin). Twelve patients responded to therapy with improved clinical symptoms and no report of recurrence, indicating successful treatment. One patient (#64) did not respond to anti-amoebic drugs and a corneal graft was performed. This patient wore hard lenses and while travelling on a motorcycle an insect had entered his right eye. He rubbed his eye while wearing a contact lens, which resulted in corneal trauma. The clinical signs of *Acanthamoeba* keratitis followed soon after and included severe pain, photophobia and stromal infiltrates. Within 20 days, he was diagnosed with *Acanthamoeba* keratitis and anti-amoebic therapy was initiated but showed no response. However, the symptoms cleared after a successful corneal graft, as indicated above.

Isolation of *Acanthamoeba* from the environmental samples in Iran

To determine the distribution of *Acanthamoeba* in the environmental samples in Iran, 12 *Acanthamoeba* were isolated from water samples obtained from different cities (Table 1). *Acanthamoeba* were isolated based on morphological characteristics of trophozoites and cysts. Overall, these results suggested a wide environmental distribution of *Acanthamoeba* from various geographic locations in Iran. The identity of both clinical and environmental isolates was further confirmed using PCR analysis as described in Methods (data not shown). *Acanthamoeba* were successfully cultured in PYG medium containing penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) at 30 °C, except for one environmental isolate (8-III).

Molecular typing of clinical isolates of *Acanthamoeba* from Iran

In this study, 13 *Acanthamoeba* keratitis cases were identified. Among these, eight (61·5 %) isolates belonged to the T4 genotype, two (15·3 %) belonged to T3 and three (23 %) belonged to the T2 genotype (Table 2). This is the first time that *Acanthamoeba* isolates belonging to the T2 genotype have been linked with *Acanthamoeba* keratitis. Of interest, patient #64, who was non-responsive to anti-amoebic therapy, was infected with a T2 isolate. To determine the pathogenic potential of the three T2 isolates (#54, #64 and #65), *in vitro* cytotoxicity assays were performed. We observed that two *Acanthamoeba* isolates (#64 and #65) produced more than 90 % HCEC cytotoxicity and were considered as potential pathogens. However, #54 exhibited minimal HCEC cytotoxicity (< 10 %) and was considered as a weak or non-pathogen. Overall, these data indicated that the majority of keratitis-causing isolates in Iran belong to the T4 genotype.

T2 is a widely distributed genotype in the environmental samples isolated from Iran

To determine the environmental distribution of *Acanthamoeba* in Iran, amoebae were isolated from 12 water samples. Out of these, seven (58·3 %) belonged to the T2 genotype and four belonged to T4 (33·3 %), while the *Rns* sequence of one (8-III) (8·3 %) was not determined (Table 2). These findings indicated that T2 is a widely distributed genotype (followed by T4) in the water samples tested from Iran.

Clinical and environmental distribution of *Acanthamoeba* from other geographic locations

For comparison of clinical isolates, 11 *Acanthamoeba* keratitis isolates from the UK were used (Khan *et al.*, 2002, plus the six new *Acanthamoeba* keratitis cases tested in the present study; Table 1). Of these, nine isolates (81·8 %) belonged to the T4 genotype, one belonged to T3 (9·1 %) and one belonged to T11 (9·1 %) (Table 2). In total, 24 *Acanthamoeba* keratitis isolates from the UK and Iran were tested. Of these 17 belonged to T4 (70·8 %), three belonged to T2 (12·5 %),

Table 2. Comparison of the clinical and environmental distribution of *Acanthamoeba* in the UK and Iran

Country	No. of samples	Genotype (%)
(a) Clinical isolates		
UK	11	1 T3 (9·1)
		9 T4 (81·8)
		1 T11 (9·1)
Iran	13	3 T2 (23)
		2 T3 (15·3)
		8 T4 (61·5)
Total	24	17 T4*
		3 T2
		3 T3
		1 T11
Association of T4 with clinical cases		
T4*	Not T4	Total
17	7	24
(b) Environmental isolates		
UK	9	2 T2 (22·2)
		1 T3 (11·1)
		4 T4 (44·4)
		2 T7 (22·2)
Iran	12	7 T2 (58·3)
		4 T4 (33·3)
		1 ND
Total	21	8 T4†
		9 T2
		1 T3
		2 T7
		Total: 20
Association of T4 with environment		
T4†	Not T4	Total
8	12	20

*$\chi^2 = 4{\cdot}167$ with 1 degree of freedom. The two-tailed P value equals 0·0412, indicating significant association of T4 with clinical cases.

†$\chi^2 = 0{\cdot}800$ with 1 degree of freedom. The two-tailed P value equals 0·3711, indicating no significant association of T4 in the environmental isolates.

three belonged to T3 (12·5 %) and one belonged to T11 (4·1 %), confirming that T4 is the predominant genotype ($\chi^2 = 4{\cdot}167$, $P = 0{\cdot}0412$) in *Acanthamoeba* keratitis.

For comparison of the environmental isolates, nine *Acanthamoeba* isolates from the UK were tested as previously described (Khan *et al.*, 2002; Khan & Paget, 2002). Among these, four isolates (44·4 %) belonged to T4, two belonged to T2 (22·2 %), one belonged to T3 (11·1 %) and two belonged to T7 (22·2 %) (Table 2). It is important to note that, except for the T4 isolates, the majority of amoebae were isolated from soil samples. Of interest, both T7 isolates were from soil samples.

