

## Discrimination between Clinically Relevant and Nonrelevant *Acanthamoeba* Strains Isolated from Contact Lens-Wearing Keratitis Patients in Austria

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**Eighteen cases of *Acanthamoeba*-associated keratitis among contact lens wearers seen at the Department of Ophthalmology, Karl-Franzens-University, Graz, Austria, between 1996 and 1999 are reviewed. The amoebae were proven to be the causative agents in three patients. The aim of our study was to discriminate between clinically relevant and nonrelevant isolates and to assess the relatedness of the isolates to published strains. Altogether, 20 strains of free-living amoebae, including 15 *Acanthamoeba* strains, 3 *Vahlkampfia* strains, and 2 *Hartmannella* strains, were isolated from clinical specimens. The virulent *Acanthamoeba* strains were identified as *A. polyphaga* and two strains of *A. hatchetti*. To our knowledge this is the first determination of keratitis-causing *Acanthamoeba* strains in Austria. Clinically relevant isolates differed markedly from nonrelevant isolates with respect to their physiological properties. 18S ribosomal DNA sequence types were determined for the three physiologically most-divergent strains including one of the keratitis-causing strains. This highly virulent strain exhibited sequence type T6, a sequence type not previously associated with keratitis. Sequence data indicate that *Acanthamoeba* strains causing keratitis as well as nonpathogenic strains of *Acanthamoeba* in Austria are most closely related to published strains from other parts of the world. Moreover, the results of our study support the assumption that pathogenicity in *Acanthamoeba* is a distinct capability of certain strains and not dependent on appropriate conditions for the establishment of an infection.**

Within the past few years free-living amoebae of the genus *Acanthamoeba* have gained increasing clinical relevance mainly as causative agents of a very often seriously progressing keratitis. The first ocular infections with acanthamoebae were diagnosed in 1974 (21). Since then the occurrence of keratitis due to *Acanthamoeba* (*Acanthamoeba* keratitis) has been escalating in correlation with the increasing number of contact lens wearers. Contaminated contact lens care systems usually are the first step in *Acanthamoeba* keratitis pathogenesis. The most prevalent risk factors are contact lens wear, poor hygiene, and a compromised corneal barrier. Users of extended-wear lenses are at special risk. Nevertheless, about 10 to 15% of cases of *Acanthamoeba* keratitis occur in persons who do not wear contact lenses (15).

The prognosis for *Acanthamoeba* keratitis, when the disease is diagnosed at an early stage and treated adequately, is rather good, but fast and reliable diagnosis is of crucial importance. Clinical signs and symptoms of *Acanthamoeba* keratitis are easily confused with fungal or viral keratitis. Initial improvement or stabilization in response to topical antibacterial, antiviral, antifungal, or corticosteroid therapy can occur, altering the clinical picture and thus complicating diagnosis. Clinical diagnosis should be based on the presence of keratitis with severe pain and photophobia, stromal infiltrates, radial keratoneuritis, and sometimes pseudodendritiform epithelial lesions. Cysts or trophozoites, found in corneal scrapings, on contact lenses, and inside of lens storage cases, are confirmatory. Agar

culture is the mainstay for laboratory detection of *Acanthamoeba* (12).

Various *Acanthamoeba* species have been reported to be able to cause keratitis: *A. castellanii*, *A. polyphaga*, *A. hatchetti*, *A. culbertsoni*, *A. rhyodes*, *A. lugdunensis*, *A. quina*, and *A. griffini* (26). Although isolates can easily be recognized as belonging to the genus *Acanthamoeba* by their polygonal cysts, accurate species determination is still problematic. An important step forward in the differentiation of acanthamoebae was the division of the genus into three morphological groups by Pussard and Pons (24). Since then a number of attempts to enable a more precise identification have been made. A very promising method for identification and phylogenetic studies is the analysis of the 18S rRNA gene sequence. Recently Stothard et al. (27) identified 12 *Acanthamoeba* sequence types, the vast majority of keratitis-causing strains belonging to sequence type T4. However, a final system does not yet exist, and, moreover, representatives from same species differ with respect to their pathogenicities.

