Acta Parasitologica, 2004, 49(3), 246–252; ISSN 1230-2821 Copyright © 2004 W. Stefański Institute of Parasitology, PAS

Isolation of *Acanthamoeba* isolates belonging to T2, T3, T4 and T7 genotypes from environmental samples in Ankara, Turkey

Abdullah Kilic[1], Mehmet Tanyuksel[1], James Sissons[2], Samantha Jayasekera[2] and Naveed A. Khan[2*]

[1]Gulhane Military Medical Academy School of Medicine, Division of Medical Parasitology, Etlik, Ankara, Turkey; [2]School of Biological and Chemical Sciences, Birkbeck College, University of London, London, WC1E 7HX, England, U.K.

Abstract

Acanthamoeba keratitis is a blinding infection that is becoming increasingly important in human health. Early diagnosis is a prerequisite for successful treatment and requires identification of *Acanthamoeba* at the genotypic level. The genus *Acanthamoeba* consists of both pathogenic and non-pathogenic species and has been recently classified into 13 different genotypes, T1-T12 and T14. More importantly, 95% of *Acanthamoeba* isolates that produce keratitis belong to T4 genotypes. In this study, we attempted to determine whether predominance of T4 isolates in *Acanthamoeba* keratitis is due to greater virulence or greater prevalence. We isolated 18 *Acanthamoeba* isolates from environmental samples in Ankara, Turkey and determined their pathogenic potential by means osmotolerance, temperature tolerance and *in vitro* cytotoxicity assays using corneal epithelial cells. Ribosomal DNA sequencing revealed that 10 isolates belong to T2, 5 belong to T3, 2 belong to T4 and one belongs to T7 genotype. As expected, T3 and T4 isolates exhibited the most pathogenic traits and were osmotolerant, temperature tolerant and exhibited severe corneal epithelial cell cytotoxicity indicating their pathogenic potential. Overall these data indicate that high frequency of T4 isolates in keratitis cases may well be due to their greater virulence. This is the first report presenting environmental distribution of *Acanthamoeba* in Ankara, Turkey.

Key words

Acanthamoeba, Protozoa, environmental isolates, genotypes, Turkey

Introduction

Acanthamoeba is an opportunistic protozoan parasite that can cause serious human infections including keratitis, which is frequently associated with contact lens use or fatal granulomatous amoebic encephalitis (GAE) that occurs in immunocompromised populations. *Acanthamoeba* infections have remained significant with continuous rise despite advances in antimicrobial chemotherapy and supportive care (reviewed in Khan 2003, Marciano-Cabral and Cabral 2003). This is due to our incomplete understanding of the precise mechanisms associated with the pathogenesis of *Acanthamoeba* infections. Additional factors include delayed diagnosis and resistance of *Acanthamoeba* to antimicrobial chemotherapy.

Identification of risk factors associated with *Acanthamoeba* infections is crucial for disease prevention. Previous studies have identified the wide environmental distribution of *Acanthamoeba*, which is a key predisposing factor in *Acanthamoeba* infections (Stehr-Green *et al.* 1989, Ma *et al.* 1990, De Jonckheere 1991, Kilvington and White 1994, Rodriguez-Zaragoza and Magana-Becerra 1997, Khan and Paget 2002). However, the genus *Acanthamoeba* consists of both pathogenic and non-pathogenic isolates and their differentiation is important for clinical diagnosis. Evolutionary studies have led to the identification of 13 different genotypes (T1-T12 and T14) (Stothard *et al.* 1998, Gast 2001), however, attempts to correlate pathogenicity with certain species/genotypes are under investigations in many laboratories. To date, studies have shown that 95% *Acanthamoeba* isolates that produce keratitis belong to the T4 genotype suggesting that pathogenicity may be limited to certain closely related genotypes (Stothard *et al.* 1998, Walochnik *et al.* 2000, Khan *et al.* 2002). In addition, Booton *et al.* (2002) tested 13 *Acanthamoeba* isolated from clinical samples, contact lenses, lens cases

*Corresponding author: n.khan@sbc.bbk.ac.uk

or lens case solutions in Hong Kong. The results revealed that 12 belong to T4 genotype and one to T3 isolate. Again, in a recent study, De Jonckheere (2003) has determined T4 as the sole keratitis-producing genotype in all nine *Acanthamoeba* keratitis cases in Belgium. However, whether predominance of T4 genotype in keratitis is due to greater virulence or greater prevalence is not known. In this study, we determined the environmental distribution of *Acanthamoeba* in Ankara, Turkey. We tested 30 environmental samples (soil and water) for the presence of *Acanthamoeba* using simple plating assays as well as polymerase chain reaction and their pathogenic potential determined with physiological and *in vitro* cytotoxicity assays using human corneal epithelial cells. *Acanthamoeba* genotypes were determined using 18S rDNA sequencing.

