Genotypic Identification of *Acanthamoeba* sp. Isolates Associated With an Outbreak of *Acanthamoeba* Keratitis

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Purpose: To determine whether increased rates of *Acanthamoeba* keratitis (AK) are due to changes in municipal water treatment or to emergence of a more pathogenic strain of *Acanthamoeba*.

Methods: Previous sequence analysis of the 18S ribosomal DNA of *Acanthamoeba* isolates resulted in the identification of 15 different genotypic classes. These analyses indicate that AK cases are associated predominantly (~97%) with a single genotype (designated T4) of *Acanthamoeba* and rarely with other genotypes (eg, T3 and T11). In this study, we test the hypothesis that a new or more pathogenic genotype of *Acanthamoeba* is the cause of the recent surge in AK.

Results: We determined the genotype of 15 *Acanthamoeba* sp. isolates from AK cases associated with this outbreak using sequence analysis of a region of the 18S ribosomal DNA. Our results indicate that these isolates are predominantly genotype T4 (87%), with the remaining isolates being genotype T3 (13%). Both genotypes have previously been observed in AK cases.

Conclusions: There is no support for the hypothesis that the current AK outbreak is associated with infection by a new more pathogenic *Acanthamoeba* genotype. In addition, these results offer support for the hypothesis that the increased AK incidence may be because of changes in water treatment protocols leading to increased bacterial colonization of the water supply and subsequent increases of already present *Acanthamoeba* sp, ultimately culminating in an increase of AK cases.

Key Words: *Acanthamoeba*, keratitis, epidemiology, genotype identification

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The protistan genus *Acanthamoeba* consists of approximately 25 named species of free-living amoeba that are ubiquitous in nature and have been isolated from a variety of environments including soil, fresh and brackish water, beach sand, air, hot tubs, and recreational swimming pools.[1–4] *Acanthamoeba* has a biphasic lifestyle consisting of a mobile trophozoite form with characteristic acanthopodia readily visible under light microscopy. A second form, the cyst, is also observed in *Acanthamoeba*. The ability to encyst enables the amoeba to survive in deteriorated or harsh environmental conditions. These cysts can survive for long periods and under severe conditions, including in water where chlorine is used as a disinfectant.[5]

In addition to its natural distribution, some strains and/or species of *Acanthamoeba* have been found to be opportunistically pathogenic. The predominant clinical manifestations of *Acanthamoeba* infection have been cases of *Acanthamoeba* keratitis (AK) infection, a painful and potentially sight-threatening disease. In North America, AK cases are predominantly associated with contact lens wear.[6–8] Other less common opportunistic infections caused by *Acanthamoeba* include those found in the lungs, skin, sinuses, and brain.[2,3,9] Infections of *Acanthamoeba* in the brain lead to nearly universally fatal cases of granulomatous amoebic encephalitis.[2,3,9] AK infections are found in otherwise healthy individuals, whereas nonkeratitis infections are associated with immunocompromised individuals, often in patients with AIDS.[2]

In the present study, we examine the genotypes of *Acanthamoeba* strains isolated from AK cases associated with an outbreak of infections in the Chicago metropolitan area. From June 1, 2003 until November 30, 2005, 40 cases of AK were studied in previous epidemiological analyses that concluded that the rate of AK cases was significantly higher than historical case numbers predicted.[10,11] The rate was also nearly 10 times the expected number of cases based on the number of cases that have been observed in the United States between 1973 and 1988.[12] In addition, Joslin et al[11] found that there was a significant difference among the distribution of cases in urban and suburban counties in the outbreak area. Specifically, suburban counties had a higher relative rate of infection compared with an urban county (Cook County, IL).[11]

It was hypothesized in the Joslin et al (2006) study that the cause of this increased rate of AK infection was an alteration in the amount and type of water treatment chemicals, following changes in Environmental Protection Agency guidelines that mandated a reduction of potentially carcinogenic disinfection by-products in drinking water.[11] Alternatively, it is

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possible that a new, potentially more pathogenic or virulent, strain of *Acanthamoeba* could be responsible for the increase in cases of AK. Therefore, in the current study, we have employed genotypic analysis of the AK-derived *Acanthamoeba* isolates to test the hypothesis that the observed outbreak has resulted from a more pathogenic, or novel, genotype of *Acanthamoeba* or alternatively, whether the genotypes observed in this outbreak have been seen in previous studies.