We report on 18 cases of *Acanthamoeba*-associated keratitis seen at the Department of Ophthalmology, Karl-Franzens-University, Graz, Austria, between 1996 and 1999; in three cases the amoebae were the disease-causing agents. The aim of our study was to discriminate between clinically relevant and nonrelevant isolates and to assess the relatedness of our isolates to published strains by 18S ribosomal DNA (rDNA) sequence similarities.

### MATERIALS AND METHODS

**Patients.** Clinical specimens of keratitis patients presenting at the Department of Ophthalmology were on a routine basis investigated for *Acanthamoeba* spp. During 1996 to 1999 specimens from 18 keratitis patients, 10 women (55.6%) and 8 men (44.4%), yielded *Acanthamoeba*. The definitive diagnosis of *Acanthamoeba* keratitis on the basis of typical clinical signs, no response to antibacterial or

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TABLE 1. Clinical data of patients with *Acanthamoeba*-associated keratitis<sup>a</sup>

Patient code	Sex <sup>b</sup>	Age (yr)	Contact lens type	Clinical signs
1OAP	F	37	Soft	Keratoconjunctivitis RE
<b>2HAP</b>	<b>F</b>	<b>15</b>	<b>Soft</b>	<b>Keratitis LE</b>
3PYP	M	20	RE, soft; LE, rigid gas permeable	Keratitis LE
4REP	M	29	Soft	Keratoconjunctivitis LE
5STP	F	25	Soft	Keratitis denticratis RE
6DOP	M	23	Soft	Keratitis
7TOP	F	54	Soft	Keratoconjunctivitis
8PRP	M	15	Soft	Keratitis LE
9GUP	F	26	Soft	Corneal infiltration RE
10DRP	F	18	Soft	Keratitis superficialis RE
<b>11DSP</b>	<b>M</b>	<b>41</b>	<b>RE, soft; LE, rigid gas permeable</b>	<b>Keratitis superficialis LE</b>
12JOP	M	43	Rigid gas permeable	Ulcer corneae LE
13PTP	F	43	Soft	Keratitis superficialis RE
14SRP	M	26	Soft	Keratitis RE
<b>15SOP</b>	<b>M</b>	<b>39</b>	<b>Rigid gas permeable</b>	<b>Keratitis RE</b>
16KVP	F	20	Soft	Keratoconjunctivitis LE
17SHP	F	19	Rigid gas permeable	Corneal infiltrates LE
18MAP	F	30	Soft	Keratitis RE

<sup>a</sup> Cases in which acanthamoebae were confirmed as causative agents are boldface. In all other cases, acanthamoebae were detected only in lens storage cases. RE, right eye; LE, left eye.

<sup>b</sup> M, male; F, female.

antiviral treatment, and detection of acanthamoebae in the corneal epithelium was verified for three patients (16.7%).

All 18 patients were contact lens wearers, and for all patients cysts were detectable in contact lens cases by lactophenol cotton blue staining (Table 1). Thirteen patients (72.2%) wore soft contact lenses, 3 (16.7%) wore rigid gas-permeable lenses, and 2 (11.1%) wore both types. Patients' ages ranged from 15 to 54 years (mean, 29 years). The most prevalent clinical signs we observed were chronic keratitis and keratoconjunctivitis; clinical signs for *Acanthamoeba* keratitis, namely, presence of keratitis with severe pain and photophobia, stromal infiltrates, radial keratoneuritis, and sometimes pseudodendritic epithelial lesions, were seen in seven patients (38.9%). In three of these patients the definitive diagnosis of *Acanthamoeba* keratitis was verified. Of the 18 patients, only these 3 showed no response to antibacterial or antiviral treatment. Moreover, these were the only three cases with acanthamoebae detectable in corneal scrapings.

The *Acanthamoeba* keratitis patients were a 15-year-old female wearer of soft daily-wear lenses (2HAP), a 41-year-old male with rigid gas-permeable lenses in the afflicted left eye (11DSP), and a 39-year-old male wearer of rigid gas-permeable lenses in both eyes (15SOP). In two of these cases the initial diagnosis had been herpes simplex virus keratitis, which did not improve despite antiviral therapy. The time between clinical onset and correct diagnosis ranged from 1 to 5 weeks. Antiamoebic treatment consisted of local application of propanidine isethionate (Brolene eyedrops), hexamidine isethionate (Desomedine eyedrops), and bacitracin plus neomycin (Nebacetin ointment). No perforating keratoplasty was necessary. The first patient recovered, with a best corrected visual acuity of 20/20; the visual acuity of the second patient was 20/60 in the affected eye; the third patient still had corneal erosions, maybe caused by the toxicity of the medication.