Materials and methods

Isolation of Acanthamoeba *from the environmental samples*

We collected 28 soil samples and two water samples from and around Military Medical Hospital, Ankara, Turkey. Most of soil samples were taken from plant pots in different departments of the hospital. *Acanthamoeba* were isolated from these environmental samples as previously described (Khan and Paget 2002). Briefly, soil samples (ca. 2 g) were dissolved in 20 ml of distilled H_2O and 100 µl of each sample was inoculated onto non-nutrient agar plates seeded with *Klebsiella aerogenes*. Water samples were collected (ca. 500 ml) and filtered through 0.45 µm pore size filter. Filters were inoculated on non-nutrient agar plates seeded with *K. aerogenes*. Plates were incubated at 30°C and observed daily for *Acanthamoeba* growth. *Acanthamoeba* were identified based on morphological characteristics.

Following isolation, *Acanthamoeba* isolates were grown axenically as previously described (Khan *et al.* 2001). Briefly, non-nutrient agar containing *Acanthamoeba* cysts were inoculated on non-nutrient agar plates seeded with UV-killed *K. aerogenes*. Once transformed into trophozoites, agar pieces containing *Acanthamoeba* trophozoites were inoculated into PYG medium [proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v) and glucose 1.5% (w/v)] and incubated at 30°C for 7–21 days.

Additionally, several known *Acanthamoeba* isolates belonging to genotypes T1, T2, T3, T4 and T7 were obtained from the Culture Collection of Algae and Protozoa (CCAP) or American Type Culture Collection (ATCC), or kindly provided by Dr. S. Kilvington (Leicester University, England) and Dr. A. Rivas (La Laguna University, Canary Islands, Spain) as described in Table I. *Acanthamoeba* were routinely grown without shaking in 15 ml of PYG medium in T-75 tissue culture flasks at 30°C as previously described (Khan *et al.* 2001).

Osmotolerance and temperature tolerance assays

Previous studies have shown that osmotolerance and temperature tolerance are physiological markers that can be used to differentiate pathogenic and non-pathogenic *Acanthamoeba* isolates (Khan *et al.* 2001). To determine the pathogenic potential of *Acanthamoeba* isolates tested in this study, osmolarity and temperature tolerance assays were performed as previously described (Khan *et al.* 2001). For osmolarity assays, *Acanthamoeba* were inoculated on non-nutrient agar plates overlaid with *K. aerogenes* and containing 1 M mannitol (0.25 osmolar). Plates were incubated at 30°C for up to 96 h. For temperature tolerance assays, *Acanthamoeba* were inoculated onto non-nutrient agar plates overlaid with *K. aerogenes* and incubated at 37°C for up to 96 h. Growth of the organisms was observed microscopically (Khan *et al.* 2001).

Human corneal epithelial cell cultures

Human corneal epithelial cells were routinely grown in SHEM medium [foetal bovine serum (15% w/v), gentamycin (40 µg/ml), insulin (5 µg/ml), cholera toxin (0.1 µg/ml), vitamins, Hanks F12 (40%), Dulbecco's modified Eagle medium (DMEM, 40%), DMSO (0.5%), epidermal growth factor (10 ng/ml)] (Invitrogen, Paisley, England)] at 37°C in a 5% CO_2 incubator as previously described (Araki-Sasaki *et al.* 2000). Epithelial cells used in different experiments were cultured using similar conditions.

Cytotoxicity assay

To examine the pathogenic potential of each isolate used in this study, cytotoxicity assays were performed as previously described (Khan and Tareen 2003). Briefly, immortalised human corneal epithelial cells were grown to confluency in

Table I. Known *Acanthamoeba* isolates used in the present study

No.	*Acanthamoeba* species	Genotype	Strain	Source
1	*A. castellanii*	T1	[a]ATCC 50494	[c]GAE
2	*A. palestinensis*	T2	[b]CCAP 1547/1	soil, Israel
3	*A. griffini*	T3	[b]CCAP 1501/4	marine, beach, USA
4	*A. divionensis*	T4	[a]ATCC 50238	fish, France
5	*A. castellanii*	T4	[a]ATCC 50492	keratitis, India
6	*A. astronyxis*	T7	[a]ATCC 30137	soil, USA

[a]ATCC – American Type Culture Collection, [b]CCAP – Culture Collection of Algae and Protozoa, England, [c]GAE – granulomatous amoebic encephalitis.

