Acta Microbiologica et Immunologica Hungarica 65 (1), pp. 73–79 (2018)
DOI: 10.1556/030.65.2018.007
First published online February 22, 2018

FIRST REPORT OF *ACANTHAMOEBA* GENOTYPE T8 HUMAN KERATITIS

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(Received: 23 June 2017; accepted: 15 November 2017)

Acanthamoeba has a worldwide distribution in the environment and it is capable of causing a painful sight-threatening disease of the cornea designated as *Acanthamoeba* keratitis (AK). Nowadays, the cases of AK have surged all over the world along with its disease burden due to increasing use of contact lenses used not only for optical correction but also for cosmetic purposes. In our present work, epithelial abrasion of a 27-year-old female soft contact lens wearer with keratitis was examined. Genotype identification was carried out with a real-time fluorescence resonance energy transfer polymerase chain reaction (PCR) assay based on sequence analysis of the 18S rRNA gene. Genotyping allowed the identification of a T8 group isolate. The analysis confirmed the importance of a complete diagnostic protocol, including a PCR assay, for the clinical diagnosis of AK from human samples. *Acanthamoeba* T8 should be considered as potential causative organism in keratitis in human.

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

Introduction

Acanthamoeba has a worldwide distribution in the environment [1–4]. *Acanthamoeba* keratitis (AK) infections occur after water exposure or a history of swimming in lakes, following contact with soil or plants, or due to contact lens wear for optical or cosmetic purposes [5–7]. Based on rRNA gene sequences, the genus *Acanthamoeba* is divided into 20 different genotypes to date (T1–T20). The first description of amoebic keratitis (AK) and granulomatous amebic encephalitis (GAE) in 1975 was performed by Jones et al. [8].

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Certain species of the genus Acanthamoeba can cause amoebic keratitis in healthy humans [9]; in immunocompromised individuals, it can cause the fatal GAE [10].

In the past few years, from ecological diagnostic and clinical therapy viewpoints, a demand raised for a polymerase chain reaction (PCR) method, which can be used for *Acanthamoeba* spp. studies [11]. Real-time PCR allows specific detection of the amplification by binding with fluorescence-labeled probes, and further downstream analysis is not required. This considerably reduces the time required for detection. In addition, the closed reaction tube minimizes the chance of cross-contamination and the assay output is quantitative and qualitative [12].

Our aim was to present genotypic characterization of an *Acanthamoeba* spp. isolated from human corneal epithelium and contact lens storage case of a patient with clinical signs of *Acanthamoeba* keratitis.

Materials and Methods

Isolation

Acanthamoeba was isolated from human corneal epithelium (following focal corneal abrasion) of a patient with clinical signs of AK (multifocal stromal infiltrates and ring infiltrate) and from contact lens storage case of the same patient.

Culture-confirmed detection method

The corneal epithelial sample and fluid from contact lens storage case 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 were studied by light microscopy, according to Page [13]. Plates were monitored for growth of amoeba microscopically, from 72 to 96 h for the presence of *Acanthamoeba* spp. cysts and trophozoites under 320× and 400× magnification.

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 (Germany), according to the instructions

of the manufacturer. The DNA amplification was performed using genus-specific primers and genus-specific-fluorescence resonance energy transfer hybridization probes, previously described by Orosz et al. [14]. Each experiment included one reaction mixture without DNA as a negative control; positive control and each specimen were 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 on Applied Biosystems 3500 Genetic Analyzer.

The 5′-NTR and VP1 gene sequences were subjected to nucleotide–nucleotide BLAST analysis [15] 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) [16]. 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) [17].

Results

Cultivation

Microscopically, the cultivation was successful for both samples. Both investigated samples revealed *Acanthamoeba* that was able to grow at 36 °C, which is the approximate temperature of the human host (Figure 1). Further examination of the obtained results was carried out by FRET PCR.

Molecular analysis

PCR amplification from both corneal epithelium and fluid from contact lens storage was succesfull. Thereafter, the *Acanthamoeba* positive samples, detected by PCR method, were sequenced to identify the species. Sequence analysis using a BLAST search indicated an identity of >98% with *Acanthamoeba* 18r rRNA gene reference sequences. All obtained sequences of amoeba isolates from the case belonged to the T8 genotype *Acanthamoeba* spp. (NCBI *Acanthamoeba* healy MF065931.1). Neighbor-joining analysis inferred relationships between the PCR

