FREE-LIVING AMOEBAE: CHARACTERISATION, INACTIVATION AND PATHOGENICITY, WITH SPECIAL REFERENCE TO HUMAN OCULAR INFECTION IN SOUTH AFRICA

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DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

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_____1st_____ day of _____October_____ 2010

In loving memory of my husband

ROBERT NISZL

1958 – 2001

ABSTRACT

In this thesis, collection and other details of strains of *Acanthamoeba* and *Mastigina* species are recorded for archival purposes for southern African research reference. This information is mainly derived from 14 of 40 southern African patients suspected of having acanthamoebic keratitis.

The high prevalence in the environment of Gauteng of *Acanthamoeba*, including highly virulent strains, is demonstrated.

The pathogenicity of clinical and environmental *Acanthamoeba* and *Mastigina* isolates was compared *in vitro*, using two different mammalian cell lines. The time taken by the amoebae to destroy cell cultures completely was shown to be largely dependent on the size of the amoebic inoculum. The use of mammalian cell cultures was shown to be an accurate, rapid and repeatable means of assaying the cytopathic effect of strains of *Acanthamoeba*.

The effects of contact lens disinfecting solutions on strains of *Acanthamoeba* and *Mastigina* were assessed. Not all solutions used in the study were effective: some intended for hard and gas-permeable contact lenses proved more satisfactory than those for soft contact lenses. Manufacturers should be aware of the killing time for *Acanthamoeba* and *Mastigina* species by contact lens solutions, and should provide appropriate guidelines for their use.

The sensitivity to drugs of isolates of *Acanthamoeba* and *Mastigina* was compared. *Mastigina* species was sensitive to amphotericin B, mefloquine-hydrochloride and polymyxin B sulphate, whereas polyhexamethylene biguanide

or chlorhexidine should be the drug of choice for keratitis when *Acanthamoeba* is isolated.

Light and electron microscopy of cysts was used to characterise strains of *Acanthamoeba* and *Mastigina*. The author obtained consistently satisfactory results using a simple technique that was adapted to facilitate embedding amoebae for transmission electron microscopy. Morphological similarities were apparent between cysts of several isolates.

Cellulose acetate and polyacrylamide gel electrophoresis were conducted to characterise strains of *Acanthamoeba* and a strain of *Mastigina*. In some instances, diversity in isoenzyme patterns for some enzymes was found in certain strains assigned to the same species, while in other cases similarities were apparent.

Restriction fragment length polymorphisms, using nine enzymes, differentiated seven strains of *Acanthamoeba* into five groups. These results confirm those obtained electrophoretically.

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PUBLICATIONS

The following publications have resulted from the PhD-related research that was carried out. Additional papers are, at the time of writing (March 2010), envisaged.

As regards joint authorship, the candidate did the planning and practical work involved and drafted publications 2–11 (i.e. all except no. 1). The supervisor then contributed editorially and intellectually. In the case of no. 10, Mrs J.M.G. van Deventer provided technical assistance. Professor R.B. Veale advised concerning cell culture for no. 11 and also read and approved the manuscript.

1. MARKUS, M.B. & NISZL, I.A. 1990. Contact lens warning in relation to *Acanthamoeba*. *South African Medical Journal* **78**: 286.

2. NISZL, I.A. & MARKUS, M.B. 1989. Processing of free-living amoebae for transmission electron microscopy. *Stain Technology* **64**: 259–260.

3. NISZL, I.A. & MARKUS, M.B. 1991. Free-living protists as human pathogens. *South African Medical Journal* **80**: 520.

4. NISZL, I.A. & MARKUS, M.B. 1996. Treatment of *Acanthamoeba* keratitis. *South African Medical Journal* **86**: 566.

5. NISZL, I.A. & MARKUS, M.B. 1998. Anti-*Acanthamoeba* activity of contact lens solutions. *British Journal of Ophthalmology* **82**: 1033–1038.

6. NISZL, I.A. & MARKUS, M.B. 2001. Treatment of acanthamoebic keratitis. *Abstracts of Proceedings of the XI International Congress of Protozoology*, Salzburg, Austria, July 15-19, 2001.

7. NISZL, I.A. & MARKUS, M.B. 2002. Treatment of acanthamoebic keratitis. *Journal of the South African Veterinary Association* **73**: 155.

8. NISZL, I.A. & MARKUS, M.B. Submitted for publication. *Acanthamoeba* keratitis and occurrence of amoebae in contact lens cases and the environment in Gauteng Province, South Africa. *South African Journal of Science*.

9. NISZL, I.A. & MARKUS, M.B. Draft paper completed. *In vitro* drug sensitivity of *Acanthamoeba*. Not yet submitted.

10. NISZL, I.A., MARKUS, M.B. & VAN DEVENTER, J.M.G. 1995. Anti-*Mastigina* activities of eight contact lens solutions. *Antimicrobial Agents and Chemotherapy* **39**: 2364–2366.

11. NISZL, I.A., VEALE, R.B. & MARKUS, M.B. 1998. Cytopathogenicity of clinical and environmental *Acanthamoeba* isolates for two mammalian cell lines. *Journal of Parasitology* **84**: 961-967.

The candidate was also a co-author of the following papers, which were peripheral to the PhD-associated research:

1. DINI, L.A., COCKINOS, C., FREAN, J.A., NISZL, I.A. & MARKUS, M.B. 2000. Unusual case of *Acanthamoeba polyphaga* and *Pseudomonas aeruginosa* keratitis in a contact lens wearer from Gauteng, South Africa. *Journal of Clinical Microbiology* **38**: 826-829.

2. LEDEE, D.R., BOOTON, G.C., AWWAD, M.H., SHARMA, S., AGGARWAL, R.K., NISZL, I.A., MARKUS, M.B., FUERST, P.A. & BYERS, T.J. 2003. Advantages of using mitochondrial 16S rDNA sequences to classify clinical isolates of *Acanthamoeba*. *Investigative Ophthalmology & Visual Science* **44**: 1142-1149.

3. SCHROEDER, J.M., BOOTON, G.C., HAY, J., NISZL, I.A., SEAL, D.V., MARKUS, M.B., FUERST, P.A. & BYERS, T.J. 2001. Use of subgenic 18S rDNA polymerase chain reaction for generic and genotypic identification of *Acanthamoeba* from cases of human keratitis and sewage sludge. *Journal of Clinical Microbiology* **39**: 1903-1911.

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LIST OF ABBREVIATIONS

A	Acanthapodia
AAT	Aspartate amino transferase
ACP	Acid phosphatase
AK	Acanthamoebic keratitis
Ampho	Amphotericin B
ATCC	American Type Culture Collection
BAK	Benzalkonium chloride
BSA	Bovine serum albumen
CAE	Cellulose acetate electrophoresis
CHX	Chlorhexidine
cm	Centimetre
CM	Conditioned medium
CNS	Central nervous system
CPE	Cytopathic effect
CSF	Cerebrospinal fluid
CV	Contractile vacuole
CW	Cyst wall
d	Days
D	Extruded cell debris in space between ectocyst and endocyst
DME	Dulbecco's modification of Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
DPD	Diethyl-p-phenylene-diamine sulphate
Ec	Ectocyst
EDTA	Disodium ethylene-diamine tetracetic acid

En	Endocyst
EST	Esterases
F	Food vacuole
Fe	Female
FBS	Foetal bovine serum
FG	Flagellum
FI	Fibrils
Fig.	Figure
Figs	Figures
Fluco	Fluconazole
g	Grams
G	Golgi apparatus
GAE	Granulomatous amoebic encephalitis
GlcNAc	N-acetyl-D-glucosamine
G6PD	Glucose-6-phosphate dehydrogenase
GPI	Glucose-phosphate isomerase
h	Hours
H & E	Haematoxylin and eosin
HK	Hexokinase
IDH	Isocitrate dehydrogenase
Keto	Ketoconazole
L	Lipid vacuoles
LDH	Lactate dehydrogenase
Lt	Left
M	Mitochondria
Ma	Male
MAC	Minimal amoebicidal concentration

MC	Mammalian cell
MCC	Minimal cysticidal concentration
MDH	Malate dehydrogenase
ME	Malic enzyme
Meflo	Mefloquine hydrochloride
mg	Milligrams
MIC	Minimal inhibitory concentration
Mico	Miconazole
min	Minutes
ml	Millilitres
mm	Millimetres
MMIC	Minimal motility inhibition concentration
MPI	Mannose-phosphate isomerase
mtDNA	Mitochondrial DNA
n	Nucleolus
N	Nucleus
Nata	Natamycin
Nb	Nucleolar-like bodies
Nm	Nuclear membrane
NNA	Non-nutrient agar
NRK	Normal rat kidney
O	Ostiole
ODH	Octanol dehydrogenase
Op	Operculum
OsO_4	Osmium tetroxide
PAGE	Polyacrylamide gel electrophoresis

PAM	Primary amoebic meningoencephalitis
PAPB	Polyaminopropyl biguanide
PAS	Periodic acid Schiff
PCR	Polymerase chain reaction
Pent	Pentamidine isethionate
6PGD	6-Phosphogluconate dehydrogenase
PGM	Phosphoglucomutase
PHMB	Polyhexamethylene biguanide
PL	Plasmalemma
Poly	Polymyxin B sulphate
Prop	Propamidine isethionate
PYG	Peptone-yeast extract glucose
R	Rough endoplasmic reticulum
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
Rt	Right
R.T.	Room temperature
S	Spongy or lamellate network in space between ectocyst and endocyst
S.A.	South Africa
SCGYEM	Serum casein glucose yeast extract medium
SGE	Starch-gel electrophoresis
SNO	Human oesophageal squamous cell carcinoma
SOD	Superoxide dismutase
sp.	Species (singular)
spp.	Species (plural)

Sulphi	Sulphisoxazole
TEM	Transmission electron microscopy
U.K.	United Kingdom
U.S.A.	United States of America
UV	Ultraviolet
Wt/Vol	Weight/volume
Yr	Years

LIST OF SYMBOLS

%	Per cent
?	No information
°C	Degrees centigrade
μl	Microlitres
μm	Micrometers

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CHAPTER 1 – INTRODUCTION

1.1 Historical review

Considering the diversity of topics covered in this thesis, and in view of the fact that it is based partly on published papers, a classical, old-style historical literature review has intentionally not been included. Instead, the more modern approach has been adopted of citing relevant literature in individual chapters, the contents of some of which correspond largely to publications containing research results (Markus, 1988).

1.2 Free-living amoebae and human disease

Recognition of the extent of human disease caused by free-living amoebae is relatively recent. In the late 19th Century, amoebae were identified in cases of dysentery and organ and tissue abscesses, such as the jaw infection reported by Flexner (1892). In the early 1900s, Vahlkampf (1905), Naegler (1909), Hartmann (1910) and other researchers gave accurate descriptions of so-called limax, or slug-like amoebae. The genus *Acanthamoeba* was subsequently established after Castellani (1930) had found that *Acanthamoeba* could grow in yeast and bacterial cultures.

In 1957, amoebae were found to produce cytopathic effects (previously mistakenly attributed to a virus) on cultures of kidney cells taken from monkeys (Jahnes *et al*., 1957). Subsequently, during a series of animal experiments originally designed to assess a polio vaccine, Culbertson (1961) showed the neuropathogenicity of these organisms in experimental animals. He predicted "the possible role of these amoebae as causal agents of undiagnosed granulomatous disease". Soon after,

Fowler & Carter (1965) identified the first well-documented human cases of amoebic meningoencephalitis in Australia. In the 1960s, reports of acute, fatal human brain infections attributable to free-living amoebae came from Czechoslovakia and the United States (Butt, 1966; Cerva *et al*., 1968). The organism responsible was eventually identified as a species of *Naegleria*.

At least four genera of amoebae, which are protozoa, contain species that are human pathogens. *Entamoeba histolytica* is a true (mainly intestinal) parasite, but which can produce brain, lung or liver abscesses. *Naegleria*, *Acanthamoeba* and *Balamuthia* are free-living amoebae that can give rise to fatal central nervous system (CNS) disease, referred to as primary amoebic meningoencephalitis (PAM) in the case of *Naegleria* infections, and granulomatous amoebic encephalitis (GAE) in *Acanthamoeba* and *Balamuthia* infections. *Acanthamoeba* does not appear to exhibit any organ specificity in secondary infections as, in mice inoculated intranasally, amoebae were cultured from organs other than those from which they were originally isolated (Mazur *et al*., 1999). In AIDS patients, *Acanthamoeba* can cause disseminated infections. In the immunocompetent human host, *Acanthamoeba* is known to be a cause of sinusitis (Sukthana *et al*., 2005; Dickson *et al*., 2009).

More superficially, *Acanthamoeba* can also produce infection in humans in the skin (Murakawa *et al*., 1995), and the cornea, where the consequences can be sight-threatening (Martinez & Visvesvara, 1997). Initial reports regarding the ophthalmic importance of amoebae involved the association of *Acanthamoeba* with uveitis (Schlaegel & Culbertson, 1972). Animal experiments using intraocular inoculation produced uveitis and optic neuritis (Schlaegel & Culbertson, 1972). The clinical correlation of these findings was demonstrated in

a fatal human case of granulomatous amoebic encephalitis in a seven-year-old boy, in whom *Acanthamoeba* cysts were identified in the ciliary body (Jones *et al.*, 1975). Vahlkampfid amoebae (Kirkness *et al*., 1993) and *Hartmannella vermiformis* (Kennedy *et al*., 1995) have also been implicated in corneal infections in contact lens wearers. Corneal infection has become an important research topic (Kinnear, 2004; van der Bijl *et al.*, 2004a; van der Bijl *et al.*, 2004b; Alizadeh *et al.*, 2007; Alsam *et al.*, 2008; Omaña-Molina *et al.*, 2010; Ren *et al.*, 2010). Mechanisms associated with pathogenesis and pathophysiology are incompletely understood. Immunologically-related predisposition to acanthamoebic corneal infection might result from extended wearing of contact lenses (Li & Sun, 2008).

1.3 Environmental distribution of free-living amoebae

Free-living amoebae of the genera *Acanthamoeba* and *Naegleria* are ubiquitous in nature, and have a world-wide distribution (Martinez & Visvesvara, 1997). *Acanthamoeba* occurs very widely in the biosphere, being the most frequently isolated free-living amoeba, and possibly the most common free-living protozoon (Page, 1988). So far, *B. mandrillaris* has not been isolated from nature, but it is suspected that these amoebae have a similar habitat to *Acanthamoeba* and *Naegleria* (Martinez & Visvesvara, 1997).

Pathogenic *Acanthamoeba* and *Naegleria* have been isolated from a range of environments. These include tap water (Jeong *et* al., 2007; Boost *et al.*, 2008; Edagawa *et al.*, 2009; Bonilla-Lemus *et al*., 2010), frozen lakes (Brown & Cursons, 1977), thermal effluents (De Jonckheere *et al*., 1975; De Jonckheere & van de Voorde, 1977; De Jonckheere, 1978; Gianiazzi *et al*., 2010), sewage (Jenkins, 1977; Sawyer *et al*., 1977; Soh *et al*., 1977; Pahren *et al*., 1979; Griffin,

1983), dental irrigation systems (Barbeau, 2007; Trabelsi *et al*., 2010), swimming pools (Cain *et al.*, 1981; Gogate & Deodhar, 1985; Madrigal Sesma *et al*., 1985; Kilvington *et al*., 1991b; Caumo *et al*., 2009), freshwater lakes (John & De Jonckheere, 1985; Kyle & Noblet, 1985), marine environments (Sawyer, 1980; Daggett *et al*., 1982), hot springs (Gianiazzi *et al*., 2010) and artificially-heated waters (Stevens *et al*., 1977). *N. fowleri* cysts are able to survive at 4°C for eight months with retention of virulence (Warhurst *et al.*, 1980). Pens & Rott (2008) impregnated filter paper with *Acanthamoeba* cysts, which remained viable for at least a year at room temperature, as did cysts kept at 4^{o}C (Campbell *et al*., 2008). At 10 years of cryostorage, viability of *Acanthamoeba* was 32% (John & John, 2006). It has been reported that cysts of *Acanthamoeba* can survive desiccation for more than 20 years (Sriram *et al.*, 2008). *Acanthamoeba* and *Naegleria* have also been isolated from soil and from dust in the air (Schuster & Visvesvara, 2004b). Moreover, processed sewage wastes often contain residual pathogenic cysts of protozoa; and clinical disease has resulted from contact with night soil, raw sludge or raw waste water (Burge & Marsh, 1978; Burge *et al*., 1978; Ibrahim *et al.*, 2007). Limax amoebae and their cysts are particularly abundant in the solid phase of secondary treatment processes for sewage sludge (Jenkins, 1977). Sawyer & Bodammer (1983) found that three species of amoebae were present in samples of active sewage and samples from dredge and acid-waste dumpsites.

Among living organisms, *Acanthamoeba* has been isolated from vegetables, mushrooms, cultured cells, fish, reptiles, birds and mammals (De Jonckheere, 1991). In humans, species of *Acanthamoeba* have been recovered from nasal cavities, throats and intestines as well as from infected tissues, including cerebral tissue, lung tissue, skin wounds and corneas (De Jonckheere, 1987b).

Disease-causing bacteria such as *Legionella pneumophila, Burkholderia cepacia* and *Vibrio cholerae* have been shown to survive inside amoebae (Kilvington & Price, 1990; Thom *et al*., 1992; Marolda *et al*., 1999). Furthermore, amoebae are known to prolong the survival of a variety of *Campylobacter* spp. in co-culture (Axelsson-Olsson *et al*., 2005; Snelling *et al*., 2008; Axelsson-Olsson *et al.*, 2010). This phenomenon has important public health implications. *Acanthamoeba* spp. have often been found to contaminate bacterial, fungal and mammalian cell cultures in laboratories (Warhurst, 1989; Martinez & Visvesvara, 1997).

Acanthamoeba and *Naegleria* are widely distributed in South Africa (Lastovica, 1980). There is, however, little information on the relation of these protozoans to human infection in South Africa (Grabow, 1986), despite the recorded occurrence of fatal granulomatous amoebic encephalitis and primary amoebic encephalitis in this country (Rutherfoord, 1986; Schoeman *et al*., 1993); and despite the reported occurrence of acanthamoebic keratitis (Maske *et al.*, 1989).

Pioneering work in South Africa on *Acanthamoeba* and *Naegleria* was carried out by Lastovica (1971; 1972; 1974; 1975; 1976; 1977a; 1977b; 1977c; 1977d; 1977e; 1980; 1982; Lastovica & Elsdon-Dew, 1971; Lastovica & Williams, 1974). Lastovica (1980) isolated seven strains of *Naegleria fowleri* and a strain of *Acanthamoeba* from a heavily polluted section of the Eerste Rivier, Cape, South Africa. These isolates were capable of growth at 43°C; and strains of *Naegleria*, which is an amoeboflagellate, were capable of transformation (the process of changing from the amoeboid to the flagellate form). Lastovica (1980) identified these strains as potential pathogens according to a comparison with reference cultures of pathogenic *Naegleria* and *Acanthamoeba*.

As far as the author has been able to ascertain, nothing is known about the distribution of *Balamuthia* in South Africa at present.

1.4 Taxonomy of free-living amoebae

The taxonomy of free-living amoebae is not well established (De Jonckheere, 1987c). The genera *Naegleria*, *Acanthamoeba* and *Balamuthia* belong to different orders and families (Page, 1988).[1] All three genera of amphizoic amoebae have both trophozoite and cyst forms, the sizes of which vary among the different species. Historically, the morphology of trophozoites and cysts has been used to assist in distinguishing genera from one another. Among species of *Acanthamoeba*, species distinctions are less precise and less agreed upon than for *Naegleria* (Page, 1988).

1.4.1 *Naegleria*

Naegleria is placed in the family Vahlkampfiidae, which is characterised by a promitotic division of the nucleus, where the nucleolus and nuclear membrane persist throughout the cell division. The nucleolus elongates and forms a dumb-bell-shaped structure before dividing into nucleoli (Martinez & Visvesvara, 1997).

Naegleria has a flagellate stage in its life cycle in addition to the trophozoite and cyst stages. Trophozoites, measuring approximately 15 – 25 μm, exhibit rounded pseudopodia or lobopodia that are related to motility of the vegetative form (Martinez & Visvesvara, 1997). When rounded, as occurs within the CNS tissue in PAM, trophozoites measure about 8 –12 μm in diameter. The cytoplasm is granular, with multiple mitochondria, lysosomes and vacuoles. The nucleus

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1. See Appendix I.

is usually centrally placed and contains clumps of compact, finely granular chromatin as well as a dense, spherical nucleolus. The flagellate form is usually pear-shaped and biflagellated. Occasionally, however, multiple flagella (up to ten) have been seen. The flagellate organism is a non-feeding stage, and reverts to the trophic stage. In freshwater bodies there may be an active shuffling of *Naegleria* amoebae from the benthos to the surface microlayers by means of the non-feeding, swimming flagellate phenotype (Preston & King, 2003). The phenotypic transformation of *Naegleria gruberi* from amoebae to flagellates that occurs when cells are placed in a nutrient-free aqueous environment is dependent on transcription and translation (Fulton, 1980). Cysts of *Naegleria* are spherical, 8 –12 μm in diameter, and composed of a dense wall having one or two flat pores.

Morphological differences between members of the genus *Naegleria* are so minimal that the number of different species which have been described has remained low (De Jonckheere, 1987c).

1.4.2 *Acanthamoeba*

Sawyer & Griffin (1975) proposed the family Acanthamoebidae for amoebae with the characters of the genus *Acanthamoeba*. In the Acanthamoebidae, both trophozoites and cysts are characterised by a single nucleus that has a large, dense, centrally-located nucleolus and a centriole-like body present in mitosis, with metamitotic division. The family Acanthamoebidae comprises two genera, namely *Acanthamoeba* and *Protacanthamoeba* (Page, 1988).

The genus *Protacanthamoeba* has no pre-formed exit pores in the cyst, and excystment occurs through a break in the wall. Preformed pores through which excystment occurs in *Acanthamoeba* can usually be seen only with the aid of

electron microscopy or special staining. Therefore, *Protacanthamoeba* can be mistaken for *Acanthamoeba* (De Jonckheere, 1987c).

Pathogenic *Acanthamoeba* spp. have sometimes been misnamed *Hartmannella* in the literature. Page (1988) separated *Acanthamoeba* and *Hartmannella* at the ordinal level by creating a new order, Acanthopodida, which includes the family Acanthamoebidae. He placed *Hartmannella* in the family Hartmannellidae, order Euamoebida (Visvesvara, 1991).[2] Until recently, it was thought that *Hartmannella* is non-pathogenic, but this genus has been implicated, together with *Acanthamoeba*, in a severe case of drug-resistant keratitis (Inoue *et al*., 1998).

Trophozoites of the genus *Acanthamoeba* possess an abundant cytoplasm in which there are multiple elongated mitochondria, lysosomes, ribosomes and vacuoles. A*canthamoeba* trophozoites can be distinguished easily from those of other genera by their spiny or filose pseudopodia, called acanthapodia. However, trophozoites of *Acanthamoeba* and *B. mandrillaris* are morphologically similar in fixed tissue sections, and measure from 15 – 35 μm in diameter. The walls of *Acanthamoeba* cysts are double and are not smooth, and the ectocyst is usually wrinkled (Ma *et al*., 1990).

Species identification of *Acanthamoeba* has been debated for years. Identification is difficult because it is based on minor morphological features that are not as clear as those for the genus. For example, it is based on size and subtle differences in the appearance of the cyst stage (Visvesvara, 1991). Difficulties

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2. See Appendix I.

can be experienced in expressing the morphological characters objectively. Differentiation of species based on morphology alone may not always be correct, since the features of cysts within a given species may vary according to cultural conditions, as was shown for *A. castellanii* by Stratford & Griffiths (1978). There appeared to be a gradation, for a single strain of *A. castellanii*, from the type of cyst with a wrinkled cyst wall, which was produced in monoxenic culture and in replacement medium, to the smooth, refractile cells lacking a distinct wall, and which were produced in a growth medium supplemented with $MgCl_2$ (Stratford & Griffiths, 1978).

Efforts have been made to include other features in the classification of *Acanthamoeba* in addition to morphology, such as isoenzyme profiles (Daggett *et al*., 1982; Costas & Griffiths, 1984) and restriction fragment length polymorphisms (RFLPs) of the mitochondrial DNA (Bogler *et al*., 1983). There seems to be no correlation between isoenzyme patterns and morphological types (Visvesvara, 1991). Isoenzyme analysis has shown that some species names are synonyms, while other strains belong to as yet undefined species (De Jonckheere, 1987c). Strains that have been assigned to the same species, especially *A. castellanii* and *A. polyphaga*, do not always have similar enzyme patterns (Costas & Griffiths, 1980). According to a study of mitochondrial DNA done by Bogler *et al*. (1983), a number of strains previously considered to be *A. castellanii*, *A. polyphaga*, or other species, have similar RFLPs. Therefore, the authors suggest that these strains may all belong to one species. However, Visvesvara (1991) suggests that the strains used in the study concerned might have been contaminated.

Basing his research principally on cyst structure, Page (1967) recognised four

different species in the genus *Acanthamoeba*, namely *A. castellanii, A. astronyxis, A. polyphaga* and *A. palestinensis*. Since then the number of different *Acanthamoeba* species has been increasing constantly.

The most detailed study of cyst morphology of *Acanthamoeba* is that of Pussard & Pons (1977), in which *Acanthamoeba* was divided into three major morphological groups. However, these authors used only one strain each of the majority of species investigated, so that interclonal variation, which does occur, could not always be taken into account (Page, 1988). Pussard & Pons (1977) concluded that there are 18 different species of *Acanthamoeba*, eight of which they described for the first time. They divided *A. castellanii* and *A. polyphaga*, as described by Page (1967), into *A. castellanii, A. lugdunensis, A. rhysodes* and *A. divionensis*. Likewise, they separated Page's (1967) *A. polyphaga* into *A. mauritaniensis, A. quina* and *A. triangularis*. Subsequent isoenzyme analysis showed that some of the new species names, such as *A. paradivionensis* and *A. pustulosa*, are not valid (De Jonckheere, 1983).

The division of the genus *Acanthamoeba* into three major morphological groups has been confirmed serologically. *A. astronyxis* of group I and *A. culbertsoni* and *A. palestinensis* of group III have proved to be antigenically more distinct from species of group II than the species within group II (Willaert, 1976). The suggestion has even been made, based on mitochondrial DNA studies, that group II might be a single species (Bogler *et al*., 1983). However, the morphology of the cysts in group II does not allow sharp demarcation. Some strains appear to be transitory between two species, and distinction made on a morphological basis is, therefore, subjective (De Jonckheere, 1987c).

Group I consists of four species of *Acanthamoeba*, with an average cyst diameter of greater than 18 μm. The cyst is characterised by a star-like endocyst enclosed by a more or less round ectocyst, with the ectocyst and endocyst being widely separated (Visvesvara, 1991). All known isolates in this group are considered to be non-pathogenic (De Jonckheere, 1980), but a human case is known where the amoeba in the brain reacted with *A. astronyxis* antiserum.

Group II is the largest group and includes ten species. The ectocyst and endocyst are either close together or widely separated. The ectocyst may be thick or thin and is usually wrinkled or mamillated; the endocyst may be stellate, polygonal, triangular, round or oval, and does not usually have well-developed arms or rays. The mean diameter of the cyst is usually less than 18 μm. The criterion for distinguishing between *A. polyphaga* and *A. castellanii* is that the cysts of the latter have six or more corners or pores in optical section, whilst those of the former have fewer than six. A wide variation in pore number is to be found even in cloned isolates (Warhurst, 1985). The distinction between *A. polyphaga* and *A. rhysodes* is even less clear (Warhurst, 1985). Using isoenzyme electrophoresis, Costas & Griffiths (1980) arranged strains of *Acanthamoeba* into 12 groups, with the isoenzyme evidence not supporting the differentiation between *A. castellanii, A. rhysodes* and *A. polyphaga*. More studies are needed before the conflicts over identification of species in the genus *Acanthamoeba*, particularly those in group II, can be resolved.

Acanthamoeba cysts belonging to group III are characterised by a mostly round endocyst (which may have three to five corners that are not sharp) surrounded by a thin ectocyst, which is sometimes either gently rippled or unrippled. The mean diameter of the cyst is usually less than 18 μm. Five species are included in this

group. It may not be possible to identify the species within this group by morphology alone.

Molecular techniques are now being used regularly for *Acanthamoeba* in environmental and clinical samples (Booton *et al.*, 2004; Zhang *et al.*, 2004; Mubareka *et al.*, 2006; Yera *et al.*, 2006; De Jonckheere, 2007; Dhivya *et al.*, 2007; Boost *et al.*, 2008; Dendana *et al.*, 2008; Goldschmidt *et al.*, 2008; Ozkoc *et al.*, 2008; Thompson *et al.*, 2008; Xuan *et al.*, 2008; Yera *et al.*, 2008; Booton *et al.*, 2009; Edagawa *et al.*, 2009; Goldschmidt *et al.*, 2009; Kong, 2009; Ledee & Byers, 2009; Niyyati *et al.*, 2009a; Niyyati *et al.*, 2009b: Rivera & Adao, 2009; Costa *et al.*, 2010; Kandori *et al.*, 2010; Nagyová *et al.*, 2010; Niyyati *et al.*, 2010; Zhao *et al.*, 2010).

1.4.3 *Balamuthia*

Trophozoites of *Balamuthia* show both conventional lobopodial morphology and a spider-like appearance, with projecting determinate pseudopodia (Martinez & Visvesvara, 1997). *Balamuthia* trophozoites are typically uninucleate; the nuclear envelope remains intact through early mitotic events, but apparently breaks down late in mitosis. It is difficult to differentiate *Balamuthia* from *Acanthamoeba* spp. in tissue sections on the basis of light microscopic morphology (Visvesvara *et al.*, 1997). However, it may be possible to distinguish *Balamuthia* from *Acanthamoeba* on the basis of nuclear morphology, as *Balamuthia* trophozoites can have more than one nucleolus in the nucleus, whereas *Acanthamoeba* trophozoites have only one (Visvesvara *et al.*, 1997).

Cysts of *Balamuthia* spp. are found in tissue sections, but it is difficult to distinguish these cysts from those of *Acanthamoeba* spp. at the light microscopic

level (Visvesvara *et al*., 1997). However, the cyst wall is seen to be tripartite ultrastructurally: it has an outer thin irregular layer, the ectocyst; a thick, electron-dense inner layer, the endocyst; and a middle amorphous fibrillar layer, the mesocyst (Martinez & Visvesvara, 1997).

Balamuthia amoebae are antigenically distinct from those of *Acanthamoeba* spp. and can easily be distinguished by immunofluorescence assay. Therefore, electron microscopy, immunohistochemical techniques or both are necessary to identify *Balamuthia* organisms in tissue sections (Visvesvara *et al*., 1997).

Unlike *Acanthamoeba* and *Naegleria, Balamuthia* will not grow on bacteria-coated, non-nutrient agar (NNA) plates but will grow on tissue culture monolayers of mammalian cells (Visvesvara *et al*., 1993).

1.5 Pathogenic effects

1.5.1 *Acanthamoebic keratitis*

In the first reported case of acanthamoebic keratitis, *A. polyphaga* cysts and trophozoites were found on smears and on a blood agar plate that contained corneal scrapings from a Texas rancher who had splashed himself in the eye with tap water from a contaminated river source (Jones *et al.*, 1975). Nagington *et al*. (1974) recorded the first two eye infections caused by free-living amoebae to be reported in the United Kingdom (U.K.). Warhurst & Thomas (1975) subsequently identified these strains as *A. polyphaga* and *A. castellanii*. Very few additional cases were reported during the next decade. However, in 1984–1985, the number of reported cases began to increase, apparently as a consequence of the use of contact lenses.

Acanthamoebic keratitis is being diagnosed with increasing frequency in various parts of the world (Martinez & Visvesvara, 1997; Acharya *et al.*, 2007; Bryant *et al.*, 2007; Foulks, 2007; Thebpatiphat *et al.*, 2007; Ku *et al.*, 2009; Ormonde, 2009; Verani *et al.*, 2009; Joslin *et al.*, 2010). The first South African case of acanthamoebic keratitis was reported two decades ago, and was detected by non-cultivation techniques (Maske *et al*., 1989). In a preliminary survey carried out in 1988, *Acanthamoeba* was cultured from the contact lens cases of persons who did not have ocular disease (Markus & Niszl, 1990). Increased awareness of the ability of *Acanthamoeba* to cause serious ocular disease is needed in South Africa (Markus & Niszl, 1990; Niszl & Markus, 1998).

At least eight species of *Acanthamoeba* have been implicated in corneal infections, namely *A. castellanii, A. culbertsoni, A. hatchetti, A. griffini, A. lugdunensis, A. polyphaga, A. quina* and *A. rhysodes* (Ma *et al*., 1990; Ledee *et al*., 1996; Niederkorn *et al.*, 1999a).[3] Two new species of *Acanthamoeba*, *A. jacobsi* (Sawyer *et al*., 1992) and *A. stevensoni* (Sawyer *et al*., 1993), have been identified as potential pathogens, but these species have not, as yet, been implicated in keratitis. Molecular techniques are elucidating the situation (Booton *et al.*, 2009; Goldschmidt *et al.*, 2009; Kong, 2009).

Evidence of the exposure of humans to ocular infection is shown by the presence of *Acanthamoeba* spp. in samples of water drawn from bathroom taps and from dust around a washbasin (Seal *et al*., 1992). Samples *et al.* (1984) found that amoebae isolated from a keratitis patient's cornea, hot bath water and garden were morphologically and immunologically identical. Likewise, domestic tap water

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3. See Appendix XI.

has been implicated in a case of acanthamoebic keratitis in the U.K. by restriction endonuclease analysis of whole-cell DNA, which showed that strains of *Acanthamoeba* isolated from a patient's cornea, contact lens storage container, saline rinsing solution, and cold-water kitchen tap were indistinguishable (Kilvington *et al*., 1990). Domestic tap water could also be a source of ocular *Acanthamoeba* infection in South Africa, as the organism occurs commonly in tap water and swimming pools in Gauteng Province (Markus & Niszl, 1990). Contact lens care systems could also become contaminated with *Acanthamoeba* cysts from the air (Kingston & Warhurst, 1969).

Mergeryan (1991) concluded that air seems to be the most likely vehicle for transmission; and the presence of *Acanthamoeba* in the air is undoubtedly the primary explanation for the frequent presence of the organism in the nasal cavities of healthy individuals. Therefore, cornea and conjunctiva are also exposed to *Acanthamoeba* cysts in the same way. Mergeryan (1991) has indicated that contamination through contact with water, for example in daily washing, is less significant in terms of migration of *Acanthamoeba* to the nasal mucosa or eyes, except in the case of swimming (especially diving) in water which is likely to contain a high concentration of *Acanthamoeba* trophozoites.

Indications of possible ocular acanthamoebic infection in humans include: variable epithelial defect; uveitis; hypopyon; ring infiltrate; corneal thinning; descemetocoele formation; excessive ocular pain; or cases in which a clinical diagnosis of herpes simplex keratitis has been made. Although usually painful, acanthamoebic keratitis can be painless (Camposampiero *et al*., 2009; Elabjer *et al*., 2009). The evidence is that contact lens wearers, in particular users of disposable lenses, appear to run a higher risk of contracting acanthamoebic

keratitis, for more than one reason (Bacon *et al*., 1993b; Joslin *et al*., 2007; Lindsay *et al*., 2007; Pens *et al*., 2008; Hasler *et al*., 2009; Hasler *et al*., 2010; Lee *et al*., 2010). In a review of 72 consecutive cases of acanthamoebic keratitis (77 eyes), 64 patients were contact lens wearers. Twenty-eight of these were users of disposable lenses (Bacon *et al*., 1993b). In addition, Stehr-Green *et al*. (1987) found that patients with acanthamoebic keratitis were significantly more likely than controls to use home-made saline instead of commercially-prepared saline to store their lenses, and that they were also more likely than controls to disinfect their lenses less frequently than is recommended by lens manufacturers. Bilateral acanthamoebic keratitis is an infection-related consequence of the wearing of contact lenses (e Sousa *et al*., 2008; Voyatis & McElvanney, 2007; Wilhelmus *et al*., 2008). Contact lenses can cause mechanical or hypoxic trauma and more amoebae bind to the surface of injured corneas than to the normal corneal surface, with mannose-containing glycoproteins possessing binding sites for *Acanthamoeba* (Jaison *et al*., 1998; Alizadeh *et al*., 2007). It might do no harm to mechanically rub contact lenses during cleaning/disinfection, even if it does not do any good (Butcko *et al*., 2007).

Acanthamoeba trophozoites and cysts are killed by steam and gas sterilisation, which are routine sterilisation techniques for surgical instruments (Meisler *et al*., 1985). The cyst form of *Acanthamoeba* is, however, resistant to freezing, desiccation, antimicrobials and most standard contact lens cleaning and disinfection methods (Ludwig *et al*., 1986; Niszl & Markus, 1998; Johnston *et al*., 2009).

Since 1984, an effective biguanide-propamidine combination has been used as treatment for acanthamoebic keratitis. Further advances as regards treatment

(experimental and otherwise) and testing of potential anti-acanthamoebic agents are gradually being made (Dudley *et al.*, 2007; Lee *et al.*, 2007a; 2007b; 2007c; McBride *et al.*, 2007; Polat *et al.*, 2007a; Polat *et al.*, 2007c; Topalkara *et al.*, 2007; Vural *et al.*, 2007; Fürnkranz *et al.*, 2008; Gooi *et al.*, 2008; Lim *et al.*, 2008; Lin *et al.*, 2008; Polat *et al.*, 2008; Ródio *et al.*, 2008; Zhu *et al.*, 2008; Agahan *et al.*, 2009; Dart *et al.*, 2009; Derda *et al.*, 2009; Goze *et al.*, 2009; Sacramento *et al.*, 2009; Walochnik *et al.*, 2009; Bang *et al.*, 2010; Cariello *et al.*, 2010; Martin-Navarro *et al.*, 2010a; Roberts & Henriquez, 2010). Cases are being diagnosed increasingly early and, consequently, the time taken to effect a medical cure has become shorter (Bacon *et al*., 1993a; 1993b). Early/rapid diagnosis is important (Claerhout *et al.*, 2004; Lorenzo-Morales *et al.*, 2007; Kovačević *et al.*, 2008; Dua *et al.*, 2009; Shiraishi *et al.*, 2009; Ueki *et al.*, 2009). A relatively new diagnostic technique for acanthamoebic keratitis is confocal microscopy, which is proving to be useful (Kanavi *et al.*, 2007; Matsumoto *et al.*, 2007; Kobayshi *et al.*, 2008; Tu *et al.*, 2008; Alomar *et al.*, 2009; Erie *et al.*, 2009; da Rocha-Azevedo *et al.*, 2009; da Rocha-Azevedo *et al.*, 2010).

1.5.2 Balamuthia *infections*

In contrast to *Naegleria*, *Balamuthia* causes a subacute to chronic infection of the CNS and is capable of infecting both healthy and immunosuppressed hosts (Visvesvara *et al.*, 1993; Schuster & Visvesvara, 2004a; Schuster & Visvesvara, 2004b; Visvesvara *et* al., 2007; Matin *et al*., 2008; Siddiqui & Khan, 2008). *Acanthamoeba*, in contrast, appears to favour the immunocompromised host (Denney *et al*., 1997). Recavarrenarce *et al.* (1999) have described amoebiasis caused by *Balamuthia mandrillaris* as a fatal disease. It primarily affects the nasal pyramid or the skin, producing granulomatous amoebic lesions. The amoebae spread from the primary nasal lesion to the meninges, where they

infiltrate blood vessels. Thrombotic amoebic angiitis produces infarcts of the central nervous substance that then become infiltrated by amoebae. The primary cutaneous lesion can persist for weeks or even months. The disease is chronic and can develop over a period of between a few weeks and approximately 2 years (Schuster & Visvesvara, 2004b).

The finding that *B. mandrillaris* has caused infection in some healthy children has raised the possibility that humans may lack the ability to mount an immune response to this amoebic species (Huang *et al.*, 1999). However, anti-*B. mandrillaris* antibodies which did not react with other amoebae, have been found in the serum of adults and one- to five-year-old children (Huang *et al.*, 1999).

1.6 Objectives and research methodology

1.6.1 *Objectives*

The following research objectives were identified:

1) to ascertain whether free-living amoebae, *Acanthamoeba* in particular, occur in Gauteng Province, South Africa (as anticipated);
2) to isolate amoebae from corneal scrapings (and/or contact lenses or contact lens solutions) in suspected cases of acanthamoebic keratitis and to culture them axenically;
3) to obtain an indication of the pathogenicity of isolates;
4) to establish whether pathogenic amoebae occur in sewage sludge in South Africa (as they do in other parts of the world);
5) to characterise amoebae isolated from corneal scrapings, contact lenses, contact lens solutions and sewage sludge;
6) to test contact lens solutions used in South Africa to determine whether they are effective in killing southern African and overseas corneal isolates of

Acanthamoeba, as well as a local strain of *Mastigina*;

7) to determine the effectiveness of drugs *in vitro* on isolates of *Acanthamoeba* and a local strain of *Mastigina*;
8) to evaluate the cytopathogenicity of strains of *Acanthamoeba* and a strain of *Mastigina* by comparing the cytopathic effect of southern African corneal and sewage strains with that for strains isolated from cases of keratitis from a different continent(s).

1.6.2 *Methodology*

1) The following procedures were used for characterising strains of *Acanthamoeba* and a strain of *Mastigina*:
    a) Samples from infected corneas, contact lens cases, sewage sludge, swimming pools and tap water were used for isolating organisms.
    b) Cysts were examined, measured and photographed under a light microscope.
    c) Temperature tolerance tests were conducted on isolates at both 37°C and at 40°C.
    d) Transmission electron microscopy (TEM) was done on certain strains. Due to initial problems experienced with processing amoebae for TEM, the author tested and applied a method used for isolated cells and cell organelles (Bullock, 1987) to the amoebae.
    e) The growth of certain amoebic isolates with the addition of various substances (benomyl, diminazine, niclosamide and benomyl with niclosamide) was compared, as this has been shown to provide an indication of the identity of amoebae (De Jonckheere, Undated).
    f) Cellulose acetate isoenzyme electrophoresis (CAE) was carried out using both local and overseas isolates. CAE results were compared with those

obtained for certain strains using polyacrylamide gel electrophoresis (PAGE).

g) Restriction endonuclease digestion of whole-cell DNA was done on both local and overseas isolates.

2) Organisms were checked in respect of growth at 37°C and 40°C simultaneously, to obtain an indication of their potential pathogenicity.
3) Eleven contact lens solutions were tested for efficacy against strains of *Acanthamoeba* and a *Mastigina* isolate.
4) Twelve drugs were used to establish their anti-acanthamoebic and anti-*Mastigina* potential.
5) Two different types of mammalian cell lines were used to evaluate the cytopathogenicity *in vitro* of *Acanthamoeba* isolates and that of a strain of *Mastigina*.

CHAPTER 2 – ISOLATION OF AMOEBAE

2.1 Prevalence of *Acanthamoeba*

Free-living amoebae of the genus *Acanthamoeba* are ubiquitous in nature and are widely distributed in South Africa (Lastovica, 1980). The first South African case of acanthamoebic keratitis was reported more than two decades ago, and was detected by non-cultivation techniques (Maske *et al*., 1989). CNS disease caused by the organism had been diagnosed in the country a few years previously (Rutherfoord, 1986).

Acanthamoebic keratitis is much more prevalent in various countries than is generally appreciated (Theodore *et al*., 1985). Increased awareness is needed of the ability of *Acanthamoeba* species to give rise to serious ocular infection in southern Africa (Markus & Niszl, 1990; Niszl & Markus, 1998). Some cases are probably misdiagnosed as herpes infections of the eye. *Acanthamoeba* species have been isolated, in different parts of the world, from numerous contact lens cases and contact lens solutions used by individuals with acanthamoebic keratitis (Jones, 1986; Usa *et al*., 2009). Synergeyes hybrid contact lenses have recently been implicated in *Acanthamoeba* keratitis in six eyes of three patients (Lee & Gotay, 2010). In a study in the U.K., 89% (64/72) of acanthamoebic infections were associated with the use of contact lenses, with the ratio of soft to rigid contact lenses being 2.7 : 1 (Bacon *et al*., 1993b).

Contact lens wearers are apparently often told by contact lens practitioners, both locally and elsewhere, to rinse their lenses in tap water prior to use (Larkin *et al*., 1990). This practice should be discouraged. Furthermore, wearers of soft contact

lenses are frequently in the habit of making up their own saline, using salt tablets and non-sterile distilled water or tap water (Ma *et al*., 1990).

Since June 1990, the author has examined, mainly by means of laboratory cultivation techniques, corneal scrapings, as well as contact lens cases and often the contact lenses of 40 southern African patients suspected of having acantha-moebic keratitis. (Case histories of these patients are given in Appendix III.) *Acanthamoeba* was isolated from 13 infected eyes and *Mastigina* was associated with one infected eye (Niszl & Markus, 1991; Niszl *et al*., 1995). The amoebic isolates from these patients are now reference strains which are available internationally for research purposes. Some of the isolates have been deposited in the American Type Culture Collection (ATCC), Rockville, Maryland, U.S.A. (The ATCC numbers appear in Tables 2.1 and 2.2.)

Water was sampled from taps and swimming pools in Gauteng Province to determine whether exposure to these water sources might predispose a contact lens wearer to the risk of acanthamoebic infection. In addition, fluid was taken from the lens cases of asymptomatic contact lens wearers to see whether they showed evidence of amoebic contamination.

Processed sewage wastes often contain residual pathogenic cysts of protozoa; various reported outbreaks of disease appear to be related to the use of night soil, raw sludge or raw waste water (Burge & Marsh, 1978; Burge *et al*., 1978). Limax amoebae and their cysts are particularly abundant in the solid phase of secondary treatment processes of sewage sludge (Jenkins, 1977). Sawyer & Bodammer (1983) found that three species of amoebae were present in collection sites from active sewage, dredge and acid-waste dumpsites. There appears to be an

association between the presence of *Acanthamoeba* in marine sediments and the sites of oceanic sludge dumping (Daggett *et al*., 1982). Since acanthamoebae are often prevalent in sewage sludge in other parts of the world (Daggett *et al*., 1982), the author wished to establish whether the organism could be isolated from sewage sites in Gauteng. Samples were collected accordingly.

2.2 Methodological implications for culturing *Acanthamoeba*

There are various ways in which *Acanthamoeba* can be detected in the cornea, but a culture-proven diagnosis is the most reliable. The presence of a corneal lesion and cysts on the corneal surface provide support for a diagnosis of acanthamoebic keratitis, since *Acanthamoeba* is an uncommon contaminant in patients who do not have acanthamoebic keratitis (Johns *et al*., 1989).

It is imperative to culture for *Acanthamoeba,* because in cases in which a smear is negative, the culture may be positive, or *vice versa*. In a smear, acanthamoebic trophozoites are more difficult to detect than cysts, because the trophozoites resemble leucocytes and are more susceptible to drying and other environmental conditions than are cysts (Ma *et al*., 1990).

Amoebae have particular nutritional requirements (Balamuth & Visvesvara, 1970), but various media can be used to culture them (Cerva, 1969; Band & Balamuth, 1974; O'Dell & Brent, 1974; Cursons *et al*., 1979). There are primarily two types of cultivation employed for amoebae in which artificial cultural media are used, namely monoxenic and axenic cultures.

Monoxenic culture, in which trophozoites feed on *Escherichia coli* or other bacteria on an agar surface, is the easiest and most effective method for culturing

amoebae. In axenic culture, enriched nutrient broth medium is used without the addition of bacteria.

Certain chemicals have been found to suppress the growth of particular free-living amoebae, which makes possible the isolation of specific organisms such as *Acanthamoeba* (De Jonckheere, Undated). These chemicals were incorporated into agar plates for some environmental and a few contact lens samples, to ascertain whether they resulted in the exclusive isolation of certain genera of free-living amoebae.

Griffin (1972) found a relationship between the ability to tolerate high temperatures, and virulence in *Naegleria* and *Acanthamoeba* – with avirulent strains being unable to grow at normal or raised body temperatures. However, Stevens *et al*. (1977) established that a number of non-pathogenic amoebae were capable of growth at 45°C, indicating that temperature tolerance is not, in fact, necessarily an indicator of pathogenicity (see also Chomicz *et al*., 2010). Nevertheless, incubating the material does result in fewer non-pathogenic isolates being cultured, with less risk of losing isolates because of overgrowth by non-pathogenic strains (Stevens *et al*., 1977). De Jonckheere (1980) concluded that although ability to grow at 40°C in the presence of bacteria points to virulence in an *Acanthamoeba* isolate, its virulence can actually be measured only in cell cultures or laboratory animals. Growth of local and overseas amoebic isolates was checked at 37°C and at 40° to obtain an indication of their virulence. In addition, the cytopathogenicity of both local and overseas strains was tested in cell cultures (Chapter 10; Niszl *et al.*, 1998). Strain variability of the amoebae/clones must also be considered in this regard.

2.3 Materials and methods

2.3.1 *Culturing of amoebae*

2.3.1.1 Monoxenic culture

E. coli bacteria that were grown on agar slopes suspended in nutrient broth[4] were seeded on to the surface of 1.5 % non-nutrient agar (NNA)[5] to provide food for the amoebae. For the inoculation on to NNA plates of samples such as clinical specimens (corneal scrapings or anterior chamber fluid [fluid from the space inside the eye between the iris and the cornea's innermost surface, the endothelium])[6] or environmental or water samples, the specimen was placed on the *E. coli* on the agar surface without streaking out or cutting into the agar. The temperature at which the different samples were incubated is given under the relevant section (Chapters 2.3.3; 2.3.5 – 2.3.9). The plates were sealed with adhesive tape to prevent dehydration, and were examined daily for up to 14 days for acanthamoebic trophozoites or cysts. Plates were examined under an inverted microscope, or the plate was inverted and screened under a compound microscope (100 x magnification).

Sub-cultures of amoebae were prepared by cutting out a small block of agar containing amoebae, and placing it face down on the surface of another (fresh) agar plate.

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4. See Appendix II.
5. See Appendix II.
6. See Appendix IX.

2.3.1.2 Axenic culture

In order to adapt strains of amoebae to axenic medium, a piece of agar containing cloned trophozoites (see below) was placed in peptone-yeast extract glucose (PYG) broth or serum casein glucose yeast extract medium (SCGYEM) containing Ampicillin.[7] These trophozoites were placed in screw-capped 80 cm^2 (260 ml) Nunc tissue culture flasks and incubated at 30°C. Sub-cultures of amoebae that had adapted to axenic medium were grown in 500 ml broth, without the addition of Ampicillin, in 5-litre flasks at 30°C on a shaker set at 100 rpm.

2.3.2 *Cloning of amoebae*

Amoebae were cloned by diluting a suspension of cysts in sterile amoeba saline (De Jonckheere *et al.*, 1974),[8] spreading them on agar under a microscope and selecting individual cysts by using low magnification. Agar blocks approximately 2 mm^3 in size and bearing a single cyst were cut out and transferred face-down to a fresh agar plate seeded with *E. coli* bacteria, to ensure monoclonal selection.

2.3.3 *Culturing of corneal scrapings*

When acanthamoebic infection was suspected, the corneal areas involved were scraped as thoroughly and as deeply as feasible by the attending ophthalmologist to maximise the chances of recovery of amoebae in cultures or smears, as recommended by Theodore *et al*. (1985). Specimens were plated directly in most cases. When this was not possible, scrapings were transported at ambient temperature in sterile amoeba-saline (Gradus *et al.*, 1989) and plated out as soon as possible. Since it often takes days for *Acanthamoeba* to grow in culture,

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7. See Appendix II.
8. See Appendix II.

examination of the patient's contact lens by light microscopy, before cultural confirmation, sometimes proved helpful in establishing a clinical diagnosis of acanthamoebic keratitis (Johns *et al.*, 1989).

For slide preparation, a thin smear was made from the corneal scraping, as cysts could become buried in a thick smear and might not be visible. The scraping was teased out and spread on to the slide with a sterile scalpel blade. The blade was not wiped with alcohol, as any trace of it could inhibit or kill *Acanthamoeba* trophozoites or cysts. The number of scrapings taken from keratitis cases is limited by the need to avoid undue damage to the cornea. However, a rapid diagnosis can often be made by microscopic examination of a good smear preparation while the culture results are awaited.

Corneal scrapings from patients with suspected acanthamoebic keratitis[9] were plated on to 1.5% NNA plates seeded with *E. coli* bacteria as food for the amoebae, and incubated at 30°C. Whole contact lenses and solutions from contact lens cases, if available, were also placed on plates. The plates were examined daily for up to 14 days for the presence of trophozoites or cysts of *Acanthamoeba* species. Amoebae were cloned by dilution, agar plating and microscopic selection of individual cysts (De Jonckheere *et al*., 1974).