24-well plates. *Acanthamoeba* isolates (5×10^5 amoebae/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. Cell monolayers were observed periodically for cytopathic effects for up to 24 h. At the end of this incubation period, cytopathic effects were assessed visually after hematoxylin staining. In addition, supernatants were collected and cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release (cytotoxicity detection kit; Roche Applied Science, Lewes, East Sussex, England). Briefly, conditioned media of co-cultures of *Acanthamoeba* and corneal epithelial cells were collected and percentage LDH was calculated as follows: sample value-control value/total LDH release-control value × 100 = % cytotoxicity. Control values were obtained from corneal epithelial cells incubated in RPMI alone. Total LDH release was determined from corneal epithelial cells treated with 1% Triton X-100 for 30 min at 37°C.

Polymerase chain reaction analyses

Total DNA was extracted using the insta-gene matrix (Bio-rad Laboratories, Hemel Hempstead, England) as previously described (Khan *et al.* 2001) and used as template for the PCR analysis. Genus-specific primers were used for the PCR amplification of 18S rDNA as previously described (Kong and Chung 1996). Primer sequences were 5'-TTTGAATTCGCTCCAATAGCGTATATTAA and 5'-TTTGAATTCAGAAAGAGCTATCAATCTGT. PCR was performed in 50 µl containing 1.25 U Taq polymerase (Amersham Biosciences, Little Chalfont, Buckinghamshire, England), 0.1–1.0 ng DNA (measured with Gene Quant, Amersham Biosciences), 200 µM dNTPs, 4 mM $MgCl_2$, and 0.5 µM primer. The PCR reactions were performed at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min for 30 cycles, and 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. Known isolates of *Acanthamoeba* belonging to genotypes T1, T2, T3, T4 and T7 were used as positive controls.

18S ribosomal DNA sequencing

For 18S rDNA sequencing, amplified DNA were directly sequenced as previously described (Khan *et al.* 2002). Phylogenetic analyses were carried out using maximum parsimony, minimum evolution and maximum likelihood optimality criteria, implemented in PHYLIP (Felsenstein 1989). Transition:transversion ratios and mutation shape parameters (γ) were estimated by maximum likelihood heuristic searches. Estimates of node support were obtained by performing 500 bootstrap replicates.

Results

Isolation of Acanthamoeba *from both soil and water samples*

To determine the distribution of *Acanthamoeba* in the environmental samples in Ankara, Turkey, both soil and water samples were tested using simple plating assays. *Acanthamoeba* were identified in all environmental samples tested, based on morphological characteristics of trophozoites and cysts. However, 12 samples (from plant pots) were heavily contaminated with fungus and other contaminants and were discarded. The remaining 18 samples were used for axenic culturing. Agar pieces containing *Acanthamoeba* were transferred through a succession of non-nutrient agar plates seeded with UV-killed *K. aerogenes* to eliminate contaminants. Additionally, *Acanthamoeba* were successfully grown in axenic cultures in PYG media within 7–21 days. Axenically grown *Acanthamoeba* were tested for PCR, *in vitro* cytotoxicity and other assays.

PCR analyses were performed using genus-specific primers to specifically identify *Acanthamoeba* at the genus level. We observed an expected PCR product of 910–1170 bp

