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Cornea & External Disease

Acanthamoeba Keratitis in China: Genotypic and Clinical Correlations

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Purpose: To investigate the relationship between *Acanthamoeba* genotypes, clinical manifestations, and outcomes in *Acanthamoeba* keratitis (AK) patients.

Methods: This retrospective study included 159 culture-confirmed AK patients. Patients' data were collected, including demographics, initial diagnosis, treatments, and clinical features. The genotype of *Acanthamoeba* was identified through sequencing the Diagnostic Fragment 3 (DF3) region in the small ribosomal subunit RNA genes. The phylogenetic tree was constructed using the ClustalW model and maximum likelihood method. Cases with "poor outcome" were defined based on specific clinical criteria, including corneal perforation, keratoplasty, other eye surgery, duration of anti-amoebic therapy $\geq$8.0 months, and final visual acuity $\leq$20/80. "Better outcome" cases were the remainder. The correlation between T4 subtypes, clinical phenotypes, and clinical prognosis were further analyzed.

Results: In this study, AK was primarily attributed to the T4A genotype, with a positive correlation between geographical and genetic distances. The primary clinical associated with T4 subtypes was deep stromal infiltration. Results was also showed a significant association between T4 subtypes and clinical outcomes ($P = 0.021$). Further analysis revealed that T4C was closely associated with a better prognosis ($P = 0.040$) and T4D with worse outcomes ($P = 0.013$).

Conclusions: In China, AK was predominantly caused by the T4A subtype. Geographical distance positively correlated with genetic distance. Clinical prognosis varied among different subtypes, notably in T4C and T4D.

Translational Relevance: This study demonstrated the association between T4 subtypes and clinical phenotypes, as well as the effects of T4 subtypes on clinical prognosis.

Introduction

Acanthamoeba keratitis (AK) is a severe corneal infection caused by the *Acanthamoeba* parasite, resulting in intense eye pain and visual impairment.[1] Among infectious keratitis cases, AK has the poorest prognosis, posing a high risk of blindness and significantly impacting patients' health and quality of life. The primary risk factors for AK, both in developed and developing countries, are the use of contact lenses[2] and cornea trauma.[3,4] First reported in 1974,[5] the incidence of AK has been increasing globally.[3,6] As of 2021, the global annual incidence of AK had reached 23,651 cases, with India reporting the highest rates and Tunisia and Belgium reporting the lowest.[7]

Acanthamoeba, an opportunistic pathogenic protozoan, is commonly found in environments such as lakes, soil, and tap water. It was first isolated by Castellani[8] in 1930 from yeast culture contaminants. *Acanthamoeba* has a life cycle consisting of trophozoite and cyst stages. In 1977, Pussar and Pons[9] classified *Acanthamoeba* into three groups (I-III) based on cyst size and morphology. However, cyst

characteristics can be influenced by culture conditions and subjective factors. Therefore Gast et al.[10] proposed a classification method using nuclear small ribosomal subunit RNA genes (*Rns*) sequences in 1996, with Diagnostic Fragment 3 (DF3) in the *Rns* region proving to be a powerful tool for identifying *Acanthamoeba* genotypes, assessing genetic relationships, and evaluating phylogenetic differences among strains. Currently, 23 *Acanthamoeba* genotypes (T1-T23) have been identified based on the complete 18S rRNA gene sequence.[11–19]

T4, the most prevalent *Acanthamoeba* genotype in the environment, is a leading cause of corneal infections.[7] Seven subtypes were further categorized as T4A, T4B, T4C, T4D, T4E, T4F, and T4Neff. Corsaro et al.[20] recently introduced T4H as a novel subtype within the T4 category. Corneal infections linked to *Acanthamoeba* have been associated with variations in the 18S rRNA gene sequence.[11] Arnalich-Montiel et al.[21] compared clinical manifestations, response to treatment, surgical interventions, and outcomes between T4 and non-T4 genotype AK patients, highlighting differences in pathogenicity among genotypes. Among T4 subtypes, by analyzing the patients' clinical presentation and prognosis, previous studies showed that T4/26 and T4/27 *Acanthamoeba* genotype were more virulent.[22] In contrast, another study revealed that no significant correlation was found between the pathogenesis of a specific genotype or species and the clinical outcome.[23] Therefore data on the relationship between T4 subtypes and clinical manifestations remain inconsistent and require validation. This study aims to identify *Acanthamoeba* genotypes in AK patients, explore the link between genetic and geographical distances, and, by focusing on the T4 genotype and its subtypes, assess their relationship in clinical manifestations and prognosis.

Methods

Subjects

This retrospective hospital-based study included AK patients with positive cultures from January 2014 to July 2023, which was approved by the Medical Ethics Committee (TRECKY2021-024). The patients with mixed infection were excluded, and 159 *Acanthamoeba* isolates were collected from corneal lesions of *Acanthamoeba* infection. According to the Helsinki Declaration, all patients were informed of the procedure and consented to participate in the current study. These cases came from 25 provinces spanning two distinct geographical regions in China: Northern China (Beijing, Gansu, Hebei, Heilongjiang, Henan, Inner Mongolia, Jilin, Liaoning, Shaanxi, Shandong, Shanxi, Tianjin, Xinjiang), Southern China (Anhui, Chongqing, Fujian, Guangdong, Guizhou, Hubei, Hunan, Jiangsu, Shanghai, Sichuan, Yunnan, Zhejiang).

Patient data, including demographics, duration of symptoms, initial diagnosis and treatments, and clinical features before treatment and at the end of follow-up, were collected. Risk factors for AK, such as contact lens use, injury, and exposure to tap water or sewage, were also documented.