For staining or sub-culturing, positive cultures were handled in a biological safety cabinet in order to prevent environmental contamination with the cysts as well as to protect the researcher.

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9. See Appendix III.

In several cases, *Acanthamoeba* was not suspected by the clinician; and other micro-organisms were initially cultured for. When acanthamoebic keratitis was suspected, culturing for *Acanthamoeba* and other micro-organisms was done simultaneously, to avoid delaying the diagnosis. Bacterial infections, such as those caused by *Pseudomonas* spp. (Dini *et al.*, 2000), streptococci or *Propionibacterium* spp., may accompany acanthamoebic keratitis, especially if the contact lenses have been washed in contaminated, home-made saline (Ma *et al*., 1990).

2.3.4 *Measurement of cysts and trophozoites*

Cysts and trophozoites were measured using a Union vernier ocular micrometer.

2.3.5 *Temperature tolerance trials*

For temperature tolerance trials, which are relevant to infrageneric characterisation of *Acanthamoeba* spp. and which can give an indication of virulence (De Jonckheere, 1980), trophozoites and cysts were incubated at both 37°C and at 40°C for 14 days, after which organisms were plated out and incubated at 30°C to ascertain whether amoebae were still viable. Control plates were kept at 30°C for the duration of the experiment, and amoebic viability monitored.

2.3.6 *Examination of contact lens cases*

For examination of contact lens cases, two drops (40 μl) of fluid were taken, using a sterile pipette, from both the left and right sides of each of 22 contact lens cases belonging to lens wearers having no symptoms of ocular disease. This fluid was cultured on NNA, as described above. Plates were incubated at 30°C. Two control plates (for the left and right sides of the lens case, respectively) were

prepared using sterile distilled water from contact lens cases that had been rinsed in boiling water.

2.3.7 *Sampling of tap water*

Tap water samples from 50 different areas in Gauteng Province, selected for convenience, were examined for amoebae.

A 10 ml sample, immediately followed by a 750 ml sample, was collected in a sterile glass bottle from a cold-water bathroom tap first thing in the morning, and processed the same afternoon. (If amoebae have multiplied overnight in the water in a tap, they can be isolated readily from the 10 ml sample. However, a larger sample can often be more useful.)

Containers were agitated to ensure thorough mixing of the water. Half of each sample was filtered, using slight suction, through sterile, 0.45 μm pore size (25 mm diameter) cellulose nitrate membranes in the case of the 10 ml samples, and 5 μm pore size (47 mm diameter) cellulose nitrate membranes for the 750 ml samples. Filtration rather than centrifugation was used, because amoebae can be recovered far more easily when samples are processed by filtration (Winiecka-Krusnell & Linder, 1998). The membranes were inverted on to NNA streaked with *E. coli* bacteria and incubated at both 25°C and 37°C. The plates were sealed with tape to prevent dehydration, and monitored daily for 14 days for amoebic growth.

Control experiments were carried out by autoclaving the remaining half of each sample, after which processing was done in the same manner as for the experimental samples.

2.3.8 *Sampling of swimming pool water*

A 750 ml sample of water was taken from each of 13 swimming pools in Gauteng, and treated in the same manner as the tap water samples. Chlorine levels in the swimming pool samples were determined using diethyl-p-phenylene-diamine sulphate (DPD) tablets (Lovibond). Isolates were tested for growth at 25°C and at 37°C (Table 2.5). Controls were set up as above.

2.3.9 *Sampling of sewage sludge*

Sewage sludge samples were taken at the final stage of treatment from four different sewage works in Gauteng, namely, Goudkoppies, Northern Sewage Works, Southern Sewage Works and Vlakplaats. Cultures were prepared by inoculating one bacteriological loopful of sediment on to the surface of an agar plate that had previously been streaked with *E. coli* bacteria. The plate was incubated at 30°C.

For temperature tolerance trials, trophozoites and cysts of isolates were incubated at 37°C and at 40°C, respectively, for 14 days, after which they were plated out and incubated at 30°C to ascertain whether they were still viable. Control plates of amoebae were kept at 30°C for the duration of the experiment, and their viability monitored.

2.3.10 *Incorporation of chemicals into agar*

The chemicals listed below and noted in Table 2.7 were incorporated into agar plates to assess their effect on suppressing the growth of certain amoebae (De Jonckheere, Undated):

- Benomyl (10 mg/l) allows for isolation only of members of the family Vahlkampfiidae;

- Berenil (used for trypanosomiasis and piroplasmosis, as it inhibits DNA synthesis) (10 mg/l) allows for isolation only of *Naegleria*;
- Niclosamide (a molluscicide which also acts against Platyhelminthes and inhibits the growth of all Vahlkampfiidae) (20 mg/l) allows for the isolation of *Acanthamoeba*;
- Benomyl (20 mg/l) and Niclosamide (20 mg/l) allows for the isolation of *A. culbertsoni* and *A. comandoni.*

Controls consisted of plating each isolate on to separate NNA plates (with no chemicals incorporated) seeded with *E. coli*.

2.4 Results

2.4.1 *Monoxenic culture*

Cysts present in the specimen excysted in the favourable environment of the NNA plates on which bacteria were growing (Figs 2.1 and 2.2). As the amoebae multiplied rapidly and engulfed bacteria, they moved to the periphery of the area of bacterial growth, usually within two or three days of incubation. Trophozoites were usually found growing away from the area of the bacterial inoculum. Trophozoites can be identified by observing a suspected trophozoite for the presence of a contractile vacuole, which disappears and reappears after a few seconds (Figs 2.3 and 2.4). This feature differentiates a trophozoite from an artefact. Cyst formation began within two or three more days, when the supply of bacteria was exhausted (Figs 2.5 and 2.6). Cysts are often arranged in clusters which, to an inexperienced observer, may look like crystals (Figs 2.5 and 2.6).

2.4.2 *Axenic culture*

The strains of *Acanthamoeba* adapted well to growth in PYG medium, but not to

SCGYEM.[10] Trophozoites of strain ATCC 50686 growing in a flask in PYG medium are shown in Figure 2.7.

2.4.3 *Patients with keratitis*

The case histories of the 14 keratitis patients from whom organisms were cultured, are summarised in Table 2.1. Detailed case histories of these 14 patients (where available) as well as the case histories of keratitis patients from whom corneal scrapings were taken and no *Acanthamoeba* was isolated, are presented in Appendix III. *Acanthamoeba* was associated with 13 infected eyes and a strain of *Mastigina* was identified (by T.A. Nerad of the American Type Culture Collection – ATCC – Rockville, Maryland, U.S.A.) in one infected eye (Table 2.1; Niszl & Markus, 1991; Niszl *et al*., 1995).[11]

2.4.4 *Measurement of cysts and trophozoites and temperature tolerance trials*

The measurements of cysts and trophozoites and the results of temperature tolerance trials are presented in Table 2.2. All the isolates grew at 37°C. Most of them encysted at 40°C, but multiplied again when replated at 37°C. All control amoebae survived for the duration of the experiment.

2.4.5 *Contact lens cases*

The results of the examination of contact lens cases are given in Tables 2.3 and 2.4. Photographs of isolates from one of the hard contact lens cases can be seen in Figures 2.8 – 2.14. None of the controls for any of the hard or soft contact lens experiments showed any growth of amoebae after day 14.

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10. See Appendix II.

11. See Appendix III.

2.4.6 *Tap water*

Amoebae were found in one of the 10 ml samples and in 14 of the 750 ml samples. Multiplication of organisms occurred at both 25°C and at 37°C. Growth of several of the strains took place at 37°C, which indicates that they could be pathogenic (De Jonckheere, 1980). Further testing would be required to confirm this. In most cases, reproduction of amoebae could be seen to have taken place after three to five days in culture. Many double-walled cysts and trophozoites with spiky acanthapodia, typical of *Acanthamoeba*, were observed. None of the controls showed any growth of amoebae after day 14.

2.4.7 *Swimming pool water*

Results for the 13 swimming pool samples taken are given in Table 2.5. Amoebae were cultured from 92% of the local swimming pools. Some of the amoebic isolates were able to grow at both 25°C and 37°C, indicating that they could possibly be virulent, whilst other isolates grew only at 25°C. Amoebae were never isolated from control samples.

2.4.8 *Sewage sludge*

Acanthamoebae were recovered from all four sewage stations. These isolates were cloned. Measurements and temperature tolerance results are given in Table 2.6. Trophozoites multiplied at 40°C, unlike most corneal isolates, which encysted at this temperature. Amoebic controls, kept at 30°C, were viable for the duration of the experiment. None of the controls showed growth of amoebae after 14 days for any of the experiments done.

2.4.9 *Incorporation of chemicals into agar*

When benomyl was incorporated into the agar, amoebae from the family Vahlkampfiidae were isolated from all samples except those from Southern Sewage (Table 2.7). The genus *Naegleria* (found when diminazine was included in agar) was isolated from some of the samples. Strains of *Acanthamoeba* were also found in most of the samples (Table 2.7). It is of interest to note that in some instances, the species of *Acanthamoeba* isolated was *A. culbertsoni* or *A. comandoni* (inclusion of benomyl and niclosamide), whereas in other cases, strains of *Acanthamoeba* (inclusion of niclosamide) did not grow when benomyl and niclosamide were incorporated into the agar. All control plates showed growth of amoebae throughout the experiment.

Table 2.1. Isolates of *Acanthamoeba* and *Mastigina* species from southern African cases of keratitis.

Strain*; isolation date; patient's age (Yr); sex	Geographic source of infection	Source of isolate	Affected eye; type of contact lens	Contact lens cleaner	Possible source of infection	Presenting features	Diagnostic interval (days from onset of disease to treatment)	Aetiological agent
ATCC 50676 (SAWE 90/1); 90/07/06; 35; Fe	Namibia or S. A.	Cornea; Lt and Rt lens case	Rt; Disposable	Home-made saline with use of non-sterile distilled water	Hot water spring; home-made saline; disposable lenses	Painful eye; corneal ulcer diagnosed as herpes simplex with *Candida* infection; hypopyon	2 months	*Acanthamoeba mauritaniensis* (Schroeder *et al*., 2001; Ledee *et al*., 2003)
ATCC 50677 (SAWE 92/2); 92/08/03; ?; Ma	East London, S. A.	Cornea	?; Soft	?	?	?	?	*Acanthamoeba mauritaniensis* (Schroeder *et al*., 2001; Ledee *et al*., 2003)
ATCC 50678 (SAWE 93/3); 93/12/29; 28; Fe	Johannesburg, S. A.	Cornea; Lt and Rt contact lenses; fluid from contact lens case	Rt; Disposable	Sterile saline	Saline; disposable contact lenses; no disinfection of contact lenses; lenses worn while bathing and washing hair	Painful eye, "scratchy" when contact lens inserted; white stromal ring; broken vessels in cornea	7 weeks	*Acanthamoeba mauritaniensis* (Schroeder *et al*., 2001; Ledee *et al*., 2003)

Strain*; isolation date; patient's age (Yr); sex	**Geographic source of infection**	**Source of isolate**	**Affected eye; type of contact lens**	**Contact lens cleaner**	**Possible source of infection**	**Presenting features**	**Diagnostic interval (days from onset of disease to treatment)**	**Aetiological agent**
ATCC 50679 (SAWE 94/4); 94/03/23; 20s; Fe	Margate or Pretoria, S. A.	Cornea	Rt; disposable and soft	Commercial cleaner; sterile saline	Disposable lenses; washed lens case out with tap water; swam in the sea with lenses inserted	Foreign body sensation; clinically diagnosed as viral herpes; brittle epithelium over cornea; thickened nerve endings and blood vessels over limbus; large, round central lesion, with full thickening of epithelium	2 months	*Acanthamoeba mauritaniensis* (Schroeder *et al*., 2001; Ledee *et al*., 2003)
ATCC 50680 (SAWE 94/5); 94/08/09; 18; Ma	Botswana or Pretoria, S. A.	Cornea	Lt; disposable	Sterile saline	Disposable lenses; no disinfection of lenses	Corneal ulcer; initially thought to be fungal in origin	1 month; there was a history of ulcers	*Acanthamoeba mauritaniensis* (Schroeder *et al*., 2001; Ledee *et al*., 2003)
ATCC 50681 (SAWE 95/6); 95/02/17; 43; Fe	Hartebeespoort Dam or Pretoria, S. A.	Cornea	Rt; disposable	None	Disposable lenses: no sterilising; swam with lenses inserted	Painful red eye; corneal oedema	3 weeks	*Acanthamoeba mauritaniensis* (Schroeder *et al*., 2001; Ledee *et al*., 2003)

Strain*; isolation date; patient's age (Yr); sex	**Geographic source of infection**	**Source of isolate**	**Affected eye; type of contact lens**	**Contact lens cleaner**	**Possible source of infection**	**Presenting features**	**Diagnostic interval (days from onset of disease to treatment)**	**Aetiological agent**
ATCC 50682 (SAWL 91/3); 91/07/29; 28; Fe	Johannesburg, S. A.	Lt and Rt sides of contact lens case	Rt; soft	?	?	Corneal ulcer	?	*Acanthamoeba mauritaniensis* (Schroeder *et al*., 2001; Ledee *et al*., 2003)
ATCC 50683 (SAWL 91/4); 91/08/07; ?; Fe	Johannesburg, S. A.	Lt and Rt sides of contact lens case	?; soft	Commercial contact lens cleaner	?	?	?	*Acanthamoeba mauritaniensis* (Schroeder *et al*., 2001; Ledee *et al*., 2003)
ATCC 50684 (SAWL 93/1); 93/07/20; 29; Fe	Pretoria, S. A.	Contact lens	Lt; disposable	Commercial contact lens cleaner and saline	Disposable lenses and saline	?	?	*Acanthamoeba mauritaniensis* (Schroeder *et al*., 2001; Ledee *et al*., 2003)
SAWE 95/7; 95/09/01; 30; Fe	Pretoria, S. A.	Cornea	?; not a contact lens wearer	None	? as patient was not a contact lens wearer	?	?	*Acanthamoeba* species

Strain*; isolation date; patient's age (Yr); sex	**Geographic source of infection**	**Source of isolate**	**Affected eye; type of contact lens**	**Contact lens cleaner**	**Possible source of infection**	**Presenting features**	**Diagnostic interval (days from onset of disease to treatment)**	**Aetiological agent**
SAWE 96/8; 96/03/08; 20; Ma	Pretoria, S. A.	Cornea	Rt; disposable	None	Disposable lenses; no disinfection	Clinically diagnosed as herpes or fungal infection; ring ulcer	2 months	*Acanthamoeba* species
SAWE 97/10; 97/01/30; 15; Fe	Port Elizabeth or Pretoria, S. A.	Cornea	Lt; soft	?	Soft contact lens wearer; swam in sea and in lagoon while wearing lenses	Corneal ring infiltrate	7 weeks	*Acanthamoeba* species
SAIMR 96/9; 96/04/02; 31; Fe	Johannesburg, S. A.	Contact lens	Rt; soft	Non-sterile tap water	Contact lenses rinsed with tap water	Described in Dini *et al*. (2000)	?	*Acanthamoeba polyphaga* (Dini *et al*., 2000)

Strain*; isolation date; patient's age (Yr); sex	**Geographic source of infection**	**Source of isolate**	**Affected eye; type of contact lens**	**Contact lens cleaner**	**Possible source of infection**	**Presenting features**	**Diagnostic interval (days from onset of disease to treatment)**	**Aetiological agent**
SAWL 91/2; 91/05/27; ?; Fe	Johannesburg, S. A.	Lt and Rt contact lenses and lens case; bottle used for home-made saline	Rt; disposable and soft	Home-made saline and boiling of soft lenses	Non-sterile bottle used for home-made saline; disposable contact lenses	Chronic corneal ulcers; clinically diagnosed as herpes simplex infection; epithelial erosions and underlying peripheral infiltrate	2 months (ulcers had occurred on and off for 8 months previously)	*Mastigina* species

Key: ? = no information; Fe = female; Lt = left; Ma = male; Rt = right; Yr = years

*The ATCC numbers are those of strains that have been deposited in the American Type Culture Collection.

Table 2.2. Measurements and temperature tolerance test results for organisms isolated from keratitis patients.

Table 2.2. Measurements and temperature tolerance test results for organisms isolated from keratitis patients.

Strain[*]	Trophozoites viable at 37°C	Trophozoites viable at 40°C	Cysts viable at 40°C	Size range of cysts (μm)[#]	Mean cyst diameter (μm)[#]
Ac/PHL/4[β]	+	++	+	10.7–14.2	11.5
Ac/PHL/9[β]	+	++	+	11.7–14.4	12.8
Ac/PHL/17[β]	+	++	+	15.0–18.0	16.0
Ac/PHL/22[β]	+	++	+	11.5–16.4	14.8
Ac/PHL/23[β]	+	++	+	11.9–17.6	15.7
ATCC 30868[β] CCAP 1501/2g	+	++	+	13.7–18.3	16.0
ATCC 30873[β] CCAP 1501/3d	+	++	+	10.5–15.0	13.7
ATCC 50676[**]	+	++	+	10.9–14.6	12.4
ATCC 50677	+	++	+	10.1–11.9	11.3
ATCC 50678	+	++	+	11.3–17.2	14.6
ATCC 50679	+	++	+	10.3–14.1	12.3
ATCC 50680	+	++	+	11.3–16.8	14.9
ATCC 50681	+	++	+	8.0–12.5	10.6
ATCC 50682	+	- -	+	7.6–11.8	10.1
ATCC 50683	+	- -	+	10.1–11.9	11.2
ATCC 50684	+	++	+	10.1–15.6	12.7
RYD	+	++	+	10.1–13.3	12.0
SAIMR 96/9[***]	+	++	+	13.5–15.2	14.1
SAWE 95/7	+	++	+	14.1–16.6	15.5
SAWE 96/8	+	++	+	14.0–17.8	16.1
SAWE 97/10	+	++	+	12.8–18.0	15.1

Table 2.2 (cont.)

SAWL 91/2	+ (but poorly)	- -	-	8.6–10.5	9.8
435/89	+	++	+	12.7–15.2	13.7
452/89	+	++	+	13.3–17.4	15.8

Key:

* The ATCC numbers are for strains that have been deposited in the American Type Culture Collection (ATCC).

** Amoebae were seen in fresh preparations from the eye (Dr. C. Heney, pathologist, personal communication; Amoils & Heney (1999)). The researcher (author of this thesis) confirmed the diagnosis by culture.

*** This strain is the subject of a paper by Dini *et al*. (2000). The initial isolation was made at the South African Institute for Medical Research.

Based on measurements of ten cysts.

β The source of this isolate is given in Appendix IV.

+ = growth occurred.

- = no growth detected.

++ = trophozoites encysted after 15d at 40°C and multiplied again when re-plated at 30°C.

- - = trophozoites died after 15d at 40°C, i.e. they did not grow again when re-plated at 30°C after 15d at 40°C.

Table 2.3. Hard contact lens cases* sampled for amoebae.

Method of cleaning	No. of contact lens cases examined	Results
Commercial chemical solution for disinfection, soaking and insertion of lenses; tap water for rinsing	6	+ve (2) (for right lenses)
Tap water used for cleaning; lenses left soaking in tap water; tap water for insertion of lenses. Protein remover used occasionally	1	+ve (1) (for both left and right lenses)
Commercial chemical solution for disinfection; rinsed in sterile, distilled water or boiled tap water; soaking in sterile saline; sterile, distilled water for insertion of lenses	2	-ve

Key:

* These fluid samples were taken from contact lens wearers without symptoms of ocular disease.

+ve = growth of amoebae occurred; the number in brackets indicates the number of contact lens case fluids that were positive.

-ve = no growth of organisms occurred.

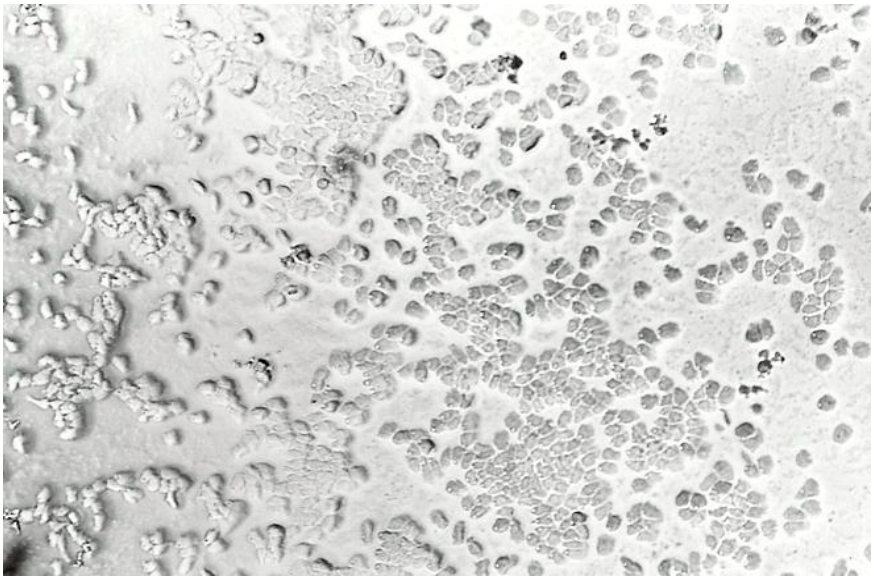
Table 2.4. Soft contact lens cases* sampled for amoebae.

Method of cleaning	**No. of contact lens cases examined**	**Results**
Cleaning, soaking and insertion of lenses with saline made up with non-sterile distilled water and salt tablets; boiling of lenses every night for disinfection	4	-ve
Commercial chemical solution for disinfection, cleaning, soaking and insertion of lenses	4	-ve
Commercial chemical solution for cleaning; preserved saline for soaking and insertion of lenses	4	-ve
Commercial chemical solution for cleaning; sterile saline for soaking and insertion of lenses; boiling of lenses every night	1	-ve

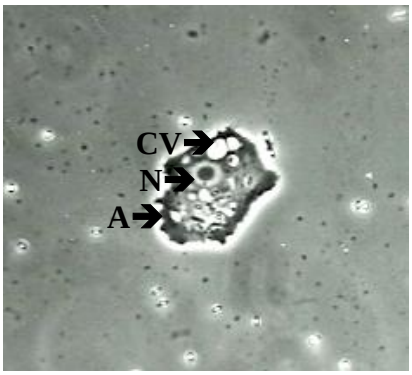
Key:

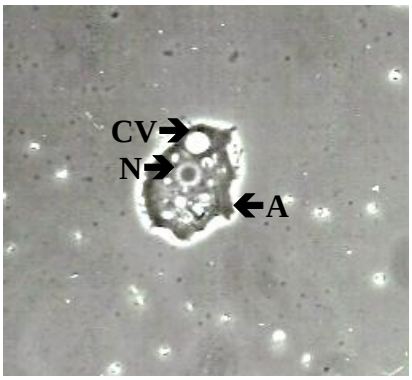
* These fluid samples were taken from contact lens wearers without symptoms of ocular disease.

-ve = no growth of organisms occurred.

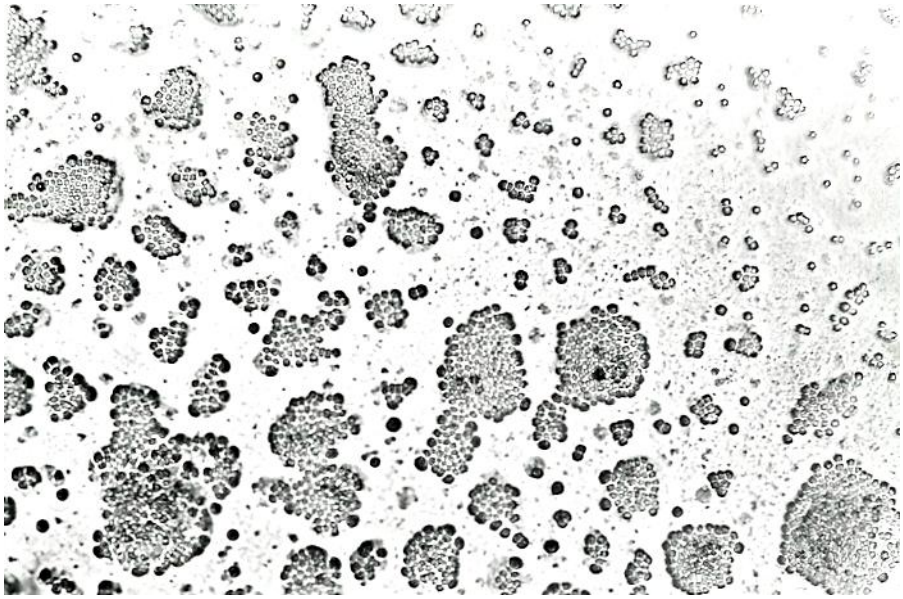


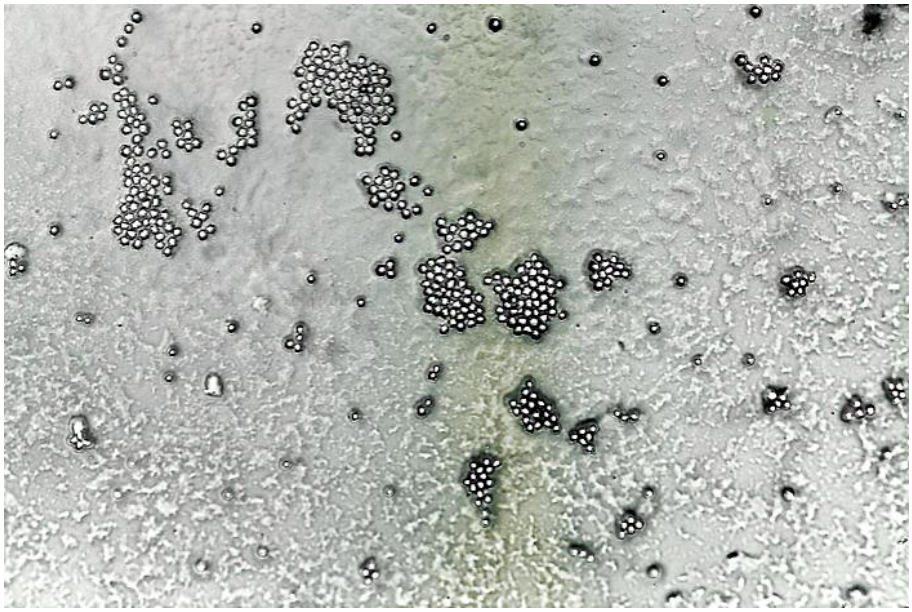
Figures 2.1 and 2.2. Trophozoites of strain ATCC 50676 on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. The amoebae multiply rapidly and engulf the bacteria as they move to the periphery of the area of bacterial growth. (x 110)





Figures 2.3 and 2.4. Trophozoites of strain SAWS 87/4. (Phase contrast.) The contractile vacuole (CV) is situated close to the nucleus (N) which contains a well-defined nucleolus. The disappearance and reappearance of this vacuole after a few seconds helps to differentiate the trophozoite from an artifact. Spiky acanthapodia (A), a distinguishing characteristic of the genus *Acanthamoeba*, are apparent. (x 420)





Figures 2.5 and 2.6. Cysts of strain ATCC 50676, formed on a non-nutrient agar plate seeded with *Escherichia coli.* Cyst formation begins when the supply of bacteria is exhausted. Note the arrangement of most of the cysts in clusters which, to an inexperienced observer, may look like crystals. (x 110)

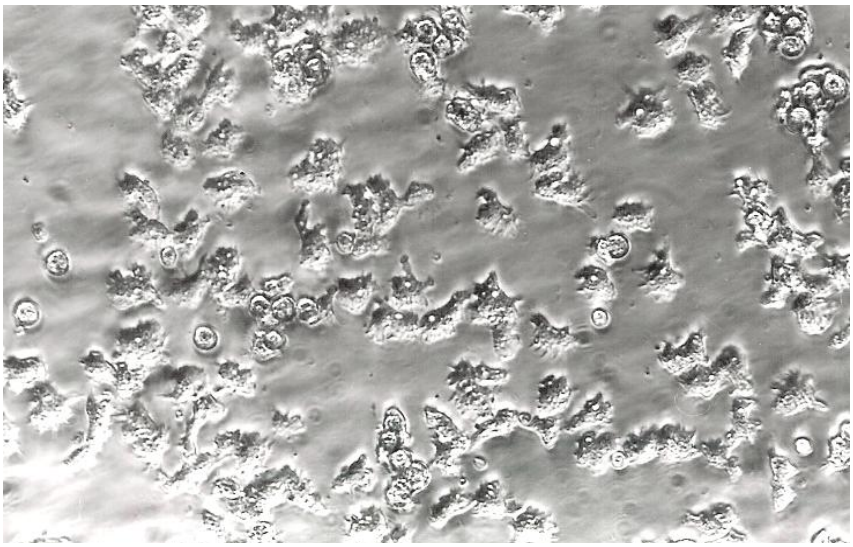


Figure 2.7. Trophozoites of strain ATCC 50686 growing in PYG medium in a flask. (Phase contrast.) Contractile vacuoles are clearly visible in several trophozoites. (x 220)

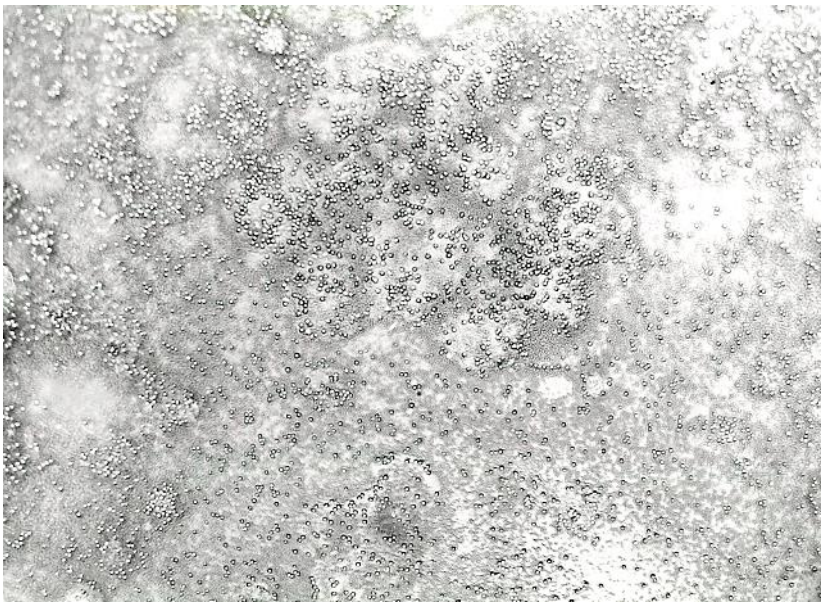
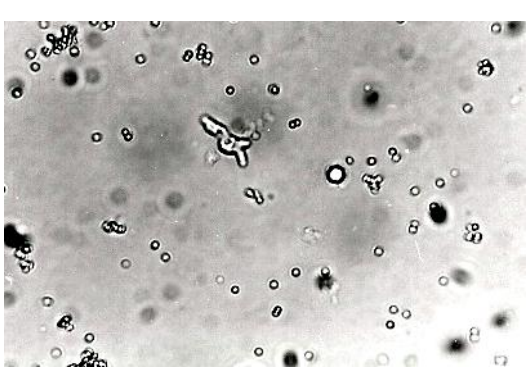
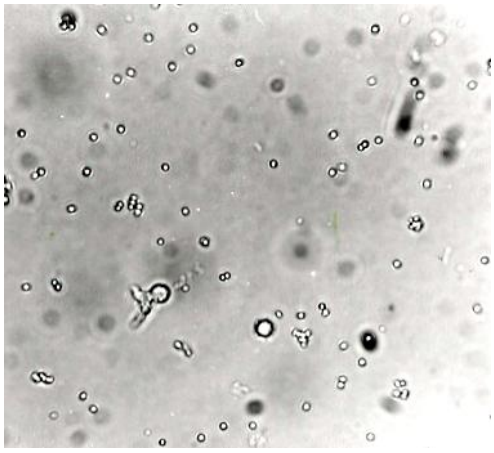


Figure 2.8. A plate of cysts grown from the contact lens case of an asymptomatic hard contact lens wearer. Amoebae were grown on non-nutrient agar plates seeded with *Escherichia coli* bacteria. (x 110)





Figures 2.9 and 2.10. Excysting trophozoite and an intact cyst isolated from the contact lens case of an asymptomatic hard contact lens wearer. (x 440)

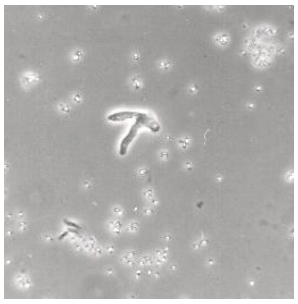


Figure 2.11. (x 440)

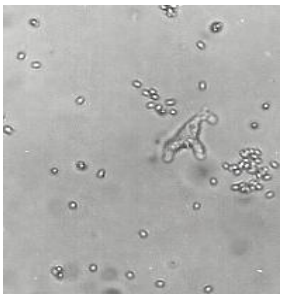


Figure 2.12. (x440)

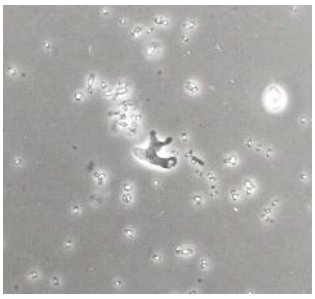


Figure 2.13. (x 440)

Figure 2.14. (x 1050)

Figures 2.11 to 2.14. Trophozoites from the contact lens case of an asymptomatic hard contact lens wearer. Pseudopodia and a contractile vacuole are visible. *Escherichia coli* bacteria can be seen around the trophozoites.

Table 2.5. Water samples from swimming pools in Gauteng Province.

Pool no.	Growth of amoebae at 25°C	Growth of amoebae at 37°C	Method of pool cleaning
1	+	+	Salt
2	+	+	Salt
3	+	+	Salt
4	+	+	Electrode
5	+	+	Electrode
6	+	-	Electrode
7	+	+	Granular Chlorine* (3 μg/ml)
8	+	+	Granular Chlorine* (1 μg/ml)
9	+	-	Granular Chlorine* (4 μg/ml)
10	-	-	Granular Chlorine* (8.5 μg/ml)
11	+	+	Granular Chlorine* (2 μg/ml)
12	+	+	Granular Chlorine* (2 μg/ml)
13	+	+	Granular Chlorine* (3 μg/ml)

Key:

* Chlorine was added directly to pool water.

+ = growth of amoebae.

- = no growth of amoebae.

Table 2.6. Measurements and temperature tolerance test results for *Acanthamoeba* species from sewage sludge.

Strain*	**Tropho-zoites viable at 37°C**	**Tropho-zoites viable at 40°C**	**Cysts viable at 40°C**	**Size range of cysts (μm)#**	**Mean cyst diameter (μm)#**	**Source of isolate**
ATCC 50685 *A. lenticulata***	+	++	+	15.4–18.6	17.1	Northern Sewage Works
ATCC 50686 *A. lenticulata***	+	++	+	16.6–21.5	18.6	Southern Sewage Works
ATCC 50687 A. *lenticulata***	+	++	+	15.6–19.9	18.0	Vlakplaats Sewage Works
SAWS 87/4 *Acanthamoeba* species	+	++	+	13.0–15.1	13.9	Goud-koppies Sewage Works

Key:

* The ATCC numbers are for those strains that have been deposited in the American Type Culture Collection (ATCC).

** As identified by Schroeder J.M., Booton, G.C., Hay J., Niszl I.A., Seal D.V., Markus M.B., Fuerst P.A. & Byers T.J. (2001).

These figures are based on measurements of ten cysts.

+ = growth of organisms occurred.

++ = trophozoites multiplied within 15d at 40°C.

Table 2.7. Incorporation of chemicals into agar.

	Chemical			
Sample	**Benomyl (selects for Vahlkampfiidae)**	**Berenil (selects for *Naegleria* species)**	**Niclosamine (selects for *Acanthamoeba* species)**	**Benomyl + Niclosamine (selects for *A. culbertsoni* and *A. comandoni*)**
Contact Lens Case 1	+	-	-	-
Contact Lens Case 2	+	+	-	-
Fish Pond 1	-	-	+	-
Fish Pond 2	+	+	-	-
Fish Pond 3	-	-	+	+
Fish Pond 4	+	-	-	-
Tap water 1	+	+	-	-
Tap water 2	+	-	-	-
Tap water 3	-	-	+	-
Tap water 4	+	-	-	-
Tap water 5	+	+	-	-
Tap water 6	+	-	-	-
Tap water 7	-	-	+	+
Northern Sewage 1	-	-	+	+
Northern Sewage 2	+	-	-	-
Southern Sewage 1	-	-	+	-
Vlakplaats Sewage 1	+	-	-	-
Vlakplaats Sewage 2	-	-	+	-

Key:

\+ = growth of amoebae.

\- = no growth of amoebae.

2.5 Discussion

2.5.1 *Ocular disease*

Thirteen of the 14 patients with keratitis caused by *Acanthamoeba* or *Mastigina* species (Niszl & Markus, 1991; Table 2.1), were contact lens wearers. Six of these 13 individuals had disposable lenses, two used both disposable and soft contact lenses and five wore soft contact lenses only. Bacon *et al*. (1993b) also found that most cases of acanthamoebic keratitis occurred in people who were contact lens wearers. These authors had managed 72 cases of the disease (involving 77 eyes). Of those patients, 64 were contact lens wearers, 28 of whom had used disposable contact lenses. The risk of microbial keratitis appears to increase with the use of extended-wear soft contact lenses (Cheng *et al*., 1999).

The shortest diagnostic interval (counted in days from the onset of ocular disease to the commencement of anti-acanthamoebic treatment) for patients in the study was 21 days. However, the diagnostic interval was as much as 60 days between onset and the initiation of specific treatment for acanthamoebic keratitis in most patients. Not all ophthalmologists in southern Africa consider the condition in their differential diagnosis of corneal keratitis, and this can cause a considerable delay in recognition of the problem. Bacon *et al*. (1993a; 1993b) believe that the biggest single variable still affecting the progression and outcome of acanthamoebic keratitis is the length of time between onset of the first symptoms and the institution of an anti-amoebic regimen. Although acanthamoebae cause superficial disease initially, deeper stromal invasion[12] and encystment occur with

12. See Appendix IX.

time, and in association with keratocyte depletion. Bacon *et al*. (1993a; 1993b) established that if the disease is diagnosed later in the infection, there is likely to be deeper corneal involvement than occurs in early symptomatic infection. This is probably the reason why corneal scrapings can be culture-negative when taken some time after manifestation of initial symptoms, and why a corneal biopsy specimen is then required to obtain viable amoebae from the deeper stroma (Ficker *et al*., 1993).

Bacon *et al*. (1993b) demonstrated that superficial corneal involvement and perineural infiltrates were common in cases of acanthamoebic keratitis diagnosed less than 30 days after the first symptoms appeared (early presentation). Moreover, they observed that ring infiltrates and ulceration with stromal lysis characterised those cases presenting at 30 – 60 days (intermediate) or after 60 days (late). Furthermore, these groups progressed more frequently to hypopyon, scleritis, glaucoma and cataract formation. Several keratitis patients in this (the present) researcher's study who were correctly diagnosed more than 30 days after onset of disease had corneal ulcers, initially thought to be of viral or fungal origin (Table 2.1). In contrast, Bacon *et al*. (1993b) found that the most common initial clinical diagnosis in Britain has, because of increased awareness, changed from herpes simplex infection to acanthamoebic keratitis. Moreover, the average diagnostic interval at Moorfields Eye Hospital in the U.K. dropped from 180 days in 1985 to 9.3 days in 1992 (Bacon *et al*., 1993b).

Acanthamoebae were not grown from the corneal scrapings of some of the patients researched, yet amoebae were isolated by culture from contact lenses used in the patients' infected eyes and/or in the contact lens solutions. This

finding confirms John *et al*.'s (1989) warning to check contact lenses for amoebae. The reason is that corneal scrapings can give negative results, even though amoebae may be visible on the contact lens. Cohen *et al*. (1987) mention that in early cases, when there is little involvement of the corneal surface, corneal smears usually give negative results, despite the fact that acanthamoebae may be found by culturing from the contact lenses.

The laboratory staining, plating and cultivation techniques needed in order to isolate *Acanthamoeba*, require certain expertise. This became evident recently in South Africa, when a local pathology laboratory was unable to recognise trophozoites of *Acanthamoeba*, and valuable time was lost before treatment could be instituted. It is particularly important to distinguish acanthamoebic from bacterial or fungal keratitis by laboratory confirmation of a clinical diagnosis, because the treatments are so different. Therefore, every effort should be made to obtain corneal material, contact lenses and their cases, as well as the cleaning solutions used by patients, for culture (Bacon *et al*., 1993b).

The morphology of cysts alone (especially fixed material) is not sufficient as a basis for species identification, because it varies according to the culture conditions (Hanssens *et al*., 1985). In fixed tissues, the trophozoite of *Acanthamoeba* can resemble a macrophage, except that the nucleus is always spherical, with a large and eosinophilic nucleolus. In addition, the size ratio of nucleus to cytoplasm is smaller in amoebae compared to macrophages.

Nine of the corneal isolates have been identified (in a separate study) as *A. mauritaniensis*, which belongs to genotype T4 (Schroeder *et al.*, 2001). The others have not yet been characterised. Schroeder *et al.* (2001) and Stothard *et al*.

(1998) placed 39 of 40 *Acanthamoeba* isolates from human eyes or contact lens cases into their genotype T4 (Ledee *et al*., 2003).

2.5.2 Temperature tolerance

Growth at 37°C, and particularly at 40°C, gives an indication of the possible pathogenicity of strains. The temperature tolerance results provided an indication of the virulence of the southern African isolates, and this was checked in mammalian cell cultures (Chapters 10 and 11; Niszl *et al.*, 1998).

2.5.3 *Contact lens cases*

Amoebae were found in fluid taken from hard contact lens cases, and it is interesting to note that the wearers concerned had used tap water for rinsing or storing their contact lenses (Tables 2.3 and 2.4). These individuals were not suffering from keratitis at the time of sampling. This situation can obtain if the cornea is not sufficiently abraded to allow amoebae to invade, or when the strains of organisms present are non-pathogenic.

2.5.4 *Tap water*

Amoebae that were morphologically indistinguishable from those belonging to the genera *Naegleria* or *Acanthamoeba* were isolated from 28% of the 50 separate 750 ml tap water samples. These amoebae were not tested for pathogenicity. The collections were made during winter, at which time Lastovica (1980) found that certain amoebae could not easily be isolated from ponds in South Africa. It follows that a study carried out during the warmer months might lead to the recovery of amoebae from a greater number of samples of tap water.

Pathogenic strains of *Naegleria* were isolated from a domestic water supply in

South Australia (Anderson & Jamieson, 1972). However, it is mainly members of the genus *Acanthamoeba* that have been detected in tap water, which is compatible with the finding that *Acanthamoeba* is more resistant to chlorination than *Naegleria* (De Jonckheere & van de Voorde, 1976). Neither virulent nor avirulent strains of *Acanthamoeba* are destroyed by the chlorine levels normally found in drinking water or swimming pools, and pathogenic strains have been found to be even more resistant to chlorine than non-pathogenic ones (De Jonckheere & van de Voorde, 1976).

2.5.5 *Swimming pool water*

The pools with low concentrations of chlorine (4 μg/ml and less) contained live amoebae. However, the single water sample from one of the swimming pools tested, which had a chlorine concentration of 8.5 μg/ml, harboured no amoebae. These results correspond with those of De Jonckheere & van de Voorde (1976), who determined that a 4 μg/ml free chlorine concentration does not destroy *Acanthamoeba* after three hours of contact time. It was not only directly-chlorinated swimming pools that had viable amoebae in them, but also those in which salt and electrode methods of cleaning were employed. This researcher's sampling of swimming pools was carried out in the winter; a study during the warmer months might lead to the recovery of greater numbers of amoebae from swimming pools.

2.5.6 *Sewage sludge*

Three of the four isolates from sewage sludge were identified (in a separate study) as *A. lenticulata* (Schroeder *et al*., 2001), a highly virulent species (De Jonckheere

& Michel, 1988) belonging to the T5 genotype (Schroeder *et al*., 2001). These amoebic isolates were kept in axenic culture and after seven years were found to be cytopathogenic *in vitro* (Niszl *et al*., 1998). None of the 15 isolates of the T5 genotype examined so far by Schroeder *et al*. (2001) have been associated with human disease, although four were taken from human nasal passages and two of these were pathogenic to mice (De Jonckheere & Michel, 1988). It is unknown, however, whether there is any significant epidemiological relationship between the T5 genotype, water, humans and sewage.

2.5.7 *Incorporation of chemicals into agar*

The inclusion of certain chemicals into NNA proved a useful way of separating the different genera. The morphological observations in this study confirm these groupings.

2.5.8 *General comments*

Not all of the cold chemical sterilisation methods that are commonly employed and recommended by many optometrists, kill species of *Acanthamoeba* (Chapter 3; Ludwig *et al*., 1986; Niszl & Markus, 1998). Therefore, contact lens wearers who use ineffective techniques of disinfection, such as those involving home-made saline or unboiled water from taps in southern Africa, are exposing themselves to the risk of acanthamoebic infection. The *Acanthamoeba* organism has been isolated from the human oral cavity (Wang & Feldman, 1967), so it follows that contact lens wearers are also at risk if they wet their contact lenses with saliva, as was shown by a case of acanthamoebic keratitis described by Mannis *et al*. (1986). An effective way of killing cysts and trophozoites of *Acanthamoeba* is to boil contact lenses, if they are of a type that will not be

damaged by the process (Ludwig *et al.*, 1986). However, this would have to be done daily or every second day rather than weekly, as is often prescribed.

2.5.9 *Conclusions*

Eye-care practitioners in southern Africa need to become aware of the signs and symptoms of acanthamoebic keratitis, so that the interval between diagnosis and treatment with appropriate drugs that might be effective against southern African strains of the organism (Niszl & Markus, 1996; Gatti *et al.*, 1998), can be shortened. Because of the high prevalence of cytopathic strains amongst local environmental and ocular isolates of *Acanthamoeba* (Chapter 10; Niszl *et al*., 1998), contact lens users in the region need to be told both about the possible dangers of using home-made saline and about the presence of potentially pathogenic amoebae in tap water and swimming pools. Contact lenses should be removed before swimming, and rinsing or splashing of contact lenses with non-sterile water should be avoided.

CHAPTER THREE – ANTI-*ACANTHAMOEBA* ACTIVITY OF CONTACT LENS SOLUTIONS

3.1 The variable efficacy of contact lens solutions in preventing acanthamoebic infection

Acanthamoebic keratitis is being reported with increasing frequency in contact lens wearers in various parts of the world. Evidence of the ease with which human infection can occur is provided by the isolation of species of *Acanthamoeba* from water drawn from bathroom taps and from dust around a washbasin (Seal *et al*., 1992). Domestic tap water has been implicated in a case of acanthamoebic keratitis in the U.K. (Kilvington *et al*., 1990). Tap water could also be a source of disease in South Africa, where the organism occurs commonly in tap water and swimming pools (Markus & Niszl, 1990). In addition, strains of *Acanthamoeba* have been isolated from the contact lens storage cases of asymptomatic contact lens wearers (Niszl & Markus, Submitted for publication). Since *Acanthamoeba* species are ubiquitous in the environment, lens care systems could possibly even become contaminated with cysts from the air (Kingston & Warhurst, 1969). The presence in contact lens cases and solutions of bacteria originating from tap water or elsewhere predisposes people to ocular acanthamoebic infection (Larkin *et al*., 1990; Bottone *et al*., 1992; Seal & Hay, 1992; Clark *et al.*, 1994; Seal *et al.*, 1999; Shoff *et al.*, 2008). The amoebae feed on bacteria and multiply, with the result that large potential amoebic inocula may be present.

The time when the highly resistant cysts of *Acanthamoeba* are most likely to be killed appears to be while the contact lenses are in storage/soaking and

disinfecting solutions. The reason is that lenses are usually exposed to the active ingredients in these solutions for a minimum period of a few hours (usually overnight). However, even though a patient may comply with general contact lens wear and care procedures recommended by lens manufacturers and healthcare professionals, a solution that does not kill *Acanthamoeba* may not protect the wearer against ocular infection by this organism (Stehr-Green *et al*., 1987; Tzanetou *et al.*, 2006; Joslin *et al*., 2007; Polat *et al.*, 2007b; Kilvington *et al*., 2008; Hasler *et al*., 2009; Lonnen *et al.*, 2009; Wanachiwanawin *et al.*, 2009; Hasler *et al*., 2010). Moore *et al*. (1987) expressed their concern at the inability of a number of chemical sterilisation methods currently in use to inactivate *Acanthamoeba* if the contact lens becomes contaminated. Ludwig *et al*. (1986) reported that heat disinfection of contact lenses is superior to cold chemical disinfection. Experimental findings concerning the efficiency of chemical disinfection systems are inconclusive because the results vary (Ludwig *et al*., 1986; Davies *et al*., 1988; Brandt *et al*., 1989; Penley *et al*., 1989; Davies *et al*., 1990; Borazjani and Kilvington, 2005). This could be due to variation in the susceptibility to contact lens solutions of different species and isolates of *Acanthamoeba* (Ludwig *et al*., 1986; Davies *et al*., 1988; Lindquist *et al*., 1988), or to differences in the experimental procedures employed (Davies *et al*., 1988; Davies *et al*., 1990). Standardised testing of the efficacy of contact lens disinfection systems against *Acanthamoeba* is, therefore, needed (Meisler & Rutherford, 1991). It is extremely important that strain variability be recognised in connection with anti-amoebic treatments.

The purpose of the experimental work described in this chapter was to assess the effectiveness of chemical storage/soaking and disinfecting solutions on strains of *Acanthamoeba* isolated in southern Africa, compared with their efficacy on

reference strains from the U.K. No such work has, to the author's knowledge, been carried out previously on any southern African isolate of the protozoon. Since the active ingredients of solutions available in southern Africa are similar to those used elsewhere in the world, a comparative study is not only of interest to the international scientific community but also to contact lens practitioners and wearers everywhere. For example, use of solutions which are effective against a wide spectrum of *Acanthamoeba* strains would be desirable for contact lens wearers travelling from a particular country to another, as well as for those resident at home.

3.2 Materials and methods

3.2.1 *Culturing of organisms*

The following ten *Acanthamoeba* isolates were used: Ac/PHL/23; ATCC 30868; ATCC 30873; ATCC 50676; ATCC 50677; ATCC 50678; ATCC 50679; ATCC 50680; ATCC 50684; ATCC 50686.[13]

The cloning of amoebae was carried out as described in Chapter 2.

Amoebae were axenically cultured at 30°C in antibiotic-free, peptone-yeast extract glucose broth (Lasman & Feinstein, 1986)[14] in screw-capped, 80 cm^2 (260 ml) Nunc tissue culture flasks. To obtain large numbers of organisms for experimentation, subcultures of *Acanthamoeba* trophozoites were grown in 500 ml broth in 5-litre flasks on a shaker set at 100 rpm for 42 hours.

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13. See Appendix IV.

14. See Appendix II.

Amoebic cysts were obtained by using trophozoites in constant pH encystment medium (Neff *et al*., 1964b; Niszl & Markus, 1998).[15] Log phase axenic cultures of trophozoites (incubated at 30°C on a gyratory shaker set at 100 rpm) were centrifuged at 740 x g for five minutes. The supernatant was carefully decanted, replaced with fresh encystment medium, and centrifugation repeated. This last step was repeated once more before the amoebic pellet was finally resuspended in 100ml of encystment medium in a 500 ml siliconised, sterile glass flask. Incubation in encystment medium was at 30°C on a shaker set at 100 rpm for 48 hours. After that time, more than 90% of cysts were mature, as determined by phase contrast microscopy (Davies *et al*., 1990).

Early exponentially growing trophozoites and cysts were harvested from axenic cultures by centrifugation in their medium at 740 x g for five minutes, and the medium was decanted to leave a pellet of trophozoites or cysts. The harvested organisms were washed three times in amoeba saline and centrifuged at 740 x g for five minutes after each wash. Trophozoites were used immediately, and cysts were stored in fresh amoeba saline at 4°C and used within seven days.

3.2.2 *Chemical disinfection*

Contact lens storage/soaking and disinfecting solutions (Table 3.1) were purchased from local retail stores or donated by the manufacturers. All solutions were used before their stated expiry date. Solutions were chosen so as to include a range of active ingredients for soft, hard and gas-permeable lenses (Table 3.1).

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15. See Appendix II.

