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Laboratory Investigation of *Acanthamoeba* Keratitis

S. KILVINGTON,[1]* D. F. P. LARKIN,[2] D. G. WHITE,[1] AND J. R. BEECHING[3]

Public Health Laboratory, Royal United Hospital, Combe Park, Bath BA1 3NG,[1] Department of Ophthalmology, Bristol Eye Hospital, Bristol,[2] and School of Biological Sciences, Bath University, Bath,[3] England

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Following the diagnosis of *Acanthamoeba* keratitis in a contact lens wearer, the antimicrobial susceptibility of the clinical isolate and the environmental source of the infection were investigated. Contrary to previous reports, in vitro antimicrobial testing showed that the infecting strain was inherently resistant to propamidine isethionate. Restriction endonuclease digestion analysis of *Acanthamoeba* whole-cell DNA of strains isolated from the patient's cornea, contact lens storage container, saline rinsing solution, and kitchen cold-water tap showed that the isolates were identical. This implicates, for the first time, domestic tap water as the source of *Acanthamoeba* sp. in this infection. It is therefore recommended that the use of homemade saline solutions and the rinsing of contact lenses in tap water be strongly discouraged.

Acanthamoeba is a genus of small free-living amoebae characterized by a life cycle of active trophozoite and dormant cyst stages (21, 22). The resistance of the cyst form to extremes of temperature (2, 9), disinfection (5, 9, 13), and desiccation (10) accounts for the isolation of the organism from virtually all soil and aquatic environments (21, 22).

Acanthamoeba spp. are opportunistic pathogens of humans (15). Infection of the cornea by *Acanthamoeba* spp. is increasingly being recognized as a severe sight-threatening ocular infection (8, 18). Several hundred cases have been reported, with, in one report, 85% of 208 infections associated with contact lens wear (23). *Acanthamoeba* spp. have been cultured from the lens storage solutions of symptomatic and asymptomatic patients (12, 17). Poor hygiene practices, notably the preparation of homemade saline rinsing solutions and rinsing of lenses with tap water, have been identified as a major risk factor leading to infection in lens wearers (23, 24).

Because the cyst form of *Acanthamoeba* infection is resistant to most antimicrobial agents at concentrations achievable in the cornea and tolerated by the ocular surface, treatment is exceedingly difficult. Prolonged medical therapy with antifungal agents or propamidine isethionate may yield a cure (28) or control the disease sufficiently to allow corneal transplantation a chance of success (4).

The conventional method of diagnosis of *Acanthamoeba* keratitis is by culture of corneal biopsy material on nonnutrient agar seeded with a lawn of *Escherichia coli* (NNA-*E. coli*) (26). *Acanthamoeba* spp. are readily identified by the morphological appearances of the trophozoite and cyst forms (21, 26). In vitro susceptibility testing can be performed on *Acanthamoeba* spp. isolated by this method (3, 7, 19, 28). The trophozoites can be adapted to axenic (bacteria-free) growth in liquid media and characterized by restriction endonuclease digestion of whole-cell DNA to detect restriction fragment length polymorphisms (RFLPs) on agarose gel electrophoresis (16). This technique is a highly specific means of differentiating morphologically identical *Acanthamoeba* strains isolated from keratitis cases and the environment (S. Kilvington, abstract presented at the 5th International Conference on Biology and Pathogenicity of Free Living Amoebae, Brussels, Belgium, 1989).

* Corresponding author.

In this paper, we describe the laboratory investigation of a case of *Acanthamoeba* keratitis by using in vitro drug susceptibility testing and restriction endonuclease digestion of *Acanthamoeba* whole-cell DNA. The demonstration that isolates from the patient's cornea, contact lens storage container, saline rinsing solution, and kitchen cold-water tap shared identical RFLPs implicates, for the first time, domestic tap water as the source of this pathogen in keratitis.