Clinical and Laboratory Evaluation

After assessing visual acuity, all patients underwent slit-lamp biomicroscopy examinations. Typical clinical signs, including pseudodendritic lesions, perineural infiltrate, multifocal stromal infiltrates, deep stromal infiltrates, ring infiltrates, neovascularization, hypopyon, groove-shaped corneal melting, and more, would be observed. Based on the AK grading system established by Carnt et al.,[24] the disease stages in these cases were categorized into three groups according to clinical presentation: Stage 1 included corneal epitheliopathy alone (Fig. 1A); Stage 2 had corneal epithelial defects, perineural infiltrates or stromal infiltrates, in addition to stage 1 findings (Fig. 1B); Stage 3 involved a corneal stromal ring infiltrate along with one or more features of stage 2 disease (Fig. 1C). Laboratory investigations for AK included corneal scrapings, optical microscope observation after Giemsa staining (Fig. 1D), and cultures on non-nutrient agar plates covered with *Escherichia coli* that were performed in the Department of Ocular Microbiology at the Beijing Institute of Ophthalmology (Fig. 1E).

For most AK cases, in vivo confocal microscopy (IVCM) examination was conducted using the new Rostock Cornea Module of the Heidelberg Retina Tomograph (HRT III) (Heidelberg Engineering GmbH, Heidelberg, Germany). The laser source used in the HRT III/RCM is a diode laser with a wavelength of 670 nm. Images consist of 384 × 384 pixels covering an area of 400 × 400 µm with transversal optical resolution of approximately 1 µm/pixel and an acquisition time of 0.024 s (Heidelberg Engineering). Each of the confocal images was reviewed separately by two experienced IVCM specialists (Z.W. and Q.L.). A protocol for IVCM characteristics of *Acanthamoeba* cysts, trophozoites, and dendritic cells were established according to the previous study.[25] Typical cysts were present with highly reflective spot,

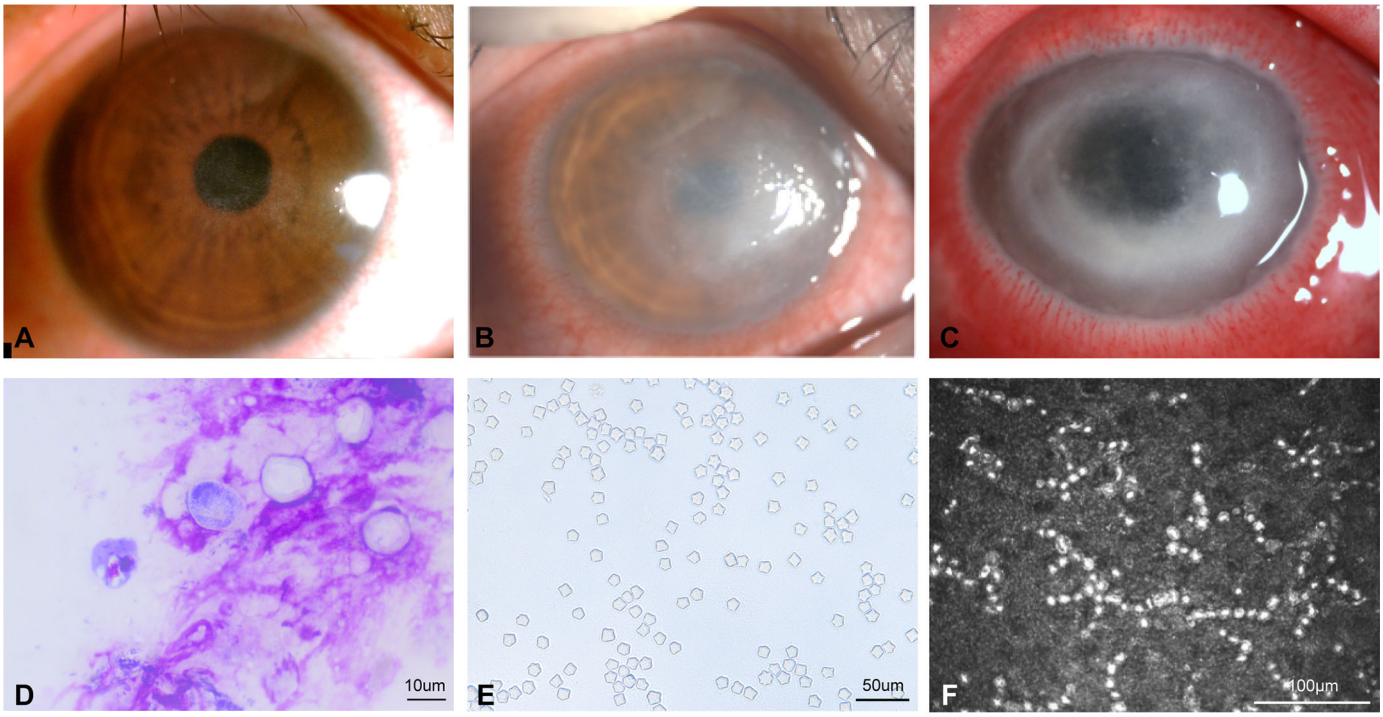

Figure 1. Clinical stages and diagnosis of *Acanthamoeba* keratitis. (**A**–**C**) Slit lamp photographs depicting *Acanthamoeba* keratitis. (**A**) Corneal epitheliopathy only; (**B**) stromal infiltrate; (**C**) ring infiltrate. (**D**–**F**) Laboratory and imaging investigations for AK. (**D**) Giemsa staining of corneal scraping revealing *Acanthamoeba* cysts (scale bar: 10 µm). (**E**) *Acanthamoeba* cysts in cultures of samples from clinical patients (scale bar: 50 µm). (**F**) IVCM examination, showing typical cysts with highly reflective spots, rings, or double-wall structures in a linear pattern (scale bar: 100 µm).

ring, or double-wall structure in a linear pattern (Fig. 1F).