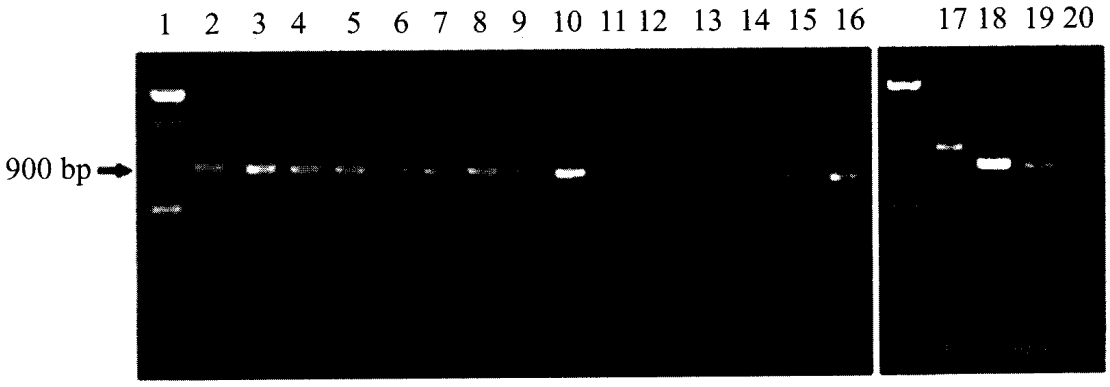

Fig. 1. Agarose gel showing amplification of 18S rDNA of *Acanthamoeba* species in the order as presented in Table I. Lane 1 – 100 bp DNA ladder; lane 2 – *A.* sp. (O14); lane 3 – *A.* sp. (RC); lane 4 – *A.* sp. (ID15); lane 5 – *A.* sp. (ID16); lane 6 – *A.* sp. (O5); lane 7 – *A.* sp. (O15); lane 8 – *A.* sp. (O16); lane 9 – *A.* sp. (O17); lane 10 – *A.* sp. (G16); lane 11 – *A.* sp. (ID19); lane 12 – *A.* sp. (ID1); lane 13 – *A.* sp. (ID20); lane 14 – *A.* sp. (N11); lane 15 – *A.* sp. (U12); lane 16 – *A.* sp. (U13); lane 17 – *A.* sp. (U14); lane 18 – *A.* sp. (SPW1); lane 19 – *A.* sp. (SPW2) and lane 20 is negative control

Table II. Characteristics of *Acanthamoeba* isolated from soil and water samples in Ankara, Turkey

Acanthamoeba sp.	Growth at 37°C	Osmotolerance growth assays	Corneal epithelial cell cytotoxicity (%)	Pathogenicity	Genotype
Soil samples*					
Operation room 14	++	++	86.8 ± 5.6	pathogen	T4
Rehabilitation center	++	++	92.5 ± 6.3	pathogen	T4
Infectious disease 15	++	++	75.4 ± 2.9	pathogen	T3
Infectious disease 16	++	++	71.7 ± 3.6	pathogen	T3
Orthopedics 5	++	++	80.6 ± 5.2	pathogen	T3
Orthopedics 15	++	++	56.8 ± 4.0	pathogen	T3
Orthopedics 16	++	++	65.2 ± 3.8	pathogen	T3
Orthopedics 17	++	++	54.5 ± 5.5	pathogen	T2
Garden 16	weak	–	22.8 ± 2.2	weak pathogen	T2
Infectious disease 19	++	weak	61.5 ± 4.7	pathogen	T2
Infectious disease 1	weak	weak	22.3 ± 1.8	weak pathogen	T2
Infectious disease 20	++	weak	38.4 ± 3.3	weak pathogen	T2
Neurology 11	++	weak	31.6 ± 2.4	weak pathogen	T2
Urology 12	++	++	56.4 ± 3.5	pathogen	T2
Urology 13	++	++	67.0 ± 4.2	pathogen	T2
Urology 14	–	–	5.7 ± 0.7	non-pathogen	T7
Water samples					
Spring hot water 1	++	weak	57.4 ± 3.8	pathogen	T2
Spring hot water 2	weak	weak	29.8 ± 1.4	weak pathogen	T2

++Represents growth; weak growth represents observation of only few trophozoites; corneal epithelial cell cytotoxicity represents percentage of LDH release as described in Materials and methods; source of new isolates is indicated in *Acanthamoeba* species column as soil or water sample. *Most of soil samples were taken from plant pots in different departments of the hospital.

in all environmental samples tested (Fig. 1). Overall these results suggested the wide environmental distribution of *Acanthamoeba* from and around Military Medical Hospital, Ankara, Turkey.

