JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1991, p. 310–314
0095-1137/91/020310-05$02.00/0

Vol. 29, No. 2

Differentiation of *Acanthamoeba* Strains from Infected Corneas and the Environment by Using Restriction Endonuclease Digestion of Whole-Cell DNA

S. KILVINGTON,[1]* J. R. BEECHING,[2] AND D. G. WHITE[1]

Public Health Laboratory, Combe Park, Bath BA1 3NG,[1] and School of Biological Sciences, University of Bath, Claverton Down, Bath BA2 7AY,[2] England

Received 2 July 1990/Accepted 19 November 1990

Restriction endonuclease digestion of *Acanthamoeba* whole-cell DNA was used to study the relationship between 33 morphologically identical strains from keratitis cases (30 strains), contact lens storage containers (2 strains), and soil (1 strain). Samples digested with *Bgl*II, *Eco*RI, or *Hin*dIII and separated by agarose gel electrophoresis contained detectable mitochondrial DNA restriction fragment length polymorphisms (RFLPs). By comparing RFLPs, the strains could be assigned to seven multiple-strain and three single-strain groups. The largest of these contained nine strains, eight of which were isolated in keratitis cases in various locations worldwide and may indicate a group particularly associated with keratitis. Restriction endonuclease analysis of whole-cell DNA is proposed as a valuable technique for detecting mitochondrial DNA RFLPs in the differentiation of morphologically identical *Acanthamoeba* strains and may therefore be useful in resolving the complex taxonomy of the genus, which has hitherto been founded on subjective morphological criteria.

Acanthamoebae are small free-living amoebae with a life cycle characterized by a trophozoite stage and a cyst stage (25, 26). These organisms are common in the environment and have been isolated from soil, natural and man-made aquatic sites, and the atmosphere (16, 25, 27). Acanthamoebae are increasingly being recognized as a cause of severe sight-threatening keratitis (12, 28). Since the disease was first recognized (13), several hundred cases have been reported, mostly from the United States (29). Contact lens wearers appear most at risk from infection (20, 28), accounting for approximately 85% of cases (29). Poor hygiene practices, notably the preparation of homemade saline rinsing solutions, are recognized risk factors among contact lens wearers (20, 28).

Although *Acanthamoeba* keratitis may be routinely diagnosed by the culture or microscopic examination of clinical material (32), the identification of species involved in the disease is unclear. The taxonomic classification of the members of the genus *Acanthamoeba* is derived from morphological observations of the trophozoite and cyst forms of these organisms (25, 26). The most detailed study is that of Pussard and Pons, who recognized 18 species that were assigned to three morphological groups (26). While this classification clearly defines the genus, the variation in cyst morphology seen even within cloned strains makes the identification of many described species a subjective and unreliable process (11, 31, 33). This has prompted the investigation of other methods for the differentiation of acanthamoebae. Isoenzyme electrophoresis (8, 11) and restriction endonuclease digestion of mitochondrial DNA (mtDNA) or whole-cell DNA (3, 4, 7, 19) have proved most useful in this respect. These methods have all demonstrated wide interstrain variation within species and also similarities between strains of separate species (7, 11).

While such studies have highlighted the inadequacies of the present taxonomic classification of the acanthamoebae, the strains examined have mainly been of environmental origin and unknown pathogenicity. The relationship between 33 morphologically identical *Acanthamoeba* strains from patients with keratitis (30 strains), contact lens storage containers (2 strains), and soil (1 strain) was therefore investigated by restriction endonuclease digestion of whole-cell DNA. The detection of mtDNA restriction fragment length polymorphisms (RFLPs) that enabled the strains to be assigned to seven multiple-strain and three single-strain groups has prompted this report.

(Parts of these results were first presented at the Vth International Conference on Biology and Pathogenicity of Free Living Amoebae, which was held at the Institute of Hygiene and Epidemiology, Brussels, Belgium, in August 1989.)