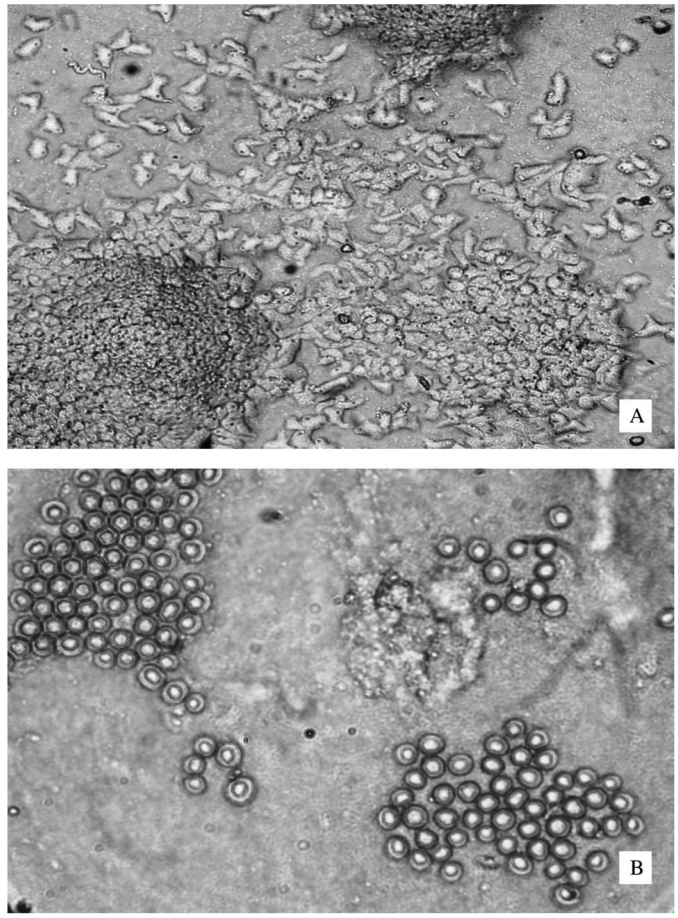

Figure 1. Photomicrograph of *Acanthamoeba* trophozoites and cysts with 320× (A) and 400× (B) magnification. Photographer: Erika Orosz

products isolated from corneal scrapings and reference strains obtained from NCBI GenBank, shown in Figure 2, respectively.

Discussion and conclusions

Acanthamoeba identification can be rapidly achieved 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 [18, 19].

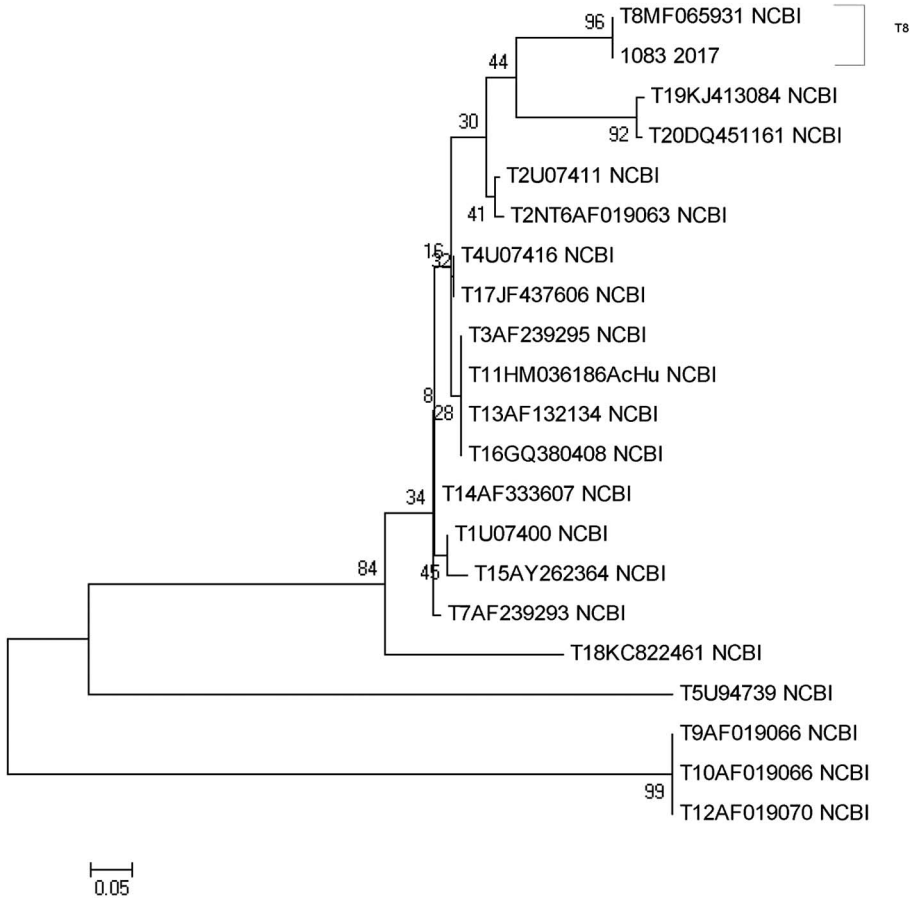

Figure 2. Phylogenetic relations of *Acanthamoeba* species PCR product 1083_2017 and reference strains from NCBI GenBank inferred by neighbor-joining analysis from pairwise comparisons (180-bp fragments)

A more precise differentiation of genotype is beneficial for better understanding of *Acanthamoeba* taxonomy and also facilitates the correct identification.

These organisms have gained attention from the broad scientific community studying environmental biology, molecular biology, and biochemistry. The culture-confirmed detection method is slow (96 h) and can detect *Acanthamoeba* only at genus level, whereas the molecular genotype analysis is faster (real-time PCR 4 h). Based on rRNA gene sequences, the genus *Acanthamoeba* is divided into 20 different genotypes to date (T1–T20). Each genotype exhibits 5% or more sequence divergence [9, 20, 21].

In conclusion, in this study, we reveal the genetic diversity of *Acanthamoeba* spp. existing in keratitis patients using the nuclear small subunit ribosomal RNA (18S rRNA). To the best of our knowledge, this is the first report on T8 genotype *Acanthamoeba* spp., causing AK in human. The presence of *Acanthamoeba* T8 should also be considered as potential causative organism of contact lens-associated keratitis.

Conflict of Interest

There is 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 presentation of the topic is independent and the presentation of the content is product-neutral.

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