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Molecular and Physiological Evaluation of Subtropical Environmental Isolates of *Acanthamoeba* spp., Causal Agent of *Acanthamoeba* Keratitis

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ABSTRACT. Previous molecular examination of *Acanthamoeba* spp. has resulted in the determination of distinct genotypes in this genus (designated T1-T12, T14). Genotype T4 has been responsible for the majority of cases of *Acanthamoeba* keratitis. Here we examine the relative abundance of environmental T4 isolates on beaches and ask whether they have temperature and salinity tolerances that could enhance pathogenicity. Twenty-four *Acanthamoeba* strains were isolated from beach sand (n = 20), soil (n = 3), and tap water (n = 1) in south Florida. Phylogenetic analysis identified 19 of 24 isolates as T4, the *Acanthamoeba* keratitis-associated genotype. The remaining isolates were genotype T5 (4) and T11 (1). Nearly all beach isolates were genotype T4, whereas the tap water and soil isolates were mostly T5. All amoebae grew at 0, 1.0, and 2.0% salt and 19 of 20 beach isolates also grew at 3.2%. No soil or tap-water acanthamoebae reproduced at 3.2%. All isolates grew at 37 °C and two (T5) at 42 °C. Little correlation existed between beach location, salt-tolerance, and genetic relatedness. Overall, the large majority of environmental isolates obtained were genotype T4, suggesting it may be the most common genotype in this environment and could be a potential source of *Acanthamoeba* keratitis infections.

Key Words. Amoeba, evolution, phylogenetics, ribosomal RNA.

THE naked free-living amoebae (those amoeba lacking tests, walls or tecta with openings) are a diverse assemblage of protists that inhabit almost every environmental niche, including fresh and salt water, soils, ocean sediments, air, plants, sewage, and animal tissues (John 1993; Marciano-Cabral et al. 2000; Marciano-Cabral and Cabral 2003). Many have a dual life cycle alternating between a vegetative trophozoite stage and a dormant cyst stage, depending on environmental conditions. A few species are pathogens of humans, including *Naegleria fowleri*, *Balamuthia mandrillaris*, and *Acanthamoeba* spp. Infections from these amoebae are rare, but when they occur in the brain, death often occurs (De Jonckheere 2002; Marciano-Cabral and Cabral 2003; Martinez and Visvesvara 1997; Visvesvara et al. 1993). In otherwise healthy individuals, *Acanthamoeba* can cause amoebic keratitis (AK), a very painful and sight-threatening infection of the cornea (Seal et al. 1998). In immunocompromised individuals, it can cause the fatal brain infection granulomatous amoebic encephalitis (GAE), as well as infections of the skin and organ tissues. Recently, three other genera of free-living amoebae (*Vahlkampfia*, *Hartmannella*, and *Sappinia*) have also been implicated in human disease (Aitken et al. 1996; Dua et al. 1998; Inoue et al. 1998; Kennedy et al. 1995).

Although opportunistically pathogenic amoebae are classified as freshwater or soil protists, they may be able to survive in marine conditions, especially as the distinction between marine and freshwater amoebae is becoming increasingly gray (Hauer et al. 2001). A recent study of a south Florida beach showed that naked amoebae (marine forms) averaged 4,236 amoebae cm^{-3} in wet sand (Rogerson et al. 2000). The present study documents the presence of free-living (freshwater) amoebae in beach sand, and focuses on acanthamoebae since these are the most frequently encountered of all the potentially problematic amoebae. Depending on environmental conditions, *Acanthamoeba* can be found in either of two stages, a mobile trophozoite form characterized by distinctive acanthapodia and a stellate cyst stage, which is resistant to degradation. While it is unlikely that the presence of *Acanthamoeba* in beach sand poses a serious health hazard, their presence should be investigated since sand particles trapped beneath a contact lens could easily abrade the corneal epithelium exposing it to bacteria and amoebae that may be adhered to the lens.

The genus *Acanthamoeba* contains more than 20 named species, which comprise three morphological groups based on various, mostly cyst, characteristics (Pussard and Pons 1977). Molecular analyses of the nuclear small subunit (ssu) ribosomal RNA gene (*Rns*) have identified thirteen *Acanthamoeba* genotypes, T1–T12 and T14 (Gast 2001; Stothard et al. 1998). These genotypes support the morphological group designations of the genus, but have not supported all named species of the genus as monophyletic clades. The genotype designations have been supported by more recent analyses using the mitochondrial small subunit ribosomal RNA gene (Ledee et al. 2003). One genotype, designated T4, has been found in nearly all AK infections. Rarely, genotypes T3 (Ledee et al. 1996) and T11 (Khan et al. 2002), and in one case genotype T6 (Walochnik et al. 2000a) have been observed in AK infections. Other infections caused by *Acanthamoeba* spp. (eg. infections of the lung, skin, and brain) have been caused by a wider number of *Acanthamoeba* genotypes including T1, T4, T10, and T12 (Stothard et al. 1998).

In an attempt to determine the quantity of sequence data from the rDNA gene that are required to establish a genotype, we identified three highly informative regions of the *Rns* that could produce phylogenetic trees that are as robust as those based on the entire gene. These were designated diagnostic fragments 1, 2, and 3 (DF1, DF2, and DF3). Further, we found that while not as robust for phylogenetic tree building, a single highly variable and highly informative region (DF3) could be used to identify genotypes rapidly (Booton et al. 2002; Schroeder et al. 2001). That is, genotype classification based on DF3 is the same as those based on the entire ssu rDNA sequence (Booton et al. 2002). Thus, the sequence of the DF3 region was used here to identify the *Rns* genotypes of the isolates of *Acanthamoeba* spp.

In addition to genotype as a marker for potential pathogenicity of *Acanthamoeba* spp., the ability to grow in salt concentrations similar to those found in tear fluid (ca. 10 parts per thousand [ppt], or 1.0%) and the ability to grow, or tolerate, body physiological temperatures might also be important fac-

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tors in pathogenicity. Thus, we have studied the response of the *Acanthamoeba* beach isolates to different salinities (0, 1.0, 2.0 and 3.2%) and temperatures (37, 40 and 42 °C).

