Genotyping of *Acanthamoeba* isolates and clinical characteristics of patients with *Acanthamoeba* keratitis in China

Ge Zhao, Shiyong Sun, Jing Zhao and Lixin Xie

Correspondence

Lixin Xie

lixin_xie@yahoo.com

State Key Lab Cultivation Base, Shandong Provincial Key Lab of Ophthalmology, Shandong Eye Institute, Qingdao, PR China

Acanthamoeba keratitis (AK) is a sight-threatening corneal infection, the epidemiology of which is related to the specific genotype of *Acanthamoeba*. In this study, the genotypes of 14 *Acanthamoeba* isolates, each from a patient with AK, were identified according to the highly variable DF3 region in the 18S rRNA gene at Shandong Eye Institute, PR China, from 2000 to 2009, and the clinical characteristics of these patients were analysed. All 14 amoebae were genotype T4, representing nine different DF3 sequence types, seven of which were newly identified. Cornea infestation was the main risk factor for these 14 AK patients. Amoebic cysts could be detected in all corneal scrapes. Corneal ulcers were located mainly at the corneal centre, accompanied by eye pain, and some appeared with a Wessely ring. Surgery was carried out on all patients. *Acanthamoeba* genotypes T4/26 and T4/27 were found to cause a more severe keratitis, whilst the others showed no significant differences in clinical characteristics. In conclusion, the majority of the keratitis-causing *Acanthamoeba* isolates were genotype T4, with *Acanthamoeba* genotypes T4/26 and T4/27 from PR China causing a more severe keratitis.

Received 12 October 2009

Accepted 4 January 2010

INTRODUCTION

Acanthamoeba keratitis (AK) is a sight-threatening corneal infection caused by free-living amoebae of the genus *Acanthamoeba*, ubiquitous organisms found in a variety of environments. In recent years, the number of AK cases has been increasing in developed countries due to the increased use of contact lenses (Patel & Hammersmith, 2008; Thebpatiphat *et al.*, 2007). In developing countries, however, the main risk factor is corneal trauma. Corneal infection induced by *Acanthamoeba* is related to the genotype, especially the sequence of the 18S rRNA gene (*Rns*; Stothard *et al.*, 1998). The genus-specific *Rns* amplicon ASA.S1 includes two regions: conserved stem 29 and highly variable stem 29-1. The latter, also named DF3 (diagnostic fragment 3), is presently more often chosen for genotype identification (Booton *et al.*, 2002; Schroeder *et al.*, 2001). To date, 15 genotype clades of *Acanthamoeba* have been identified using *Rns* DNA genotyping, isolates from six of which (T3, T4, T5, T6, T11 and T15) have been confirmed to cause corneal infection. Up to 90% of isolates from AK patients belong to the T4 genotype (Booton *et al.*, 2002; Di Cave *et al.*, 2009; Ledee *et al.*, 2009; Schroeder *et al.*, 2001; Walochnik *et al.*, 2000).

Therefore, *Acanthamoeba* genotyping is a useful tool for the investigation of taxonomic and epidemiological relationships, allowing correlation between infectious isolates and the disease phenotype.

This study was undertaken to identify the genotypes of *Acanthamoeba* isolates from AK patients at our institution using *Rns* DNA genotyping and to compare them with the genotypes of strains from other countries and areas. In addition, the relationship between *Acanthamoeba* genotypes and clinical characteristics of AK was evaluated.

METHODS

Patients, isolation and culture. Fourteen amoebal isolates were obtained from the eyes of 14 patients with AK at Shandong Eye Institute, PR China, from 2000 to 2009 (Table 1). All 14 patients were farm workers. Their ages ranged from 23 to 54 years (mean 39 years). For the aetiological diagnosis of each patient, corneal ulcer scrapings were first searched for amoebic cysts or trophozoites. The samples of scrapings or corneal buttons were then transferred to non-nutrient agar plates overlaid with live *Escherichia coli* and cultured at 37 °C for 10 days until amoebic cysts or trophozoites could be detected under a microscope.

PCR analysis. The genomic DNA of 14 amoebae was extracted by a method described previously (Zhang *et al.*, 2004). A PCR assay was performed with the genus-specific primers JDP1 (5'-GGCC-CAGATCGTTTACCGTGAA-3') and JDP2 (5'-TCTCACAAGCTGCT-

Abbreviation: AK, *Acanthamoeba* keratitis.

The GenBank/EMBL/DDBJ accession numbers for the DF3 sequences of the 14 amoebal isolates in this study are GQ342612–GQ342625.