Pathogenic Acanthamoeba: *differentiated based on osmotolerance and temperature tolerance assays*

Previously, we and others have shown that pathogenic *Acanthamoeba* exhibit growth at high temperatures and increased osmolarity (De Jonckheere 1983, Khan *et al.* 2002) and these physiological determinants can be used to differentiate pathogenic and non-pathogenic *Acanthamoeba*. To determine the pathogenic potential of *Acanthamoeba* isolated from the environment in Ankara, Turkey, osmotolerance and temperature tolerance assays were performed as described above. In addition, we used clinical isolates of *Acanthamoeba* as positive controls and known non-pathogens (based on cytotoxicity assays) as negative controls. We determined that 12 out of 18 isolates (66.6%) exhibited growth at high temperature (37°C) and high osmolarity (0.25 osmolar) and were considered as potential pathogens. Additionally, 5 out of 18 (27.7%) exhibited few trophozoites and limited growth and considered as weak potential pathogens, while one isolate (5.5%) exhibited no growth and was considered as non-pathogen (Table II).

Pathogenic Acanthamoeba *producing corneal epithelial cell cytotoxicity*

To determine whether potential pathogenic *Acanthamoeba* isolated from the environment in Ankara, Turkey can directly produce host cell damage, *in vitro* cytotoxicity assays were performed by incubating *Acanthamoeba* with human corneal epithelial cell monolayers. We determined that potential pathogenic *Acanthamoeba* were able to disrupt host cell monolayers within 24 h incubations (Table II). However, non-pathogens had no effect on host cells and weak potential pathogens had limited host cell monolayer disruptions, further confirming results from temperature tolerance and osmotolerance assays. To determine whether corneal epithelial cell monolayer disruptions represent actual cell death, cytotoxicity assays were performed by measuring LDH release. Lactate dehydrogenase is a stable cytoplasmic enzyme present in all cells and is released into the culture supernatant upon plasma membrane damage. We observed that potential pathogenic *Acanthamoeba* were able to produce severe host cell cytotoxicity within 24 h (Table II). *Acanthamoeba* isolates exhibiting less than 39% cell death together with weak growth in plating assays were considered "weak potential pathogens" (Table II). In contrast, non-pathogenic *Acanthamoeba* isolates did not exhibit cytotoxicity on corneal epithelial cells (Table II). Moreover, these data supported the results from osmotolerance and temperature tolerance assays. Based on these data, all *Acanthamoeba* isolates were divided into three groups, potential pathogens, potential weak-pathogens and non-pathogens.

Acanthamoeba *isolates belonging to T2, T3, T4 and T7 genotypes*

For genotype identifications, partial 18S rDNA sequencing was performed. Phylogenetic relationships within the genus

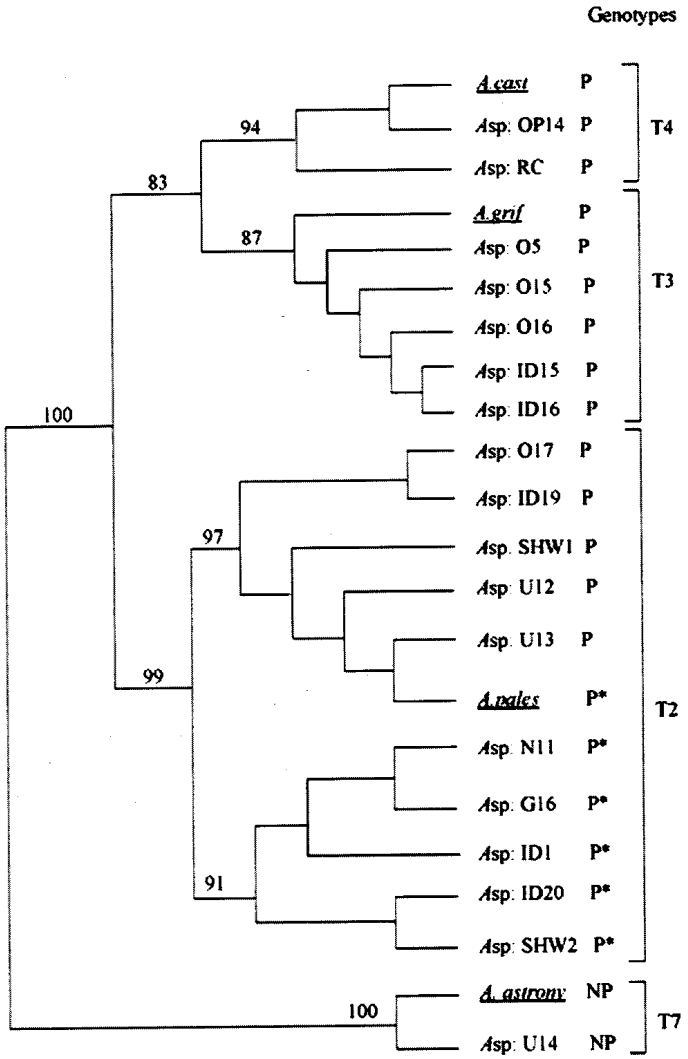

Fig. 2. Phylogenetic analysis based on 18S rDNA sequence. P – potential pathogen, NP – non-pathogen and P* – weak potential pathogens. Numbers represent bootstrap values based on 500 replicates. Values below 50 are omitted. One reference isolate for each genotype is underlined

