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CLINICAL COURSE OF *ACANTHAMOEBA* KERATITIS BY GENOTYPES T4 AND T8 IN HUNGARY

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Genus *Acanthamoeba* is an opportunistic protozoan that is widely distributed in the environment. Within this genus, numerous species are recognized as human pathogens, potentially causing *Acanthamoeba* keratitis (AK). AK is a corneal disease, associated predominantly with contact lens (CL) wear; its epidemiology is related to the specific *Acanthamoeba* genotypes. This study reports seven CL wearer, *Acanthamoeba* PCR-positive patients with AK, diagnosed between January 2015 and 2018. Patients had the diagnosis of AK 1.36 months after first symptoms. Genotyping allowed the identification of six isolates of the T4 and one of the T8 genotypes. At first presentation, pseudendritiformic epithelopathy/dirty epithelium (four eyes, 57.1%), multifocal stromal infiltrates (five eyes, 71.4%), ring infiltrate (three eyes, 42.8%), and perineuritis (one eye, 14.3%) were observed. AK was healed without later recurrence in two eyes (28.5%) using triple-topical therapy, in three eyes (42.8%) following additional penetrating keratoplasty. In one patient (14.3%), AK recurred following successful application of triple-therapy and was treated successfully with repeated triple-topical therapy and in one patient (14.3%), no follow-up data were available after diagnosis. We could not observe correlation of genotype and clinical course or the necessity of corneal transplantation in our case series.

Keywords: *Acanthamoeba* keratitis, real-time FRET PCR, sequence analysis, clinical course

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Introduction

Acanthamoeba is a genus of free-living amebae widely distributed in various ecological environments. The spectrum ranges from natural biotopes, such as soil, plants, air, dust, freshwater, fishes, sea water, drinking water, swimming pools, and contact lenses (CLs). In addition, they have been isolated from animals such as fish and mammals and also from humans [1–3].

The first cases, which clearly established *Acanthamoeba* as a causative agent of disease in humans, were published in the early 1970s [4]. The pathogenesis of AK in humans is currently studied and different *Acanthamoeba* genotypes have been reported from all over the world [5].

The traditional *Acanthamoeba* classification has used morphological characteristics, such as morphology, size, and shape [6]. Modern classification uses a molecular biological approach based on 18S ribosomal RNA (rRNA) gene to classify *Acanthamoeba* isolates as one of the 20 known genotypes (T1–T20). Detection of *Acanthamoeba* can be rapidly achieved using real-time molecular methods. Each genotype exhibits 5% or more sequence divergences between different genotypes [7]. For diagnostic purposes, the detection of *Acanthamoeba* at the genus level is sufficient to recognize whether an individual is infected or not. *Acanthamoeba* keratitis (AK) is mainly present in CL wearers, especially in case of prolonged use of CLs, unappropriate hygienic conditions (contact of the lenses with tap water, swimming pool, dust, etc.) or in case of corneal trauma. AK is mainly caused by isolates with T4 genotype [8–10]; however, T2, T3, T5, T6, T8, T9, T11, T13, and T15 genotype species have also been identified in patients with AK, as shown in Table I [11–18].

Following an appropriate clinical and also laboratory diagnosis (confocal microscopy, polymerase chain reaction, histology, and microbiological culture) and having *Acanthamoeba* in culture, it is still not possible to arrive at a conclusion in which topical treatment could be effectively used.

Table I. Non-T4 genotype *Acanthamoeba* in different countries (literature data)

Acanthamoeba genotypes	Report countries
T2	Iran [12]
T3	Iran [12], England [12], Austria, USA, Spain [15, 18], and Mexico [17]
T5	Austria [16] and USA [14]
T6	Austria [16]
T8	Hungary [26]
T9	Thailand [3]
T10	Austria [16]
T11	Austria [16], USA [11], and Spain [15]
T15	Italy [13]

Our aim was to analyze the effect of *Acanthamoeba* genotype, isolated from human corneal scrapings and fluid from CL storage, on clinical course of AK.

Materials and Methods

We retrospectively collected diagnostic and clinical data of patients with AK [polymerase chain reaction (PCR) and/or culture positive], with AK diagnosis between January 2015 and 2018 at the Department of Ophthalmology of Semmelweis University.