**Isolation and culture.** Contact lenses, corneal scrapings, and swabs from contact lens cases were transferred to nonnutrient agar plates covered with 100 µl of a 24-h-old culture of *Escherichia coli* in brain heart infusion medium. The plates were sealed and incubated at 30°C for 14 days and examined every 48 h for amoebal growth. Positive cultures were diluted in order to eliminate coexisting ciliates, flagellates, bacteria, and fungi by harvesting amoebae at a noncontaminated site of the plate with a sterile cotton-tipped applicator and transferring the amoebae to a fresh plate. All isolates were cloned with the use of a micromanipulator and incubated at various temperatures (30, 34, 37, and 42°C). They were examined daily by phase-contrast microscopy, and amoebal growth and temperature tolerances were recorded.

**Identification and characterization.** Amoebae were identified as belonging to one of the cyst morphological groups (*Acanthamoeba* sp. groups I to III) established by Pussard and Pons (24), and species determination was performed according to the identification key of Page (23). Differentiation was achieved mainly on the basis of cyst size, number of opercula, and temperature tolerance.

Moreover, all isolates were examined for their cytopathic effects to a human cell line (HEp-2). Amoebae were axenized by harvesting cysts from the plate cultures, incubating them in 3% HCl overnight in order to eliminate the bacteria,

and transferring the amoebae into liquid culture. As a liquid medium we used proteose peptone-yeast extract-glucose (23). We cultured the amoebae in 150-cm<sup>2</sup> tissue culture flasks (Corning Costar, Bodenheim, Germany) at 30°C. Trophozoites were harvested from the axenic cultures by centrifugation (500 × g for 7 min) and transferred onto a monolayer of HEp-2 cells in an amoeba/cell ratio of 1/10. The amoebae were designated as highly cytopathic when the monolayer was completely lysed after 24 to 48 h.

**Molecular biology analysis.** 18S rDNA sequence analysis was performed for three isolates most divergent with respect to their physiological properties (strains 4RE, 9GU, and 11DS) in order to determine the differences of these strains from and their relatedness to the published strains from other parts of the world. The 4RE and the 9GU strains were derived from contact lens cases of non-*Acanthamoeba* keratitis patients, while the 11DS strain was isolated from the corneal scraping of *Acanthamoeba* keratitis patient 11DSP.

For molecular biology investigations amoebae (~10<sup>6</sup> cells) were harvested from actively growing axenic cultures by centrifugation at 500 × g for 7 min. Whole-cell DNA was isolated by a modified UNSET procedure (14). Briefly, the pellet was resuspended in 500 µl of UNSET lysis buffer, overlaid with 500 µl of phenol-chloroform-isoamylalcohol (PCI), and shaken gently for 5 h. DNA was extracted by multiple PCI extraction, precipitated in alcohol, air dried and resuspended in 30 µl of sterile double-distilled water. The 18S rRNA gene was amplified using the SSU1 and SSU2 primers (9), complementary to the 5' and 3' ends of the gene, respectively, and a standard amplification program (30 cycles; 95°C for 1 min, 50°C for 2 min, 72°C for 3 min). Amplification of the 18S rRNA gene was visualized with ethidium bromide in an agarose gel electrophoresis. The amplified gene was sequenced stepwise by direct sequencing from the PCR product using the Thermo Sequenase II sequencing kit (Amersham Pharmacia Biotech GmbH, Vienna, Austria) and subsequent construction of complementary internal primers. Sequences were obtained from both strands. Sequencing was carried out in a 310 ABI PRISM automated sequencer (PE Applied Biosystems, Langen, Germany).

Sequence data were processed with the GeneDoc (22) sequence editor, and sequences were compared to the ones of published strains using a BLAST search (2). ClustalX (29) was used for pairwise alignment and calculation of the percentage of sequence dissimilarity.

**Nucleotide sequence accession numbers.** Sequence data reported in this paper were deposited in GenBank and are available under the following reference numbers: strain 4RE, AF251937; strain 9GU, AF251938; strain 11DS, AF251939.