Prognosis Assessment

In this study, all AK patients were treated medically at first, with a combination of 0.02% chlorhexidine and 0.02% polyhexamethylene biguanide, which were initially used hourly for the first week and tapered slowly over the following four weeks to dosing four times a day. According to the response to medication, maintenance therapy was performed with monotherapy (0.02% chlorhexidine) four times a day for three to six months. For uncontrolled AK cases with deep stroma infiltrates or hypopyon, surgical interventions involving penetrating keratoplasty, lamellar keratoplasty, or deep anterior lamellar keratoplasty were performed. During the follow-up period, cases with "poor outcomes" were defined based on specific clinical criteria, including corneal perforation, keratoplasty, other eye surgery (excluding biopsy), duration of anti-amoebic therapy ≥8.0 months (based on the seventy-fifth percentile of the entire sample), and final visual acuity ≤20/80. "Better outcome" cases were the remainder.[24]

Acanthamoeba Genotyping and Phylogenetic Analysis

After identifying *Acanthamoeba* trophozoites or cysts through corneal scraping and staining, the samples were transferred to the non-nutrient agar plates coated with *Escherichia coli* and then left at 28°C for 14 days. Subsequently, *Acanthamoeba* trophozoites and cysts were collected via centrifugation at 1500 rpm for five minutes, and DNA extraction was performed using the FastPure Microbiome DNA Isolation Kit (cat. DC502-01, Vazyme, Nanjing, China).

The DF3 region in *Rns* of *Acanthamoeba* was amplified by polymerase chain reaction (PCR) to identify its genotype, using specific primer JDP1 (5′-GGCCCAGATCGTTTACCGTGAA) and JDP2 (5′-TCTCACAAGCTG CTAGGGGAGTCA). The PCR reaction was conducted in 20 µL volumes, comprising 10 µL 2x Flash Hot Start MasterMix (Dye) (CW3007, CWBIO, Beijing, China), 1 µL of each primer, 2 µL of DNA templates, and 6 µL of double-distilled water. PCR commenced at 95°C for five minutes, followed by 35 cycles of 95°C for 60 seconds, 62°C for 45 seconds, and 72°C for 45 seconds. The final cycle included an elongation step at 72°C for five minutes.

PCR amplification products were verified via 1.5% agarose gel electrophoresis. Reference sequences for existing *Acanthamoeba* genotypes were obtained from the website created by The Ohio State University (https://u.osu.edu/acanthamoeba/). The phylogenetic tree was constructed using the ClustalW model and the maximum likelihood method in MEGA11. The online tool ITOL was used to edit the phylogenetic tree. Genetic distance calculations were also performed using MEGA11.

Statistical Analysis

Statistical Analysis in this article was conducted using R 3.5.3. Genotype comparisons in geographical distribution and clinical profiles (e.g., clinical manifestation, disease prognosis) were conducted using R*C analysis. The differences were further analyzed by chi-square test; if theoretical numbers $T < 5$ but $T \geq 1$, and $n \geq 40$, continuity-corrected χ^2 test was used; if there is a theoretical number $T < 1$ or $n < 40$, Fisher's test was used. Correlations between geographical and genetic distance were examined using Pearson's correlation analysis.

Results

Clinical Characteristics

A total of 159 culture-positive AK patients were included in the study. The demographic and clinical characteristics are summarized in Table 1. Among these patients, 88 were male, and 71 were female (the ratio of male to female was 1.2:1). The mean age was 39.9 ± 18.9 years (ranging from three to 79 years). Of the 159 AK patients. Among the 159 AK patients, 35 (22.0%) cases had a documented history of contact lens use, 22 (13.8%) cases reported a past incident of corneal trauma, seven (4.4%) cases had a documented exposure to tap water or sewage, two (1.3%) cases had a history of ocular surgery, 10 (6.3%) cases explicitly denied any known risk factors, and for the rest the specific risk factors remained unknown.

The average time to AK onset for the 159 patients was 54.0 ± 47.7 days. Of these, 139 patients (87.4%) had external eye images, and most were in the middle and late stages during their initial visit. Among the 139 patients, more than half of the patients presented with stage 2 at the initial visit (54.0%), followed by stage 3 (40.3%), and only a few patients were classified as stage 1 (5.7%). Furthermore, the most predominant clinical manifestation was deep stromal infiltration (72.7%), followed by ring infiltration (40.3%), and neovascularization (31.7%). On the contrary, pseudo-dendritic lesion was the rarest manifestation, appearing in 3.6% of AK patients.

