Molecular identification of T4 and T5 genotypes in *Acanthamoeba* keratitis patients

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ABSTRACT

Acanthamoeba keratitis (AK) is a rare but sight-threatening ocular infection. Outbreaks have been associated with contaminated water and contact lens wear. The epidemiology and pathology may be associated with unique genotypes. We determined the Rns genotype for 37 clinical isolates from 23 patients presenting at the University of Miami Bascom Palmer Eye Institute with confirmed AK infections in 2007. The genus-specific ASA.S1 amplicon allowed for rapid genotyping of the non-axenic cultures. Of the 37 isolates 36 were of the T4 genotype. Within this group thirteen unique DF3 sequences were identified; three of which were not in GenBank. The thirty-seventh isolate was a T5, the first in the US and second worldwide to be found in AK. For five patients with isolates from the cornea and contact lens/cases identical sequences within each patient cluster was observed, confirming the link between contact lens contamination and AK infection. Genotyping is an important tool in the epidemiological study of AK. In this study it allowed for detection of new stains and provided an etiological link between source and infection. Additionally, it can allow for accurate categorizing of physiological differences, such as strain virulence, between isolates and clades.
INTRODUCTION

The genus Acanthamoeba is comprised of a group of free-living amoebae that are responsible for causing Acanthamoeba keratitis (AK), a rare but sight threatening corneal infection. In recent years the number of AK case has been on the increase, especially among wearers of contact lenses, who make up 85-90% of the AK cases (23, 30, 34, 38). Diagnosis of AK is problematic due to clinical features which are similar to herpetic, bacterial and fungal infections. For example, the stromal ring infiltrate associated with AK is only observed ~6% of the time in early cases and ~16% of the time in late cases (2, 3, 4, 13). AK can be the primary infection or present as a suprainfection in combination with other infectious organisms, like bacteria or fungi, thereby complicating diagnosis and treatment. The encystment capability of Acanthamoeba also confounds treatment due to the recalcitrant nature of the cyst to most treatment options allowing amoebae reemergence after treatment cessation.

Genotyping Acanthamoeba is a useful tool for studying taxonomic and epidemiological relationships, thereby, allowing correlations between the infectious isolates and disease phenotypes, such as virulence factors, drug susceptibility and/or species-clinical outcome correlations, to be explored. The gene targeted most often in Acanthamoeba genotyping is the nuclear small-subunit rRNA gene (Rns), and utilizing a 5% sequence dissimilarity cutoff point 15 or more genotype clades, designated T1, T2, T3, etc., have been identified (11, 12, 14, 16, 28). Isolates from 6 of the genotypic clades (T3, T4, T5, T6, T11 and T15) are confirmed as causative agents in AK (9, 12, 18, 20, 27, 28, 33, 35). The most prevalent Acanthamoeba genotype in both clinical and environmental samples is the T4 genotype (6, 7). Within the genotype clades, multiple
species designations can be observed. This is primarily due to the traditional classification method reliance on changeable morphological characteristics, such as cyst morphology, creating inconsistent species identification (24, 31). Therefore, it was proposed that each genotypic clade be equated with a single species (28). For example, all isolates in the T4 clade could be reclassified as *Acanthamoeba castellanii* since the T4 genotype includes the type strain for that species.

In this study 37 isolates from corneal scrapes, contact lenses, and lens cases of 23 patients from 2006 - 2008 presenting with AK at the Anne Bates Leach Eye Hospital, Bascom Palmer Eye Institute, University of Miami were examined to access the *Rns* genotypes responsible for the infections. *Acanthamoeba* can be rapidly genotyped by targeting a highly variable region designated diagnostic fragment 3 (DF3) within the genus specific *Rns* ASA.S1 amplicon (5, 25), therefore, this region was chosen for analysis. The genotypes identified in this study were also compared to strains identified in other studies in order to examine prevalence of the DF3 sequence types within genotype clades(5, 36, 37).

**MATERIALS AND METHODS**

**Cultures.** Thirty-seven *Acanthamoeba* cultured from corneal scrapings, biopsies, contact lenses, or lens cases were recovered from 23 patients presenting with AK to the University of Miami Bascom Palmer Eye Institute between January 2006 and February 2008 (Table 1). Patients’ age ranged from 14 to 83. The risk factor for all patients involved the use of contact lenses. Diagnosis of AK was based on detection of cyst or trophozoites in corneal sample smear and/or growth on non-nutrient agar plates overlaid with live *E. coli.*
Genotyping. *Acanthamoeba* were harvested from agar plates and rinsed in phosphate-buffered saline (pH 7.4) and DNA extracted using the UNSET method (17). PCR amplification of the *Rns* amplicon ASA.S1 was generated using the genus-specific primer set JDP1 (5’-GGCCCAGATCGTTTACCGTGAA-3’) and JDP2 (5’-TCTCACAAGCTGCTAGGGGAGTCA-3’), which encodes the highly variable diagnostic fragment 3 (DF3) region (25). Two or more PCR products were pooled or independently sequenced using the amplification primers JDP1 and JDP2, in addition to the conserved primers 892 (5’-CCAAGAATTTCACCTCTGAC-3’) and 892C (5’-GTCAGAGGTGAAATTCTTGG-3’). Sequencing of the PCR products was performed by Genewiz, Inc (South Plainfield, NJ). The DF3 sequence designation is based on nomenclature described by Booton et al. (5). The first part is the *Rns* genotype of the isolate. The second part is a unique code assigned to a specific DF3 sequence type. The Booton et al. (5) study identified 10 DF3 sequence types. The numbers used to define the DF3 sequence type in this study is a continuation of that system.

