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Identification and Distribution of *Acanthamoeba* Species Genotypes Associated with Nonkeratitis Infections

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***Acanthamoeba* is a free-living protozoan genus found in a wide variety of natural habitats, including water, soil, and air. Pathogenic isolates of *Acanthamoeba* are medically relevant as the causative agent of sight-threatening *Acanthamoeba* keratitis (AK), serious infections of other organs, and fatal granulomatous amebic encephalitis. Previous work employing DNA sequences of nuclear and mitochondrial small-subunit rRNA genes (SSU rRNA genes) determined the genotypic diversity of *Acanthamoeba* and found that many named species of *Acanthamoeba* are associated with particular genotypes. These studies also concluded that nearly all AK infections result from a single molecular genotype: T4. Here, we asked whether *Acanthamoeba* clinical isolates from non-AK infections are also associated with particular genotypes. DNA sequence determination of nuclear SSU rRNA genes was employed for genotypic identification of 29 isolates of *Acanthamoeba* from non-AK infections. Sequence analysis demonstrates that T4 is the predominant genotype in non-AK infections, including those in brain, cerebrospinal fluid, nasal passages, skin, and lung. Rare genotypes (T1, T10, and T12) have been isolated from brain infections. We conclude that genotype T4 is the primary genotype in non-AK *Acanthamoeba* infections, as was the case in AK infections. However, the genotypes that were isolated from brains have not been observed in environmental isolates of *Acanthamoeba*, and their natural ecological niche is unknown.**

The genus *Acanthamoeba* is a group of nearly 25 named species that has a worldwide distribution. Members of *Acanthamoeba* are naked (i.e., lacking tests, walls, or tecta with openings), free-living amebae that inhabit a wide range of natural ecological niches, including fresh and brackish water, beach sand, soil, air, etc. (4, 13, 16, 17). *Acanthamoeba* also has been isolated from humans and animals (16, 18). In addition to its natural distribution, *Acanthamoeba* can be opportunistically pathogenic, being identified as the causative agent of a painful and sight-threatening infection of the cornea, *Acanthamoeba* keratitis (AK) (22). These infections can occur in otherwise healthy, nonimmunocompromised individuals. In developed countries, AK infections are usually associated with contact lens wear. Improper sterilization procedures have been identified as the cause of a large percentage of such AK infections (2, 22, 25). In developing nations, AK infections are not generally associated with contact lens wear; by contrast, most cases are the result of ocular trauma (6, 23).

Acanthamoeba is also responsible for life threatening infections in patients with immunodeficiency disease. These include documented infections of the skin, nasal passages, lung, and brain (16, 17, 18, 19). In the brain, *Acanthamoeba* causes a severe encephalitis termed granulomatous amebic encephalitis, which is nearly always fatal (18). Variations in the pathogenicity of different *Acanthamoeba* strains have been recognized in laboratory studies, but the relevance of these results to human disease is unclear (7).

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Traditional taxonomy of *Acanthamoeba* has used morphological characteristics such as cyst morphology and trophozoite size and shape as classification characters (20). Species of *Acanthamoeba* are categorized into three morphological groups based largely on the cyst morphology of the species. Molecular analyses, including several of our own studies using nuclear and mitochondrial small-subunit (SSU) rRNA genes, support the morphological group structure of the genus (10, 15, 24). Our work focuses on the SSU rRNA genes of the nucleus (*Rns*) and the equivalent gene from the mitochondrial genome (*rns*) (15, 24). Data collected over the last decade now allow us to quickly analyze a clinical or environmental sample using molecular methods to determine and classify the *Acanthamoeba* sequence genotype (3, 4). Sequence similarities between isolates using these two genes are used to determine phylogenetic relationships between strains and to explore possible correlation with disease phenotypes. The molecular analyses thus far suggest that 15 or more genotypes exist, designated T1, T2, etc. (1, 9, 11, 12, 24, 27, 29). Characterization of genotypes is an active area of investigation, since it is dependent upon the statistical criteria employed to distinguish genotypes and the expanding number of analyzed isolates. Although the major morphological groups are supported by molecular analyses, a number of the named species have not been observed as unique monophyletic entities when examined by molecular methods.