Tests were initially done on both cysts and trophozoites of strain ATCC 50676, using eight different contact lens solutions (Tables 3.2 and 3.3). The only exception was in the case of Steropt, which was tested only on cysts because Steropt is no longer available locally (i.e. in South Africa). Consequently there was only sufficient solution for experiments on cysts, which were found to be more resistant to the solutions; and because trophozoites which are kept in any solution for longer than approximately 24 hours will encyst (De Jonckheere & Van De Voorde, 1976; Penley *et al*., 1989). For these reasons, Steropt was tested on cysts rather than on trophozoites. Subsequent experiments were conducted only on the cysts of the other strains that were studied for the same reasons. Eleven solutions (including some that had appeared on the local market subsequent to the carrying out of the initial experiments) were tested on all the strains. Most of these are shown in Figure 3.1, which does not include the results for Oxysept 1 Step because it is used for only two hours with a neutralising tablet. Experiments were carried out in triplicate. A 1 ml amount of each solution was dispensed aseptically into labelled 15 ml plastic screw-cap centrifuge tubes, which could be used for spinning down directly at a later stage without the risk of losing trophozoites or cysts during transfer to a tube. In the case of Softab, the solution was made up according to the manufacturer's instructions (that is, one Softab in 10 ml non-preserved saline). Control tubes were prepared containing only amoeba saline, with other controls comprising only lens solutions. All tubes were left overnight to allow impregnation to take place. This is because certain chemicals have been shown to interact with storage containers by a surface adsorption process (Richardson *et al*., 1977), which could possibly dilute the amount of active ingredient present. Overnight impregnation with each solution was an attempt to deal with this problem. Tubes were kept in the dark during

impregnation and for the duration of the experiment, to prevent possible deactivation of solutions in light, and to simulate conditions in a lens case.

Following impregnation and immediately before commencement of the experiment, solutions and amoeba saline were pipetted off and 1 ml of fresh fluid was added to each tube. A 10 μl trophozoite suspension containing approximately 1×10^4 organisms and a 10 μl cyst suspension containing approximately 1×10^4 cysts, as had been recommended (Osato *et al*., 1991), was pipetted into each tube. Both trophozoite and cyst counts were done on an Adams haemocytometer. In the case of Oxysept 1 Step, the neutralising tablet (5200 U catalase per tablet) was added at the same time as the solution, and left in the tube for the duration of the disinfection period, according to the manufacturer's instructions. Care was taken to ensure that the trophozoites and cysts were re-dispersed throughout the solution and did not remain clumped in a pellet. The caps of the tubes were tightened and sealed with Parafilm (American Can Company) to prevent evaporation of liquid, and the tubes were left at room temperature in the dark until they were ready to be processed.

Three sets of control tubes were prepared. The first contained amoeba saline alone, the second set amoeba saline plus 10 μl amoeba suspension, and the third lens solution alone. Control tubes were treated in exactly the same way as the experimental tubes. At various times (Fig. 3.1; Tables 3.2 and 3.3), one tube of lens solution with amoebae and one of each control tube were gently shaken to loosen organisms that might be adhering to the tube, and centrifuged at 740 x g for five minutes. The supernatant was discarded, and the pellet was washed twice in amoeba saline, except in the case of Oxysept 1 (3% hydrogen peroxide), where the solution was first neutralised for ten minutes in Oxysept 2 (containing 520

units catalase per ml with 0.001% thiomersal and 0.1% disodium edetate as preservatives), according to the manufacturer's instructions. This was followed by a wash with amoeba saline.

After the last centrifugation, 200 μl of amoeba saline was left in the bottom of each tube. This material was then spread on to a plate (20 by 90 mm) of NNA seeded with *E. coli*, and the plates were sealed with masking tape to prevent the agar from drying out. Plates were incubated at 30°C for 14 days and were examined daily for *Acanthamoeba* trophozoites by means of an inverted microscope. For initial tests done on strain ATCC 50676 (Tables 3.2 and 3.3), the number of colonies on the plates was compared with those on the control plates, and assessed as a percentage of growth. If no trophozoites were observed at the end of 14 days, the inoculum used for the plate was considered non-viable. For all other strains tested (Fig. 3.1), the viability of organisms was assessed in terms of their ability (or otherwise) to excyst and multiply, compared to control plates.

3.2.3 *Statistical methods*

A comparison test run on a SAS Version 6.1 computer package was used for statistical analysis. To evaluate differences between the responses of the various *Acanthamoeba* strains to contact lens solutions, 95% confidence intervals were constructed using the mean (arithmetic average). In this analysis, overlapping confidence intervals indicate no significant difference, whereas those not overlapping indicate a significant difference.

Table 3.1. Contact lens solutions tested on *Acanthamoeba.*

Solution trade name	Manufac-turer	Active ingredient(s)[a]	Preservative(s)	Type(s) of lens
Bausch & Lomb Multi-Purpose Solution®!	Bausch & Lomb	Polyaminopropyl biguanide (DYMED) (0.0005); sodium borate (1.20); sodium chloride (4.9); poloxamine 1107 (10.00); boric acid (6.40)	0.11% (wt/vol) Disodium edetate	Soft
Clean-N-Soak®!	Allergan	Miranol 2 MCA (buffered) (16.0)	0.004% Phenyl-mercuric nitrate in 0.01% disodium edetate	Gas-permeable and hard
Complete®!	Allergan	Polyhexamethylene biguanide (0.001)		Soft
Duracare®#	Allergan	0.004% Polyvinyl alcohol	0.004% Benzalkonium chloride; 0.004% Sodium Edetate	Gas-permeable
Hydrocare Cleaning/Soaking Solution®!	Allergan	Alkyl triethanol ammonium chloride (0.3)	0.002% Thiomersal	Soft
Optifree®!	Alcon		0.001% m/v Polyquad, i.e. polyquaternium-1; 0.05% (wt/vol); sodium edetate	Soft
Optisoak®!	Alcon	0.75 g polyvinyl alcohol; 0.005 g polysorbate 80; 0.65 g hydroxy-ethyl cellulose (all per 100 ml) + sodium chloride + sodium phosphate	Polyquad, i.e. polyquaternium-1 0.005% (wt/vol); sodium edetate 0.1% (wt/vol)	Gas-permeable and hard
Oxysept 1®@	Allergan	Hydrogen peroxide (31.0)		Soft
Oxysept 1 Step®+	Allergan	3% Hydrogen peroxide		Soft
Soflens Soaking Solution®#	Bausch & Lomb	0.03% Alkyl triethanol ammonium chloride	0.002% Thiomersal + surfactant in special polymer vehicle	Soft
Softab®!	Alcon	Sodium dichloroisocyanurate (0.065); available chlorine in an effervescent base (0.04)		Soft

Solution trade name	**Manufac-turer**	**Active ingredient(s)[a]**	**Preservative(s)**	**Type(s) of lens**
Steropt Soaking and Cleansing Solution®&	Steropt Remedia Medical	Phenylethanol; sodium chloride (concentration not stated)	Benzalkonium chloride; disodium edetate (concentration not stated)	Hard
Total®#	Allergan	Polyvinyl alcohol (25.0)	0.004% Benzalkonium chloride	Gas-permeable and hard
Transoak®&	*	0.01% (wt/vol) Benzalkonium chloride	0.2% (wt/vol) Disodium edetate	Gas-permeable and hard
Transol Wetting Solution®**	Chauvin Pharmaceu-ticals Ltd.	1 g Polyvinyl alcohol/50 ml	0.004% Benzalkonium chloride; 0.02% (wt/vol) disodium edetate	Gas-permeable and hard

Key:

* Smith & Nephew were manufacturers of Transoak when experiments on cysts and trophozoites were done on strain ATCC 50676 (Tables 3 and 4). Transoak was marketed under the name of Chauvin Pharmaceuticals Ltd for experiments done on cysts of all other strains (Fig. 1).

** Minimum soaking period is not stated, as this is a wetting solution, not intended for soaking lenses. It was included because its constituents are similar to those of other soaking solutions tested, although a different company manufactures it.

[a] All data in parentheses are in mg/ml.

[!] Minimum stated soaking period for solution is four hours.

[#] Minimum stated soaking period for solution is overnight (one manufacturer states that this is approximately six to eight hours).

[@] Minimum stated soaking period for solution is 20 minutes.

[+] Minimum stated soaking period for solution is two hours.

[&] Minimum soaking period is not stated.

3.3 Results

Information on the effect of different contact lens storage/soaking and disinfecting solutions on *Acanthamoeba* trophozoites and cysts is given in Figure 3.1, which represents the result of three experiments. Each experiment gave reproducible results. (Detailed results are given in Appendix V.) All the control tubes containing amoeba saline plus trophozoites or cysts showed growth of organisms for all the contact times tested. The amoeba saline control was negative for growth, as did all the pure solutions tested.

Transoak was the most effective contact lens solution tested on strain ATCC 50676, with no live trophozoites present at one hour (Table 3.2), and only 25% of cysts viable at one hour (Table 3.3). All cysts were dead at five hours. Duracare showed some effectiveness against trophozoites of strain ATCC 50676, as they were all killed within the first hour (Table 3.2). Cysts were still viable at five hours, but dead at 12 hours (Table 3.3). The killing time for cysts of strain ATCC 50676 by Oxysept 1 and Steropt was similar to that for Duracare, but it took longer for trophozoites to be inactivated by Oxysept 1 than by Duracare. Clean and Soak, Hydrocare, Soflens and Softab did not eliminate cysts of strain ATCC 50676 within seven days. The effect of Softab, in particular, proved to be negligible, as viable cysts were still present after 90 days.

The most effective of the gas permeable and hard contact lens solutions tested on cysts of all strains was Transoak (Fig. 3.1). Although viable cysts were still present at two hours, all had been inactivated by four hours.

Oxysept 1 was the most effective of the soft contact lens solutions tested. It killed cysts of strains ATCC 30873 (CCAP 1501/3d), ATCC 50677, ATCC 50679 and ATCC 50686 within four hours, whereas cysts of strains Ac/PHL/23, ATCC 30868 (CCAP 1501/2g), ATCC 50676, ATCC 50680 and ATCC 50684 were killed within eight hours (Fig. 3.1). However, viable cysts of strain ATCC 50678 were still present after exposure to Oxysept 1 for eight hours; but were non-viable after three days of exposure to this solution. Oxysept 1 Step, which is used for a period of two hours with a neutralising tablet, had no cysticidal effect within this time on any of the strains of *Acanthamoeba* tested.

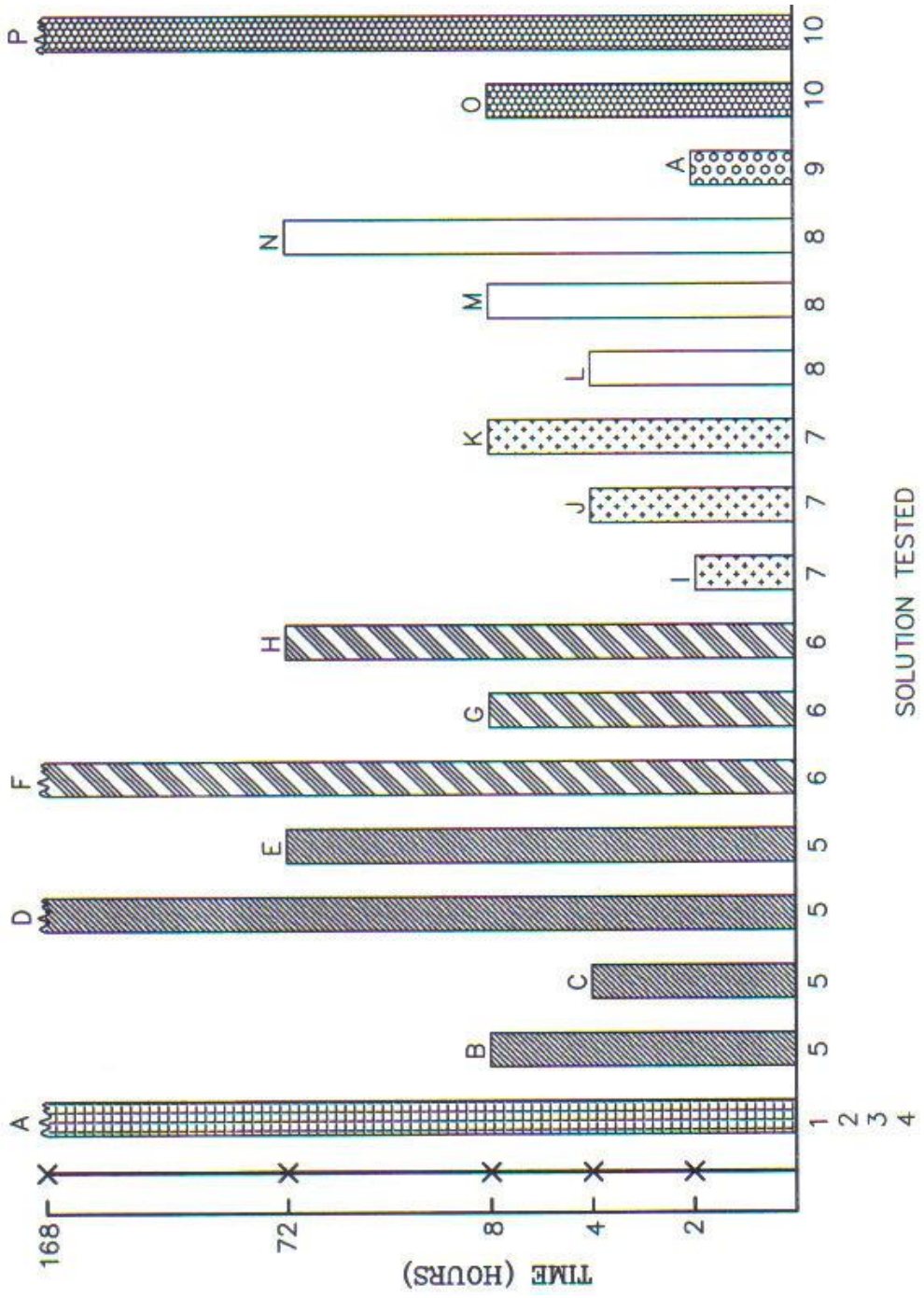
Cysts of strains ATCC 30868 (CCAP 1501/2g) and ATCC 50679 were killed within eight hours in Total, but cysts of all other strains were still viable after exposure to this solution for eight hours.

The time taken for the other solutions (Bausch & Lomb Multipurpose, Complete, Duracare, Hydrocare, Optifree, Optisoak and Transol) to kill cysts of all ten strains was longer than eight hours in most cases. Viable cysts were still present at seven days in several cases (Fig. 3.1).

3.3.1 *Statistical analysis*

Statistically significant differences were detected between the following strains with regard to their susceptibility to contact lens solutions (Fig. 3.2): Ac/PHL/23 vs ATCC 30873; ATCC 30868 vs ATCC 30873; ATCC 30868 vs ATCC 50677; ATCC 30868 vs ATCC 50680; and ATCC 30868 vs ATCC 50686. No significant differences were found between any of the other strains.

Statistical analyses could not be done on Bausch and Lomb Multipurpose Solution, Complete, Optifree, Optisoak and Transol, where all (or most, in the case of Transol) of the strains of *Acanthamoeba* were still viable at 168 hours. Nor could statistical analyses be done for Transoak, where all the amoebae of all strains were killed within four hours. There were statistically significant differences in the cysticidal activity of the following contact lens solutions (Fig. 3.3): Duracare vs Oxysept 1; Hydrocare vs Oxysept 1; Hydrocare vs Total; and Oxysept 1 vs Total. Differences between Duracare vs Hydrocare and Duracare vs Total solutions, were not significant.


TIME (HOURS)
168
72
8
4
2
A
B
C
D
E
F
G
H
I
J
K
L
M
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SOLUTION TESTED

Figure 3.1. Viability of cysts of ten strains of *Acanthamoeba* after exposure to contact lens disinfecting solutions. All 10 strains of *Acanthamoeba* were tested against each solution. The bars represent the variation in time needed to complete killing of the cysts by different solutions. The jagged edges at the tops of the bars indicate that cysts were still viable at that time, but not at the next testing time. Each bar is the consensus result of three repeated experiments. Each experiment gave reproducible results.

Key:

A = all ten strains of *Acanthamoeba*.
B = Ac/PHL/23; ATCC 50677; ATCC 50678; ATCC 50679; ATCC 50680.
C = ATCC 30868; ATCC 50676.
D = ATCC 50684.
E = ATCC 30873; ATCC 50686.
F = ATCC 30873; ATCC 50676; ATCC 50679; ATCC 50684.
G = Ac/PHL/23; ATCC 30868; ATCC 50678; ATCC 50686.
H = ATCC 50677; ATCC 50680.
I = ATCC 30873; ATCC 50677; ATCC 50679; ATCC 50686.
J = Ac/PHL/23; ATCC 30868; ATCC 50676; ATCC 50680; ATCC 50684.
K = ATCC 50678.
L = ATCC 30868; ATCC 50679.
M = Ac/PHL/23; ATCC 30873; ATCC 50677; ATCC 50678; ATCC 50680; ATCC 50684; ATCC 50686.
N = ATCC 50676.
O = ATCC 50679.
P = Ac/PHL/23; ATCC 30868; ATCC 30873; ATCC 50676; ATCC 50677; ATCC 50678; ATCC 50680; ATCC 50684; ATCC 50686.
X = exposure time (hours) of cysts to solution.
1 = Bausch & Lomb Multi-Purpose Solution.
2 = Complete.
3 = Optifree.
4 = Optisoak.
5 = Duracare.
6 = Hydrocare.
7 = Oxysept.
8 = Total.
9 = Transoak.
10 = Transol.

Table 3.2. Inactivation of trophozoites of *Acanthamoeba* strain ATCC 50676 by contact lens solutions.

	Result* after contact time							
Solution	**10 min**	**1 h**	**5 h**	**12 h**	**24 h**	**2 d**	**3 d**	**7 d**
Clean-N-Soak	4+	4+	4+	4+	4+	4+	-	-
Duracare	2+	-	-	-	-	-	-	-
Hydrocare	4+	4+	4+	4+	4+	4+	4+	4+
Oxysept 1	4+	4+	-	-	-	-	-	-
Soflens	4+	4+	4+	4+	4+	4+	4+	4+
Softab	4+	4+	4+	4+	4+	4+	4+	4+
Transoak	2+	-	-	-	-	-	-	-
Control (amoeba saline + trophozoites)	4+	4+	4+	4+	4+	4+	4+	4+
Control (amoeba saline)	-	-	-	-	-	-	-	-

Key:
*Experiments were conducted in triplicate and results were the same for all three trials.
2+ = 50% of trophozoites viable.
4+ (control) = 100% of trophozoites viable.
- = no growth of organisms.

Table 3.3. Inactivation of cysts of *Acanthamoeba* strain ATCC 50676 by contact lens solutions.

Result* after contact time

Solution	10 min	1 h	5 h	12 h	24 h	2 d	3 d	7 d	14 d	30 d	90 d
Clean-N-Soak	4+	4+	4+	4+	4+	4+	4+	4+	-	-	-
Duracare	4+	2+	1+	-	-	-	-	-	-	-	-
Hydrocare	4+	4+	4+	4+	4+	4+	4+	4+	-	-	-
Oxysept 1	4+	2+	1+	-	-	-	-	-	-	-	-
Soflens	4+	4+	4+	4+	4+	4+	4+	4+	-	-	-
Softab	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Steropt	4+	2+	1+	-	-	-	-	-	-	-	-
Transoak	4+	1+	-	-	-	-	-	-	-	-	-
Control (amoeba saline + trophozoites)	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Control (amoeba saline)	-	-	-	-	-	-	-	-	-	-	-

Key:

*Experiments were conducted in triplicate and results were the same for all three trials.

1+ = 25% of cysts viable.

2+ = 50% of cysts viable.

4+ (control) = 100% of cysts viable.

- = no growth of organisms.

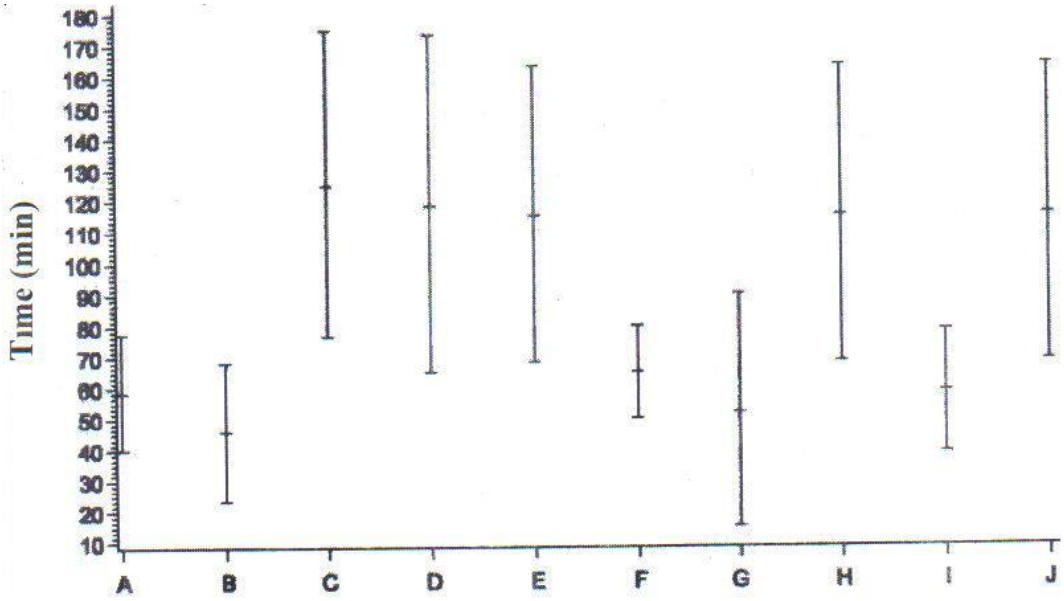

Figure 3.2. Statistical comparison of the susceptibility of strains of *Acanthamoeba* to 6 contact lens solutions (Clean-N-Soak, Duracare, Oxysept 1, Hydrocare, Soflens and Total), using 95% confidence intervals.

Key:

A = Ac/PHL/23.
B = ATCC 30868.
C = ATCC 30873.
D = ATCC 50676.
E = ATCC 50677.
F = ATCC 50678.
G = ATCC 50679.
H = ATCC 50680.
I = ATCC 50684.
J = ATCC 50686.

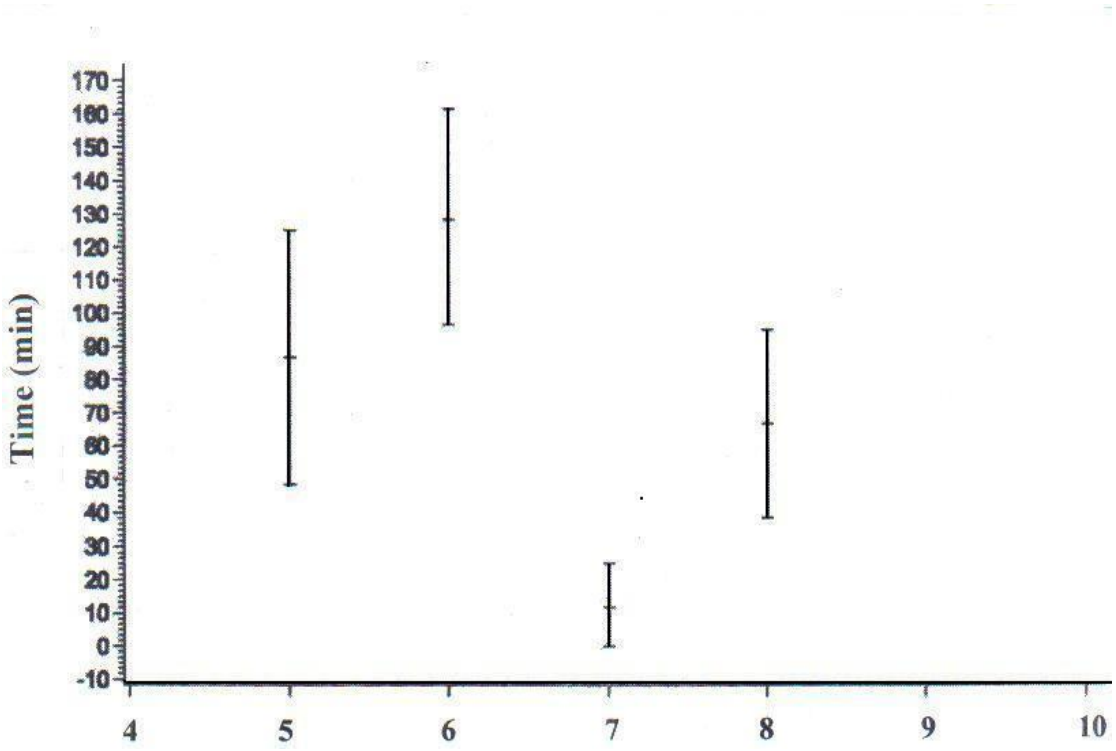

Figure 3.3. Statistical comparison of the effectiveness of contact lens solutions on strains of *Acanthamoeba* (Ac/PHL/23, ATCC 30868, ATCC 30873, ATCC 50676, ATCC 50677, ATCC 50678, ATCC 50679, ATCC 50680, ATCC 50684 and ATCC 50686), using 95% confidence intervals.

Key:

5 = Duracare.
6 = Hydrocare.
7 = Oxysept 1.
8 = Total.

3.4 Discussion

A. castellanii (ATCC 30868) was found to be the strain most commonly showing a statistically significant difference to other isolates, in that less time was required for the contact lens solutions to kill this strain than the other isolates tested. It should be noted that Silvany *et al*. (1990) found that complete inhibition of a strain of *A. castellanii* was generally achieved sooner than total inactivation of a strain designated as *A. polyphaga*.

Overall, it was found that solutions for hard and gas permeable lenses were more effective against *Acanthamoeba* cysts than those for soft contact lenses. These results are similar to those of other authors (Connor *et al*., 1991; Hiti *et al.*, 2006).

3.4.1 *Benzalkonium chloride*

Transoak (0.01% benzalkonium chloride–BAK–and 0.2% disodium edetate) proved to be the most effective solution tested for anti-*Acanthamoeba* activity. It destroyed cysts of all strains within four hours. Lower concentrations of BAK do not appear to be as efficient, since Duracare, Total and Transol, all of which contain 0.004% BAK and polyvinyl alcohol, generally took longer than eight hours to kill cysts of the isolates tested. In the case of Steropt (the concentration of BAK is not stated by the manufacturers), cysticidal activity had not been achieved at five hours, but only by 12 hours. This researcher's results with 0.004% BAK are similar to those of other authors (Penley *et al*., 1989; Zanetti *et al*., 1995). However, Silvany *et al*., (1990), using the same strain of *A. castellanii* (ATCC 30868/CCAP 1501/2g) as Penley *et al*. (1989), as well as a strain of *A. polyphaga*, found that 0.004% BAK was effective against cysts and trophozoites within one hour, whilst 0.001% BAK was effective within eight hours. A 0.003%

BAK solution did not inactivate cysts of *Acanthamoeba* within the testing time (Connor *et al*., 1991; Hugo *et al*., 1991).

Higher levels (0.01% to 4%) of BAK are known to cause significant corneal toxicity, but few effects have been noted at a concentration of 0.003%, which is that used in several solutions for rigid gas-permeable contact lenses. There is a progressive increase in damage at concentrations between 0.001% and 0.01%, as determined by scanning electron microscopy (Burstein, 1980; Begley *et al*., 1992). In addition, 0.01% BAK causes instability of the tear film (Burstein, 1980), which makes the advisability of having all-in-one solutions questionable (Phillips & Stone, 1989). BAK at a concentration of 0.01% should be considered for use as an effective disinfecting solution for *Acanthamoeba* only if this solution is not used for insertion of lenses into the eye, and if binding of the BAK to the contact lenses does not occur. The BAK solution should be washed off by a wetting solution before the contact lens is inserted into the eye.

3.4.2 *Hydrogen peroxide*

Hydrogen peroxide disinfection has been thought to be a predisposing factor for acanthamoebic keratitis (Cohen *et al*., 1987). Different strains of *Acanthamoeba* tested in this study show varying susceptibilities to this solution. Cysts of some strains were killed within four hours, whilst viable cysts of other strains were still present at eight hours. The results with 3% hydrogen peroxide on strain Ac/PHL/23(SHI) are similar to those of Kilvington (1990) for this strain. The findings of different researchers with regard to the cysticidal activity of 3% hydrogen peroxide vary according to the strain of *Acanthamoeba* (Ludwig *et al*., 1986; Silvany, 1987; Lindquist *et al*., 1988; Brandt *et al*., 1989; Connor *et al*., 1989; Davies *et al*., 1990; Silvany *et al*., 1990; Connor *et al*., 1991; Silvany *et al*.,

1991; Bilgin *et al*., 1993; Zanetti *et al*., 1995; Mowrey-McKee & George, 2007; Johnston *et al.*, 2009). The taxonomic identity of the various isolates is academic, however. What is important from a practical point of view is to know what will kill the most resistant strain of *Acanthamoeba* which might be present – for example, in a lens case – irrespective of its genetic relationships. Davies *et al*. (1990) found that whereas AOSept (3% hydrogen peroxide with 0.85% sodium chloride) did not work, Oxysept (3% hydrogen peroxide) had measurable anti-acanthamoebic activity. The authors suggested that this is possibly due to either the difference in pH between the two solutions or to the presence of a neutralising catalytic disc in AOSept which renders the solution ineffective against *Acanthamoeba*. The presence of different stabilising ingredients may also result in slightly different amoebicidal effects for different products containing the same concentration of hydrogen peroxide (Zanetti *et al*., 1995).

In this study, Oxysept 1 Step (3% hydrogen peroxide) was found to be totally ineffective for inactivating cysts of the strains of *Acanthamoeba* tested. Hiti *et al.* (2002; 2005) also found that one-step hydrogen peroxide systems are not effective for killing *Acanthamoeba* cysts but the 2 two-step systems tested by them completely destroyed the cysts of two strains of *Acanthamoeba*. It should be noted that using a one-step hydrogen peroxide system, Bilgin *et al*. (1993) achieved only a 55% rate of disinfection for *Pseudomonas* sp., although the rate was 100% for other bacteria.

3.4.3 *Alkyl triethanol ammonium chloride*

Hydrocare (0.3 mg/ml alkyl triethanol ammonium chloride) and Soflens (0.03% alkyl triethanol ammonium chloride) both showed similar, unsatisfactory anti-*Acanthamoeba* activity against strain ATCC 50676. Hydrocare was also

ineffective against cysts of the other strains of *Acanthamoeba* tested. The results of some previous studies are similar (Ludwig *et al*., 1986; Brandt *et al*., 1989; Penley *et al*., 1989; Connor *et al*., 1991). In contrast, Davies *et al*. (1990) and Silvany *et al*. (1990) found that Hydrocare killed cysts of *Acanthamoeba* within four hours.

3.4.4 *Miranol 2 MCA*

Clean-N-Soak (16 mg/ml miranol 2 MCA) showed very poor amoebicidal activity for strain ATCC 50676, with cysts and trophozoites surviving for up to several days.

3.4.5 *Polyaminopropyl biguanide*

Polyaminopropyl biguanide (PAPB), 0.0005 mg/ml (Bausch & Lomb Multi-Purpose solution) was found to be totally ineffective against *Acanthamoeba* cysts. Viable cysts of all the strains of amoebae tested were present after seven days of exposure to this solution. The results of other authors are in agreement (Connor *et al*., 1989; Penley *et al*., 1989; Davies *et al*., 1990; Connor *et al*., 1991; Silvany *et al*., 1991; Zanetti *et al*., 1995; Johnston *et al*., 2009). However, Silvany *et al*. (1990) determined that although a 0.00005% PAPB solution was effective at 12 hours against *A. castellanii* (strain ATCC 30868), it was not effective against *A. polyphaga* cysts, even at 24 hours. At a higher concentration (0.0015%), PAPB was found to be ineffective against *Acanthamoeba* cysts by Connor *et al*. (1991) and Hugo *et al.* (1991). However, Silvany *et al*. (1991) found that 0.0015% PAPB was effective against strains of *A. castellanii* and *A. polyphaga* grown axenically and in co-culture in less than two hours in most cases. It is relevant that a 0.0015% PAPB solution caused increased corneal staining by fluorescein,

indicating sloughing of cells, as compared to the staining for eyes used as controls (Begley *et al*., 1992).

3.4.6 *Polyhexamethylene biguanide*

Complete (0.001 mg/ml polyhexamethylene biguanide – PHMB) did not inactivate cysts of any of the strains of *Acanthamoeba* tested. Viable cysts were detected in all experiments after seven days of exposure to the solution. PHMB has been found to be ineffective against *Acanthamoeba* cysts by other researchers (Kilvington, 1990; Silvany *et al*., 1990; Shoff *et al.*, 2007; Johnston *et al.*, 2009). However, MeniCare Soft Multipurpose Solution containing 0.0001% PHMB showed effective disinfection efficacy against *Acanthamoeba* trophozoites and cysts (Heaselgrave *et al.*, 2010; Lonnen *et al.*, 2010). These conflicting results can be ascribed to reduced antimicrobial efficacy by PHMB when formulated in combination with a phosphate buffering system compared with when formulated in a borate or TRIZMA system (Lloyd *et al.*, 2001). It should be noted that PHMB is useful at higher concentrations for treating *Acanthamoeba* keratitis (Chapter 5; Larkin *et al*., 1992; Niszl & Markus, 1996; Dart *et al.*, 2009).

3.4.7 *Polyquad*

Optifree (0.001 % polyquad) and Optisoak (0.005 % polyquad) did not kill the strains of *Acanthamoeba* worked on in this study. Cysts of all strains were found to be viable after seven days of exposure to these solutions. The absence of any marked cysticidal effect of 0.001% polyquad against cysts of *Acanthamoeba* has also been demonstrated by other authors (Penley *et al*., 1989; Davies *et al*., 1990; Silvany *et al*., 1990; Connor *et al*., 1991; Silvany *et al*., 1991; Zanetti *et al*., 1995; Johnston *et al.*, 2009).

3.4.8 *Sodium dichloroisocyanurate*

Softab (0.065 mg/ml sodium dichloroisocyanurate) showed very poor amoebicidal activity for strain ATCC 50676, with cysts and trophozoites surviving for up to several days. These results for Softab (0.04 mg/ml available chlorine) on strain ATCC 50676 are confirmed by those of others, which show that cysts of *Acanthamoeba* can survive high free chlorine concentrations (De Jonckheere & Van De Voorde, 1976; Kilvington, 1990; Kilvington & Price, 1990; Seal *et al.*, 1999). Prolonged super-chlorination is required to kill *Acanthamoeba* cysts (Chang, 1978), and *Naegleria* is more sensitive to chlorine than *Acanthamoeba* (De Jonckheere & van de Voorde, 1976; Cursons *et al*., 1980). Pathogenic *A. culbertsoni* was found to be more resistant to chlorine than an avirulent strain of *Acanthamoeba* (De Jonckheere & van de Voorde, 1976).

The usefulness of chlorine as a disinfecting system for contact lenses has been questioned (Seal *et al*., 1993; Radford *et al.*, 1995). Trials of the bactericidal effect of Softab have demonstrated that the chlorine can be neutralised if a very large inoculum is used (Haldane Consultants Ltd., 1982), because chlorine reacts with organic material in general. The addition of organic matter to chlorine causes the level of chlorine to diminish rapidly, and if sufficient organic matter is present, it will fall below the level that has bactericidal effect. Haldane Consultants Ltd. (1982) concluded that Softab will effectively sterilise lenses in less than six hours, provided they are reasonably clean and that excessive amounts of organic matter are not present.

3.4.9 *General comments*

Over half of the solutions examined by Richardson *et al*. (1977) contained less than 90% of the stated preservative content. In the case of thiomersal, only 2/15

solutions were within the acceptable limits of 90–110% of declared preservative concentration; and one solution contained 170% of the stated amount. This finding is highly significant, as it could explain why researchers obtain varying results (that is, the actual concentration of active ingredient may not always be as stated by the manufacturer). BAK shows the least interaction with plastics, and loss of this preservative from solutions is probably not biologically significant (Richardson *et al*., 1977). The retention of a high level of activity in the storage container, as occurs with Transoak, possibly enhances the consistent effectiveness of BAK against cysts of different strains of *Acanthamoeba*. Richardson *et al*. (1977) commented that adsorption in storage containers appeared to occur with thiomersal and chlorbutol, in contrast with BAK and chlorhexidine gluconate, which are known to interact mainly by a surface adsorption process. The extent of the interactions was found to be dependent upon the type of plastic material used to make the container.

In initial experiments on strain ATCC 50676, the present researcher calculated the percentage of amoebae still viable; but for subsequent experiments, the viability vs non-viability of cysts was assessed instead. Even though it could be of interest to ascertain at what rate amoebae are killed off (Davies *et al*., 1990), it could be argued that the importance of this researcher's experiments lies in knowing at what stage all amoebae are non-viable, because in the present state of knowledge, it must be assumed that even one viable cyst could theoretically cause keratitis, should the eye be invaded. This might not be the case, however.

Discrepancies in results reported by various authors could possibly also be due to the age of the cysts (Hughes *et al.*, 2003). Neff *et al*. (1964a) observed that cyst wall synthesis of *Acanthamoeba* sp. occurs in two stages, which are separable in

time and location. The first stage is primarily protein synthesis; the wall is later reinforced by the addition of a second layer, made up largely of cellulose (Neff *et al*., 1964a). Visual observations indicated that cyst walls become thicker as the cyst ages. It might be more difficult for chemicals to penetrate through older and thicker walls, making the cysts more resistant to inactivation. Brandt *et al*. (1989) grew amoebae for four to six weeks on agar plates. These cysts were much older than those used by other authors – compared with, for example, cysts used within seven days (Davies *et al*., 1990). The time taken for encystment to occur is speeded up when an encystment medium is used (as was done in this study), in which case the process occurs within 20 hours. Within 48 hours, encystment is greater than 90% (Neff *et al*., 1964b; Davies *et al*., 1990).

Yet another factor that could affect results is shaking of the container in which the experiment is conducted, so that cysts or trophozoites come out of the suspension and adhere to the side of the tube or the lid. In this situation, the exposed amoeba would no longer be in contact with the solution for the full experimental period. At the time of washing and plating, the organism could be washed back into the solution and, thus, give rise to a false positive result when the amoeba multiplies. In the same way, cysts in the middle of any pellet might not be exposed to the solution for the full period. Accordingly, a sterile pipette was used to bubble amoebae gently through the solution so as to ensure that they were not left in a pellet at the bottom of the tube.

The experiment was carried out in the dark, as the potency of the disinfectants might have been affected by light. (Most lens cases and bottles containing solutions are made of coloured plastic that does not allow bright light into the solutions.)

The normal time allowed for the disinfection of lenses would be overnight, while the wearer is sleeping. Therefore, a solution that kills amoebae within five hours could be considered safe for users. In this study, the minimum stated soaking time for the solutions tested, when recommended by manufacturers (Table 3.1), was in no instance sufficient for inactivating cysts of *Acanthamoeba*. Hay and Seal (1994) also found that in most cases, *Acanthamoeba* cysts were not killed within the manufacturer's minimum recommended disinfecting time for bacteria. It is, therefore, very important that manufacturers of disinfectants are made aware of the time required to kill *Acanthamoeba*, so that users can be advised as to the appropriate length of time for soaking contact lenses in the solution concerned.

User compliance with manufacturers' recommendations concerning contact lens disinfection appears, in general, to be unsatisfactory (Willcox, 2010). Recommendations by attending optometrists and ophthalmologists should be taken more seriously by contact lens wearers. Seal & Hay (1992) have suggested that contact lens wearer compliance could be improved by the development of a compact, single- use, disposable disinfection system. Bioclen FR One Step® is supplied in a single pouch to improve user compliance with manufacturer's instructions and to make the product easier to use (Martin-Navarro *et al*., 2010b). These researchers found that this povidone-iodine based system was effective against *Acanthamoeba.*

The wiping of contact lenses with a daily cleaner can perhaps also be considered as part of the disinfection procedure. Owing to the physical action of rubbing the lenses, wiping may help to dislodge some amoebic cysts or trophozoites from contact lenses (Butcko *et al.*, 2007; Kilvington and Lonnen, 2009). However,

laboratory experience is that amoebae tend to attach so firmly to plastic culture dishes and non-siliconised glassware that vigorous rubbing would be required to remove all *Acanthamoeba* cysts that might be adhering to the contact lenses or lens case. John *et al*. (1989) and John (1991) have shown that both cysts and trophozoites stick to extended-wear soft contact lenses, and that the process of washing the lenses does not remove all the trophozoites and cysts from the contact lens surface. Consequently, the wearer needs to ensure that any amoebae introduced into the contact lens storage case or adhering to contact lenses, will be killed by the soaking solution used.

The experiments described in this chapter have shown that the killing time for cysts of the African and U.K. isolates of *Acanthamoeba* is, in general, similar. Guidelines for contact lens solution usage suggested in the U.K. would, therefore, be as appropriate for the increasing number of travellers who visit South Africa as well as for local residents in South Africa.

CHAPTER FOUR – *MASTIGINA* AND CONTACT LENS SOLUTIONS

4.1 *Mastigina* as a potential pathogen

Leptomyxid amoebae have been identified as human pathogens (Anzil *et al*., 1991). Prior to this discovery, the only free-living amoebae known to cause disease in man were *Acanthamoeba* and *Naegleria*, both of which are widely distributed in South Africa (Lastovica, 1980; Markus & Niszl, 1990; Niszl & Markus, 1991). *Sappinia diploidea* has been found to cause encephalitis in the human host and there are probably other free-living amoebae that are capable of causing disease in man (Visvesvara *et al.*, 2007). In 1991, the researcher isolated a protist which was associated with the infected eye of a patient. The organism has amoeboid, cystic and flagellate stages in its life cycle, and thus bears a superficial resemblance to polymorphic amoebae like *Phreatamoeba*, which was found and described in West Africa (Chavez *et al*., 1986). However, this isolate appears to be a species of *Mastigina*.

Mastigina organisms are of uncertain taxonomic position and appear to be related to amoebae or slime moulds (Lee *et al*., 1985; Margulis *et al*., 1990). They are ubiquitous and may have a world-wide distribution. The isolation in South Africa of large numbers of a *Mastigina* sp. (identified by T.A. Nerad of the American Type Culture Collection – ATCC – Rockville, Maryland, U.S.A.) from a contact lens case, a contact lens and a bottle kept for storage of saline for use with contact lenses, implicated this protist as the possible causative agent in a case of ocular infection (Niszl & Markus, 1991). No other micro-organisms were cultured, despite repeated testing. T.A. Nerad of the ATCC informed the author that

Mastigina organisms have also been isolated from eye wash stations in the U.S.A. It was found, using an *in vitro* test system for pathogenicity (Cursons & Brown, 1978; De Jonckheere, 1980), that the South African isolate of *Mastigina* has a cytopathic effect on cultured mammalian cells (Chapter 11), which confirms that the organism is not necessarily innocuous and that it can be regarded as a potential pathogen. It is, therefore, of interest to know how labile the organism is. Accordingly, the effects of current contact lens disinfection methods on *Mastigina* sp. were determined. This information has not, to the writer's knowledge, hitherto been available.

4.2 Materials and methods

4.2.1 *Organisms*

Cloning of the strain of *Mastigina* found, which has been deposited in the ATCC and at the Culture Collection of Algae and Protozoa in the U.K., was carried out as described for *Acanthamoeba* sp. (Chapter 2).

Mastigina organisms were grown on agar plates after cloning. The organisms thus obtained, were axenically cultured at 30°C in antibiotic-free peptone-yeast extract glucose (PYG) broth (Lasman & Feinstein, 1986)[16] in screw-cap, 80 cm^2 (260 ml) Nunc tissue culture flasks. The growth of *Mastigina* organisms in PYG medium was very poor. Early, exponentially-growing organisms were harvested from axenic cultures by centrifugation in their medium at 740 x g for five minutes, and the medium was decanted to leave a pellet of amoeboid and flagellated forms (which are referred to together in this chapter as "trophozoites", as these stages are rapidly interchangeable).

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16. See Appendix II.

The harvested organisms were washed three times in amoeba saline and centrifuged at 740 x g for five minutes after each wash. Trophozoites (amoeboid and flagellated forms, as opposed to cysts) were used immediately.

Cysts formed in PYG culture within a week, and were used from seven-day-old cultures. Cysts were harvested and treated in the same way as the trophozoites.

4.2.2 *Chemical disinfection*

Eight different contact lens storage/soaking and disinfecting solutions were purchased from local retail stores and chosen so as to include a range of active ingredients for soft, hard and gas-permeable lenses (Table 4.1). All solutions were used prior to their stated expiry date.

Experiments were carried out in triplicate, with each solution used on trophozoites and on cysts. A 1 ml amount of each solution was dispensed aseptically into labelled, 15 ml plastic screw-cap centrifuge tubes, which could be used for spinning down directly at a later stage without the risk of trophozoites or cysts being lost while being transferred to a tube. In the case of Softab, the solution was made up according to the manufacturer's instruction (that is, one Softab in 10 ml non-preserved saline). Control tubes were prepared, with some containing amoeba saline only, and others containing lens solutions alone. All tubes were left overnight to allow impregnation of the plastic to take place. Tubes were incubated in the dark during impregnation and for the duration of the experiment, to prevent possible deactivation of solutions in light, and to simulate conditions in a lens case.

Following impregnation and immediately prior to commencement of the experiment, solutions and amoeba saline were pipetted off and 1 ml of fresh fluid was added to each tube. A 10 μl trophozoite suspension containing approximately 2.8 x 10^4 trophozoites and a 10 μl cyst suspension containing approximately 3.92 x 10^4 cysts was pipetted into each tube. Both trophozoite and cyst counts were done using an Adams haemocytometer. Care was taken to ensure that the trophozoites and cysts remained dispersed throughout the solution, and were not clumped in a pellet. The caps of the tubes were tightened and sealed with Parafilm (American Can Co.) to prevent evaporation of liquid, and the tubes were left at room temperature in the dark until ready to be processed.

Three sets of control tubes were prepared. The first set contained amoeba saline alone; the second set contained amoeba saline plus a 10 μl *Mastigina* suspension; and the third set contained lens solution alone. Control tubes were treated in exactly the same way as the experimental tubes. At ten minutes, one hour, five hours, 12 hours, 24 hours, two days, three days, seven days, 14 days, 30 days and 90 days, one tube of lens solution for both trophozoites and cysts and one of each control tube were gently shaken to loosen organisms that might be adhering to the tube, and centrifuged at 740 x g for five minutes. The supernatant was discarded, and the pellet was washed twice in amoeba saline. In the case of Oxysept 1 the solution was first neutralised for ten minutes in Oxysept 2 (containing 520 U of catalase per ml), according to the manufacturer's instructions, followed by a wash with amoeba saline. After the last centrifugation, 200 μl of amoeba saline was left in the bottom of each tube. This material was then spread on to a plate (20 by 90 mm) of NNA seeded with *E. coli*. The plates were sealed with masking tape to prevent the agar from drying out. Plates were incubated at 30°C for 14 days and examined daily for the presence of *Mastigina* trophozoites by means of an

inverted microscope. The number of colonies on the plates was compared to that on the control plates, and assessed as a percentage of growth. If no trophozoites were observed at the end of 14 days, the inoculum used for the plate was considered to have been non-viable.

4.3 Results

Information on the effect of different contact lens storage/soaking and disinfecting solutions on *Mastigina* trophozoites and cysts is given in Tables 4.2 and 4.3, respectively. These tables represent the result of three experiments, all of which gave reproducible results. All the control tubes containing amoeba saline plus trophozoites or cysts showed growth of organisms for all the contact times tested. The amoeba saline control tested negative for growth, as did all the pure solutions.

Clean-N-Soak, Duracare, Soaclens and Transoak were all highly effective against trophozoites and cysts of *Mastigina*, as indicated by the fact that all organisms were eliminated within ten minutes. Oxysept was also effective against the protist, with total kill occurring within one hour for Oxysept trophozoites, and within five hours of exposure of cysts to Oxysept. Up to five hours were required to inactivate trophozoites with Hydrocare, whilst cysts were killed within 12 hours in this soft lens solution. Bausch and Lomb Soaking Solution destroyed all trophozoites within 24 hours, and inactivated cysts within two days. Softab was effective against trophozoites within 24 hours, but total inactivation of cysts had not been achieved by 90 days.

All gas permeable and hard contact lens solutions used were found to be highly effective against the strain of the *Mastigina* sp. tested, even though the composition of the solutions tested varied (Table 4.1).

Table 4.1. Contact lens solutions tested on *Mastigina* strain SAWL 91/2.

Solution trade name	Manufac-turer	Active ingredient(s)*	Preservative(s)	Type(s) of lens
Bausch & Lomb Multi-Purpose Solution®	Bausch & Lomb	Polyaminopropyl biguanide (DYMED) (0.0005); sodium borate (1.20); sodium chloride (4.9); poloxamine 1107 (10.00); boric acid (6.40)	0.11% (wt/vol) Disodium edetate	Soft
Clean-N-Soak®	Allergan	Miranol 2 MCA (buffered) (16.0)	0.004% Phenyl-mercuric nitrate in 0.01% disodium edetate	Gas permeable and hard
Duracare®	Allergan	0.004% Polyvinyl alcohol	0.004% Benzalkonium chloride; 0.004% Sodium Edetate	Gas permeable
Hydrocare Cleaning/Soaking Solution®	Allergan	Alkyl triethanol ammonium chloride (0.3)	0.002% Thiomersal	Soft
Oxysept 1®	Allergan	Hydrogen peroxide (31.0)		Soft
Soaclens Soaking and Wetting Solution®	Alcon	Polysorbate 80 (0.5); Hydroxyethyl cellulose (7.5); Polyvinyl alcohol (7.5)	0.1% Disodium edetate; 0.004% thiomersal	Hard
Softab®	Alcon	Sodium dichloroisocyanurate (0.065); available chlorine in an effervescent base (0.04)		Soft
Transoak®	Smith & Nephew	0.01% (wt/vol) Benzalkonium chloride	0.2% (wt/vol) Disodium edetate	Gas permeable and hard

Key:

*All data in parentheses are in mg/ml.

Table 4.2. Inactivation of *Mastigina* strain SAWL 91/2 trophozoites.

Contact time

Product	**10 min**	**1 h**	**5 h**	**12 h**	**24 h**	**2 d**	**3 d**	**7 d**	**14 d**	**30 d**	**90 d**
Bausch & Lomb Soaking Solution	3+	3+	1+	1+	-	-	-	-	-	-	-
Clean-N-Soak	-	-	-	-	-	-	-	-	-	-	-
Duracare	-	-	-	-	-	-	-	-	-	-	-
Hydrocare	2+	2+	-	-	-	-	-	-	-	-	-
Oxysept 1	2+	-	-	-	-	-	-	-	-	-	-
Soaclens	-	-	-	-	-	-	-	-	-	-	-
Softab	3+	2+	1+	1+	-	-	-	-	-	-	-
Transoak	-	-	-	-	-	-	-	-	-	-	-
Control (amoeba saline + trophozoites)	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Control (amoeba saline)	-	-	-	-	-	-	-	-	-	-	-

Key:

1+ = 25% of trophozoites viable.
2+ = 50% of trophozoites viable.
3+ = 75% of trophozoites viable.
4+ = control (100% of trophozoites viable).
- = no growth of organisms.

Table 4.3. Inactivation of *Mastigina* strain SAWL 91/2 cysts.

	Contact time										
Product	**10 min**	**1 h**	**5 h**	**12 h**	**24 h**	**2 d**	**3 d**	**7 d**	**14 d**	**30 d**	**90 d**
Bausch & Lomb Soaking Solution	3+	3+	2+	2+	1+	-	-	-	-	-	-
Clean-N-Soak	-	-	-	-	-	-	-	-	-	-	-
Duracare	-	-	-	-	-	-	-	-	-	-	-
Hydrocare	3+	2+	1+	-	-	-	-	-	-	-	-
Oxysept 1	2+	1+	-	-	-	-	-	-	-	-	-
Soaclens	-	-	-	-	-	-	-	-	-	-	-
Softab	3+	3+	2+	1+	1+	1+	1+	1+	1+	1+	1+
Transoak	-	-	-	-	-	-	-	-	-	-	-
Control (amoeba saline + trophozoites)	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Control (amoeba saline)	-	-	-	-	-	-	-	-	-	-	-

Key:

1+ = 25% of trophozoites viable.
2+ = 50% of trophozoites viable.
3+ = 75% of trophozoites viable.
4+ = control (100% of trophozoites viable).
- = no growth of organisms.

4.4 Discussion

Oxysept 1 (hydrogen peroxide), which eliminated trophozoites totally within one hour and cysts within five hours, was the most effective of the soft contact lens solutions tested. This was followed by Hydrocare (alkyl triethanol ammonium chloride preserved with thiomersal), which achieved total inactivation of trophozoites within five hours and cysts within 12 hours. The killing of cysts by this method probably takes too long: although the normal disinfection period for lenses would be overnight while the wearer is asleep, someone sleeping for only a short period would be at risk.