TABLE 2. Parasitological data for patients with *Acanthamoeba*-associated keratitis<sup>a</sup>

Patient code	LPCB <sup>b</sup> stain	Culture	Morphological identification	Strain code	Temperature tolerance (°C)	Cytopathic effect <sup>c</sup>
1OAP	P	P	<i>Vahlkampfia</i> sp.	1OA	30	—
<b>2HAP</b>	<b>P</b>	<b>P</b>	<b><i>A. rhyssodes</i></b>	<b>2HAA</b>	<b>37</b>	<b>—</b>
			<b><i>A. hatchetti</i></b>	<b>2HAB</b>	<b>42</b>	<b>+++</b>
3PYP	P	P	<i>V. debilis</i>	3PY	30	—
4REP	P	P	<i>A. hatchetti</i>	4RE	37	—
5STP	P	P	<i>A. hatchetti</i>	5ST	37	—
6DOP	P	P	<i>A. rhyssodes</i>	6DOA	34	—
			<i>Hartmannella</i> sp.	6DOB	30	—
7TOP	P	P	<i>A. hatchetti</i>	7TOA	37	—
			<i>V. enterica</i>	7TOB	30	—
8PRP	P	P	<i>A. triangularis</i>	8PRA	30	—
			<i>H. vermiformis</i>	8PRB	30	—
9GUP	P	P	<i>A. polyphaga</i>	9GU	37	++
10DRP	P	P	<i>A. triangularis</i>	10DR	30	—
<b>11DSP</b>	<b>P</b>	<b>P</b>	<b><i>A. hatchetti</i></b>	<b>11DS</b>	<b>42</b>	<b>+++</b>
12JOP	P	N	No growth	12JO	ND <sup>e</sup>	ND
13PTP	P	N	No growth	13PT	ND	ND
14SRP	P	P	<i>A. hatchetti</i>	14SR	37	—
<b>15SOP</b>	<b>P</b>	<b>P</b>	<b><i>A. polyphaga</i></b>	<b>15SO</b>	<b>37</b>	<b>++</b>
16KVP	P	P	<i>A. polyphaga</i>	16KV	37	++
17SHP	P	P	<i>A. rhyssodes</i>	17SH	34	—
18MAP	P	P	<i>A. lenticulata</i>	18MA	37	—

<sup>a</sup> Cases in which acanthamoebae were confirmed as causative agents are boldface. In all other cases, acanthamoebae were detected only in lens storage cases. P, positive; N, negative.

<sup>b</sup> LPCB, lactophenol cotton blue.

<sup>c</sup> ND, no data.

<sup>d</sup> The number of plus signs is a measure of the cytopathic effect. —, no cytopathic effect.

TABLE 3. Sequence identity of isolates to published strains

Isolate	% Identity	Strain	Habitat	GenBank accession no.	Sequence type	Morphological group
4RE	99.3	<i>A. hatchetti</i> BH-2	Brackish water, United States	AF019068	T11	II
9GU	98.6	<i>A. castellanii</i> Castellani	Yeast culture, United Kingdom	U07413	T4	II
11DS	97.7	<i>A. palestiniensis</i> 2802	Swimming pool, France	AF019063	T6	III

## RESULTS

**Identification and characterization of isolates.** In all, 20 strains of free-living amoebae including 15 *Acanthamoeba* strains, 3 *Vahlkampfia* strains, and 2 *Hartmannella* strains were isolated from clinical specimens (Table 2). Four specimens revealed two variant strains of free-living amoebae each (2HAP, 6DOP, 7TOP, and 8PRP), and in two cases culture was unsuccessful (12JOP and 13PTP). Fourteen of the 15 *Acanthamoeba* isolates were identified as belonging to *Acanthamoeba* sp. group II. However, several strains exhibited rather varied cyst morphologies with respect to size and number of opercula although they were derived from a clone. These strains were classified according to the average cyst morphology. One strain (18MA) was designated *A. lenticulata* (morphological group III), the cysts being rather small and round. No isolate exhibited a group I morphology. In all, *A. hatchetti* was identified six times, *A. rhyssodes* and *A. polyphaga* were each identified three times, *A. triangularis* was identified twice, and *A. lenticulata* was identified once.