In the present study, we examined *Acanthamoeba* isolates that were obtained from non-AK infections. The genotypes of these isolates were determined using nuclear rRNA (*Rns*) gene sequences.

MATERIALS AND METHODS

Isolates. Most of the 29 non-AK disease samples of *Acanthamoeba* examined in this study were isolated by G. S. Visvesvara at the Centers for Disease Control

TABLE 1. *Acanthamoeba* sp. sources and genotypic determinations[a]

Source	No. of isolates with *Acanthamoeba* genotype															
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15	Total
Environment	0	3	8	73	19	1	3	1	2	1	6	0	2[b]	7[c,d]	6	132
AK	0	0	2	83	0	1	0	0	0	0	2	0	0	0	0	88
Non-AK disease	3	0	0	23	0	0	0	0	0	2	0	1	0	0	0	29
Total	3	3	10	179	19	2	3	1	2	3	8	1	2	7	6	249
Morphological group	II	III	II	II	III	III	I	I	I	III	II	III	ND[e]	III	III	

[a] The 200 isolates included in this study include those previously examined (3, 4, 8, 9, 24, 14, 21, 27, 28, 29). The genotypes of the remainder ($n = 49$) have been determined for the current study.
[b] These two isolates were from a nasal swab and contact lens case and are treated here as environmental samples (27).
[c] Five of seven T14 isolates and five others (three T4 and two T11 isolates) were identified by ribotyping (9).
[d] Twelve isolates (three T4, two T11, and seven T14 isolates) from stool samples (9) are assumed to be environmental isolates of *Acanthamoeba.*
[e] ND, not done; isolates were not assigned to a morphological group.

and Prevention (Atlanta, GA). A few isolates represent samples obtained from the American Type Culture Collection (ATCC, Manassas, VA). Five of the non-AK isolates were previously genotyped, and genotyping of 24 isolates was accomplished in the current study (24, 27). The mitochondrial 16S rRNA genotypes of five of these 24 non-AK isolates were previously determined (15). Nuclear rRNA genotypes of new AK ($n = 18$) and environmental ($n = 7$) isolates were also determined in these analyses. The data from the 49 newly genotyped isolates were combined with the sequencing/genotyping data from 200 other *Acanthamoeba* sp. isolates that have been determined in our, and other, laboratories (1, 3, 9, 10 11, 14, 12, 21, 24, 26, 27). Most isolates were cultured axenically in optimal growth medium prior to DNA extraction (4). Following DNA extraction, PCR was used to amplify the nuclear *Rns* sequences. Nearly complete *Rns* was amplified using the primers CRN5 (5-TGGTTGATCCTGCCAGTAG) and SSU2-TRUN (5′-TGATCCCTCCGCAGGTTCAC-3′). Sequencing of *Rns* was done using conserved eukaryotic SSU rRNA gene primers previously used in our laboratory (4, 15, 21, 24). Amplification of a partial *Rns* region (ASA.S1) that contains a diagnostic fragment with respect to genotype was done using the primers JDP1 (5′-GGCCCAGATCGTTTACCGTGAA-3′) and JDP2 (5′-TCT CACAAGCTGCTAGGGGAGTCA-3′). Sequencing of ASA.S1 was done using the PCR primers and internal primers 892 (5′-CCAAGAATTTCACCTCTGA C-3′) and 892C (5′-GTCAGAGGTGAAATTCTTGG-3′). DNA sequencing of partial or nearly complete *Rns* was done with an ABI 310 automated fluorescent sequencing system using these primers and methods that have been used previously in our *Acanthamoeba* studies (4, 15, 21, 24). The sequences that were obtained were aligned with other *Acanthamoeba* sequences in our *Rns* database using the alignment program XESEE (5).

Nucleotide sequence accession number. Sequences obtained in this study have been deposited in GenBank under the accession numbers AY702019 to AY702030 and AY702982 to AY703018.

