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LABORATORY DIAGNOSIS OF *ACANTHAMOEBA* KERATITIS IN HUNGARY

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Acanthamoeba species are free-living amebae that can be found in almost every range of environments. Within this genus, numerous species are recognized as human pathogens, potentially causing *Acanthamoeba* keratitis (AK). AK is a corneal disease that is predominantly associated with contact lens use, the epidemiology of which is related to the specific genotype of *Acanthamoeba*. This study reports seven (7/16; 43.75%) positive cases. Detection of *Acanthamoeba* in corneal scrapings is based on cultivation and polymerase chain reaction (PCR) combined with the molecular taxonomic identification method. By PCR, seven samples were positive; cultivation was successful for five samples, probably because of the low quantity of samples. Genotype identification was carried out with a real-time fluorescence resonance energy transfer PCR assay based on sequence analysis of the 18S rRNA gene, and sensitivity and specificity were evaluated in comparison with traditional parasitological techniques. All seven detected *Acanthamoeba* strains belonged to the T4 genotype, the main AK-related genotype worldwide. These results confirmed the importance of a complete diagnostic protocol, including a PCR assay, for the clinical diagnosis of AK from human samples. Genotyping allowed the identification of all isolates in the T4 group, thus demonstrating the prevalence of this genotype in Hungary.

Keywords: 18S rRNA, *Acanthamoeba*, real-time PCR, hybridization probes, fluorescence resonance energy transfer PCR, sequence analysis

Introduction

Acanthamoeba is a genus of free-living amebae widely distributed in various ecological environments. These amebae are thermotolerant, and resistant to extreme temperature and pH conditions, as well as to chlorine and other disinfectant media. Unlike obligate parasites, pathogenic *Acanthamoeba* spp. can complete their life cycle and environmental performance without having to enter the human or animal host [1–4].

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Some genera of *Acanthamoeba* cause a different infection that produces *Acanthamoeba* keratitis (AK), subacute or chronic granulomatous amebic encephalitis, and skin infections. Human infections with these amebae have been reported from all over the world [5–7]. The first cases which clearly established *Acanthamoeba* as causative agents of disease in humans were reported in the early 1970s [8].

In many cases, AK infections occur after water exposure or a history of swimming in lakes, following contact with soil or plants, or while wearing contact lenses (CLs). The initial stage in the development of AK is thought to be contamination of a CL care system with an *Acanthamoeba* cyst(s) introduced from the environment, where they are widespread. Diagnosis is made on the basis of the clinical picture and laboratory investigations. Samples of corneal epithelium and any infiltrated stroma are removed under local anesthesia, and the CLs and their storage cases may also be cultured. All patients with unresponsive microbial keratitis, even those without CL use, should be tested for *Acanthamoeba*.

This study aimed to investigate the CLs and their paraphernalia of patients with keratitis for pathogenic free-living amebae.

Materials and Methods

Sample collection

Acanthamoeba was originally isolated from a human cornea. From January 2015 to December 2015, 16 patients (6 males and 10 females) were examined for their corneal scrapings.

Culture-confirmed detection method

The 16 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. Plates were monitored for growth of amebae microscopically, from 72 to 96 h, for the presence of *Acanthamoeba* spp. cysts and trophozoites under 320× and 400× magnifications [9].

Molecular methods

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 instructions of the manufacturer. 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. [10]. Each experiment included one reaction mixture without DNA as a negative control; positive control and each specimen were run in duplicate for real-time polymerase chain reaction (PCR) assay in parallel. We have used serial dilutions of *Acanthamoeba* (Gene bank accession number KC434439) strain to determine the calibration curve that the LightCycler real-time PCR device could determine the additional samples parasite number in copy numbers.

PCR products were purified with PCR Clean up-M Kit (Viogene, Sunville, CA, USA). The sequence of each amplicon was determined by cycle sequencing with primers for the 5′-NTR region and with primers with Big DYEnamic ET dye terminator kit (Amersham Pharmacia, Munich, Germany) according to the manufacturer's instruction. The electrophoresis was carried out on Applied Biosystems 3500 Genetic Analyzer.

The 5′-NTR and VP1 gene sequences were subject to nucleotide–nucleotide BLAST analysis [11] 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) [12]. 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 5 (http://www.megasoftware.net) [13].

Results

Cultivation

All investigated samples revealed *Acanthamoeba* that were able to grow at 36 °C, the approximate temperature of the human host (Figure 1). Microscopically, cultivation was successful for five samples, probably because of the low quantity of sample; 11 samples showed negative results for cultivation. Further examination of the obtained results was done by FRET PCR.

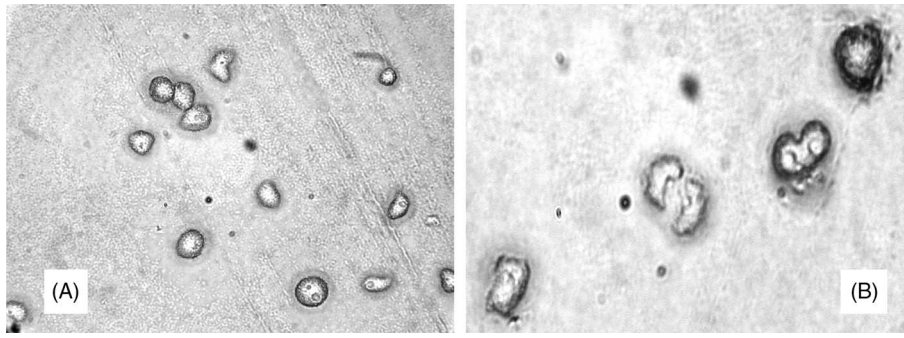

Figure 1. Photomicrograph of amebae cysts and trophozoites with 320× (A) and 400× (B) magnifications

Molecular analysis

This study reports successful PCR amplification for seven (7/16; 43.75%) positive cases. The samples for seven *Acanthamoeba*-positive patients, detected by PCR method, were sequenced to identify the species. Sequence analysis using a BLAST search indicated an identity of >98% with *Acanthamoeba* 18S rRNA gene reference sequences. It was found that all obtained sequences of amebae isolates from the cases belong to the different T4 genotypes of *Acanthamoeba* spp. Neighbor-joining analysis inferred relationships between the PCR products isolated from corneal scrapings and reference strains obtained from NCBI GenBank, shown in Figure 2, respectively.