MATERIALS AND METHODS

Corneal scrapes from patients with AK were collected by E.Y.T. of University of Illinois, Chicago (UIC), and sent to Ohio State University (OSU). These were isolates used in previous studies by Joslin et al.[10,11] Upon arrival at OSU, UIC samples were given unique OSU numbers based on arrival date, for example, 05-009, which represents the ninth overall sample received in 2005 at OSU. Next, the samples were briefly vortexed to remove any cells adhering to the sides of the tubes and several drops were plated onto a nonnutrient amoeba saline agar plate seeded with *Enterobacter aerogenes* (CDC strain #1998-68) as prey. All cultures were maintained at room temperature. To prevent the agar plates from drying out (amoebae migrate in the thin water film on the surface of the agar), all plates were sealed with Parafilm "M" (Pechiney Plastic Packaging, Chicago, IL). Additionally, several drops of the sample were placed in a petri dish containing amoeba saline. The plates were checked for growth at 3 days, 7 days, and 2 weeks. This process was repeated until positive growth appeared or until no original sample was left. Blocks of agar from plates that showed positive *Acanthamoeba* growth were then transferred to liquid culture using a Bacto-Casitone/ Serum media or amoeba saline.[13,14] DNA was extracted by using the DNeasy kit (Qiagen, Inc, Valencia, CA). After DNA extraction, polymerase chain reaction (PCR) was used to amplify the partial nuclear small subunit ribosomal DNA (ssu rDNA) sequences. DNA sequencing of the partial ssu rDNA sequence was done with an ABI 310 automated fluorescent sequencing system using a set of conserved primers and methods that have been used previously in our phylogenetic studies.[15] The sequences obtained in this study have been deposited in GenBank under the accession numbers EU168067-EU168082.

The sequences were aligned with a standard set of >130 "complete" sequences (over 2000 nucleotides in length) of the *Acanthamoeba* nuclear small subunit ribosomal RNA gene (ssuDNA) using the sequence alignment application CLUSTALX within the analysis package Mega 3.1.[16] This alignment allowed the identification of those complete *Acanthamoeba* sequences that most closely matched the sequences from each isolate currently being studied. For most isolates, the overall sequence overlap with the standard set of sequences encompassed approximately 490 of the ~2300 nucleotide long ssuDNA sequence. The phylogenetic relationships of the sequences from the Chicago outbreak were then obtained by using the phylogenetic identification of the closest complete sequence as a surrogate for the shorter sequence obtained in this study.

RESULTS

Sequencing of the nuclear ssu rDNA DF3 diagnostic region in the 15 UIC AK isolates resulted in the genotypic determination of 17 individual DF3 sequences (Table 1). Direct sequence analysis of the PCR product from 06-024 resulted in multiple peaks in the electropherogram, indicative of a mixed product. Therefore, T/A cloning of the PCR amplimer of 06-024 was performed, and subsequent sequence

TABLE 1. University of Illinois, Chicago, AK Isolates Analyzed

Taxa	UIC ID*	OSU ID†	Sample Source‡	18S ssu rDNA Genotype	GenBank Sequence Accession Number
Acanthamoeba sp.	5731	05-003	AK	T4	EU168067
Acanthamoeba sp.	5742	05-009	AK	T4	EU168068
Acanthamoeba sp.	1504	05-011	AK	T4	EU168069
Acanthamoeba sp.	4736	05-013	AK	T4	EU168070
Acanthamoeba sp.	2713	05-014	AK	T3	EU168071
Acanthamoeba sp.	1404	05-020	AK	T3	EU168072
Acanthamoeba sp.	3403	05-023	AK	T4	EU168073
Acanthamoeba sp.	6191	06-001	AK	T4	EU168074
Acanthamoeba sp.	2968	06-002	AK	T4	EU168075
Acanthamoeba sp.	8599	06-004	AK	T4	EU168076
Acanthamoeba sp.	0410	06-005	AK	T4	EU168077
Acanthamoeba sp.	4423	06-016	AK	T4	EU168078
Acanthamoeba sp.	6050	06-024	AK	T4	EU168079
Acanthamoeba sp.	6590	06-025	AK	T4	EU168080
Acanthamoeba sp.	1060	06-033	AK-liq	T4	EU168081
Acanthamoeba sp.	1060	06-034	AK-plate	T4	EU168082

*University of Illinois, Chicago identification number for clinical isolates.
†Ohio State University identification number assigned to all samples arriving at OSU for analysis. Format: (year acquired-sequential sample number, eg, 05-003).
‡AK, *Acanthamoeba* keratitis corneal scrape; AK-liq, AK sample from liquid culture; AK-plate, AK sample from agar plate.