RESULTS

In this study we have compiled the *Acanthamoeba* genotype determinations based on *Rns* from 249 (49 from this study) *Acanthamoeba* isolates, including isolates obtained from the environment ($n = 132$), from patients diagnosed with AK ($n = 88$), and from patients diagnosed with non-AK infections ($n = 29$). The genotypic identification of the isolates compiled for this study is summarized and presented in Table 1. When isolates were examined as a single group, the large majority, whether environmental, AK, or non-AK, were identified as genotype T4. We believe genotype T4 should be referred to as the *A. castellanii* species complex until future higher-resolution analyses are sufficient to more accurately differentiate between the morphologically described species within this genotype. *Acanthamoeba* genotype T4 comprises nearly 72% of the total number of isolates. Three genotypes, T3, T4, and T11, which are phylogenetically very closely related make up 75% of the total number of samples. The second most common individual genotype was T5 (*Acanthamoeba lenticulata*), which contains 19 environmental isolates (7.6% of total isolates). The remaining genotypes are relatively rare, containing one to seven isolates (about three isolates/genotype for the rare genotypes).

Genotypic identification of pathogenic isolates of *Acanthamoeba* associated with non-AK infections shows that genotype T4, which is associated with the vast majority of AK infections, is also the dominant genotype in non-AK infections. While the dominance of genotype T4 is maintained in all of the three subcategories of *Acanthamoeba* isolates (environmental, AK, and non-AK), the frequency distribution of genotypes differs by the category. Overall, genotype T4 represents 72% (179/249) of the isolates of *Acanthamoeba* examined in this study (Table 1). However, T4 is more frequent in infections of the eye. When examined as a single group, the large majority of isolates, whether environmental, AK, or non-AK, were identified as genotype T4 (55.3%, 94.3% and 79.3%, respectively). It should be noted that there are many more isolates from AK infections than isolates derived from non-AK infections.

The specific aim of the present study was the determination of the genotypic distribution of the 24 new non-AK *Acanthamoeba* sp. isolates combined with five previously characterized strains. The results from *Rns* sequencing demonstrate that the 29 non-AK isolates belong to four *Acanthamoeba* genotypes. Twenty-three isolates were categorized as genotype T4, the most commonly observed genotype and the genotype predominantly associated with AK infections. Two of these isolates have previously been classified as *A. castellanii*. The remainder ($n = 21$) have not been classified to particular species using the morphological classification system and are all referred to as *Acanthamoeba* sp. No non-AK infections were found involving either genotype T3 or T11, the other genotypes that have been encountered in AK infections.

Six non-AK isolates were found to be genotypes other than T4. Three isolates were characterized as genotype T1, corresponding to an as-yet-unnamed species of *Acanthamoeba*. Two isolates from non-AK infections were identified as genotype T10, a genotype that is correlated with the named species *Acanthamoeba culbertsoni*. Lastly, a single non-AK isolate was found to be genotype T12, correlated with the named species *Acanthamoeba healyi*. Summarized genotyping results for the non-AK *Acanthamoeba* sp. isolates are presented in Table 2.

TABLE 2. Genotypic identification of non-AK disease isolates of *Acanthamoeba* spp.