MATERIALS AND METHODS

Sampling sites and isolation of amoebae. Samples were collected during low tide from Fort Lauderdale Beach (26° 07′ 17.35″N, 80° 06′ 14.24″ W), Hollywood Beach (26° 02′ 02.56″N, 80° 06′ 50.36″ W), and Hobe Beach (25° 44′ 22.5″ N, 80° 10′ 18.7″ W) (southeastern Florida, USA) over a seven-month period (August 2001 to February 2002). On each outing, three samples were processed from each beach. Water was collected at 5 m from shore, wet sand was collected from the top 10 cm of sand located halfway between the high-tide mark and current water level, and dry sand was sampled from the top 10 cm of sand 5 m above the high-tide mark. Water samples (100 ml) were concentrated to 1 ml by centrifugation for 15 min at 300 *g*. Sand samples (200 g) were shaken vigorously for 1 min with 500 ml of phosphate-buffered saline and 50 ml of the suspension was concentrated by centrifugation as described above. Drops (ca. 100 μl) of concentrate were placed at the end of a 1-mm-wide *Escherichia coli* streak spread across the diameter of a 100 × 15 mm Petri dish containing non-nutrient agar (NNA: 15 g agar in 1 liter amoeba saline, Page 1988). After incubating for 2 wk at room temperature (ca. 23 °C) amoebae were harvested by flushing the agar surface with amoeba saline and the amoebae that grew were observed at 600× using phase contrast microscopy. Where possible, amoebae were identified by light microscopical features using the keys of Page (1983, 1988). In many cases, identification to species, or even genus, was impossible without electron microscopy so some isolates are listed as morphotypes (presumed different species).

Clonal isolation of *Acanthamoeba*. Before processing for species identification, plates were examined using a dissecting microscope to identify regions containing *Acanthamoeba*. Any amoeba resembling acanthamoebae (based on cyst morphology) was subcultured onto a fresh NNA plate with *E. coli*. If *Acanthamoeba* growth was verified on the second plate, individual cells that had migrated away from the bacterial smear were transferred to a fresh agar/*E. coli* plate. This procedure was repeated until a clonal culture was established. A total of twenty *Acanthamoeba* clones was obtained from the south Florida beach samples. For comparison, three clones of acanthamoebae were also isolated from soil near Hollywood Beach, Florida and one clone was isolated from a temperate beach in Irvine, Scotland (55.6° N, 4.6° W).

In addition to the beach and soil isolates examined in this study, other isolates of *Acanthamoeba* were studied for comparison. One clone was isolated from tap water from an apartment complex in Fort Lauderdale, Florida. This sample was obtained by insertion of a sterile swab several centimetres into a cold-water faucet, and used to scrape off some of the biofilm of the faucet. The swab head was place on NNA with *E. coli* as a food source. After incubation, acanthamoebae that grew out on the agar surface, were subsequently clonally isolated as described above, and included in this study. Six additional tapwater isolates and five corneal scrape isolates from a previous study in Hong Kong (Booton et al. 2002) were also considered in this study; these isolates were supplied from stock cultures maintained by G. Booton (OSU). Lastly, *A. polyphaga* obtained from the Culture Collection of Algae and Protists (CCAP) was included for comparison.

These novel clonal cultures of *Acanthamoeba* (as cysts) were sent to Ohio State University (OSU) for molecular typing. Upon arrival at OSU, clonal isolates were subcultured onto fresh NNA plates containing living or heat-killed *Enterobacter* spp. Cultures were then subsequently transferred to liquid culture using Optimal Growth Media (OGM) (Byers et al. 1980) or Bacto-Casitone/Serum (BCS) media (Cerva 1980), modified with the substitution of adult bovine serum for rabbit serum, in 25-cm^2 culture flasks at 27 °C.

DNA extraction and PCR. DNA was extracted as previously described (Schroeder et al. 2001) or by extraction using the DNeasy kit (Qiagen, Inc., Valencia, CA). *Rns* genotyping of environmental isolates was done using previously published procedures (Booton et al. 2002; Schroeder et al. 2001). Amplicon ASA.S1, containing DF3, was amplified by PCR using genus-specific primers JDP1 (5′-GGCCCAGATCGTTTACCGTGAA-3′) and JDP2 (5′-TCTCACAAGCTGCTAGGGGAGTCA-3′) following DNA extraction (Schroeder et al. 2001). Since these primers are genus-specific they are not affected by co-extraction of bacterial DNA from *Acanthamoeba* cultures that contain *Enterobacter* as a food source. PCR conditions were the following: an initial denaturing step of 7 min at 95 °C followed by 40 cycles of 1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C. This was followed by a final extension of 15 min at 72 °C. Amplicons of approximately 470 bp from *Acanthamoeba* cultures were visualized on a 1% agarose gel following electrophoresis of 20% of the PCR reaction and staining with ethidium bromide. The remaining PCR product was prepared for sequencing by using the Qiaquick PCR clean-up kit (Qiagen, Inc.). Samples were typically eluted in 30–50μl of Qiagen elution buffer and 3.5 μl of purified sample was routinely used for sequencing reactions.

Sequencing, alignment, and phylogenetic analysis. Direct sequencing of the PCR product was done with an ABI 310 automated fluorescent sequencing system (Applied Biosystems, Foster City, CA) using *Rns* conserved primers 892 (5′-CCAAGAATTTCACCTCTGAC-3′) and 892C (5′-GTCAGAGGTGAAATTCTTGG-3′) to determine the primary DNA sequence of DF3 of *Rns* (Schroeder et al. 2001). Sequence differences 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. Genotype determination was done by phylogenetic analysis of sequence variation of DF3. In some isolates we determined the entire *Rns* sequence for comparison to the results using DF3. In all instances in this study, as in previous work, DF3 fragment sequence analysis identified the same genotypes as identified using complete *Rns* genes. The 24 sequences obtained in this study were aligned to each other and to 80 previously determined sequences using the computer sequence alignment program ESEE (Cabot and Beckenbach 1989). These sequences are a subset of the OSU *Acanthamoeba* Nuclear Small Subunit Ribosomal DNA (SSU rDNA) Database (www.biosci.ohio-state.edu/tbyers/byers.htm). The alignment of the genotypically informative region of DF3 (105 bp) was used to calculate dissimilarity values using the Kimura 2-parameter algorithm in the phylogenetic analysis computer program MEGA2 (Kumar et al. 2001). Gaps were excluded from distance calculations using the pair-wise deletion option in MEGA2. Phylogenetic reconstruction also was done in MEGA2, which was used to produce gene trees using neighbor-joining and minimum evolution methods.

