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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

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We examined partial 18S ribosomal DNA (*Rns*) sequences of *Acanthamoeba* isolates cultured in a study of microbial keratitis in Hong Kong. Sequence differences were sufficient to distinguish closely related strains and were used to examine links between strains obtained from corneal scrape specimens, contact lenses, lens cases, lens case solutions, and home water-supply faucets of patients with *Acanthamoeba*. We also looked for evidence of mixed infections. Identification of *Acanthamoeba Rns* genotypes was based on sequences of ~113 bp within the genus-specific amplicon ASA.S1. This permitted genotype identification by using nonaxenic cultures. Of 13 specimens obtained from corneal scrapes, contact lenses, lens cases, or lens case solutions, 12 were *Rns* genotype T4 and the remaining one was *Rns* genotype T3. The sequences of corneal scrape specimens of two patients also were the same as those obtained from their contact lenses or lens case specimens. A possible triple-strain infection was indicated by three different T4 sequences in cultures from one patient's lenses. Although faucet water used by patients to clean their lenses is a possible source of infections, specimens isolated from the faucets at two *Acanthamoeba* keratitis patients' homes differed from their corneal scrape or lens specimens. The overall results demonstrate the potential of this *Rns* region for tracking *Acanthamoeba* keratitis strains in infections and for distinguishing single-strain and closely related multiple-strain infections even when other microorganisms might be present with the cultured specimens. They also confirm the predominance of *Rns* genotype T4 strains in *Acanthamoeba* keratitis infections.

In a previous study investigating microbial keratitis in Hong Kong, *Pseudomonas aeruginosa* was the most commonly isolated bacterium from keratitis patients' corneal scrape specimens (3). *Acanthamoeba* was the second most commonly isolated microbe among patients who wore contact lenses (3). That study also identified careless practice in contact lens handling, including the use of tap water for storing lenses, as a major risk factor for microbial keratitis. A previous study using the presence of a unique *Rns* intron as a marker had conclusively shown that the home water supply could be the source of an *Acanthamoeba* keratitis (AK) infection (5). Thus, in the present study we attempted to determine whether tap water was the source of any of the infections studied here. As part of an effort to track infections, we also sought to determine whether *Acanthamoeba* strains isolated in corneal scrapes also might be present on contact lenses and/or in lens cases.

In one of the AK cases examined in this study, the patient was a regular contact lens wearer and *Acanthamoeba* was isolated from corneal scrape specimens, a contact lens, and swabs taken at the home kitchen water faucet and bathroom basin drain where the lenses were routinely prepared. It seemed likely that colonization of the home water supply could be the source of the infection. In order to test this possibility, DNA typing was used to test the relatedness of acanthamoebae isolated from this patient's corneal scrape specimens, contact lenses, and kitchen and bathroom water supplies. Contact lens and water faucet isolates were available from a second patient, and these were also compared with each other. DNA *Rns* genotyping of acanthamoebae was also done for 12 other patients and/or home water faucet supplies as a further check on the occurrence of different sequence types.

Genetic identification of these Hong Kong *Acanthamoeba* specimens was based on information in the nuclear small subunit 18S rRNA gene (*Rns*) that has made it possible to distinguish 12 sequence variants referred to as genotypes *Rns* T1 to T12 (7). Previous use of *Rns* DNA genotyping indicates that nearly all acanthamoebae isolated from AK infections are due to genotype T4 (6, 7, 8).

The sequences of a number of regions in *Rns* are highly variable and can be used to differentiate genotypes. Phylogenetic trees based on three of these variable regions that have been identified are as robust as those based on the entire *Rns* sequence (6). In this study we examine one of these regions, which we have designated diagnostic fragment 3 (DF3). Trees based on this region alone are not as robust as those obtained with longer sequences, but analysis of the region is especially

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ID      Source  Seq Des.  DF3 variable region