MATERIALS AND METHODS

Patient. In the weeks following cataract surgery on the left eye, a 70-year-old female patient residing in Cardiff, United Kingdom, had a daily-wear hard contact lens fitted. The patient prepared her own saline rinsing solution for lens cleansing from kitchen tap water and table salt. No recognized lens disinfection agent was used. After 3 months of lens wear, she had an acute onset of severe ocular pain, photophobia, and visual loss. Clinical features suggested *Acanthamoeba* infection (25), and the organism was subsequently cultured from a sample of corneal epithelium. Treatment was commenced with intensive topical propamidine isethionate, with an initial improvement. However, a worsening in the patient's vision and severity of the keratitis were noted. Because this was interpreted as resistance to propamidine isethionate, in vitro drug susceptibility testing of the axenic *Acanthamoeba* isolate was undertaken. These studies showed that whereas the trophozoites were susceptible to this agent, the cysts were resistant.

Isolation of amoebae. (i) Tissue scrapings from the infected cornea were taken by using a sterile hypodermic needle and inoculated directly onto an NNA-*E. coli* plate (1.5% plain agar in distilled H_2O; living *E. coli*). (ii) Fluid from the patient's contact lens storage container and saline rinsing bottle was centrifuged at 500 × *g* for 10 min at room temperature, and the deposit was inoculated onto NNA-*E. coli* plates. (iii) Swab samples were also obtained from the kitchen and bathroom tap water outlets in the patient's home. The swab tips were vortexed in 1 ml of 1/4 strength Ringer solution (Oxoid Ltd, Basingstoke, England), and 0.5-ml volumes were spread onto NNA-*E. coli* plates and allowed to absorb to dryness at room temperature.

Inoculated plates were incubated at 30°C in sealed polythene bags and examined daily for the presence of amoebic trophozoites for up to 7 days with an inverted light microscope. *Acanthamoeba* isolates were cloned by microcapil-

lary manipulation of a single cyst onto fresh NNA-*E. coli* plates. Trophozoites from these clones were adapted to axenic culture at 30°C in serum-casein-glucose-yeast extract medium (SCGYEM) (1) modified by the inclusion of 0.1% filter-sterilized Panmede liver digest (Paines & Byrne Ltd, Greenford, England).

In vitro drug susceptibility testing. The susceptibility of the patient's keratitis isolate to the following antimicrobial agents was determined: propamidine isethionate, pentamidine isethionate, 2-hydroxystilbamidine isethionate, spiramycin (May & Baker Pharmaceuticals), miconazole nitrate (Janssen Pharmaceutical Ltd), fluconazole (Pfizer Ltd, Sandwich, England), paromomycin sulfate (Sigma Chemical Co., Poole, England), neomycin sulfate (Upjohn Ltd, Crawley, England), and polymyxin E (Pharmax Ltd, Bexley, England). These were prepared immediately before use as 1,000-μg/ml stock solutions of active component in distilled H_2O (or methanol for spiramycin). The effect of the methanol solvent on the assay results was also determined. All assays were performed in triplicate and repeated on one other occasion for propamidine isethionate, pentamidine isethionate, and neomycin sulfate.

In the trophozoite assay, serial twofold dilutions of 100 μl of the test solution were made with distilled H_2O in the wells of a tissue culture grade microtiter plate (Nunc, GIBCO, Paisley, Scotland). Control wells received distilled H_2O in place of test solution. Log-phase cultures of axenic trophozoites were adjusted to a concentration of 10^4/ml in 2× tryptic soy broth (TSB) (Difco, East Molesey, England), and 100 μl of the calibrated suspension was added to the wells. Assays were performed in TSB rather than the modified SCGYEM used to adapt the strain to axenic culture, as it was considered that the less complex TSB medium would have fewer components that could interact with the antimicrobial agents. Normal trophozoite growth occurs in this medium but at a slower rate than in modified SCGYEM.

The plates were sealed with clear adhesive film and incubated at 36°C. After 48 h, the wells were inspected with an inverted microscope. By comparing the appearance of the trophozoites in the test wells to those in controls, the degree of amoeba growth, inhibition, or destruction was recorded. The minimum trophozoite inhibitory concentration (MTIC) was defined as the lowest test concentration resulting in no replication compared with the control wells. The minimum trophozoite amoebicidal concentration (MTAC) was defined as the lowest concentration of test solution that resulted in the complete lysis or degeneration of the trophozoites.