*Acanthamoeba* spp. had been proven to be of clinical relevance in three cases (2HAP, 11DSP, and 15SOP). All of these isolates showed an *Acanthamoeba* group II cyst morphology. In one case (2HAP) two different strains of *Acanthamoeba*, *A. rhyssodes* and *A. hatchetti*, were detected. The other keratitis-causing isolates were identified as *A. hatchetti* (11DS) and *A. polyphaga* (15SO).

Temperature tolerance tests revealed that all 20 isolates of free-living amoebae grew at 30°C; 13 of them also grew at 34°C, 11 strains grew at 37°C, and 2 strains grew at 42°C. All of the thermophilic strains were among the *Acanthamoeba* isolates. The two *Vahlkampfia* strains and the *Hartmannella* strain showed no growth above 30°C. Five isolates (2HAB, 9GU, 11DS, 15SO, and 16KV) showed cytopathic effects against monolayers of HEp-2 cells. Strains 2HAB, 11DS, and 15SO had been isolated from *Acanthamoeba* keratitis patients, yet two strains (9GU and 16KV) isolated from patients without *Acanthamoeba* keratitis also exhibited moderate cytopathic effects (monolayer lysis after 3 to 4 days). From the two different *Acanthamoeba* strains isolated from patient 2HAP only one (2HAB) showed pathogenicity-related physiological characteristics. Eventually only this strain was responsible for the infection, while the other strain (2HAA), with no pathogenicity-associated characteristics, was only a concomitant.

The clinically relevant strains 2HAB, 11DS, and 15SO not only showed high-temperature tolerance and high cytopathic effects but also generally exhibited far higher growth rates than the other isolates.

**Molecular biology analysis.** All strains investigated revealed more than 97% sequence identity to classified published strains (Table 3) and could thus be assigned to one of the 12 published sequence types, as sequence types differ from one another by at least 5% (27). Nonpathogenic strain 4RE (GenBank accession no. AF251937), having been identified as *A. hatchetti*, displayed sequence type T11, with 99.3% identity to the BH-2 *A. hatchetti* strain isolated in brackish water in the United States (GenBank accession no. AF019068). The 9GU strain (GenBank accession no. AF251938), which did not cause

disease but which is pathogenic to tissue culture, showed 98.6% identity to the Castellani strain of *A. castellanii* (GenBank accession no. U07413), which has sequence type T4. This strain of *A. castellanii* has been isolated from a yeast culture in the United Kingdom and is the type strain for the species *A. castellanii*. We thus reclassified our isolate, initially classified as *A. polyphaga*, as *A. castellanii*. Most of the published *A. polyphaga* isolates show sequence type T4, as do most of the *A. castellanii* isolates. Stothard et al. (27) proposed to reclassify all sequence type T4 isolates as *A. castellanii*. Strain 11DS (GenBank accession no. AF251939), isolated from the contact lens case of a patient with serious *Acanthamoeba* keratitis, showed an 18S rDNA sequence with highest identity (97.7%) to the 2802 strain of *A. palestiniensis* with sequence type T6, isolated in a swimming pool in France (GenBank accession no. AF019063). However, the 11DS strain exhibits a typical *A. hatchetti* morphology, with most of the cysts being rectangular (Fig. 1), and the isolate showed the ability to grow at 42°C, while *A. palestiniensis* is described as showing no growth even at 37°C (23). Moreover, because *A. hatchetti* and *A. palestiniensis* are described as polyphyletic (27), we prefer not to reclassify this isolate.

The sequences of our strains clearly diverged from one another, all exhibiting different sequence types, namely, T4, T6, and T11. The 4RE and the 9GU strains were more closely related to one another (5.57% dissimilarity) than either was to the 11DS strain. The 4RE and 9GU strains showed 9.68 and 9.40% sequence dissimilarities, respectively, to the 11DS strain.

