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SHORT COMMUNICATION

First report of an *Acanthamoeba* genotype T13 isolate as etiological agent of a keratitis in humans

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Abstract Several strains of free-living amoebae (FLA) belonging to the genus *Acanthamoeba* are able to cause a painful sight-threatening disease of the cornea designated as *Acanthamoeba* keratitis (AK). In this case report, a 22-year-old woman, wearer of soft contact lenses, was treated after the initial examination, and follow-up laboratory results led to the diagnosis of *Acanthamoeba* keratitis. The patient recovered under the targeted therapy, demonstrating that the acanthamoebae were the etiological agents of the keratitis in this case. The acanthamoebae belonged morphologically to group II. Genotyping of the causative *Acanthamoeba* strain based on sequences of the PCR amplimer ASA.S1 amplified from 18S ribosomal DNA by using the genus-specific primers JDP1 and JDP2 followed. The phylogenetic comparison of ASA.S1 confirmed that the isolated *Acanthamoeba* strain is closely related to genotype T13 supported by pairwise sequence identities of 97.1–98.0 % and bootstrap support of 980 replicates with reference sequences of genotype T13. These results regarding the *Acanthamoeba* keratitis-causing isolate KaBo expands the number of known pathogenic genotypes to 12. To our knowledge, this is the first report of a T13 *Acanthamoeba* genotype being associated with keratitis in humans.

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Introduction

Acanthamoebae can be found ubiquitously in almost any habitat with a solid surface and moisture (Marciano-Cabral and Cabral 2003; Khan 2006). The spectrum ranges from natural biotopes such as freshwater and saltwater, sediments, biofilms, soil and air to artificial habitats such as compost, swimming pools, surgical instruments, contact lenses and air-conditioning units. In addition, they have been isolated from animals such as fish, amphibians, reptiles and mammals, including humans (for overview, see Marciano-Cabral and Cabral 2003; Lorenzo-Morales et al. 2007).

Acanthamoebae are well known as facultative human parasites that may cause the clinical patterns of granulomatous amoebic encephalitis (GAE), *Acanthamoeba* keratitis (AK) and cutaneous acanthamoebiasis in humans (Jager and Stamm 1972; Marciano-Cabral and Cabral 2003; Scheid et al. 2008; Lorenzo-Morales et al. 2013). AK is a serious sight-threatening disease of the cornea for which major preconditions are the use of contact lenses (especially soft lenses) combined with poor lens hygiene (Marciano-Cabral and Cabral 2003). Acanthamoebae have been isolated frequently from lens storage cases (Marciano-Cabral and Cabral 2003; Scheid et al 2008). Additionally, acanthamoebae can act as hosts of and vehicles for phylogenetically divergent microorganisms (Rowbotham 1980; Michel et al. 1995; Gómes-Couso et al. 2007; Scheid 2007; Scheid et al. 2010; Scheid and Schwarzenberger 2011, 2012).

The traditional classification of the genus *Acanthamoeba* has used morphological characteristics such as cyst morphology size and shape as classification characters, which allow us to distinguish three morphological groups or species (Pussard and Pons 1977; De Jonckheere 1987; Page 1988). Modern classification uses a molecular biological approach on the

basis of 18S ribosomal RNA (rRNA) gene (*Rns*) to classify *Acanthamoeba* isolates as one of the 18 known genotypes (Gast et al. 1996; Stothard et al. 1998; Horn et al. 1999; Gast 2001; Hewett et al. 2003; Corsaro and Venditti 2010; Nuprasert et al. 2010; Qvarnstrom et al. 2013). In this study, the *Rns* genotype with the genus-specific *Rns* ASA.S1 amplicon responsible for the infection was assessed in one strain isolated from the contact lens case of a patient presenting with AK (Schroeder et al. 2001).

Case report

A 22-year-old woman, living in southern Africa, wearer of monthly disposable soft contact lenses, presented with "severe and persisting keratitis" in her left eye. Before arriving at the German clinic, she did not show any improvement after an initial local therapy with xylometazoline and gentamycin eye drops. A concomitant reduction in visual acuity was diagnosed. Additionally, she complained of massive pain and of a foreign body sensation in the eye. The contact lenses, along with the fluid container, were sent to the laboratory of parasitology for examination and identification of a suspected *Acanthamoeba* keratitis. Acanthamoebae, morphologic group II, were found microscopically, showing the typical cyst morphology after culture, and the diagnosis of "*Acanthamoeba* keratitis" was established. Brolene® eye drops (propamidine), Lavasept® (biguanide) and Polyspectran® eye drops (polymyxin B, neomycin, gramicidin) were administered every 30 min as therapy against acanthamoebae. The patient's condition improved noticeably with this therapy. Vision returned to pre-infection levels, and the therapy was reduced to Polyspectran® eye drops only after 4 weeks. The patient recovered subsequently.