                          ***  ***    ** * **    *  **** ****  * ******** ** ******* ***   **   *     *  * *
P1      CS      T4/1      GGT ----GCGGTC GTCCTTGGCG TCTCGGTCCT TCAC-----G GGGCCGGGGC GCGGGGGCGG CTTAGCCCG- G
P6      CS      T4/1      GGT ----GCGGTC GTCCTTGGCG TCTCGGTCCT TCAC-----G GGGCCGGGGC GCGGGGGCGG CTTAGCCCG- G
P77     LC      T3/1      CG- -ATTGCGGTC GTCTTTGGTG T--CG---CT -CACA---AG GCGGCAT--- -C-GGGACGG CTTAGCTCGC G
P91     CS      T4/2      GGT ----GCGGTC ATCCTTGGCG T-T-GGT-CT TCAAA---A- --GCCA--GC GCGGGGGTGG CTTAGCCCG- G
P91     LC      T4/2      GGT ----GCGGTC ATCCTTGGCG T-T-GGT-CT TCAAA---A- --GCCA--GC GCGGGGGTGG CTTAGCCCG- G
P97     LCS     T4/3      GGT ----GCGGTC GTCCTTGGCG GTT-GGT-CT TCAAA---A- --GCCAGC-G GCGGGGGCGG CTTAGCCCG- G
P97     RCL     T4/3      GGT ----GCGGTC GTCCTTGGCG GTT-GGT-CT TCAAA---A- --GCCAGC-G GCGGGGGCGG CTTAGCCCG- G
P97     LCL     T4/4      GGT ----GCGGCT GTTCTTGGCG TC--GG--TT TC-------- -GGCC--GGC GCGGGGATGG CTTAGCCCG- G
P97     RCL     T4/4      GGT ----GCGGCT GTTCTTGGCG TC--GG--TT TC-------- -GGCC--GGC GCGGGGATGG CTTAGCCCG- G
P97     LCL     T4/5      GGT ----GCGGTC GTCCTTGGCG GTT-GGT-CT TCGAA---A- --GCCAGC-G GCGGGGGCGG CTTAGCCCG- G
P120    CS      T4/6      GGT ---TGCGGTC GTCCTTGGCG TCTCGG--TT TC-------- -GGCCGGGGC GCGGGGATGG CTTAGCCCG- G
P120    CL      T4/6      GGT ---TGCGGTC GTCCTTGGCG TCTCGG--TT TC-------- -GGCCGGGGC GCGGGGATGG CTTAGCCCG- G
P191    CL      T4/6      GGT ---TGCGGTC GTCCTTGGCG TCTCGG--TT TC-------- -GGCCGGGGC GCGGGGATGG CTTAGCCCG- G
P120    KTW     T3/2      CG- -ATTGCGGTC GTCTTTGGTG T--CG---CT -CACA---AG GCGGCAT--- -C-GGGGCGG CTTAGCTCGC A
P120    BTW     T3/3      CGC -ATTGCGGTC GTCTTTGGTG T--CG---CT -CACA---AG GCGGCA---- -CCGGGGCGG CTTAGCTCGC A
P191    TW      T3/4      CGC -ATTGCGGTC GTCTTTGGTG TGTCG---CT -CACA---AG GCGGCATCAT -C-GGGACGG CTTAGCTCGC A
P208    TW      T3/5      CG- -ATTGCGGTC GTCTTTGGTG T--CG----T CCACAGCGAT GTG-GGCGGC ATCGGGATGG CTTAGCTCGC A
P209    CS      T4/7      GGT ----GCGGTC GTCCTTGGCG TC--GG--TT TC-------- -GGCC--GGC GCGGGGGTGG CTTAGCCCG- G
C10     TW      T4/8      GGT ----GCGGTC GTCCTTGGCG TCTCGGTCCT TCAC-----G GGGCCGGGGC GCGGGGGTGG CTTAGCCCG- G
C68     TW      T4/9      GGT ----GCGGTC GTCCTTGGCG TCTCGG--TT TC-------- -GGCCGGGGT GCGGGGACGG CTTAGCCCG- G
C109    TW      T4/9      GGT ----GCGGTC GTCCTTGGCG TCTCGG--TT TC-------- -GGCCGGGGT GCGGGGACGG CTTAGCCCG- G
C124    LC      T4/10     GGT ---TGCGGTC GTCCTTGGCG TCTCGG--TT TC-------- -GGCCGGGGC GCGGGGACGG TTTAGCCCG- G
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FIG. 1. Primary sequence alignment of variable region of DF3 (stem 29-1 of 18S rRNA) of Hong Kong isolates. This alignment is derived from alignment of the entire Hong Kong DF3 sequences with the OSU *Acanthamoeba* DNA database of *Rns* primary sequences. The alignment shown is a subset of DF3 that contains the highly variable and informative section of this fragment. Abbreviations for samples are as defined in Table 1. Sequences were aligned in this figure by similarity. Asterisks denote variable positions; gaps are represented as dashes.

useful for *Rns* genotyping in cases where, as in the present study, resistance to axenization of cultures is encountered (6). DF3 encodes the highly variable stem 29-1 of *Rns* (6, 7). In the present study we amplified the genus-specific *Rns* amplicon ASA.S1 that includes DF3 (6) and used the variable DF3 sequences to identify *Rns* genotypes of the Hong Kong isolates. We also examined relationships between strains obtained from corneal scrape specimens, contact lenses, lens cases, and home water faucet supplies where possible. The use of this diagnostic region (DF3) of *Rns* permitted the genotypic identification of acanthamoebae in cultures that contained other microorganisms.