Statistical analyses. Statistical data analyses including average, variance, standard deviation, correlation, and analysis of variance (ANOVA) of migration data were performed using the computer program Statview (Abacus Concepts, Inc., Berkeley, CA).

GenBank references. Sequences determined in the current study were deposited in GenBank and are available through

accession numbers AY343661-AY343684. Other sequences used in this study are available in GenBank under the following accession numbers: *Acanthamoeba astronyxis* AF019064; *Acanthamoeba castellanii* Castellani, U07413; *A. castellanii* Ma, U07414; *A. castellanii* Neff, U07416; *A. castellanii* 0180, U07405; *A. castellanii* V014, U07401; *A. castellanii* V042, U07403; *Acanthamoeba comandoni* AF019066; *Acanthamoeba culbertsoni* A1, AF019067; *Acanthamoeba griffini* Panola Mt., AF019052; *A. griffini* Sawyer, AF019053; *A. griffini* S7, U07412; *A. griffini* TIOH37, S81337; *Acanthamoeba hatchetti* BH2, AF019068; *A. hatchetti* 2AX1, AF019060; *Acanthamoeba healyi* V013, AF019070; *Acanthamoeba lenticulata* JC1, U94739; *A. lenticulata* 7327, U94731; *A. lenticulata* 72/2, U94732; *A. lenticulata* 45, U94730; *A. lenticulata* 407–3A, U94734; *A. lenticulata* NJSP, U94738; *A. lenticulata* E18, U94735; *A. lenticulata* 53–2, U94737; *A. lenticulata* 118, U94736; *A. lenticulata* 25–1, U94740; *A. lenticulata* SAWS 87/1, AF343802; *A. lenticulata* SAWS 87/2, AF343806; *A. lenticulata* SAWS 87/3, AF343809 AF343; *Acanthamoeba. lugdunensis* Garcia, U07407; *Acanthamoeba palestinensis* Reich, U07411; *A. palestinensis* 2802, AF260719; *Acanthamoeba polyphaga* HC2, AF019056; *A. polyphaga* Jac/S2, U07415; *A. polyphaga* V029, U07402; *A. polyphaga* 1501/3C, AF239298; *A. polyphaga* 1501/3D, AF019062; *A. polyphaga* Page23, AF019061; *Acanthamoeba pustulosa* GE3a, AF019050; *Acanthamoeba rhysodes* Haas, U07406; *A. rhysodes* Singh, ARU07406; *Acanthamoeba tubiashi* AF019065; *Acanthamoeba* sp. Czech 4339, AF140711; *Acanthamoeba* sp. Czech 4706, AF140712; *Acanthamoeba* sp. Czech 43337, AF140713; *Acanthamoeba* sp. Czech 4528, AF140715; *Acanthamoeba* sp. Czech 4178, AF140716; *Acanthamoeba* sp. Czech 3668, AF140717; *Acanthamoeba* sp. Czech 4465, AF140718; *Acanthamoeba* sp. Czech 4422, AF140719; *Acanthamoeba* sp. Czech G1, AF140720; *Acanthamoeba* sp. Czech 4436, AF140721; *Acanthamoeba* sp. Diamond, AF019057; *Acanthamoeba* sp. Fernandez, U07409; *Acanthamoeba* sp. Galka JAC324, ASU07408; *Acanthamoeba* sp. Jin E5, AF019054; *Acanthamoeba* sp. Liu E1, AF019055; *Acanthamoeba* sp. Rawdon, U07410; *Acanthamoeba* sp. Rodriguez, AF019059; *Acanthamoeba* sp. Vazaldua, AF019058; *Acanthamoeba* sp. V006, U07400; *Acanthamoeba* sp V125, ASU07404; *Acanthamoeba* sp.SAWE 95/6, AF343836; *Acanthamoeba* sp. SAWE 94/5, AF343833; *Acanthamoeba* sp. SAWE 94/4, AF343830; *Acanthamoeba* sp. SAWE 93/3, AF343827; *Acanthamoeba* sp. SAWE 92/2, AF343824; *Acanthamoeba* sp. SAWE 90/1, AF343821; *Acanthamoeba* sp. SAWL 93/1, AF343818; *Acanthamoeba* sp. SAWL 91/4, AF343815; *Acanthamoeba* sp. SAWL 91/3, AF343810; *Acanthamoeba* sp: HK P1 CS, AF441812; *Acanthamoeba* sp. HK P6 CS, AF441811; *Acanthamoeba* sp. HK P91CS, AF441808; *Acanthamoeba* sp. HK P91 LC, AF441807; *Acanthamoeba* sp. HK P120 CS, AF441810; *Acanthamoeba* sp. HK P209 CS, AF441802; *Acanthamoeba* sp. HK C68, AF441803; *Acanthamoeba* sp. HK C109, AF441804; *Acanthamoeba stevensoni*, AF019069.

Migration rate (growth), salt tolerance, and temperature tolerance. *Acanthamoeba* clones were inoculated onto the end of an *E. coli* streak spanning the diameter of a 15 × 100 mm Petri dish containing non-nutrient agar adjusted to 0, 1.0, and 2.0% salt using NaCl, and 3.2% using sea water. All inoculations were repeated in triplicate and incubated at 23 °C. Growth was indirectly determined by measuring the migration rate (mm/h) of the amoebae moving along the bacterial streak, since amoebae replicated as they migrated. Measurements were obtained every 12 h (or more frequently when migration was rapid) for two weeks, or until the amoebae had exhausted the bacteria. The maximum rates of migration were calculated for each strain at the different salt concentrations.

To examine temperature tolerance, non-nutrient agar plates (at 0% NaCl), streaked with *E. coli* as described above, were inoculated with *Acanthamoeba* and incubated at 37 °C, 40 °C, and 42 °C (n = 3). After five days, the plates were examined for amoebae using a dissecting microscope.

Table **1**. Proportion of samples containing 'freshwater' naked amoebae isolated from seawater and sand collected from Hollywood, Fort Lauderdale, and Hobe beaches in south Florida.