MATERIALS AND METHODS

Amoebae. The *Acanthamoeba* strains examined in this study are listed in Table 1. The majority of strains were from cases of keratitis not reported in the literature and were identified only by the names of the patients from whom they were isolated. Where possible, a reference to a strain is cited in Table 1. The cyst forms of all the strains appeared morphologically similar when viewed by light microscopy. Strains Ac/PHL/7a through Ac/PHL/7c were three consecutive isolates from the same patient obtained over a 7-month period. By cyst morphology, strains Ac/PHL/6 (Bitzer), Ac/PHL/8 (CCAP 1501/3d), Ac/PHL/10 (Garcia), and Ac/PHL/23 (SHI) have previously been identified as *Acanthamoeba polyphaga* (13, 14, 22) and Ac/PHL/29 (CCAP 1501/1a, Neff Strain) has been identified as *Acanthamoeba castellanii* (23). The two contact lens storage case isolates, Ac/PHL/30 and Ac/PHL/31, were isolated from asymptomatic patients and found to be cytopathogenic for Vero tissue culture cells at 32°C (14). Trophozoites were adapted to axenic culture in Chang's serum-casein-glucose-yeast extract medium (1), which was modified in this study by the inclusion of 0.1% filter-sterilized Panmede liver digest

* Corresponding author.

TABLE 1. *Acanthamoeba* strains examined in this study

Isolate	Country of origin	Source[a]	Reference
Ac/PHL/1 (Ac 112)	Australia	AK	2
Ac/PHL/2 (Ac 116)	Australia	AK	2
Ac/PHL/3	England	AK	
Ac/PHL/4	England	AK	
Ac/PHL/5	The Netherlands	AK	
Ac/PHL/6 (Bitzer)	United States	AK	13
Ac/PHL/7a	England	AK	30
Ac/PHL/7b	England	AK	30
Ac/PHL/7c	England	AK	30
Ac/PHL/8 (CCAP 1501/3d)	England	AK	22
Ac/PHL/10 (Garcia)	United States	AK	13
Ac/PHL/12	England	AK	
Ac/PHL/13	England	AK	
Ac/PHL/14	England	AK	
Ac/PHL/15	Germany	AK	
Ac/PHL/16	France	AK	
Ac/PHL/17	England	AK	
Ac/PHL/18	The Netherlands	AK	
Ac/PHL/19	England	AK	
Ac/PHL/20	England	AK	
Ac/PHL/21	France	AK	
Ac/PHL/22	England	AK	
Ac/PHL/23 (SHI)	England	AK	14
Ac/PHL/24	The Netherlands	AK	
Ac/PHL/25	England	AK	
Ac/PHL/26	France	AK	
Ac/PHL/28	England	AK	
Ac/PHL/29 (CCAP 1501/1a, Neff strain)	United States	Soil	23
Ac/PHL/30	England	CLC	14
Ac/PHL/31	England	CLC	14
Ac/PHL/32	Australia	AK	
Ac/PHL/33	England	AK	15
Ac/PHL/34	England	AK	1

[a] AK, *Acanthamoeba* keratitis; CLC, contact lens container.

(Paines & Byrne Ltd., Greenford, England) and maintained in tissue culture flasks (GIBCO, Uxbridge, Middlesex, England) at 32°C.

DNA isolation. Approximately 5×10^7 trophozoites of each strain were used for DNA isolation. Late-log-phase cultures were concentrated by centrifugation at 500 × *g* for 10 min at room temperature and suspended in 5 ml of cell lysis buffer (100 mM disodium EDTA, 100 mM NaCl, 10 mM Tris hydrochloride [pH 8.0]). Proteinase K (20 mg/ml) was added to a final concentration of 300 μg/ml, and the cells were lysed in 1% Sarkosyl (20%, wt/vol). After gentle mixing by inversion, the lysates were incubated at 56°C for a period ranging from 4 h to overnight. The samples were then chilled on ice for 5 min and extracted twice by gentle mixing for 5 min with equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). After each extraction, the aqueous and organic phases were separated by centrifugation at 2,000 × *g* for 15 min at room temperature, and the upper aqueous phase was retained. Nucleic acids were precipitated by the addition of an equal volume of isopropanol and holding at −20°C for a period ranging from 1 h to overnight (18). The precipitate was pelleted by centrifugation at 2,000 × *g* for 10 min at room temperature and dissolved in 5 ml of TE buffer (10 mM Tris hydrochloride, 1 mM disodium EDTA [pH 7.5]). RNase (5 mg/ml) was added up to a final concentration of 50 μg/ml, and the mixture was incubated at 37°C for 1 h. After extraction once each with phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1), the DNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and an equal volume of isopropanol and holding at −20°C for a period ranging from 1 h to overnight. The DNA was pelleted by centrifugation, washed twice with 70% ethanol, and dried briefly under a vacuum. The DNA was dissolved in 300 μl of double-distilled H_2O and stored at −20°C until use.