MATERIALS AND METHODS

Cultures containing *Acanthamoeba* were obtained from patients and their homes in Hong Kong. The amoebae were cultured on agar plates at the Chinese University of Hong Kong but were not axenized (3). *Rns* genotyping of patient 120 (P120) isolates was done at the Chinese University of Hong Kong by Y.-W. Chu and E. Houang according to procedures described by Schroeder et al. (6). G. C. Booton and D. J. Kelly performed *Rns* genotyping of the remaining isolates at Ohio State University (OSU). DNA was extracted as previously described (4) or by extraction with a commercially available DNA extraction kit: DNeasy (Qiagen, Inc., Valencia, Calif.). Amplicon ASA.S1 was amplified by PCR with the genus-specific primers JDP1 (5′-GGCCCAGATCGTTTACCGTGAA-3′) and JDP2 (5′-TCTCACAAGCTGCTAGGGGAGTCA-3′) after DNA extraction (6). Direct sequencing of the PCR product was done with an ABI 310 automated fluorescent sequencing system (Applied Biosystems, Foster City, Calif.) with the conserved primers 892 (5′-CCAAGAATTTCACCTCTGAC-3′) and 892C (5′-GTCAGAGGTGAAATTCTTGG-3′) to determine the primary DNA sequence of DF3 of *Rns* (6). The production of two or more different ASA.S1 PCR products from a single isolate was indicated when direct sequencing of the products revealed multiple peaks in the electropherogram. When multiple sequences were observed, ASA.S1 PCR products were ligated into a plasmid vector by using a T/A Cloning Kit (Invitrogen, Inc., Carlsbad, Calif.). Positive clones were identified and sequenced with the ABI 310 system with primer 892C. The 22 Hong Kong DF3 sequences obtained in this study were aligned to 38 previously determined sequences that are a subset of the OSU *Acanthamoeba* nuclear small subunit ribosomal DNA (rDNA) Database (Department of Molecular Genetics, OSU [www.biosci.ohio-state.edu/tbyers/byers.htm]). Putative sequence differences in the newly acquired Hong Kong DF3 sequences were confirmed by repeated sequencing and by examination of the primary data electropherogram. DF3 sequences that differed consistently by a single nucleotide or more were defined as different sequences in this study. The DF3 sequence nomenclature used in this study includes two parts. The first part is the *Rns* genotype of the specimen (previously called the "sequence type" [2, 7]). The genotype is a clade determined by phylogenetic analysis of sequence variation of DF3 or analysis of the complete *Rns* gene if available (7). The DF3 fragment identifies the same genotypes as are identified by using the complete *Rns* sequence (6). The second part of the nomenclature is a unique code assigned to the specific DF3 sequence. For example, a specimen with a T4 genotype and sequence code #1 is designated T4/1. A DF3 sequence alignment for the Hong Kong specimens is provided in