Acanthamoeba are causative agents of painful eye infections, which can lead to blindness. They are one of the most ubiquitous organisms and have been classified into 15 different genotypes. In an attempt to correlate pathogenicity with certain genotypes, several studies have identified T4 as the major keratitis-causing genotype. However, whether the increased ability of T4 isolates to produce keratitis is due to their greater virulence or their greater prevalence is somewhat unclear. In this study, we tested clinical and environmental isolates of *Acanthamoeba* from a variety of sources and various geographic locations in the UK and Iran. The majority of clinical isolates tested in our study belonged to the T4 genotype, consistent with previous studies. Among the environmental isolates, T4 was again the predominant genotype in the UK, but T2 was predominant in Iran within the limited number of samples tested.

One of the interesting findings in our study was the isolation of T2 isolates from three keratitis patients (#54, #64, #65). Of these, #65 exhibited the characteristics of *Acanthamoeba* keratitis. In addition, #65 received anti-acanthamoebic chemotherapy with a successful outcome, indicating *Acanthamoeba* as the causative agent. As quoted by De Jonckheere (2003) and others, 'contact lens cases are the breeding grounds for *Acanthamoeba*', and in the absence of a mixed infection, it is reasonable to presume that the T2 isolate was the causative agent. This is the first time that *Acanthamoeba* isolates belonging to the T2 genotype have been linked with *Acanthamoeba* keratitis. This is in contrast to previous studies, which showed that a T2 isolate (CCAP 1501/3c) exhibited minimal binding and minimal cytotoxicity to the host cells (Alsam *et al.*, 2003; Khan *et al.*, 2002) and was considered as a weak or non-pathogen. There are several possible explanations for these findings: the T2 genotype may consist of both pathogenic and weakly or non-pathogenic isolates (since rRNA gene sequence information is not a determinant for pathogenicity), or T2 isolates may have diverse virulence properties and individual strains (current or new isolates) should be tested for their virulence before designating them pathogens or non-pathogens. Alternatively, a more feasible scheme will be to subdivide the T2 genotype into two separate sequence types based on their *Rns* sequences. In support of this, Stothard *et al.* (1998) have shown that, within the T2 genotype, a sequence dissimilarity of 4·9 % exists between CCAP 1501/3c and ATCC 30871. Comparison of CCAP 1501/3c with another T2 isolate (ATCC 50252) also revealed similar differences (4·5 %) (Stothard *et al.*, 1998). These differences are very close to the current cut-off limit of 5 % between different genotypes. Thus we propose that T2 isolates should be subdivided into two separate groups, T2A and T2B, with a sequence dissimilarity of 4 % or more. This may help to differentiate pathogenic and non-pathogenic isolates within this genotype. Further studies involving *Acanthamoeba* belonging to the T2 genotype with CCAP 1501/3c-like *Rns* sequences and more detailed analysis of their properties will clarify the need for two separate groups in this genotype.

Another intriguing finding was the inability of one *Acanthamoeba* isolate (#54 from the contact lens of a keratitis patient) to produce host-cell cytotoxicity, suggesting that it has weak

and/or non-pathogenic properties (< 10 % cell death, as compared with more than 70 % cell death with other clinical isolates). One explanation for this is that there may have been a mixed infection and T2 was isolated from the contact lens even though it was not the causative agent. Future studies are in progress to address these issues.

Despite the occasional cases of *Acanthamoeba* keratitis due to T2 (the present study), T3 (Ledee *et al.*, 1996), T6 (Walochnik *et al.*, 2000) and T11 (Khan *et al.*, 2002), the majority of cases are due to the T4 genotype. This was further confirmed in our study, as more than 80 % of *Acanthamoeba* isolates (UK keratitis) belonged to the T4 genotype. In addition, we also determined that T4 was widely distributed in the environmental samples isolated from the UK. These results, together with previous findings, suggest that *Acanthamoeba* isolates belonging to the T4 genotype naturally occur in the environment and present potential reservoirs and therefore sources of infection to susceptible hosts. As indicated above, the repeated appearance of T4 isolates in keratitis may be due to their greater pathogenicity (virulence properties), greater transmissibility (i.e. they may be widely distributed in the habitat where the probability of being picked up by humans is very high), or a combination of both. To this end, we determined T2 as the most abundant genotype, followed by T4, in the environmental samples from Iran, even though the number of T4 Iranian keratitis isolates was higher than that of T2 isolates (61·5 vs 23 %). These findings demonstrated that the abundance of T4 isolates in *Acanthamoeba* keratitis patients is most likely due to their greater virulence and/or properties that enhance their transmissibility, as well as their ability to bind to contact lenses and their decreased susceptibility to disinfectants. This raises additional questions about the properties of T2 and T4 genotypes that enable them to be the most widespread genotype in a given environment among 15 different genotypes and whether these properties play any role(s) in infection. Future studies that identify virulence traits and genetic markers limited to certain genotypes may help to clarify these issues.

Overall, there is a clear need for more detailed knowledge about the distribution of each genotype in different environments and for detailed analyses of direct and indirect virulence factors of environmental and clinical isolates of each genotype. These findings will help us understand the basis of differential distribution of genotypes in natural habitats, and to some extent in clinical cases, and may identify their differential properties contributing to disease, which should allow the development of pre-emptive or therapeutic measures against these serious infections.

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