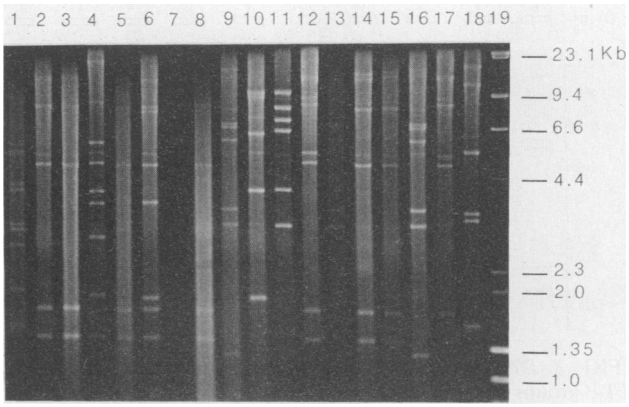

FIG. 1. Agarose gel electrophoresis of *Eco*RI digests of *Acanthamoeba* whole-cell DNA. Lanes: 1, Ac/PHL/28; 2, Ac/PHL/24; 3, Ac/PHL/23 (SHI); 4, Ac/PHL/22; 5, Ac/PHL/20; 6, Ac/PHL/19; 7, Ac/PHL/18; 8, Ac/PHL/15; 9, Ac/PHL/12; 10, Ac/PHL/10; 11, Ac/PHL/7b; 12, Ac/PHL/6 (Bitzer); 13, Ac/PHL/5; 14, Ac/PHL/4; 15, Ac/PHL/3; 16, Ac/PHL/8 (CCAP 1501/3d); 17, Ac/PHL/30; 18, Ac/PHL/29 (CCAP 1501/1a, Neff strain); 19, DNA fragments from lambda *Hin*dIII-φX 174 RF *Hae*III digestion.

Restriction endonuclease analysis. DNA samples (2 to 3 μg in 18 μl) were digested at 37°C for 2 h with 5 to 10 U of the restriction endonuclease *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Kpn*I, or *Pst*I by using appropriate reaction buffers provided with the enzymes (Northumbria Biologicals Ltd., Cramlington, Northumberland, England). The samples were loaded

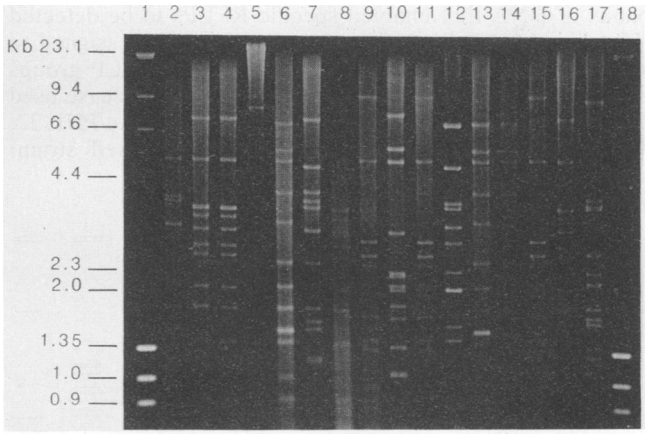

FIG. 2. Agarose gel electrophoresis of *Hin*dIII digests of *Acanthamoeba* whole-cell DNA. Lanes: 1 and 18, DNA fragments from lambda *Hin*dIII-φX 174 RF *Hae*III digestion; 2, Ac/PHL/28; 3, Ac/PHL/24; 4, Ac/PHL/23 (SHI); 5, Ac/PHL/22; 6, Ac/PHL/20; 7, Ac/PHL/19; 8, Ac/PHL/15; 9, Ac/PHL/12; 10, Ac/PHL/10 (Garcia); 11, Ac/PHL/5; 12, Ac/PHL/7b; 13, Ac/PHL/4; 14, Ac/PHL/3; 15, Ac/PHL/8 (CCAP 1501/3d); 16, Ac/PHL/30; 17, Ac/PHL/29 (CCAP 1501/1a, Neff strain).