Identity	Frequency occurrence (%)[a]		
	Wet sand	Dry sand	Seawater
Sacchamoeba	8	8	0
Hartmannella	8	8	8
Stygamoeba	24	24	0
Willartia magna	4	4	0
Naegleria-like	20	16	4
Cochliopodium	8	0	0
Acanthamoeba	32	44	0
Rhizamoeba	24	16	0
Vexillifera	16	0	0
Stachyamoeba	0	4	4
Platyamoeba linguiform	4	0	4
Thecamoeba	4	4	0
vahlkampfiid[b]	4	8	0
vannellid sp. 1[b]	8	4	0
vannellid sp. 2[b]	0	12	0
vannellid sp. 3[b]	0	0	4
vannellid sp. 4[b]	4	0	0
mayorellid sp. 1[b]	12	8	0
mayorellid sp. 2[b]	0	4	0
leptomyxid[b]	0	4	0
unidentified sp. 1[b]	4	16	8
unidentified sp. 2[b]	4	0	0
unidentified sp. 3[b]	28	12	0
unidentified sp. 4[b]	4	0	4

[a] There was no difference between amoeba populations at these three beaches, so the data are pooled. The percentage frequency occurrence in the three sampled sites is given.

[b] Amoebae that could not be identified to a particular genus by light microscopy were referred to as morphotypes, and itemized in this table by type, or as unidentified species.

RESULTS

Beach amoebae. Of 90 samples processed for 'freshwater' amoebae over the seven-month sampling period, 24 different morphotypes (= species) were identified (Table 1). These freshwater amoebae were all isolated from salty environments. The salinity of the coastal sea water was approximately 3.2%, the wet sand averaged 3.1% and the dry sand 2.3% (Bonilla, unpubl. data). No distinction was made between trophic and cyst stages, so some of these isolates may have grown from cysts in the water column or from cysts lodged in the sand.

Among the most frequently encountered isolates were several genera with possible pathogenic connections. *Acanthamoeba* spp. were present in 23 of the 60 (38%) wet or dry sand samples making it the most commonly isolated morphotype in this study. It was not, however, detected in the 30 seawater samples processed. *Naegleria*-like amoebae were present in 20% of the wet sand samples, 16% of the dry sand samples, and in 4% of the water samples. Light microscopy alone is not sufficient to unambiguously identify the genus *Naegleria* from the other morphologically similar genera of the Heterolobosea (sensu Page 1988), thus only some of these isolates are likely to belong to the genus *Naegleria*. *Hartmannella* spp. were isolated from

Table **2**. Summary of physiological and molecular results of *Acanthamoeba* sp. isolates examined in the current study.

Clone #	Location isolated[a]	Genotype[d]	Salinity migration rate (mm/hr)[b] 0%	1.0%	2.0%	3.2%	Growth[c] 37 C	Growth 40 C	Growth 42 C
Fla1	Hollywood Beach wet sand	T4	0.36	0.49	0.43	0.09	+	−	−
Fla2	Hollywood Beach wet sand	T4	0.49	0.55	0.5	0.01	+	−	−
Fla19	Hollywood Beach wet sand	T4	1.02	0.43	0.74	0.55	+	−	−
Fla5	Hollywood Beach dry sand	T4	0.82	0.79	0.64	0	+	−	−
Fla8	Ft. Lauderdale Beach wet sand	T4	0.68	0.67	0.77	0.48	+	−	−
Fla15	Ft. Lauderdale Beach wet sand	T4	0.51	0.55	0.68	0.07	+	−	−
Fla6	Ft. Lauderdale Beach dry sand	T4	0.63	0.79	0.71	0.11	+	−	−
Fla11	Ft. Lauderdale Beach dry sand	T4	0.59	1.00	0.33	0.11	+	−	−
Fla20	Ft. Lauderdale Beach dry sand	T4	0.73	0.54	0.46	0.04	+	−	−
Fla7	Hobe Beach wet sand	T4	0.51	0.36	0.2	0.01	+	−	−
Fla12	Hobe Beach wet sand	T4	0.64	0.82	0.59	0.31	+	−	−
Fla14	Hobe Beach wet sand	T4	0.69	0.68	0.7	0.17	+	−	−
Fla16	Hobe Beach wet sand	T4	0.45	0.65	0.4	0.04	+	−	−
Fla3	Hobe Beach dry sand	T5	0.27	0.22	0.11	0.07	+	+	+
Fla4	Hobe Beach dry sand	T4	0.56	0.56	0.4	0.02	+	−	−
Fla9	Hobe Beach dry sand	T4	0.62	0.93	0.74	0.62	+	−	−
Fla10	Hobe Beach dry sand	T4	0.71	0.78	0.79	0.19	+	−	−
Fla17	Hobe Beach dry sand	T4	0.58	0.83	0.32	0.07	+	−	−
Fla18	Hobe Beach dry sand	T4	0.68	0.59	0.67	0.14	+	−	−
Fla13	Hobe Beach dry sand	T4	0.86	0.64	0.5	0.05	+	−	−
Fla21	Hollywood soil	T11	0.12	0.36	0.18	0	+	+	−
Fla22	Hollywood soil	T5	0.76	0.65	0.22	0	+	+	−
Fla23	Hollywood soil	T5	1	0.88	0.5	0	+	+	−
IV	Tapwater	T5	0.64	0.54	0.15	0	+	+	+
DS	Scottish beach	T4	0.54	1.27	0.58	0.08	+	−	−
CCAP	*A. polyphaga*	n.d.	0.16	0.12	0.06	0	+	−	−
P1[e]	Corneal scrape	T4	0.5	0.43	0.41	0.02	+	−	−
P209[e]	Corneal scrape	T4	0.44	0.42	0.37	0.06	+	−	−
P6[e]	Corneal scrape	T4	0.67	0.44	0.56	0	+	−	−
P91[e]	Corneal scrape	T4	0.41	0.37	0.34	0.09	+	−	−
P120[e]	Corneal scrape	T4	0.4	0.43	0.28	0.25	+	−	−
P208[e]	Home water	T3	0.35	0.4	0.31	0.15	+	+	−
P191[e]	Home water	T3	0.6	0.84	0.68	0.21	+	−	−
P120[e]	Kitchen tap	T3	0.55	0.39	0.3	0.13	+	−	−
c10[e]	Home tap water	T4	0.32	0.28	0.19	0.02	+	−	−
c109[e]	Home tap water	T4	0.43	0.39	0.2	0.13	+	−	−
c68[e]	Home tap water	T4	0.58	0.71	0.63	0.38	+	−	−

[a] Geographical location that was the source of *Acanthamoeba* spp. isolates examined in the current study.