Table 1. Demographics and Clinical Characteristics of Cases With *Acanthamoeba* Keratitis

Parameters	Value
Gender, male (%)	88 (55.3%)
Age (years)	39.9 ± 18.9
Duration (days)	54.0 ± 47.7
Risk factors	
Contact lens use	35 (22.0%)
Injury	22 (13.8%)
Tap water or sewage exposure	7 (4.4%)
Ocular surgery	2 (1.3%)
None	10 (6.3%)
Unknown	83 (52.2%)
Visual acuity	
<20/1000	31 (62.0%)
20/1000–20/66	14 (28.0%)
>20/66	5 (10.0%)
Clinical stage	
Stage 1	8 (5.7%)
Stage 2	75 (54.0%)
Stage 3	56 (40.3%)
Clinical signs	
Pseudo-dendritic lesions	5 (3.6%)
Peripheral infiltrates	14 (10.0%)
Multifocal stromal infiltrates	30 (21.6%)
Deep stromal infiltrates	101 (72.7%)
Ring infiltrates	56 (40.3%)
Groove-shaped melting	25 (18.0%)
Neovascularization	44 (31.7%)
Hypopyon	34 (24.5%)

Genotypes of *Acanthamoeba* Isolates

The DF3 region of all 159 isolates was successfully sequenced, and a phylogenetic analysis based on this region was conducted to determine the genetic relationships. The phylogenetic tree analysis revealed three distinct clusters: T3, T4, and T11. Among these, the T4 genotype was predominant, encompassing 156 isolates, with only a minority classified as T3 (two isolates) and T11 (one isolate) (Fig. 2). Because of the limited number of T3 and T11 genotypes, subsequent investigations focused on the T4 genotype. Notably, the T4A and T4D subtypes were the most common subtypes, with the breakdown of T4 subtypes as follows (Supplementary Table S1): T4A, 49 isolates (31.4%); T4B, 19 isolates (12.2%); T4C, three isolates (1.9%); T4D, 41

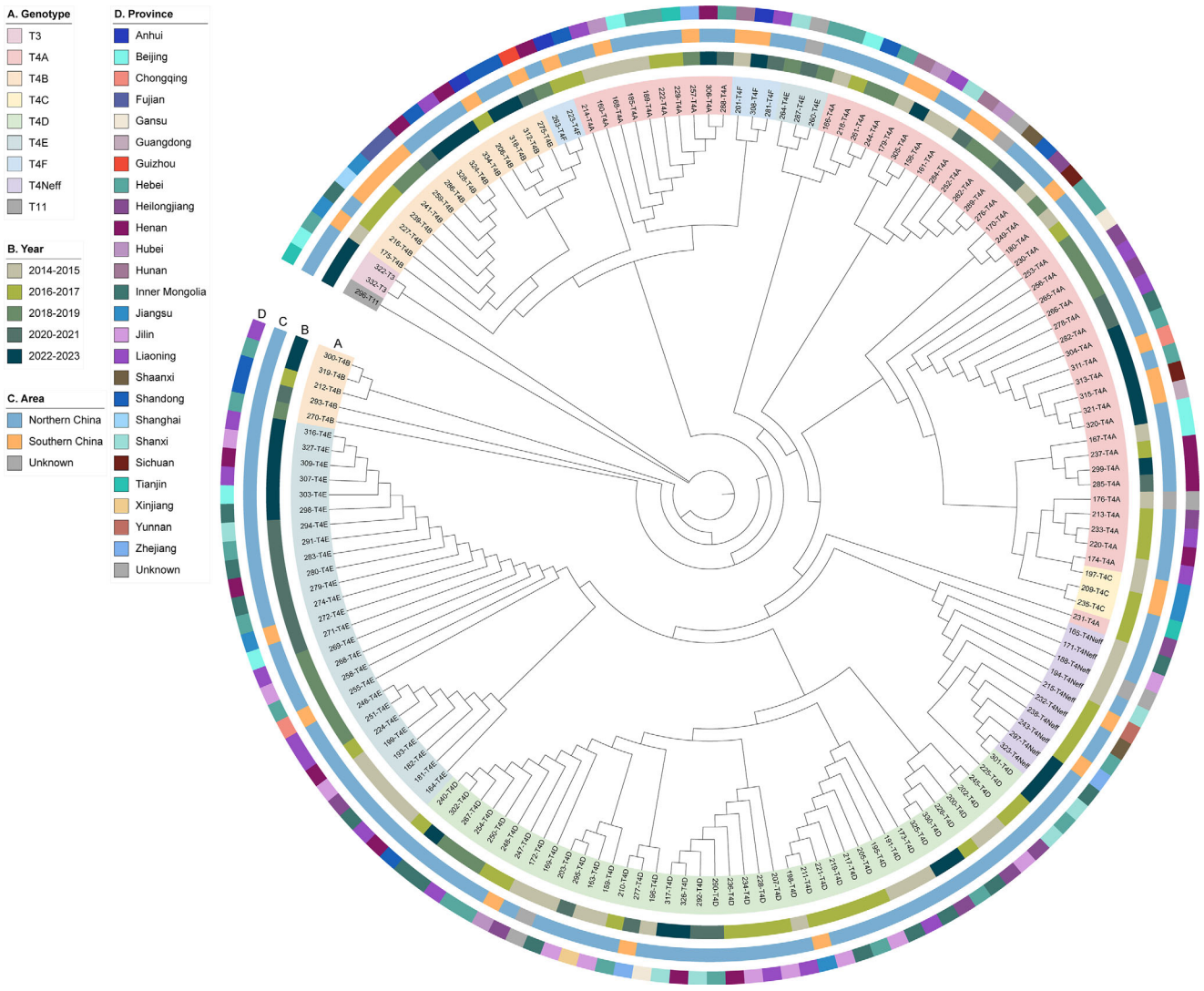

Figure 2. Phylogenetic analysis based on DF3 region among *Acanthamoeba* isolates and diverse genotypes. (**A**) The innermost colored bands represent 159 strains of *Acanthamoeba* isolated from cases with *Acanthamoeba* keratitis. A colored ring representing three different genotypes of *Acanthamoeba*. (**B**) A colored ring representing five different periods of collection years. (**C**) A colored ring showing two different geographical origins (areas) of *Acanthamoeba*. (**D**) A colored ring illustrating 25 different geographical origins (provinces) of *Acanthamoeba*.

isolates (26.3%); T4E, 29 isolates (18.6%); T4F, five isolates (3.2%); and T4Neff, 10 isolates (6.4%).