Sample collection

Acanthamoeba was isolated from corneal scrapings of seven patients [three males (43%) and four females (57%), mean age during diagnosis 30.71 years] between January 2015 and 2018.

Culture-confirmed detection method

The seven samples of corneal scrapings were then transferred to Page's agar plates overlaid with heat-killed *Escherichia coli* and cultured at 37 °C for 10 days. The morphology of trophozoites and cysts was studied by light microscopy, according to Page [19]. Plates were monitored for growth of ameba microscopically, from 72 to 96 h for the presence of *Acanthamoeba* spp. cysts and trophozoites under 320× and 400× magnification.

Molecular methods

Acanthamoeba was isolated from corneal scrapings of the patients and from fluid of CL storage case. The *Acanthamoeba* species were isolated by dilution method. For this purpose, the samples of corneal scrapings were suspended in 400-µl physiological saline solution (0.85%). After preparation, the DNA extraction was treated with High Pure PCR Template Preparation Kit (Roche, Germany) according to the manufacturer's instructions. If further processing was delayed, the isolates were stored at 4 °C for 24 h or at −20 °C for a longer period. The DNA amplification was performed using genus-specific primers and genus-specific fluorescence resonance energy transfer (FRET) hybridization probes, previously described by Orosz et al. [20]. Each experiment included one reaction mixture

without DNA as a negative control; positive control and each specimen was run in duplicate for real-time PCR assay in parallel.

PCR products were purified with PCR Clean-Up M Kit (Viogene, Sunville, CA). The sequence of each amplicon was determined by cycle sequencing with primers for the 5′-NTR region and with primers with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Germany) according to the manufacturer's instruction. The electrophoresis was carried out using Applied Biosystems 3500 Genetic Analyzer.

The 5′-NTR and VP1 gene sequences were subject to nucleotide–nucleotide BLAST analysis [21] using the online server at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast).

The unknown sequences were aligned with known published sequences of the major genotypes using the alignment program MULTALIN (http://multalin.toulouse.inra.fr/multalin) [22]. The genotypes of samples were determined based on this comparison.

The phylogenetic tree was constructed by the neighbor-joining method of genetic distance calculated by the MEGA 6 (http://www.megasoftware.net) [23].

Genotype identification was carried out using a real-time FRET PCR assay based on sequence analysis of the 18S rRNA gene, and sensitivity and specificity were evaluated in comparison with traditional parasitological techniques.

Clinical course

Observing the clinical course, we collected data on (1) time between onset of symptoms and diagnosis of AK, (2) clinical signs of AK, (3) coinfection with bacteria or fungi, (4) conservative treatment, (5) surgical treatment, and (6) outcome. For the outcome, we defined "success" if no sign of active AK was observed at least for 6 months, without the use of topical antiamebic therapy. We defined "failure" if AK persisted or recurred.

Results

Clinical data

Seven patients with AK had the diagnosis 1.36 months after first symptoms, at the Department of Ophthalmology of Semmelweis University as shown in Figure 1.

At the first presentation, pseudendritiformic epithelopathy/dirty epithelium (four eyes, 57.1%), multifocal stromal infiltrates (five eyes, 71.4%), ring infiltrate (three eyes, 42.8%), and perineuritis (one eye, 14.3%) were observed. The clinical data are summarized in Table II.