OSU/CDC ID[a]	Tissue source	Genotype	Reference
Acanthamoeba sp. strain CDC V006	Brain	T1	15, 24
Acanthamoeba sp. strain OSU 03-022/CDC V329	Brain	T1	Current study
Acanthamoeba sp. strain CDC V327	Brain	T1	Current study
Acanthamoeba sp. strain CDC V017	Sinus	T4	Current study; 15
Acanthamoeba sp. strain CDC V168	Skin	T4	Current study; 15
Acanthamoeba sp. strain CDC V328	Brain	T4	Current study; 15
Acanthamoeba sp. strain CDC V382	Skin	T4	Current study; 15
Acanthamoeba sp. strain CDC V390	Skin	T4	Current study; 15
A. castellanii CDC V0180:1	Lung	T4	15, 24
A. castellanii 40AB	Necrotic tissue	T4	28
Acanthamoeba sp. strain OSU 03-009/CDC 12741:1	Lung	T4	Current study
Acanthamoeba sp. strain OSU 03-017/CDC V221	Skin	T4	Current study
Acanthamoeba sp. strain OSU 03-019/CDC V240	Skin	T4	Current study
Acanthamoeba sp. strain OSU 03-020/CDC V245	Skin	T4	Current study
Acanthamoeba sp. strain OSU 03-021/CDC V313	Nasal	T4	Current study
Acanthamoeba sp. strain OSU 03-025/CDC V398	Sinus	T4	Current study
Acanthamoeba sp. strain OSU 03-026/CDC V411	Brain	T4	Current study
Acanthamoeba sp. strain OSU 03-027/CDC V425	Skin	T4	Current study
Acanthamoeba sp. strain OSU 03-029/CDC V501	CSF	T4	Current study
Acanthamoeba sp. strain OSU 03-030/CDC V503	CSF	T4	Current study
Acanthamoeba sp. strain OSU 03-035/CDC V522	Skin	T4	Current study
Acanthamoeba sp. strain CDC V021	Skin	T4	Current study
Acanthamoeba sp. strain CDC VE67/H7	Brain	T4	Current study
Acanthamoeba sp. strain CDC VEPV 94-3	Brain	T4	Current study
Acanthamoeba sp. strain CDC V388	Skin	T4	Current study
Acanthamoeba sp. strain OSU 03-046[b]	Brain	T4	Current study
Acanthamoeba sp. strain OSU 03-023/CDC V369	Brain	T10	Current study
A. culbertsoni CDC V409	Brain	T10	15
A. healyi CDC V013	Brain	T12	15, 24

[a] Isolates without an Ohio State University (OSU) number were obtained prior to the development of the OSU identification system. CDC, Center for Disease Control and Prevention; ID, identification.
[b] Isolate provided by F. Schuster, California Department of Health Services.

DISCUSSION

The primary focus of our earlier studies has been the genotype identification of isolates obtained from AK infections. While over 14 genotypic classes (previously called sequence types) have tentatively been identified, nearly all AK-associated strains are classified within a closely related group of genotypes sharing similar *Rns* and *rns* genotypes. These three genotypes (T3, T4, and T11) form a single monophyletic group, including a number of the nominal species of the genus *Acanthamoeba*. The vast majority (~94%) of AK strains are classified as T4. A single example of a T6 genotype associated with an AK infection has been observed (29). However, the T6 genotype isolate in that situation was obtained from a lens case, not directly from a corneal scrape. Therefore, it is possible that the genotype of the AK infection itself was of a different genotype.

In this study we found genotype T4 associated with all foci of non-AK infections. Specifically, it is found in brain, cerebrospinal fluid (CSF), nasal, skin, and lung infections. This is consistent with the frequent occurrence of genotype T4 in the environment and its apparent ability to exploit available niches opportunistically. In contrast, other rare genotypes are associated only with isolates derived from brain infections. These include genotypes T1 (*Acanthamoeba* sp.), T10 (*A. culbertsoni*), and T12 (*A. healyi*). These species are rarely, if ever, isolated from environmental sources. Their true environmental niche remains unknown, and the reasons for their ability to invade the brain, but apparently not other organ systems, remain unexplained. No non-AK infections are found to be associated with genotypes T3 or T11, genotypes that have been occasionally associated with AK infections.

The initial scheme that we proposed to discriminate genotypes was based on 5% sequence dissimilarity (24). That is, groups that differed by 5% or more in pairwise sequence comparisons for the nuclear *Rns* sequence are defined as different genotypes. This is an arbitrary figure, however. It was originally chosen using criteria that had been applied in molecular phylogenetic analyses of some bacteria and provided an initial quantitative value for genotypic determination. There are a number of closely related *Acanthamoeba* groups, including genotypes that are only slightly more divergent from each other than the 5% sequence divergence criterion that was used to establish genotypes. One closely related genotypic cluster includes genotypes T3, T4, and T11. Other closely related groups include T2/6, T10/12/14, and the morphological group I taxa T7/8/9. By observing these phylogenetic relationships, we can next examine the distribution of isolates in genotypes and genotypic clusters.