Phylogenetic Analysis. Alignments and phylogenetic reconstructions were performed using the phylogenetic computer program MEGA4 (Molecular Evolutionary Genetic Analysis software, ver. 4) (29). The evolutionary distances were computed using the Kimura 2-parameter distance algorithm (19) and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons. A total of 449 positions were used in the final dataset. The bootstrap consensus tree is inferred from 1000 replicates (10).

*Balamuthia* mandrillaris, a close phylogenetic relative to *Acanthamoeba* was used as the outgroup to root the trees. Phylogenetic reconstructed gene trees were generated using
maximum-parsimony, neighbor-joining, UPGMA or minimum evolution methods in
MEGA4 were compared. The neighbor-joining tree is displayed in Fig. 1.
The thirty-seven sequences determined in this study were deposited in GenBank under
accession numbers FJ422510 to FJ422546. The other *Acanthamoeba* sequences used in
this study are available in GenBank under the following accession numbers:
*Acanthamoeba castellanii* strain CDC:0180:1, U07405; *Acanthamoeba hatchetti* strain
2HH, AF26022; *Acanthamoeba castellanii* strain castellani, U07413; *Acanthamoeba* sp.
strain KA/E21, EF140633; *Acanthamoeba* sp. strain U/E3, AY026747; *Acanthamoeba*
sp. S36, EU146073; *Acanthamoeba* sp. S30, DQ087313; *Acanthamoeba* sp. SF2.JDP,
EU338518); *Acanthamoeba* sp. S4, DQ087320; *Acanthamoeba castellanii* strain
CDC:0184:V014, U07401; *Acanthamoeba* sp. strain BCM:0288:27, U07409;
*Acanthamoeba hatchetti* strain BH2, AF019068; *Acanthamoeba stevensoni* strain RB:F:1,
AF019069; *Acanthamoeba* sp. strain V006, U07400; *Acanthamoeba palestinensis* strain
Reich, U07411; *Acanthamoeba pustulosa* strain GE 3a, AF019050; *Acanthamoeba* sp.
strain RAC013, AB327060; *Acanthamoeba* sp. strain GAK1, AY944575; *Acanthamoeba*
*lenticulata* strain Jc-1, U94739; *Acanthamoeba lenticulata* strain PD2S, U94741;
*Acanthamoeba* sp. strain S35, EU146072; *Balamuthia mandrillaris*, AF477022.

**RESULTS**

**DF3 sequences.** The variable DF3 regions of the *Rns* genes of 37 isolates from 23
patients identified 14 unique DF3 sequences (Fig. 2; Table 1). Of the 14 sequence types
obtained 13 correspond to 36/37 (97%) of the isolates examined, and these were identical
or similar to previously described isolates of the T4 genotype (Fig. 1), herein referred to
as T4/2, T4/6, and T4/11 to T4/21. Three of the sequence types (T4/11, T4/17 and T4/19)
represent new T4 sequence not found in GenBank. The remaining isolate possessed a DF3 sequence most similar to sequences of *Acanthamoeba lenticulata* isolates, which are classified as T5 genotype.

**Rns T4 genotype isolates.** Table 1 summarizes the genotype/DF3 sequence type of all the isolates examined in this study. All 23 patients were contact lens wearers, and of these 5 (patients BP:P3, BP:P9, BP:P10, BP:14, and BP:P16) had *Rns* sequence type determined for the cultures grown from their contact lens paraphernalia and corneal scrapes. In all cases identical DF3 sequences were observed in the corneal scrape and the contact lens paraphernalia, which suggests that the contact lens paraphernalia can be a source of the infection (Table 1, Fig. 1).

Patient BP:P7 was unusual in that the sequence types of the isolated *Acanthamoeba* were different between the right and left lens case. The *Acanthamoeba* isolated from the right lens case was genotype T4/15, whereas the genotype of *Acanthamoeba* in the left lens case was T4/14. No corneal scrape specimen was available for patient BP:P7, therefore it is unknown which, if either, caused the keratitis.

Identical sequence types were observed not only within different sources from a single patient, but also between different patients. Five of the sequence types T4/2, T4/6, T4/21, T4/14, and T4/11 showed identical sequence types between different patients suggesting infection by similar if not identical *Acanthamoeba* strains. Alignments with sequences from GenBank showed the majority of the sequence types have been observed in multiple patients with keratitis worldwide.

**Rns T5 genotype isolate.** Of the 37 *Acanthamoeba* cultures examined one isolate was determined to have the rare T5 genotype. This isolate was identical in its DF3 sequence
to A. sp. RAC013, an isolate from drinking water in Osaka, Japan, and is the first case of a T5 Acanthamoeba causing AK in the United States.