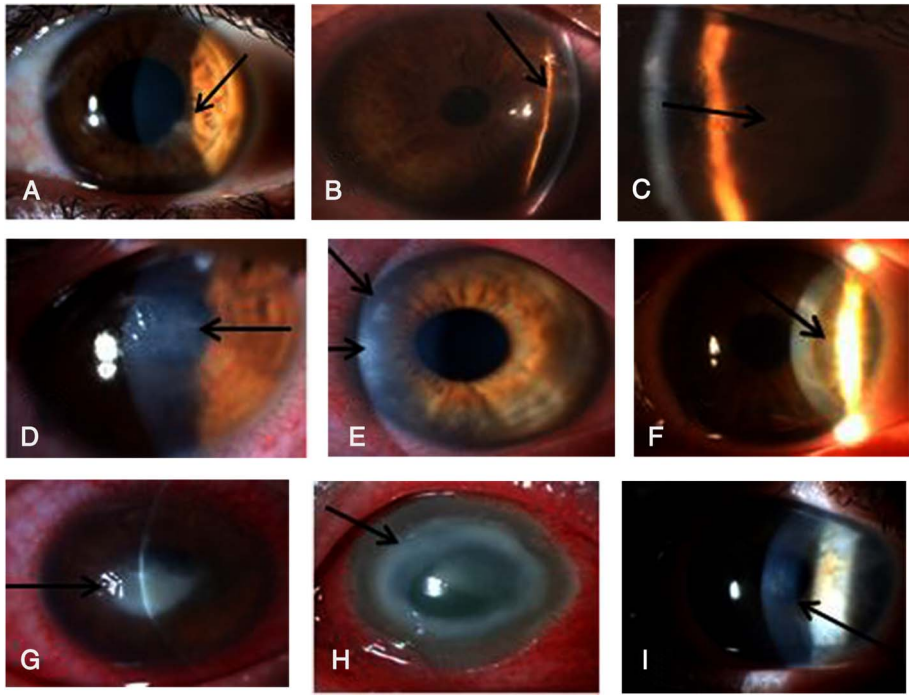

Figure 1. Clinical images of *Acanthamoeba* keratitis in patients 1–7. Patient 1: multifocalstromal infiltrates. B and C: Patient 2: perineuritis (B) (arrow) and pseudodendritiformic epitheliopathy ("dirty epithelium") (C). D: Patient 3: multifocal stromal infiltrates and incomplete ring infiltrate (arrow). E and F: Patient 4: multifocal stromal infiltrates (arrows) (E) and ring infiltrate (F) (arrow). G: Patient 5: deep stromal infiltrate (arrow) and fine slightly visible multifocal stromal infiltrates. H: Patient 6: epithelial erosion and broad ring infiltrate (arrow). I: Patient 7: multifocal stromal infiltrates (arrow)

AK was healed without later recurrence in two eyes (28.5%) using triple-topical therapy (TTT; polyhexamethylene biguanide, propamidine isethionate, and antibiotics/neomycin) in three eyes (42.8%) following additional penetrating keratoplasty.

In Patient 3, AK recurred following successful application of TTT and was treated successfully with repeated TTT. In Patient 7, no follow-up data were available, following diagnosis. We could not observe similarities in genotype and clinical course or the necessity of corneal transplantation.

Cultivation

All investigated samples revealed *Acanthamoeba* that were able to grow at 36 °C, the approximate temperature of the human host. Microscopical cultivation

Table II. Clinical data during the observation time period in our set of patients

Case no.	Age (years), sex	Time to diagnosis	Clinical signs	Coinfection	Conservative treatment	Surgical treatment	Outcome	*Acanthamoeba* genotype
1	24, female	3 weeks	MSI	–	TTT	–	Success	T4
2	30, female	2 weeks	Dirty epithelium and perineuritis	–	TTT	–	Success	T4
3	16, male	2 weeks	MSI and ring infiltrate	–	TTT	–	Failure	T4
4	20, female	10 days	MSI and ring infiltrate	–	TTT	PKP and AMT	Success	T4
5	37, male	2 months	Persistent epithelial defect and MSI	staph.	TTT	PKP and AMT	Success	T4
6	60, male	5 months	Persistent epithelial defect and ring infiltrate	–	TTT	PKP	Success	T4
7	28, female	2 weeks	MSI	–	No information available			T8

Note: Observing the clinical course, we collected data on (1) time between onset of first symptoms and diagnosis of AK, (2) clinical signs of AK, (3) coinfection with bacteria or fungi, (4) conservative treatment, (5) surgical treatment, and (6) outcome. For the outcome, we defined as "success" if no signs of AK were observed for at least 6 months, without the use of topical antiamebic therapy. We defined as "failure" if AK persisted or recurred. MSI: multifocal stromal infiltrates; staph.: *Staphylococcus*; TTT: triple-topical therapy; PKP: penetrating keratoplasty; AMT: amniotic membrane transplantation as patch.

was successful in six samples. Probably due to low quantity of corneal scraping in the seventh sample, one sample showed negative result for cultivation. Further examination of the obtained results was carried out by FRET PCR.