The cysticidal assay relies on the observation that *Acanthamoeba* cysts adhere to the well bottoms of polycarbonate microtiter plates and remain attached following drug exposure and removal by washing. Addition of a living suspension of *E. coli* to the wells and incubation result in excystment of viable organisms and replication of the emergent trophozoites. Serial twofold dilutions of 100 μl of the test solutions were prepared with distilled H_2O in the wells of an immunoassay grade microtiter plate (Nunc, GIBCO). Control wells received distilled H_2O in place of test solutions. Cysts were obtained from axenic trophozoites by using the constant pH encystment medium of Neff et al. (20) in stationary tissue culture flasks at 30°C. Cysts were adjusted to a final concentration of 10^4/ml in 1/4 strength Ringer solution, and 100-μl aliquots were added to the test and control wells. The plates were sealed with clear adhesive film and incubated at 36°C. After 48 h, the well solutions were gently removed by aspiration with a multichannel micropipette and replaced with 200 μl of 1/4 strength Ringer solution. The process was repeated twice before the addition of 100 μl of 1/4 strength Ringer solution containing live *E. coli* at an optical density at 546 nm of 0.2. The plates were resealed and incubated at 30°C. The minimum cysticidal concentration (MCC) was defined as the lowest concentration of test solution that resulted in no trophozoite excystment or replication after 7 days of incubation. A temperature of 30°C was chosen for the excystment studies, as this reduced the degree of reencystment of the emergent trophozoites which occurs at 36°C. Furthermore, it has been reported that some pathogenic acanthamoebae grow better at lower temperatures, although this was not the case with the strain studied here (6).

TABLE 1. Free-living amoebae isolated from clinical and environmental samples

Sample site	Free-living amoeba isolated (30°C)
Cornea	*Acanthamoeba* sp.
Contact lens storage container	*Acanthamoeba* sp.
Saline rinsing solution	*Acanthamoeba* sp.
Bathroom water taps	
Hot	None
Cold	*Hartmannella* sp.
Kitchen water taps	
Hot	None
Cold	*Hartmannella* sp., *Naegleria* sp., *Acanthamoeba* sp.

Restriction endonuclease digestion. Clones of *Acanthamoeba* isolates from the patient's cornea, contact lens storage container, saline rinsing solution, and kitchen cold-water tap were compared by using restriction endonuclease digestion of whole-cell DNA. Axenic trophozoites were lysed with 1% sarcosyl, digested with 300 μg of proteinase K per ml at 56°C for 18 h, and extracted twice with phenol-chloroform (14). The nucleic acids were precipitated with an equal volume of isopropanol and dissolved in distilled H_2O. Approximately 2 to 3 μg of DNA was digested with the restriction endonucleases *Bgl*II, *Eco*RI, and *Hin*dIII (Northumbria Biologicals Ltd, Northumberland, England) and separated by electrophoresis in a 0.7% agarose gel at 2 V/cm for 18 h (14). DNA standards of lambda-*Hin*dIII/ΦX-174 RF-*Hin*cII digests (Pharmacia LKB Ltd, Milton Keynes, England) were included as size markers. The gel was stained with 1.0 μg of ethidium bromide per ml and photographed under UV transillumination by using Polaroid 665 film and a Kodak Wratten #9 orange filter.

RESULTS

Isolation of amoebae. *Acanthamoeba* spp. and other free-living amoebae isolated from the clinical and environmental samples are listed in Table 1. Amoebae were identified to the genus level by the morphological appearance of the trophozoite and cyst forms (21). Identification of the *Naegleria* isolates was confirmed by the ability of the trophozoites to transform into a highly motile but temporary flagellate phase when incubated at 30°C in distilled water (21). Isolates did not grow above 37°C and were therefore identified as *N. gruberi* (21). *Acanthamoeba* spp. were cultured from the patient's cornea, contact lens storage container, saline rinsing solution, and kitchen cold-water tap (one isolate). Cysts derived from clones of these isolates were morphologically identical by light microscopy and were typical of that described for *A. polyphaga* (21).