The *Mastigina* sp. is not as resistant to contact lens solutions as the strains of *Acanthamoeba* tested by this researcher (Chapter 3; Niszl & Markus, 1998) or those strains of *Acanthamoeba* studied by other authors (Ludwig *et al*., 1986; Cohen *et al*., 1987; Lindquist *et al*., 1988; Brandt *et al*., 1989; Penley *et al*., 1989; Hugo *et al*., 1991). Soft contact lens wearers in particular should be advised on the use of appropriate disinfecting solutions which kill organisms such as *Mastigina* and *Acanthamoeba*, since not all solutions are effective. Additional protist genera might in the future be shown to be capable of infecting man, since a wide variety of them exist in nature and some of these organisms have been described in the literature as the causative agents of disease in plants and invertebrate animals.

CHAPTER FIVE – *ACANTHAMOEBA*: DRUG SENSITIVITY *IN VITRO*

5.1 Seeking a medical regimen for acanthamoebic keratitis

The results of medical therapy for acanthamoebic keratitis had, until the 1990s, been disappointing. The optimal medical regimen for this disease has yet to be properly defined (Osato *et al*., 1991), although considerable progress has been made (Dart *et al.*, 2009). Clinical cure necessitates eradication of encysted amoebae with medications known to have toxic ocular effects, in combination with surgical procedures (Osato *et al*., 1991). Differences in strain and species susceptibility require a multiple-drug regimen for the treatment of acanthamoebic keratitis, but the optimal regimen varies with each case (Osato *et al*., 1991; Kitagwa *et al.*, 2003).

Drug sensitivity tests for *Acanthamoeba* are generally not available (Casemore & Warhurst, 1992). Some investigators have reported improvement of keratitis after administration of various drugs (Wright *et al*., 1985; Ishibashi *et al*., 1990; Bacon *et al*., 1993a; Seal, 2003; Mathers, 2006). However, *Acanthamoeba* species have historically shown marked resistance to a range of antibiotics and other antimicrobial agents. Results of drug sensitivity studies are conflicting, and seem to vary according to differences in virulence and the strain of amoeba (Nagington & Richards, 1976; Ma *et al*., 1981; Samples *et al*., 1984; Theodore *et al*., 1985; Bacon *et al*., 1993b). Amoebic cysts, being double-walled, tend to be highly resistant to concentrations of most compounds that are both achievable and safe in the cornea (Jones, 1986). Furthermore, the severity and duration of keratitis, the multiplicity of antimicrobial agents administered, the use of corticosteroids and

the timing of keratoplasty make the different *in vivo* drug results difficult to interpret (Osato *et al*., 1991). Early diagnosis and aggressive medical treatment of amoebic infections with topical medications appear to be important factors in the successful medical control of acanthamoebic keratitis (Larkin *et al*., 1992; Claerhout *et al.*, 2004; Lorenzo-Morales *et al*., 2007; Kovačević *et al.*, 2008; Dart *et al.*, 2009; Dua *et al.*, 2009; Shiraishi *et al*., 2009; Ueki *et al.*, 2009).

Hay *et al*. (1994) comment that for sensitivity testing, it is not sufficient to rely on isolation of the organism from the contact lens or its storage case, since amoebae from these sources may have different sensitivities to those obtained from the cornea. This occurs particularly when the diagnosis has been made late, and there has been pre-treatment with a variety of drugs.

Some of the southern African corneal and environmental isolates used in this study have shown a high degree of cytopathogenicity when compared with overseas strains (Chapter 10; Niszl *et al.*, 1998). Because of the difficulty in treating infections caused by this organism, as well as the grave outcome if the disease is not treated early enough, a comparison of the sensitivities of isolates of *Acanthamoeba* from southern Africa and the U.K. to drugs used locally and abroad, seemed appropriate. This information is potentially of great value to travellers to southern Africa who may become infected with *Acanthamoeba*, since early treatment with the most effective drug available, is of the utmost mportance.

5.2 Materials and methods

5.2.1 *Organisms*

The following isolates were used: Ac/PHL/23; ATCC 30868; ATCC 30873; ATCC 50676; ATCC 50677; ATCC 50678; ATCC 50679; ATCC 50680; ATCC

50681; ATCC 50684; and ATCC 50686.[17]

Cloning of *Acanthamoeba* isolates was done as described in Chapter 2.

Amoebae were axenically cultured at 30°C in antibiotic-free, peptone-yeast extract glucose (PYG) broth (Lasman & Feinstein, 1986)[18] in screw-cap, 80 cm^2 (260 ml) Nunc tissue culture flasks. To obtain large numbers of organisms for experimentation, sub-cultures of *Acanthamoeba* trophozoites were grown in 500 ml broth in 5-litre flasks on a shaker set at 100 rpm for 42 hours.

Amoebic cysts were obtained from trophozoites in constant pH encystment medium, as outlined in Chapter 3 of this thesis.

For the harvesting of early exponentially-growing trophozoites and cysts, the procedures outlined in Chapter 3 of this thesis were followed.

Trophozoite and cyst counts were done with an Adams haemocytometer. Amoebae in suspension were seeded in flat-bottomed wells of micro-titre plates (96 Nunc Intermed microwell), using an automatic non-electric pipette to give a concentration of 1×10^4 amoebae/ml.

Prior to drug addition, trophozoites and cysts were left standing for 30 minutes to allow them to settle.

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17. See Appendix IV.

18. See Appendix II.

5.2.2 *Drug treatment*

Compounds were obtained from the manufacturers as pure powders, pharmaceutical preparations for injection, or stock solutions. Working stock solutions were prepared immediately before use from the powdered drug – in distilled water or other appropriate solvent (indicated with the drug). Chlorhexidine (CHX) and polyhexamethylene biguanide (PHMB) were prepared as 10 or 100 μg/ml stock solutions, and all other drugs were prepared as 1 000 or 2 000 μg/ml stock solutions. Stock solutions (except natamycin, which was in suspension, and amphotericin B, which was dissolved in dimethyl sulfoxide – DMSO), were filter-sterilised through a 0.22 μm membrane filter (Millex-GS millipore). A trial run was conducted with unfiltered drugs, to ensure that the active ingredient/s was/were not being filtered out even though they were in solution. However, the results were similar to those for the filtered drugs.

The antimicrobial agents listed were used well before their stated expiry dates, except in the case of Pimafucin (discussed below):

- Amphotericin B (Squibb Laboratories), dissolved in DMSO (the highest concentration of DMSO used was 3.4%);

- Chlorhexidine gluconate (CHX) (Hibitane gluconate 20%, Imperial Chemical Industries Plc), diluted in distilled water;

- Fluconazole (Pfizer Laboratories), both pure powder dissolved in distilled water and unpreserved, sterile 100 mg fluconazole in 50 ml saline;

- Ketoconazole (Janssen Pharmaceutica), both pure base substance and Nizoral tablets dissolved in 0.05N HCl;

- Mefloquine hydrochloride (Roche, Basle, Switzerland), dissolved in 5% ethanol;

- Miconazole (Janssen Pharmaceutica), both pure base substance dissolved in 10% isopropyl alcohol and Daktarin I.V. solution (10 mg/ml);

- Natamycin–Pimafucin tablets (Reicker Lab., Gist-Brocades) – which were two months beyond their expiry date but the only ones available locally – as a suspension in distilled water; and Natacyn (Alcon) eye drops in a suspension of 50 mg/ml water;

- Pentamidine isethionate (May Baker), dissolved in distilled water;

- Polyhexamethylene biguanide (PHMB) (Bacquacil 20%, Swimline (Pty) Ltd.), diluted in distilled water;

- Polymyxin B sulphate (Wellcome), dissolved in distilled water;

- Propamidine isethionate (May Baker), dissolved in distilled water;

- Sulfisoxazole (Roche, Basle, Switzerland), dissolved in 5% methanol.

A pilot study was conducted in which the drugs were tested three times for anti-amoebic activity on trophozoites (where applicable) and cysts, up to a

concentration of 1 000 μg/ml. Drugs that did not kill amoebae at concentrations of up to 1 000 μg/ml within 48 hours, were not tested further.

Initial experiments were conducted using certain drugs on cysts and trophozoites of strain ATCC 50676 (Table 5.1). However, CHX, pentamidine isethionate and PHMB were tested on only the cysts of other strains (Table 5.2), because it is of greater value to measure cysticidal drug levels than to measure trophozoite inhibitory concentrations, considering that cysts are uniformly less sensitive to drugs and eradication is the object of therapy (Larkin *et al*., 1992). Moreover, trophozoites which are kept in any solution for longer than approximately 24 hours, will encyst (De Jonckheere & van de Voorde, 1976; Penley *et al*., 1989).

For initial experiments done on strain ATCC 50676, the total volume per well was 350 μl, with the appropriate amount of PYG medium used to make up the balance between amoebae and drug. Thereafter, for CHX, pentamidine isethionate and PHMB tested on cysts of all other strains, amoeba saline was used to make up the balance of the 350 μl.

A concentration of solvent equivalent to that used in the maximum drug concentration studied was added to trophozoites and cysts in axenic media to one chamber, as control. Another control consisted of trophozoites and cysts in axenic medium alone. Two additional blank controls were prepared. One consisted of the maximum amount of drug used, made up to 350 μl with either PYG medium or amoeba saline. The other consisted of PYG medium or amoeba saline alone.

For initial experiments on strain ATCC 50676, the cultures were incubated at 30°C, and trophozoites were examined and a cell count done at six hours, 24

hours and 48 hours. The number of amoebae in 25 randomly chosen fields of an eyepiece grid were counted, using an inverted microscope (320x magnification). At each of these times, 20 μl from wells showing inhibition of growth was placed in tissue culture flasks containing PYG medium to give a dilution of 1:50, incubated at 30°C and observed for growth for 14 days. This plating was found to be necessary, because amoebae that looked dead under the microscope were sometimes able to grow when placed in flasks.

MMIC (minimum motility inhibition concentration) was determined for trophozoites by microscopic examination, and compared with the MMIC of controls. This concentration was the lowest at which a reaction by the amoebae could be noted visually. Certain morphological changes generally accompanied immobilisation of the trophozoites: diminution in size, rounding up, an increase in granulation and/or complete disintegration, and the appearance of a large central vacuole in amoebae that were rounding up.

MIC (minimum inhibitory concentration) for the trophozoites was defined as the lowest concentration of antimicrobial agents at which growth was 70% less than in the control cultures.

The minimum amoebicidal concentration, at which there were no viable amoebae (MAC), was also recorded.

The minimum cysticidal concentration (MCC) was obtained by taking 20 μl of cyst sample at 24 hours and at 48 hours, and testing for viability by plating, as described below.

For experiments on cysts using CHX, pentamidine isethionate and PHMB, the contents of both control and experimental wells were washed three times in a centrifuge tube containing 10 ml of amoeba saline and centrifuged at 740 x g for five minutes. After the last centrifugation, 20 μl of amoeba saline was left in the bottom of each tube. This material was then spread on to *E. coli*-seeded NNA in a Petri dish (20 by 90 mm). The dishes were sealed with masking tape to prevent the agar from drying out. Plates were incubated at 30°C for 14 days and examined daily for *Acanthamoeba* trophozoites by means of an inverted microscope. The viability of organisms was assessed in terms of their ability (or otherwise) to excyst and multiply, as compared with those on control plates.

5.2.3 *Statistical analysis*

To compare the effect of the drugs tested on the different strains of *Acanthamoeba*, an analysis of variance run on a SAS version 6.1 computer package was performed. Since the experiment was stopped at the same time for all drugs, an analysis of variance in cultures where no lifetime could be observed, could not be carried out on these results. Where variance was found, a multiple comparison test using a Bonferroni (Dunn) T test was used to identify significant differences.

5.3 Results

Drug sensitivities at different times of exposure for trophozoites (MMIC, MIC and MAC) and cysts (MCC) of strain ATCC 50676, are listed in Table 5.1. The results are expressed as the mean of experiments that were conducted in triplicate.

The following drugs were not amoebicidal or cysticidal on strain ATCC 50676 within 48 hours, in concentrations up to 1,000 μg/ml (Table 5.1): amphotericin B,

fluconazole, ketoconazole, miconazole and sulfisoxazole. In addition, polymyxin B sulphate and propamidine isethionate were not cysticidal on strain ATCC 50676 within 48 hours, in concentrations up to 1,000 μg/ml (Table 5.1).

Trophozoites rapidly exhibited sensitivity to a very low concentration of natamycin, with amoebae rounding up quite soon after the addition of the drug. A similar reaction occurred within the first six hours in higher concentrations of the other drugs. At 48 hours, MMIC values for natamycin, mefloquine hydrochloride, pentamidine isethionate and propamidine isethionate were low (Table 5.1). Polymyxin B sulphate proved ineffective against strain ATCC 50676.

Concentrations of drugs that were cysticidal for strain ATCC 50676 at 24 and 48 hours (trials run in triplicate) are shown in Table 5.1. Cysts of strain ATCC 50676 were insensitive to the initial drugs tested, with only mefloquine hydrochloride, natamycin and pentamidine isethionate achieving total kill at less than 1,000 μg/ml. These values for total kill are, nevertheless, high, with the concentration of pentamidine isethionate at 400 μg/ml; mefloquine hydrochloride at 450 μg/ml; and natamycin at 508 μg/ml (Table 5.1).

Table 5.2 shows the effect at 48 hours of PHMB, CHX and pentamidine isethionate on cysts of 11 strains of *Acanthamoeba*. Both PHMB and CHX were highly effective against cysts of all strains tested, whilst high concentrations of pentamidine were ineffective against cysts of most strains (Table 5.2). The range at which PHMB was effective against the strains of *Acanthamoeba* tested was 1.7–15 μg/ml (mean 7.03 μg/ml); while that for CHX was 7.6–18.3 μg/ml (mean

13.72). In most cases (except strains ATCC 50677 and ATCC 50686), lower concentrations of PHMB than CHX were required in order to kill 100% of cysts. In all cases, control amoebae grew well when they were subcultured. Blank controls showed no growth of amoebae within the 14-day incubation period.

5.3.1 *Statistical analysis*

When the six and 24-hour MMICs for trophozoites of strain ATCC 50676 were compared for those drugs with an observed lifetime with the six and 24 hour MACs respectively (Table 5.1), an overall significance at the 0.05 level was found. In all cases, a lower concentration of the drug was required to achieve an MMIC compared to that required to achieve an MAC.

For drugs with an observed lifetime tested on strain ATCC 50676, the 48 hour MMIC, 48 hour MIC and 48 hour MAC all differed significantly at the 0.05 level from the MCC at 48 hours. In all cases, a higher concentration of the drug was required to achieve an MCC.

An overall significance was found in respect of drugs with an observed lifetime for their MAC at six hours, and at 24 hours for trophozoites of strain ATCC 50676 (Table 5.1). A multiple comparison test was done to see where the significant differences at the 0.05 level occurred, with the following results. There was a significant difference between polymyxin B sulphate and mefloquine, natamycin, pentamidine and propamidine, with polymyxin B sulphate proving less effective than the other drugs. Mefloquine was found to differ significantly from pentamidine, with mefloquine less effective than pentamidine. A significant difference was found between natamycin and pentamidine, with natamycin proving less effective than pentamidine, although natamycin did not differ

significantly from mefloquine or propamidine. In addition, propamidine was found to differ significantly from pentamidine, with propamidine being less effective than pentamidine. The confidence limits and difference between means are given in Table 5.3.

Analysis of variance showed that for all the strains tested (Table 5.2), there was no significant difference at the 0.05 level between the MCCs of CHX and PHMB at 48 hours. There was, however, a significant difference at the 0.05 level for all the strains, where the lifetime could be observed, between the MCCs at 48 hours for CHX and pentamidine; and for PHMB and pentamidine (Table 5.4). Both CHX and PHMB were found to be significantly more effective against strains of *Acanthamoeba* than was pentamidine.

Table 5.1. ***In vitro*** **sensitivity of trophozoites of** ***Acanthamoeba*** **strain ATCC 50676 to drugs at 6, 24 and 48 hours; and cysts at 24 and 48 hours.***

	6 HOURS (μg/ml)		24 HOURS (μg/ml)		48 HOURS (μg/ml)			MCC (μg/ml)	
DRUG	**MMIC**	**MAC**	**MMIC**	**MAC**	**MMIC**	**MIC**	**MAC**	**24h**	**48h**
Ampho	-	-	-	-	-	-	>1000	>1000	>1000
Fluco!	-	-	-	-	-	-	>1000	>1000	>1000
Keto!	-	-	-	-	-	-	>1000	>1000	>1000
Meflo	20	400	11	90	11	13	48	>1000	450
Mico!	-	-	-	-	-	-	>1000	>1000	>1000
Nata!	1.5	300	0.75	70	0.25	0.5	40	800	508
Pent	40	100	7	40	2	4	12	680	400
Poly	200	500	125	400	140	175	325	>1000	>1000
Prop	55	300	10	45	4	10	28	>1000	>1000
Sulfi	-	-	-	-	-	-	>1000	>1000	>1000

Key:

* Results represent the mean of experiments conducted in triplicate.

! Both forms of this drug gave similar results.

- = drug ineffective in concentrations of up to 1000 μg/ml.
MCC = minimum cysticidal concentration.
MMIC = minimum motility inhibition concentration.
MAC = minimum amoebicidal concentration.
MIC = minimum inhibitory concentration.
Ampho = amphotericin B.
Fluco = fluconazole (pure powder in distilled water and in saline).
Keto = ketoconazole (pure base substance and Nizoral in HCl).
Meflo = mefloquine hydrochloride.
Mico = miconazole (base substance in alcohol and Daktarin).
Nata = natamycin (Pimafucin tablet and Natacyn).
Pent = pentamidine isethionate.
Poly = polymyxin B sulphate.
Prop = propamidine isethionate.
Sulfi = sulfisoxazole.

Table 5.2. *In vitro* sensitivity of cysts of strains of *Acanthamoeba* to drugs at 48 hours.*

MINIMUM CYSTICIDAL CONCENTRATION (MCC) (μg/ml)

Strain	PHMB	CHX	Pentamidine
Ac/PHL/23	5.6	11.0	930
ATCC 30868 CCAP 1501/2g	3.3	12.0	700
ATCC 30873 CCAP 1501/3d	3.0	11.3	>1000
ATCC 50676	12.0	14.1	400
ATCC 50677	10.5	7.6	960
ATCC 50678	6.6	17.3	>1000
ATCC 50679	6.8	17.2	>1000
ATCC 50680	2.5	18.3	>1000
ATCC 50681	10.3	17.6	>1000
ATCC 50684	1.7	10.0	730
ATCC 50686	15.0	14.5	>1000

Key:

*Results represent the mean of experiments conducted in triplicate

CHX = chlorhexidine.

PHMB = polyhexamethylene biguanide.

Table 5.3. Comparison between the minimum amoebicidal concentration (MAC) of drugs at 6 hours and 24 hours on trophozoites of strain ATCC 50676 with an observed lifetime.

Drug comparison	Simultaneous lower confidence limit	Difference between means	Simultaneous upper confidence limit	
Poly-Meflo	1.0578	2.0500	3.0422	***
Poly-Nata	1.6578	2.6500	3.6522	***
Poly-Pent	2.8078	3.8000	4.7922	***
Poly-Prop	1.7828	2.7750	3.7672	***
Meflo-Nata	-0.3922	0.6000	1.5922	
Meflo-Pent	0.7578	1.7500	2.7422	***
Meflo-Prop	-0.2672	0.7250	1.7172	
Nata-Prop	-0.8672	0.1250	1.1172	
Nata-Pent	0.1578	1.1500	2.1422	***
Prop-Pent	0.0328	1.0250	2.0172	***

Key:
*** indicates comparisons significant at the 0.05 level.
Meflo = mefloquine hydrochloride.
Nata = natamycin (Pimafucin tablet and Natacyn).
Pent = pentamidine isethionate.
Poly = polymyxin B sulphate.
Prop = propamidine isethionate.

Table 5.4. Overall comparison between the minimum cysticidal concentrations (MCCs) of drugs at 48 hours for all strains of *Acanthamoeba* with an observed lifetime.

Drug comparison	Simultaneous lower confidence limit	Difference between means	Simultaneous upper confidence limit	
Pent-PHMB	7.3481	7.8221	8.2961	***
Pent-CHX	7.3560	7.8300	8.3040	***
PHMB-CHX	-0.3669	0.0079	0.3826	

Key:
*** indicates comparisons significant at the 0.05 level
CHX = chlorhexidine.
Pent = pentamidine isethionate.
PHMB = polyhexamethylene biguanide.

5.4 Discussion

The results reported here are discussed primarily in relation to the state of knowledge at the time that the experiments were carried out. However, research concerning potential anti-acanthamoebic drugs is on-going (Ondarza, 2007; Dart *et al.*, 2009; Roberts & Henriquez, 2010).

Osato *et al*. (1991) have emphasised that different *in vitro* investigations have yielded inconsistent data concerning the efficacy of specific antimicrobial agents. This prevented an exchange of susceptibility data among laboratories. For this reason, these authors have proposed a standardised methodology to address the problem. The experimental techniques used in the present study followed the standardised methodology suggested by Osato *et al*. (1991), except that the test plates were incubated at 30°C instead of the proposed 35°C, because the range of strains tested are well adapted to this temperature. It is important that the end points chosen are amoebicidal and cysticidal rates of 100%, because there is no evidence of the existence of host mechanisms that can destroy residual numbers of encysted amoebae in acanthamoebic keratitis, as there is for bacterial keratitis (Nagington & Richards, 1976; Garner, 1993). This is why protracted drug treatment is indicated clinically (Nagington & Richards, 1976).

Trophozoites of strain ATCC 50676 were found to be more sensitive than cysts to the drugs tested. Statistical analysis confirmed this observation. Tirado-Angel *et al*. (1996) obtained similar results for strains tested by them.

When comparing cysts of the different strains, the author ascertained that there were slightly different sensitivities to CHX, PHMB and pentamidine, but the overall variations between the strains were not statistically significant. Elder *et al*.

(1994) ascribe the range of sensitivity to drugs of strains of *Acanthamoeba* tested by them to the natural variation in the genetic make-up of these organisms in the U.K.

5.4.1 *Polyhexamethylene biguanide*

It was found that PHMB was effective against cysts of the *Acanthamoeba* isolates used in the experiments. The range of activity of PHMB for the strains tested was 1.7–15 μg/ml (mean 7.03 μg/ml). This is well within the concentration of 0.02% (that is, 200 μg/ml) of PHMB used locally for treatment of acanthamoebic keratitis. Other researchers have also reported that PHMB is effective at low concentrations against cysts and trophozoites of *Acanthamoeba* spp. (Kilvington, 1990; Larkin *et al*., 1992; Elder *et al*., 1994; Hay *et al*., 1994; Khunkitti *et al*., 1996; Ledee *et al*., 1996; Tirado-Angel *et al*., 1996; Dart *et al.*, 2009). Gilbert *et al*. (1990) investigated the antimicrobial activities of four discrete molecular weight fractions of PHMB on a number of *E. coli* strains. They found that activity of the polymers increased in proportion to polymerisation number (n). The PHMB used in this study had an average of n = 4-6 according to the manufacturers, whereas Larkin *et al*. (1992) used PHMB with n = 2-40, and a mean of 5.5. Differences in results using PHMB could be accounted for by variations in the strains of *Acanthamoeba* used and differences in polymerisation number.

PHMB did not have a toxic effect on the ocular surface, unlike propamidine or neomycin (Larkin *et al*., 1992). However, some complications that have often been attributed to the use of biguanides might be related, rather, to inflammatory consequences of the keratitis, at least in part (Dart *et* al., 2009). The same applies to diamidines, although prolonged treatment with propamidine can lead to toxic

keratopathy (Dart *et al.*, 2009). It is noteworthy that PHMB is used as a contact lens disinfectant at a concentration of 0.00005%, which has been shown *in vitro* to be ineffective against *Acanthamoeba* cysts (Kilvington, 1990; Silvany *et al*., 1990; Niszl & Markus, 1998).

5.4.2 *Chlorhexidine*

CHX was effective against cysts of all strains of *Acanthamoeba* tested in this study, with a range of activity of 7.6–18.3 μg/ml (mean 13.72), a slightly higher range than that for PHMB. Trophozoites and cysts of *Acanthamoeba* isolates tested by other researchers were also sensitive to low concentrations of CHX (Elder *et al*., 1994; Hay *et al*., 1994; Khunkitti *et al*., 1996; Ledee *et al*., 1996; Tirado-Angel *et al*., 1996; Luo *et al.*, 2008; Dart *et al.*, 2009). Kosrirukvongs *et al.* (1999) found that CHX dramatically hastened clinical improvement in all of the eyes that they tested it on. They mention that it is a successful medical treatment that has excellent results in patients who are diagnosed early. In addition, CHX appears to be well tolerated in the eye (Hay *et al*., 1994; Kosrirukvongs *et al.,* 1999). However, Gooi *et al.* (2008) showed the persistence of viable *Acanthamoeba* cysts and trophozoites in the corneal stroma, without clinical evidence of inflammation, after one year of topical 0.02% CHX monotherapy. Interestingly, 0.004% chlorhexidine gluconate is effective as a contact lens solution, killing cysts and trophozoites of *Acanthamoeba* within four hours of exposure to this solution (Anthony *et al*., 1991).

Gatti *et al.* (1998) showed that povidone-iodine (Betadine) has better anti-amoebic activity on both trophic and cystic stages of *Acanthamoeba* spp. than does CHX. Povidone iodine at 5% can safely be inserted into the eye 4 times daily and is used for “dry eye conditions” (British National Formulary). Future testing of this drug

on southern African isolates of *Acanthamoeba* will indicate its effectiveness against these strains.

5.4.3 *Amphotericin B*

Resistance to amphotericin B was found in these experiments for strain ATCC 50676 and has been corroborated by screening other amoebic isolates (Jones *et al*., 1975; Visvesvara & Balamuth, 1975; Duma & Finley, 1976; Nagington & Richards, 1976; Ma *et al*., 1981; Wright *et al*., 1985; Osato *et al*., 1991). However, Ferrante *et al*. (1984) reported an MIC of 6.25 μg/ml and an MAC of 100 μg/ml for amphotericin B when tested on a strain of *A. culbertsoni*; and Casemore (1970) found that this drug showed marked inhibition of trophozoites of *Acanthamoeba* sp. at 100 μg/ml.

5.4.4 *Fluconazole*

Fluconazole was ineffective against strain ATCC 50676. This drug has also been found to be ineffective against other strains of *Acanthamoeba* at low concentrations (Kilvington *et al*., 1990; Schuster, 1993; Elder *et al*., 1994).

The apparent efficacy of fluconazole *in vivo*, despite its low activity *in vitro*, has caused confusion (Troke *et al*., 1990). Fluconazole is, for example, 15-fold more potent than ketoconazole in a model of vaginal candidiasis in mice, despite being 80-fold less active *in vitro*. It should be noted, in addition, that Savani *et al*. (1987) found that fluconazole penetrated the eye tissue better than the other azoles. Nagington & Richards (1976) also mention that *in vitro* activity is not necessarily indicative of *in vivo* action, as was ascertained by Jamieson (1975) for clotrimazole and *Naegleria fowleri*; nor can the results of animal experiments necessarily be extrapolated as applicable in man.

5.4.5 *Ketoconazole*

Ketoconazole was ineffective against trophozoites and cysts of the southern African strain ATCC 50676. Several authors have found that ketoconazole is amoebicidal or cysticidal only at higher concentrations (Ferrante *et al*., 1984; Samples *et al*., 1984; Driebe *et al*., 1988; Osato *et al*., 1991; Larkin *et al*., 1992; Schuster, 1993; Elder *et al*., 1994). However, Driebe *et al*. (1988) and Epstein *et al*. (1986) reported that it was inhibitory at low concentrations for certain strains of *Acanthamoeba*.

5.4.6 *Mefloquine hydrochloride*

This researcher's readings of MIC at 13 μg/ml and MAC of 48 μg/ml at 48 hours for mefloquine hydrochloride for strain ATCC 50676, are lower than those recorded by Ferrante *et al*. (1984) for a strain of *A. culbertsoni*. MCC values for the strain tested in the present study were, however, very high, with the MCC value at 24 hours greater than 1,000 μg/ml, while that at 48 hours was 450 μg/ml.

5.4.7 *Miconazole*

Miconazole was ineffective against strain ATCC 50676. These results are similar to those of other researchers who worked with different strains, where high concentrations of miconazole were required to kill amoebic trophozoites and cysts (Duma & Finley, 1976; Nagington & Richards, 1976; Stevens & Willaert, 1980; Ferrante *et al*., 1984; Wright *et al*., 1985; Kilvington *et al*., 1990; Osato *et al*., 1991; Larkin *et al*., 1992; Saunders *et al*., 1992; Elder *et al*., 1994; Asiri *et al*., 1994). Driebe *et al*. (1988) obtained similar values for *A. culbertsoni*, *A. castellanii* and *A. polyphaga*, with all the MICs at 12.5 μg/ml and the MACs between 50 and 55 μg/ml. Other results vary, depending on the strain of *Acanthamoeba* tested (Epstein *et al*., 1986).

5.4.8 *Natamycin*

Inhibition for trophozoites of strain ATCC 50676 was achieved within 48 hours with low concentrations of natamycin (0.5 μg/ml), but a high concentration (508 μg/ml) was required to kill cysts of this isolate. Jones *et al*. (1975) reported that natamycin is not inhibitory in a concentration that is non-toxic to the system. Moreover, quite high concentrations of this drug were required to kill 90% of three species of amoebae (Osato *et al*., 1991). However, Epstein *et al*. (1986) achieved inhibition of *A. castellanii* at concentrations of 6.25 mg/litre, and Ma *et al*. (1981) ascertained that their strain of *A. castellanii* was sensitive to 0.5% pimaricin (natamycin). Natamycin, however, is not always well tolerated in the eye: in suspension, this drug appears to irritate the cornea.

5.4.9 *Pentamidine isethionate*

Pentamidine isethionate was effective against trophozoites of strain ATCC 50676 (the MIC at 48 hours was 4 μg/ml and the MAC was 12 μg/ml). However, cysts of strain ATCC 50676 required a much higher concentration of pentamidine (400 μg/ml) for total kill, whilst other strains tested required in excess of 500 μg/ml. Other researchers also found that low concentrations of pentamidine were effective against trophozoites of *Acanthamoeba* (Wright *et al*., 1985; Kilvington *et al*., 1990; Kishore *et al*., 1990; Elder *et al*., 1994; Ledee *et al*., 1996). Inhibitory values of pentamidine for amoebae, as obtained by Ferrante *et al*. (1984) and Ficker *et al*. (1990), were much higher than in the author's experiments. Some researchers reported that pentamidine was inhibitory for cysts of *Acanthamoeba* at fairly low concentrations (Wright *et al*., 1985; Hay *et al*., 1994; Ledee *et al*., 1996). Elder *et al*. (1994) found that the effect of pentamidine on *Acanthamoeba* cysts varied depending on the strain, with a range of 0.97–500

μg/ml. Results for different strains of *Acanthamoeba* led Duma & Finley (1976) to conclude that pentamidine isethionate appears to be slightly effective.

5.4.10 *Polymyxin B sulphate*

Polymyxin B sulphate was not effective against trophozoites or cysts of strain ATCC 50676, with the MCC at greater than 1,000 μg/ml. Other researchers also found that high concentrations of this diamidine compound were required for an amoebicidal effect (Casemore, 1970; Nagington & Richards, 1976; Ma *et al*., 1981; Samples *et al*., 1984; Wright *et al*., 1985; Osuna *et al*., 1987; Driebe *et al*., 1988; Ficker *et al*., 1990). Ferrante *et al*. (1984), however, obtained lower amoebicidal values with polymyxin B sulphate.

5.4.11 *Propamidine isethionate*

Propamidine isethionate proved effective against trophozoites of strain ATCC 50676, with an MIC of 10 μg/ml and an MAC of 28 μg/ml, but the MCC was very high, at greater than 1,000 μg/ml. Epstein *et al*. (1986) found MICs in a range close to that obtained by these experiments. The mean MCC that has been reported for propamidine is "17 to 46μg/ml, although clinical isolates vary widely with a range of up to 500μg/ml" (Dart *et al*., 2009). This drug has been shown to have good *in vitro* activity against trophozoites of *Acanthamoeba* strains (Casemore, 1970; Nagington & Richards, 1976; Ferrante *et al*., 1984; Wright *et al*., 1985; Kilvington *et al*., 1990; Larkin *et al*., 1992; Elder *et al*., 1994; Ledee *et al*., 1996). Other researchers indicate that the required drug concentration for an amoebicidal and cysticidal effect is quite high (Driebe *et al*., 1988; Ficker *et al*., 1990; Kilvington *et al*., 1990; Osato *et al*., 1991; Dart *et al*., 2009). The effectiveness of propamidine isethionate seems to vary from very low to very high, depending on the strain of *Acanthamoeba* (Matoba *et al*., 1989; Larkin *et al*.,

1992; Elder *et al*., 1994; Hay *et al*., 1994). Saunders *et al*. (1992) reported that propamidine isethionate was not cysticidal; but it became cysticidal, even in low dilution, when combined with 30% DMSO. Resistance to propamidine of certain strains of *Acanthamoeba* that cause keratitis, has been recorded (Kilvington *et al*., 1990). Ledee *et al*. (1998) have shown, using 18S rRNA gene analysis, that propamidine-resistant amoebae obtained during drug therapy came from the same strain as amoebae obtained prior to therapy. Ledee *et al.* (1998) concluded that drug resistance was probably due to a genetic or physiological change that occurred during therapy.

This drug resistance may account for unsuccessful chemotherapeutic treatment of infections, which makes surgical intervention necessary (Cohen *et al*., 1987). Ficker *et al*. (1990) have noted that resistant isolates were temperature-sensitive mutants which could not grow at temperatures above 30°C, and this could explain "culture-negative" results in some cases of clinical recurrence when incubation of laboratory samples had been performed at 37°C only. Also, diamidine compounds like propamidine isethionate have been shown to stimulate *Acanthamoeba* encystment at inhibitory and sub-inhibitory concentrations (Kim *et al*., 1987). Incidentally, the exact mechanisms involved in encystment have yet to be clarified (Chávez-Munguia *et al.*, 2007; Moon *et al.*, 2007; de Moraes & Alfieri, 2008; Kilvington *et al.*, 2008; Köhsler *et al.*, 2008; Lorenzo-Morales *et al.*, 2008; Moon *et al.*, 2008; Leitsch *et al.*, 2010). This propensity may undermine medical treatment, and account for the intensive and prolonged therapy necessary to destroy the more resistant forms. Moreover, propamidine isethionate is said to be potentially toxic to the corneal epithelium, and reports of apparent toxicity have occurred (Yeoh *et al*., 1987). Prolonged treatment of acanthamoebic keratitis with this topical drug has also allegedly caused the development of

corneal abnormality: these changes may be confused with those caused by active acanthamoebic infection (Johns *et al*., 1988; Dart *et al.*, 2009).

5.4.12 *Sulphisoxazole*

Sulphisoxazole (a sulphonamide) had no effect on trophozoites or cysts of strain ATCC 50676. However, Ferrante *et al*. (1984) reported that sulphisoxazole was the only sulphur drug which caused destruction of *A. culbertsoni* within the concentration range tested (that is, an MIC of 0.78125 μg/ml and an MAC of 50 μg/ml). Casemore (1970) found that the sulphonamides gave variable inhibition results with *Acanthamoeba* sp.

5.4.13 *General comments*

There has been debate as to whether CHX or PHMB should be the drug of choice for acanthamoebic keratitis (Elder & Dart, 1995; Seal *et al*, 1995) but both are effective (Dart *et al.*, 2009). This researcher's results showed that the difference in sensitivity to PHMB and CHX by strains of *Acanthamoeba* was not statistically significant (Niszl & Markus, 2002). Lee *et al.* (2007c) and Lim *et al.* (2008) found that PHMB and CHX showed a similar amoebicidal efficacy. Other researchers found that PHMB was effective at lower concentrations than CHX, and *vice versa* (Elder *et al*., 1994; Hay *et al*., 1994; Seal *et al*., 1995; Tirado-Angel *et al*., 1996). Khunkitti *et al*. (1998) used trophozoites and cysts of *A. castellanii* to observe the effects of PHMB and CHX on cell ultrastructure and surface structure, using both transmission and scanning electron microscopy. PHMB caused a greater degree of structural and membrane damage: the cytoplasmic contents were severely depleted and clusters of densely-stained precipitates appeared on the cell surface (Khunkitti *et al.,* 1998; Lee *et al.,* 2007c).

Cryotherapy has been found to enhance the cysticidal activity of anti-infective agents tested against cysts of *A. castellanii* and *A. polyphaga* (Matoba *et al*., 1989). Although the exact mechanism of this greater killing effect has yet to be determined, it is surmised that the cyst wall is mechanically disrupted, which facilitates entry of the anti-infective agents into the cyst and ultimately results in death of amoebae.

Drug resistance may develop as a result of low-dose, single-drug anti-amoebic therapy (Hay *et al*., 1994). Variance in strain and species susceptibility has necessitated the recommendation of a multiple-drug regimen for the treatment of amoebic keratitis, but the optimal regimen varies with each case (Osato *et al*., 1991; Kitagwa *et al*., 2003). Since an effective biguanide-propamidine treatment combination has been introduced, and cases have been diagnosed increasingly early, there has been a consequent decrease in the time taken for medical cure (Bacon *et al*., 1993b). Notable in the context of combination therapy *in vivo*, are the combination of PHMB and propamidine (Larkin *et al*., 1992; Duguid *et al*., 1997; Dart *et al*., 2009; Hasler *et al*., 2009); neomycin and propamidine (Varga *et al*., 1992); neomycin, dibromopropamidine and propamidine (Wright *et al*., 1985); and *in vitro*, DMSO and propamidine isethionate (Saunders *et al*., 1992). Studies by Hay *et al*. (1994) showed additive effects with cationic antiseptics (CHX or PHMB) plus propamidine or neomycin, and slight synergy between the antiseptics and pentamidine. Tirado-Angel *et al*. (1996) did not find a marked increase in kill rate for amoebae with a combination of PHMB and CHX as compared with the individual biguanides. Nevertheless, at sub-amoebicidal concentrations, the combination was significantly more effective against *A. castellanii* and *A. polyphaga* than the individual biguanides (Tirado-Angel *et al*., 1996). The synergistic activity of the combination may be related to the smaller biguanide,

CHX, altering the membrane, resulting in enhanced binding and uptake of the more toxic PHMB (Gilbert *et al*., 1990). Since useful anti-acanthamoebic drugs may not have universal activity against all amoebae, combination therapy should always be employed to allow the possibility of an additive anti-amoebal effect and to prevent the emergence of resistance (Hay *et al*., 1994).

Cases do occur where viable amoebae are recovered from corneas after prolonged, intensive combination treatments with neomycin and propamidine, and also with PHMB and propamidine, despite good *in vitro* and clinical results (Elder *et al*., 1994). Drug resistance did not occur (Elder *et al*., 1994), although this phenomenon has been reported for some drugs (Ficker *et al*., 1990). Larkin *et al*. (1992) also had a patient with acanthamoebic keratitis where the strain of *Acanthamoeba* had a low MCC for PHMB and yet there was there was medical treatment failure. The authors suggest that this failure could be due to the absence of some necessary factor in the host's immune response, or to insufficient drug penetration. Dart *et al.* (2009) state that the diamidines and biguanides "are currently the most effective cysticidal anti-amoebics *in vitro* and their use is supported by a substantial case series".

Early diagnosis and aggressive medical treatment of amoebic corneal infections with topical medications appear to be important factors in the successful medical control of acanthamoebic keratitis (Bacon *et al*., 1993b; Claerhout *et al.*, 2004; Lorenzo-Morales *et al.*, 2007; Kovačević *et al.*, 2008; Dart *et al.*, 2009; Dua *et al.*, 2009; Shiraishi *et al.*, 2009; Ueki *et al.*, 2009). The results of the present researcher's experiments indicate that, as for the overseas isolates tested, PHMB or CHX should be the drugs of choice unless *in vitro* drug sensitivity results can be obtained. Since medical treatment is not always effective, greater emphasis

should be placed on educating patients in the use and disinfection of contact lenses (Stehr-Green *et al*., 1989).

CHAPTER SIX – *MASTIGINA* SP.: *IN VITRO* DRUG SENSITIVITY

6.1 The need to find a treatment for *Mastigina*

A strain of *Mastigina* (SAWL 91/2) that was associated with keratitis in a patient in South Africa, was found to have a cytopathic effect on cultured mammalian cells (Chapter 11). This is confirmation that the organism is not necessarily innocuous and that it can be regarded as a potential pathogen. It was, therefore, of interest to assess the sensitivity of this protostelid to drugs, as this had not, to the writer's knowledge, been investigated previously.

6.2 Materials and methods

6.2.1 *Organisms*

Cloning of the strain of *Mastigina* found was carried out as described for *Acanthamoeba* sp. (Chapter 2).

For the culturing and harvesting of *Mastigina* organisms, the procedures outlined in Chapter 4 of this thesis were followed.

Trophozoite and cyst counts were done using an Adams haemocytometer. Organisms in suspension were seeded in flat-bottomed wells of microtitre plates (96 Nunc Intermed microwell), using an automatic, non-electric pipette to give a concentration of 10^4 organisms/ml.

Prior to drug addition, *Mastigina* organisms were left standing for 30 minutes to allow them to settle.

6.2.2 *Drug treatment*

Compounds were obtained as pure powders, pharmaceutical preparations for injection, or stock solutions from the manufacturers. Working stock solutions (2 mg/ml) were prepared immediately before use from the powdered drug in distilled water or other appropriate solvent (indicated with the drug). Stock solutions (except natamycin, which was in suspension; and amphotericin B, which was dissolved in dimethyl sulfoxide – DMSO), were filter-sterilised through a 0.22 μm membrane filter (Millex-GS millipore). A trial run was conducted with unfiltered drugs to ensure that the active ingredients were not being filtered out despite the fact that they were in solution. However, results were similar to those for the filtered drugs.

The antimicrobial agents were used well before their stated expiry dates, except in the case of Pimafucin (discussed below):

- Amphotericin B (Squibb Laboratories), dissolved in DMSO (the highest concentration of DMSO used was 3.4%);

- Fluconazole (Pfizer Laboratories), both pure powder dissolved in distilled water and unpreserved, sterile 100 mg fluconazole in 50 ml saline;

- Ketoconazole (Janssen Pharmaceutica), pure base substance dissolved in 0.05N HCl;

- Mefloquine hydrochloride (Roche, Basle, Switzerland), dissolved in 5% ethanol;

- Miconazole (Janssen Pharmaceutica), both pure base substance dissolved in 10% isopropyl alcohol and Daktarin I.V. solution (10 mg/ml);

- Natamycin - Pimafucin tablet (Reicker Lab., Gist-Brocades) – the only tablets available locally were two months beyond their expiry date – as a suspension in distilled water;

- Pentamidine isethionate (May Baker), dissolved in distilled water;

- Polymyxin B sulphate (Wellcome), dissolved in distilled water;

- Propamidine isethionate (May Baker), dissolved in distilled water; and

- Sulfisoxazole (Roche, Basle, Switzerland), dissolved in 5% methanol.

A pilot study was conducted and the drugs were tested in triplicate for anti-*Mastigina* activity up to a concentration of 500 μg/ml. Drugs not causing death of organisms in concentrations of up to 500 μg/ml within 48 hours were not tested further.

The total volume per well was 350 μl, the appropriate amount of PYG medium having made up the balance, together with *Mastigina* organisms and drug. To one chamber, as control, a concentration of solvent equivalent to that used in the maximum drug concentration studied was added to *Mastigina* organisms in axenic media. Another control consisted of organisms in axenic medium alone. The cultures were incubated at 30°C and examined at 24 hours and at 48 hours. At 24

hours, 20 μl was taken from each well, and the remaining fluid was removed at 48 hours. The fluid was placed in tissue culture flasks containing PYG medium to give a dilution of 1:50, incubated at 30°C and observed for growth for 14 days. This plating was found to be necessary, as organisms that looked dead under the microscope were sometimes able to grow when placed in flasks.

MMIC (minimum motility inhibition concentration) was determined for trophozoites by microscopic examination and compared with the controls. This concentration was the lowest at which a reaction by the trophozoites could be noted visually. Rounding up of trophozoites accompanied immobilisation, and if no visible reaction was seen at 24 hours, it was assessed at 48 hours.

The minimum drug concentration, in which no viable trophozoites (MTC) or cysts (MCC) occurred, was recorded.

6.2.3 *Statistical analysis*

To compare the effect of the different drugs tested on trophozoites and cysts of the strain of *Mastigina*, an analysis of variance run on a SAS Version 6.1 computer package was performed. For cultures where variance was found, a multiple comparison test using a Bonferroni (Dunn) T test was used to see where the significant differences occurred.

6.3 Results

Fluconazole and sulfisoxazole did not kill trophozoites or cysts of *Mastigina* organisms within 48 hours in concentrations of up to 500 μg/ml. Furthermore, miconazole was not cysticidal in concentrations of up to 500 μg/ml.

MMICs are given in Table 6.1, which also reflects whether organisms could be observed at 24 or 48 hours.

MTCs at 24 hours and 48 hours for the drugs tested, are listed in Table 6.1. The results are expressed as the mean of three experiments. As can be seen from Table 6.1, the trophozoite forms of *Mastigina* were highly sensitive to polymyxin B sulphate, with total killing occurring at 4 μg/ml within 24 hours and at 1.5 μg/ml within 48 hours. These forms also responded to amphotericin B, with complete inactivation occurring within 24 hours at 5 μg/ml. A degree of sensitivity of *Mastigina* trophozoites to mefloquine hydrochloride was observed; total kill was achieved within 24 hours at 15 μg/ml. Much higher concentrations of the other drugs tested, were required to kill the trophozoites within 48 hours.

Table 6.2 lists MCCs at 24 hours and 48 hours for the drugs tested. The results are expressed as a mean of the three experiments. Cysts showed a high degree of sensitivity to polymyxin B sulphate, with 4 μg/ml required for 100% killing of *Mastigina* cysts within 24 hours, whereas a concentration of 3.5 μg/ml was required at 48 hours for a similar effect (Table 6.2).

Amphotericin B achieved complete inactivation of cysts at 10 μg/ml in 24 hours, and cysts were killed by mefloquine within 24 hours at 20 μg/ml. High concentrations of the other drugs tested were required to kill *Mastigina* cysts (Table 6.2).

6.3.1 *Statistical analysis*

A significant difference at the 0.05 level was found for trophozoites of *Mastigina* between the MMIC at 24 hours and the MTC at 24 hours (Table 6.1), with higher concentrations of drugs required to achieve the MTC effect than the MMIC effect.

A significant difference at the 0.05 level was found for the drugs tested for MTC at 48 hours (Table 6.1). The Bonferroni (Dunn) T tests showed that the results were significant for all the different drugs tested (Table 6.3).

For those drugs with observable lifetimes, a significant difference at the 0.05 level was found for the MCCs at 24 and at 48 hours (Table 6.2). The Bonferroni (Dunn) T tests indicated that the significant differences at the 0.05 level for the MCCs at 24 and at 48 hours were observable only between certain drugs (Table 6.4).

Table 6.1. *In vitro* sensitivity of *Mastigina* sp. trophozoites to antimicrobial drugs at 24 and 48 hours.

Drug	MMIC (μg/ml)*	MTC at 24 hours (μg/ml)	MTC at 48 hours (μg/ml)
Amphotericin B	0.2 (24 hrs)	5	5
Fluconazole	>500	>500	>500
Ketoconazole	5 (24 hrs)	125	80
Mefloquine	0.2 (48 hrs)	15	15
Miconazole	>500 (48 hrs)	>500	100
Natamycin	10 (24 hrs)	60	55
Pentamidine	20 (24 hrs)	130	110
Polymyxin B	0.2 (24 hrs)	4	1.5
Propamidine	30 (24 hrs)	200	65
Sulfisoxazole	>500 (48 hrs)	>500	>500

Key:

*The first concentration at which an effect can be observed at 24 or 48 hours is indicated, with the time in parentheses.

MMIC = minimum motility inhibition concentration.

MTC = minimum trophozoite-killing concentration.

Table 6.2. ***In vitro*** **sensitivity of** ***Mastigina*** **sp. cysts to antimicrobial drugs at 24 and 48 hours.**

Drug	**MCC at 24 hours (μg/ml)**	**MCC at 48 hours (μg/ml)**
Amphotericin B	10	10
Fluconazole	>500	>500
Ketoconazole	130	120
Mefloquine	20	20
Miconazole	>500	>500
Natamycin	85	85
Pentamidine	300	165
Polymyxin B	4	3.5
Propamidine	350	100
Sulfisoxazole	>500	>500

Key:
MCC = minimum cysticidal concentration.

Table 6.3. Confidence limits and differences between means for drugs tested for minimum trophozoite-killing concentrations (MTCs) at 48 hours on a strain of *Mastigina.*

Drug comparison	Simultaneous lower confidence limit	Difference between means	Simultaneous upper confidence limit	
Ampho-Keto	-0.80064	-0.75000	-0.69936	***
Ampho-Meflo	-0.15064	-0.10000	-0.04936	***
Ampho-Mico	-1.00064	-0.95000	-0.89936	***
Ampho-Nata	-0.55064	-0.50000	-0.44936	***
Ampho-Pent	-1.10064	-1.05000	-0.99936	***
Ampho-Prop	-0.65064	-0.60000	-0.54936	***
Keto-Meflo	0.59936	0.65000	0.70064	***
Keto-Mico	-0.25064	-0.20000	-0.14936	***
Keto-Nata	0.19936	0.25000	0.30064	***
Keto-Pent	-0.35064	-0.30000	-0.24936	***
Keto-Poly	-0.75064	-0.70000	-0.64936	***
Keto-Prop	0.09936	0.15000	0.20064	***
Meflo-Poly	-1.40064	-1.35000	-1.29936	***
Meflo-Pent	-1.00064	-0.95000	-0.89936	***
Meflo-Mico	-0.90064	-0.85000	-0.79936	***
Meflo-Prop	-0.55064	-0.50000	-0.44936	***
Meflo-Nata	-0.45064	-0.40000	-0.34936	***
Mico-Poly	-0.55064	-0.50000	-0.44936	***
Mico-Pent	-0.15064	-0.10000	-0.04936	***

Mico-Prop	0.29936	0.35000	0.40064	***
Mico-Nata	0.39936	0.45000	0.50064	***
Nata-Poly	-1.00064	-0.95000	-0.89936	***
Nata-Pent	-0.60064	-0.55000	-0.49936	***
Nata-Prop	-0.15064	-0.10000	-0.04936	***
Pent-Poly	-0.45064	-0.40000	-0.34936	***
Pent-Prop	0.39936	0.45000	0.50064	***
Poly-Prop	0.79936	0.85000	0.90064	***
Poly-Ampho	1.39936	1.45000	1.50064	***

Key:
*** indicates significance at the 0.05 level.
Ampho = amphotericin B.
Keto = ketoconazole (pure base substance and Nizoral in HCl).
Meflo = mefloquine hydrochloride.
Mico = miconazole.
Nata = natamycin (Pimafucin tablet and Natacyn).
Pent = pentamidine isethionate.
Poly = polymyxin B sulphate.
Prop = propamidine isethionate.

Table 6.4. Confidence limits and differences between means for drugs having significantly different minimum cysticidal concentrations (MCCs) at 24 hours and at 48 hours.

Drug comparison	Simultaneous lower confidence limit	Difference between means	Simultaneous upper confidence limit	
Ampho-Pent	-4.0437	-2.2250	-0.4063	***
Ampho-Prop	-3.9687	-2.1500	-0.3313	***
Ampho-Poly	-3.8187	-2.0000	-0.1813	***
Ampho-Keto	-2.9687	-1.1500	0.6687	
Ampho-Nata	-2.5687	-0.7500	1.0687	
Ampho-Meflo	-1.9187	-0.1000	1.7187	
Keto-Pent	-2.8937	-1.0750	0.7437	
Keto-Prop	-2.8187	-1.0000	0.8187	
Keto-Poly	-2.6687	-0.8500	0.9687	
Keto-Nata	-1.4187	0.4000	2.2187	
Keto-Meflo	-0.7687	1.0500	2.8687	
Meflo-Pent	-3.9437	-2.1250	-0.3063	***
Meflo-Prop	-3.8687	-2.0500	-0.2313	***
Meflo-Poly	-3.7187	-1.9000	-0.0813	***
Meflo-Keto	-2.8687	-1.0500	0.7687	
Meflo-Nata	-2.4687	-0.6500	1.1687	
Nata-Pent	-3.2937	-1.4750	0.3437	
Nata-Prop	-3.2187	-1.4000	0.4187	
Nata-Poly	-3.0687	-1.2500	0.5687	

Pent-Prop	-1.7437	0.0750	1.8937	
Pent-Poly	-1.5937	0.2250	2.0437	
Poly-Ampho	0.1813	2.0000	3.8187	
Poly-Meflo	0.0813	1.9000	3.7187	
Prop-Meflo	0.2313	2.0500	3.8687	
Prop-Ampho	0.3313	2.1500	3.9687	

Key:
*** indicates significance at the 0.05 level.
Ampho = amphotericin B.
Keto = ketoconazole (pure base substance and Nizoral in HCl).
Meflo = mefloquine hydrochloride.
Nata = natamycin (Pimafucin tablet and Natacyn).
Pent = pentamidine isethionate.
Poly = polymyxin B sulphate.
Prop = propamidine isethionate.