Regarding regional characteristics, the distribution of T4 subtypes in each province was illustrated in Figure 3A and Supplementary Table S2. T4A was widespread, observed in 17 provinces (68.0%), whereas T4C was found in only two provinces (8.0%). Seven T4 subtypes were present in both Northern and Southern China. In the northern region, T4A and T4D were the predominant genotypes, each accounting for 30.3%, followed by T4E (21.3%), T4B (9.9%), T4Neff (5.7%), T4F (1.7%) and T4C (0.8%). In the southern region, T4A also prevailed (34.5%), followed by T4B (24.2%), T4D and T4F (10.3% each), T4C, T4E, and T4Neff (6.9% each) (Fig. 3B). When comparing the two geographical regions of China, T4D was more common in North China ($P = 0.028$), whereas T4F was more prevalent in South China ($P = 0.049$) (Fig. 3C). Furthermore, a small but significant positive correlation was observed between geographic distance and genetic distance ($R = 0.05$, $P < 0.001$) (Fig. 3D), indicating that geographical distance contributed to genetic distance differences. The larger the geographic distance, the larger the genetic distance and the further the kinship between *Acanthamoeba* strains.

Additionally, the temporal evolution of T4 subtypes among clinical isolates from 2014 to 2023 is depicted in Figure 4. In 2014–2015, T4A was the dominant

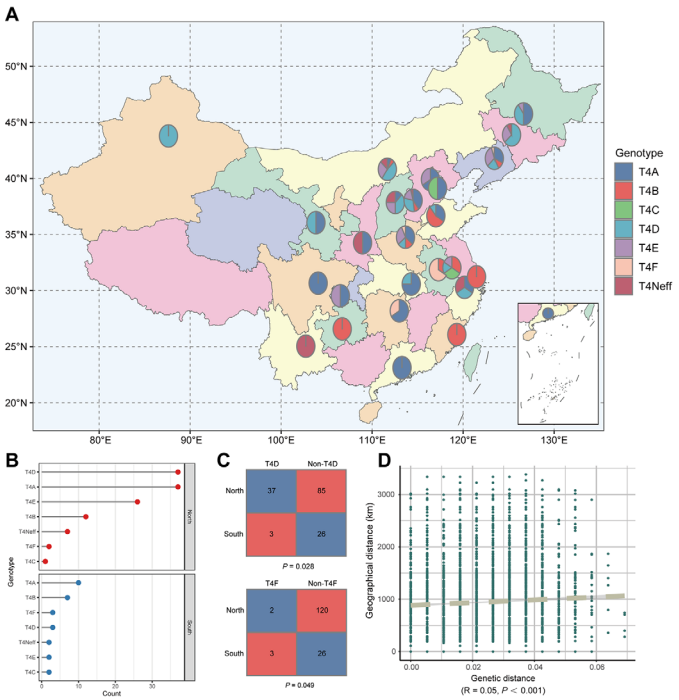

Figure 3. The relationship between regional distribution and *Acanthamoeba* T4 subtypes. (**A**) The distribution of T4 subtypes in 25 different geographical origins (provinces). (**B**) The distribution of T4 subtypes in 6 different geographical origins (areas). (**C**) The χ^2 test of clinical outcomes in T4D and T4F genotype. (**D**) The relationship between geographical and genetic distance in T4 subtypes.

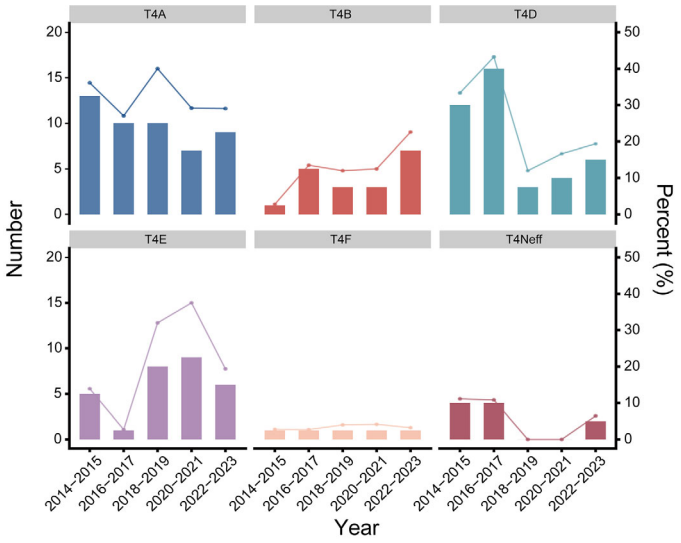

Figure 4. Variations in *Acanthamoeba* T4 subtypes over time. The bar graph corresponds to the left y-axis, while the line graph corresponds to the right y-axis.

subtype, comprising 35.1% of the isolates, followed by T4D (32.5%), T4E (13.5%), T4Neff (10.8%), T4B (2.7%), T4C (2.7%), and T4F (2.7%). Throughout the years, T4A consistently remained the most prevalent subtype, except for the period between 2016–2017 and 2020–2021. T4B exhibited a rising trend, increasing from 2.7% to 22.6% during the study period. T4C consistently had a low prevalence, with only three isolates identified in 2015, 2016, and 2017. The prevalence of T4D fluctuated between 12.0% and 41.0%, showing an initial increase until 2017, followed by a sharp decline until 2019, and subsequently, it showed an upward trend again. Similarly, the percentage of T4E fluctuated between 2.6% and 37.5%, with a declining trend until 2017, followed by a significant increase until 2021 and then decreased. T4F remained relatively stable, whereas T4Neff showed no consistent trend, with fluctuations from year to year.