[b] Distances migrated by *Acanthamoeba* spp. cultures in mm/hr across a NNA plate (23 °C) as a measure of growth of the culture, in 0, 1.0, 2.0, and 3.2% salinity agar. NaCl was used for salt concentrations of 1.0, and 2.0%. Saltwater was used for 3.2% experiments. All experiments were repeated 3× and averages determined.

[c] Growth of *Acanthamoeba* spp. by inoculation of ameba on NNA plates streaked with *E. coli*. Plates were examined after five days with a dissecting microscope to determine growth of ameba culture; scored as + for growth, or − for no growth.

[d] *Acanthamoeba* spp. genotype determination based on sequence analysis of diagnostic fragment 3 (DF3), a subset of the nuclear small subunit ribosomal RNA gene.

[e] Genotype determination of these *Acanthamoeba* spp. isolates was determined previously in Booton et al. 2002.

both sand and sea water, and vahlkampfiids were found in the sand. On one occasion, a large leptomyxid amoeba, reminiscent of *Balamuthia*, was isolated from dry sand at Hobe Beach. In all the above cases, it must be stressed that these genera are all common in fresh water and soils and that opportunistic infections by species within these genera are extremely rare. Amoebae were isolated more frequently from sand samples compared to water samples ($p < 0.001$). There was no statistical difference between the frequency of amoebae isolated from wet sand compared to dry sand ($p = 0.22$). As discussed, 24 different species (or morphotypes not identifiable to species) were identified. However, it should be noted that these results are not a direct reflection of the abundance of these organisms in these natural environments since these taxa were identified following enrichment and culture under laboratory conditions. Nonetheless, the results are an estimate of amoebae species diversity in these environments at the time of sampling.

Molecular analysis. *Acanthamoeba* spp. isolates were characterized with respect to the *Rns* genotype using diagnostic fragment 3 (DF3) (Booton et al. 2002). Those results are summarized in Table 2. Overall, the twenty beach samples from Florida contained 19 T4 isolates and one T5 isolate. The T4 sequences fell into six groups of identical DF3 sequences, with the number of isolates of the same sequence ranging from one to six. Three groups of T4 beach isolates possessed DF3 sequences not observed previously in our rDNA database. Two of these groups included multiple isolates of the same sequence, whereas one ''group'' was a single unique sequence. The remaining T4 beach isolates were identical to other sequences previously observed and currently in our database. Eighteen of

the 19 T4 strains were closely related to each other, and were members of two clades of the phylogenetic gene tree (Fig. 1). Within these two clades, three terminal branches represented only Florida beach isolates, with one, four, and five isolates. The remaining two groups of beach isolate sequences were identical to previous *Acanthamoeba* isolates, most of which were obtained from AK infections. Genotype T4 beach isolate Fla19 was the most divergent of the T4 beach isolates, but was identical in the DF3 region to *A. castellanii* strain V014, an AK isolate. The only non-T4 isolate obtained from a beach sample was a T5 isolate obtained from a dry sand sample on Hobe Beach.

In addition to the sub-tropical beach samples, we also examined three soil samples from Hollywood, Florida. Two of these isolates were T5 genotype and were identical in sequence to the single T5 isolate that we obtained from the dry sand of Hobe Beach, discussed above. The remaining soil isolate was a T11 genotype. A single tapwater isolate from Florida also was a genotype T5. However, it was a novel T5 sequence for this region, unlike any other sequence previously in our database, and different from the other T5 genotypes determined in this study. The beach isolate from Scotland was a genotype T4, unlike any of the Florida beach isolates, but identical in sequence to the type-strain of the species, *A. castellanii* (ATCC 50374).

Physiological properties. A rapid migration rate was equated with rapid growth as evidenced by the high density of cells at the advancing front. All acanthamoebae grew at 0, 1.0 and 2.0% salt, and 81% of the clones showed some growth at 3.2% salt (Table 2). Generally, the T3, T4, and T5 types all grew well at 0 and 1.0% salt (Fig. 2). However, at the higher salinities (2.0 and 3.2%), the T3 and T4 genotypes performed better than the T5 and T11 genotypes (Fig. 2). Genotype T4 isolates had a slightly higher migration rate at 1.0%, which is close to the physiological conditions found in the human eye. There was, however, considerable variation in the response of the different clones tested making generalizations difficult. For example, at 0% salt, migration rates varied from 0.36 to 1.02 mm/h. Even so, some trends were apparent from the data. Considering just the beach isolates, which showed significant variation between salinity treatments (ANOVA $p < 0.001$), mean migration rates were similar over the salinity range 0 to 2.0% (0.54–0.67 mm/h) (Table 2). Mean migration rates were lower at 3.2% salt (0.15 mm/h), but still non-zero in all but one beach isolate. No significant differences in migration rates were found comparing acanthamoebae from wet sand vs. dry sand. Similar trends in salt tolerance were found in the case of the corneal isolates and tap water isolates although overall mean migration rates (and hence growth) were consistently lower than for the beach isolates. The three soil isolates migrated (i.e. grew) well at 0 and 1.0%, and modestly at 2.0%. However, all failed to migrate (or grow) at 3.2%. (Table 2).

With respect to temperature tolerance, all isolates tested grew at 37 °C, but only six of 37 amoebae tested grew at 40 °C and only two isolates grew at 42 °C (Table 2). Interestingly, none of the T4 isolates grew at the higher temperatures. Instead, all of the T5 isolates, one of the T3 isolates and the single T11 isolate tested grew at 40 °C. At the highest temperature, the only isolates that grew were two T5 isolates showing that this genotype is the most thermotolerant of the isolates tested.