Fig. 2. *Rns* DF3 linearized neighbour-joining tree. The tree was constructed using 1000 bootstrap replications. The T1, T3, T4, T5 and T11 designations shown on the tree correspond to strains determined previously to be of that particular genotype (Ledee *et al.*, 2009; Zhang *et al.*, 2004). **, Isolates from this study. Bar, Nucleotide changes.

AKSI003, AKSI009 and AKSI012 were identified as T4/24, suggesting that the corneal infection in these patients may have been caused by the same or by closely related strains.

All the amoebal isolates in this study belonged to genotype T4, which is consistent with the findings of previous studies (Booton *et al.*, 2002; Ledee *et al.*, 2009; Yera *et al.*, 2008; Zhang *et al.*, 2004). T4 has been confirmed to be the predominant *Acanthamoeba* genotype in AK. Using the NCBI BLAST software to analyse the DF3 sequences, we found that the genotype of AKSI006 (T4/6) was the same as that of an *Acanthamoeba* isolate from AK patients in Hong Kong (Booton *et al.*, 2002). The genotype of AKSI010 (T4/13) was the same as that of an isolate in the USA (Ledee

et al., 2009). The DF3 sequences of three isolates were identical to that of an isolate in north China (GenBank accession no. AY393972; Zhang *et al.*, 2004), herein designated T4/24. Furthermore, one DF3 sequence was identical to that of an isolate from India (GenBank accession no. AF534154; Pasricha *et al.*, 2003), designated T4/22. It is thus clear that our study reported genotypes of *Acanthamoeba* isolates that have also been reported in studies from north China and from other countries. Moreover, *Acanthamoeba* genotype T4/24 was found to be the most common acanthamoebal clinical isolate in China. However, other *Acanthamoeba* genotypes such as T3 were not identified in our study. The reason for this might have been an insufficient sample size.

Clinical characteristics of AK caused by *Acanthamoeba* genotype T4

The major clinical characteristics of the 14 AK patients are listed in Table 1. Most of the patients had significant risk factors (64.3 %), and their corneas were infected to different extents. The risk factors for these AK patients included intraocular foreign bodies (five cases, 35.7 %) and corneal trauma (two cases, 14.3 %). As these patients were all farm workers, chips or dust might enter their eyes, and twigs or pebbles might injure their corneas during their daily work. These conditions would result in corneal infection with the ubiquitous *Acanthamoeba*. In addition, a weakened immunity caused by the common cold (AKSI002) or insufficient sleep (AKSI012) was the risk factor in two cases (14.3 %), which indicates that *Acanthamoeba* is distributed everywhere and can infect the cornea whenever the immune defence is weakened, as reported by van Klink *et al.* (1996).

In this study, amoebic cysts were detectable in all of the corneal scrapes under a microscope, but only two cases were culture-positive from the scrapes. The other amoebae were isolated from the pathological corneal buttons obtained during surgery. Due to missed diagnosis, misdiagnosis or some other reason in the initial stage of the disease, all AK patients had their treatment delayed for different periods of time, with the longest delay being 150 days (AKSI009) and the shortest being 16 days (AKSI006). Except for AKSI014 with an ulcer site at the temple side, most of the ulcers were located in the corneal centre (92.9 %), and the ulcer of AKSI011 extended to the whole cornea. The corneal stroma around the ulcer was infiltrated and oedematous. The ulcer size was between 4 and 8 mm in diameter in ten eyes (71.4 %), and a Wessely ring was present in six eyes. Moreover, 11 patients (78.6 %) suffered from eye pain of varying degrees, which was due to radial invasion of the corneal nerves by the amoebae (Moore *et al.*, 1986). Eye pain seems to be one of the characteristic symptoms of AK, whilst the Wessely ring is not specific for AK, as it also appears in fungal and herpes simplex keratitis.

The pathogenic conditions of these 14 AK patients were rather severe; therefore, in addition to basic medication, surgery was required to be performed as the ultimate treatment: ophthalmectomy (two cases), penetrating keratoplasty (three cases) and lamellar keratoplasty (nine cases). The occurrence of such severe cases might be attributed to the following factors. Firstly, the early clinical features of AK are similar to those of corneal infections by other pathogens, and laboratory examination often does not include screening for *Acanthamoeba*, so the misdiagnosis rate of AK is high in the early stages. Secondly, lack of related knowledge results in a delay in obtaining medical attention in these patients. Thirdly, there are currently no particularly effective anti-amoebic agents. Lastly, the *Acanthamoeba* isolates in this study were all genotype T4, which is the most virulent genotype (Ledee *et al.*, 2009).