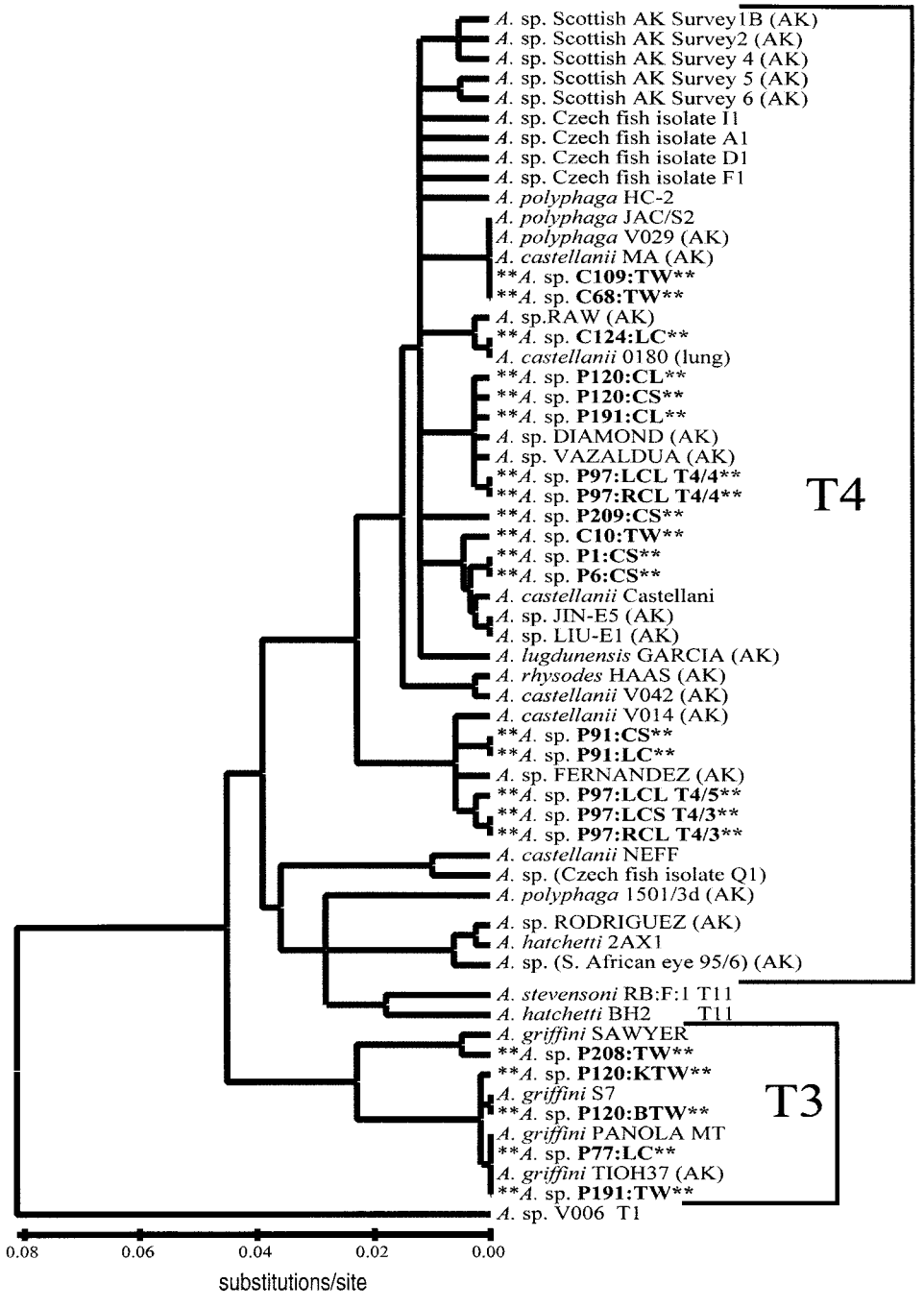

FIG. 2. 18S rDNA DF3 linearized neighbor-joining gene tree with Hong Kong isolates. Sequences from Hong Kong isolates are shown in bold font. "(AK)" identifies specimens previously isolated from AK patients. The T1, T3, T4, and T11 designations shown on the tree correspond to strains previously determined to be of that particular genotype (6, 7).

Fig. 1 and an alignment of the Hong Kong DF3 sequences with those from other *Acanthamoeba* strains is available from G. C. Booton. Phylogenetic reconstruction produced gene trees by using maximum-parsimony, neighbor-joining, and minimum evolution methods in the phylogenetic computer program MEGA2 (Molecular Evolutionary Genetic Analysis software, ver. 2.1 [S. Kumar et al., Arizona State University, Tempe; http://www.megasoftware.net/]). *Acanthamoeba* sp. strain V006, an *Rns* genotype T1 strain that is an outgroup to sequence types T3, T4, and T11, was used to root the trees. Figure 2 is a linearized neighbor-joining tree, obtained by using the Kimura two-parameter distance algorithm, produced in MEGA2. The Hong Kong isolates' source, determined *Rns* genotypes, and DF3 sequences are listed in Table 1. The isolates also are identified in the tree (Fig. 2).