6.4 Discussion

Trophozoites and cysts of the strain of *Mastigina* studied were much more sensitive to drugs than strains of *Acanthamoeba* (Chapter 5). Generally, *Acanthamoeba* cysts are highly resistant to drugs (Ma *et al*., 1981; Kilvington *et al*., 1990; Osato *et al*., 1991), whereas complete inactivation of *Mastigina* cysts was achieved by reasonably low concentrations of polymyxin B sulphate, amphotericin B and mefloquine hydrochloride. The thicker cyst walls of *Acanthamoeba* (compared with the cyst walls of *Mastigina*) possibly reduce the accessibility of certain drugs to the organism.

It is interesting to note that there are significant differences between the MTCs of all the drugs with an observed lifetime. Trophozoites were killed with the lowest concentration of polymyxin B sulphate, the most effective of all the drugs tested. In addition, a significant difference was found between the MMIC and MTC of *Mastigina* at 24 hours, with the MMICs being much lower than the MTCs. It is to be expected that a lower drug concentration is required to inhibit the trophozoites than would be required to kill them. For certain drugs, the MCC at 24 hours was significantly higher than the MCC at 48 hours. It appears that certain drugs take a longer time to have an effect.

The patient from whose contact lenses the *Mastigina* organisms were isolated, had originally been treated with corticosteroids, which had had no effect. She was subsequently treated with antibiotics and propamidine isethionate, after which the infected eye improved remarkably. The samples of the patient's contact lenses and the fluid from the lens case taken after the latter treatment, tested negative. Even though propamidine isethionate was not the most effective drug against the

strain of *Mastigina* tested in this series of experiments, the combination of therapies applied proved to be useful in treating the patient.

Trials covering an even broader spectrum of drugs would be of interest, to allow the effectiveness of a greater range of antimicrobial and antifungal drugs against this protostelid to be established.

CHAPTER SEVEN – CHARACTERISATION OF *ACANTHAMOEBA* BY LIGHT MICROSCOPY

7.1 Differentiation of *Acanthamoeba* species based on their properties and morphology

The genus *Acanthamoeba* is well characterised, because ultrastructural characteristics of the cysts of species of *Acanthamoeba* enable them to be differentiated from those of *Naegleria* and other genera of protozoa.

The status of species within the genus *Acanthamoeba* is, however, still not well clarified (Page, 1976). In the past, the differentiation of species was based largely on cyst morphology, but classification in this way does not adequately reflect the diversity of these organisms (Griffiths *et al*., 1978). Amoebae exhibit different characteristics depending on which culture medium is used (Cline *et al*., 1983), and variations in the properties and morphology of cysts of a species of *Acanthamoeba* have been found (Stratford & Griffiths, 1978). When the structure and behaviour of cysts of a single strain of *A. castellanii* produced under different cultural conditions was investigated by Stratford & Griffiths (1978), there appeared to be a gradation from cysts with wrinkled walls, giving each cyst (produced in monoxenic culture and in replacement medium), a stellate appearance in section to smooth, refractile cells (produced in a growth medium supplemented with $MgCl_2$) lacking a distinct wall. All the cyst types exhibited depressed metabolic activity, and differed only in the degree to which they were resistant to heating and to low temperatures (Stratford & Griffiths, 1978).

Trophozoites of *Acanthamoeba* are also not useful for distinguishing between species of this genus, because trophozoites of different species are generally quite similar (Visvesvara & Balamuth, 1975). For example, trophozoites of *A. castellanii* (Singh and Neff strains), *Acanthamoeba* sp. (Lilly strain), *A. polyphaga* and *A. terricola* are similar except for slight differences in size. Trophozoites of *A. astronyxis* and *A. comandoni* resemble those of other species of this genus, but are distinctly larger and broader, and have thicker acanthopodia (Visvesvara & Balamuth, 1975).

While pathogenic *Naegleria* cysts tend to exhibit a rough-textured surface compared with those of *N. gruberi*, which is non-pathogenic (Lastovica, 1974), no structures in the trophozoites or cysts of *Acanthamoeba* have been found that can be linked with pathogenicity (Lastovica, 1977a; Lastovica, 1977b).

Several stains can be used for cysts and trophozoites of *Acanthamoeba*. These include Giemsa-Wright or haemacolour, Wheatley trichrome, fluorescein-conjugated lectin, Gomori-methenamine silver, Periodic Acid Schiff (PAS), immunofluorescent antibody (both trophozoites and cysts can be stained with rabbit antiserum to *Acanthamoeba*) and stains for electron microscopy (Chapter 8; Niszl & Markus, 1989; Ma *et al*., 1990).

Calcofluor white with methylene blue, used for the detection of fungi, is an effective staining technique for *Acanthamoeba*, with cysts being stained green and

trophozoites or young cysts bright orange (Wilhelmus *et al*., 1986).[19] Examination requires a fluorescence microscope. No permanent smear can be saved for future reference because the fluorescence fades with time. This method has also been successfully applied to paraffin-embedded sections. According to Marines *et al*. (1987), calcofluor white appears to be reliable for diagnosing acanthamoebic keratitis, as there are usually no false positives (except for the possible presence of cotton or lint on slides) or false negatives. Moore and McCulley (1989) found that when, in certain cases, no *Acanthamoeba* was detected in samples stained with haematoxylin and eosin (H & E), PAS or methenamine silver, calcofluor white staining of the same sample gave positive results. Insler *et al*. (1988), however, cite an instance where they obtained negative results using corneal scrapings stained with calcofluor white, yet biopsy and cultures confirmed acanthamoebic keratitis.

Fungiflora Y is a stain that was originally developed to show the presence of fungi. Chitin and cellulose are components of the cell wall of fungi but *Acanthamoeba* cysts also contain the latter. Fungiflora Y has an affinity for chitin and cellulose and it has recently been used to reveal cysts of *Acanthamoeba* in frozen sections of corneal scrapings (Shiraishi *et al*., 2009).

Species of *Acanthamoeba* can be identified in tissue sections by means of monoclonal or polyclonal antibodies. However, the usefulness of serological tests in the diagnosis of infections due to these amoebae is limited. Much work needs to be done to delineate the immune response in acanthamoebic keratitis, and to

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19. See Appendix VI.

ascertain the extent (if any) of false-positive and false-negative results before these tests can be considered diagnostic.

As has been indicated elsewhere in this thesis, molecular techniques are currently being used to detect *Acanthamoeba*; and a number of references have been provided in this regard.

Light microscopy was used to examine the different strains of *Acanthamoeba* described in Chapter 2. Some of the isolates were also stained in an attempt to correlate morphological observations with non-morphological results for speciation.

7.2 Materials and methods

Amoebae were cultured on NNA plates seeded with *E. coli* bacteria, as described in Chapter 2. Photographs of amoebae growing on agar plates were taken under a compound microscope, with the Petri dish inverted (Figs 2.1; 2.2; 2.5; 2.6; 2.8).

For light microscopic photographs of amoebae, organisms were scraped from the agar surface using a flamed loop. Trophozoites were taken from fresh plates, and cysts were scraped off older plates. Amoebae were placed in sterile amoeba saline,[20] covered with a coverslip sealed with Vaseline, and photographed under a compound microscope. Fixation and staining procedures for H & E, calcofluor white and Weigert's Iron Haematoxylin, are described in Appendix VI.

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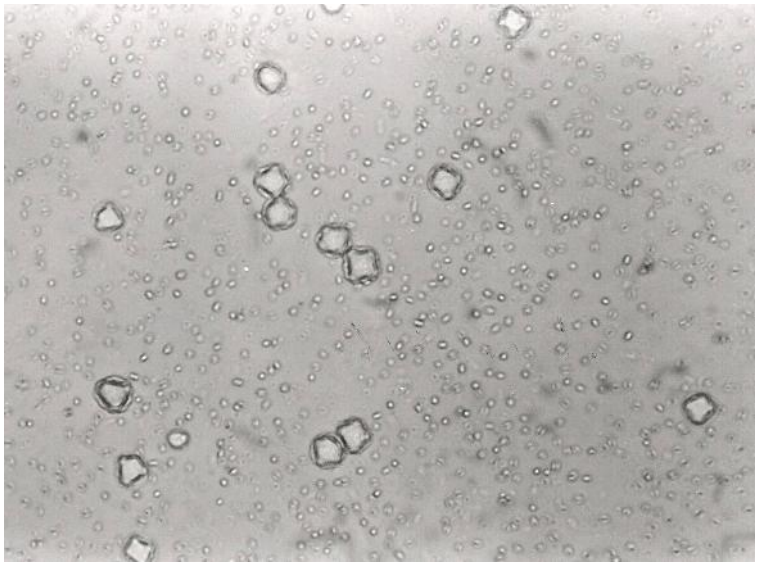
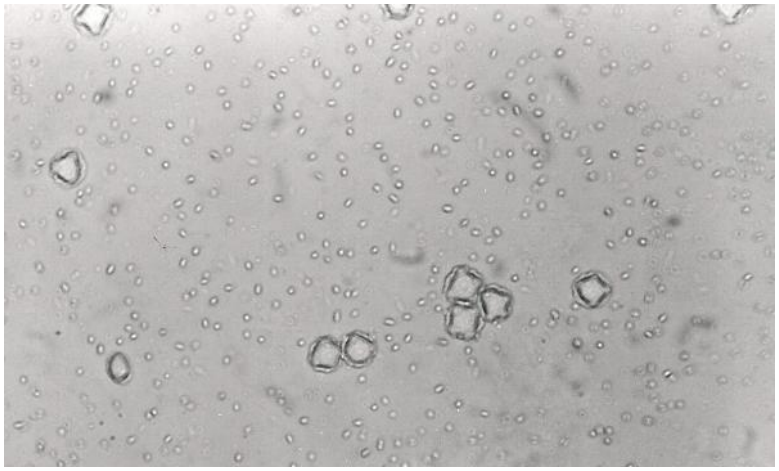
20. See Appendix II.

7.3 Results

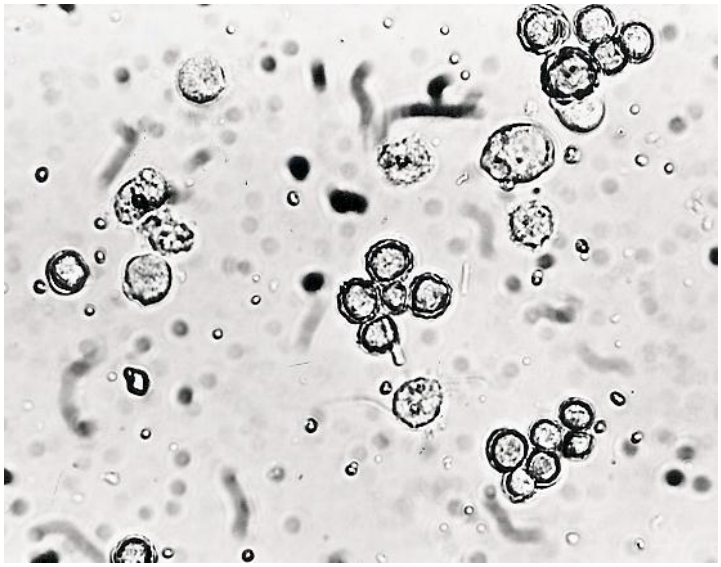
Photographs of amoebae on NNA in Petri dishes are presented in Figures 2.1; 2.2; 2.5; 2.6 and 2.8. Light microscopic photographs of the different strains of amoebae can be seen in Figures 7.1 – 7.54. Amoebae stained with H & E, calcofluor white, iron haematoxylin and trichrome are shown in Figures 7.17 – 7.21; 7.35 – 7.36; and 7.46 – 7.52.

Cysts of some of the corneal isolates tested were morphologically similar both to each other and to corneal isolates from overseas. The sewage isolates, strains ATCC 50685, ATCC 50686 and ATCC 50687, were morphologically similar (Figs 7.31 –7.37). Their sizes were also similar, having means of 17.1 μm, 18.6 μm and 18.0 μm respectively (Table 2.6). Strains ATCC 30868 and SAWS 87/4 were not similar to each other or to any of the other strains (Figs 7.11; 7.12; 7.44 – 7.52).

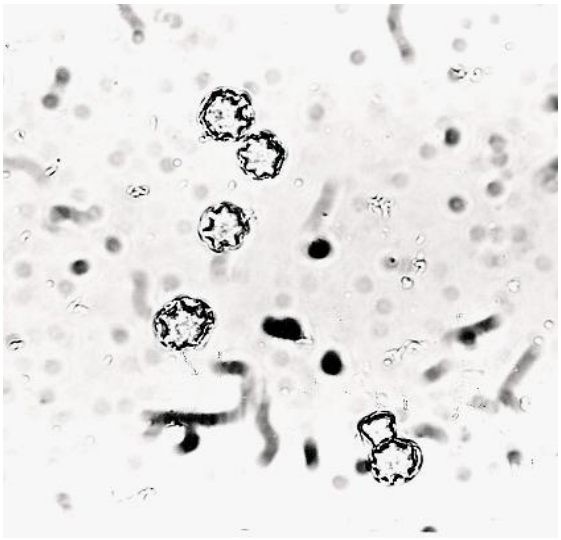


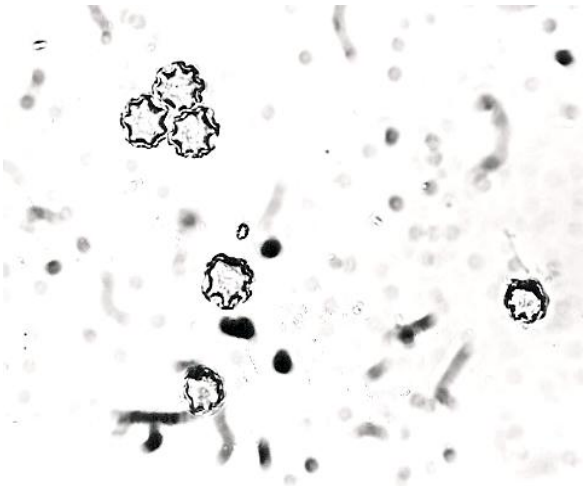


Figures 7.1 and 7.2. Cysts of *Acanthamoeba* strain Ac/PHL/4, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 450)



Figures 7.3 and 7.4. Cysts of *Acanthamoeba* strain Ac/PHL/9, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 450)





Figures 7.5 and 7.6. Cysts of *Acanthamoeba* strain Ac/PHL/17, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 450)

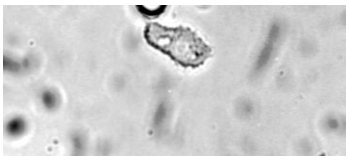


Figure 7.7. A trophozoite of *Acanthamoeba* strain Ac/PHL/22, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Acanthapodia and a contractile vacuole are visible in the trophozoite. (x 450)

Figure 7.8. Cysts of *Acanthamoeba* strain Ac/PHL/22, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 450)

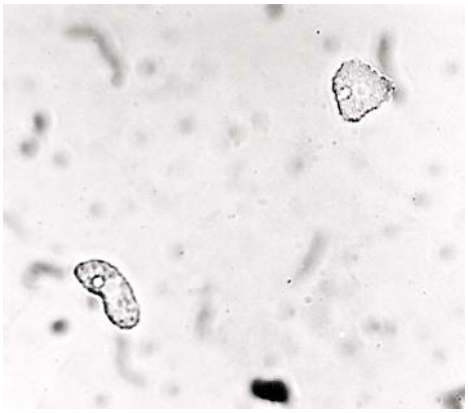


Figure 7.9. Two trophozoites of *Acanthamoeba* strain Ac/PHL/23, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Contractile vacuoles are visible in the trophozoites. (x 450)

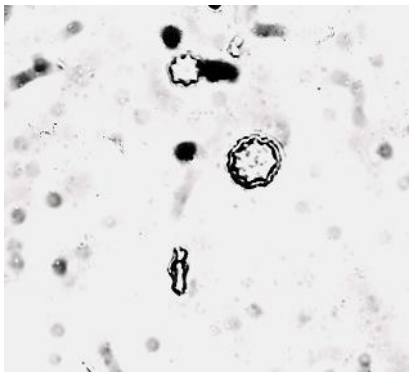
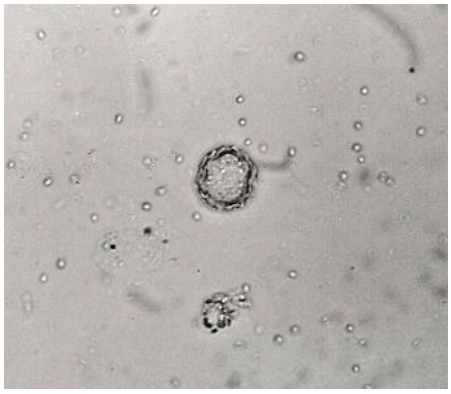
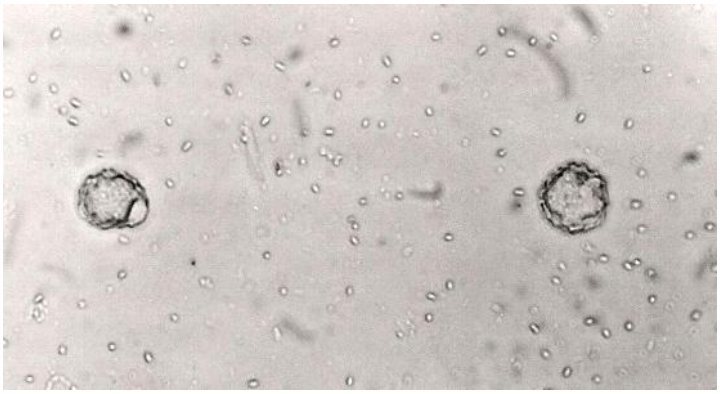
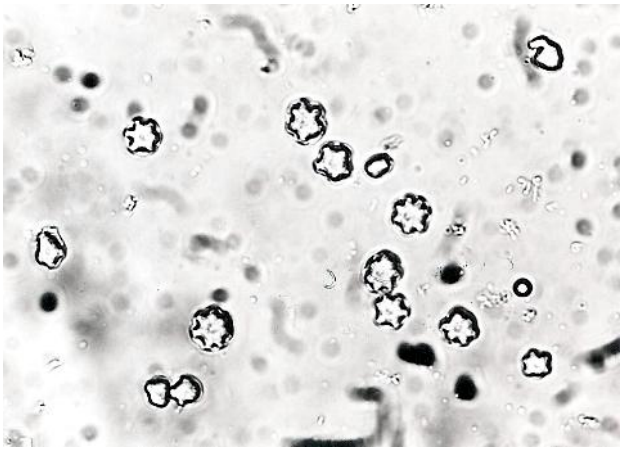


Figure 7.10. Cysts of *Acanthamoeba* strain Ac/PHL/23, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 450)

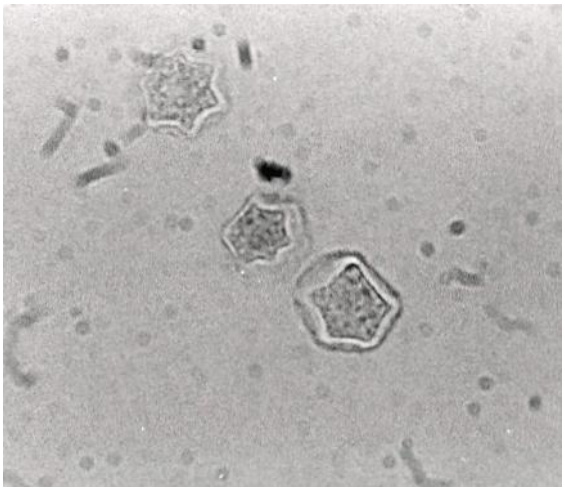


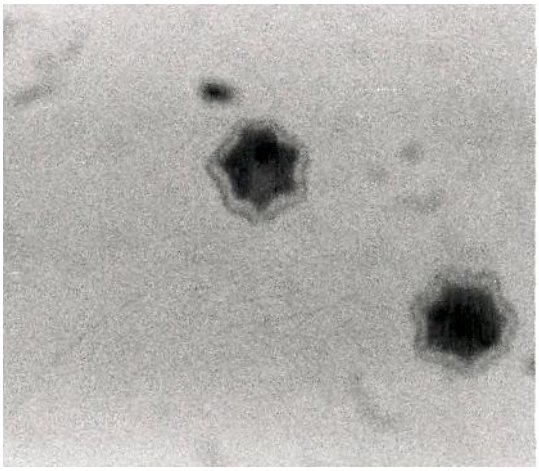


Figures 7.11 and 7.12. Cysts of *Acanthamoeba* strain ATCC 30868, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 450)



Figures 7.13 and 7.14. Cysts of *Acanthamoeba* strain ATCC 30873, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 450)





Figures 7.15 and 7.16. Cysts of *Acanthamoeba* strain ATCC 50676, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 1160)

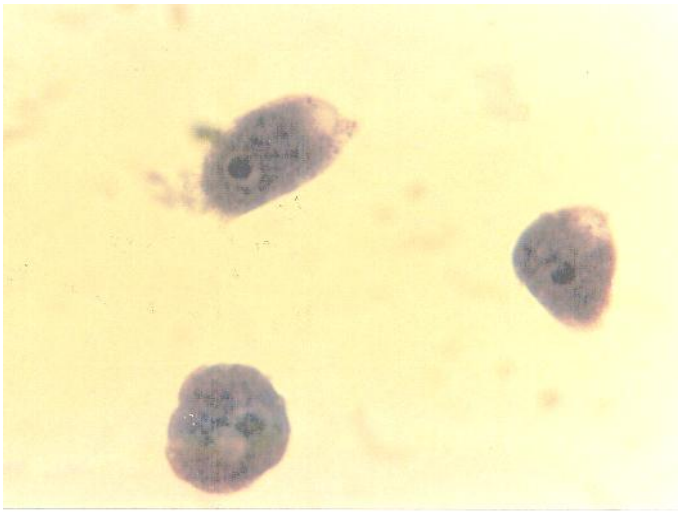


Figure 7.17. Trophozoites of *Acanthamoeba* strain ATCC 50676, stained with haematoxylin and eosin. Amoebae were grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Nucleoli, which have stained dark blue, and contractile vacuoles are visible in the trophozoites. (x 1160)

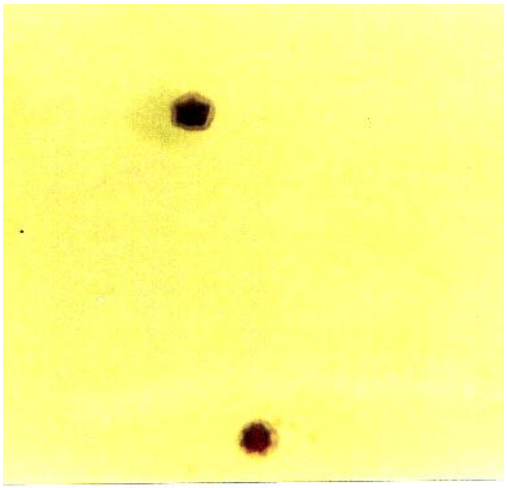


Figure 7.18. (x 450)

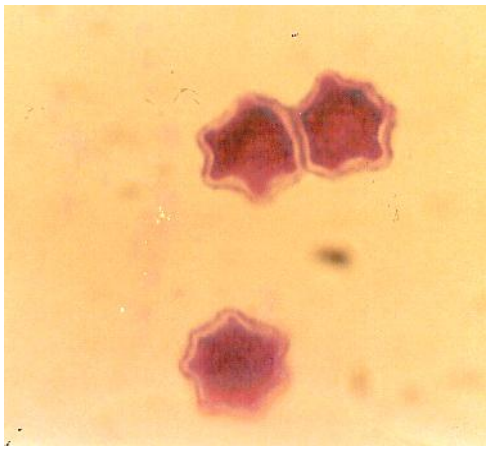


Figure 7.19. (x 1160)

Figures 7.18 and 7.19. Cysts of *Acanthamoeba* strain ATCC 50676, stained with haematoxylin and eosin. Amoebae were grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. The contents of the cyst have stained red whilst the ectocyst is light pink in colour. The shapes of the ectocyst and endocyst are clearly visible.

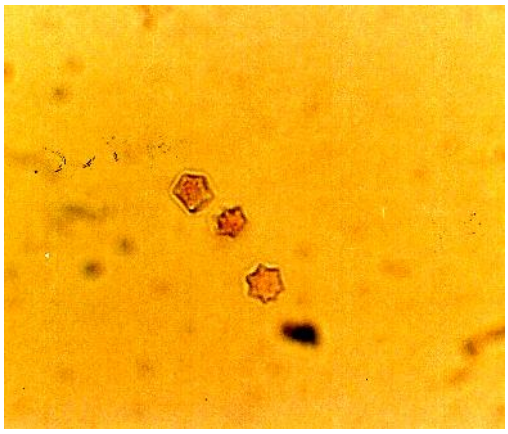


Figure 7.20. Cysts of *Acanthamoeba* strain ATCC 50676 stained, with trichrome stain. Amoebae have been grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. The contents of the cyst have stained light pink whilst the ectocyst has not taken up any stain. The shapes of the ectocyst and endocyst are clearly visible. (x 450)

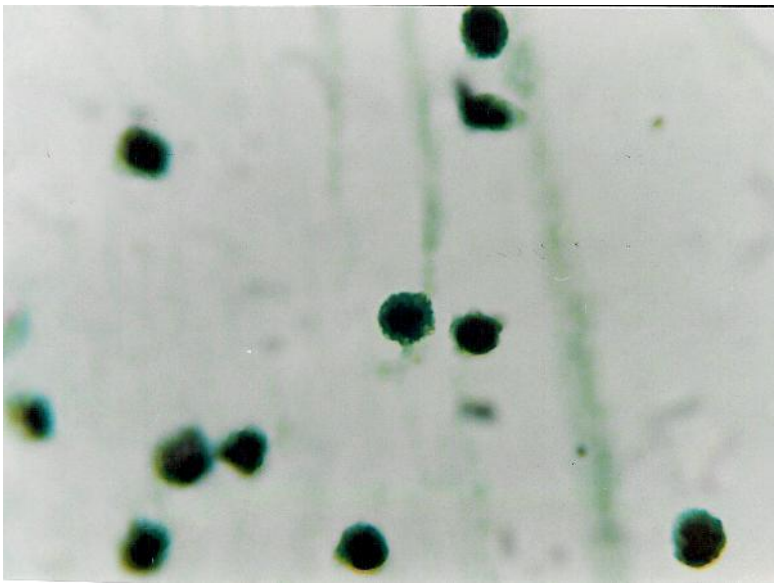


Figure 7.21. Cysts of *Acanthamoeba* strain ATCC 50676, stained with iron haematoxylin. Amoebae were grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. The contents of the cyst have stained a dark green colour, whilst the ectocyst is lighter green. (x 500)

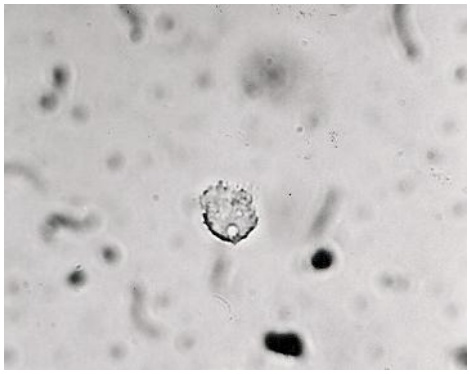


Figure 7.22. Trophozoite of *Acanthamoeba* strain ATCC 50677, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Acanthapodia and a contractile vacuole are visible. (x 450)

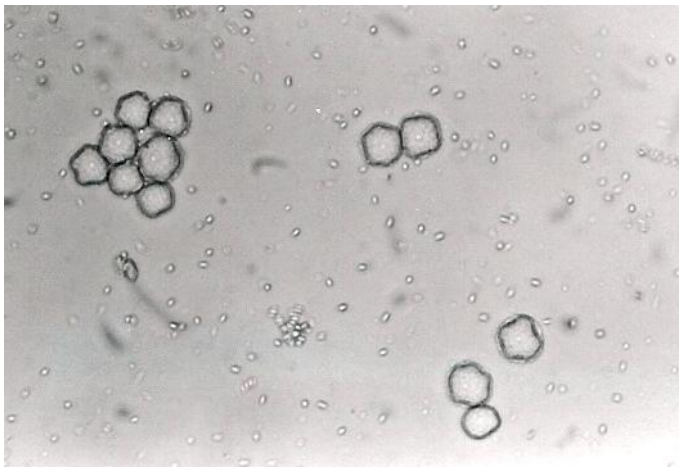


Figure 7.23. Cysts of *Acanthamoeba* strain ATCC 50677, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 450)

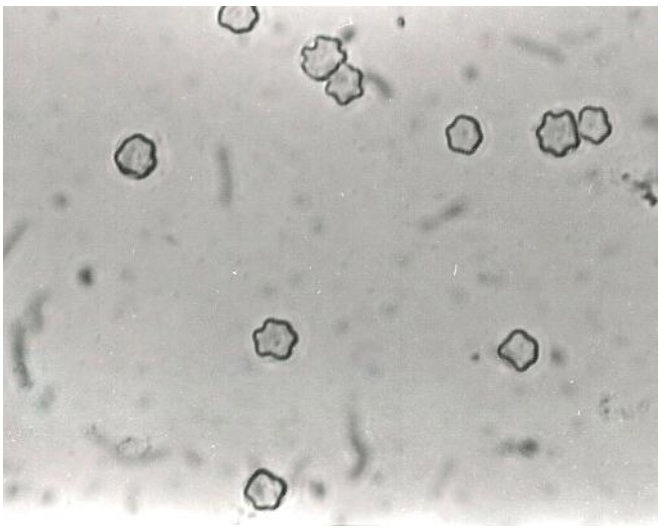


Figure 7.24. Cysts of *Acanthamoeba* strain ATCC 50678, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are visible. (x 450)

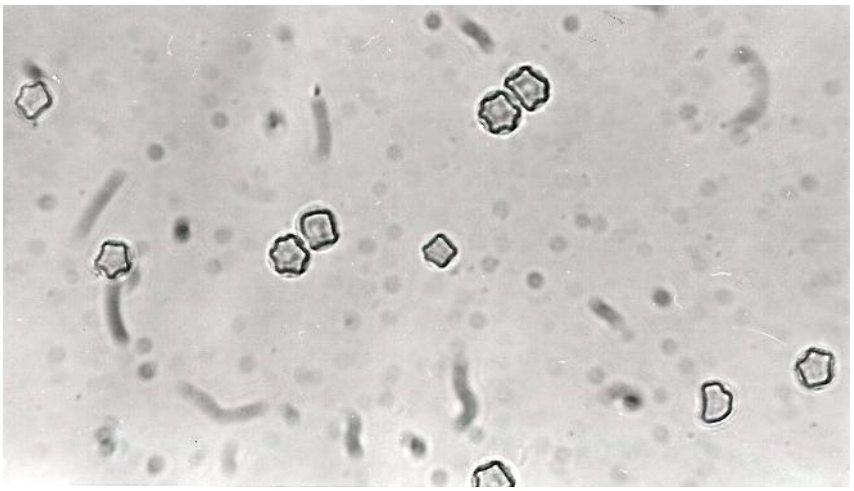


Figure 7.25. Cysts of *Acanthamoeba* strain ATCC 50679, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 450)

Figure 7.26. Cysts of *Acanthamoeba* strain ATCC 50680, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are visible. (x 450)

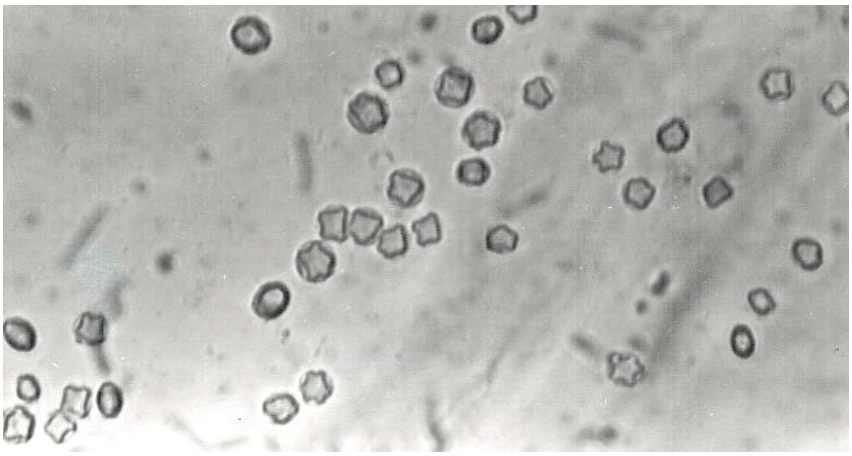


Figure 7.27. Cysts of *Acanthamoeba* strain ATCC 50681, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 450)

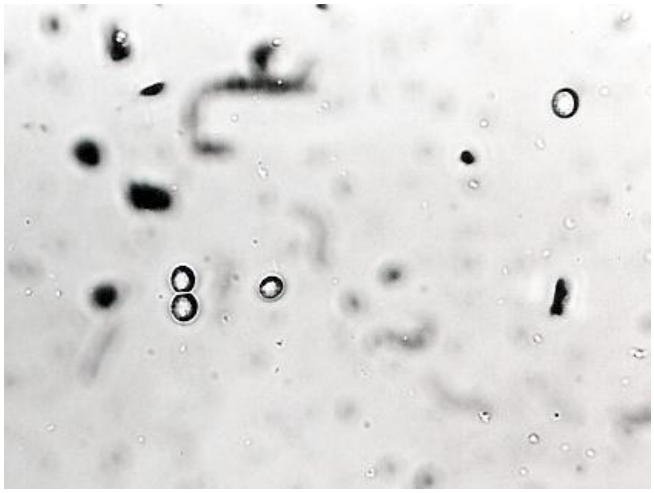


Figure 7.28. Cysts of *Acanthamoeba* strain ATCC 50682, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are visible. (x 450)

Figure 7.29. Cysts of *Acanthamoeba* strain ATCC 50683, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 450)

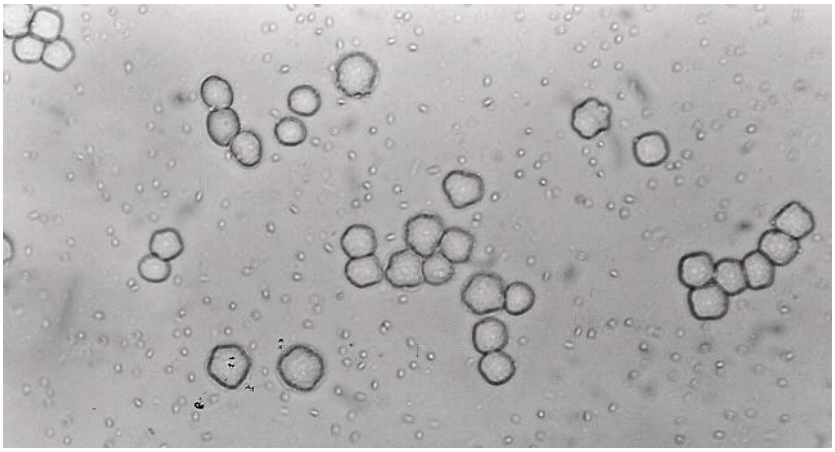
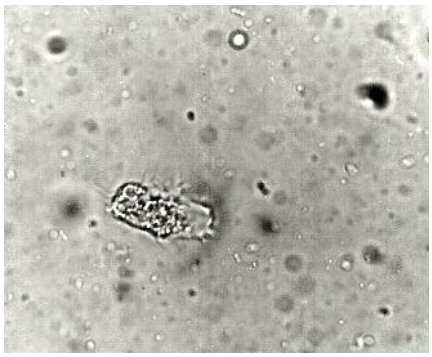


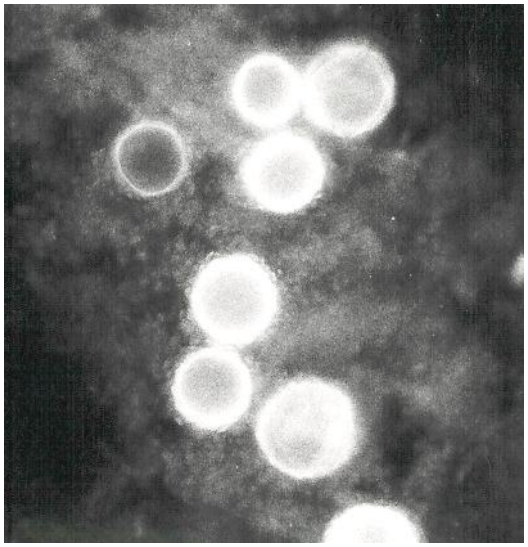
Figure 7.30. Cysts of *Acanthamoeba* strain ATCC 50684, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. (x 450)

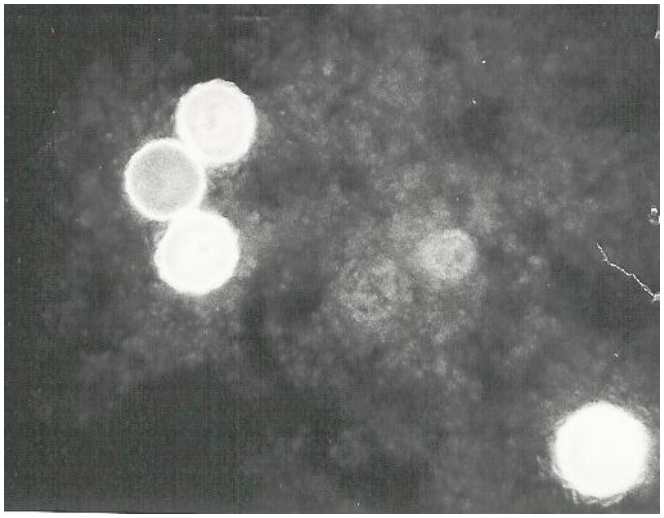
Figure 7.31. Cysts of *Acanthamoeba* strain ATCC 50685, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 450)



Figures 7.32. and 7.33. Trophozoites of *Acanthamoeba* strain ATCC 50686, grown in SCYGEM. Spiky acanthapodia are clearly visible. (x 450)

Figure 7.34. Cysts of *Acanthamoeba* strain ATCC 50686, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 450)





Figures 7.35 and 7.36. Cysts of *Acanthamoeba* strain ATCC 50686, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria, stained with calcofluor white and viewed under a fluorescence microscope. Both the ectocyst and endocyst are clearly visible. (x 650)

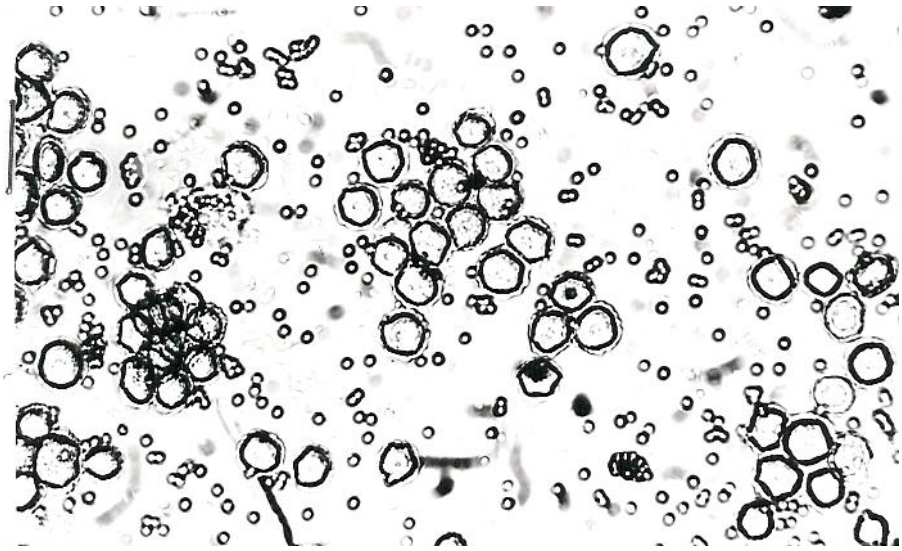


Figure 7.37. Cysts of *Acanthamoeba* strain ATCC 50687, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. (x 450)

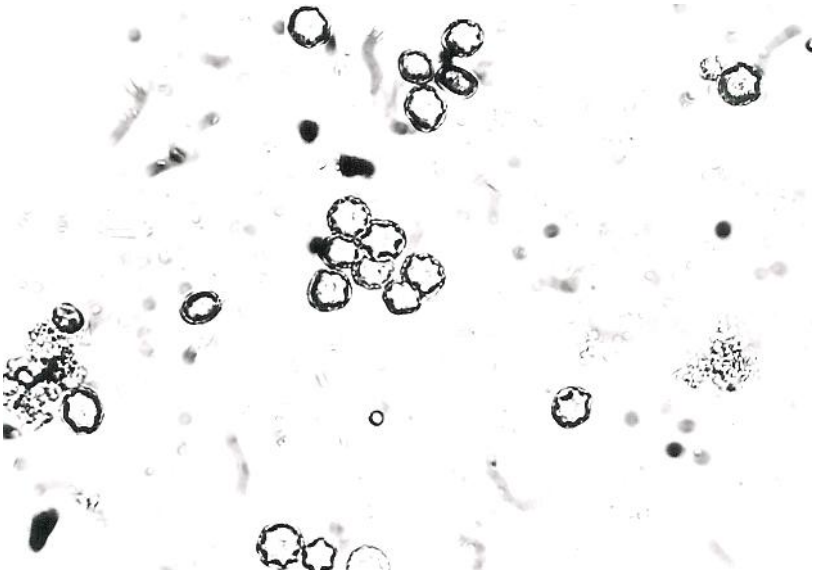


Figure 7.38. Cysts of *Acanthamoeba* strain RYD, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 450)

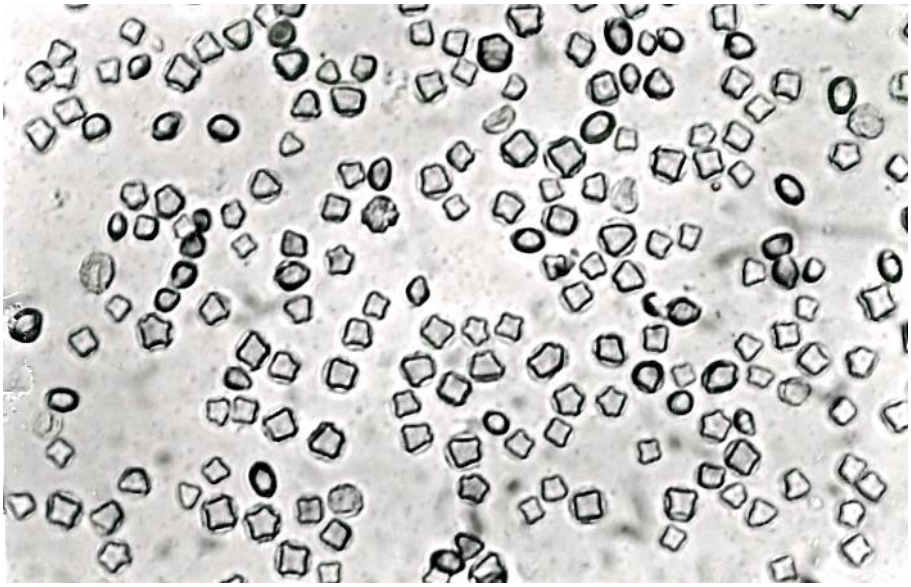


Figure 7.39. Cysts of *Acanthamoeba* strain SAIMR 96/9, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. The ectocyst and endocyst are clearly visible. (x 450)

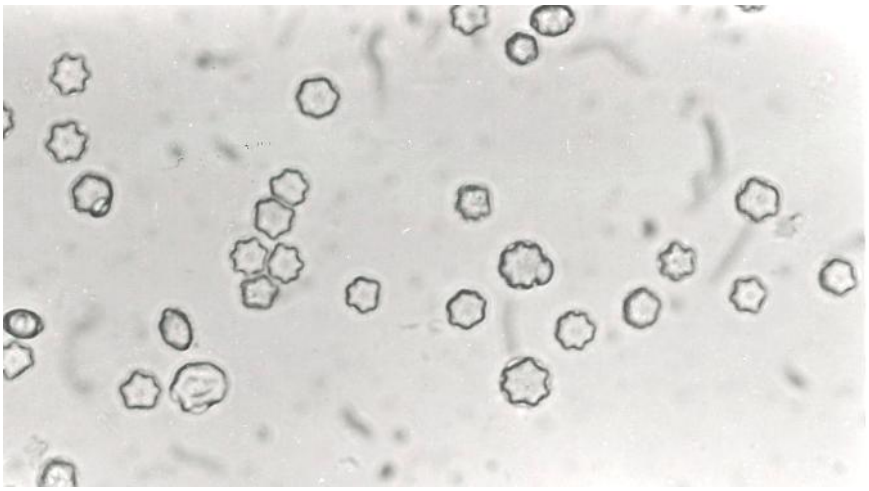
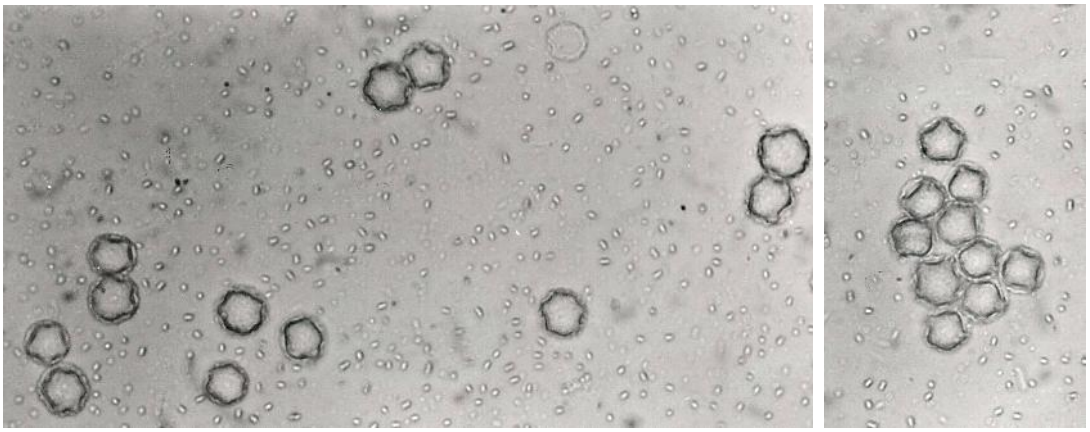


Figure 7.40. Cysts of *Acanthamoeba* strain SAWE 95/7, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 450)



Figures 7.41 and 7.42. Cysts of *Acanthamoeba* strain SAWE 96/8, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. The ectocyst and endocyst are clearly visible. (x 450)

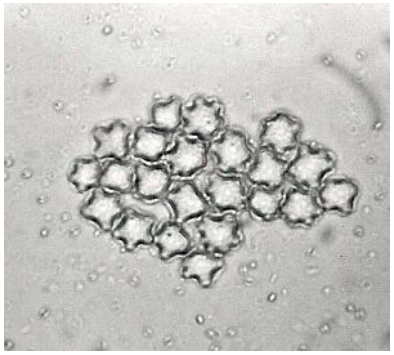


Figure 7.43. Cysts of *Acanthamoeba* strain SAWE 97/10, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 450)

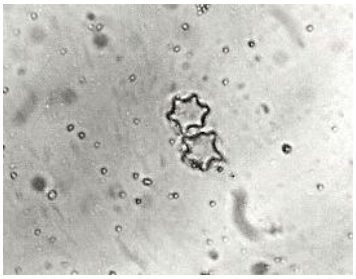


Figure 7.44. (x 450)

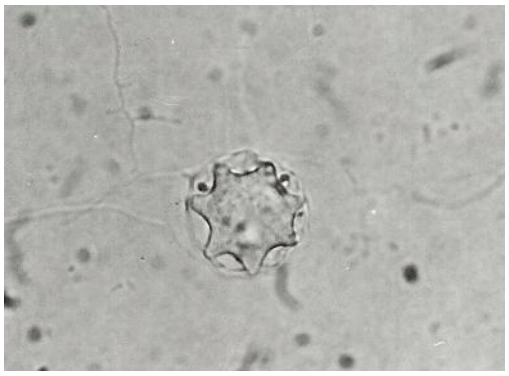


Figure 7.45. (x 1200)

Figures 7.44 and 7.45. Cysts of *Acanthamoeba* strain SAWS 87/4, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible.

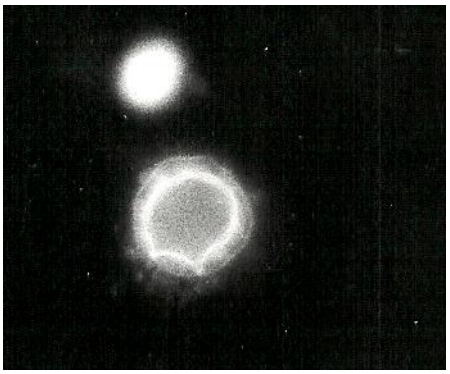


Figure 7.46. (x 1200)

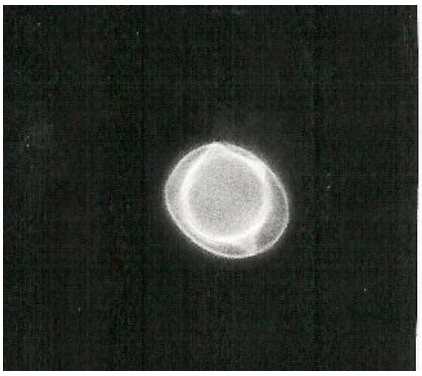


Figure 7.47. (x 1200)

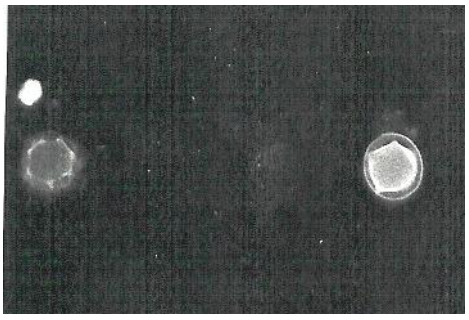


Figure 7.48. (x 600)

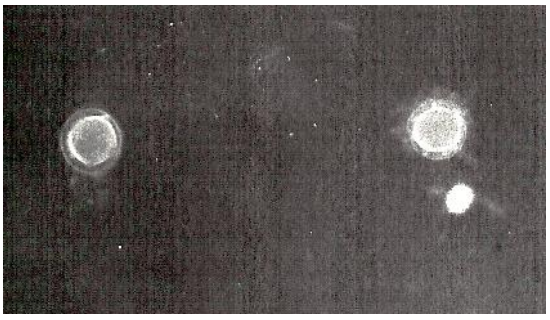


Figure 7.49. (x 600)

Figures 7.46 to 7.49. Cysts of *Acanthamoeba* strain SAWS 87/4, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria, stained with calcofluor white and viewed under a fluorescence microscope. Both the ectocyst and endocyst are clearly visible.

Figure 7.50. (x 1200)

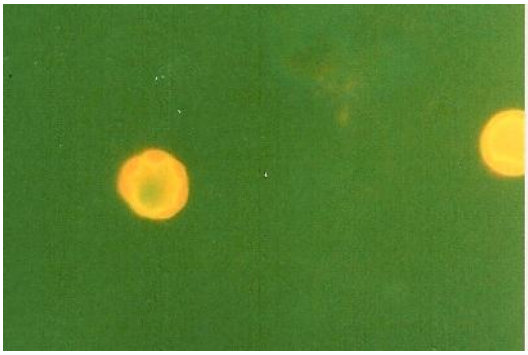


Figure 7.51. (x 700)

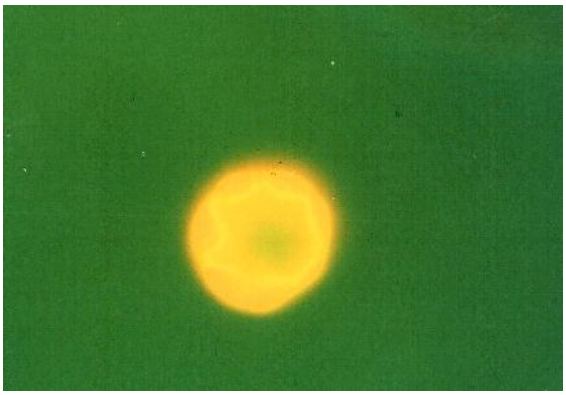


Figure 7.52. (x 1500)

Figures 7.50 to 7.52. Cysts of *Acanthamoeba* strain SAWS 87/4, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria, stained with calcofluor white and viewed under a fluorescence microscope. UV filters for fluorescein staining have been used (excitation 490 nm, emission 520 nm), hence the dull orange/yellow colour. Both the ectocyst and endocyst are clearly visible.