Correlation Between Clinical Phenotypes and T4 Subtypes

Among all T4 subtypes, deep stromal infiltration was identified as the most common clinical manifestation (73.5%), whereas pseudodendritic lesions were the least common (2.9%), consistent with the overall findings. Further details about the distribution of clinical signs within T4 subtypes were provided in Table 2 and Figure 5. T4A was characterized by ring infiltrates (26.1%) and multifocal stromal infiltrates (26.1%). Both T4B and T4D were primarily associated with ring infiltrates (41.2% and 61.7%, respectively). T4C displayed neovascularization (33.3%) and perineural infiltrates (33.3%). T4E was characterized by ring infiltrates (33.3%) and neovascularization (33.3%). T4F predominantly exhibited ring infiltrates (50.0%), neovascularization (50.0%), and groove-shaped corneal melting (50.0%), while T4Neff was characterized by hypopyon (75.0%). Further statistical tests revealed that, apart from ring infiltrates ($P = 0.030$) and hypopyon ($P = 0.027$), there were no statistically significant differences between the following clinical phenotypes and T4 subtypes: pseudodendritic lesions ($P = 0.938$), perineural infiltrate ($P = 0.461$), multifocal stromal infiltrates ($P = 0.657$), deep stromal infiltrates ($P = 0.660$), groove-shaped corneal melting ($P = 0.135$), and neovascularization ($P = 0.234$). Furthermore, there were no statistically significant differences observed between the T4 subtypes and clinical staging ($P = 0.109$).

Based on the predefined outcome criteria, patients were categorized into two groups: those with good outcomes and those with poor outcomes (as shown in Fig. 6A). The average follow-up time was 6.9 months, with a median of 4.0 months. Among the 108 patients who were followed up, 80% experienced a poor outcome, while 20% had a good outcome. Notably, there was a statistically significant associ-

Table 2. The Distribution of Clinical Signs in *Acanthamoeba* Keratitis Among Different T4 Subtypes

Subtypes	No.	Distribution of Clinical Stage (Case Number)			Distribution of Clinical Signs (Case Number)							
		Stage 1	Stage 2	Stage 3	DSI	RI	NV	Hypopyon	MSI	GCM	PI	PDL
T4A	46	3 (6.5%)	31 (67.4%)	12 (26.1%)	35 (76.1%)	12 (26.1%)	10 (21.7%)	8 (17.4%)	12 (26.1%)	6 (13.0%)	5 (10.9%)	1 (2.2%)
T4B	17	1 (5.9%)	9 (52.9%)	7 (41.2%)	13 (76.5%)	7 (41.2%)	4 (23.5%)	3 (17.7%)	5 (29.4%)	1 (5.9%)	2 (11.8%)	1 (5.9%)
T4C	3	0 (0)	3 (100)	0 (0)	2 (66.7%)	0 (0)	1 (33.3%)	0 (0)	0 (0)	0 (0)	1 (33.3%)	0 (0)
T4D	34	3 (8.8%)	10 (29.4%)	21 (61.8%)	26 (76.5%)	21 (61.7%)	13 (38.2%)	11 (32.4%)	5 (14.7%)	10 (29.4%)	3 (8.8%)	1 (2.9%)
T4E	24	1 (4.2%)	15 (62.5%)	8 (33.3%)	15 (62.5%)	8 (33.3%)	8 (33.3%)	5 (20.8%)	7 (29.2%)	3 (12.5%)	1 (4.2%)	1 (4.2%)
T4F	4	0 (0)	2 (50)	2 (50)	2 (50.0%)	2 (50.0%)	2 (50.0%)	0 (0)	0 (0)	2 (50.0%)	1 (25.0%)	0 (0)
T4Neff	8	0 (0)	4 (50)	4 (50)	7 (87.5%)	4 (50.0%)	5 (62.5%)	6 (75.0%)	1 (12.5%)	2 (25.0%)	1 (12.5%)	0 (0)
Total	136	8 (5.9%)	74 (54.4%)	54 (39.7%)	100 (73.5%)	54 (39.7%)	43 (31.6%)	33 (24.3%)	30 (22.1%)	24 (17.6%)	14 (10.3%)	4 (2.9%)

DSI, deep stromal infiltrates; GCM, groove-shaped corneal melting; MSI, multifocal stromal infiltrates; NV, neovascularization; PDL, pseudodendritic lesions; PI, perineural infiltrates; RI, ring infiltrates.