Sequences determined in the current study were deposited in GenBank and are available under accession numbers AF441793 to AF441814. Other *Acanthamoeba* sequences used in this study are available in GenBank under the following accession numbers: *A. castellanii* strain Castellani, U07413 (2); *A. castellanii* strain Ma, U07414 (2); *A. castellanii* strain Neff, U07416 (2); *A. castellanii* 0180, U07405 (2); *A. castellanii* V014, U07401 (2); *A. castellanii* V042, U07403 (2); *A. griffini* Panola Mt., AF019052 (7); *A. griffini* Sawyer, AF019053

TABLE 1. Source, *Rns* genotypes, and unique DF3 sequences of Hong Kong *Acanthamoeba* keratitis cultures and controls

Culture designation[a]	Culture source	*Rns* genotype/DF3 sequence
Patient samples		
P1:CS	Corneal scrape	T4/1
P6:CS	Corneal scrape	T4/1
P77:LC	Lens case	T3/1
P91:CS	Corneal scrape	T4/2
P91:LC	Lens case	T4/2
P97:LCS	Lens case solution	T4/3
P97:LCL	Left contact lens	T4/4, T4/5
P97:RCL	Right contact lens	T4/3, T4/4
P120:CS	Corneal scrape	T4/6
P120:CL	Contact lens	T4/6
P120:KTW	Kitchen faucet	T3/2
P120:BTW	Bathroom basin	T3/3
P191:CL	Contact lens	T4/6
P191:TW	Faucet	T3/4
P208:TW	Faucet	T3/5
P209:CS	Corneal scrape	T4/7
Control samples		
C10:TW	Faucet	T4/8
C68:TW	Faucet	T4/9
C109:TW	Faucet	T4/9
C124:LC	Lens case	T4/10

[a] Sample abbreviations: P, patient; C, control; CS, corneal scrape; LC, lens case; LCS, lens case solution; LCL, left contact lens; RCL, right contact lens; KTW, kitchen tap water; BTW, bathroom tap water; CL, contact lens; TW, tap water.

(7); *A. griffini* S7, U07412 (2); *A. griffini* TIOH37, S81337 (5); *A. hatchetti* BH2, AF019068 (7); *A. hatchetti* 2AX1, AF019060 (7); *A. lugdunensis* Garcia, U07407 (2); *A. polyphaga* HC2, AF019056 (7); *A. polyphaga* Jac/S2, U07415 (2); *A. polyphaga* V029, U07402 (2); *A. polyphaga* 1501/3D, AF019062 (7); *A. rhysodes* strain Haas, U07406 (2); *Acanthamoeba* sp. strain Czech A1, AF140711 (1); *Acanthamoeba* sp. strain Czech D1, AF140713 (1); *Acanthamoeba* sp. strain Czech F1, AF140721 (1); *Acanthamoeba* sp. strain Czech I1, AF140719 (1); *Acanthamoeba* sp. strain Czech Q1, AF140715 (1); *Acanthamoeba* sp. strain Diamond, AF019057 (7); *Acanthamoeba* sp. strain Fernandez, U07409 (2); *Acanthamoeba* sp. strain Jin E5, AF019054 (7); *Acanthamoeba* sp. strain Liu E1, AF019055 (7); *Acanthamoeba* sp. strain Rawdon, U07410 (2); *Acanthamoeba* sp. strain Rodriguez, AF019059 (7); *Acanthamoeba* sp. strain S. African AK 95/6, AF343836 (6); *Acanthamoeba* sp. strain Vazaldua, AF019058 (7); *Acanthamoeba* sp. strain V006, U07400 (2); *Acanthamoeba* sp. strain Scottish AK Survey 1B-*Acanthamoeba* sp. strain Scottish AK Survey 6, AF343559 to AF343563 (6); and *A. stevensoni* RB:F:1, AF019069 (7).

RESULTS AND DISCUSSION

DF3 sequences. DF3 sequence determination from the 20 isolates examined resulted in 22 DF3 sequences. Isolates from patient 97 (P97) had multiple sequences (Fig. 1; Table 1). Of the 22 sequences obtained, 15 were unique (~68%). Of the 15 unique sequences, 10 were phylogenetically similar to other genotype T4 *Rns* isolates previously identified, whereas the remaining 5 unique sequences were similar to genotype T3 isolates. No sequences were obtained that were similar to any *Acanthamoeba Rns* genotype other than T3 or T4. Once obtained, the DF3 sequences allowed us to address our initial hypothesis regarding the genotype and potential source of AK infections. Initially, we examined sequences that were from corneal scrape and contact lens isolates.