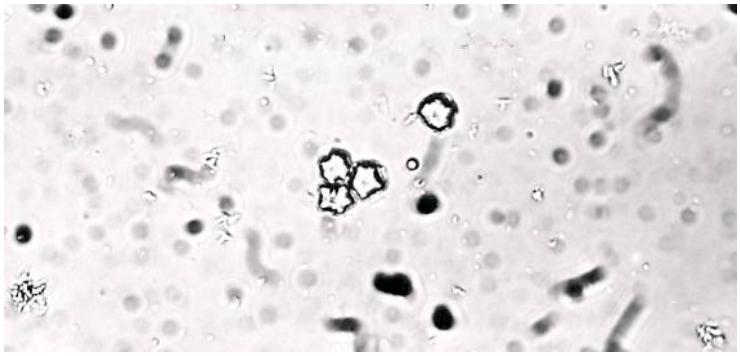


Figure 7.53. Cysts of *Acanthamoeba* strain 435/89, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. The ectocyst and endocyst are clearly visible. (x 450)

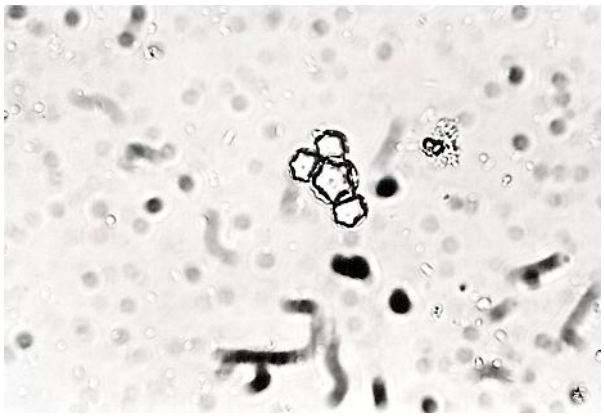


Figure 7.54. Cysts of *Acanthamoeba* strain 452/89, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. Both the ectocyst and endocyst are clearly visible. (x 450)

7.4 Discussion

Page (1988) mentioned that variation, even within a clone, and the difficulty of expressing the characters objectively, are two problems encountered when using morphological characters for taxonomy or identification. The most detailed study of cyst morphology is that of Pussard & Pons (1977). However, they had before them only one strain of each of the majority of species investigated, so that inter-clonal variation could not always be taken into account. Variation within the clone is apparent in the photograph of cysts of *A. castellanii*, the original Castellani strain (CCAP 1501/2a) (Page, 1988). In some instances, the endocyst appears roundish or squarish in shape, with the number of endocyst arms varying greatly.

The distinction between *A. castellanii* and *A. polyphaga* on morphological grounds is very uncertain (Warhurst, 1985). A morphological criterion for possibly distinguishing between *A. polyphaga* and *A. castellanii* is that cysts of the latter have six or more corners or pores in optical section, whilst those of the former have fewer than six. Even in cloned isolates, wide variation in pore number is found. The distinction between *A. polyphaga* and *A. rhysodes* is even less clear (Warhurst, 1985). Page (1976) considers *A. rhysodes* (Singh, 1952) to be a synonym for *A. castellanii*. Visvesvara and Balamuth (1975) found that although both the *A. castellanii* [Singh] strain and the *A. culbertsoni* [Lilly] strains produced cysts with variable structure, the majority of cysts had morphological features more or less characteristic of the strain they represented. Griffiths *et al.* (1978) devised physiological tests to compare 15 strains representing five species of *Acanthamoeba*. The similarity between strains of *A. castellanii* and *A. polyphaga* was greater than that between strains of *A. polyphaga* alone, and the

strains of *A. rhysodes* showed a greater average similarity to those of *A. palestinensis* than they did to each other. Consequently, even the tests that gave the best differentiation between the species did not allow an unequivocal separation to be made on physiological grounds. Willaert (1976) has suggested that on the basis of their antigenic relationships, *A. castellanii, A. polyphaga* and *A. rhysodes* are a homogeneous group whereas *A. palestinensis* and *A. astronyxis* could both be considered as distinct from *A. castellanii.*

Several of the southern African corneal and contact lens isolates were morphologically similar. Nine of them have been identified by Schroeder *et al.* (2001) as *A. mauritaniensis*, belonging to the T4 genotype. The present author (i.e. of this thesis) was a participant in that study. The T4 lineage is referred to as the "*A. castellanii* complex", with rDNA sequences not yet a sufficiently valid basis to allow for differentiation among species within that complex (T.J. Byers, personal communication). The T4 genotype appears to be commonly associated with human eyes, considering that Schroeder *et al*. (2001) and Stothard *et al*. (1998) placed 39 of 40 *Acanthamoeba* isolates from human eyes or contact lens cases in this genotype.

Kilvington *et al.* (1991a) described the cyst forms of strains Ac/PHL/4, Ac/PHL/17, Ac/PHL/22 and Ac/PHL/23 as being morphologically similar when viewed by light microscopy. The present researcher also saw the morphological similarity between some of these strains (donated by Dr S. Kilvington), and noted that they were, likewise, similar to some of the southern African corneal isolates. Kilvington *et al.* (1991a) mention that these cysts are typical of group II *Acanthamoeba* spp., and resemble *A. polyphaga* or possibly *A. castellanii* (the

differentiation of these species by this means being considered subjective). Previously, using cyst morphology, researchers have identified strains ATCC 30873 (CCAP 1501/3d) and Ac/PHL/23 (SHI) as *A. polyphaga* (Jones *et al*., 1975; Kilvington, 1989). However, when using restriction endonuclease digestion of whole-cell DNA of 33 strains of *Acanthamoeba*, Kilvington *et al*. (1991a) placed only strains Ac/PHL/17 and Ac/PHL/23 in the same restriction fragment length polymorphism (RFLP) group. Strains Ac/PHL/4, Ac/PHL/22 and ATCC 30873 (CCAP 1501/3d) all fell into different and separate RFLP groups. Kilvington *et al*. (1991a) comment that it is unclear whether *Acanthamoeba* mtDNA RFLPs indicate intra- or inter-species differences.

Three of the four sewage isolates, namely strains ATCC 50685, ATCC 50686 and ATCC 50687, were morphologically identical. Schroeder *et al*. (2001) found that the DNA sequences of these isolates were identical to those of *A. lenticulata*, belonging to the genetic lineage designated as T5. The rDNA sequences of *A. lenticulata* are so distinctive that there can be no confusion with any of the other sequence types that they have observed to date (T.J. Byers, personal communication). *A. lenticulata* is included in group III of the Pussard & Pons (1977) groupings. It may not be possible to identify the species within this group by morphology alone. *A. lenticulata* is confirmed to be a species containing only pathogenic strains, which grows easily at 40°C (De Jonckheere, 1983). Therefore, it came as a surprise to De Jonckheere & Michel (1988), working in Germany, that several isolates from nasal mucosa in healthy individuals belonged to the pathogenic *A. lenticulata*. Although this species is morphologically related to *A. culbertsoni* and both species are pathogenic for mice, they are serologically different and yield distinctive zymograms (Molet & Ermolieff-Braun, 1976).

Molet & Ermolieff-Braun (1976) record the size of *A. lenticulata* cysts as being 11–18 μm. The average diameter of cysts of two of the strains of this species was found by the present researcher to be 18 μm or greater, but cysts can vary considerably in size in different media (T.J. Byers, personal communication). The diameter of cysts of *Acanthamoeba* isolated from freshwater fishes was found to be as much as 50% greater when formed in liquid medium as when they are formed on agar surfaces, although the differences were more commonly about 20–30% greater (T.J. Byers, personal communication).

Strain ATCC 30868 has been identified as *A. castellanii* (Nagington & Richards, 1976). Cysts of this strain appeared to be morphologically distinct from those of other strains.

Costas & Griffiths (1986) described 37 strains of *Acanthamoeba*, using 69 physiological characteristics. Thirty-six distinct patterns were generated by the use of numerical profiles, but it was possible to distinguish, within this diversity, 20 sub-generic groups that, although not completely congruent with the morphological species, were consistent in relation to other non-morphological characteristics. These authors found that seven species which are morphologically distinguishable (*A. royreba, A. terricola, A. griffini, A. palestinensis, A. astronyxis, A. hatchetti* and *A. lenticulata*) are also distinguishable by their physiological characteristics. In contrast, of the other species studied, *A. castellanii, A. polyphaga* and *A. rhysodes* are represented by more than one profile, or show some degree of overlap, or both. Of these three groups, even *A. rhysodes*, which has the lowest average difference between its constituent strains,

is more heterogeneous than the groups divided by their physiological characteristics. The strains of *A. castellanii* show the same degree of relationship to the strains of the other species as they do to each other. This must cast doubt on the validity of using morphological characteristics to distinguish between species of *Acanthamoeba*, as it is difficult to see how these may be appropriate for some species but not for others (Costas & Griffiths, 1986). This leads to the conclusion that *Acanthamoeba* is a coherent, well-founded genus that is morphologically distinguishable from other amoeboid forms (Costas & Griffiths, 1986). However, below the generic level, there appears to be little justification for the recognition of morphological species, according to these authors.

Isoenzyme studies and/or RFLP analyses (Chapters 12 and 13) have been carried out on most of the strains for which there is, as yet, no information in the literature. However, these results, based on biochemical data and morphological criteria for conventional specific classification, respectively, do not necessarily correlate – a phenomenon also observed by other researchers (Page, 1988; Badenoch *et al*., 1995; Visvesvara, 1991). Therefore, subgeneric taxonomic designations have not been attempted for all the strains that are the subject of this thesis. With time and advances in knowledge, many current specific identifications may in retrospect prove to be meaningless, particularly in such equivocal cases as those referred to above. However, the isolates used in this study have been deposited in the American Type Culture Collection (ATCC) so that they are readily available to other workers.

The present researcher concludes that, although a few of the southern African strains of *Acanthamoeba* studied are obviously morphologically distinct from other strains, the differentiation of species based on morphology alone, is not possible.

CHAPTER EIGHT – CHARACTERISATION OF *ACANTHAMOEBA* BY ELECTRON MICROSCOPY

8.1 Differentiation of *Acanthamoeba* species based on their ultrastructural features

Trophozoites of different species of *Acanthamoeba* are difficult to identify because of the absence of distinctive morphological features (Page, 1967; Bowers & Korn, 1968). The shape and structure of cysts, on the other hand, has been considered to differ markedly among the species (Page, 1967), and these ultastructural features have been said to be useful in identifying and classifying free-living amoebae (Page, 1985). Therefore, the author examined the ultrastructure of cysts and trophozoites of some of the southern African *Acanthamoeba* isolates, i.e. electron microscopy was used in order to assist in characterising these strains. Electron microscopy has also been used to study changes in cyst morphology resulting from exposure to chlorhexidine (Luo *et al.*, 2008) and, *inter alia*, povidone iodine (Roongruangchai *et al.*, 2009).

Problems can occur during the processing of amoebic trophozoites or cysts for transmission electron microscopy if the amoebae do not persist as a solid pellet when solutions are changed. Published methods for pelleting amoebae include centrifugation at each step (Bowers & Korn, 1968) and the addition of amoebae to heated agar (Lasman, 1977). The first method was found to be time-consuming, while the second technique requires agar to be kept and centrifuged at a temperature high enough to maintain it in liquid form. At the same time, overheating must be avoided, as this could damage the amoebae. This researcher tried using agar with a low melting point, but processing was successful only

when a heated centrifuge was available. It was not always satisfactory to place tubes containing amoebae inside centrifuge tubes filled with warm water, because if the water is not hot enough, the agar starts to solidify during centrifugation.

Consistently satisfactory results were obtained using a simple technique that facilitates embedding of amoebae for transmission electron microscopy (Niszl & Markus, 1989). This method, described in the section that follows, was based on a suggestion by Bullock (1987) for isolated cells and cell organelles.

8.2 Materials and methods

After fixation with glutaraldehyde,[21] amoebae were centrifuged into a pellet. The researcher sometimes stained the amoebae with toluidine blue during the third buffer wash. Most of the buffer was pipetted off. Next, a 40% bovine serum albumen (BSA) solution (diluted with buffer) was mixed with the amoebae before centrifugation was repeated. Excess BSA was pipetted off and one drop of 25% glutaraldehyde was added to gel the BSA containing the amoebae. When the stain was used, amoebae could be seen clearly. The gelled BSA was cut into small pieces, which were treated like bits of tissue. Post-fixation with 1% osmium tetroxide darkens the material, so that ultimately it is clearly visible inside the blocks of embedding resin.[22]

The methodology for staining semi-thin and ultrathin sections is in Appendix VII.

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21. See Appendix VII.

22. See Appendix VII.

8.3 Results

Electron micrographs of strains of *Acanthamoeba* are presented in Figures 8.1 – 8.42. Some of the amoebic cysts that are covered by this chapter were not DNA typed. Consequently, there is no generic, specific or strain detail for the amoebae concerned.

8.3.1 *Amoeba isolated from tap water from Johannesburg, Gauteng Province (Figs 8.1 – 8.3)*

Transmission electron microscopy shows that cysts of this strain are characterised by a prominent double-layered wall consisting of an outer, fibrous, electron-dense ectocyst and an inner, less electron-dense endocyst (Figs 8.1 – 8.3). Endocysts of this strain are almost round in shape. The ectocyst has an irregular appearance and the distance between the ectocyst and the endocyst is variable. The space between the ectocyst and endocyst is filled in some regions with a spongy network or with cytoplasmic debris (Figs 8.1 – 8.2). The endocyst wall is thicker than that of the ectocyst (Figs 8.1 and 8.2). The nucleus is more or less centrally placed, with an electron-dense nucleolus. Mitochondria are present around the nucleus (Fig. 8.2). There is a round, electron-dense body near the nucleus (Figs 8.1 – 8.3). Membrane-bound lipid vacuoles are apparent.

8.3.2 *Amoeba isolated from tap water in Roodepoort, Gauteng Province (Figs 8.4 – 8.8)*

Both an ectocyst and an endocyst are clearly visible, with a zone of variable width separating the wrinkled ectocyst from the endocyst. The endocyst is closely applied to the cell membrane. The space between the ectocyst and endocyst is filled in some regions with a spongy network or with cytoplasmic debris (Fig. 8.4). The inner and outer walls fuse at irregular intervals, forming gaps (or ostioles), and there is an operculum-like structure in the cyst wall that contains

neither endocyst nor ectocyst (Figs 8.4 – 8.8). The number of ostioles varies. Many phagosomes of various sizes, containing whole bacteria or debris, are found in the cytoplasm of the trophozoites.

8.3.3 *Amoeba isolated from the swimming pool at the University of the Witwatersrand, Johannesburg, Gauteng Province* *(Figs 8.9 – 8.11)*

Cysts – When viewed by transmission electron microscopy, cysts of this strain are seen to be characterised by a prominent double-layered wall comprising an outer, fibrous, electron-dense ectocyst and an inner, less electron-dense round/oval endocyst (Fig. 8.9). An interphase nucleus is apparent, containing a large, centrally-placed nucleolus having electron-dense material in it (Fig. 8.9). The nucleus is round or oval in shape. Membrane-bound lipid vacuoles and mitochondria are present in the cytoplasm.

Trophozoites – A well-defined nucleus is visible, as is the nuclear membrane and a densely-staining nucleolus. Many phagosomes of various sizes, containing whole bacteria, debris and/or myelinated structures, are found in the cytoplasm of the trophozoites (Figs 8.10 and 8.11). Rough endoplasmic reticulum is visible (Fig. 8.11).

8.3.4 *Amoeba isolated from the contact lens case of a hard contact lens wearer* *(Figs 8.12 – 8.15)*

Cysts – Transmission electron microscopy reveals that cysts of this strain are characterised by a prominent double-layered wall consisting of an outer, fibrous, electron-dense ectocyst and an inner, less electron-dense, almost round endocyst (Figs 8.12 – 8.14). The endocyst walls are thicker than the ectocyst walls (Figs 8.12 and 8.14). The ectocyst has an irregular shape, and the distance between the ectocyst and the endocyst varies. The space between the ectocyst and endocyst is filled in some regions with a spongy network or with cytoplasmic debris (Figs

8.12 – 8.14). Membrane-bound lipid vacuoles are present in the cytoplasm (Figs 8.12 – 8.15). Mitochondria surround the nucleus (Figs 8.14 and 8.15). An interphase nucleus is apparent, containing a large, centrally placed nucleolus with electron-dense material (Fig. 8.12 – 8.15).

8.3.5 Acanthamoeba *strain ATCC 50676, isolated from a keratitis patient (Figs 8.16 – 8.19)*

Cysts – Ultrastructurally, cysts of this strain are characterised by a prominent double-layered wall comprising an outer, fibrous, electron-dense ectocyst and an inner, less electron-dense endocyst (Figs 8.16 – 8.18). Both the ectocyst and endocyst of this strain have an irregular shape, and the amount of space between the ectocyst and the endocyst varies. The space between the ectocyst and endocyst is filled with electron-dense material. The inner and outer walls fuse at irregular intervals, forming gaps, or ostioles; and there is an operculum-like structure in the cyst wall that contains neither endocyst nor ectocyst (Fig. 8.19). A large, centrally-located nucleus with a densely-staining nucleolus is apparent in the cysts (Figs 8.17 and 8.18). Membrane-bound lipid vacuoles, concentrated mainly around the periphery of the cyst, are present in the cytoplasm (Figs 8.16 – 8.19).

8.3.6 Acanthamoeba *strain ATCC 50685, isolated from Northern Sewage Works, Gauteng Province (Figs 8.20 – 8.26)*

Cysts – Characteristic of the fine structure of cysts of this strain, are a prominent double-layered wall consisting of an outer, fibrous, electron-dense ectocyst and an inner, less electron-dense endocyst (Figs 8.20 – 8.23). A zone of variable width separates the wrinkled ectocyst from the endocyst. The endocyst is closely applied to the cell membrane and is round or oval in shape (Figs 8.20 – 8.23). The space between the ectocyst and endocyst is filled in some regions with a spongy or fibrous network (Figs 8.20 – 8.23). The inner and outer walls fuse at irregular intervals, forming gaps, or ostioles, with an operculum-like structure in

the cyst wall that contains neither endocyst nor ectocyst (Fig. 8.23). A round nucleus with an electron-dense nucleolus is apparent (Fig. 8.22). Numerous membrane-bound lipid vacuoles, as well as phagosomes of various sizes containing whole bacteria or debris, are found in the cytoplasm (Figs 8.20 – 8.23). Mitochondria and rough endoplasmic reticulum are visible (Figs 8.20 – 8.23).

Trophozoites - Numerous phagosomes of various sizes, containing whole bacteria, vesicles, granules, myelinated bodies or debris, are found in the cytoplasm (Figs 8.24 – 8.26). Mitochondria and acanthapodia are visible (Figs 8.24; 8.25).

8.3.7 Acanthamoeba *strain ATCC 50686, isolated from Southern Sewage Works, Gauteng Province* *(Figs 8.27 – 8.32)*

Cysts – Both an ectocyst and an endocyst are clearly visible, with a zone of variable width separating the wrinkled ectocyst from the endocyst. The endocyst is closely applied to the cell membrane, and is round or square in shape (Figs 8.27 – 8.30). The space between the ectocyst and endocyst is filled in some regions with a spongy or fibrous network (Figs 8.27 – 8.30). The inner and outer walls fuse at irregular intervals, forming gaps, or ostioles, with an operculum-like structure in the cyst wall that contains neither endocyst nor ectocyst (Fig. 8.28). The number of ostioles varies. Numerous membrane-bound lipid vacuoles as well as phagosomes of various sizes containing whole bacteria or debris are found in the cytoplasm (Figs 8.27 – 8.30). Mitochondria and rough endoplasmic reticulum are visible (Figs 8.27; 8.29; 8.30).

Trophozoites - Numerous phagosomes of various sizes, containing whole bacteria, vesicles, granules, myelinated bodies or debris, are found in the cytoplasm (Figs 8.31; 8.32). Mitochondria and rough endoplasmic reticulum are visible (Figs

8.31; 8.32). The nucleus, containing an electron-dense nucleolus, is apparent (Fig. 8.31).

8.3.8 Acanthamoeba *strain SAWS 87/4, isolated from Goudkoppies Sewage Works, Gauteng Province* *(Figs 8.33 – 8.42)*

Cysts – The endocyst is approximately star-shaped, with several truncated rays, while the ectocyst is interrupted by gaps (ostioles) located at the edges of the rays (Figs 8.33 – 8.35; 8.38; 8.40 – 8.41). The centre of the ostiole is occupied by the thick, arched operculum, a segment of closely apposed ectocyst and endocyst (Figs 8.33; 8.34; 8.40; 8.41). The operculum is composed of a granular material comparable in density to that of the endocyst. Vesicles enclosed by plasma membrane containing cytoplasmic material are found in the space between the cyst wall and cell membrane; some of them are embedded in the wall material. A clear zone of variable width, which contains fragments of membranes or membrane-bound vesicles, separates the ectocyst from the endocyst (Figs 8.33 – 8.38). Numerous phagosomes containing vesicles, granules and myelinated bodies are apparent (Figs 8.34 – 8.39). Membrane-bound lipid vacuoles are present in the cytoplasm (Figs 8.34 – 8.39). Mitochondria are also visible in the cytoplasm (Figs 8.36; 8.37; 8.39). The interphase nucleus, containing a large nucleolus with electron-dense material, is apparent (Figs 8.33; 8.34; 8.36; 8.39). A clear, centrally located space is sometimes seen in the nucleolus (Figs 8.36; 8.39).

Pre-cystic stage – The electron-dense endocyst, closely applied to the plasmalemma, is visible in the pre-cystic stage (Fig. 8.42). A clear, centrally located space is visible within the electron-dense nucleolus. Mitochondria and phagocytic vesicles can be seen in the cytoplasm (Fig. 8.42).

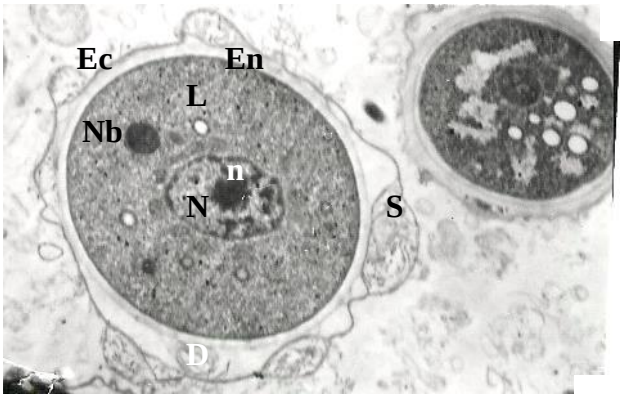


Figure 8.1. (x 8000)

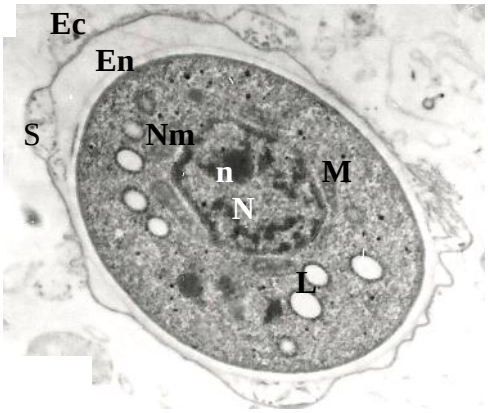


Figure 8.2. (x 10000)

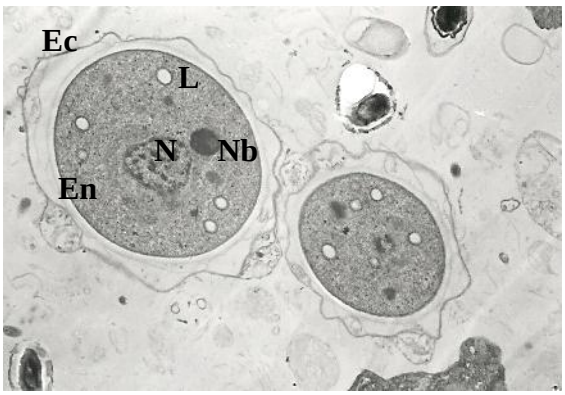
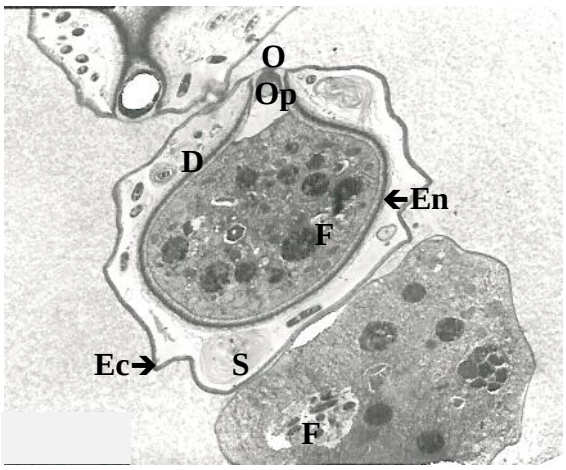


Figure 8.3 (x 6000)

Figures 8.1 to 8.3. Transmission electron micrographs of cysts of amoebae isolated from tap water from Johannesburg, Gauteng Province. The prominent double-layered wall comprising an outer, fibrous, electron-dense ectocyst (Ec) and an inner, less electron-dense endocyst (En), is apparent. The space between the ectocyst and endocyst is filled in some regions with a spongy network (S) or with cytoplasmic debris (D). The nucleus (N), nucleolus (n), nuclear membrane (Nm), mitochondria (M) and membrane-bound lipid vacuoles (L) are visible. A round, electron-dense body, possibly a nucleolar-like body (Nb), is present near the nucleus.



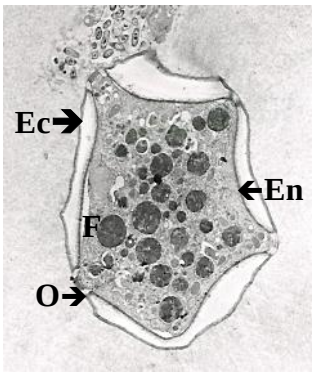


Figure 8.4. (x 3000) **Figure 8.5 (x 3000)**

Figures 8.4 and 8.5. Transmission electron micrographs of cysts of *Acanthamoeba* isolated from tap water from Roodepoort, Gauteng Province. Both an ectocyst (Ec) and an endocyst (En) are clearly visible, with a zone of variable width separating the wrinkled ectocyst from the endocyst. The endocyst is closely applied to the cell membrane. The space between the ectocyst and endocyst is filled in some regions with a spongy network (S) or with cytoplasmic debris (D) (Fig. 8.4). The inner and outer walls fuse at irregular intervals, forming gaps, or ostioles (O), with an operculum-like (Op) structure in the cyst wall that contains neither endocyst nor ectocyst. The number of ostioles varies. Many phagosomes or food vacuoles (F) of various sizes, containing whole bacteria or debris, are found in the cytoplasm.

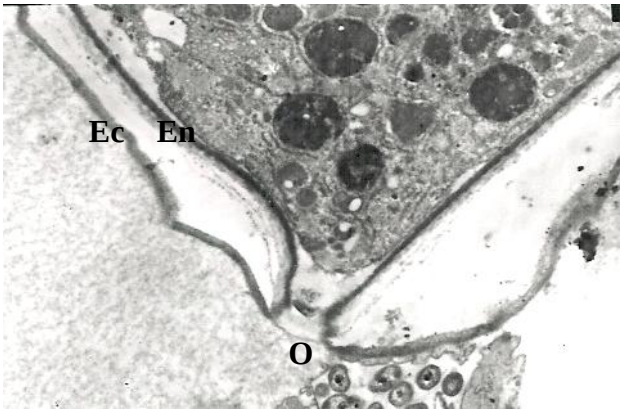


Figure 8.6. (x 6000)

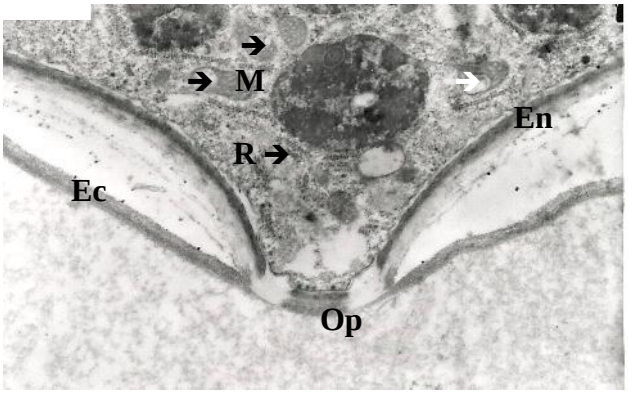


Figure 8.7. (x 10000)

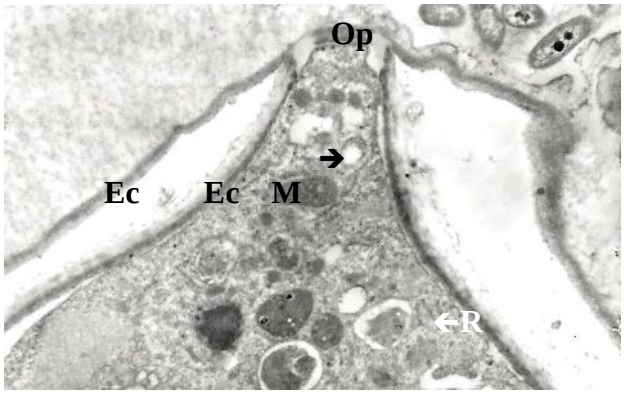


Figure 8.8 (x 8000)

Figures 8.6 – 8.8. Transmission electron micrographs of ostioles in cysts of *Acanthamoeba* isolated from tap water from Roodepoort, Gauteng Province. The inner and outer walls fuse at irregular intervals, forming gaps, or ostioles (O), with an operculum-like structure (Op) in the cyst wall that contains neither endocyst nor ectocyst. (Figs 8.7 and 8.8). Rough endoplasmic reticulum (R) is visible in this region (Figs 8.7 and 8.8). Note the clear space (arrows) in some of the mitochondria (M) (Fig. 8.7).

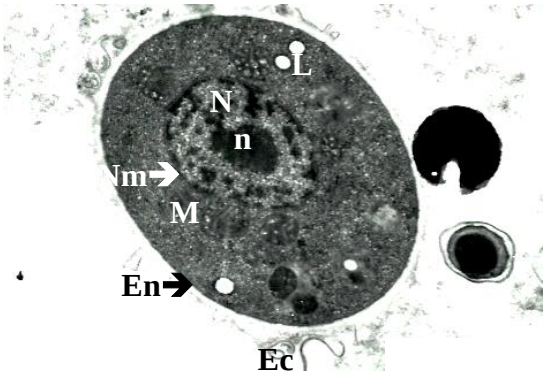
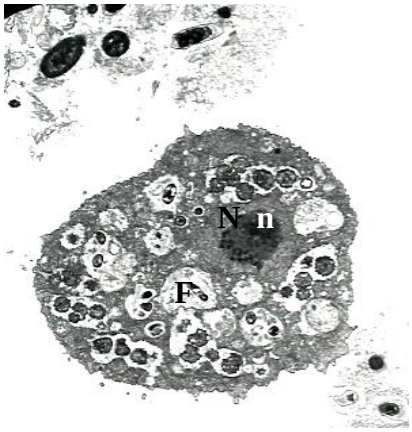


Figure 8.9. Transmission electron micrograph of a cyst of an amoeba isolated from the swimming pool at the University of the Witwatersrand, Johannesburg, Gauteng Province. The double-layered wall, consisting of an outer, fibrous, electron-dense ectocyst (Ec) and an inner, less electron-dense endocyst (En), is apparent. The nucleus (N), nucleolus (n), nuclear membrane (Nm), mitochondria (M) and membrane-bound lipid vacuoles (L) are visible. (x 10000)



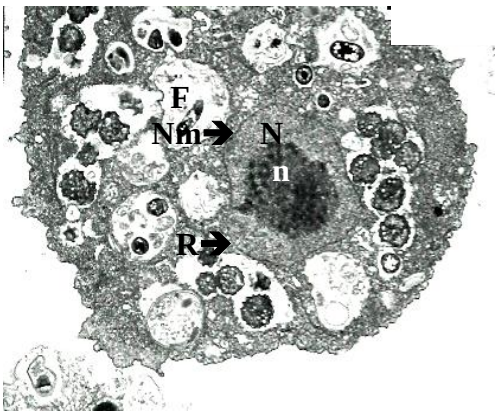


Figure 8.10 (x 3000) **Figure 8.11 (x 5000)**

Figures 8.10 to 8.11. Transmission electron micrographs of a trophozoite of an amoeba isolated from the swimming pool at the University of the Witwatersrand, Johannesburg, Gauteng Province. A well-defined nucleus (N) with a nuclear membrane (Nm) and intensely staining nucleolus (n) is visible. Many phagosomes or food vacuoles (F) of various sizes, containing whole bacteria, debris and/or myelinated structures, are found in the trophozoite. Rough endoplasmic reticulum (R) is visible.

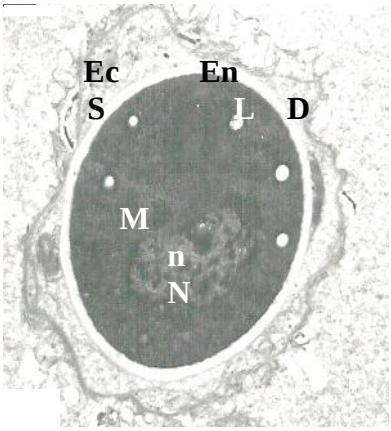


Figure 8.12. (x 8000)

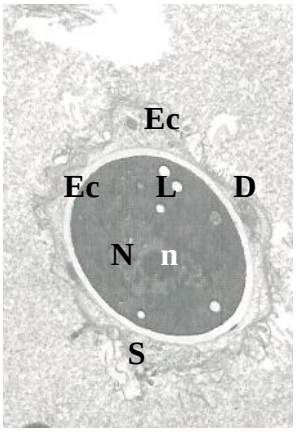


Figure 8.13. (x 6000)

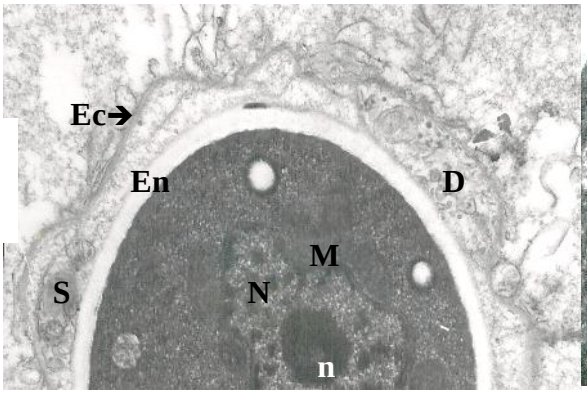


Figure 8.14 (x 15000)

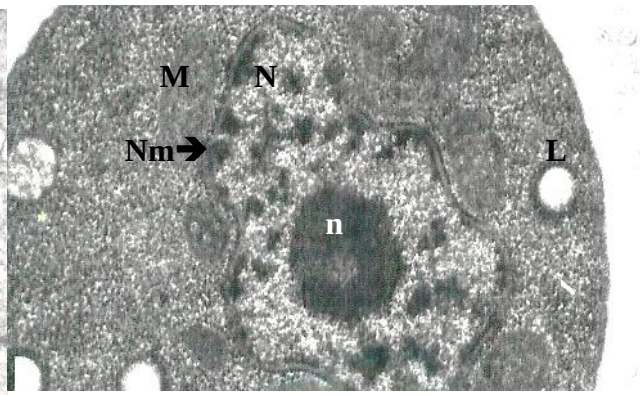
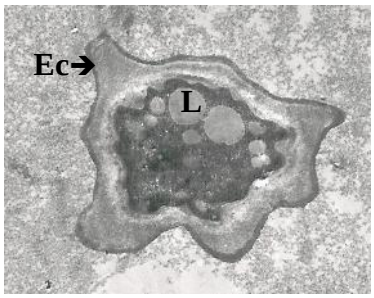


Figure 8.15. (x 25000)

Figures 8.12 to 8.15. Transmission electron micrographs of cysts of amoebae isolated from the contact lens case of a hard contact lens wearer. The prominent double-layered wall, comprising an outer, fibrous, electron-dense ectocyst (Ec) and a thicker, inner, less electron-dense endocyst (En), is apparent. The space between the ectocyst and endocyst is filled in some regions with a spongy network (S) or with cytoplasmic debris (D) (Figs 8.12 – 8.14). The nucleus (N), nuclear membrane (Nm) and electron-dense nucleolus (n) are visible. Mitochondria (M) can be seen around the nucleus, and membrane-bound lipid vacuoles (L) are visible.



F igure 8.16. (x 10000)

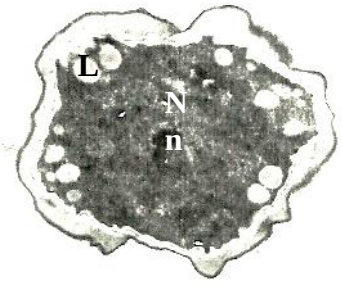


Figure 8.17. (x 6000)

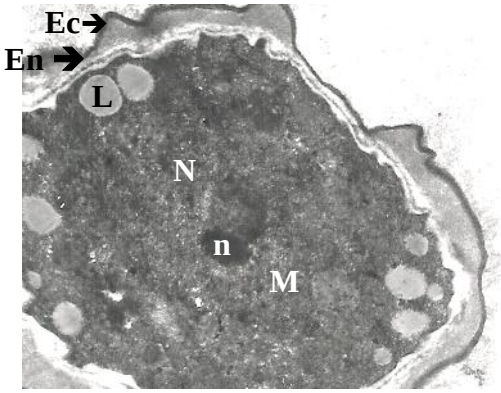


Figure 8.18. (x 10000)

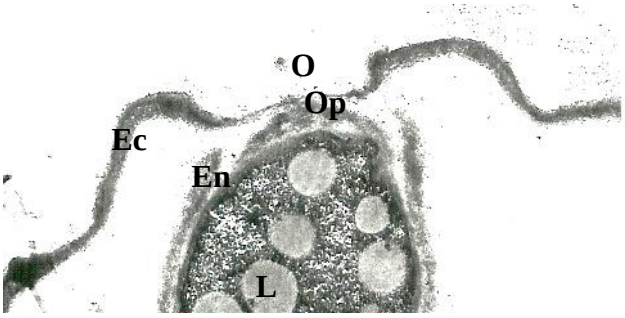


Figure 8.19. (x 15000)

Figures 8.16 to 8.19. Transmission electron micrographs of cysts of *Acanthamoeba* strain ATCC 50676, isolated from a keratitis patient. The prominent, double-layered wall, consisting of an outer, fibrous, electron-dense ectocyst (Ec) and an inner, less electron-dense endocyst (En), is apparent. The space between the ectocyst and endocyst is filled with electron-dense material. The inner and outer walls fuse at irregular intervals, forming gaps, or ostioles (O), with an operculum-like structure (Op) in the cyst wall that contains neither endocyst nor ectocyst (Fig. 8.19). A large, centrally located nucleus (N) with a densely staining nucleolus (n) is apparent in the cysts (Figs 8.17 and 8.18). Membrane-bound lipid vacuoles (L), concentrated mainly around the periphery of the cytoplasm, are present in the cyst. Mitochondria (M) can be seen around the nucleus.

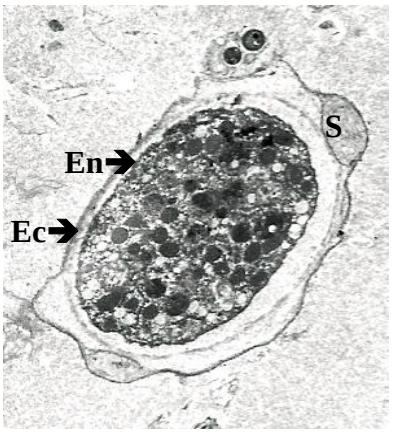


Figure 8.20 (x 5000)

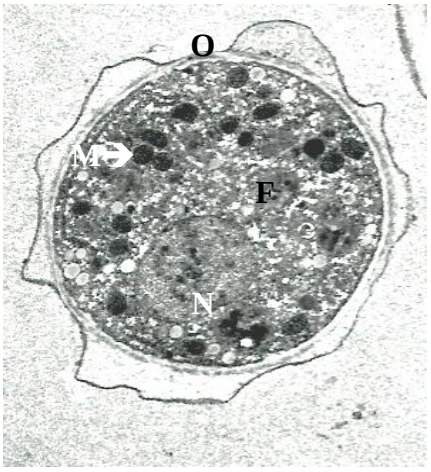


Figure 8.21 (x 6000)

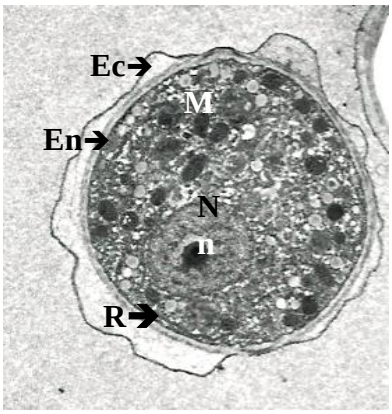


Figure 8.22 (x 5000)

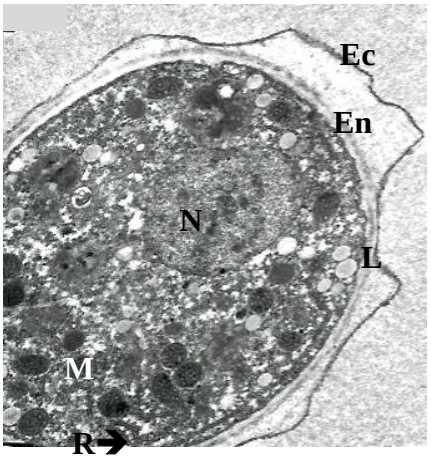


Figure 8.23 (x 8000)

Figures 8.20 to 8.23. Transmission electron micrographs of cysts of *Acanthamoeba* strain ATCC 50685, isolated from Northern Sewage Works, Gauteng Province. The prominent, double-layered wall, comprising an outer, fibrous, electron-dense ectocyst (Ec) and an inner, less electron-dense endocyst (En), is visible. A zone of variable width separates the wrinkled ectocyst from the endocyst. The space between the ectocyst and endocyst is filled in some regions with a spongy or fibrous network (S). Ostioles (O) and a round nucleus (N) with an electron-dense nucleolus (n) are apparent (Fig. 8.22). Numerous membrane-bound lipid vacuoles (L), as well as phagosomes (F) of various sizes containing whole bacteria or debris, are found in the cytoplasm. Mitochondria (M) and rough endoplasmic reticulum (R) are visible.

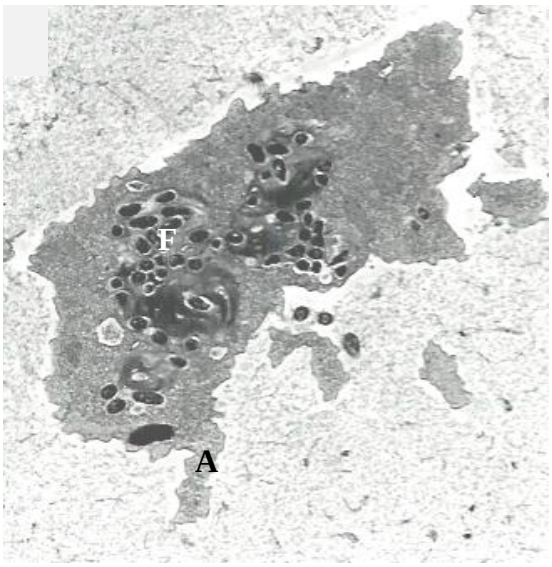


Figure 8.24 (x 3000)

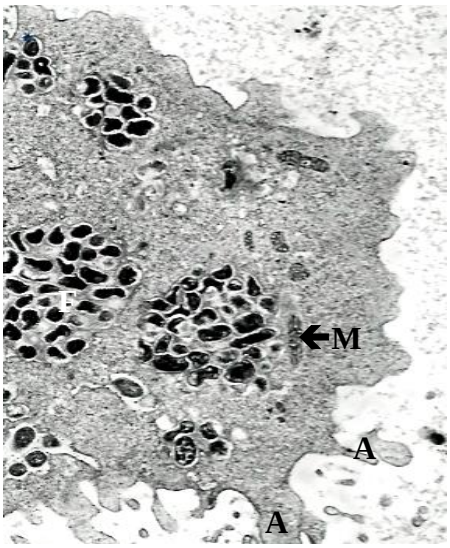


Figure 8.25 (x 5000)

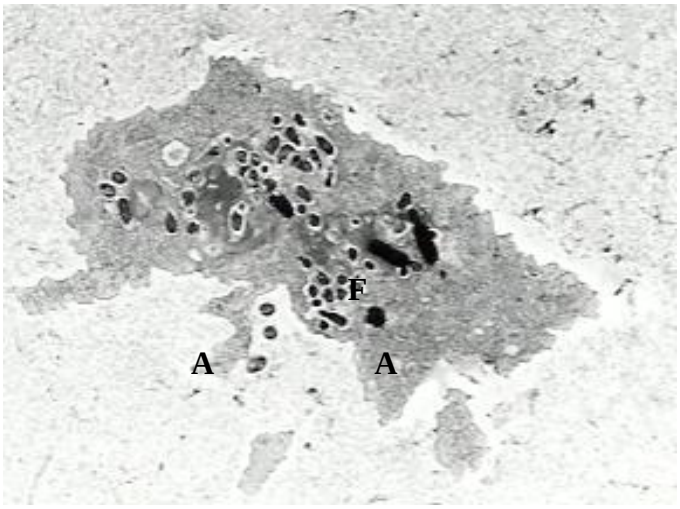


Figure 8.26 (x 2000)

Figures 8.24 to 8.26. Transmission electron micrographs of trophozoites of *Acanthamoeba* strain ATCC 50685, isolated from Northern Sewage Works, Gauteng Province. Numerous phagosomes (F) of various sizes and containing whole bacteria, vesicles, granules, myelinated bodies or debris are found in the cytoplasm. Acanthapodia (A) (Figs 8.24 – 8.26) and mitochondria (M) (Fig. 8.25) are visible.

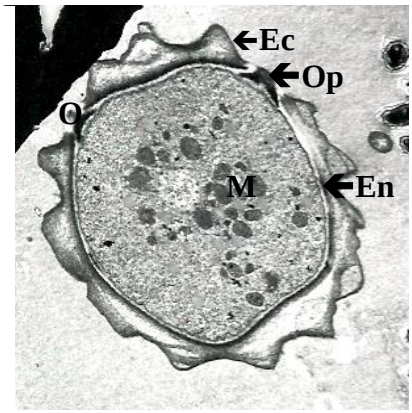


Figure 8.27 (x 4000)

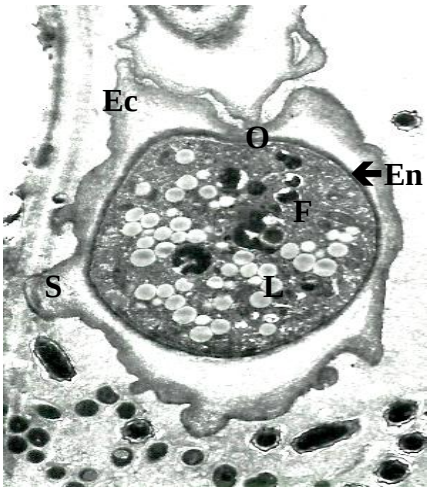


Figure 8.28 (x 5000)

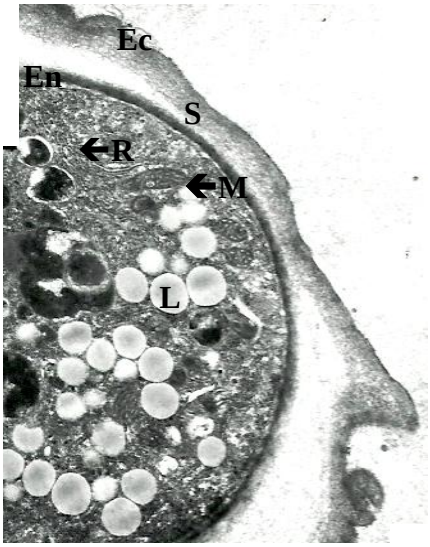


Figure 8.29 (x 10000)

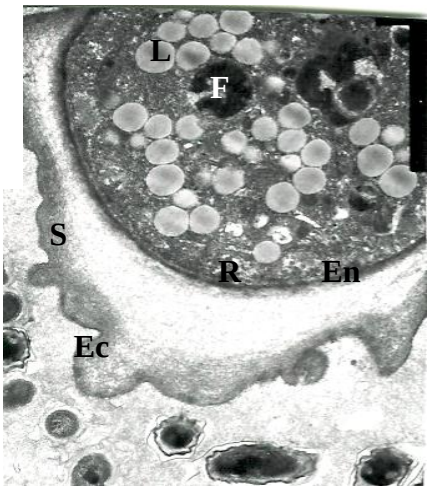
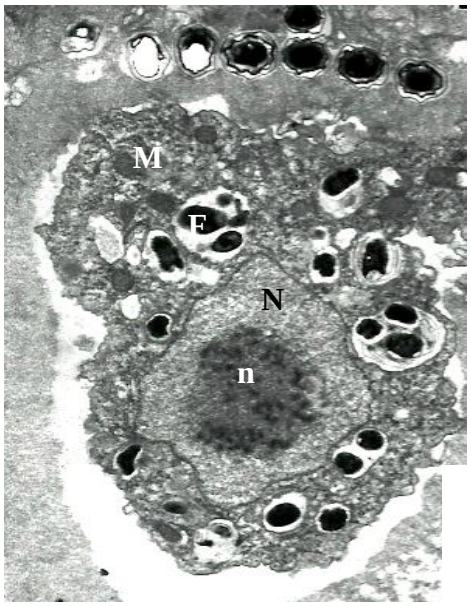


Figure 8.30 (x 8000)

Figures 8.27 to 8.30. Transmission electron micrographs of cysts of *Acanthamoeba* strain ATCC 50686, isolated from Southern Sewage Works, Gauteng Province. Both an ectocyst (Ec) and an endocyst (En) are clearly visible, with a zone of variable width separating the wrinkled ectocyst from the endocyst. The endocyst is closely applied to the cell membrane and is round/square in shape. The space between the ectocyst and endocyst is filled in some regions with a spongy or fibrous network (S). Ostioles (O), with an operculum-like structure (Op) in the cyst wall that contains neither endocyst nor ectocyst, are apparent (Fig. 8.27). Numerous membrane-bound lipid vacuoles (L), as well as phagosomes (F) of various sizes and containing whole bacteria or debris, are found in the cytoplasm. Mitochondria (M) and rough endoplasmic reticulum (R) are visible.



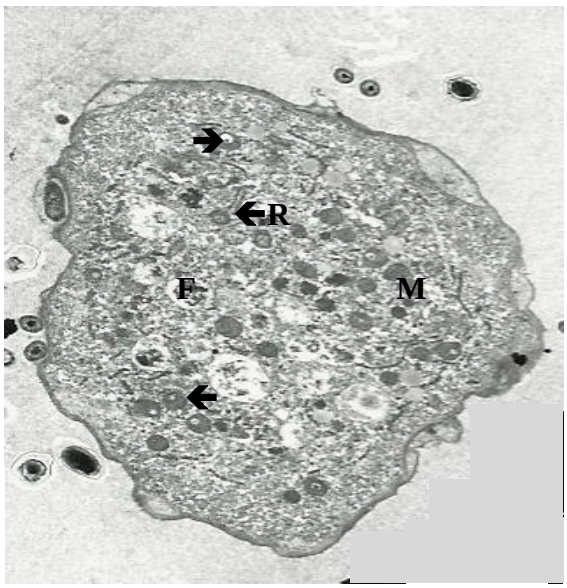


Figure 8.31 (x 6000) **Figure 8.32 (x 4000)**

Figures 8.31 to 8.32. Transmission electron micrographs of trophozoites of *Acanthamoeba* strain ATCC 50686, isolated from Southern Sewage Works, Gauteng Province. Numerous phagosomes (F) of various sizes, containing whole bacteria, vesicles, granules, myelinated bodies or debris, are found in the cytoplasm. Mitochondria (M) and rough endoplasmic reticulum (R) are visible. The conspicuous, electron-dense nucleolus (n) enveloped by evenly granular nuclear chromatin in the nucleus (N), is apparent (Fig. 8.31). Note the clear space in some of the mitochondria (arrows) (Fig. 8.32).