Molecular methods

This study reports successful PCR amplification for seven (four females and three males) positive cases. The samples for *Acanthamoeba*-positive patients, detected by PCR method, were sequenced to identify the species (NCBI Bank: Patient_1-KF873021_T4, Patient_2-KP337296_T4, Patient_3-KU356846_T4, Patient_4-KU356848_T4, Patient_5-KR494236_T4, Patient_6-KJ094693_T4, and Patient_8-MF065931_T8). Sequence analysis using a BLAST search indicated an identity of >98% with *Acanthamoeba* 18S rRNA gene reference sequences. It was found that the obtained sequences of amebae isolates from the cases belonged to the T4 and T8 genotypes *Acanthamoeba* spp. neighbor-joining analysis inferred relationships between the PCR products isolated from corneal scrapings and reference strains obtained from NCBI GenBank are shown in Figure 2, respectively.

Discussion and Conclusions

Detection of *Acanthamoeba* can be rapidly achieved using real-time FRET molecular methods. For diagnostic purposes, the detection of *Acanthamoeba* at the genus level is sufficient to recognize whether an individual is infected or not [24, 25].

Literature describes T4 genotype *Acanthamoeba*, as the most common in the environment; however, in AK, prevalence of T4 genotype is even more common. The molecular analysis conducted in this study confirmed, in our series of patients, the T4 genotype to be the most frequent cause of AK. In addition, one isolate was T8 genotype and was first associated with AK [26]. These results are consistent with previous findings indicating that T4 is worldwide predominant in AK [27, 28].

Nevertheless, a heterogeneous virulence of different *Acanthamoeba* strains has been observed in our case series, for different *Acanthamoeba* strains. In our opinion, different reference sequences of T4 or other *Acanthamoeba* genotypes may also be important in prognosting disease progression.

For an appropriate diagnosis, ophthalmological signs of AK must be known. These are “dirty epithelium” (pseudodendritiformic epitheliopathy), non-healing epithelial defects or ulcer, mono- or multifocal stromal infiltrates, perineuritis, ring