DISCUSSION

Several species of 'freshwater' free-living amoebae can be pathogenic in humans, including *Naegleria fowleri* (associated with primary amoebic meningoencephalitis, PAM), *Balamuthia mandrillaris* (granulomatous amoebic encephalitis, GAE), and *Acanthamoeba* spp. (GAE and amoebic keratitis, AK) (Martinez and Visvesvara 1997; Visvesvara, Schuster, and Martinez 1993). Our beach survey clearly showed that acanthamoebae are commonly isolated from salt-water environments, as well as other genera that have recently been suggested to be associated with keratitis (i.e., *Vahlkampfia*, *Naegleria*, and *Hartmannella*) (Aitken et al. 1996; Dua et al. 1998; Kennedy et al. 1995). The diseases described above caused by these amebae are all rare and unlikely to pose a significant risk to beach users even if clinically important species were present. The one exception is *Acanthamoeba*, found in 38% of the sand samples examined. The abrasive nature of sand particles may cause damage to the corneal epithelium, especially when sand gets under a contact lens, providing an entry route for acanthamoebae to initiate infection. The possibility that beach sand provides a reservoir for potentially pathogenic amoebae has never before been considered, probably because it is assumed that high salinity water is too harsh an environment for these 'freshwater' protists.

However, the data must be viewed in perspective. The most common of these infections, *Acanthamoeba* keratitis, is a rare condition with an estimated incidence rate of only 0.33 cases per 10,000 contact lens wearers per year (Lam et al. 2002). Amoebae naturally inhabit environments shared by humans. Sera and tears collected from healthy human subjects have shown that between 50% and 100% of the population have specific antibodies for potentially pathogenic amoebae (Alizadeh et al. 2001; Walochnik et al. 2001). Clearly, humans are in frequent contact with disease-causing microorganisms and immunity from prior exposure usually protects against infection. However, there are examples where physical stress can lead to *Acanthamoeba* infection, as in the case of contact lens wear. Improper contact lens disinfection regimes allow *Acanthamoeba* to contaminate the lens storage case (Houang et al. 2001) resulting in the attachment of the organism to the lens surface material (Beattie et al. 2003; Seal et al. 1995; Simmons et al. 1996; Tomlinson et al. 2000). Upon insertion into the eye, the contact lens disrupts tear flow over the eye, leaving a thin layer of fluid trapped between the lens and the ocular surface; any *Acanthamoeba* present in this layer can penetrate the corneal epithelium causing infection. In non-contact lens wearers, the primary risk factors for AK infection are through ocular trauma and/or eye contact with water (Chang and Soong 1991; Illingworth and Cook 1998; Sharma et al. 1990).

It was surprising that so many amoebae (24 presumed species) were isolated from beach sand onto media appropriate for the growth of freshwater isolates. Both wet sand and dry sand are high salinity environments (albeit fluctuating) averaging 3.1 and 2.3% salt, respectively (Bonilla, unpubl.). Since most freshwater amoebae can form resistant cysts, it is possible that all the isolates were from cysts concentrated in the sand. It is also possible that some of the isolates were marine amoebae. Recent studies (Hauer et al. 2001) have shown that some amoebae have remarkable salt tolerance, making the distinction between freshwater and marine amoebae increasingly gray. The distinction between freshwater and marine amoebae is further blurred by the acanthamoebae that were singled out for study. These soil/freshwater protists were isolated from both wet and dry sand with equal frequency, and all grew well between 0 and 2.0% salt, with most also showing some growth at 3.2% salt. In fact, when comparing the mean migration rate (index of growth) of all the acanthamoebae examined, amoebae preferred media at 1.0% salt. The higher abundance of *Acanthamoeba* in sand relative to water and their tolerance to high salinities suggests that they are active in sand, rather than surviving as cysts, probably because sand affords a protected habitat rich in prey bacteria.