were examined with maximum parsimony, minimum evolution and maximum likelihood optimality criteria. The tree topology was identical regardless of the method used. The potential pathogens, weak potential pathogens and non-pathogens were within separate clusters on the tree (Fig. 2). The partial 18S rDNA sequence was also compared with the reference species of each genotype. Results revealed that *Acanthamoeba* isolates from soil samples belong to T2, T3, T4 and T7 genotypes, while *Acanthamoeba* isolates from two water samples belong to T2 genotypes (Fig. 2 and Table II). Assays for pathogenicity indicated that all *Acanthamoeba* isolates belonging to T3 and T4 genotypes are potential pathogens (Table II). However, 5 out of 10 *Acanthamoeba* isolates belonging to T2 isolates did not exhibit the full range of pathogenic traits observed in the other strains and were considered weak potential pathogens (Table II). In contrast, *Acanthamoeba* isolates belonging to T7 did not exhibit any pathogenic trait and were considered non-pathogen.

Discussion

Previously, researches have repeatedly identified T4 as a predominant genotype in *Acanthamoeba* keratitis cases, however, question of their more abundance in the environment or whether T4 isolates are more virulent remained unanswered. In this study we attempted to determine the environmental distribution of *Acanthamoeba* and identified their genotype. Although limited in number of samples, our results revealed the environmental distribution of *Acanthamoeba* isolates belonging to T2, T3, T4 and T7 genotypes indicating that higher frequency of T4 isolates in *Acanthamoeba* keratitis cases may well be due to their greater virulence than greater prevalence. However, presence of only a limited number of *Acanthamoeba* genotypes (T2, T3, T4 and T7) in our study is somewhat surprising. This could be due to the fact that we tested a limited area for the collection of samples. Further studies with large environmental sample collection from diverse sources and representing diverse geographic locations may identify wider genotypic distribution as well as identification of potential new genotypes.

Initially, we tested 30 soil and water samples for *Acanthamoeba* and it is intriguing that 100% of these samples exhibited *Acanthamoeba* presence, clearly indicating their ubiquitous nature. This is the first report describing wide environmental distribution of *Acanthamoeba* in Ankara, Turkey indicating our frequent exposure to these organisms. Therefore, it is not surprising that Chappell *et al.* (2001) observed the presence of serum antibodies against *Acanthamoeba* antigens in more than 80% of human populations in the USA. With the wide environmental distribution of *Acanthamoeba* together with increasing importance of these parasites in human health and problems associated with treatment, it is obvious that we should suspect these pathogens in patients presented with keratitis, for early diagnosis, worldwide.

As indicated above, the genus *Acanthamoeba* consists of both pathogenic and non-pathogenic species and differentiation of pathogenic *Acanthamoeba* is important for the clinical diagnosis. To this end, recent studies have established the T4 isolates as the predominant genotype responsible for *Acanthamoeba* keratitis. However, it is not clear whether T4 isolates are the most ubiquitous genotype in the environment and thus are most likely to encounter humans to produce infections. Our results showed that presence of *Acanthamoeba* in the environment is not limited to T4 genotype and it is likely that T4 isolates have certain properties that make them more virulent, thus their higher frequency in keratitis. For example, we have recently shown that *Acanthamoeba* isolates belonging to T4 genotypes exhibit significant higher binding and produced severe cytotoxicity on host cells as compared to T2, T3, T7 or T11 genotypes (Alsam *et al.* 2003). Furthermore, differences in extracellular protease activities (Khan *et al.* 2000), presence of acanthopodia and formation of amoebastomes (Khan 2001, 2003), have been observed. Further support comes from a recent study by Walochnik *et al.* (2001), who tested sera from 20 individuals and observed anti-acanthamoebic IgG, IgM