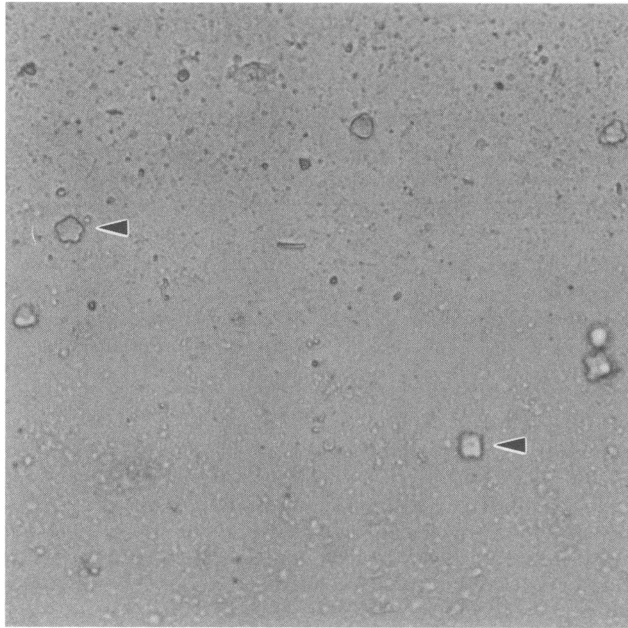

FIG. 1. *Acanthamoeba* cysts (indicated by arrows) adhering to the surface of the patient's hard contact lens (magnification, ×200).

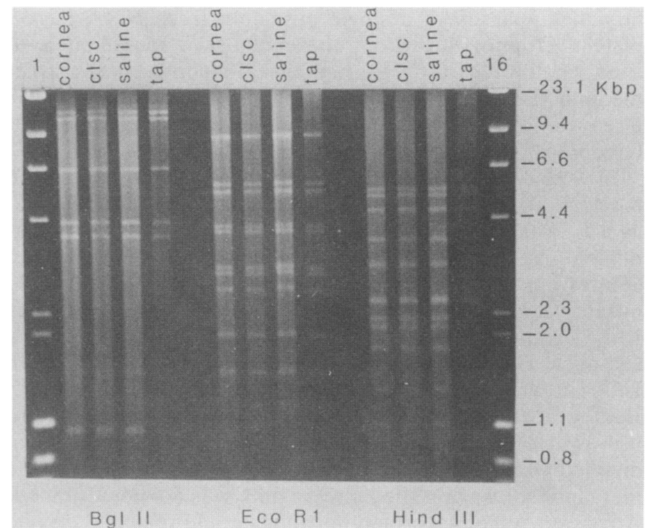

FIG. 2. Restriction endonuclease digestion profiles of *Acanthamoeba* whole-cell DNA from cloned strains isolated from the cornea, contact lens storage container (clsc), saline rinsing solution (saline), and kitchen cold-water tap (tap). Lanes 1 and 16, DNA size markers of lambda-*Hin*dIII/ΦX-174 RF-*Hin*cII digests.

Direct microscopic examination of the patient's hard contact lens also revealed the presence of cysts adhering to the lens surface (Fig. 1).

In vitro drug susceptibility testing. The trophozoite and cyst in vitro antimicrobial assay results for the corneal strain are shown in Table 2. The diamidine compounds, pentamidine isethionate, propamidine isethionate, and 2-hydroxystilbamidine isethionate, were most active against the trophozoites, with a MTAC at 48 h of ≤3.9 μg/ml. Paromomycin sulfate also showed good activity, with a MTAC of 15.6 μg/ml. In contrast, the cysts were found to be markedly more resistant, with MCCs for pentamidine isethionate, propamidine isethionate, 2-hydroxystilbamidine isethionate, and paromomycin sulfate of 31.25, 125, 250, 62.5 μg/ml, respectively. Fluconazole, miconazole nitrate, spiramycin, neomycin sulfate, and polymyxin E showed little or no activity against either trophozoite or cyst.