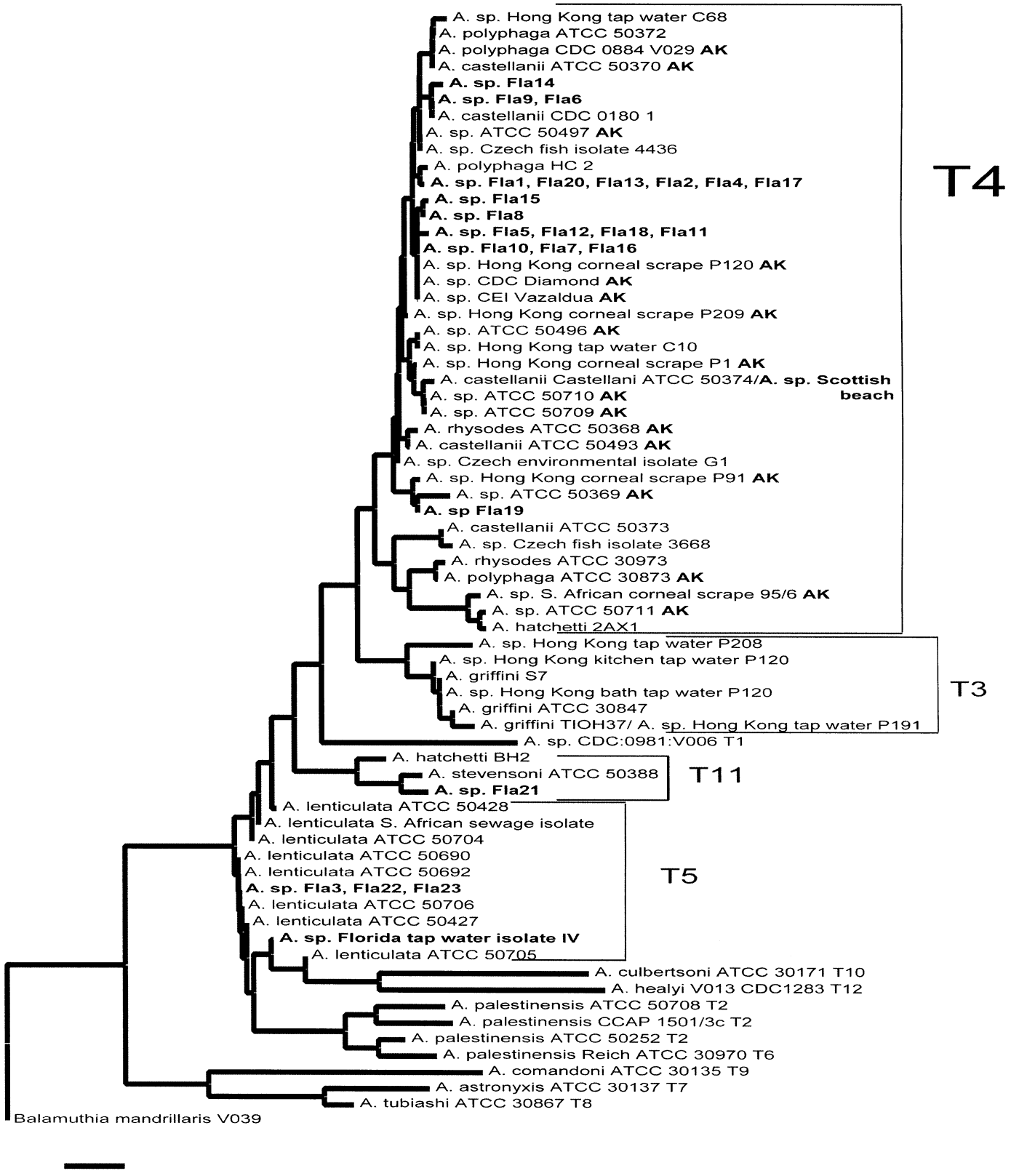

Fig. **1.** *Rns* DF3 phylogenetic gene tree of Florida and other *Acanthamoeba* isolates. Genotypes are grouped by type, eg. T4, T11. The tree is a neighbor-joining tree using distances calculated from the number of pair-wise comparisons. Scale bar below tree represents five nucleotide differences. T4 *Acanthamoeba* keratitis isolates are designated **AK** on terminal branches. The tree is rooted with *Balamuthia mandrillaris* as the outgroup taxa.

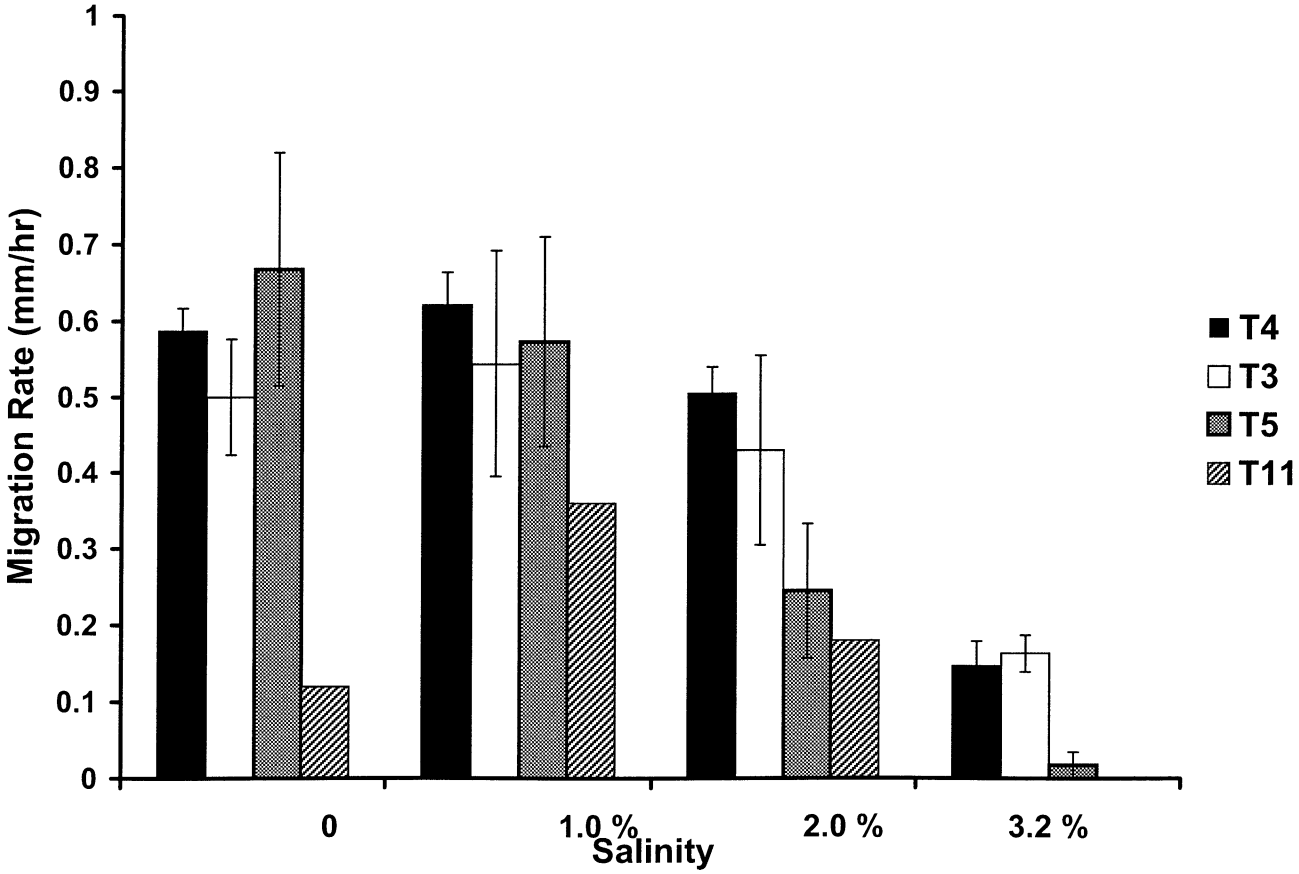

Fig. **2.** Comparison of migration rates, which is used in the present study as a measure of growth rates, of the different genotypes (T3, T4, T5, and T11) of acanthamoebae grown at different salinities (0, 1.0, 2.0, and 3.2%)

A major aim of the present research was to determine whether beach acanthamoebae have any clinical relevance, by documenting their genotypes and physiological responses (to temperature and salinity). The results suggest that in addition to tap water or lens case solutions as the usual suspect sources of AK infections, natural, commonly encountered sources must also be considered since most keratitis causing acanthamoebae are T3 and T4 genotypes. The results presented here are the first attempts to determine the genotypes of naturally occurring *Acanthamoeba* spp. isolates from a sub-tropical beach environment (including adjacent soils). Growth analyses showed that the large majority of these isolates had the ability to grow at physiological salt and temperature conditions. Genotype analysis classified most of these samples as T4, the genotype most commonly associated with *Acanthamoeba* keratitis infections. It is possible that T4 is the most common environmental genotype, as it was in the case of the sub-tropical beach environment studied here. This result is consistent with the assumption that the relative abundance of T4 isolates accounts for this genotype's prevalence in acanthamoebal infections and that genotype is relevant to pathogenic potential. On the other hand, isolates from other sources (Florida soil and tap water) yielded three T5 and one T11 genotypes. Although there have never been any cases of AK attributed to T5 strains, a T11 genotype was recently isolated from a keratitis patient (Khan et al. 2002).