In the largest genotypic cluster (T3/4/11; $n = 197$), environmental and AK isolates occur equally (44%). However, only 12% of all T3/4/11 isolates have been associated with non-AK infections. Within this group, T4 represents the most frequent genotype recovered from non-AK infections, presumably because they are the most frequent genotype in the environment. Alternatively, genotype T4 may be more common following culture because it is better adapted to conditions of culture and

emerges from an initial mixture of different genotypes as the predominant genotype. However, some recent DNA extractions and PCR performed directly from brain tissue, without possible culture enhancement, have also produced genotype T4 following sequence analysis (G. C. Booton, unpublished data). Because of the sensitivity of PCR methods, future analyses employing direct extraction and analysis of infected tissue compared with results from these same samples following *Acanthamoeba* culture, DNA extraction, and genotype determination will be better suited to test whether culture enhancement, or selection, of T4 is a serious concern.

Genotype T1 contains only three isolates. However, all are associated with non-AK infections. The natural environmental distribution of *Acanthamoeba* genotype T1 is unknown, and its normal ecological niche must be unique or occur rarely. No environmental isolates have been recovered of this genotype. The cluster of genotypes T10/12/14 has some similarities to T1, but with an interesting difference. Genotypes T10 and T12, while rare, are similar to T1 in that, when they have been found in disease, they are associated only with non-AK infections. However, the related genotype T14 has never been found in a disease isolate, although it has been found in human fecal samples. This observation has to be viewed cautiously, because T14 isolates from stool samples are assumed to be environmental and not pathogenic. Similarly, our environmental designation of genotype T13, which comprises nasal swab and lens case isolates, is based on samples from this genotype not being isolated from an infection, and this too must be viewed with caution. The two isolates of genotype T13 were originally described as two distinct genotypes (12). However, pairwise sequence comparison of these two isolates shows that their sequences are more than 98% similar and that they should be grouped together as a single genotype, which is what we have done.

The other genotypic groups seem to be rarely associated with disease but rather are associated with environmental sources. The rare genotypic group T2/6 shows a mixed composition, with most (80%) of the isolates being classified as from environmental sources. But, as noted above, the single T6 AK isolate was not directly derived from diseased tissue and may actually be of environmental origin. Genotype T5 (*A. lenticulata*) is a common genotype that shows 100% association with environmental isolation. Similarly, genotype T15, which is very dissimilar from other genotypes and is associated with the species *Acanthamoeba jacobsi*, has only been found in environmental isolates (100%). In addition, species of *Acanthamoeba* morphological group I (*Acanthamoeba astronyxis* [T7], *Acanthamoeba tubiashi* [T8], and *Acanthamoeba comandoni* [T9]) have been isolated only from environmental sources (100%).

In summary, we have examined the largest sample of non-AK *Acanthamoeba* infection isolates studied to date to determine the genotypic distribution of isolates involved in these serious, often fatal, infections. Comparisons of the nuclear SSU rRNA genes sequences indicate that the majority of the non-AK isolates are genotype T4 (the AK-associated genotype). However, other rare, more distantly related genotypes are also observed in non-AK infections. The non-T4 genotypes found in non-AK infections are not observed in AK infections. In addition, two of these non-AK pathogenic genotypes have not yet been found in environmental isolates. Further, we show that some groups of phylogenetically closely related genotypes are predominantly associated with either environmental, AK, or non-AK isolates. The natural source of rare *Acanthamoeba* genotypes associated with brain infections is still unknown. Expanded environmental screening of diverse ecological niches of *Acanthamoeba* spp. is required and may eventually reveal these hidden, but potentially dangerous, habitats.