By analysing the clinical characteristics of these AK patients and the genotypes of the pathogenic amoebal isolates, we found that genotype T4/26 and T4/27 amoebae caused more severe AK. The ulcers in the eyes of these two patients were approximately 9 mm and extended to the whole cornea within 20 days, and these patients required ophthalmectomy as their final treatment. The reason for this may be that genotype T4/26 and T4/27 amoebae are more virulent. The symptoms of keratitis caused by representatives of the remaining seven subgenotypes of *Acanthamoeba* T4 showed no significant differences in their clinical characteristics.

In conclusion, most *Acanthamoeba* infections of the cornea are due to *Acanthamoeba* genotype T4, and those with *Acanthamoeba* genotype T4/26 and T4/27 appear to be more severe in China.

ACKNOWLEDGEMENTS

This study was supported in part by the National Natural Science Foundation of China (30630063) and Qingdao Municipal Science and Technology Bureau (02KGYSH-01). The authors thank Ms Ping Lin for her editorial assistance.

REFERENCES

- Booton, G. C., Kelly, D. J., Chu, Y. W., Seal, D. V., Houang, E., Lam, D. S. C., 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. *J Clin Microbiol* **40**, 1621–1625.
- Di Cave, D., Monno, R., Bottalico, P., Guerriero, S., D'Amelio, S., D'Orazi, C. & Berrilli, F. (2009). *Acanthamoeba* T4 and T15 genotypes associated with keratitis infections in Italy. *Eur J Clin Microbiol Infect Dis* **28**, 607–612.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: an integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Ledee, D. R., Iovieno, A., Miller, D., Mandal, N., Diaz, M., Fell, J., Fini, M. E. & Alfonso, E. C. (2009). Molecular identification of T4 and T5 genotypes in isolates from *Acanthamoeba* keratitis patients. *J Clin Microbiol* **47**, 1458–1462.
- Moore, M. B., McCulley, J. P., Kaufman, H. E. & Robin, J. B. (1986). Radial keratoneuritis as a presenting sign in *Acanthamoeba* keratitis. *Ophthalmology* **93**, 1310–1315.
- Pasricha, G., Sharma, S., Garg, P. & Aggarwal, R. K. (2003). Use of 18S rRNA gene-based PCR assay for diagnosis of *Acanthamoeba* keratitis in non-contact lens wearers in India. *J Clin Microbiol* **41**, 3206–3211.
- Patel, A. & Hammersmith, K. (2008). Contact lens-related microbial keratitis: recent outbreaks. *Curr Opin Ophthalmol* **19**, 302–306.
- Schroeder, J. M., Booton, G. C., Hay, J., Niszl, I. A., Seal, D. V., Markus, M. B., Fuerst, P. A. & Byers, T. J. (2001). Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *acanthamoebae* from humans with keratitis and from sewage sludge. *J Clin Microbiol* **39**, 1903–1911.
- 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. *J Eukaryot Microbiol* **45**, 45–54.

Thebpatiphat, N., Hammersmith, K. M., Rocha, F. N., Rapuano, C. J., Ayres, B. D., Laibson, P. R., Eagle, R. C., Jr & Cohen, E. J. (2007). *Acanthamoeba* keratitis: a parasite on the rise. *Cornea* **26**, 701–706.

van Klink, F., Taylor, W. M., Alizadeh, H., Jager, M. J., Van Rooijen, N. & Niederkorn, J. Y. (1996). The role of macrophages in *Acanthamoeba* keratitis. *Invest Ophthalmol Vis Sci* **37**, 1271–1281.

Walochnik, J., Haller-Schober, E., Kolli, H., Picher, D., Obwaller, A. & Aspöck, H. J. (2000). Discrimination between clinically relevant and

nonrelevant *Acanthamoeba* strains isolated from contact lens-wearing keratitis patients in Austria. *J Clin Microbiol* **38**, 3932–3936.

Yera, H., Zamfir, O., Bourcier, T., Viscogliosi, E., Noël, C., Dupouy-Camet, J. & Chaumeil, C. (2008). The genotypic characterisation of *Acanthamoeba* isolates from human ocular samples. *Br J Ophthalmol* **92**, 1139–1141.

Zhang, Y., Sun, X., Wang, Z., Li, R., Luo, S., Jin, X., Deng, S. & Chen, W. (2004). Identification of 18S ribosomal DNA genotype of *Acanthamoeba* from patients with keratitis in North China. *Invest Ophthalmol Vis Sci* **45**, 1904–1907.