This is not the first study to consider links between physiological features, genotypes and pathogenicity. Morphological characteristics (Khan 2001), physiological features (De Jonckeere 1980; Khan et al. 2001, 2002; Walochnik et al. 2000a, b), virulence in laboratory animals (De Jonckeere 1980), extracellular proteases (Khan 2003; Khan et al. 2000, 2002), identification of genetic markers (Howe et al. 1997), and mitochondrial sequences and mtDNA RFLP (Ledee et al. 2003; Yagita and Endo 1990) have all been used to differentiate putative pathogenic species and strains of *Acanthamoeba* from non-pathogenic strains.

Sawyer (1970, 1971) was the first to show that some *Acanthamoeba* are salt-tolerant. He isolated *A. griffini* from water at between 2.4% and 2.8% salt and showed that it could grow between 0% and 3.2%. He also demonstrated that a strain of *A. polyphaga* could grow in both freshwater and marine conditions. Although slow to grow initially on marine agar, after four or five generations rapid growth was restored for this isolate (Sawyer 1970). This suggests that the slow rates of growth at 3.2% in the present study may have increased if we had acclimatized the amoebae to higher salinities.

A correlation between temperature tolerance (De Jonckeere 1980; Khan et al. 2001, 2002; Walochnik et al. 2000a, b) or osmotolerance (Khan 2003; Khan et al. 2001, 2002) and pathogenicity has been demonstrated. For example, Khan et al. (2002) found that some pathogenic T3 and T4 isolates were all osmotolerant (using 1 M mannitol in non-nutrient agar) and grew at 37 °C, whereas they also found that some strains failed to grow at 37 °C or grew slowly (Khan et al. 2002). However, the majority of strains used in their study were obtained from culture collections, and the results of the present study with the Culture Collection of Algae and Protozoa (CCAP) strain of *A. polyphaga* suggests that there might be some limitation in using

old laboratory strains for physiological comparisons. Most of the beach isolates migrated (i.e. grew) faster than strains that had been in culture for extended periods of time. Overall, beach isolates that were tested within a few weeks of isolation, migrated 0.6- to 0.7 mm/h compared to migration rates 0.4- to 0.5 mm/h for the other strains tested. The CCAP strain of *A. polyphaga*, which has been in culture for some 20 yr, had a maximum migration rate of only 0.16 mm/h.

Walochnik et al. (2000b) examined the thermotolerance of clinically relevant strains of acanthamoebae and concluded that potentially pathogenic strains have high temperature tolerance and high growth rates. In contrast, Kilvington and White (1994) concluded that some species of *Acanthamoeba* do not grow at or above 35 °C. These results are at odds with the findings from the present study since all clones grew at 37 °C. Moreover, the most thermotolerant strains (tolerating at least 40 °C and one strain 42 °C) were T5 genotypes that are not known to be pathogens, but commonly occur in sewage. One T3 genotype and one T11 genotype did grow at 40 °C and all T4's grew at 37 °C. In terms of temperature tolerance, 37 °C might be the most relevant since the temperature of the eye is only around 34 °C. However, it should be realized that scrapings from infected eyes have often been exposed to drugs, such as propamidine and neomycin, which can induce drug resistance and temperature sensitivity when the isolate does not grow well above 32 °C (Ficker et al., 1990; Hay et al. 1994; Ledee et a.l 1998).

In determining the compatibility of the abundant T4 beach isolates with the ocular environment, it is important to consider the physiochemical characteristics of the tear film and anterior ocular surface tissue. The pH of human tears has been variously measured from 6.93 to 7.83 (Andres et al. 1988). The osmolarity of normal tears averages 304.4 ± 0.4 mOsmol/L and ranges between 299 and 309 (Gilbard and Rossi 1994). The proportion of electrolytes in normal tears are measured in mmol/L as follows: sodium, 133.2 ± 0.2; potassium, 24.0 ± 0.2; calcium, 0.8 ± 0.04; magnesium, 0.61 ± 0.03, and the bicarbonate anions as 52.8 ±0.2 (Gilbard and Rossi 1994). Others have measured similar levels for sodium, but lower levels for potassium (17), calcium (0.32), magnesium (0.35) and bicarbonate anions (12.4) (Ubels and Williams 1994). The value for chloride is reported to be 141.3 mmol/L (Ubels and Williams 1994). A comparison of electrolyte values in normal tears with those in plasma (Midler 1975) indicates similar levels for bicarbonate and sodium but lower levels for chloride (102) and potassium (5). Full-strength sea water (around 3.5 % salinity) has a similar pH to tears (around 7.4), and although the osmolarity has never been measured directly, it has been estimated to be 24.5 atmospheres at 25 °C based on freezing point-depression measurements (Pilson 1998). This is equivalent to that of a 1-M solution of sucrose in water and is considerably higher than the range reported for the eye. In terms of levels of electrolytes, sea water has sodium (468.9 mmol/L), potassium (10.2), calcium (10.3), magnesium (52.8), bicarbonate anion (ca. 2.0), and chloride (548.9) (Pilson 1998). Levels of electrolytes in tears are comparable to levels in sea water when salt water is diluted to around 1.0% salinity, as is often the case in sand. The only major exceptions are the levels of magnesium and bicarbonate, which are much lower and higher in tears than sea water, respectively. Thus, there are similarities between the dilute seawater and ocular environments, which would be consistent for the T4 genotype and would support the hypothesis that it causes AK by its predominance in the environment.

In summary, in this study we have investigated naturally occurring genotypes of *Acanthamoeba* in one type of environment, sub-tropical beaches in Florida. We have shown that the most commonly observed genotype in this natural environment (T4) was the same genotype that has been found in the vast majority of *Acanthamoeba* keratitis infections. Thus, the results suggest that beaches could be a potential source of AK infections, particularly since the abrasive nature of sand particles can provide corneal trauma of the type sometimes associated with *Acanthamoeba* infections.

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