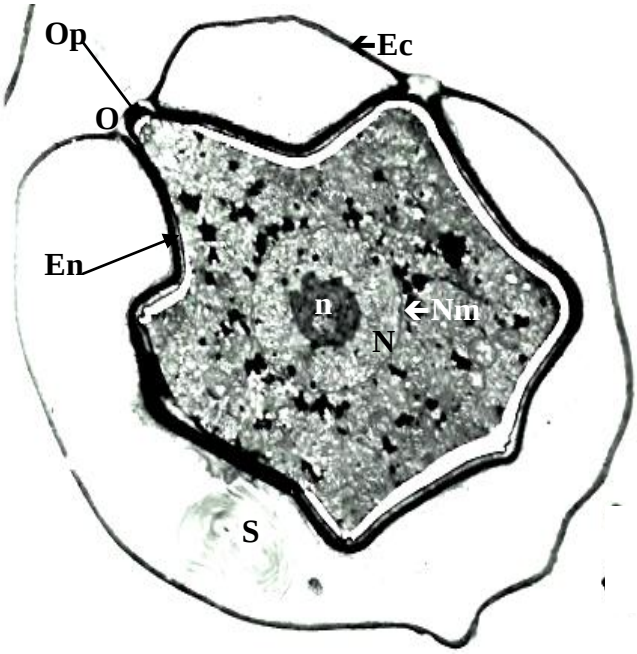


Figure 8.33. (x 3000)

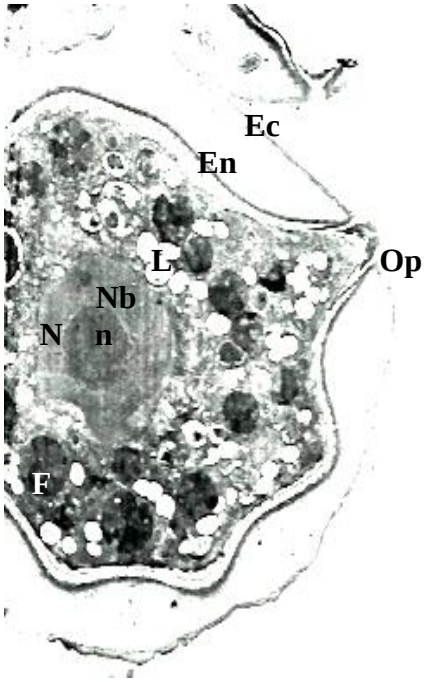


Figure 8.34. (x 3000)

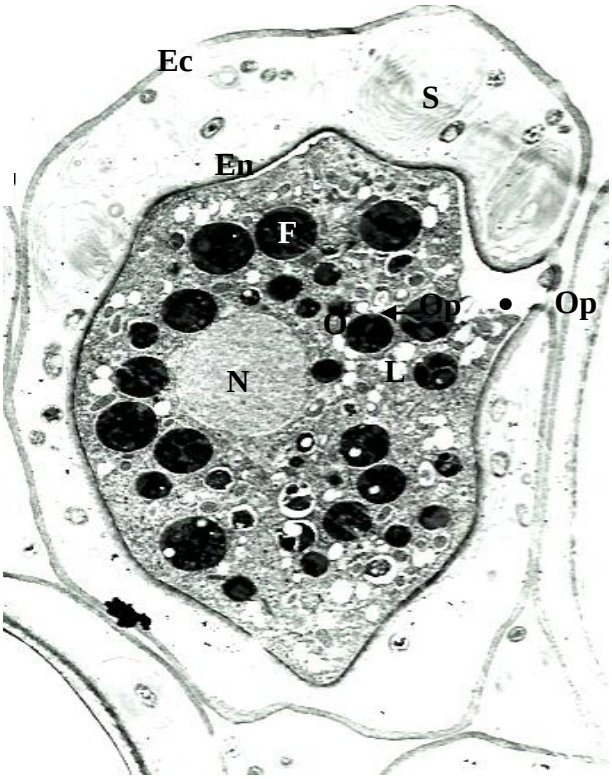


Figure 8.35. (x 3000)

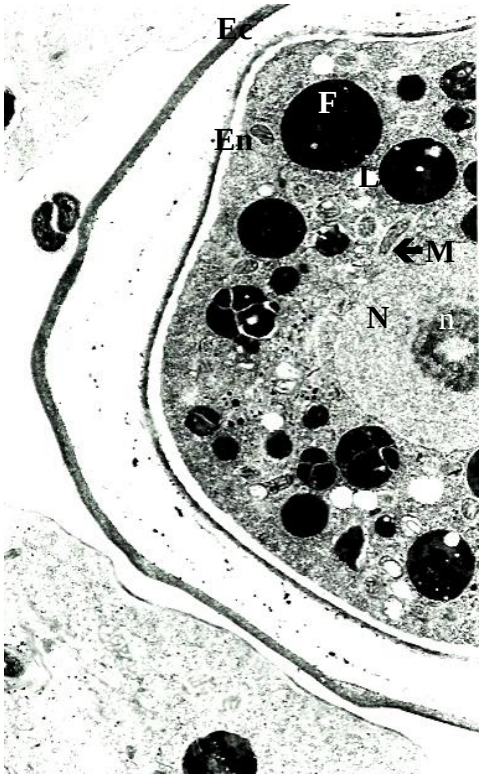


Figure 8.36. (x 5000)

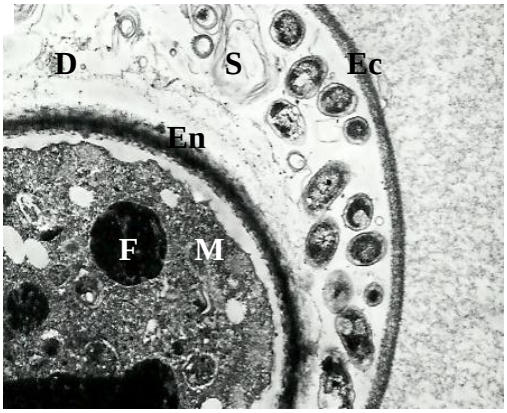


Figure 8.37. (x 5000)

Figure 8.38 (x 6000)

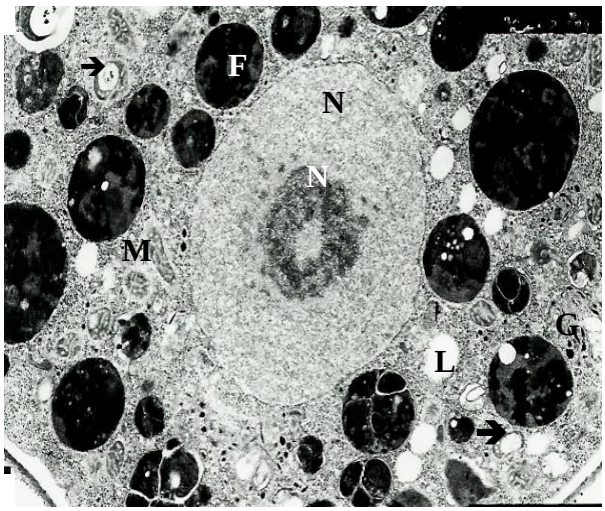


Figure 8.39 (x 8000)

Figure 8.40 (x 20000)

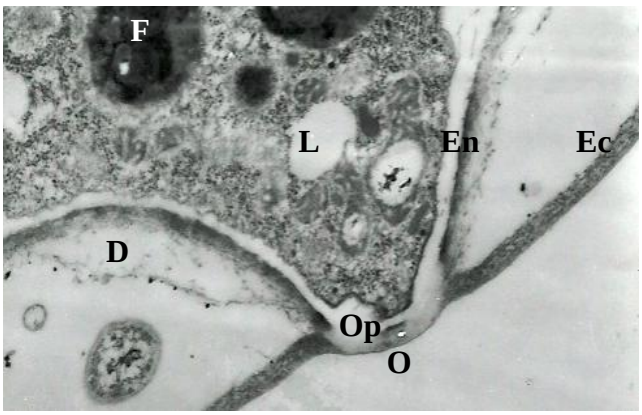


Figure 8.41 (x 15000)

Figures 8.33 to 8.41. Transmission electron micrographs of cysts of *Acanthamoeba* strain SAWS 87/4, isolated from Goudkoppies Sewage Works, Gauteng Province. The endocyst (En) is approximately star-shaped, with several truncated rays, and the ectocyst is interrupted by gaps and ostioles (O) with opercula (Op), located at the edges of the rays (Figs 8.33 – 8.35; 8.38; 8.40 – 8.41). A clear zone of variable width, which contains fragments of membranes or membrane-bound vesicles (S), separates the ectocyst (Ec) from the endocyst (Figs 8.33 – 8.38). Numerous phagosomes are apparent, containing vesicles, granules and myelinated bodies (Figs 8.34 – 8.39). Membrane-bound lipid vacuoles (L) are present in the cytoplasm (Figs 8.34 – 8.39). Mitochondria (M) are also visible in the cytoplasm (Figs 8.36; 8.37; 8.39). The interphase nucleus (N) enveloped by a nuclear membrane (Nm) is apparent. A large, conspicuous nucleolus (n) with electron-dense material can be seen (Figs 8.33; 8.34; 8.36; 8.39). Note the conspicuous nucleolus (karyosome) enveloped by evenly granular nuclear chromatin. Sometimes a clear, centrally located space is visible inside the nucleolus, giving the nucleolus a ring-shaped appearance (Figs 8.36; 8.39). The Golgi apparatus (G) is apparent, and many small vesicles appear to be budding off the Golgi cisternae (Fig. 8.39). Note the clear space in some of the mitochondria (arrows) (Fig. 8.39).

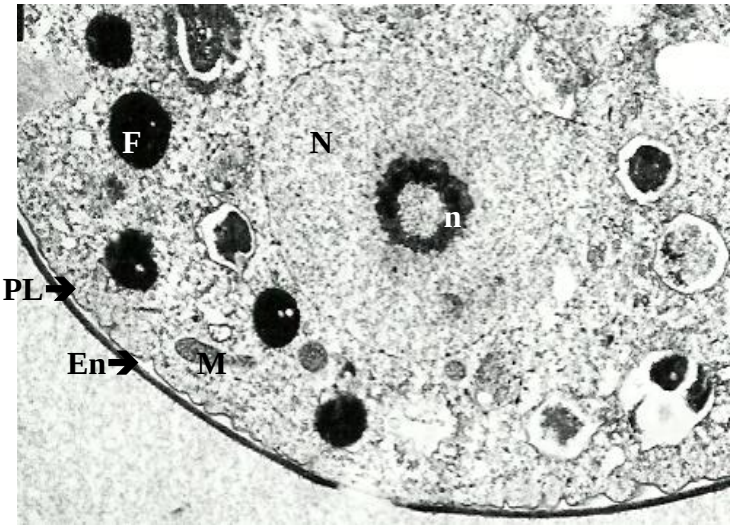


Figure 8.42. Pre-cystic stage of *Acanthamoeba* strain SAWS 87/4, isolated from Goudkoppies Sewage Works, Gauteng Province. The electron-dense endocyst (En), closely applied to the plasmalemma (PL), is visible in the pre-cystic stage. A clear, centrally located space is visible within the electron-dense nucleolus (n). Mitochondria (M) and phagocytic vesicles (F) are present in the cytoplasm. (x 5000)

8.4 Discussion

Results using LR White (London Resin Company) as the embedding medium for amoebae, proved satisfactory. Penetration of this low-viscosity resin is rapid, which is advantageous if immediate examination of tissue is required for diagnostic purposes in cases of suspected *Naegleria* or acanthamoebic infection in humans.

By transmission electron microscopy, *Acanthamoeba* cysts were seen to be characterised by a prominent, double-layered wall comprising an outer, fibrous, electron-dense ectocyst and an inner, less electron-dense endocyst. The two layers were usually separated, but joined at one or more points in the cyst wall to form opercula in ostioles. Material, probably cytoplasmic debris, was present between the two layers. Lastovica (1977b) described cysts of *Acanthamoeba* that were viewed under the transmission electron microscope in a similar way. Stratford & Griffiths (1978) state that autolysosomal material was embedded in the wall layers, and that plugs of electron-dense material closed the pores through which excystation occurs. These were clear discontinuities in the wall structure.

The present author found, as did Lastovica (1977b), that the cyst cytoplasm was electron-dense and contained lipid-like bodies. The extremely dense cytoplasm of encysted cells in both light and electron micrographs suggests that the volume change is partially the result of dehydration and shrinkage of the material during preparation for microscopy (Bowers & Korn, 1969; Stratford & Griffiths, 1978). Membrane-bound vacuoles containing cytoplasmic debris (autolysosomes) were prominent in the encysting amoebae (Lastovica, 1977b; Chávez-Munguía *et al.*, 2005). Other features observed in the cyst cytoplasm were nuclei, mitochondria and rough endoplasmic reticulum. Lastovica (1977b) found that the ultrastructure

of virulent and avirulent *Acanthamoeba* cysts was similar, and that no structure linked to pathogenicity, such as virus-like particles, could be observed.

Page (1976) distinguished between *A. castellanii* and *A. polyphaga*, using as his criterion differences in cell structure, which were based essentially on the degree of contact between the wall layers, and on whether the wall is wrinkled or smooth. Stratford & Griffiths (1978) examined by transmission electron microscopy the cyst types produced by *A. castellanii* in four different media. All the cystic forms of *A. castellanii* and *A. polyphaga* described by Page (1976) were produced by Stratford & Griffiths (1978) in a single strain of *A. castellanii*, simply by modifying the environment in which they were formed. When *A. castellanii* cysts were produced in replacement medium and in monoxenic culture, there were no discernible organelles, with the possible exception of some membrane-bound structures that were suggestive of autophagosomes (Stratford & Griffiths, 1978).

Using scanning electron microscopy, Lastovica (1977b) found that cyst exteriors of *Acanthamoeba* had a characteristic wrinkled appearance, due to deep interconnecting ridges that surrounded depressions in the cyst wall. These ridges were most prominent in older cysts. Ostioles marked by the presence of opercula were conspicuous in some of these depressions. During excystment, opercula were displaced, leaving large apertures in the empty cysts (Lastovica, 1977b).

By means of transmission electron microscopy, Lasman & Feinstein (1986) found that encystment is accompanied by a marked decrease in cell size, indicating a significant loss of plasma membrane during this period. The appearance of vesicles at the cell surface and in the space between the cyst wall and cell membrane may indicate that reduction in cell size is achieved in part through

vesicles simply being pinched off from the cell membrane (Lasman & Feinstein, 1986). It is also possible that the removal of excess cytoplasm during encystment takes place by this means (Lasman & Feinstein, 1986). The encysting cell has an irregularly circular outline at first, which then becomes more polygonal (Page, 1967). In the early stages of encystment, the outer surface (where the cyst is forming) is smooth and somewhat flexible. Later, the outer surface or wall is seen to be more irregular and somewhat wrinkled.

8.4.1 *Rough endoplasmic reticulum*

Rough endoplasmic reticulum was noted in several cysts and trophozoites. Lasman (1982) mentions that the appearance of whorls of endoplasmic reticulum in *A. astronyxis* may be correlated with protein synthesis that occurs during the course of encystment. It has been suggested that membrane whorls may also be associated with cellular compartmentalisation, resulting in the isolation of different metabolic activities within the cell. Such structures may be involved in degradative processes, considering that disintegrating cytoplasmic organelles can be seen in the centre of whorls during encystment. The degradation of cellular components and macromolecules provides material for cyst wall synthesis, generates energy and allows the removal of excess cytoplasm during cyst formation (Lasman, 1982). Whorls may be formed as a response of cytoplasmic membranes to a change in the degree of hydration of the cytoplasmic matrix. This may also be the case in encysting amoebae, since it is known that during encystment, alterations in water content occur (Lasman, 1982).

8.4.2 *Mitochondria*

Mitochondria were found in cysts and trophozoites in several of the strains of *Acanthamoeba* that were examined by transmission electron microscopy. In some cases (strains ATCC 50686; SAWS 87/4; and *Acanthamoeba* sp. from tap water from Roodepoort, Gauteng), clear regions were present in the mitochondria. Bowers and Korn (1969) found similar clear regions in *A. castellanii* (Neff strain), and suggested that these could be where an amorphous granule had been present. They ascertained that the embedding medium frequently fails to penetrate the concretion, so that it drops out of the section, leaving a space. These characteristics suggest that it is inorganic material. By the time the ectocyst is complete, many mitochondria have also developed a large spherical droplet lying adjacent to, or surrounding, the granule and its concretion (Bowers & Korn, 1969).

Lasman (1982) found variations in mitochondrial shape in *A. astronyxis*. These alterations may reflect changes in the cellular activity that occurs during encystment. Lasman (1982) explains that modifications in mitochondrial shape similar to those seen by her have been found in various glandular cells, and were related to an increase in the synthetic and secretory activities of the cells.

8.4.3 *Nucleolus*

It has been reported that the nucleolus decreases markedly in size during cyst formation (Ray & Hayes, 1954; Bowers & Korn, 1969). Transformations of the nucleolus, described by Ray & Hayes (1954), indicate that nucleolar material was extruded into the cytoplasm. Although Lasman (1982) observed nucleolar changes, she found no evidence of extrusion of nucleolar material. A nucleolar-like body was visible in the cysts of amoebae isolated from Johannesburg tap

water. Nucleolar-like bodies, similar to those described in *A. palestinensis* by Lasman (1977), appeared in the nucleus of encysting cells. A large droplet which developed in association with the nucleolus in *A. castellanii* may be analogous to the nucleolar-like bodies found in other species (Bowers & Korn, 1969).

8.4.4 *Cyst wall*

The composition of the ectocyst and the endocyst seem to vary, depending on the species. This researcher found that both the ectocyst and endocyst in the strains studied appeared to be fibrous. Lemgruber *et al*. (2010) have shown that filamentous molecules are dispersed in the intercyst space, connecting the endocyst to the ectocyst. Lasman (1982) described both the ectocyst and endocyst in *A. astronyxis* as having a granular appearance, with the granules of the ectocyst being much finer than those of the endocyst. The operculum seems to be composed of the same material as the endocyst, and is relatively close to the plasma membrane (Lasman, 1982), as is the case for strains ATCC 50685, 50686 and SAWS 87/4. In *A. castellanii*, on the other hand, the ectocyst is fibrous and the endocyst has a finely granular appearance, with fibrils embedded in the granular matrix (Bowers & Korn, 1968). The operculum in this species consists of the closely-apposed ectocyst and endocyst, and is separated from the plasma membrane by a wider space. Thus far, no differences have been found between the ectocyst and endocyst in *A. palestinensis* (Lasman, 1977). Whether the differences described in this paragraph are species specific or caused by various conditions of growth and induction procedure is, as yet, not clear (Lasman, 1982).

8.4.5 *Centrosphere*

No structure resembling a centrosphere was found in the strains of *Acanthamoeba* examined in this study. However, Lasman (1982) provides evidence for the

occurrence of a centrosphere in *A. astronyxis*. It was found to be an association of a well-developed Golgi complex and an electron-dense structure from which microtubules radiate. The existence of the centrosphere is considered by some scientists to be debatable (Page, 1967; Sawyer & Griffin, 1971), since this organelle was not demonstrated in earlier ultrastructural studies of *A. astronyxis* (Deutsch, 1960; Threadgold, 1976).

8.4.6 *Golgi complex*

Densely-staining, small vesicles appear to bud off the Golgi cisternae in strain SAWS 87/4. Both the form and the distribution of the Golgi complex change during encystment (Bowers & Korn, 1969). The Golgi complex of the trophozoite quite often occurs as large aggregates of cisternal stacks; however, the Golgi complex of encysting cells is more widely distributed in smaller aggregates (Bowers & Korn, 1969).

8.4.7 *Autolysosomes*

Large basophilic granules are a prominent component of the cytoplasm of encysting amoebae (Bowers & Korn, 1969). These granules can be readily identified by the use of the electron microscope as vacuoles with dense content that includes mitochondria, lipid droplets and glycogen. By cytochemical methods, the vacuoles can be shown to contain acid phosphatase through a positive staining reaction. This reaction and the recognisable cytoplasmic contents are the criteria that define these vacuoles as autolysosomes (Bowers & Korn, 1969). In dehydrated, mature cysts, cytoplasmic components cannot be identified within the autolysosomes, and crystalline structures are present (Bowers & Korn, 1969). The contents of some of these autolysosomes are deposited in the developing cyst wall.

The results of the author's examination of the ultrastructure of strains of amoebae are in agreement with the observations of other researchers. The generic status of *Acanthamoeba* cysts can easily be determined by electron microscopy of the cyst wall. One cannot, however, exclusively use ultrastructural characteristics for identification at the specific level.

CHAPTER NINE – CHARACTERISATION OF *MASTIGINA* SP. BY TEMPERATURE TOLERANCE, SIZE AND LIGHT AND ELECTRON MICROSCOPY

9.1 The difficulty in establishing the generic position of *Mastigina*

Mastigina sp. is included in the superclass Mastigophora, class Zoomastigophorea, family Mastigamoebidae. This is because the flagellated form appears to be the usual one, and there is no indication thus far of transformation of all members of a population to an amoeboid form under defined conditions (Wickerham & Page, 1970).

The matter of generic position *per se* is somewhat more complicated. Wickerham & Page (1970) have employed the genus *Mastigina* to include amoeboid flagellates with a more or less limax-shaped locomotive form, equipped with one or more flagella. Although T.A. Nerad accepts *Mastigina* as a valid genus, there is some controversy over whether or not this genus is a junior synonym of *Mastigamoeba* (T.A. Nerad, personal communication). The debate will continue, since there are no cultures available for comparative molecular studies (T.A. Nerad, personal communication).

Mastigina appears to be very closely related to the protostelid slime moulds. The morphology of the flagellated stage in true protostelids is indistinguishable from that of *Mastigina*. It is possible that plasmodial formation may be induced only

under certain conditions. T.A. Nerad believes, however, that there may be a taxon of organisms which has either lost the ability to form plasmodia or perhaps has never had that ability (personal communication).

Mastigina sp. was implicated as the possible causative agent in a case of ocular infection in a patient in South Africa (Table 2.1). It was, therefore, of interest to the researcher to examine the morphology of this organism, using both light and electron microscopy. Although *Mastigina* is not classified like free-living amoebae that are well known pathogens, it is included in this thesis, nevertheless, being an amoeba-like free-living organism that might cause ocular disease.

9.2 Materials and methods

9.2.1 *Culturing, cloning and temperature tolerance of* Mastigina

Mastigina was cultured on NNA plates seeded with *E. coli* bacteria at 30°C, as described in Chapter 2. Cloning of these organisms was carried out as described for *Acanthamoeba* sp. (Chapter 2). The researcher attempted to adapt *Mastigina* to peptone-yeast extract glucose (PYG) broth as for *Acanthamoeba* (Chapter 2), and assessed growth of *Mastigina* sp. at 25°C, 37°C and 40°C.

9.2.2 *Measurement of* Mastigina *organisms*

Mastigina organisms were measured using a Union vernier ocular micrometer.

9.2.3 *Light microscopic studies of* Mastigina

For light microscopic studies of *Mastigina*, trophozoites were taken from fresh plates, while cysts were taken from older plates. A flamed loop was used to scrape *Mastigina* from the agar surface, and organisms were placed in sterile distilled water.

9.2.4 *Electron microscopic studies of* Mastigina

The processing of *Mastigina* organisms for electron microscopy followed the procedures outlined for *Acanthamoeba* in Chapter 8.

The process of staining semi-thin and ultrathin sections is presented in Appendix VII.

9.3 Results

9.3.1 *Culturing and temperature tolerance of* Mastigina

The rate of multiplication of *Mastigina* sp. at 30°C on NNA plates seeded with *E. coli* bacteria was not as rapid as that of *Acanthamoeba*, and growth of the organisms was no faster at 25°C. The trophozoites multiplied very slowly at 37°C. Both cysts and trophozoites of *Mastigina* sp. died at 40°C. The growth of *Mastigina* organisms in PYG medium was very poor.

9.3.2 Measurement of Mastigina *cysts*

The size range of the *Mastigina* cysts was 8.6 μm – 10.5 μm, and trophozoites extended to a length of 30 μm.

9.3.3 *Light microscopic studies of* Mastigina *(Figs 9.1 – 9.3)*

Trophozoites move by means of lobose pseudopodia, but convert to a flagellate form when transferred to liquid medium at 30°C. These converted forms are unlike the flagellates in *Naegleria*, as they resemble worms and move in a worm-like fashion. Their movement is slow compared to that of *Naegleria* flagellates. One very long flagellum, or sometimes one long flagellum and a shorter, non-motile one that is held very tightly appressed to the body, are formed at the

anterior end (direction of movement). The anterior end is tapered, with the nucleus close to the tip. The posterior end is formed into acanthapodia-like spikes, and is much broader than the anterior end (Fig. 9.1). These flagellates occasionally coil themselves up and change direction, always moving with the flagellum in front. This typical morphology, found in the strains held in culture in the American Type Culture Collection (T.A. Nerad, personal communication), is similar to that observed in the southern African strain.

Cysts are circular with a smooth wall, and appear to be surrounded by a gelatinous coat, as seen by phase contrast microscopy (Figs 9.2; 9.3).

9.3.4 *Electron microscopic studies of* Mastigina

Electron micrographs of *Mastigina* sp. are presented in Figures 9.4 – 9.25.

Cysts (Figs 9.4 – 9.7) – The cysts are spherical or ovoid and smooth-walled. The cyst wall appears to be of almost uniform thickness around the entire cyst. Vacuoles are apparent in the wall (Fig. 9.6). The interphase nucleus contains one large, electron-dense nucleolus (Figs 9.4 – 9.6). The cytoplasm is dense, and mitochondria are visible near the nucleus (Figs 9.4 – 9.6). Several large, lighter patches are apparent in the cytoplasm (Fig. 9.4). Phagocytic vacuoles containing debris are visible (Fig. 9.6).

Trophozoites (Figs 9.8 – 9.25) – The interphase nucleus contains one large, electron-dense nucleolus, within which a clear, centrally located space is sometimes seen (Figs 9.10 and 9.18). Mitochondria are visible, some of which contain a densely-staining core (Figs 9.8; 9.9; 9.12; 9.14; 9.15; 9.20) or sometimes surround the nucleus (Figs 9.10; 9.15; 9.18; 9.20). Phagosomes that sometimes

contain debris are visible (Figs 9.8; 9.9; 9.11 – 9.17; 9.20; 9.23). Rough endoplasmic reticulum is apparent (Figs 9.8; 9.9; 9.17; 9.19; 9.21) and fibrils are visible (Figs 9.12; 9.22; 9.24; 9.25). Parts of the flagella can be seen in Figures 9.13 and 9.16.

9.4 Discussion

Strains of *Mastigina* very similar to the South African strain investigated in this study have been isolated from the following sites (T.K. Sawyer & T.A. Nerad, personal communications).

- An airborne contaminant was taken from the eastern shore of Maryland, USA.
- Several strains were obtained from sewage dump sites in the Atlantic Ocean. These strains are probably not euryhaline but, rather, freshwater ones whose cysts tolerate seawater. No attempt has yet been made to culture them in seawater (T.A. Nerad, personal communication).
- A contaminant from a culture of *Tillina magna* (ATCC 50128) was deposited by Dr. D. Lynn of Guelph, Canada.
- A strain from a soil sample was obtained from the Dominican Republic.
- Several strains which appear to be freshwater strains that have washed into the sea, were extracted from deep water sites in the Atlantic Ocean.
- Several strains were taken from eye-wash stations.

Wickerham & Page (1970) isolated a strain of *Mastigina* from the frass of a pine tree growing in Spain. The isolation of *Mastigina* from so many diverse habitats gives an indication of its wide distribution. Sampling of different areas in South Africa could possibly lead to the isolation of more strains of *Mastigina* from various sources in the environment.

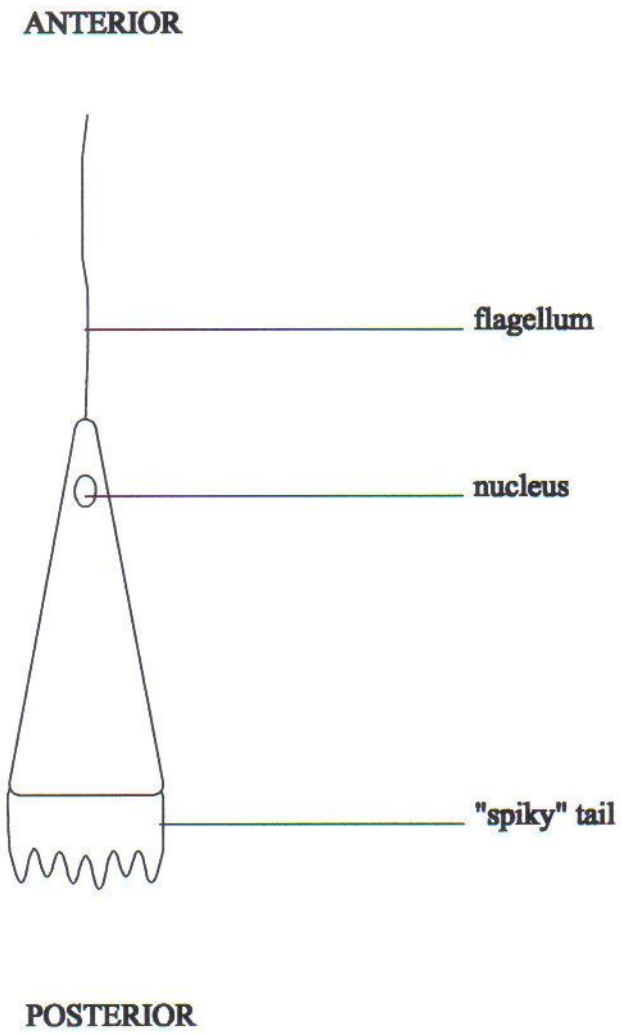
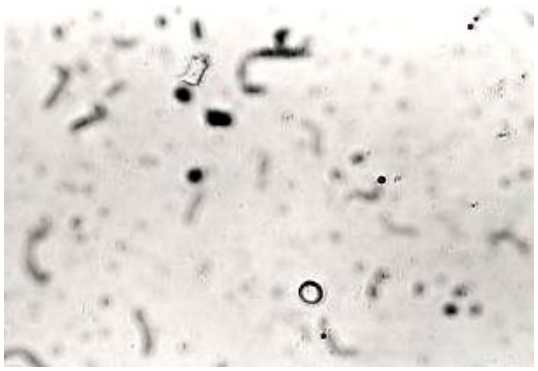


Figure 9.1. Diagrammatic representation of the flagellate form of *Mastigina* sp.



Figures 9.2 and 9.3. Light microscopic photographs of trophozoites and cysts of *Mastigina* strain SAWL 91/2, grown on a non-nutrient agar plate seeded with *Escherichia coli* bacteria. (x 450)

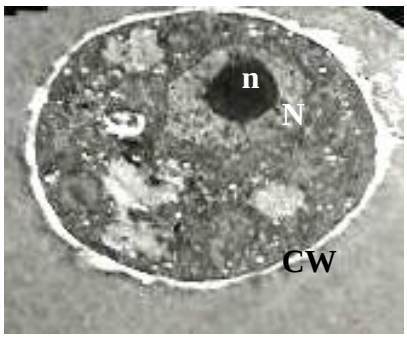


Figure 9.4. (x 6000)

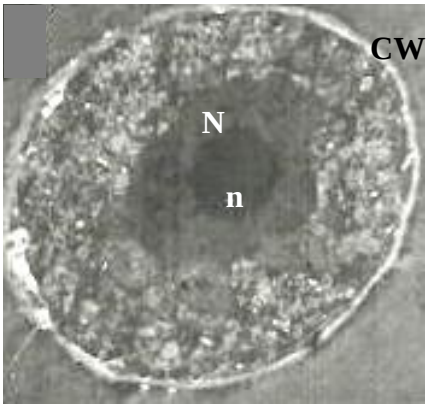


Figure 9.5 (x 8000)

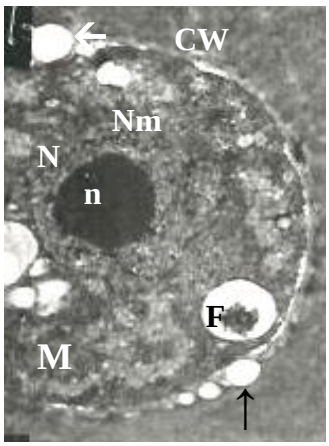


Figure 9.6 (x 10000)

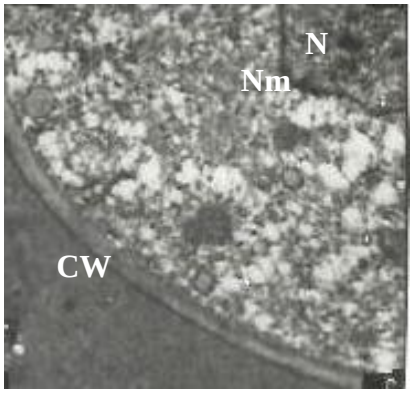


Figure 9.7 (x 15000)

Figures 9.4 to 9.7. Transmission electron micrographs of cysts of *Mastigina* strain SAWL 91/2. The cyst wall (CW) appears to be of almost uniform thickness around the entire cyst. Vacuoles (arrows) are apparent in the wall (Fig. 9.6). The nucleus (N), with a densely staining nucleolus (n), can be seen in Figs 9.4 – 9.6. The cytoplasm is dense, and mitochondria are visible near the nucleus (Figs 9.4 – 9.6). Several large, lighter patches are apparent in Figure 9.4. Phagocytic vacuoles (F) containing debris are visible (Fig. 9.6).

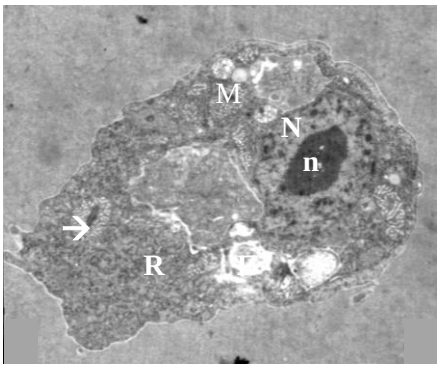


Figure 9.8. (x 5000)

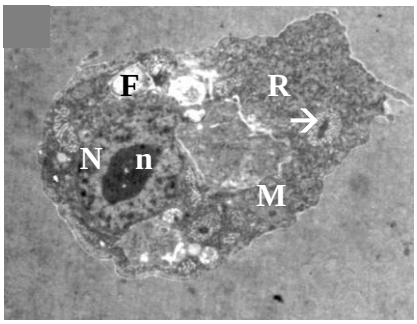


Figure 9.9 (x 6000)

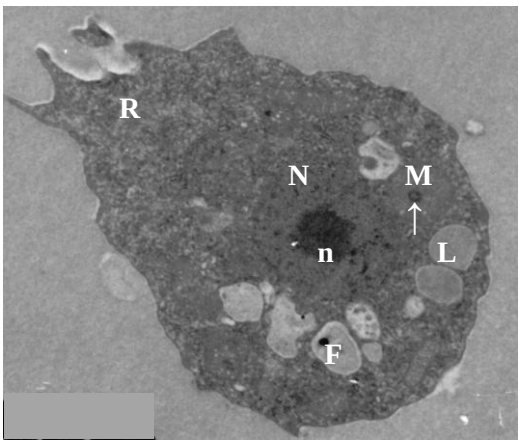


Figure 9.10 (x 6000)

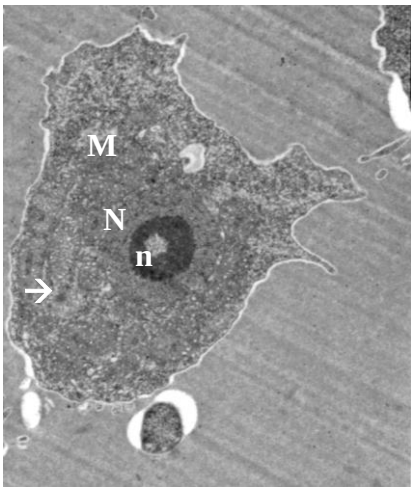


Figure 9.11 (x 8000)

Figures 9.8 – 9.11. Transmission electron micrographs of trophozoites of *Mastigina* strain SAWL 91/2. The nucleus (N), with a densely staining nucleolus (n), is apparent. The nucleolus in Figure 9.11 is ring-shaped. Mitochondria (M) are visible, and some of the mitochondria contain a densely-staining core (arrows). Mitochondria can be seen surrounding the nucleus in Figure 9.10. Phagosomes (F) containing debris are visible. Rough endoplasmic reticulum (R) (Figs 9.8 – 9.10) and lipid vacuoles (L) (Fig. 9.10) are apparent.

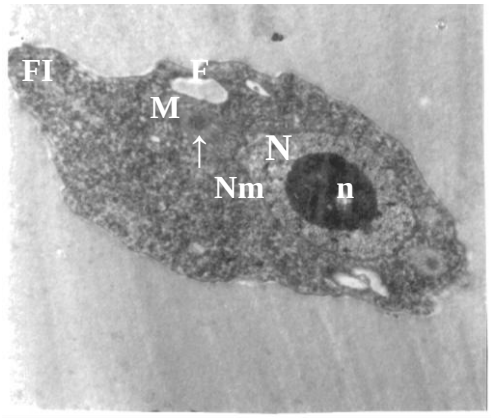


Figure 9.12. (x 8000)

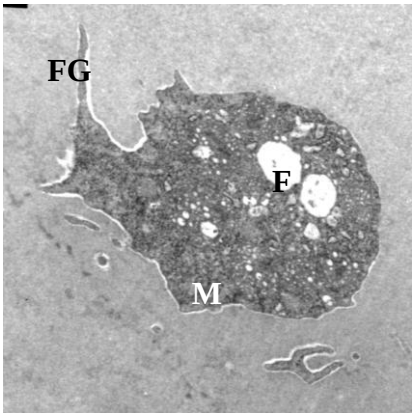


Figure 9.13. (x 6000)

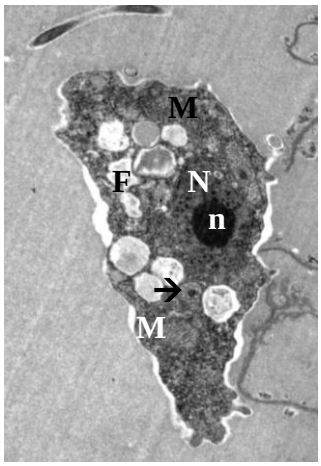


Figure 9.14. (x 6000)

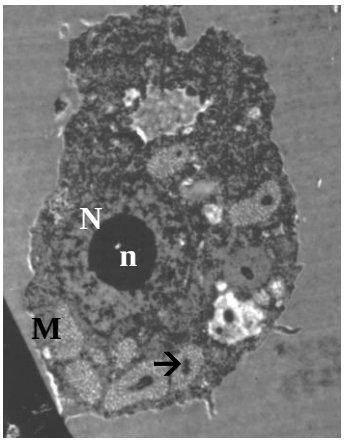


Figure 9.15. (x 6000)

Figures 9.12 to 9.15. Transmission electron micrographs of trophozoites of *Mastigina* strain SAWL 91/2. The nucleus (N), having a densely-staining nucleolus (n) and nuclear membrane (Nm), is apparent (Figs 9.12, 9.14 – 9.15). Mitochondria (M) are visible, and some of the mitochondria contain a densely-staining core (arrows) (Figs 9.12, 9.14; 9.15). Mitochondria surround the nucleus (Fig. 9.15). Phagosomes (F) containing debris are visible. Fibrils (FI) (Fig. 9.12) and parts of the flagellae (FG) (Fig. 9.13) are apparent.

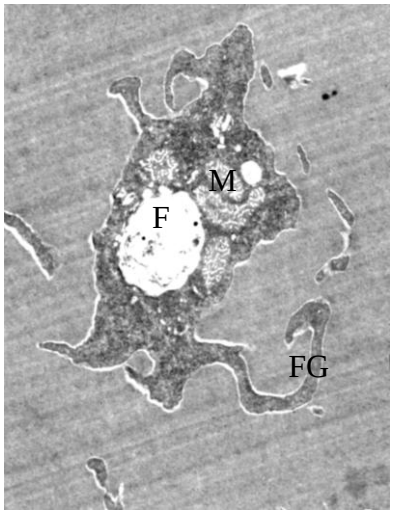


Figure 9.16. (x 6000)

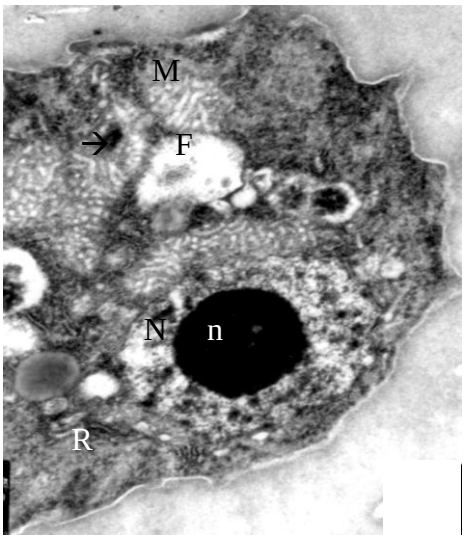


Figure 9.17. (x 15000)

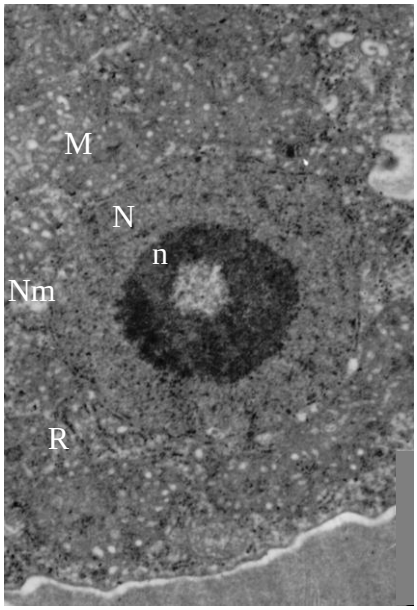


Figure 9.18. (x 15000)

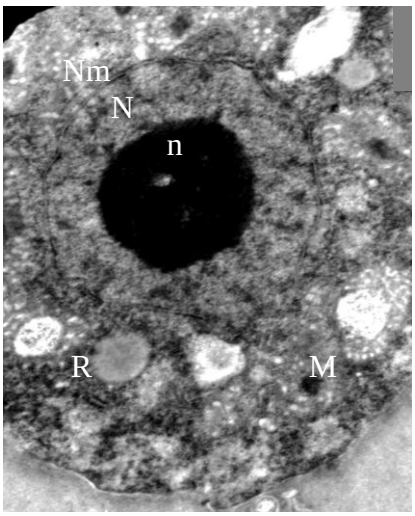


Figure 9.19. (x 15000)

Figures 9.16 to 9.19. Transmission electron micrographs of trophozoites of *Mastigina* strain SAWL 91/2. The nucleus (N) with a densely staining nucleolus (n) and nuclear membrane (Nm) are apparent (Figs 9.17 – 9.19). The nucleolus is sometimes ring-shaped (Fig. 9.18). Mitochondria (M) are visible. Mitochondria are surrounding the nucleus (Figure 9.18). Phagosomes (F) containing debris are visible (Figs 9.16 – 9.17). Rough endoplasmic reticulum (R) is evident (Figs 9.17 and 9.19). Parts of the flagella (FG) are visible in Figure 9.16.

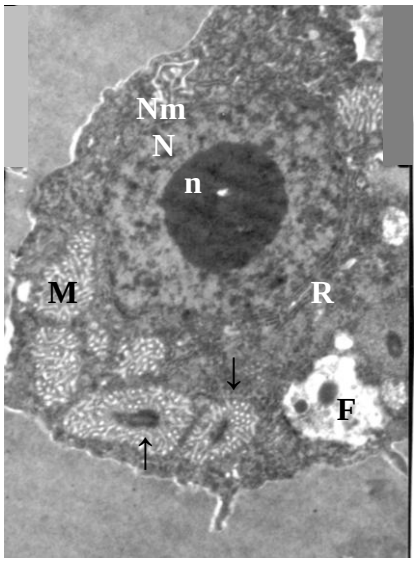


Figure 9.20. (x 10000)

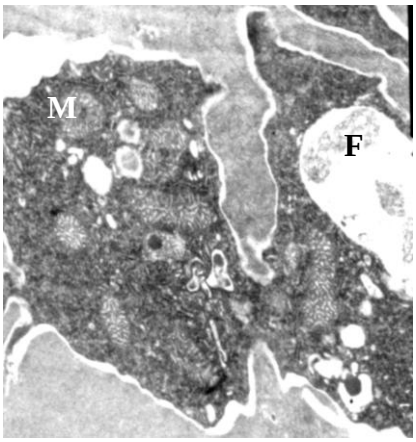


Figure 9.21. (x 8000)

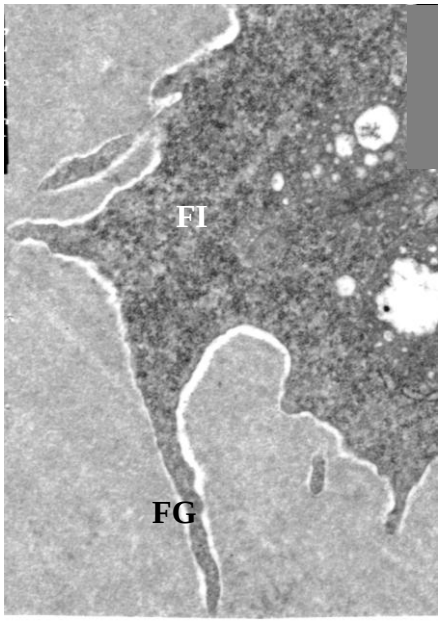


Figure 9.22. (x 15000)

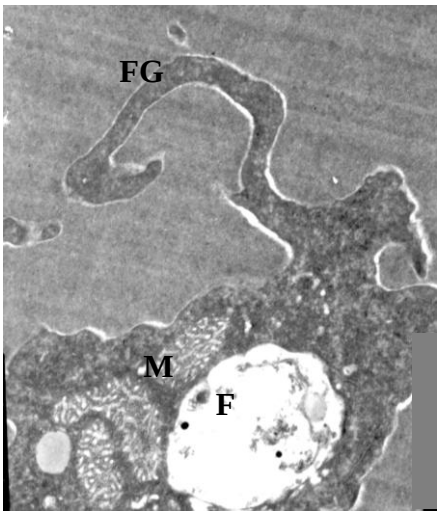


Figure 9.23. (x 10000)

Figures 9.20 – 9.23. Transmission electron micrographs of trophozoites of *Mastigina* strain SAWL 91/2. The nucleus (N) with a densely staining nucleolus (n) and nuclear membrane (Nm) are apparent (Fig. 9.20). Mitochondria (M) with anastomosing tubular cristae are visible, and some of the mitochondria contain a densely-staining core (Fig. 9.20). Mitochondria surround the nucleus (Figure 9.20). Phagosomes (F) containing debris are visible. Rough endoplasmic reticulum (R) is evident (Fig. 9.21). Fibrils (FI) (Fig. 9.22) and sections of the flagella (FG) (Figs 9.22 and 9.23) are visible. The trophozoite in Figure 9.21 is in the process of dividing.

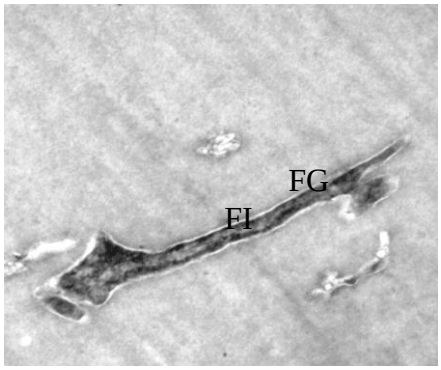


Figure 9.24. (x 15000)

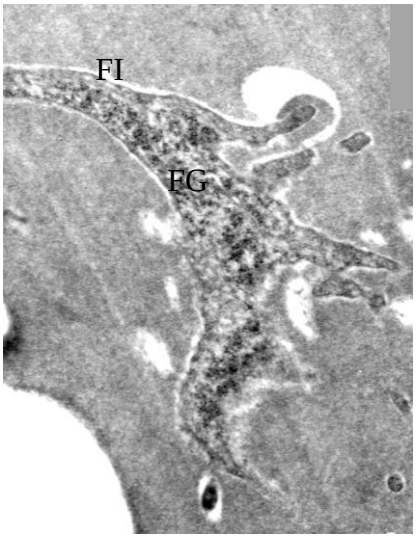


Figure 9.25. (x 10000)

Figures 9.24 – 9.25. Transmission electron micrographs of flagella of trophozoites of *Mastigina* strain SAWL 91/2. Fibrils (FI) are visible in the flagella (FG).

The strain of *Mastigina* tested did not grow very well in the presence of *E. coli* bacteria. The researcher inadvertently isolated *Mastigina* on bacterial cultures because she was attempting to culture *Acanthamoeba* from the corneal scrapings, contact lens cases and/or contact lens solutions of patients with ocular disease. *Mastigina* feeds on some species of bacteria and yeasts: of the yeasts, it feeds preferentially on those that produce mucoid colonies (Wickerham & Page, 1970). For general cultivation of *Mastigina* on solid medium, malt extract agar is better than yeast extract-malt extract agar (Wickerham & Page, 1970). However, for cultivation in liquid medium, yeast extract-malt extract medium is preferred, as *Mastigina* develops rapidly on dead or living yeast cells in shaken cultures, and the trophozoites may convert quantitatively to cysts (Wickerham & Page, 1970). For any further studies of this organism, or when attempting further isolations of *Mastigina*, the writer would attempt to culture it differently, following the recommendations of Wickerham & Page (1970). Those authors mention that the locomotive form of *Mastigina*, averaging 27 μm in length, resembles that of a limax amoeba, with a vesiculate nucleus at the anterior end. Cells are capable of simultaneous movement by pseudopodia and flagella, and the trophozoite emerges from the cyst by breaking the wall (Wickerham & Page, 1970).

This researcher found an electron-dense area in the centre of mitochondria of the *Mastigina* trophozoites. The area concerned is possibly similar to the concentrate described by Bowers & Korn (1969) in the centre of mitochondria of trophozoites in encysting *A. castellanii* cells. This concentrate, usually less than 0.1 μm in diameter, is completely electron-opaque. Frequently, the embedding medium fails to penetrate the concretion, so that it drops out of the section. These characteristics suggest that it is inorganic material (Bowers & Korn, 1969). Schuster (1965) found that the core in mitochondria of a slime mould

contained DNA. Further investigations are required to establish the nature of the core in mitochondria of the species of *Mastigina* investigated in the research recorded here.

CHAPTER TEN – CYTOPATHOGENICITY OF CLINICAL AND ENVIRONMENTAL *ACANTHAMOEBA* ISOLATES FOR TWO MAMMALIAN CELL LINES

10.1 Distinguishing between pathogenic and non-pathogenic species of *Acanthamoeba* by testing their cytopathogenicity

The severity of the infections caused by *Acanthamoeba* is such that there is a need for a rapid and accurate means of distinguishing between pathogenic and non-pathogenic species of this genus (Cursons & Brown, 1978).

Simple morphological identification of *Acanthamoeba* species provides no measure of pathogenicity (De Jonckheere, 1980), although pathogenicity does appear to be associated closely with the Pussard-Pons groupings of species (Badenoch *et al*., 1995). Despite the fact that growth at high temperatures and readiness to multiply axenically are used as indicators of potential pathogenicity, each strain has to be tested in cell culture or in an animal model to determine its virulence (De Jonckheere, 1980; Ren & Wu, 2010). One of the reasons is that strains of *Acanthamoeba* can show reduced temperature tolerance after long-term axenic culture (Pumidonming *et al*., 2010). However, animal models show marked differences as regards host susceptibility (De Jonckheere, 1980). Mice, for example, exhibit considerable variation in their response to infection with *Acanthamoeba*, a feature which is further complicated by inconsistencies caused by variations in the inoculation technique (Cursons & Brown, 1978). Attempts to correlate cytopathic potential and mouse pathogenicity for *Naegleria fowleri* and *N. gruberi* have failed (De Jonckheere & van de Voorde, 1977; Marciano-Cabral

et al., 1982; Fulford *et al*., 1985; John & John, 1989). A reason that has been suggested to explain this failure is that the pathogenicity of amoebae may be determined more by their ability to proliferate in the host and to escape host defences than by unique virulence factors (Marciano-Cabral *et al*., 1982).

In the case of *Acanthamoeba*, determination of the cytopathic effect (CPE) in cell culture is reported to have become the most reliable means of ascertaining whether or not a strain is pathogenic (Cursons & Brown, 1978; De Jonckheere, 1980). Cytopathogenicity of human and environmental acanthamoebic isolates for cell cultures has, overall, been shown to mirror the pathogenicity in animals. For example, Badenoch *et al*. (1995) found an 86% correlation between the virulence of *Acanthamoeba in vivo* and *in vitro*. Hitherto, the choice of cell line has not appeared to be important (Cursons & Brown, 1978; Larkin *et al*., 1991), although it has been said that HeLa cells are unsuitable because both pathogenic and non-pathogenic amoebae have a CPE on this cell line (Chang, 1971). Certainly, HeLa cells are markedly affected when *Acanthamoeba* is tested *in vitro* (Martin-Navarro *et al*., 2010c).