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analysis resulted in the identification of a distinct DF3 sequence, which was genotype T4. Additional sequences were not found after cloning; however, the mixed pherogram suggests that more products were present. Overall, 14 of the 17 AK sequences (82%) were determined to be genotype T4 based on sequence alignment and overall sequence similarity analysis using our *Acanthamoeba* rDNA database. Similarly, 2 of the sequences (18%) were determined to be genotype T3, also based on sequence alignment of the DF3 region. Both genotypes have previously been observed in AK cases. In addition, DNA sequences are overwhelmingly similar to previously sequenced isolates of their 2 respective genotypes. Among the 17 sequences obtained from isolates obtained in this study, 16 could be related to a specific monophyletic subgroup within the phylogenetic tree of the set of complete sequences (Table 1). A single ambiguous isolate sequence represented a generic T4 type sequence but was equally similar to a number of subgroups within T4 and could not be placed more accurately.

DISCUSSION

After the recognition of *Acanthamoeba*'s role in keratitis infections in 1973, there were slightly over 200 cases of AK documented in the United States between 1973 and 1988.[12] The rate of infection of contact lens wearers has been estimated in the United States to range from 1.65 to 2.01 per 1,000,000 contact lens users.[10,17,18] The identification of particular *Acanthamoeba* species that are responsible for AK and other infections has been an active area of investigation since the recognition of this amoeba as the etiological agent in these infections. Traditional taxonomy of *Acanthamoeba* has used morphological markers such as cyst morphology and trophozoite size and shape as classification characters; however, this classification is questionable as morphology of cysts and trophozoites changes with culture conditions.[19] Taxa of *Acanthamoeba* have been categorized into 3 morphological groups based largely on the cyst morphology of the species. Molecular analyses using nuclear and mitochondrial ssuDNA have supported the 3 morphological groupings, however some species have been shown to polyphyletic in these analyses.[20–22] In our laboratory, we have worked extensively on the analysis of the ssu rDNA of the nucleus and the equivalent gene from the mitochondrial genome and have analyzed more than 200 clinical and environmental isolates using these genes.[20,21] Work by other laboratories has increased the number of isolates from which sequences have been determined to over 500.[22–28] This database allows us to quickly analyze a clinical or environmental sample using molecular methods to determine and classify the *Acanthamoeba* sequence genotype.[1,15]

Sequence similarities between isolates using the nuclear and mitochondrial ssuDNA have been used to determine phylogenetic relationships between strains and to explore possible correlation with disease phenotypes. The molecular analyses thus far suggest that 15 genotypic classes exist, designated T1, T2, T3, …, T15.[21–27] Different *Acanthamoeba* 18S rDNA genotypes are distinguished from one another by a 5% or greater sequence dissimilarity between isolates. The final number of genotypes is an active area of investigation because it is dependent upon the statistical criteria employed to distinguish genotypes and the expanding number of analyzed isolates. Although the major morphological groups and some of the named species are supported by molecular analyses, a number of the named taxa are not supported as unique monophyletic entities when examined using molecular methods.[21]

Nearly all AK cases examined in our laboratory (and by several other investigators) are because of infections involving a closely related group of strains sharing similar ribosomal genotypes. Although at least 15 genotypic classes have been identified, nearly all AK-associated strains are classified within a closely related group of genotypes.[21] This group includes genotypes classified as T3, T4, and T11 (and the vast majority of AK strains, >90%, are classified as genotype T4).[21] In addition to the AK isolates, the genotypes T3, T4, and T11 also contain environmental isolates.[21] Furthermore, these 3 genotypes form a single monophyletic group, including a number of the nominal species of the genus *Acanthamoeba*.[21] There have been rare examples of other genotypes (T5 and T6) that have been identified in AK infections.[25]

As discussed previously, *Acanthamoeba* is capable of infecting other tissues and organs in addition to the eye, for example, skin, sinus, liver, and brain.[2,3,9] Our phylogenetic comparisons of the nuclear ssuDNA sequences obtained from these non-AK *Acanthamoeba* isolates indicated that although the majority of these non-AK infection isolates are genotype T4 (the most common AK and environmental genotype), other rare genotypes were isolated from these non-AK infections (eg, T1, T10, and T12).[29] In addition, 2 of these non-AK pathogenic genotypes (T10 and T12) have not yet been observed in environmental isolates.[29] More recent reports have identified genotype T5 isolates in disseminated non-AK infections, which as mentioned above have also rarely been found in AK infections.[25,30,31]

