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

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*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. Corneal infection 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.

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*; Stothers 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; Walochek 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 aetiologic 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-3').
AGGGGAGTCA-3′) to amplify the highly variable DF3 region in the 18S rRNA gene.

Genotyping and phylogenetic analysis. Direct sequencing of PCR products was performed with the conserved primer 892C (5′-GTCAGAGGTGAAATTCTTGG-3′) to determine the primary DNA sequence of DF3. The DF3 sequence designation was based on the nomenclature described by Booton et al. (2002): the first part is the Rns genotype of the isolate; the second part is a unique code assigned to a specific DF3 sequence type. Twenty-seven DF3 sequence types have been identified so far (Booton et al., 2002; Ledee et al., 2009). The numbers used to define DF3 sequence types in this study were a continuation of that system. DF3 sequence alignment of the 14 amoebae with other Acanthamoeba species available in GenBank was performed using CLUSTAL 2.0. Phylogenetic reconstructions were performed using the phylogenetic computer program MEGA3 (Kumar et al., 2004). Evolutionary distances were computed using the Kimura two-parameter distance algorithm, and the bootstrap consensus tree was inferred from 1000 replicates. Acanthamoeba sp. strain V006, an Rns genotype T1 strain, was used as the outgroup to root the tree. Phylogenetic trees were generated using neighbour-joining evolution methods in MEGA3.

RESULTS AND DISCUSSION

DF3 sequences

DF3 sequence determination of the 14 amoebal isolates resulted in 14 DF3 sequences (Table 1, Fig. 1), nine of which were unique (64 %). All of the nine sequence types were phylogenetically similar to previously described Acanthamoeba isolates with Rns genotype T4 (Fig. 2). The specific DF3 sequence type of AKSI006 was identical to the previously described T4/6, and that of AKSI010 was identical to T4/13. The remaining seven specific DF3 sequence types were designated T4/22–T4/28 according to the nomenclature described above. DF3 sequence alignment of the 14 amoebae with other Acanthamoeba species available in GenBank was performed using CLUSTAL 2.0. Phylogenetic reconstructions were performed using the phylogenetic computer program MEGA3 (Kumar et al., 2004). Evolutionary distances were computed using the Kimura two-parameter distance algorithm, and the bootstrap consensus tree was inferred from 1000 replicates. Acanthamoeba sp. strain V006, an Rns genotype T1 strain, was used as the outgroup to root the tree. Phylogenetic trees were generated using neighbour-joining evolution methods in MEGA3.

Table 1. Clinical characteristics of AK patients and Rns genotyping results of amoebal isolates

<table>
<thead>
<tr>
<th>Code</th>
<th>Culture source</th>
<th>Risk factors*</th>
<th>Treatment delay (days)</th>
<th>Ulcer site</th>
<th>Ulcer size (mm)†</th>
<th>Wessely ring</th>
<th>Eye pain</th>
<th>Operation type‡</th>
<th>Rns genotype/DF3 sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKSI001</td>
<td>Corneal scrape</td>
<td>ND</td>
<td>60</td>
<td>Corneal centre</td>
<td>8</td>
<td>Yes</td>
<td>Yes</td>
<td>PKP</td>
<td>T4/22</td>
</tr>
<tr>
<td>AKSI002</td>
<td>Corneal button</td>
<td>IE</td>
<td>30</td>
<td>Corneal centre</td>
<td>5</td>
<td>No</td>
<td>Yes</td>
<td>PKP</td>
<td>T4/23</td>
</tr>
<tr>
<td>AKSI003</td>
<td>Corneal button</td>
<td>FBEE</td>
<td>30</td>
<td>Corneal centre</td>
<td>5</td>
<td>No</td>
<td>No</td>
<td>LKP</td>
<td>T4/24</td>
</tr>
<tr>
<td>AKSI004</td>
<td>Corneal button</td>
<td>Trauma</td>
<td>40</td>
<td>Corneal centre</td>
<td>4</td>
<td>Yes</td>
<td>Yes</td>
<td>LKP</td>
<td>T4/25</td>
</tr>
<tr>
<td>AKSI005</td>
<td>Corneal button</td>
<td>FBEE</td>
<td>60</td>
<td>Corneal centre</td>
<td>6</td>
<td>Yes</td>
<td>Yes</td>
<td>LKP</td>
<td>T4/25</td>
</tr>
<tr>
<td>AKSI006</td>
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<td>FBEE</td>
<td>16</td>
<td>Corneal centre</td>
<td>7</td>
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<td>No</td>
<td>LKP</td>
<td>T4/6</td>
</tr>
<tr>
<td>AKSI007</td>
<td>Corneal scrape</td>
<td>ND</td>
<td>124</td>
<td>Corneal centre</td>
<td>2.5</td>
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<td>Yes</td>
<td>LKP</td>
<td>T4/25</td>
</tr>
<tr>
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<td>FBEE</td>
<td>16</td>
<td>Corneal centre</td>
<td>9</td>
<td>Yes</td>
<td>Yes</td>
<td>Enucleation</td>
<td>T4/26</td>
</tr>
<tr>
<td>AKSI009</td>
<td>Corneal button</td>
<td>Trauma</td>
<td>150</td>
<td>Corneal centre</td>
<td>8</td>
<td>No</td>
<td>Yes</td>
<td>LKP</td>
<td>T4/24</td>
</tr>
<tr>
<td>AKSI010</td>
<td>Corneal button</td>
<td>ND</td>
<td>20</td>
<td>Corneal centre</td>
<td>6</td>
<td>Yes</td>
<td>Yes</td>
<td>LKP</td>
<td>T4/13</td>
</tr>
<tr>
<td>AKSI011</td>
<td>Corneal button</td>
<td>ND</td>
<td>19</td>
<td>Corneal centre</td>
<td>9.5</td>
<td>No</td>
<td>Yes</td>
<td>Enucleation</td>
<td>T4/27</td>
</tr>
<tr>
<td>AKSI012</td>
<td>Corneal button</td>
<td>IE</td>
<td>90</td>
<td>Corneal centre</td>
<td>5</td>
<td>Yes</td>
<td>Yes</td>
<td>LKP</td>
<td>T4/24</td>
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<td>AKSI013</td>
<td>Corneal button</td>
<td>ND</td>
<td>80</td>
<td>Corneal centre</td>
<td>6</td>
<td>No</td>
<td>Yes</td>
<td>PKP</td>
<td>T4/25</td>
</tr>
<tr>
<td>AKSI014</td>
<td>Corneal button</td>
<td>FBEE</td>
<td>90</td>
<td>Temple side</td>
<td>2</td>
<td>No</td>
<td>No</td>
<td>LKP</td>
<td>T4/28</td>
</tr>
</tbody>
</table>

*ND, Not determined; IE, immunity-weakened; FBEE, foreign body entering eye.
†Maximum ulcer diameter.
‡PKP, Penetrating keratoplasty; LKP, lamellar keratoplasty.

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Fig. 1. Primary sequence alignment of a variable region of DF3 (stem 29-1 of the 18S rRNA gene) of isolates from AK patients at Shandong Eye Institute by CLUSTAL 2.0. The alignment shown is a subset of DF3 that contains the highly variable and informative section of this fragment. Sequences are aligned by similarity. Asterisks denote variable positions; gaps are represented as dashes.
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.

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.
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 (Lede *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.

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