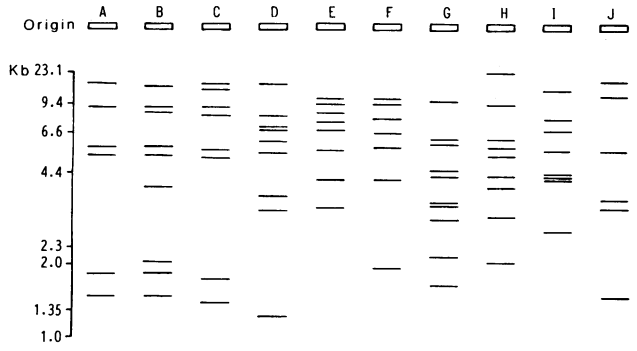

FIG. 3. Differentiation of *Acanthamoeba* strains by *Eco*RI RFLP groups (see Table 3).

onto horizontal 0.7% agarose gels (20 by 20 by 0.5 cm) prepared in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM disodium EDTA [pH 8.2]) for electrophoresis in TBE buffer at 1.5 V/cm overnight (18). DNA standards of lambda *Hin*dIII and φX 174 RF *Hae*III digests (Pharmacia LKB Ltd., Milton Keynes, England) were included as size markers. Gels were stained with 1 μg of ethidium bromide per ml in distilled H_2O for 1 h, destained in distilled water for 20 min, and photographed under shortwave UV transillumination with Polaroid 665 film and a Kodak Wratten 23A orange filter.

Analysis of results. The sizes of the RFLPs in kilobases were estimated with the GELPROG computer program written by K. Merrifield at the Institute of Cancer Research, Chester Beatty Laboratories, London, England.

RESULTS

Digestion of *Acanthamoeba* whole-cell DNA with the restriction endonucleases BamHI, *Kpn*I, and *Pst*I resulted in only a smearing of DNA after agarose gel electrophoresis and staining with ethidium bromide (result not shown). Increasing the restriction endonuclease concentration and incubation time did not alter this effect. In contrast, samples digested with *Bgl*II, *Eco*RI, and *Hin*dIII produced discrete bands of DNA that enabled specific RFLPs to be detected (Fig. 1 and 2). This permitted the strains to be assigned to seven multiple-strain and three single-strain RFLP groups that were consistent for each restriction endonuclease used (Fig. 3 to 5). Of the 33 strains examined, only Ac/PHL/22, Ac/PHL/25, and Ac/PHL/29 (CCAP 1501/1a, Neff strain) showed unique RFLPs. With *Eco*RI, the RFLPs of strains assigned to groups A and C appeared closely related, but they were clearly separated when *Hin*dIII and *Bgl*II were used. The most common RFLP profile belonged to the nine strains assigned to group A, of which eight were from keratitis cases in Australia (Ac/PHL/1 and Ac/PHL/2), the United States (Ac/PHL/6 [Bitzer]), England (Ac/PHL/17, Ac/PHL/23 [SHI], and Ac/PHL/34), Germany (Ac/PHL/15), and the Netherlands (Ac/PHL/24). This was followed by group F (five strains), group G (four), groups B and C (three each), groups D and E (two each, assuming Ac/PHL/7a, Ac/PHL/7b, and Ac/PHL/7c of group D are the same strain), and groups H, I, and J (one each). These findings are summarized in Table 2.