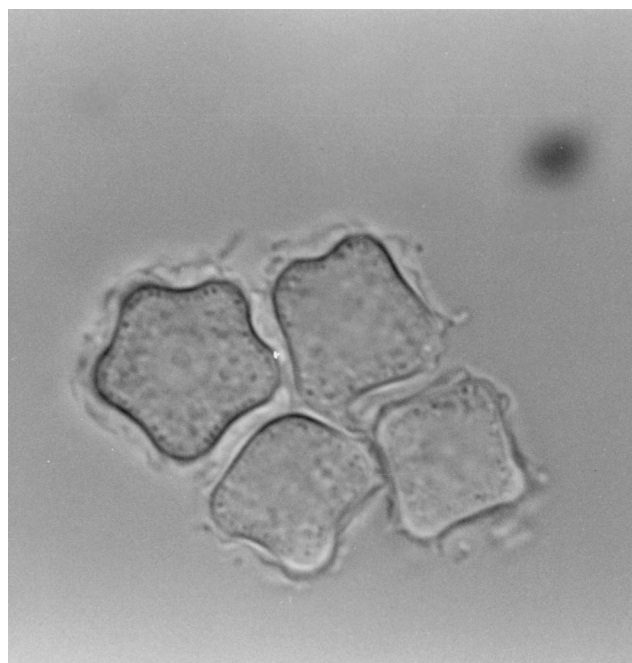


FIG. 1. Cysts of the 11DS isolate with typical *A. hatchetti* morphology, as shown by phase-contrast microscopy. Magnification,  $\times 1,000$ .

## DISCUSSION

**Identification and characterization of isolates.** Altogether 15 strains of *Acanthamoeba*, 3 strains of *Vahlkampfia*, and 2 strains of *Hartmannella* were isolated. During the last few years it has become apparent that *Vahlkampfia* and *Naegleria* (11) as well as *Hartmannella* (1, 16) can cause keratitis or at least be associated with keratitis. However, neither the *Hartmannella* strain nor the *Vahlkampfia* strains were of clinical relevancy in our study.

In two cases the amoebae could not be grown in vitro. This may partially be due to the fact that patients had already been treated for bacteria, and antibacterial and antifungal treatment is at least partly effective against free-living amoebae. On the other hand, contact lens disinfectants, even if not used properly, compromise amoebal viability. Moreover, amoebae penetrate the cornea during the course of infection, protruding up to Descemet's membrane. It might therefore in some cases be impossible to isolate viable amoebae by scraping. Several studies report on unsuccessful attempts to culture amoebae from clinical specimens (15, 26).

In general most of the acanthamoebae isolated were identified as belonging to *Acanthamoeba* sp. group II, which also is reported to be the most prevalent group of these microorganisms (23). In a former study we could demonstrate that in the area of Vienna *Naegleria* seems to be the predominant genus in environmental habitats, whereas probing of shower heads and tap water mainly revealed amoebae of the genera *Acanthamoeba* and *Hartmannella* (our unpublished data). This supports the assumption that, in *Acanthamoeba* keratitis, infection is primarily acquired via contaminated contact lenses and lens cases, the amoebae deriving mainly from tap water, rather than by outdoor swimming. Domestic tap water as source of *Acanthamoeba* sp. in *Acanthamoeba* keratitis was described for the first time in 1990 (17). Interestingly two of the *Acanthamoeba* keratitis patients ( $n = 3$ ) discussed here wore rigid gas-permeable contact lenses in the afflicted eyes. Usually wearers of soft lenses are more likely to acquire *Acanthamoeba* keratitis, as the hydrophilic material seems to support attachment and survival of cysts. However, several cases of *Acanthamoeba* keratitis in wearers of rigid gas-permeable contact lenses have been reported (4, 25). A case of *Acanthamoeba* keratitis occurring in a wearer of daily-disposable contact lenses has also been documented recently (31). We did not observe any correlation to age or sex, and in none of the three patients did surgical treatment become necessary. In a study from the United Kingdom severe visual loss was seen in about 15% of the patients (25).

Morphological determination was rather difficult in some cases as cysts, although all deriving from one clone, had varied morphologies, and generally intraspecific polymorphism is rather common among acanthamoebae (3). The four *Acanthamoeba* strains isolated from the contact lens cases of the three *Acanthamoeba* keratitis patients were identified by cyst morphology as *A. rhyssodes*, *A. polyphaga*, and two strains of *A. hatchetti*. All of these species are known to cause keratitis (26). To our knowledge this is the first determination of keratitis-causing strains in Austria. In 1989, Huber-Spitz et al. described a case of *Acanthamoeba* keratitis in a 37-year-old woman in Austria (13). They detected cysts of *Acanthamoeba* in the corneal epithelium, but the amoeba was neither isolated nor identified to the species level.