Material and methods

Isolation and cultivation

Pieces of contact lenses and fluid from their storage case were transferred to non-nutrient agar (NNA) plates, according to Page (1988). The strain was subcultured using a piece of isolation agar on a fresh plate (Khan 2006; Scheid et al. 2008). The cultivation was carried out xenically with 1.2 % NNA (Page 1988). Incubation was performed at 20 °C. *Enterobacter cloacae* served as the food organism.

Morphological classification

For morphological determination of the amoebae, trophozoites and cysts were taken from NNA plates seeded with *E. cloacae*. The microscopical identification was done using the Zeiss Axioskop 2 plus (Carl Zeiss Microscopy GmbH, Jena, Germany). The amoebae were identified as being from one of the three cyst morphological groups established by Pussard and Pons (1977). Differentiation was achieved mainly on the basis of endo- and ectocyst shape and thickness, their distance from each other and average cyst size. The last criterion was acquired by averaging the diameter of 100 cysts of each isolate with the documentation and archiving software DISKUS 4.30 (Hilgers, Koenigswinter, Germany) (Scheid 2013).

DNA extraction, PCR and DNA sequencing

The amoebae were taken from the NNA plates using amoebal saline (according to Page 1967). The DNA was extracted and purified using a QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The template DNA was amplified using the primers JDP1 and JDP2 (Schroeder et al. 2001). The PCR products were subsequently purified and sequenced. Sequencing of ASA.S region was performed in both directions with the same primers using the ABI 3730 XL DNA Analyzer (Applied Biosystems, Foster City, USA).

Phylogenetic analysis

The raw sequence data were read, analysed and assembled using a DNA Baser Sequence Assembler (Heracle BioSoft S.R.L., Pitesti, Romania). Twenty-five reference sequences of 18 genotypes (T1–T18) from GenBank of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) were used to compare and analyse the sequence data of the strain KaBo. This comparison was made with reference sequences of Gast et al. (1996), Stothard et al. (1998), Horn et al. (1999), Gast (2001), Hewett et al. (2003), Edagawa et al. (2009), Corsaro and Venditti (2010), Nuprasert et al. (2010) and Qvarnstrom et al. (2013).

To align the sequences, they were all trimmed to start with the oligonucleotide array AAAATTAGAGTGTT and to end with TGGTGAA, CGGTGAA or TAAAGGA, respectively. Afterwards, consensus sequences of 26 ASA.S1 fragments were multiple aligned with ClustalX (Larkin et al. 2007; Thompson et al. 1994).

The phylogenetic analysis was accomplished with MEGA version 6 (Tamura et al. 2013). The tree was constructed using the neighbour-joining algorithm based on evolutionary distances calculated from maximum composition likelihood method estimated with 1,000 bootstrap samplings (Felsenstein 1985; Tamura et al. 2004). Pairwise sequence identities were calculated using BioEdit 7.2.2 (Bio Biosciences, Carlsbad, USA).

Nucleotide sequence accession number

The new nucleotide sequence is available in the GenBank of the National Center for Biotechnology Information under the reference number KJ476522.

Results

Morphological determination

According to Pussard and Pons (1977), strain KaBo displayed the cyst shape and size of *Acanthamoeba* morphological group II with an average cyst diameter of 7.92±0.8 μm (Fig. 1). The trophozoites showed the genus characteristic acanthophodia (Fig. 2).

Molecular biological determination

ASA.S1 was successfully amplified and sequenced for the examined strain. To align the 26 sequences for multiple alignment, it was necessary to limit them to the stated start and ending oligonucleotide array; therefore, the whole amplimer could not be included in the analysis. Nonetheless, the sequence length was sufficient to perform a valid genotyping. Thus, based on hierarchical cluster analysis, the sequence of the clinical sample KaBo was grouped with reference sequences of genotype T13 (Fig. 3). Bootstrap support (BS) for terminal knot of the sample showed a high value of 980 replicates (Fig. 3). Pairwise sequence identities between strain KaBo and RAC147 (AB327095.1), RAC055 (AB327077.1) and UWET39 (AF132136.1) were 98, 97.1 and 97.3 %. Sequence identities between strain KaBo and the other used sequences ranged from 63 to 91 %.