All corneal scrape and contact lens isolates from AK patients were *Rns* genotype T4. Table 1 summarizes the genotype results obtained from the Hong Kong isolates. The DF3 regions of the *Rns* genes of five corneal scrape specimens from patients with AK were amplified and sequenced. Sequences of the variable regions of DF3 in the Hong Kong samples are presented in Fig. 1. All five corneal scrape isolates (P1:CS, P6:CS, P91:CS, P120:CS, and P209:CS) were identified as *Rns* genotype T4 (Fig. 2). Likewise, the isolates obtained from contact lenses of AK patients P97, P120, and P191 were genotype T4. This is in agreement with recent results of other studies that have shown that the majority of AK cases are associated with this genotype (6, 7, 8). Patients P1 and P6 had identical corneal scrape DF3 sequences, suggesting infection with the same or closely related strains, but different sequences were found in corneal scrape isolates from each of the three other patients.

The determined genotype sequence of the contact lens specimen of patient P120 was T4/6, which was identical to that of this patient's corneal scrape isolate sequence (as would be expected). It also was identical to the lens isolate of P191, suggesting that these two patients might have been infected by the same strain, although no corneal scrape specimen was available for P191. No corneal scrape specimen was available for patient P97 either, but this patient was unique in that two DF3 sequences were obtained from the contact lenses from each eye. Specimens with sequences T4/3 and T4/4 were cultured from the left lens, and specimens with sequences T4/4 and T4/5 were cultured from the right lens. Because both lens specimens shared sequence T4/4, it is likely that they originally came from a common source such as the lens case. However, only sequence T4/3 was obtained from P97's lens case solution. Thus, T4/4 and T4/5 specimens possibly either came from a different source such as airborne cysts, or had been in, but were subsequently eliminated from, the lens case or were present in the case but escaped detection.

***Rns* genotype T3 was only found in one lens case and in several water faucet samples.** Although the large majority of AK cases have involved amoebae with genotype T4, a few cases have involved the closely related genotype T3 (5, 7). In the present study contact lens case specimens were available from three AK patients and one non-AK control. The lens case or lens case solution isolates from patients P91 and P97, and from control subject C124, were all genotype T4. The lens case isolate from patient P77 was unique in being the only genotype T3 specimen isolated in this study from any source other than water faucets. Unfortunately, there were no other specimens from this patient. The lens case and corneal scrape isolates from P91 both had sequence T4/2. Thus, the lens case was again a likely source of the infection, although a direct infection of the eye which subsequently contaminated the case cannot be ruled out.

When the T3/2 and T3/3 sequences of specimens from AK patient P120's kitchen faucet and bathroom basin were determined, both clustered phylogenetically with *Rns* genotype T3 strains (Fig. 2). Thus, neither specimen could be the source of this patient's *Rns* genotype T4 eye infection. In addition to being distinct from the P120 corneal scrape specimen, the two water faucet isolates were also different from one another. Therefore, more than one *Acanthamoeba* strain probably was present in the patient's home water supply. Our inability to

detect the strain that infected the eye of this patient could be due to its absence from the water supply, at least at the time of sampling. In this case, the infection may have originated at some other point in the sequence of steps linking the eye and the water supply. Alternatively, we simply may have failed to culture it. Answering the question of whether home water supplies are the usual source of AK infections will require a larger study with more complete data sets. It also will require the cloning of amoebae when multiple sequences are detected to determine whether the sequences are from different strains or simply different alleles from a single strain.

***Rns* genotypes T3 and T4 were equally common in home faucet water.** The relative abundance of *Acanthamoeba* isolates with genotype T4 *Rns* sequences in the environment has been proposed as one explanation for the preponderance of genotype T4 in AK infections (7). No population study has been done to test this hypothesis but, in this regard, observations of the genotypes found in home water faucets in this study are of interest. Water faucet cultures were obtained from the homes of three AK patients and three non-AK controls. The *Rns* genotypes obtained from the AK patients' homes were all T3, and the genotypes from the control homes were all T4. Although the results are limited, they suggest that the occurrence of T3 strains in home water supplies, at least, may be more common than the very low frequency of these strains in AK infections. It is interesting in this regard that the single case in which an AK infection has been unequivocally linked to the home water supply involved a T3 strain in Scotland (5).

The relatively rapid DNA typing method used here can identify individual strains and trace potential paths of AK infections. In cases where it is critical to have a higher level of confidence about a result in which DF3 sequences are identical, cultures can be axenized and the complete *Rns* sequence can be determined and compared.

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