**DISCUSSION**

The genotyping data obtained in this study of amoeba isolated from AK patients further confirms T4 as the predominant genotype, a trend observed in previous studies (5, 36, 37). A comparison of genotypes from this study with other studies that investigated multiple AK isolates revealed that the T4/6 and T4/2 genotypes were shared with the study of Hong Kong isolates (5). The T4/2, T4/12 and T4/13 genotypes were in common between our study and the Zhang et al. (37) results for North China, and only the T4/2 genotype was common between our study and Yera et al. (36) study from France. Although based on limited datasets, the T4/2 genotype appears to be the geographically predominate sequence type.

With the worldwide prevalence of the T4 genotype regardless of region it is not surprising that 90% of Acanthamoeba isolates associated with AK are genotype T4. What is of particular interest is that the second most abundant environmental clade, T5, is dramatically underrepresented in AK cases (6, 7). This study is only the second study to describe a T5 causing AK and the first in the United States. It is unlikely a lack of exposure that explains the low infection rate, as the T5 genotype has been detected in human mucosa without amoebic infection (8). Further complicating the issue is experimental animal and tissue culture models that have shown T5 isolates capable of a high degree of pathogenicity (32, 33). Additionally, studies comparing T4 and T5 resistance to multipurpose contact lens cleaning solutions (MPS), interestingly, show the T5 genotype possesses a better resistance (15, 26). It is possible that the majority of T5
Acanthamoeba may not be pathogenic to humans, but as the number of people that wear contact lenses continue to grow the risk of encountering pathogenic T5 isolates may increase.

An interesting observation was the lack of the T3 genotype in this study. Several studies, that have determined genotypes of Acanthamoeba from AK and contact lens/cases each identified the presence of T3 genotypes, which based on environmental distribution, is less prevalent than T5 (5, 7, 36, 37). Also, like the T5, T3 isolates can show more resistance to MPS than isolates of the T4 genotype (26). Understanding what makes T4 more virulent to humans is an important area of study. Multiple factors contribute to Acanthamoeba pathogenicity, such as extracellular protease production and amoeba-cell surface adherence ability. In studies that examined pathogenicity predictive factors the T3, T5 and T4 genotypes all displayed high pathogenicity (1, 21, 32, 33), although the T3 results were not always consistent between isolates. The T4 genotypes did show increased cell surface binding compared to T3 (1), however, it is essential to realize the small number of T3 and T5 genotypes examined in these studies compared to the T4. These observation do suggest a different rationale must exist to explain the under representation observed with T3 and T5 genotypes. It should be noted that these studies used in vitro cell culture models to compare the pathogenicity of isolates, which emphasizes the need for a good clinical animal model.

Obviously, there are certain properties within the T4 genotype that make them more virulent. Therefore, the need for accurate genotyping of Acanthamoeba from different environments along with an analysis of their virulence factors, and in clinical AK cases an examination of outcome would greatly enhance and stimulate research. Also, the
integration of a PCR based assay in the detection of *Acanthamoeba*, in addition to
genotypic information that can be obtained, offers a rapid diagnostic tool. Utilized along
side the conventional method of smear examination an AK diagnosis can be ideally
accomplished in less than a day and would be more cost effective than fluorescence or in
vivo confocal microscopy based methods. The use of a PCR based assay offers all the
hallmarks of a good diagnostic test: high sensitivity, high specificity and high positive
and negative predictive values (22, 35).
REFERENCES

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   18S ribosomal DNA typing and tracking of 

   Molecular and physiological evaluation of subtropical environmental isolates of 


genotypes from environmental freshwater samples in the Nile Delta region, Egypt. *Acta Trop.* **100:**63-69.


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Fig. 1. *Rts* DF3 linearized neighbor-joining gene tree. Isolates from this study are shown in bold font with asterisks. The tree was constructed using 1000 bootstrap replications. The T1, T2, T4, T5 and T11 designations shown on the tree correspond to strains previously determined to be of that particular genotype (25, 28).

Fig. 2. Primary sequence alignment of a subset area of the highly variable and informative region of DF3 (stem 29-1, 18S rRNA) of BPEI isolates. Sequences are aligned by similarity. Gaps are represented by dashes.
TABLE 1. Isolates used for sequencing of \textit{Rns} ASA.S1 amplicon, \textit{Rns} genotype, Accession number.

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<th>Culture Source</th>
<th>\textit{Rns} Genotype/DF3 Sequence \textsuperscript{b}</th>
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Culture designation: BP, Bascom Palmer; P, Patient. Acronyms - RCS, Right Corneal Scrap; CB, Corneal Button; LCS, Left Corneal Scrap; LLC, Left Lens Case; RLC, Right Lens Case; LC, Lens Case; LCL, Left Contact Lens; RCL, Right Contact Lens.

The DF3 sequence nomenclature used in this study is the same as reported by Booten et al. (5). The first part is the Rns genotype and the second part is a unique code assigned to the specific DF3 sequence. [2] describes second isolates obtained from the same source.
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