The results of the current study clearly demonstrate that these isolates are of previously known genotypes: T3 and T4. The sequences determined do not represent novel genotypes; therefore, there is no support for the hypothesis that the AK cases in the Chicago area outbreak are the result of infections caused by a new *Acanthamoeba* genotype(s). In fact, there is very high sequence similarity between the DF3 primary sequences of the Chicago-area isolates and previously sequenced T3 and T4 genotype isolates derived from other AK cases. Specifically, the observation of high sequence similarity in this region with other isolates does not provide support for the hypothesis that these isolates represent more pathogenic *Acanthamoeba* of previously unknown genotypes. Therefore, the results of this genetic study have conclusively shown that these Chicago-area AK infections are the result of infection by *Acanthamoeba* of previously known genotypes. Because novel *Acanthamoeba* genotypes are not the cause of the current outbreak, alternative explanations must be explored to explain the ongoing increased rate of AK infection observed in the Chicago area.

One general alternative hypothesis suggests that the increased number of AK cases may be because of an increased abundance of *Acanthamoeba* in the water supply.[11] The question remains as to the possible reasons for an increased abundance of *Acanthamoeba*. One possibility is based upon

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the relatively recent changes that have occurred in the chemical disinfectant treatment of municipal water supplies. Environmental Protection Agency guidelines have resulted in the reduction of chemicals whose breakdown products have been shown to be carcinogenic. The decrease in these water treatment disinfectant chemicals may permit increased bacterial colonization of the water and water supply surfaces. The decreased levels of disinfectant can directly lead to an increased population density of grazing *Acanthamoeba* that use these bacteria as a food source, resulting in an overall increase of *Acanthamoeba* abundance in the water supply. This scenario may ultimately culminate in an increased number of AK cases because of the general inability of currently available contact lens disinfectant solutions to overcome the increased protozoan load.[32,33] Regardless of whether this outbreak is determined to be linked to the water supply, the results of this study have unambiguously identified the Chicago-area isolates as genotypically very similar to previously sequenced AK isolates. This lends further support to the alternative hypothesis that the increased AK cases in the Chicago area may ultimately be linked to mandated changes in the chemical treatment of the household water supply, permitting expansion of already present *Acanthamoeba* populations of known AK-associated genotypes.