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REFERENCES

1. **Alves, J. M. P., C. X. Gusmão, M. M. G. Teixeira, D. Freitas, A. S. Foronda, and H. T. Affonso.** 2000. Random amplified polymorphic DNA profiles as a tool for the characterization of Brazilian keratitis isolates of the genus *Acanthamoeba*. Braz. J. Med. Biol. Res. **33:**19–26.
2. **Beattie, T. K., A. Tomlinson, A. K. McFadyen, D. V. Seal, and A. Grimason.** 2003. Enhanced attachment of *Acanthamoeba* to extended-wear silicone hydrogel contact lenses: a new risk factor for infection? Ophthalmology **110:**765–771.
3. **Booton, G. C., D. J. Kelly, Y.-W. Chu, D. V. Seal, E. Houang, D. S. C. Lam, T. J. Byers, and P. A. Fuerst.** 2002. 18S ribosomal DNA typing and tracking of *Acanthamoeba* species isolates from corneal scrape specimens, contact lenses, lens cases, and home water supplies of *Acanthamoeba* keratitis patients in Hong Kong. J. Clin. Microbiol. **40:**1621–1625.
4. **Booton, G. C., A. Rogerson, T. D. Bonilla, D. V. Seal, D. J. Kelly, T. K. Beattie, A. Tomlinson, F. Lares-Villa, P. A. Fuerst, and T. J. Byers.** 2004. Molecular and physiological evaluation of subtropical environmental isolates of *Acanthamoeba* spp., causal agent of *Acanthamoeba* keratitis. J. Eukaryot. Microbiol. **51:**192–200.
5. **Cabot, E. L., and A. T. Beckenbach.** 1989. Simultaneous editing of multiple nucleic acid and protein sequences with ESEE. Comput. Appl. Biosci. **5:**233–234.
6. **Chang, P. C., and H. K. Soong.** 1991. *Acanthamoeba* keratitis in non-contact lens wearers. Arch. Ophthalmol. **109:**463–464.
7. **DeJonckheere, J.** 1980. Growth characteristics, cytopathic effect in cell culture, and virulence in mice of 36 type strains belonging to 19 different *Acanthamoeba* spp. Appl. Environ. Microbiol. **39:**681–685.
8. **Dyková, I., J. Lom, J. M. Schroeder-Diedrich, G. C. Booton, and T. J. Byers.** 1999. *Acanthamoeba* strains isolated from organs of freshwater fishes. J. Parasitol. **85:**1106–1113.
9. **Gast, R. J.** 2001. Development of an *Acanthamoeba*-specific reverse dot-blot and the discovery of a new ribotype. J. Eukaryot. Microbiol. **48:**609–615.
10. **Gast, R. J., D. R. Ledee, P. A. Fuerst, and T. J. Byers.** 1996. Subgenus systematics of *Acanthamoeba*: four nuclear 18S rDNA sequence types. J. Eukaryot. Microbiol. **43:**498–504.
11. **Hewitt, M. K., B. S. Robinson, P. T. Monis, and C. P. Saint.** 2003. Identification of a new *Acanthamoeba* 18S rRNA gene sequence type, corresponding to the species *Acanthamoeba jacobsi* Sawyer, Nerad, and Visvesvara, 1992 (Lobosea: Acanthamoebidae). Acta Protozool. **42:**325–329.
12. **Horn, M., T. R. Fritsche, R. K. Gautom, K-M. Schleifer, and M. Wagner.** 1999. Novel bacterial endosymbionts of *Acanthamoeba* spp. related to *Paramecium caudatum* symbiont *Caedibacter caryophilus*. Environ. Microbiol. **1:**357–367.
13. **John, D. T.** 1993. Opportunistically pathogenic free-living amebae, p. 143–246. *In* J. P. Kreier and J. R. Baker (ed.), Parasitic protozoa, 2nd ed., vol. 3. Academic Press, San Diego, Calif..
14. **Khan, N. A., E. L. Jarroll, and T. A. Paget.** 2002. Molecular and physiological differentiation between pathogenic and nonpathogenic *Acanthamoeba*. Curr. Microbiol. **45:**197–202.
15. **Ledee, D. R., G. C. Booton, M. H. Awwad, S. Sharma, R. K. Aggarwal, I. A. Niszl, M. B. Markus, P. A. Fuerst, and T. J. Byers.** 2003. Advantages of using mitochondrial 16S rDNA sequences to classify clinical isolates of *Acanthamoeba*. Investig. Ophthalmol. Vis. Sci. **44:**1142–1149.
16. **Marciano-Cabral, F., and G. A. Cabral.** 2003. *Acanthamoeba* spp. as agents of disease in humans. Clin. Microbiol. Rev. **16:**273–307.
17. **Marciano-Cabral, F., R. Puffenbarger, and G. A. Cabral.** 2000. The increasing importance of *Acanthamoeba* infections. J. Eukaryot. Microbiol. **47:**29–36.
18. **Martinez, A. J., and G. S. Visvesvara.** 1997. Free-living amphizoic and opportunistic amebas. Brain Pathol. **7:**583–598.
19. **Moura, H., S. Wallace, and G. S. Visvesvara.** 1992. *Acanthamoeba healyi* n. sp. and the isozyme and immunoblot profiles of *Acanthamoeba* spp., groups 1 and 3. J. Protozool. **39:**573–583.
20. **Pussard, M., and R. Pons.** 1977. Morphologie de la paroi kystique et tax-