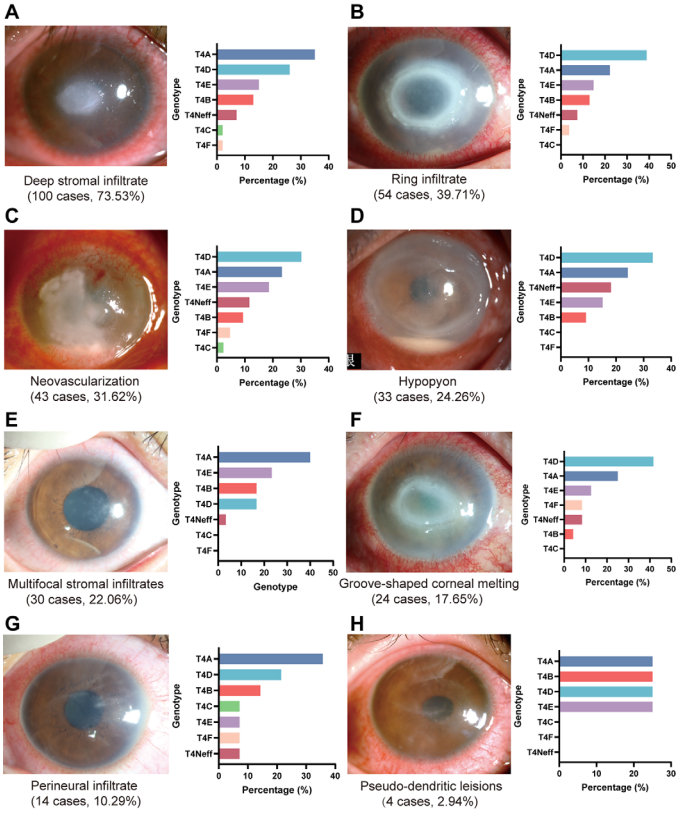

Figure 5. Distribution of clinical signs in *Acanthamoeba* keratitis across T4 subtypes. (**A–H**) Representative images of *Acanthamoeba* keratitis patients displaying various clinical signs and the corresponding proportions within T4 subtypes. (**A**) Deep stromal infiltrates. (**B**) Ring infiltrates. (**C**) Neovascularization. (**D**) Hypopyon. (**E**) Multifocal stromal infiltrates. (**F**) Groove-shaped corneal melting. (**G**) Perineural infiltrates. (**H**) Pseudodendritic lesions.

ation between T4 subtypes and clinical outcomes ($P = 0.021$). Furthermore, additional statistical analysis revealed that T4C and non-T4C ($P = 0.040$), as well as T4D and non-T4D ($P = 0.013$), showed significant differences (as illustrated in Fig. 6B). These findings indicate that T4C was closely associated with a good prognosis, whereas T4D was closely associated with a poor prognosis.

Discussion

AK is a rare corneal infection with a poor prognosis.[1] Using DF3 fragments in this study efficiently determined *Acanthamoeba* genotypes. This not only improves researchers' knowledge of how the pathogen spreads and evolves but also enables the identification of connections between genotypes and clinical outcomes.[26,27] In this study, we successfully sequenced the DF3 region of all 159 isolates and conducted a phylogenetic analysis to determine genetic relation-

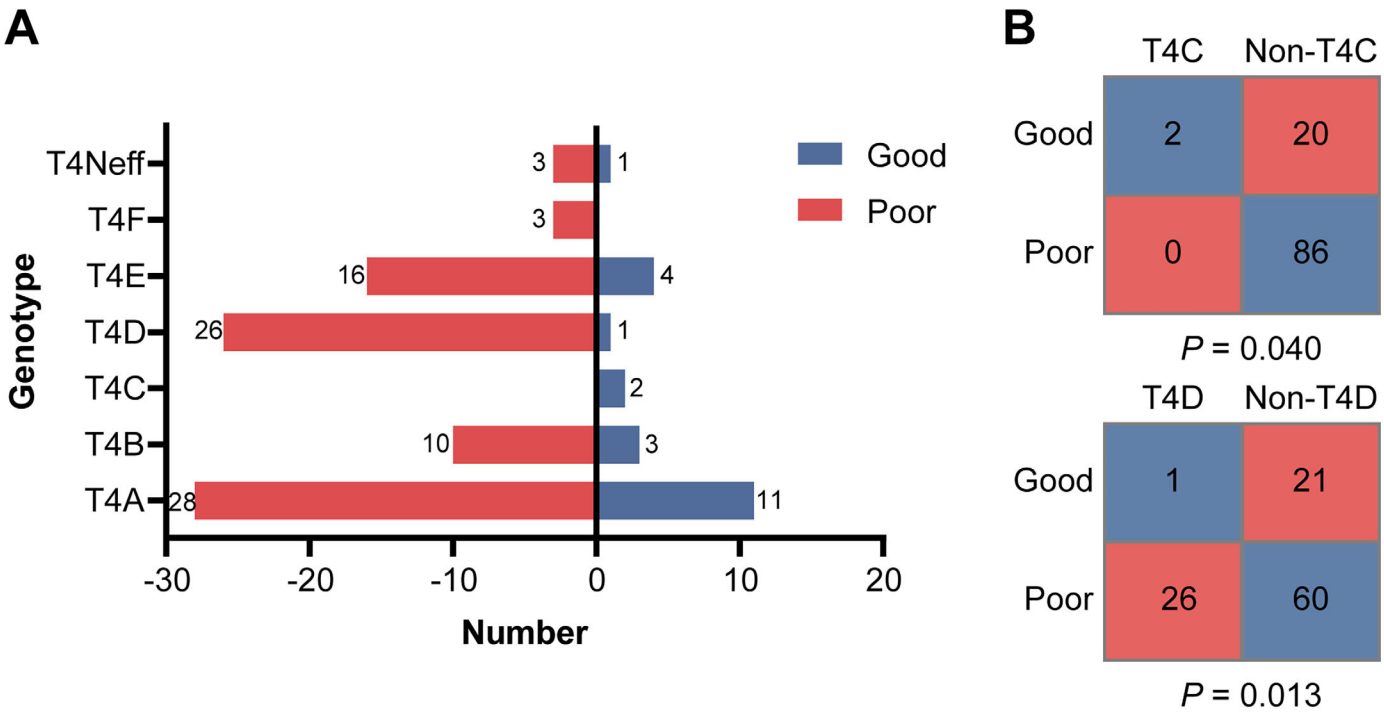

Figure 6. The distributions of clinical outcomes in T4 subtypes. (**A**) Clinical outcomes distributions in *Acanthamoeba* keratitis in T4 subtypes. (**B**) Chi-square test of clinical outcomes in T4C and T4D subtypes.