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REFERENCES

1. Booton GC, Rogerson A, Bonilla TD, et al. Molecular and physiological evaluation of subtropical environmental isolates of *Acanthamoeba* spp., causal agent of *Acanthamoeba* keratitis. *J Eukarot Microbiol*. 2004;51: 192–200.
2. Marciano-Cabral F, Cabral GA. *Acanthamoeba* spp. as agents of disease in humans. *Clin Microbiol Rev.* 2003;16:273–307.
3. Marciano-Cabral F, Puffenbarger R, Cabral GA. The increasing importance of *Acanthamoeba* infections. *J Eukaryot Microbiol*. 2000; 47:29–36.
4. John DT. Opportunistically pathogenic free-living amoebae. In: Kreier JP, Baker JR, eds. *Parasitic Protozoa*. Vol 3. 2nd ed. San Diego, CA: Academic Press; 1993:143–246.
5. Schuster FL. Cultivation of pathogenic and opportunistic free-living amebas. *Clin Microbiol Rev.* 2002;15:342–354.
6. Beattie TK, Tomlinson A, McFayden AK, et al. Enhanced attachment of *Acanthamoeba* to extended-wear silicone hydrogel contact lenses: a new risk factor for infection? *Ophthalmology.* 2003;110:765–771.
7. Schroeder JM, Booton GC, Hay J, et al. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoeba* from humans with keratitis and from sewage sludge. *J Clin Microbiol*. 2001;39:1903–1911.
8. Tomlinson A, Simmons P, Seal DV, et al. Salicylate inhibition of *Acanthamoeba* attachment to contact lenses: a way to reduce the risk of infection. *Ophthalmology.* 2000;107:112–117.
9. Martinez AJ, Visvesvara GS. Free-living amphizoic and opportunistic amebas. *Brain Pathol*. 1997;7:583–598.
10. Joslin CE, Tu EY, Shoff M, et al. The association of contact lens solution use and *Acanthamoeba* keratitis. *Am J Ophthalmol*. 2007;144:169–180.
11. Joslin CE, Tu EY, Shoff ME, et al. Epidemiological characteristics of a Chicago-area *Acanthamoeba* keratitis outbreak. *Am J Ophthalmol*. 2006;142:212–217.
12. Stehr-Green JK, Bailey TM, Visvesvara GS. The epidemiology of *Acanthamoeba* keratitis in the United States. *Am J Ophthalmol*. 1989;107: 331–336.
13. Page FC. *A New Key to Freshwater and Soil Gymnamoebae With Instructions for Culture*. Cumbria, United Kingdom: Freshwater Biological Association; 1988.
14. Cerva L. *Naegleria fowleri*: trimethoprim sensitivity. *Science*. 1980; 209:1541.
15. Booton GC, Kelly DJ, Chu Y-W, et al. 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*. 2002; 40:1621–1625.
16. Kumar S, Tamura K, Nei M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform*. 2004;5:150–163.
17. Schawnberg DA, Snow KK, Dana MR. The epidemic of *Acanthamoeba* keratitis: where do we stand? *Cornea*. 1998;17.1:1–2.
18. Mathers WD, Suphin JE, Folberg R, et al. Outbreak of keratitis presumed to be caused by *Acanthamoeba*. *Am J Ophthalmol*. 1996;121:128–142.
19. Pussard M, Pons R. Morphologie de la paroi kystique et taxonomie du genre *Acanthamoeba (Protozoa: Amoebida)*. *Protistologica*. 1977;8:557–598.
20. Ledee DR, Booton GC, Awwad MH, et al. Advantages of using mitochondrial 16S rDNA sequences to classify clinical isolates of *Acanthamoeba*. *Invest Ophthalmol Vis Sci*. 2003;44:1142–1149.
21. Stothard DR, Schroeder-Diedrich JM, Awwad MH, et al. The evolutionary history of the genus *Acanthamoeba* and the identification of eight new 18S rRNA gene sequence types. *J Eukaryot Microbiol*. 1998; 45:45–54.
22. Gast RJ, Ledee DR, Fuerst PA, et al. Subgenus systematics of *Acanthamoeba*: four nuclear 18S rDNA sequence types. *J Eukaryot Microbiol*. 1996;43:498–504.
23. Horn M, Fritsche TR, Gautom RK, et al. Novel bacterial endosymbionts of *Acanthamoeba* spp. related to *Paramecium caudatum* symbiont *Caedibacter caryophilus*. *Environ Microbiol*. 1999;1:357–367.
24. Alves JMP, Gusmão CX, Teixeira MMG, et al. Random amplified polymorphic DNA profiles as a tool for the characterization of Brazilian keratitis isolates of the genus *Acanthamoeba*. *Braz J Med Biol Res*. 2000; 33:19–26.
25. Walochnik J, Obwaller A, Aspöck H. Correlations between morphological, molecular biological, and physiological characteristics in clinical and nonclinical isolates of *Acanthamoeba* spp. *Appl Environ Microbiol*. 2000; 66:4408–4413.
26. Walochnik J, Haller-Schober E, Kolli H, et al. Discrimination between clinically relevant and non-relevant *Acanthamoeba* strains isolated from contact lens-wearing keratitis patients in Austria. *J Clin Microbiol*. 2000; 38:3932–3926.
27. Hewitt MK, Robinson BS, Monis PT, et al. 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*. 2003;42:325–329.
28. NCBI database of available (published and unpublished) *Acanthamoeba* nuclear 18S ribosomal DNA sequences (ssuDNA, full and partial). Available at: http://www.ncbi.nlm.nig.gov. Accessed March 20, 2008.
29. Booton GC, Visvesvara GS, Byers TJ, et al. Identification and distribution of *Acanthamoeba* species genotypes associated with nonkeratitis infections. *J Clin Microbiol*. 2005;43:1689–1693.
30. Spanakos G, Tzanetou K, Miltsakakis D, et al. Genotyping of pathogenic Acanthamoebae isolated from clinical samples in Greece: report of a clinical isolate presenting T5 genotype. *Parasitol Int*. 2006;55:147–149.
31. Barete S, Combes A, de Jonckheere JF. Fatal disseminated *Acanthamoeba lenticulata* infection in a heart transplant patient. *Emerg Infect Dis*. 2007; 13:736–738.
32. Shoff M, Rogerson A, Schatz S, et al. Variable responses of *Acanthamoeba* strains to three multipurpose lens cleaning solutions. *Optom Vis Sci*. 2007;84:202–207.
33. Shoff M, Joslin CE, Tu EY, et al. Efficacy of contact lens systems against recent clinical and tap water *Acanthamoeba* isolates. *Cornea*. 2008;27: 713–719.

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