

## Genotypic Identification of Non-Keratitis Infections Caused by the Opportunistically Pathogenic Ameba Genus *Acanthamoeba*

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The genus *Acanthamoeba* is an assemblage of approximately 20 named species of free-living amoebae inhabiting a wide range of ecological niches, and has a worldwide distribution. Individual *Acanthamoeba* have been isolated from soil, fresh and saltwater, humans, domestic and feral animals. In addition to natural habitats, *Acanthamoeba* has been identified as the cause of several diseases. It acts as an opportunistic pathogen in the painful and sight-threatening condition *Acanthamoeba keratitis* (AK) in otherwise healthy individuals. *Acanthamoeba* is also responsible for life threatening infections in patients with immune defense deficiencies (IDD). *Acanthamoeba* strains have also been associated with other infections, including those of the skin, eye, and brain (granulomatous amoebic encephalitis, GAE). These are generally observed in immunocompromised individuals. Variations in the pathogenicity of *Acanthamoeba* strains have been recognized in various laboratory studies, but the relevance of these results to human disease is unclear.

Traditionally, *Acanthamoeba* has been classified using morphological markers such as cyst morphology and trophozoite size and shape. Originally, taxa of *Acanthamoeba* were categorized into 3 morphological groups based largely on the cyst morphology of the species. More recent molecular analyses, including several of our own studies using the nuclear and mitochondrial small subunit ribosomal RNA genes (ssu rDNA), supported the morphological group structure of the genus [1–4]. Our work has focused on the small subunit ribosomal RNA gene of the nucleus (*Rns*) and the equivalent gene from the mitochondrial genome (*rns*). Data collected over the last decade now allow us to quickly analyze a clinical or environmental sample using molecular methods and determine and classify the sequence genotype. Sequence similarities between isolates for the 2 genes have been examined to determine relationships between strains and to explore possible correlations to disease phenotypes. The molecular analyses suggest that 12–15 genotype groups exist, designated T1, T2, etc. [3,4]. The final number of genotypes is an active area of investigation, since it is dependent upon statistical criteria employed to distinguish types. Although the major morphological groups are supported by molecular analyses, a number of the named species have not been supported as unique monophyletic entities when examined by molecular methods.

The primary focus of our earlier studies has been on the genotype of isolates obtained from AK infections. Thus far, nearly all AK cases examined in our lab (as well as in several other labs around the world) are due to infections involving a closely related group of strains sharing similar *Rns* and *rns* genotypes. While over 14 genotypic classes (sequence types) have been identified, AK-associated strains all are classified within a closely related group of genotypes. This group includes genotypes classified as T4, T3, and T11 (most AK strains are classified as T4). Further, these three genotype classes form a single monophyletic group, including a number of nominal species within the genus *Acanthamoeba*.

In the present study we examine *Acanthamoeba* isolates that have been obtained from various non-AK human infections. The genotypes of these isolates have been determined using both nuclear and mitochondrial ribosomal RNA gene sequences. Phylogenetic comparison of the sequences indicates that, while many of the non-AK

disease isolates are genotype T4 (the AK associated genotype), other more distantly related genotypes are also observed in non-AK infections.

### MATERIALS AND METHODS

Most *Acanthamoeba* cultures in this study were obtained from isolates collected by G.S. Visvesvara as part of the work of the CDC. A few isolates represent samples obtained from the American Type Culture Collection (ATCC). Most isolates were cultured axenically in Optimal Growth Medium (OGM) prior to DNA extraction. Following DNA extraction, PCR was used to amplify the nuclear *Rns* sequence. DNA sequencing of the entire *Rns* gene was done with an ABI 310 automated fluorescent sequencing system using a set of conserved primers that have been used previously in our phylogenetic studies. The sequences that were obtained were aligned to other *Acanthamoeba* sequences in our *Rns* database. Phylogenetic reconstruction to classify isolates (not shown) was performed by maximum parsimony and neighbor-joining, using the phylogenetic package MEGA2, in order to identify the genotypic class into which an isolate was placed.

### RESULTS AND DISCUSSION

We analyzed genotypes from over 150 *Acanthamoeba* isolates obtained from the environment (70 isolates), from patients diagnosed with AK (62 isolates), and from patients diagnosed with non-AK infections (17 isolates). Results of genotyping are presented in Table 1. Approximately 61% of the 70 environmental isolates were classified as being in the T4/T3/T11 group of genotypes (33 T4 isolates, 7 T3 isolates and 3 T11 isolates). In the environment, the second most frequent genotypic class (28%) of *Acanthamoeba* was found to be genotype T5, which represents the species *Acanthamoeba lenticulata*. Isolates were also obtained from 6 other less frequent genotypic classes (T2, T6, T7, T8, T9, and T10). All 62 isolates that we have analyzed from individuals diagnosed with *Acanthamoeba keratitis* (AK) fall into the T4/T3/T11 group of genotypes, with 58 of the AK isolates being T4.

Seventeen isolates have been obtained from human non-AK *Acanthamoeba* infections (Table 1). Fifteen of these isolates were obtained from patients diagnosed with GAE. Three of the 15 isolates from GAE patients were non-T4 strains. The unusual strains were found to be associated with genotype T1, T10, and T12 (one each). The remaining 2 non-GAE and non-AK human isolates were found to be T4 genotypes.

Strains of *Acanthamoeba* isolated from human infections have been found to be predominantly members of the phylogenetic group designated the T4 genotype (or rarely the closely associated T3 or T11 genotypic classes). These represent the most frequent genotypes found in environmental samples. However, the T4 genotypic class is significantly over-represented in human infections (representing 84% of infections compared to 46% of environmental isolates). Further, however, among non-AK infections, some non-T4 genotypes were found, types that do not appear in AK infections. So far, these non-T4 genotypes have been associated specifically with GAE infections, although they make up only about 20% of GAE infections, which have been analyzed. We are currently analyzing the sequences from an additional 15 GAE isolates to increase the database for non-AK infections.

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Table 1. Sequence types observed for *Acanthamoeba* isolates.

Sequences type:	T1	T2	T3	T3/4/11	T5	T(misc) <sup>a</sup>	T10	T11	T12
Environmental samples	0	3	7	23	19	5 <sup>b</sup>	0	3	0
AK-isolates	0	0	2	58	0	0	0	2	0
Non-AK-isolates	1	0	0	14	0	0	1	0	1

<sup>a</sup> T(misc) = T6, T7, T8, T9, and T10.

<sup>b</sup> 1 strain from each T(misc) type.

One interesting result from our analysis concerns the second most frequent environmental genotype, genotype T5, which represents the morphological species *Acanthamoeba lenticulata*. Genotype T5 has never been isolated from a human infection. Many of the environmental samples, which have been typed as T5, however, come from sites, such as sewage treatment plants, which would normally be thought of as potential sources of infectious disease. Since genotype T5 represents more than one-quarter of all environmental isolates, it is significantly under represented in infection isolates. It may be that *Acanthamoeba lenticulata* is a non-pathogenic species.

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