and IgA immunoreactivities for both virulent and avirulent *Acanthamoeba* clearly indicating our exposure to both pathogenic and non-pathogenic *Acanthamoeba*. These findings suggest that both pathogenic and non-pathogenic *Acanthamoeba* belonging to different genotypes are distributed in the environment, however, given the access and the appropriate conditions, only pathogenic *Acanthamoeba* can cause serious infections. This is further supported by clinical studies of Aitken *et al.* (1996), who isolated three amoebal genera, *Vahlkampfia*, *Hartmannella* and *Acanthamoeba* from the contact lens storage case of an amoebic keratitis patient. However, viable *Vahlkampfia* and *Hartmannella*, but no *Acanthamoeba*, were isolated from the corneal biopsy indicating *Vahlkampfia* and *Hartmannella* as the causative agents. These findings further indicate the distribution of both pathogenic and non-pathogenic *Acanthamoeba*, however, virulence properties may well be limited to certain genotypes of *Acanthamoeba*.

It is interesting to note that in our study, 66.6% of *Acanthamoeba*, isolated from the environmental samples were potential pathogens based on osmotolerance, temperature tolerance and *in vitro* cytotoxicity assays using human corneal epithelial cells. Moreover, all T3 and T4 isolates (100%) exhibited pathogenic traits supporting the previous notion that pathogenicity may be limited to a certain closely related genotypes. Additional studies using a larger number of environmental samples from diverse sources worldwide will further determine the significance of these findings. However, it is important to indicate that environmental conditions may be selective in growth of pathogenic *Acanthamoeba*. For example, seawater may inhibit the growth of weak or non-pathogens due to their inability to grow at high osmolarity, thus higher frequency of pathogenic *Acanthamoeba* in these samples is expected.

In conclusion, we have shown for the first time, the environmental distribution of both pathogenic and non-pathogenic *Acanthamoeba* belonging to T2, T3, T4 and T7 genotypes in Ankara, Turkey suggesting that higher frequency of T4 isolates in keratitis cases is most likely due to their virulence properties than predominance in the environment.

Acknowledgements. We are grateful to Dr. Sim Webb, Norwich Eye Research Group, School of Biological Sciences, University of East Anglia, England, UK for providing immortalised human corneal epithelial cells; Dr. Simon Kilvington, Department of Microbiology and Immunology, University of Leicester, Leicester, England, and Dr. Antonio Ortega Rivas, Department of Parasitology, La Laguna University, Spain for providing *Acanthamoeba* isolates. This work was supported by grants from the FEMS Fellowship Programme, The Royal Society and The Nuffield Foundation.

References

Aitken D., Hay J., Kinnear F.B., Kirkness C.M., Lee W.R., Seal D.V. 1996. Amebic keratitis in a wearer of disposable contact lenses due to a mixed *Vahlkampfia* and *Hartmannella* infection. *Ophthalmology*, 103, 485–494.

Alsam S., Kim K.S., Stins M., Rivas A.O., Sissons J., Khan N.A. 2003. *Acanthamoeba* interactions with human brain microvascular endothelial cells. *Microbial Pathogenesis*, 35, 235–241.

Araki-Sasaki K., Aizawa S., Hiramoto M., Nakamura M., Iwase O., Nakata K., Sasaki Y., Mano T., Handa H., Tano Y. 2000. Substance P-induced cadherin expression and its signal transduction in a cloned human corneal epithelial cell line. *Journal of Cellular Physiology*, 182, 189–195.

Booton G.C., Kelly D.J., Chu Y.W., Seal D.V., Houang E., Lam D.S., Byers T.J., Fuerst P.A. 2002. 18S ribosomal DNA typing and tracking of *Acanthamoeba* species isolates from corneal scrape specimens, contact lenses, lens cases, and home water supplies of *Acanthamoeba* keratitis patients in Hong Kong. *Journal of Clinical Microbiology*, 40, 1621–1625.

Chappell C.L., Wright J.A., Coletta M., Newsome A.L. 2001. Standardized method of measuring *Acanthamoeba* antibodies in sera from healthy human subjects. *Clinical and Diagnostic Laboratory Immunology*, 8, 724–730.

De Jonckheere J.F. 1983. Growth characteristics, cytopathic effect in cell culture and virulence in mice of 36 type strains belonging to 19 different *Acanthamoeba* spp. *Applied and Environmental Microbiology*, 39, 681–685.