The four strains identified as *A. polyphaga* by cyst morphology were separated into groups A (Ac/PHL/6 [Bitzer] and Ac/PHL/23 [SHI]), B (Ac/PHL/8 [CCAP 1501/3d]), and E (Ac/PHL/10 [Garcia]). *A. castellanii* (Ac/PHL/29 [CCAP 1501/1a, Neff strain]) showed unique RFLPs from all the strains tested.

The sums of the sizes (in kilobases) of the RFLPs detected in strains Ac/PHL/7a, Ac/PHL/23 (SHI), Ac/PHL/28, and Ac/PHL/29 (CCAP 1501/1a, Neff strain) by digestion with *Bgl*II, *Eco*RI, and *Hin*dIII are shown in Table 3.

DISCUSSION

By either cyst morphology or biochemical analysis, *A. castellanii, A. hatchetti, A. lugdunensis, A. polyphaga,* and *A. rhysodes* of morphological group II (26) and *A. culbertsoni* of morphological group III have been described as causing keratitis. The 33 strains examined in this study formed cysts typical of group II *Acanthamoeba* spp. and resembled *A. polyphaga* or possibly *A. castellanii* (25, 26), as the differentiation of these species by this means is considered subjective (11, 31, 33). Using restriction endonuclease digestion of *Acanthamoeba* whole-cell DNA, we were able to detect RFLPs that differentiated these strains into seven multiple-strain and three single-strain groups labeled A through J directly by agarose gel electrophoresis. Of these, group A contained the largest number of strains (nine), of which eight were isolated from keratitis cases in various locations worldwide. This group may therefore indicate the type most frequently associated with keratitis through either a greater prevalence in the environment or increased virulence for corneas.

Although *Acanthamoeba* keratitis has been successfully

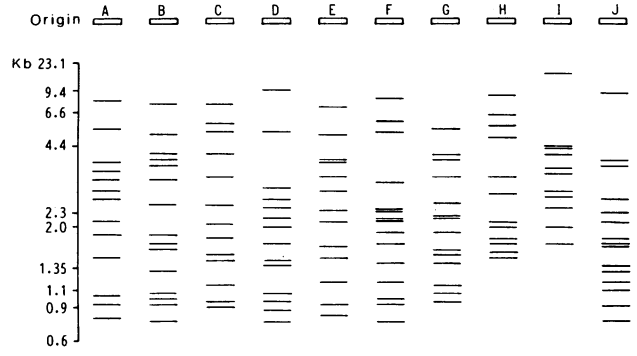

FIG. 4. Differentiation of *Acanthamoeba* strains by *Hin*dIII RFLP groups (see Table 3).

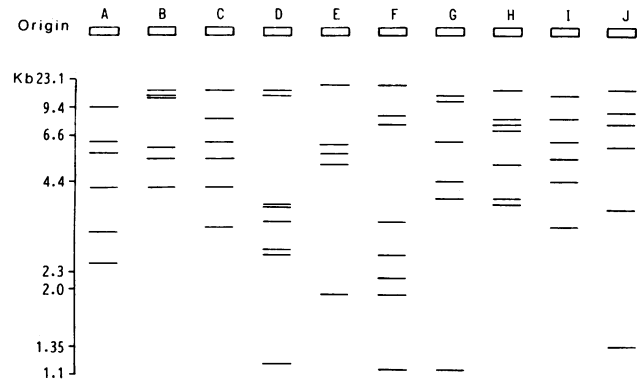

FIG. 5. Differentiation of *Acanthamoeba* strains by *Bgl*II RFLP groups (see Table 3).

TABLE 2. RFLP group assignment of *Acanthamoeba* strains

RFLP group	*Acanthamoeba* strain
A	Ac/PHL/1 (Ac 112), Ac/PHL/2 (Ac 116), Ac/PHL/6 (Bitzer), Ac/PHL/17, Ac/PHL/15, Ac/PHL/24, Ac/PHL/23 (SHI), Ac/PHL/30, Ac/PHL/34
B	Ac/PHL/5, Ac/PHL/8 (CCAP 1501/3d), Ac/PHL/12
C	Ac/PHL/3, Ac/PHL/4, Ac/PHL/20
D	Ac/PHL/7a, Ac/PHL/7b, Ac/PHL/7c, Ac/PHL/18
E	Ac/PHL/10 (Garcia), Ac/PHL/31
F	Ac/PHL/13, Ac/PHL/14, Ac/PHL/16, Ac/PHL/19, Ac/PHL/21
G	Ac/PHL/26, Ac/PHL/28, Ac/PHL/32, Ac/PHL/33
H	Ac/PHL/22
I	Ac/PHL/25
J	Ac/PHL/29 (CCAP 1501/1a, Neff strain)