onomie du genre *Acanthamoeba* (*Protozoa*: *Amoebida*) Protistologica **8:**557–598.

21. **Schroeder, J. M., G. C. Booton, J. Hay, I. Niszl, D. V. Seal, M. B. Markus, P. A. Fuerst, and T. J. Byers.** 2001. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of acanthamoebae from humans with keratitis and from sewage sludge. J. Clin. Microbiol. **39:**1903–1911.
22. **Seal, D. V., A. J. Bron, and J. Hay.** 1998. Ocular infection. Investigation and treatment in practice. Martin Dunitz Ltd., London, United Kingdom.
23. **Sharma, S., M. Srinivasan, and C. George.** 1990. *Acanthamoeba* keratitis in non-contact lens wearers. Arch. Ophthalmol. **108:**676–678.
24. **Stothard, D. R., J. M. Schroeder-Diedrich, M. H. Awwad, R. J. Gast, D. R. Ledee, S. Rodriguez-Zaragoza, C. L. Dean, P. A. Fuerst, and T. J. Byers.** 1998. The evolutionary history of the genus *Acanthamoeba* and the identification of eight new 18S rRNA gene sequence types. J. Eukaryot. Microbiol. **45:**45–54.
25. **Tomlinson, A., P. Simmons, D. V. Seal, and A. K. McFadyen.** 2000. Salicylate inhibition of *Acanthamoeba* attachment to contact lenses: a way to reduce the risk of infection. Ophthalmology **107:**112–117.
26. **Walochnik, J., M. Duchêne, K. Seifert, A. Obwaller, T. Hottkowitz, G. Wiedermann, H. Eibl, and H. Aspöck.** 2002. Cytotoxic activities of alkylphosphocholines against clinical isolates of *Acanthamoeba* spp. Antimicrob. Agents Chemother. **46:**685–701.
27. **Walochnik, J., E. Haller-Schober, H. Kolli, O. Picher, A. Obwaller, and H. Aspöck.** 2000. Discrimination between clinically relevant and non-relevant *Acanthamoeba* strains isolated from contact lens-wearing keratitis patients in Austria. J. Clin. Microbiol. **38:**3932–3936.
28. **Walochnik, J., A. Hassl, K. Simon, G. Benyr, and H. Aspöck.** 1999. Isolation and identification by partial sequencing of the 18S ribosomal gene of free-living amoebae from necrotic tissue of *Basiliscus plumifrons* (Sauria: Iguanidae). Parasitol. Res. **85:**601–603.
29. **Walochnik, J., A. Obwaller, and H. Aspöck.** 2000. Correlations between morphological, molecular biological, and physiological characteristics in clinical and nonclinical isolates of *Acanthamoeba* spp. Appl. Environ. Microbiol. **66:**4408–4413.