**Clinical relevance of strains.** Remarkably, in the majority of keratitis cases we investigated, the acanthamoebae were of no clinical relevance. Although *Acanthamoeba* keratitis has become of increasing importance within the last 10 years, corre-

lating to the greater number of contact lens wearers, it is still a rare disease. The annualized incidence of *Acanthamoeba* keratitis is estimated as 0.14 per 100,000 individuals (25).

Also other studies revealed that the majority of people do not develop a keratitis in spite of coming into contact with acanthamoebae; the prevalence of acanthamoebae and other free-living amoebae in asymptomatic contact lens wearers has been reported frequently (8, 18). Even from the nasal mucosae of healthy individuals different strains of *Acanthamoeba* spp. could be isolated (20). Apart from such widely accepted risk factors as extended contact lens wear and microlesions in the cornea, certainly the immune status of the patient may play a fundamental role in the course of infection and may result in enhanced susceptibility to developing an *Acanthamoeba* keratitis. It was shown that nearly 50% of healthy individuals carry antibodies against *Acanthamoeba*, probably due to the ubiquity of this microorganism (28).

Moreover, apart from differences in predisposition of patients, one can assume that amoeba strains vary in pathogenicity. Mazur et al. demonstrated in an animal model a clear correlation between the occurrence of eye infections and the degree of virulence of the strains after installation into the brain (19), which suggests that pathogenicity is not so much dependent on environmental conditions but rather represents a distinct characteristic of certain strains. Nevertheless the initial infective dose is certainly of considerable importance. The factors which prime amoebae for pathogenicity are poorly understood. Temperature tolerance (10) and cell culture pathogenicity (3, 5, 6) seem to be related to pathogenicity potential. Temperature tolerance is widely accepted to be a prerequisite for pathogenicity in granulomatous amoebic encephalitis caused by *Acanthamoeba*, as the human body temperature is 37°C. However, the human eye has a mean temperature of only around 34°C. Yet data presented here indicate that clinically relevant strains not only show higher temperature tolerances but also generally yield far higher growth rates, which certainly is of major importance for the establishment of infection. Temperature tolerance among keratitis-causing *Acanthamoeba* strains is rather conspicuous (3, 7). Moreover, the keratitis-causing acanthamoebae described here were highly cytopathic to HEp-2 cells, producing complete destruction of the monolayer in 1 to 2 days. Altogether, there seems to be a correlation between clinical relevance and pathogenicity-related physiological characteristics.

The occurrence of free-living amoebae of nonpathogenic strains in contact lens cases, however, is still of medical interest, as they can harbor bacteria inside their cysts, protecting them from disinfectants, and thus function as vectors. In a recent study we showed that viable *Pseudomonas aeruginosa*, one of the major ocular pathogens, can survive in and be reisolated from cysts of *Acanthamoeba* (30).

**Molecular biology analysis.** Interestingly, the three investigated strains showing the most-diverse physiological capacities also differed with respect to their 18S rDNA sequences, exhibiting three different sequence types. The two strains with no clinical relevancy, both classified as group II acanthamoebae, showed sequence type T4 (strain 9GU) and sequence type T11 (strain 4RE). Sequence types T3, T4, and T11 are most closely related, and all represent morphological group II (27). T4 is reported to be the sequence type isolated most frequently. Moreover, the majority of keratitis-causing *Acanthamoeba* strains are reported to belong to sequence type T4 (27).

The keratitis-causing and highly cytopathic strain 11DS, identified as *A. hatchetti*, morphological group II, exhibited sequence type T6. Sequence type T6 is represented by a single strain, which had been identified morphologically as *A. pales-*

*tiniensis* belonging to morphological group III. Stothard et al. also report on a strain presumed to be group II but with a group III sequence. The authors assume that *A. palestinensis* and *A. hatchetti* are polyphyletic (27). However, a final system does not yet exist, and most probably diversification will still be necessary. T6 isolates have not previously been reported to cause keratitis. Also no keratitis-causing strain is known to exhibit sequence type T2, which is the sequence type most closely related to sequence type T6.

Altogether the results of our study support the assumption that pathogenicity in *Acanthamoeba* seems to be a distinct capability of certain strains and not so much dependent on appropriate conditions for the establishment of an infection. Moreover, data presented here indicate that *Acanthamoeba* keratitis-causing strains as well as nonpathogenic strains of *Acanthamoeba* in Austria are most closely related to published strains from other parts of the world.

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