TABLE 3. Total sizes of *Acanthamoeba* RFLPs

Acanthamoeba strain	Size of RFLPs (kb) with indicated restriction endonuclease			
	Sum			Mean ± SD
	*Bgl*II	*Eco*RI	*Hin*dIII	
Ac/PHL/7a	37.5	43.3	38.1	39.6 ± 2.6
Ac/PHL/23 (SHI)	42.6	39.6	39.2	40.5 ± 1.5
Ac/PHL/28	42.2	37.1	39.5	39.5 ± 2.6
Ac/PHL/29 (CCAP 1501/1a, Neff strain)	43.3	40.8	36.1	40.1 ± 3.7

treated with propamidine isethionate (35), the disease frequently necessitates surgical intervention (6, 34). Reports on the susceptibility of *Acanthamoeba* strains to chemotherapeutic agents and contact lens disinfectants often give conflicting results (5, 6, 9, 17, 21, 35). Strain Ac/PHL/33 of group G is inherently resistant to propamidine isethionate (15), while strain Ac/PHL/23 (SHI) of group A is susceptible (unpublished observations). This suggests that innate differences in susceptibility to antimicrobial agents occur between *Acanthamoeba* strains. Whether such variation correlates with RFLP group assignments of strains merits further investigation. Acquired drug resistance can also complicate the treatment of *Acanthamoeba* keratitis. Strains Ac/PHL/7a, Ac/PHL/7b, and Ac/PHL/7c represent three consecutive isolates from the same patient over a 7-month period. Ac/PHL/7c is resistant to propamidine isethionate, while Ac/PHL/7a and Ac/PHL/7b are susceptible (34). Since all three showed identical RFLPs, this result indicates that resistance was acquired by the original infecting strain during the course of treatment.

RFLP profiles may also be useful in identifying pathogenic *Acanthamoeba* strains. The virulence of acanthamoebae has been shown to attenuate during axenic culture (30) and may account for the description of both pathogenic and nonpathogenic strains within species (10). With the exception of group J, which contains only an environmental strain, Ac/PHL/29 (CCAP 1501/1a, Neff strain), the RFLP profiles defined here are those of pathogenic strains associated with keratitis. Therefore, environmental strains with RFLPs of groups A through I could be assumed to be potential human pathogens.

Byers et al. (4) previously studied the relationship of 13 *Acanthamoeba* strains, including Ac/PHL/29 (CCAP 1501/1a, Neff strain), by restriction endonuclease digestion of purified mtDNA. The number and positions of the *Eco*RI RFLPs for this strain are identical to those obtained in this study. Bogler et al. (3) estimated the size of the mtDNA genome for this strain to be 40.6 kb, which is in accord with the value of 40.1 kb found in our study. It is therefore concluded that restriction endonuclease digestion of *Acanthamoeba* whole-cell DNA gives rise to RFLPs originating from mtDNA. The reason for the failure of the restriction endonucleases *Bam*HI, *Kpn*I, and *Pst*I to cleave the mtDNA in the *Acanthamoeba* whole-cell DNA samples is not known. This failure may be due to the absence of endonuclease sequence recognition sites on the mtDNA or due to methylation of the sites, which would render them resistant to digestion (24).

In conclusion, restriction endonuclease digestion of whole-cell DNA is a potent technique for differentiating morphologically identical *Acanthamoeba* strains by the detection of mtDNA RFLPs. Although this study was not intended to be a taxonomic investigation of the acanthamoebae, four strains of *A. polyphaga* and one of *A. castellanii* that had been identified by cyst morphology were included. Of these, only two of the four strains of *A. polyphaga* showed identical RFLPs, while the one strain of *A. castellanii* was found to have a unique RFLP profile. It is therefore unclear whether *Acanthamoeba* mtDNA RFLPs indicate intra- or interspecies differences. Future studies will address this problem with a larger number of strains of the presently described species of the genus.