Discussion and Conclusion

Detection of *Acanthamoeba* can be rapidly achieved by using real-time molecular methods. For diagnostic purposes, the detection of *Acanthamoeba* at the genus level is sufficient to recognize whether an individual is infected or not. Based on rRNA gene sequences, the genus *Acanthamoeba* is divided into 20 different genotypes (T1–T20) to date. Each genotype exhibits 5% or more sequence divergences between different genotypes [14].

All seven detected *Acanthamoeba* strains belonged to the T4 genotype, the main AK-related genotype worldwide. A more precise differentiation of genotype would be beneficial for the better understanding of taxonomy of *Acanthamoeba* and this may also facilitate the correct identification.

These organisms have gained attention from the broad scientific community studying AK. The culture-confirmed detection method is slow (96 h) and can

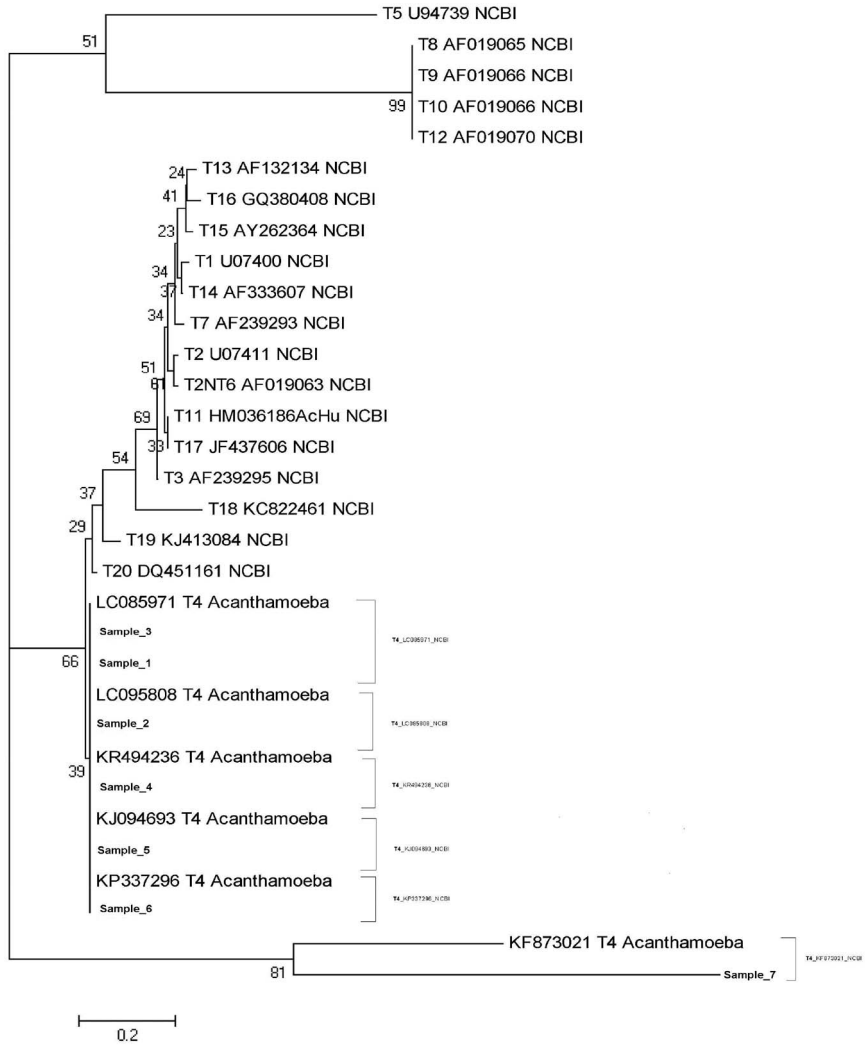

Figure 2. Phylogenetic relations of *Acanthamoeba* species PCR product sample_1, sample_2, sample_3, sample_4, sample_5, sample_6, sample_7, and reference strains from NCBI GenBank inferred by neighbor-joining analysis from pairwise comparisons (180-bp fragments)

detect *Acanthamoeba* only at genus level, while the molecular genotype analysis is faster (real-time PCR within 4 h) and allows further detection at species level too.

This study represents the first characterization of *Acanthamoeba* genotypes in Hungary, in isolates obtained from patients showing keratitis-related symptoms.

In conclusion, AK has emerged as a clinical problem only during the last 45 years and has been one of the most difficult corneal diseases. The lack of appropriate hand washing and the use of non-sterile home-made saline solutions for CLs are also linked to the infection [15, 16]. Recently, treatment has become more effective and diagnosis is often made earlier than in the past, due to increased familiarity with the condition. Our results support previous report on *Acanthamoeba* genotype T4. The symptoms of keratitis caused by representatives of *Acanthamoeba* genotype T4 showed no significant differences in their clinical characteristics.

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