TABLE 2. In vitro drug susceptibility results of the *Acanthamoeba* corneal isolate

Antibiotic	Concn[a] (μg/ml) of active compound		
	Trophozoites		Cysts, MCC
	MTIC	MTAC	
Pentamidine isethionate	0.98	1.95	31.25
Propamidine isethionate	1.95	3.9	125
2-Hydroxystilbamidine isethionate	0.98	1.95	250
Fluconazole	7.8	62.5	>500
Miconazole nitrate	62.5	125	500
Neomycin sulfate	125	250	>500
Paromomycin sulfate	3.9	15.6	62.5
Spiramycin	3.9	250	>500
Methanol diluent	>10,000	>10,000	>10,000
Polymyxin E	15.6	62.5	>500

[a] MTIC, Minimum trophozoite inhibitory concentration at 48 h; MTAC, minimum trophozoite amoebicidal concentration at 48 h; MCC, minimum cysticidal concentration at 48 h.

The MTIC, MTAC, and MCC results for propamidine isethionate, pentamidine isethionate, and neomycin sulfate were consistent on repeated testing. It was also noted that trophozoite encystment was induced by propamidine isethionate at both the MTIC and sub-MTIC of 1.95 and 0.98 μg/ml, respectively. By counting the number of cysts in the test and control wells by using low-power microscopy of random fields, we determined that the MTIC and sub-MTIC wells contained 46.5 and 64% more cysts than the controls.

Restriction endonuclease digestion analysis. Restriction endonuclease digestion of *Acanthamoeba* whole-cell DNA with *Bgl*II, *Eco*RI, or *Hin*dIII gave rise to prominent DNA bands on gel electrophoresis, enabling RFLPs to be detected (Fig. 2). Isolates from the patient's cornea, contact lens storage container, saline rinsing solution, and kitchen cold-water tap showed identical RFLPs with respect to each endonuclease used.

DISCUSSION

Acanthamoeba keratitis is a severe sight-threatening infection particularly associated with contact lens wear (18, 24). Propamidine isethionate, shown to have potent in vitro activity against *Acanthamoeba* strains (3, 7, 19, 28), has been used to successfully treat infections (28). In the case described here, it was found that while the trophozoites were susceptible to 3.9 μg of this agent per ml, the cysts were resistant to at least 62.5 μg/ml, which is well above values reported by other workers (3, 7, 19, 28). As the strain was isolated prior to commencement of propamidine isethionate treatment, this suggests that drug-resistant strains of *Acanthamoeba* spp. exist which cause keratitis. This may account for the unsuccessful chemotherapeutic treatment of infections that necessitate surgical intervention (4, 27).

As in other microbial infections, isolation and susceptibility testing therefore appear to be fundamental for *Acanthamoeba* diagnosis and therapy. The in vitro assay methods described here are simple to perform and reproducible and should be more widely available to clinicians. Although

assays were performed with axenic cultures, testing can be accomplished in association with heat-killed *E. coli* (65°C for 20 min) by using trophozoites or cysts taken directly from NNA-*E. coli* plates. As was observed in this study, diamidine compounds like propamidine isethionate have been shown to stimulate *Acanthamoeba* encystment at inhibitory and subinhibitory concentrations (10; T. J. Byers, Rev. Infect. Dis., in press). This feature may undermine medical treatment and account for the intensive and prolonged therapy necessary to destroy the more resistant cyst forms.

Because pharmacological and surgical treatment of *Acanthamoeba* keratitis so frequently results in failure, prevention of this infection is important. The detection of RFLPs is a potent technique for differentiating morphologically identical *Acanthamoeba* strains (Kilvington, abstract presented). The demonstration here that strains from the patient's cornea, contact lens storage container, homemade saline rinsing solution, and kitchen cold-water tap shared common RFLPs implicates for the first time the last as the source of *Acanthamoeba* spp. in this infection. It is therefore recommended that the use of homemade saline solutions and the rinsing of lenses in tap water be strongly discouraged.

If *Acanthamoeba* keratitis is diagnosed early in disease, medical treatment may result in a cure. In this regard, clinicians must suspect *Acanthamoeba* infection in at-risk patients with suggestive clinical signs. Furthermore, microbiologists must be in a position to culture the organism from clinical material. This method of diagnosis has the advantage over histological methods of allowing susceptibility testing.

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