ACKNOWLEDGMENTS

We are grateful to P. Christy, J. De Jonckheere, D. Seal, P. Wright, and D. Warhurst for supplying many of the strains used in this study and also to the anonymous referees of the manuscript for their constructive comments.

REFERENCES

1. **Aufy, S. S. Kilvington, P. G. Mann, and D. C. Warhurst.** 1986. Improved selective isolation of *Naegleria fowleri* from the environment. Trans. R. Soc. Trop. Med. Hyg. **80**:350–351.
2. **Badenoch, P. R., A. M. Johnson, P. E. Christy, and D. J. Coster.** 1990. Pathogenicity of *Acanthamoeba* and a Corynebacterium in the rat cornea. Arch. Ophthalmol. **108**:107–112.
3. **Bogler, S. A., C. D. Zarley, L. L. Burianek, P. A. Fuerst, and T. J. Byers.** 1983. Interstrain mitochondrial DNA polymorphism detected in *Acanthamoeba* by restriction endonuclease analysis. Mol. Biochem. Parasitol. **8**:145–163.
4. **Byers, T. J., S. A. Bogler, and L. L. Burianek.** 1983. Analysis of mitochondrial DNA variation as an approach to systematic relationships in the genus *Acanthamoeba*. J. Protozool. **30**:198–203.
5. **Casemore, D. P.** 1970. Sensitivity of *Hartmannella* (*Acanthamoeba*) to 5-fluorocytosine, hydroxystilbamidine and other substances. J. Clin. Pathol. **23**:649–652.
6. **Cohen, E. J., C. J. Parlato, J. J. Arentsen, G. I. Genvert, R. C. Eagle, M. R. Wieland, and P. R. Laibson.** 1987. Medical and surgical treatment of *Acanthamoeba* keratitis. Am. J. Ophthalmol. **103**:615–625.
7. **Costas, M., S. W. Edwards, D. Lloyd, A. J. Griffiths, and G. Turner.** 1983. Restriction enzyme analysis of mitochondrial DNA of members of the genus *Acanthamoeba* as an aid to