De Jonckheere J.F. 1991. Ecology of *Acanthamoeba*. *Reviews in Infectious Diseases*, 13, S385–S387.

De Jonckheere J.F. 2003. Epidemiological typing of *Acanthamoeba* strains isolated from keratitis cases in Belgium. *Bulletin de la Société Belge d'Ophthalmologie*, 287, 27–33.

Felsenstein J. 1989. PHYLIP – phylogeny inference package, vers. 3.2. *Cladistics*, 5, 164–166.

Gast R.J. 2001. Development of an *Acanthamoeba*-specific reverse dot-blot and the discovery of a new ribotype. *Journal of Eukaryotic Microbiology*, 48, 609–615.

Khan N.A. 2001. Pathogenicity, morphology and differentiation of *Acanthamoeba*. *Current Microbiology*, 43, 391–395.

Khan N.A. 2003. Pathogenesis of *Acanthamoeba* infections. *Microbial Pathogenesis*, 34, 277–285.

Khan N.A., Paget T.A. 2002. Molecular tools for speciation and epidemiological studies of *Acanthamoeba*. *Current Microbiology*, 44, 444–449.

Khan N.A., Tareen N.K. 2003. Genotypic, phenotypic, biochemical, physiological and pathogenicity-based categorisation of *Acanthamoeba* strains. *Folia Parasitologica*, 50, 97–104.

Khan N.A., Jarroll E.L., Paget T.A. 2001. *Acanthamoeba* can be differentiated clinically by the polymerase chain reaction and simple plating assays. *Current Microbiology*, 43, 204–208.

Khan N.A., Jarroll E.L., Paget T.A. 2002. Molecular and physiological differentiation between pathogenic and non-pathogenic *Acanthamoeba*. *Current Microbiology*, 45, 197–202.

Khan N.A., Jarroll E.L., Panjwani N., Cao Z., Paget T.A. 2000. Proteases as markers of differentiation of pathogenic and non-pathogenic *Acanthamoeba*. *Journal of Clinical Microbiology*, 38, 2858–2861.

Kilvington S., White D.G. 1994. *Acanthamoeba*: biology, ecology and human disease. *Reviews in Medical Microbiology*, 5, 12–20.

Kong H.H., Chung D.I. 1996. PCR and RFLP variation of conserved region of small subunit ribosomal DNA among *Acanthamoeba* isolates assigned to either *A. castellanii* or *A. polyphaga*. *Korean Journal of Parasitology*, 34, 127–134.

Ma P., Visvesvara G.S., Martinez A.J., Theodore F.H., Daggett P.M., Sawyer T.K. 1990. *Naegleria* and *Acanthamoeba* infections: review. *Reviews in Infectious Diseases*, 12, 490–513.

Marciano-Cabral F., Cabral G. 2003. *Acanthamoeba* spp. as agents of disease in humans. *Clinical Microbiology Reviews*, 16, 273–307.

Rodriguez-Zaragoza S., Magana-Becerra A. 1997. Prevalence of pathogenic *Acanthamoeba* (Protozoa: Amoebidae) in the atmosphere of the city of San Luis Potosi, Mexico. *Toxicology and Industrial Health*, 13, 519–526.

Stehr-Green J.K., Baily T.M., Visvesvara G.S. 1989. The epidemiology of *Acanthamoeba* keratitis in the United States. *American Journal of Ophthalmology*, 107, 331–336.

Stothard D.R., Schroeder-Diedrich J.M., Awwad M.H., Gast R.J., Ledee D.R., Rodriguez-Zaragoza S., Dean C.L., Fuerst P.A., Byers T.J. 1998. The evolutionary history of the genus *Acanthamoeba* and the identification of eight new 18S rRNA gene sequence types. *Journal of Eukaryotic Microbiology*, 45, 45–54.

Walochnik J., Obwaller A., Aspock H. 2000. Correlations between morphological, molecular biological, and physiological characteristics in clinical and nonclinical isolates of *Acanthamoeba* spp. *Applied and Environmental Microbiology*, 66, 4408–4413.

Walochnik J., Obwaller A., Haller-Schober E.M., Aspock H. 2001. Anti-*Acanthamoeba* IgG, IgM, and IgA immunoreactivities in correlation to strain pathogenicity. *Parasitology Research*, 87, 651–656.

(Accepted June 8, 2004)