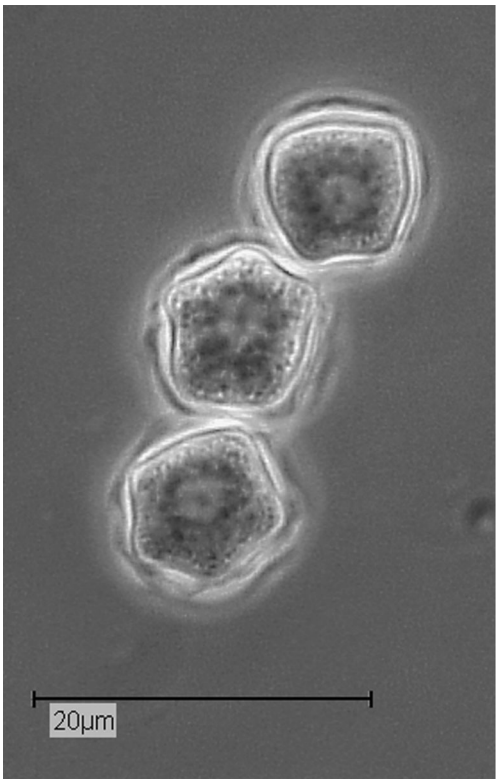

Fig. 1 Cysts of strain KaBo at object slide using light microscopy (phase contrast). *Scale bar* represents 20 μm

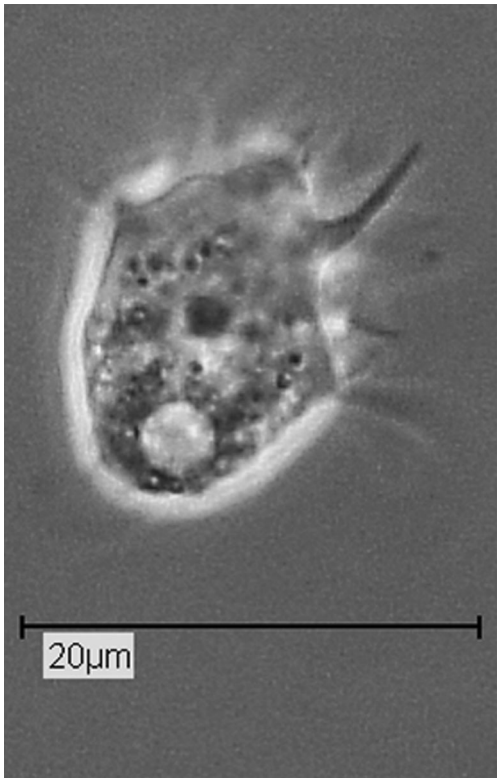

Fig. 2 Trophozoite of strain KaBo using light microscopy (phase contrast). *Scale bar* represents 20 μm

The evolutionary history was inferred using the neighbour-joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length=0.91734959 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 26 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 535 positions in the final data set. Evolutionary analyses were conducted in MEGA 6 (Tamura et al. 2013). The sequence of our strain KaBo is highlighted. Reference sequences are characterised by accession number, species and genotype.