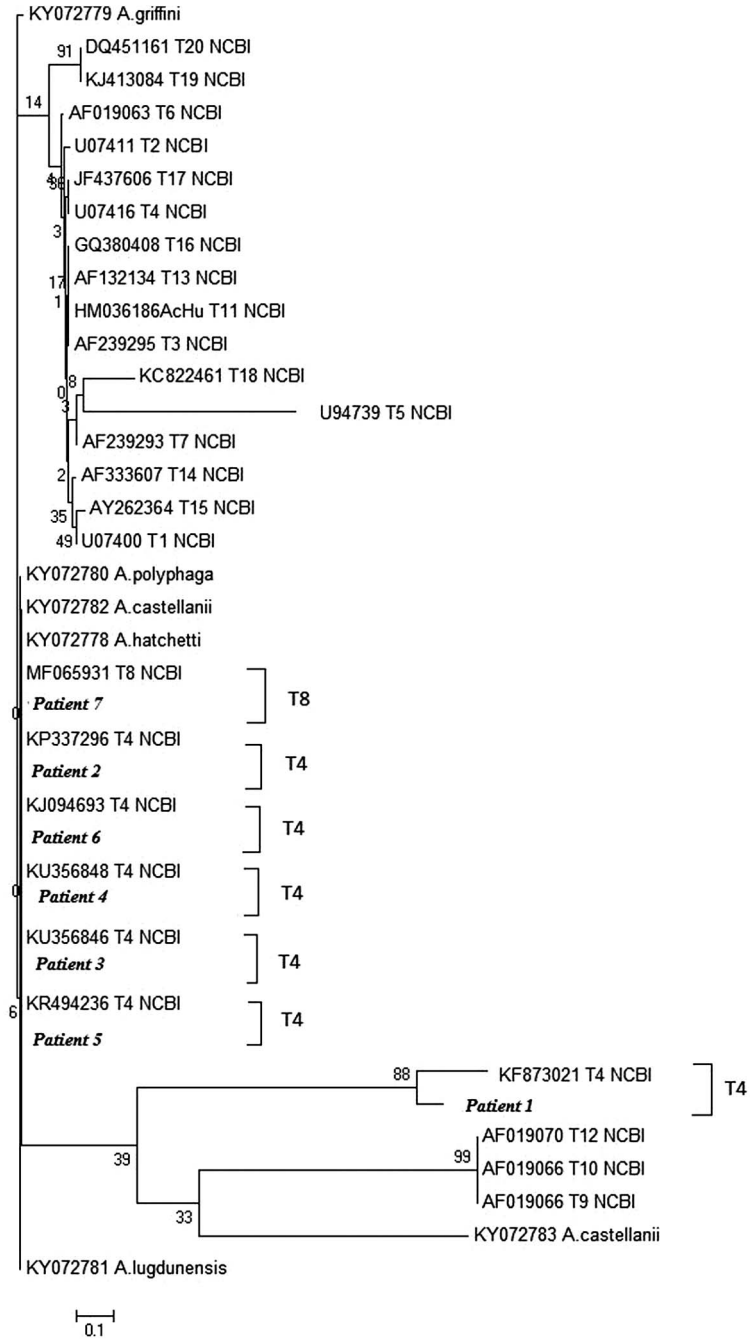

Figure 2. Phylogenetic relations of *Acanthamoeba* species PCR product Patient_1, Patient_2, Patient_3, Patient_4, Patient_5, Patient_6, Patient_7 and reference strains from NCBI GenBank inferred by neighbor-joining analysis from pairwise comparisons (180-bp fragments)

infiltrate, and in later stages anterior syneciae, iris atrophy, secondary glaucoma, mature cataract, scleritis and chorioretinitis, or even blindness. To date, about 75% of ophthalmologists miss the appropriate clinical diagnosis in AK. Faulty typical diagnosis is herpetic keratitis in most of the cases; however, bacterial or mycotic keratitis may also be suggested [29–33]. However, cooperation between clinicians and experts in laboratory diagnostics is indispensable, for adquate treatment *in time* of these patients.

Nowadays, there is no standardized treatment of AK, as no previous randomized controlled studies have been performed in this rare and often heterogeneous corneal disease. In addition, *Acanthamoeba* cysts are often resistant or become resistant during treatment to all available topical therapeutic options. The therapeutic regimen used in the recent times includes biguanides (pethylene biguanide 0.02% and chlorhexidine 0.02%), diamidine (propamidine isethionate 0.1% and hexamidine 0.1%), and antibiotics/neomycin as TTT. Antimycotic eye drops, propidium iodide, and miltefosine may also be effective against *Acanthamoeba* strains. Besides conservative treatment, in advanced cases or in cases without response to topical therapy, penetrating keratoplasty, amniotic membrane transplantation, or crosslinking treatment may be performed [34].

To the best of our knowledge, this is the first study to analyze a correlation between *Acanthamoeba* genotype and clinical course. However, we could not determine a relationship between both of them in these series of patients. To date, there is no standard treatment of AK; however, we also did not find a study analyzing relationship between genotype and *Acanthamoeba* susceptibility to different agents. In our opinion, this also needs development in the following years.

There are only case series on safety and effectivity of medical and surgical treatment of AK and to date there are no randomized controlled clinical studies. In Hungary, we suggest topical application of polyhexamethylene biguanide, propamidine isethionate, and antibiotics/neomycin as TTT in case of AK [35].

During the first 2 days, a “surprise attack” or “flash war” is initiated with polyhexamethylene biguanide and propamidine isethionate every quarter to half an hour day and night. Then, until the sixth day, polyhexamethylene biguanide and propamidine isethionate are applied every hour and only over the day. Following 4 weeks, eyedrop use is reduced to every 2 h. In addition, antibiotics/neomycin 5× a day is also applied for some week.

Worldwide incidence of AK increases, presumably due to the increasing use of CLs [36, 37]. To the best of our actual knowledge, combination therapy using diamidine, biguanide, and antibiotics should be continued in descending dosis until 1 year.

In Hungary, AK has been developed through *Acanthamoeba* genotypes T4 and T8 in the past 3 years. Analyzing seven patients, we could not determine a

relationship between *Acanthamoeba* genotype and clinical course of the disease. We suggest the development of an international database on AK causative isolates for better understanding of the disease course and better treatment of these patients.

Conflict of Interest

The authors declare no conflict of interest. EO assures that there are no links with a company whose product is mentioned in the article or a company that distributes a competing product. The authors also state that the presentation of the topic is independent and the presentation of the content is product-neutral.

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