ships. In China, AK was mainly attributed to the T4A genotype, and there was a positive correlation between geographical and genetic distance. Clinically, T4A was characterized by ring infiltrates and multifocal stromal infiltrates, while T4B and T4D were primarily associated with ring infiltrates. Furthermore, different subtypes of *Acanthamoeba* infection had varying prognosis, with T4C linked to better outcomes and T4D associated with worse outcomes. This study represented a comprehensive investigation of AK, shedding light on its genetic diversity and clinical manifestations. To date, this study represented the largest series on AK, examining the relationship between clinical profiles and genotyping using DF3 region detection. Additionally, it explored T4 subtypes and their impact on clinical prognosis.

This study revealed that *Acanthamoeba* can affect a wide age range, from three to 79 years. Hence, it is vital to enhance health protection, promote health awareness, and offer health education across age groups. In a clinical study conducted in China from 1991 to 2013, ocular trauma was the most common risk factor (53.1%), followed by contact lens usage (29.8%).[3] However, the present findings differed from previous findings, indicating that contact lens use (22.0%) has now surpassed corneal trauma (13.8%) as the most important trigger for AK. This shift could be attributed to the growing popularity of contact lenses and improved hygiene practices among lens users.

From 159 clinical isolates, our study identified three *Acanthamoeba* genotypes (T3, T4, T11) and seven T4 subtypes (T4A, T4B, T4C, T4D, T4E, T4F, T4Neff). The prevalent genotypes in this region of China were T4 and its T4A subtype, consistent with global trends where T4 was the most common causative genotype, and T4A was the dominant subtype.[7] However, the evolutionary tree of the DF3 fragments revealed that the T4 subtypes were not closely clustered, suggesting potential unusual evolutionary events such as gene mutation and gene recombination. Furthermore, our study included AK patients from 25 provinces, indicating the widespread geographical distribution of *Acanthamoeba* and its adaptability to diverse ecological environments. We also proposed that the T4D subtype may be better adapted to cold, dry northern environments, whereas the T4F subtype may thrive in warm, humid southern areas, based on observations in Southern and Northern China. Furthermore, the genetic variation in *Acanthamoeba* was influenced by geographical distance, emphasizing its significant role in genetic differentiation.

Among T4 subtypes in AK patients, the most common clinical presentation was deep stromal infiltrations, in line with previous findings,[2] which suggested

that corneal *Acanthamoeba* infection often damaged deep corneal tissues through invasion, multiplication, and inflammation. Although perineural infiltrates, multifocal stromal infiltrates, ring infiltrations, and groove-shaped melting were considered as characteristic clinical features,[3,28] there were no statistically significant differences in subtype distribution. Therefore the relationship between clinical manifestations and genotypes were diverse and complex, suggesting that genotypes were not the sole determinant of clinical presentation.

In our study, 80% of AK patients experienced poor outcomes, potentially because of the delayed diagnosis,[29] a high misdiagnosis rate,[6] and the limited treatment options for AK.[30] Further analysis showed a connection between T4C and better outcomes, while T4D was associated with a worse outcome, differing from prior research. Zhao et al.[22] found that genotypes T4/26 and T4/27 (equivalent to T4A) were associated with severe keratitis in 14 AK patients, while another study involving 30 AK patients (including 26 T4 genotype isolates—T4A, T4B, T4D, T4E) found there was no significant correlation between the isolates and the clinical outcomes.[23] This discrepancy may be due to sample size and individual differences. Moreover, most *Acanthamoeba* derived from clinical and environmental origins contain various endosymbionts (bacteria, viruses), whose impact on pathogenicity remains unclear.[31] In vitro studies by Fritsche et al.[32] and Iovieno et al.[33] suggested that the presence of endosymbionts may enhance pathogenic potential, but Fritsche et al.[34] found the opposite. Hence, the factors that affect pathogenicity of *Acanthamoeba* and clinical prognosis of AK patients should be investigated in further study.

This study had several limitations. First, because of the number of cases in the T4C group, although a meaningful result was detected, the small sample size made it difficult to determine whether this observed clinical outcome was universal. To draw more accurate conclusions and validate our findings, a larger sample size study is needed. Second, the study was conducted at a single regional medical center, which could introduce selection bias. Last, in the analysis of clinical manifestations and prognosis, only 136 AK patients with complete information were included; thus this study was also limited by its retrospective design. Therefore a multicenter prospective study is needed to address these limitations, offering larger sample sizes, reducing single-center bias, enhancing external validity, and improving result reliability.

In conclusion, AK was predominantly caused by the T4 genotype (T4A). In China, T4D and T4F subtypes were distributed differently according to geography. Geographical distance was positively correlated with genetic distance. The deep stromal infiltration was the main clinical manifestation of AK, and prognosis varied among subtypes, with T4D having a worse outcome and T4C a better one. To enhance result reliability, future research should consider conducting a multicenter prospective study.

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