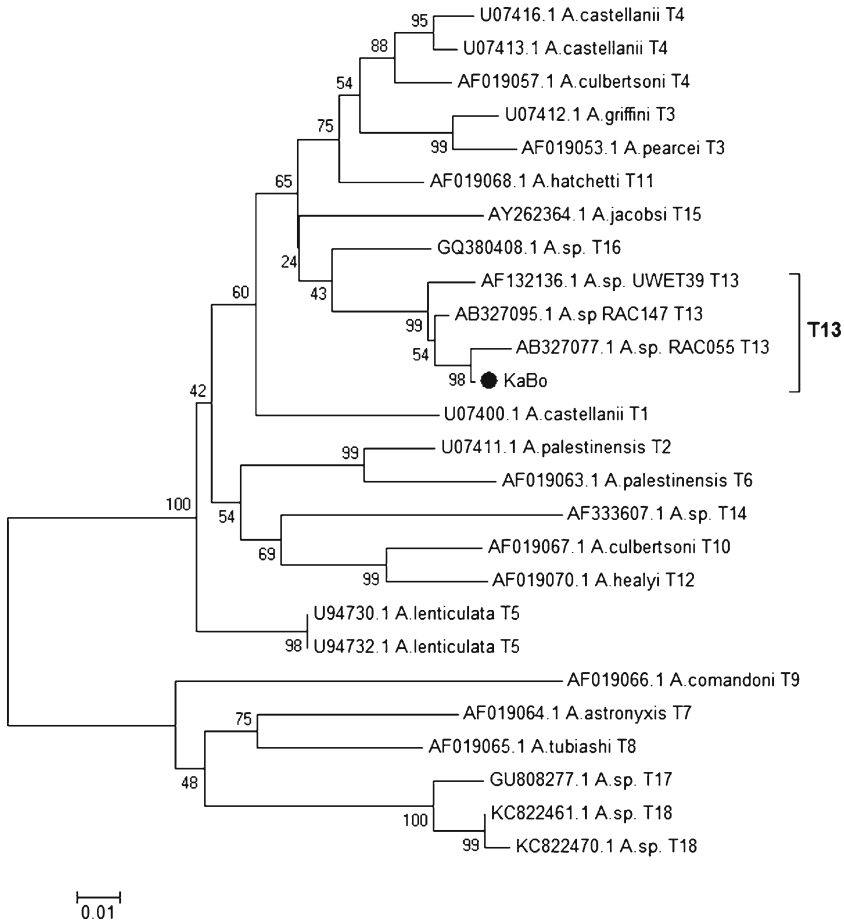

Fig. 3 Phylogenetical relationship of strain KaBo with 25 reference sequences of *Acanthamoeba* genotypes T1–T18

Discussion

Acanthamoebae gain most attention because of their pathogenicity for humans. Jager and Stamm (1972) and Nagington et al. (1974) were the first to identify *Acanthamoeba* as the agent of brain abscesses and eye infections. After these initial findings, the efforts to explore these eukaryotic single-celled organisms have rapidly increased. Since then, results of numerous studies, focussing on the pathogenicity, epidemiology and taxonomy of the disease-causing *Acanthamoeba* strains, have been published (Moore et al. 1985, 1987; Bacon et al. 1993; Schaumberg et al. 1998; Walochnik et al. 2000; Marciano-Cabral and Cabral 2003; Lorenzo-Morales et al. 2005; Clarke and Niederkorn 2006; Ertabaklar et al. 2007; Scheid et al. 2008; Xuan et al. 2008; Nagyová et al. 2010; Magnet et al. 2012; Risler et al. 2013).

The traditional morphological classification to determine an *Acanthamoeba* species based primarily on cyst shape and size became inconsistent in recent years. Hereafter, the discrepancy of the morphological approach from the molecular biological findings led to a revision of species classification, which started with the analysis of parts of the 18S rRNA gene (Johnson et al. 1990). Gast et al. (1996) followed this strategy and identified the first four *Acanthamoeba* genotypes with statistical evaluations. Due to this approach 18 genotypes are known today, which have been labelled as T1–T18. Considering the fact that a classified *Acanthamoeba* species according to Pussard and Pons (1977) or Page (1988) did not always match with the determined genotype, the modern classification scheme is now leaving the traditional classification scheme. Acanthamoebae are therefore not classified to the species level, but to one of the 18 genotypes. This is reasonable, since Stothard et al. had already shown in 1998 that isolates with the same genotype were classified as different species and that several isolates with the same morphological species description belonged to several genotypes.

Thus, we limited our focus only to the morphological group for the *Acanthamoeba* sp. strain KaBo and did not try to name a species. It is confirmed that Acanthamoebae of morphological group II are usually the most frequently isolated free-living amoebae from environmental and clinical samples (Stothard et al. 1998; Walochnik et al. 2000; Booton et al. 2005; Khan 2006).

Up to now, *Acanthamoeba* strains of genotypes T1, T2, T3, T4, T5, T6, T10, T11, T12, T15 and T18 have been classified as human pathogenic (Gast et al. 1996; Stothard et al. 1998; Horn et al. 1999; Gast 2001; Hewett et al. 2003; Maghsood et al. 2005; Corsaro and Venditti 2010; Nagyová et al. 2010;

Nuprasert et al. 2010; Siddiqui and Khan 2012; Qvarnstrom et al. 2013; Risler et al. 2013). In this study, comparison of a substantial part of the 18S rDNA gene confirmed that the *Acanthamoeba* strain KaBo is closely related to RAC147, RAC055 and UWET39 and, therefore, to genotype T13. Furthermore, the strain KaBo is distinctly separated from members of the other existing sequence types, with high bootstrap support (Fig. 3) and low pairwise sequence identities. In consequence, the *Acanthamoeba* keratitis-causing isolate KaBo expands the number of pathogenic *Acanthamoeba* genotypes to 12. To our knowledge, this is the first report of an AK caused by a genotype T13 isolate. The acanthamoebae were demonstrated to be the etiological agents of the keratitis, while the targeted therapy led to a subjective and objective improvement in health of the keratitis patient. Hitherto, genotype T13 was isolated exclusively from environmental samples and healthy persons, but not from infected persons, which might be attributed to the small number of T13 isolates (Horn et al. 1999; Booton et al. 2005; Edagawa et al. 2009; Siddiqui and Khan 2012; Maciver et al. 2013).

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