taxonomy. FEMS Microbiol. Lett. **17**:231–234.
8. **Costas, M., and A. J. Griffiths.** 1980. The suitability of starch gel electrophoresis of esterases and acid phosphatases for the study of *Acanthamoeba* taxonomy. Arch. Protistenkd. **123**:272–279.
9. **Davies, D. J. G., Y. Anthony, B. J. Meakin, S. Kilvington, and D. G. White.** 1988. Anti-acanthamoeba activity of chlorhexidine and hydrogen peroxide. Trans. Br. Contact Lens Assoc. **5**:80–82.
10. **De Jonckheere, J. F.** 1980. Growth characteristics, cytopathic effect in cell culture, and virulence in mice of 36 type strains belonging to 19 different *Acanthamoeba* spp. Appl. Environ. Microbiol. **39**:681–685.
11. **De Jonckheere, J. F.** 1983. Isoenzyme and total protein analysis by agarose isoelectric focusing, and taxonomy of the genus *Acanthamoeba*. J. Protozool. **30**:701–706.
12. **Jones, D. B.** 1986. *Acanthamoeba*—the ultimate opportunist? Am. J. Ophthalmol. **102**:527–530.
13. **Jones, D. B., G. S. Visvesvara, and N. M. Robinson.** 1975. *Acanthamoeba polyphaga* keratitis and *Acanthamoeba* uveitis associated with a fatal meningoencephalitis. Trans. Ophthalmol. Soc. U.K. **95**:221–232.
14. **Kilvington, S.** 1989. Moist-heat disinfection of pathogenic *Acanthamoeba* cysts. Lett. Appl. Microbiol. **9**:187–189.
15. **Kilvington, S., D. F. P. Larkin, D. G. White, and J. R. Beeching.** 1990. Laboratory investigation of *Acanthamoeba* keratitis. J. Clin. Microbiol. **28**:2722–2725.
16. **Kingston, D., and D. C. Warhurst.** 1969. Isolation of amoebae from the air. J. Med. Microbiol. **2**:27–36.
17. **Ludwig, I. H., D. M. Meisler, I. Rutherford, F. E. Bican, R. H. S. Langston, and G. S. Visvesvara.** 1986. Susceptibility of *Acanthamoeba* to soft contact lens disinfection systems. Invest. Ophthalmol. Visual Sci. **27**:626–628.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **McLaughlin, G. L., F. H. Brandt, and G. S. Visvesvara.** 1988. Restriction fragment length polymorphisms of the DNA of selected *Naegleria* and *Acanthamoeba* amebae. J. Clin. Microbiol. **26**:1655–1658.
20. **Moore, M. B., J. P. McCulley, C. Newton, L. M. Cobo, G. N. Foulks, D. M. O'Day, K. J. Johns, W. T. Driebe, L. A. Wilson, R. J. Epstein, and D. J. Doughman.** 1987. *Acanthamoeba* keratitis: a growing problem in soft and hard contact lens wearers. Ophthalmology **94**:1654–1661.
21. **Nagington, J., and J. E. Richards.** 1976. Chemotherapeutic compounds and *Acanthamoeba* from eye infections. J. Clin. Pathol. **29**:648–651.
22. **Nagington, J., P. G. Watson, T. J. Playfair, J. McGill, and B. R. Jones.** 1974. Amoebic infection of the eye. Lancet **ii**:1537–1540.
23. **Neff, R.** 1957. Purification, axenic cultivation and description of a soil amoeba, *Acanthamoeba* sp. J. Protozool. **4**:176–182.
24. **Nelson, M., and M. McClelland.** 1989. The effect of site-specific methylation on restriction-modification enzymes. Nucleic Acids Res. **15**:(Suppl):r219–r230.
25. **Page, F. C.** 1976. An illustrated key to freshwater and soil amoebae. Freshwater Biological Association scientific publication no. 34. Freshwater Biological Association, Ambleside, England.
26. **Pussard, M., and R. Pons.** 1977. Morphologie de la paroi kystique et taxonomie du genre *Acanthamoeba* (Protozoa, Amoebida). Protistologica **13**:557–598.
27. **Singh, B. N.** 1975. Pathogenic and non-pathogenic amoeba. Macmillan Press Ltd., London.
28. **Stehr-Green, J. K., T. M. Bailey, F. H. Brandt, J. H. Carr, W. M. Bond, and G. S. Visvesvara.** 1987. *Acanthamoeba* keratitis in soft contact lens wearers. A case control study. JAMA **258**:57–60.
29. **Stehr-Green, J. K., T. M. Bailey, and G. S. Visvesvara.** 1989. The epidemiology of *Acanthamoeba* keratitis in the United States. Am. J. Ophthalmol. **107**:331–336.
30. **Stevens, A. R., and W. O'Dell.** 1974. In vitro growth and virulence of *Acanthamoeba*. J. Parasitol. **60**:884–885.
31. **Stratford, M. P., and A. J. Griffiths.** 1978. Variations in the properties and morphology of cysts of *Acanthamoeba castellanii*. J. Gen. Microbiol. **108**:33–37.
32. **Visvesvara, G. S., D. B. Jones, and N. M. Robinson.** 1975. Isolation, identification, and biological characterization of *Acanthamoeba polyphaga* from a human eye. Am. J. Trop. Med. Hyg. **24**:784–790.
33. **Warhurst, D. C.** 1985. Pathogenic free-living amoebae. Parasitol. Today **1**:24–28.
34. **Wright, P., L. Ficker, and D. Seal.** 1990. *Acanthamoeba* keratitis—resistance to medical therapy. Invest. Ophthalmol. Visual Sci. **31**(Suppl.):421.
35. **Wright, P., D. Warhurst, and B. R. Jones.** 1985. *Acanthamoeba* keratitis successfully treated medically. Br. J. Ophthalmol. **69**:778–782.