Studies involving different isolates of *Acanthamoeba* have shown the importance of routinely monitoring the virulence of strains of pathogenic amoebae that have been maintained in the laboratory for a long time (Stevens & O'Dell, 1974). Currently, the suggestion is that periodic passage of strains through animals may be necessary for the retention of a high level of virulence (Stevens & O'Dell, 1974).

The main purpose of the research recorded in this chapter was to ascertain how *Acanthamoeba* interacted with mammalian cells (MCs) *in vitro* in a particular

experimental situation, so as to assess the potential of the system for future work. A secondary consideration in carrying out the study was the fact that no information on the pathogenicity of southern African strains of *Acanthamoeba* has hitherto been obtained experimentally. In recent years, the laboratory in which this research was carried out has isolated several strains of *Acanthamoeba* from cases of keratitis and from the environment. Here, a report is provided on a comparison of the virulence of a small number of environmental isolates of *Acanthamoeba* spp. from southern Africa with some isolates from cases of keratitis in Africa, Asia and the U.K. Furthermore, strains isolated relatively recently are compared with others that have been maintained in axenic culture for several years. The significance of the amoeba:target cell ratio in relation to the comparative virulence of strains is also considered.

10.2 Materials and methods

10.2.1 *Amoebae*

The following *Acanthamoeba* isolates were used: Ac/PHL/9; Ac/PHL/17; Ac/PHL/22; Ac/PHL/23; ATCC 30868; ATCC 30873; ATCC 50676; ATCC 50677; ATCC 50684; ATCC 50685; ATCC 50686; ATCC 50687; RYD; and 435/89.[23]

Amoebae were cloned as described in Chapter 2.

Trophozoites were adapted to axenic culture in peptone-yeast extract-glucose broth (PYG)[24] (Lasman & Feinstein, 1986) at 37°C. Exponentially growing

23. See Appendix IV.

24. See Appendix II.

trophozoites were harvested from axenic cultures by low-speed centrifugation (740 g for five minutes), washed three times in saline and suspended in Dulbecco's modification of Eagle's medium (DME) containing one per cent foetal bovine serum (FBS). Counts of trophozoites were performed using a haemocytometer.

10.2.2 *Cell culture*

Two quite different MC lines were used in the cytopathogenicity experiments. These were a normal rat kidney (NRK) cell line (Highveld Biological, Johannesburg, South Africa), which is an indicator line that is widely used for assaying various factors in medium conditioned by other cells (Rizzino, 1987), and a human oesophageal squamous cell carcinoma (SNO) line, as an example of a human keratinocyte (Bey *et al*., 1976). Both MC lines were maintained at 37°C in DME supplemented with five per cent FBS. Log-phase cultures were used in all experiments without the addition of antibiotics; 90 x 10^3 cells (unless otherwise stated) in DME containing one per cent FBS were seeded into a 3 cm tissue culture dish and allowed to attach, forming a subconfluent layer. Unless otherwise stated, at no stage were the cell layers allowed to become confluent. The FBS was reduced to the minimum level that still supported NRK and SNO cell growth, that is one per cent, thus limiting the nutrients in the medium that the amoebae could feed on. Cell numbers were determined using a haemocytometer.

Initially, trial inoculations of amoebae were performed in order to maximise numbers while maintaining optimal assay conditions (Fig. 10.1; Table 10.1). As a result, two concentrations of amoebae were decided upon, namely 30 x 10^3/ml and 60 x 10^3/ml, giving amoeba:cell ratios of 1:1 and 2:1, respectively. Routinely, a total volume of 3 ml per dish was used in all experiments, and all

tests were performed at 37°C. Each experiment was run in duplicate. Experiments identifying strains of amoebae as apparently non-cytopathic were repeated six times on separate occasions.

10.2.3 *Controls*

Control dishes of both MC lines and amoebae were subjected to precisely the same experimental conditions. Inoculated experimental dishes and non-inoculated controls were examined twice daily (at 0h600 and 18h00) for the first three days and then daily thereafter, and the CPE was scored. The experiment was allowed to proceed until the control MC cultures became contact inhibited (usually after seven to eight days) or the amoebae encysted (after about eight days).

10.2.4 *Direct/indirect feeding*

Conditioned medium (CM) was produced by plating MC as above, and allowing them to grow to near confluency without changing the medium. The CM was decanted, centrifuged (5000 g), filtered (0.45 μm) and used to culture amoebae at the concentrations stated above. Amoebae cultured in fresh DME with one per cent FBS served as controls. All experiments were performed in duplicate. The time to encystment for both control and experimental amoebae was recorded.

10.2.5 *Direct/indirect CPE*

Strains of amoebae were suspended in DME containing one per cent FBS in the absence of MC cells. This was done to ascertain whether amoebae secreted anything during the period of co-culture that might cause MC disintegration. Trophozoites were allowed to encyst, after which the medium was aspirated, centrifuged and filtered as above, and the supernatant used to culture the MC lines. Controls consisted of MC in DME with one per cent FBS. Both test and

control experiments were performed in duplicate. Cell cultures were examined daily, and any differences in morphology or behaviour (or both) between the cells in the control and experimental dishes were recorded.

10.2.6 *Effect of cell density*

The possible role that the space between cells might play in CPE was studied by plating out different concentrations of both cell types. In other words, NRK cells were plated at 45 x 10^3 and 90 x 10^3, and SNO cells were plated at 90 x 10^3 and 450 x 10^3. (Because SNO cells are smaller, the amount of space between 450 x 10^3 SNO cells approximates that between 90 x 10^3 NRK cells.) These MC concentrations were then exposed to 60 x 10^3 amoebae of strains Ac/PHL/17, ATCC 30868 and ATCC 50684, respectively.

10.3 Results

10.3.1 *Amoeba:tissue culture cell ratio*

Experiments using a broad range of amoebic concentrations (Table 10.1) showed clearly that the time taken to complete the destruction of 90 x 10^3 NRK cells was dependent on the number of amoebae inoculated into the culture, that is, the amoeba:cell ratio. As is made explicit in Figure 10.1 and Table 10.1, large numbers of amoebae were able to destroy the entire plate of cells within two days, whereas the lowest numbers of amoebae had very little effect. As a result, two concentrations of trophozoites, namely 30 x 10^3/ml and 60 x 10^3/ml (Fig. 10.1; Table 10.2), were chosen for the subsequent experiments, ensuring consistent and accurate recordings of CPE.

10.3.2 *Cytopathic effect on normal rat kidney cells*

Co-culture of *Acanthamoeba* and MC demonstrated clear differences between the various amoebic strains (Fig. 10.2; Table 10.2). Certain isolates (ATCC 50677, ATCC 50685, ATCC 50686 and ATCC 50687), when inoculated at 60 x 10^3 amoebae/ml, showed a CPE within the first few hours. Compared to the controls, the NRK cells became spindle-like and contained numerous cytoplasmic granules (Figs 10.3 – 10.9). Once MC destruction had commenced, the trophozoites were commonly seen to form a "front" that accelerated the destruction of the MC cultures (Figs 10.10; 10.11). Strains ATCC 50677 and ATCC 50687 had a distinct CPE on NRK cells at concentrations as low as 30 x 10^3 amoebae/ml. Nine strains, namely Ac/PHL/22, Ac/PHL/23, RYD, ATCC 50676, ATCC 50677, ATCC 50685, ATCC 50686, ATCC 50687 and 435/89, caused complete destruction of the NRK cell monolayer within the seven-day observation period when inoculated at 60 x 10^3 amoebae/ml. A similar result was recorded at 30 x 10^3 amoebae/ml for all of these isolates except RYD. It became apparent that at low amoeba:MC ratios, certain strains were able to destroy the cells, but their rate of proliferation was low (ATCC 50686 at 10 x 10^3 and 20 x 10^3 amoebae/ml on 90 x 10^3 NRK cells – Table 10.1; and RYD at 30 x 10^3 amoebae/ml on 90 x 10^3 NRK cells – Table 10.2). This permitted the MC cultures to outgrow the amoebae and eventually restore the monolayer. Therefore, the amoebae were outcompeted in terms of the growth rate of the cells, with the result that they rounded up and died once there were no cell edges for them to attack. Strains Ac/PHL/9 and ATCC 30873 had a clear CPE on NRK cells, even though they were unable to destroy the monolayer completely within seven days. Ac/PHL/17 had a weak CPE on NRK cells, and ATCC 30868 and ATCC 50684 showed no CPE on the monolayer within the seven-day period at either 30 x 10^3 or 60 x 10^3 amoebae/ml (Table 10.2). NRK cells did not support certain strains of amoebae,

namely Ac/PHL/4, ATCC 50678, ATCC 50679, ATCC 50682, ATCC 50683 and 452/89 (Table 10.2), and the amoebae started rounding up. This was also the case for strain ATCC 50680: the cells reacted initially because the metabolic by-products of the amoebae disturbed them and the cells then re-established when the amoebae encysted. A similar result was observed even when 80,000 amoebae of strains ATCC 50682 and ATCC 50683 were used (Table 10.2).

Strains of amoebae showing either no CPE (for example ATCC 30868 and ATCC 50684) or total CPE (such as ATCC 50677 and ATCC 50686) were re-examined on half the number of NRK cells, that is, 45×10^3 (where these cells did not grow to confluency – Table 10.3), or on confluent dishes of NRK or SNO cells (Table 10.4) respectively. These trials demonstrated no change from the results originally obtained (see also "Effect of cell density", below).

10.3.3 *Verification of cytopathic effect on a human epithelial cell line*

Because of the variations in CPE reported for HeLa and Vero cell lines (Chang, 1971; John & John, 1989), the SNO cell line was used to confirm the above results (Fig. 10.2, Table 10.5). SNO is a squamous cell carcinoma line that retains most of its differentiated characteristics (Bey *et al*., 1976).

A comparison between NRK and SNO cells shows that across all the strains of amoebae tested, trophozoites were able to destroy SNO cells more rapidly than the same number of NRK cells. CPE caused the SNO cells to clump, round up and detach from the surface of the dish (Figs 10.12 – 10.15). Interestingly, the ATCC 50684 strain had no effect on either cell line, even when the number of NRK cells was reduced (Table 10.3). ATCC 30868, which did not affect 90×10^3 NRK cells, was able to destroy the same number of SNO cells when inoculated on to

the plates at 60 x 10^3 amoebae/ml (Fig. 10.2, Table 10.5). However, ATCC 30868 was able to destroy NRK cells if half the number of NRK cells was used, that is 45 x 10^3 (Table 10.3). In general, trophozoites caused SNO cells to detach from the substrate more easily than NRK cells, which displayed the ability to remain firmly attached until almost completely destroyed. SNO cells did not support strains Ac/PHL/4, ATCC 50678, ATCC 50679, ATCC 50680, ATCC 50682 and ATCC 50683. Metabolic by-products of strain ATCC 50680 disturbed the SNO cells initially, but these cells were able to re-establish once the amoebae started encysting.

10.3.4 *Direct/indirect feeding*

In order to address the question of whether the MC cultures secrete a substance on which amoebae feed, or whether amoebae feed directly on the MC cells themselves, a series of experiments was performed using CM to culture amoebae (Table 10.6). In all cases, amoebae in the experimental (CM) groups behaved similarly to those in the control groups (no CM), in that encystment occurred in each group after the same number of days. It would appear, therefore, that the MC cultures were not secreting into the medium any substances able to sustain the growth of trophozoites.

10.3.5 *Direct/indirect cytopathic effect*

Amoebic CM was tested on NRK cells to determine whether the MC response recorded (CPE) was the result of active destruction of MC cultures or a reaction to some substance secreted by the amoebae. Strains Ac/PHL/9, Ac/PHL/23, ATCC 30868, RYD, ATCC 50677 and ATCC 50685, representing isolates having a range of CPEs (see above), were tested. The cells grew to confluency in all cases, with the NRK cells in the experimental group remaining indistinguishable from

those in the control group. These data led the author to conclude that the CPE described above is the result of mechanical destruction of cells by the trophozoites. However, one cannot exclude the possibility of a non-mechanical cause(s) such as enzyme secretion by the amoebae.

10.3.6 *Effect of cell density*

Prompted by the apparent differences in the interactions between certain amoebae and the NRK as opposed to the SNO cell line, the researcher performed experiments to assess the role of MC spacing on CPE. Confluent plates of both NRK and SNO cells were destroyed by the cytopathic ATCC 50677 and ATCC 50686 strains, with the time course to total destruction being proportional to the number of cells present (Table 10.4). Moreover, the time taken for confluent plates of SNO and NRK cells to be completely destroyed by strains ATCC 50677 and ATCC 50686, was similar (Table 10.4).

Strain Ac/PHL/17 was able to eliminate 45 x 10^3 NRK cells much faster than 90 x 10^3 NRK cells, and destroyed both 90 x 10^3 and 450 x 10^3 SNO cells (Table 10.3). ATCC 30868 was able to kill 45 x 10^3 NRK and 90 x 10^3 SNO cells, whereas it had no effect on 90 x 10^3 NRK or 450 x 10^3 SNO cells (Table 10.3). The ATCC 50684 strain of *Acanthamoeba* had no CPE on either the NRK or SNO cell lines, regardless of the numbers of MC present (Table 10.3).

Table 10.1. Effect of various densities of *Acanthamoeba* (strains ATCC 50676 and ATCC 50686) on normal rat kidney (NRK) cells.

	Days						
No. amoebae/ml	**1**	**2**	**3**	**4**	**5**	**6**	**7**
500 ATCC 50676	0	0	0	0	0	0	0
500 ATCC 50686	0	0	0	0	0	0	0
3 000 ATCC 50676	0	0	0	0	0	0	0
3 000 ATCC 50686	0	0	0	0	0	0	0
10 000 ATCC 50676	1+	1+	1+	2+	2+	2+	2+
10 000 ATCC 50686	1+	1+	1+	0	0	0	0
20 000 ATCC 50676	1+	1+	2+	2+	2+	2+	2+
20 000 ATCC 50686	1+	1+	1+	0	0	0	0
30 000 ATCC 50676	1+	2+	2+	3+	3+	3+	3+
30 000 ATCC 50686	1+	3+	5+	5+	6+	6+	6+
50 000 ATCC 50676	2+	3+	5+	5+	5+	5+	5+
50 000 ATCC 50686	3+	3+	4+	4+	4+	4+	5+
60 000 ATCC 50676	2+	3+	5+	5+	6+	6+	6+
60 000 ATCC 50686	3+	5+	6+	6+	6+	6+	6+
75 000 ATCC 50676	3+	5+	5+	5+	6+	6+	6+
75 000 ATCC 50686	5+	5+	6+	6+	6+	6+	6+
115 000 ATCC 50676	3+	5+	5+	6+	6+	6+	6+
115 0040 ATCC 50686	3+	5+	6+	6+	6+	6+	6+
130 000 ATCC 50676	3+	5+	6+	6+	6+	6+	6+
130 000 ATCC 50686	5+	6+	6+	6+	6+	6+	6+
150 000 ATCC 50676	3+	5+	6+	6+	6+	6+	6+
150 000 ATCC 50676	3+	5+	6+	6+	6+	6+	6+

Unless specifically indicated, all control amoebae remained in good condition throughout the seven-day experimental period.

Key:

0	=	no CPE (normal cell monolayer).
1+	=	slight CPE with few detached cells and cells looking "spindly".
2+	=	25% of cells have been destroyed by amoebae or no longer adhere to base of dish.
3+	=	50% of cells have been destroyed by amoebae or no longer adhere to base of dish.
4+	=	75% of cells have been destroyed by amoebae or no longer adhere to base of dish.
5+	=	small patches of cells remain.
6+	=	only amoebae and cellular debris remain.

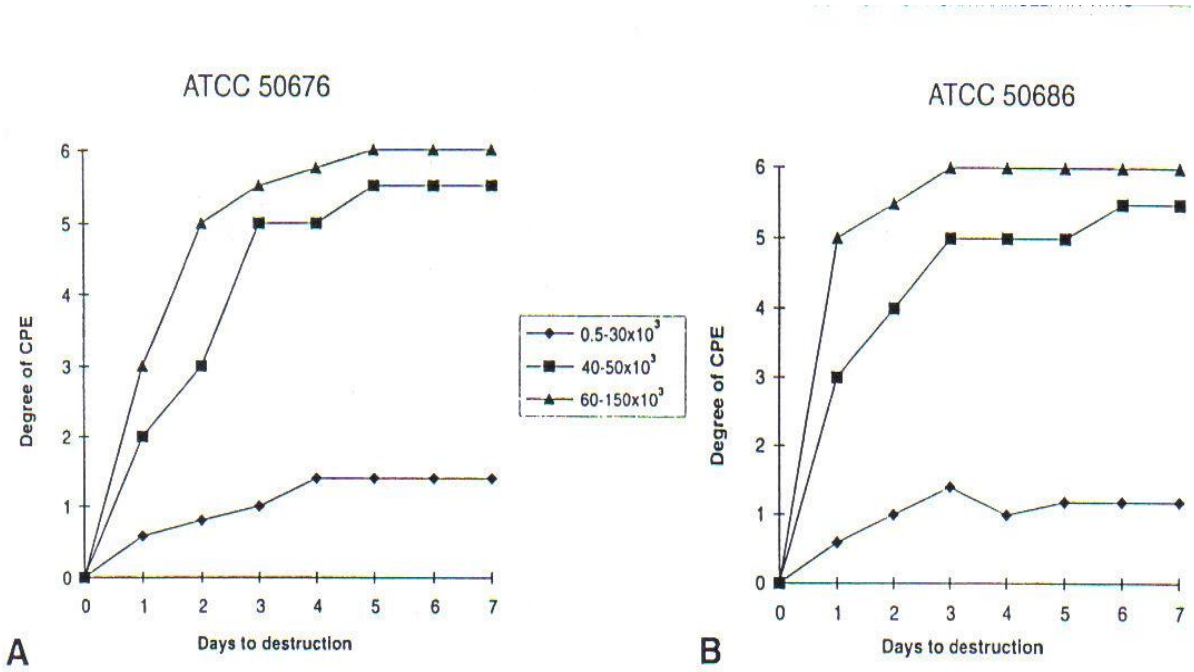


Figure 10.1. Effect of two different *Acanthamoeba* strains, ATCC 50676 and ATCC 50686, on normal rat kidney (NRK) cells. Aliquots of 90 x 10^3 NRK cells were exposed to a wide range of *Acanthamoeba* densities (0.5-150 x 10^3). In summarising the extensive data, it was found that the cytopathic effect (CPE) results for both strains, when averaged out, fell mainly into the three groups indicated by the graphs. The results identified amoebae numbers of 30 x 10^3 and 60 x 10^3 as those to be used routinely in all subsequent trials. The degree of CPE was recorded as follows:

Key:

0 = no CPE (normal cell monolayer).
1 = slight CPE (only small numbers of cells detached; and cells generally had a "spindly" appearance).
2 = 25% cell destruction or cells no longer adhering to substrate.
3 = 50% cell destruction or cells no longer adhering to substrate.
4 = 75% cell destruction or cells no longer adhering to substrate.
5 = only small patches of cells remaining.
6 = only amoebae and cell debris remaining.

Table 10.2. Cytopathic effect of strains of *Acanthamoeba* on 90 x 10^3 normal rat kidney (NRK) cells.

		Days for CPE to develop									
Strain	**Size of inoculum (amoebae x10^3/ml)**	**1 am**	**1 pm**	**2 am**	**2 pm**	**3 am**	**3 pm**	**4**	**5**	**6**	**7**
Ac/PHL/4	30	0	0	0	0	0	0	0	1+	1+	1+
	60	0	0	0	0	0	0	0	1+	1+	1+
Ac/PHL/9	30	0	0	1+	1+	1+	1+	1+	1+	1+	1+
	60	0	0	2+	3+	4+	4+	5+	5+	5+	5+
Ac/PHL/17	30	0	0	0	0	0	1+	1+	1+	1+	1+
	60	0	0	0	1+	1+	1+	1+	1+	1+	1+
Ac/PHL/22	30	0	0	3+	4+	4+	5+	5+	6+	6+	6+
	60	0	0	4+	5+	5+	6+	6+	6+	6+	6+
Ac/PHL/23	30	0	0	0	0	0	1+	2+	4+	5+	6+
	60	0	0	0	1+	2+	3+	4+	5+	6+	6+
ATCC 30868	30	0	0	0	0	0	0	0	0	0	0
	60	0	0	0	0	0	0	0	0	0	0
ATCC 30873	30	0	0	0	1+	1+	1+	1+	1+	1+	2+
	60	0	1+	1+	1+	2+	2+	2+	3+	3+	3+
ATCC 50676	30	0	1+	2+	2+	2+	2+	3+	3+	3+	3+
	60	0	2+	3+	4+	5+	5+	5+	6+	6+	6+
ATCC 50677	30	2+	4+	6+	6+	6+	6+	6+	6+	6+	6+
	60	3+	5+	6+	6+	6+	6+	6+	6+	6+	6+
ATCC 50678	30	0	0	0	0	1+	1+	1+	1+	1+	1+
	60	0	0	0	1+	1+	1+	1+	1+	1+	1+
ATCC 50679	30 60	0 0	0 0	0 0	0 0	0 0	0 0	0 1+	1+ 1+	1+ 1+	1+ 1+

Strain	**Size of inoculum (amoebae x10^3/ml)**	**1 am**	**1 pm**	**2 am**	**2 pm**	**3 am**	**3 pm**	**4**	**5**	**6**	**7**
ATCC 50680	30	2+	3+	2+	2+	1+	0	0	0	0	0
	60	3+	3+	2+	2+	1+	0	0	0	0	0
ATCC 50682	30	0	0	0	0	0	0	0	1+	1+	1+
	60	0	0	0	0	0	0	0	1+	1+	1+
	80	0	0	1+	1+	1+	1+	1+	1+	0	0
ATCC 50683	30	0	0	0	0	0	0	0	1+	1+	1+
	60	0	0	0	0	0	0	0	1+	1+	1+
	80	0	0	1+	1+	1+	1+	1+	1+	0	0
ATCC 50684	30	0	0	0	0	0	0	0	0	0	0
	60	0	0	0	0	0	0	0	0	0	0
ATCC 50685	30	0	4+	5+	6+	6+	6+	6+	6+	6+	6+
	60	1+	4+	5+	6+	6+	6+	6+	6+	6+	6+
ATCC 50686	30	0	1+	3+	3+	5+	5+	5+	6+	6+	6+
	60	1+	3+	5+	6+	6+	6+	6+	6+	6+	6+
ATCC 50687	30	1+	3+	4+	6+	6+	6+	6+	6+	6+	6+
	60	1+	4+	6+	6+	6+	6+	6+	6+	6+	6+
RYD	30	0	0	1+	1+	1+	2+	2+	0	0	0
	60	0	0	2+	3+	3+	3+	4+	5+	6+	6+
SAWL 91/2	30	0	0	0	0	0	0	0	1+	1+	1+
	60	0	0	0	0	0	0	0	1+	1+	1+
435/89	30	0	1+	1+	2+	2+	2+	3+	6+	6+	6+
	60	0	2+	3+	4+	4+	5+	5+	6+	6+	6+
452/89	30	0	0	0	0	0	0	0	0	1+	1+
	60	0	0	0	0	0	0	0	0	1+	1+

Unless specifically indicated, all control amoebae remained in good condition throughout the seven-day experimental period.

See Table 10.1 for explanation of symbols.

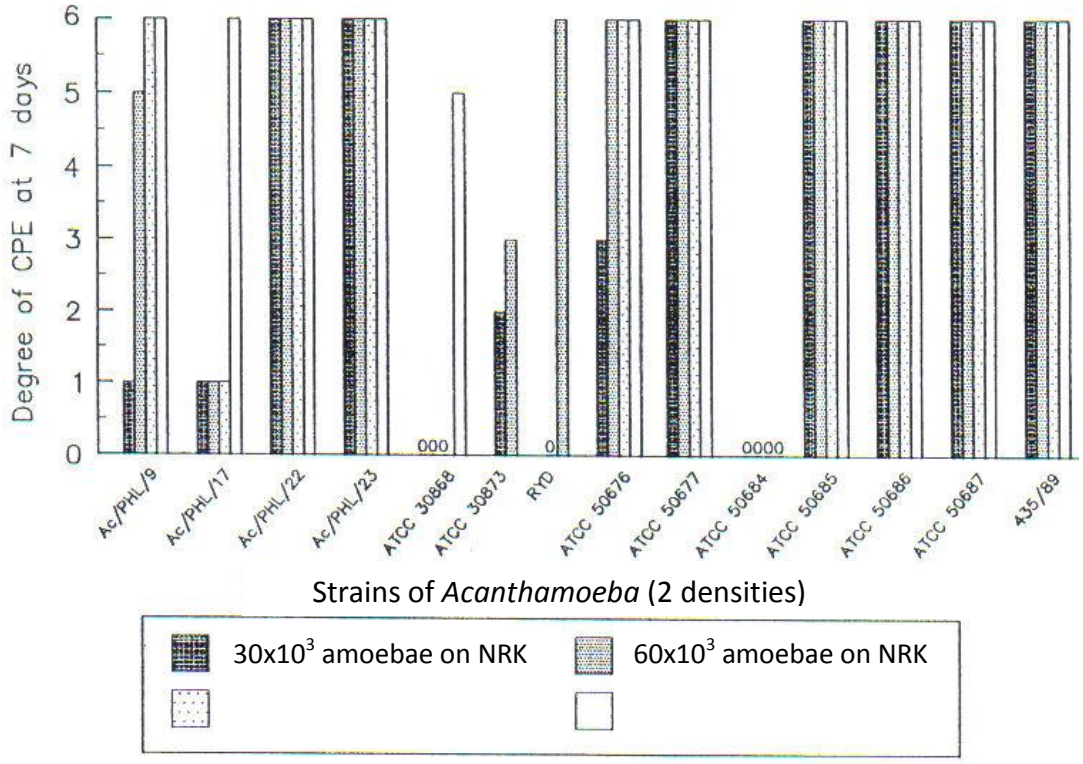


Figure 10.2. Effect of *Acanthamoeba* strains on cultured mammalian cells. Aliquots of 90 x 10^3 normal rat kidney (NRK) and/or human oesophageal squamous cell carcinoma (SNO) cells were exposed to different densities of the various strains of *Acanthamoeba*. The degree of cytopathic effect (CPE) is shown at day seven (the rating of CPE is as described in the explanation for Fig. 10.1). The control amoebae remained in excellent condition throughout the experimental period.

Key:
0 = no observed CPE over the seven-day period.

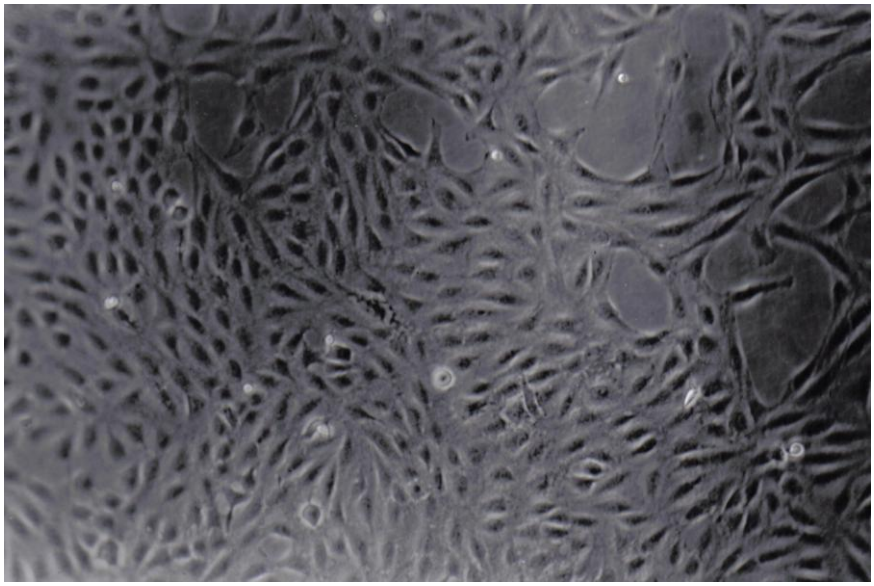
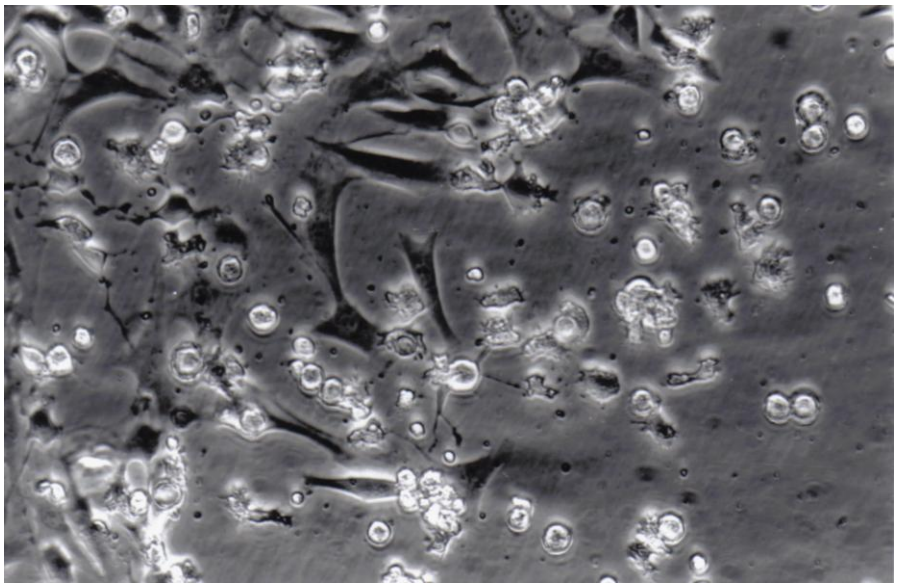
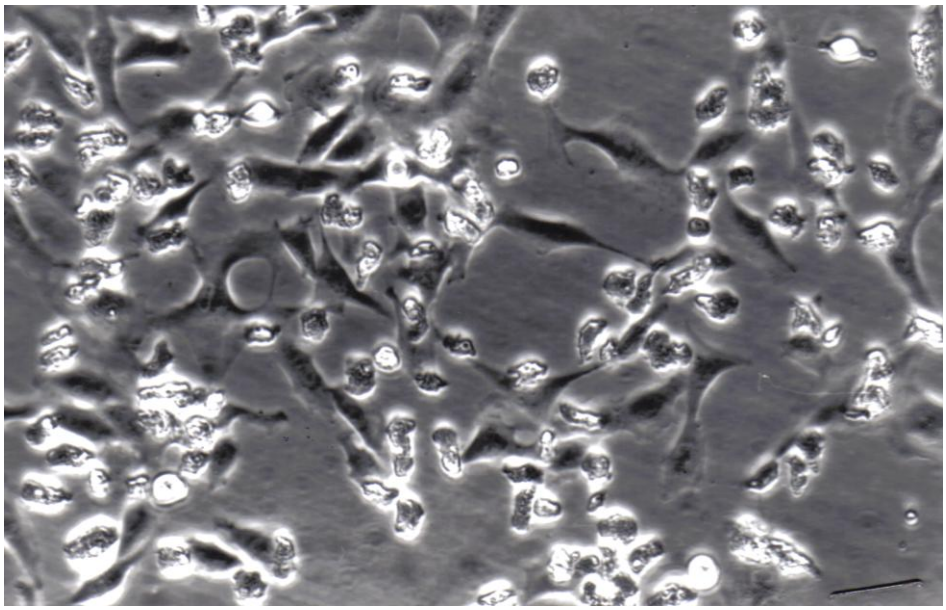


Figure 10.3. Control normal rat kidney (NRK) cells. (x 110)





Figures 10.4 and 10.5. Normal rat kidney (NRK) cells, showing the cytopathic effect (CPE) of *Acanthamoeba* strain ATCC 50676. The reaction of NRK cells to the presence of trophozoites was invariably that the cells became spindle-shaped and developed a granular cytoplasm. Note also how the cells rounded up and disintegrated; and the resultant accumulation of debris in the culture. (x 220)

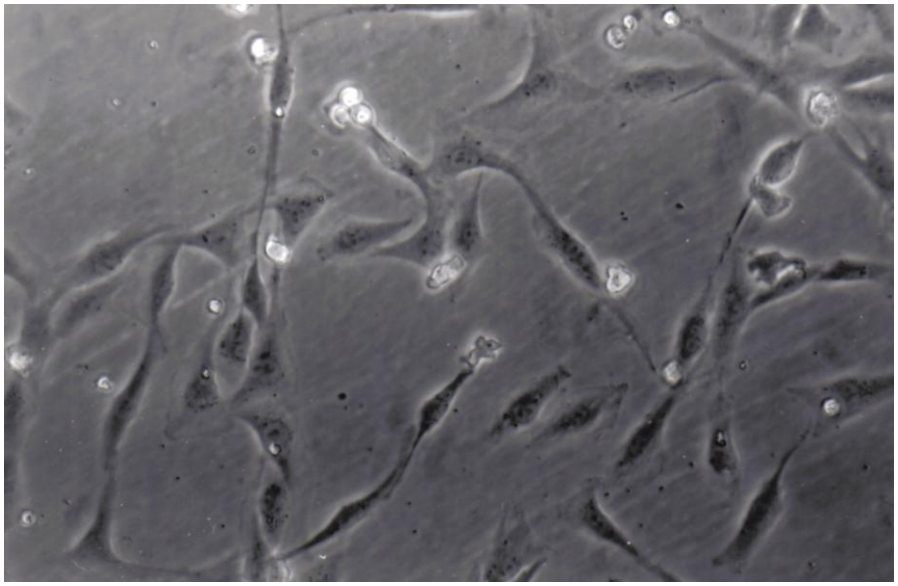


Figure 10.6. Normal rat kidney (NRK) cells, showing the cytopathic effect (CPE) of *Acanthamoeba* strain ATCC 50678. The reaction of NRK cells to the presence of trophozoites was invariably that the cells became spindle-shaped and developed a granular cytoplasm. Note also how the cells rounded up and disintegrated; and the resultant accumulation of debris in the culture. (x 220)

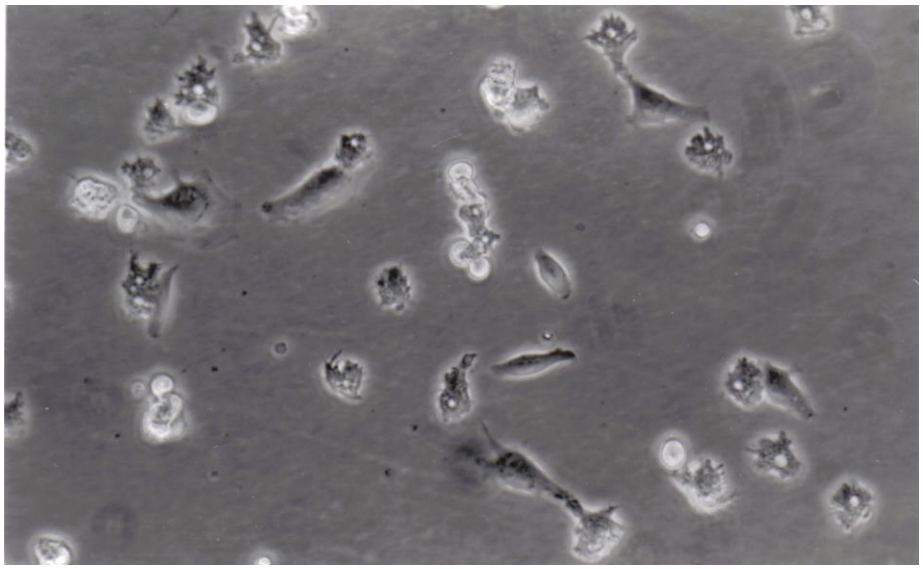
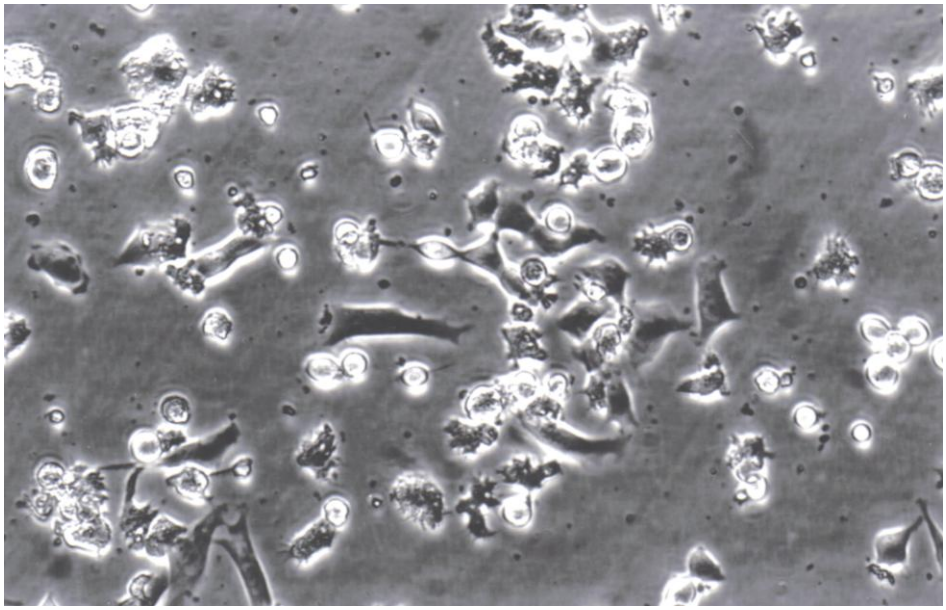
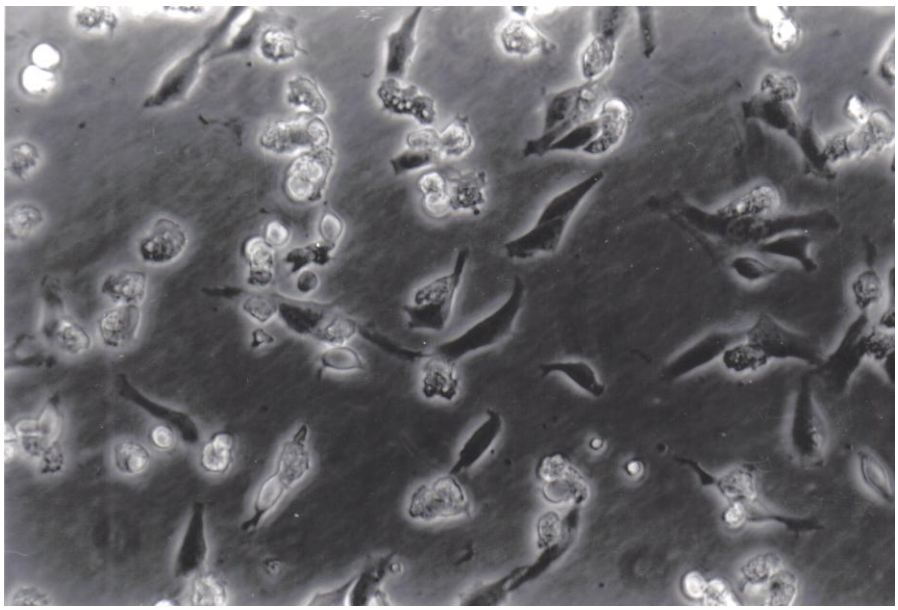


Figure 10.7. Normal rat kidney (NRK) cells, showing the cytopathic effect (CPE) of *Acanthamoeba* strain ATCC 50685. The reaction of NRK cells to the presence of trophozoites was invariably that the cells became spindle-shaped and developed a granular cytoplasm. Note also how the cells rounded up and disintegrated; and the resultant accumulation of debris in the culture. (x 220)





Figures 10.8 and 10.9. Normal rat kidney (NRK) cells, showing the cytopathic effect (CPE) of *Acanthamoeba* strain ATCC 50686. The reaction of NRK cells to the presence of trophozoites was invariably that the cells became spindle-shaped and developed a granular cytoplasm. Note also how the cells rounded up and disintegrated; and the resultant accumulation of debris in the culture. (x 220)

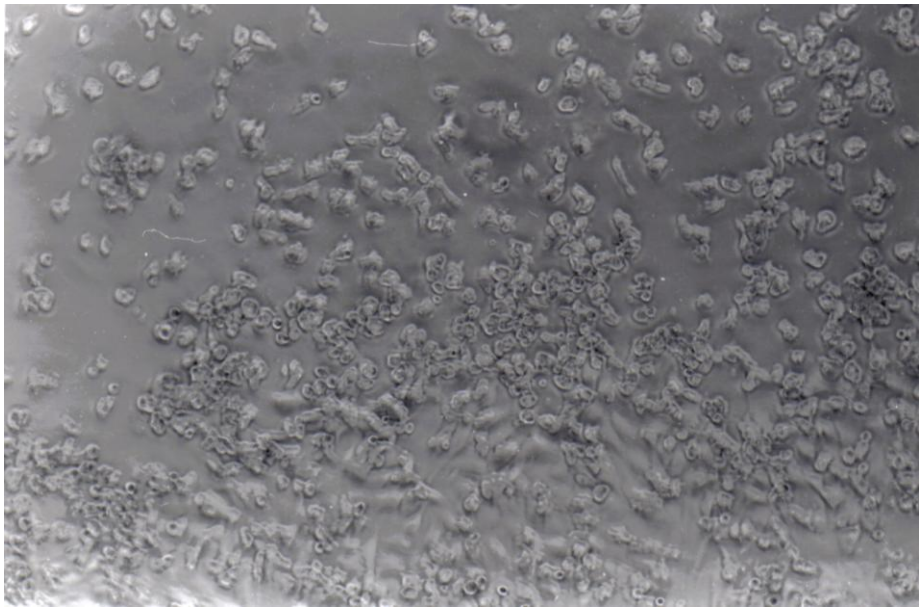


Figure 10.10. Destruction of normal rat kidney (NRK) cells by *Acanthamoeba* trophozoites of strain Ac/PHL/23. Note the "destruction front" resulting from the activity of the trophozoites. (x 110)

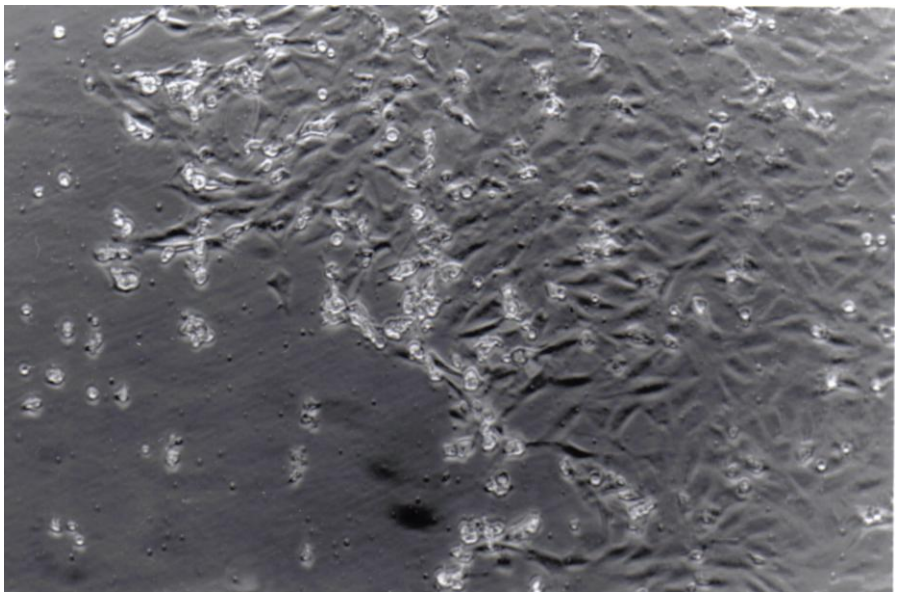


Figure 10.11. Destruction of normal rat kidney (NRK) cells by *Acanthamoeba* trophozoites of strain ATCC 50676. Note the "destruction front" resulting from the activity of the trophozoites. (x 110)

Table 10.3. Cytopathic effect of strains of *Acanthamoeba* on different densities of normal rat kidney (NRK) and squamous cell carcinoma (SNO) cells.

	Days								
60 000 amoebae/ml	**1 am**	**1 pm**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Ac/PHL/17									
45 000 NRK	1+	1+	6+	6+	6+	6+	6+	6+	6+
90 000 SNO	1+	2+	6+	6+	6+	6+	6+	6+	6+
450 000 SNO	0	0	3+	5+	6+	6+	6+	6+	6+
ATCC 30868									
45 000 NRK	0	0	0	1+	4+	4+	4+	4+	4+
90 000 SNO	0	0	0	1+	3+	4+	5+	5+	5+
450 000 SNO	0	0	0	0	0	0	0	0	0
ATCC 50684									
45 000 NRK	0	0	0	0	0	0	0	0	0
90 000 SNO	0	0	0	0	0	0	0	0	0
450 000 SNO	0	0	0	0	0	0	0	0	0

Unless specifically indicated, all control amoebae remained in good condition throughout the eight-day experimental period.

See Table 10.1 for explanation of symbols.

Table 10.4. Cytopathic effect of strains of *Acanthamoeba* on confluent plates of normal rat kidney (NRK) cells (1,2 x 10^5) and squamous cell carcinoma (SNO) cells (2 x 10^5).

	Day 1 am	**Day 1 pm**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
ATCC 50677 with NRK	0	1+	4+	5+	5+	6+
ATCC 50686 with NRK	1+	1+	5+	5+	5+	6+
ATCC 50677 with SNO	0	1+	2+	3+	5+	6+
ATCC 50686 with SNO	1+	1+	4+	4+	5+	6+

Unless specifically indicated, all control amoebae remained in good condition throughout the five-day experimental period.

See Table 10.1 for explanation of symbols.

Table 10.5. Cytopathic effect of strains of *Acanthamoeba* on squamous cell carcinoma (SNO) cells.

		Days for CPE to develop									
Strain	**Size of inoculum (amoebae x10^3/ml)**	**1 am**	**1 pm**	**2 am**	**2 pm**	**3 am**	**3 pm**	**4**	**5**	**6**	**7**
Ac/PHL/4	30	0	0	0	1+	1+	2+	2+	2+	2+	2+
	60	0	0	0	1+	1+	2+	2+	2+	2+	2+
Ac/PHL/9	30	2+	3+	6+	6+	6+	6+	6+	6+	6+	6+
	60	3+	4+	6+	6+	6+	6+	6+	6+	6+	6+
Ac/PHL/17	30	60	0	0	0	0	0	1+	1+	1+	1+
	60	1+	2+	6+	6+	6+	6+	6+	6+	6+	6+
Ac/PHL/22	30	0	1+	4+	5+	6+	6+	6+	6+	6+	6+
	60	2+	4+	6+	6+	6+	6+	6+	6+	6+	6+
Ac/PHL/23	30	0	0	0	1+	3+	4+	6+	6+	6+	6+
	60	0	0	2+	3+	5+	6+	6+	6+	6+	6+
ATCC 30868	30	0	0	0	0	0	0	0	0	0	0
	60	0	0	0	0	1+	1+	3+	4+	5+	5+
ATCC 50676	30	1+	2+	6+	6+	6+	6+	6+	6+	6+	6+
	60	1+	3+	6+	6+	6+	6+	6+	6+	6+	6+
ATCC 50677	30	5+	6+	6+	6+	6+	6+	6+	6+	6+	6+
	60	5+	6+	6+	6+	6+	6+	6+	6+	6+	6+
ATCC 50678	30	0	0	2+	2+	3+	3+	3+	3+	3+	3+
	60	0	2+	2+	2+	3+	3+	3+	3+	3+	3+
ATCC 50679	30	0	1+	1+	1+	1+	1+	1+	1+	1+	1+
	60	0	1+	1+	1+	1+	1+	1+	1+	1+	1+
ATCC 50680	30	2+	3+	4+	4+	3+	2+	2+	1+	1+	1+
	60	3+	3+	4+	4+	3+	2+	2+	1+	1+	1+

Strain	**Size of inoculum (amoebae x10^3/ml)**	**1 am**	**1 pm**	**2 am**	**2 pm**	**3 am**	**3 pm**	**4**	**5**	**6**	**7**
ATCC 50682	30	0	0	0	0	0	0	0	1+	1+	1+
	60	0	0	0	0	0	0	0	1+	1+	1+
ATCC 50683	30	0	0	0	0	0	0	0	1+	1+	1+
	60	0	0	0	0	0	0	0	1+	1+	1+
ATCC 50684	30	0	0	0	0	0	0	0	0	0	0
	60	0	0	0	0	0	0	0	0	0	0
ATCC 50685	30	2+	5+	6+	6+	6+	6+	6+	6+	6+	6+
	60	3+	5+	6+	6+	6+	6+	6+	6+	6+	6+
ATCC 50686	30	5+	6+	6+	6+	6+	6+	6+	6+	6+	6+
	60	5+	6+	6+	6+	6+	6+	6+	6+	6+	6+
ATCC 50687	30	2+	4+	5+	6+	6+	6+	6+	6+	6+	6+
	60	3+	4+	6+	6+	6+	6+	6+	6+	6+	6+
435/89	30	0	1+	2+	2+	3+	6+	6+	6+	6+	6+
	60	1+	3+	4+	4+	5+	5+	6+	6+	6+	6+

Unless specifically indicated, all control amoebae remained in good condition throughout the seven-day experimental period.

See Table 10.1 for explanation of symbols.

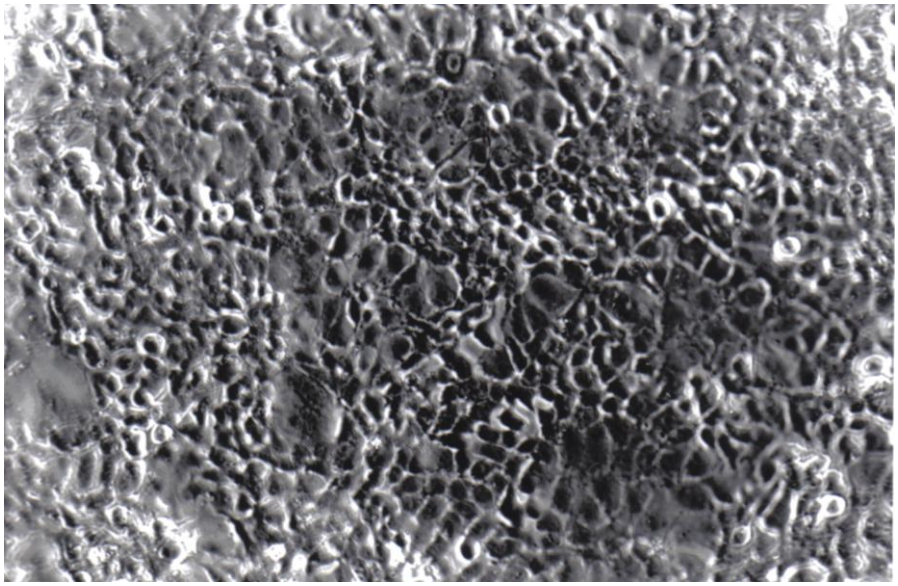


Figure 10.12. Control human oesophageal squamous cell carcinoma (SNO) cells. (x 110)

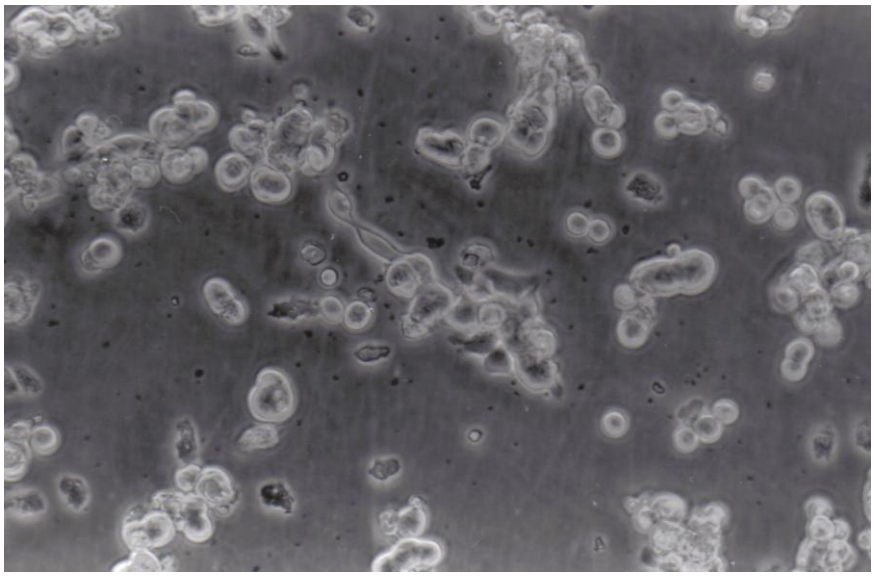
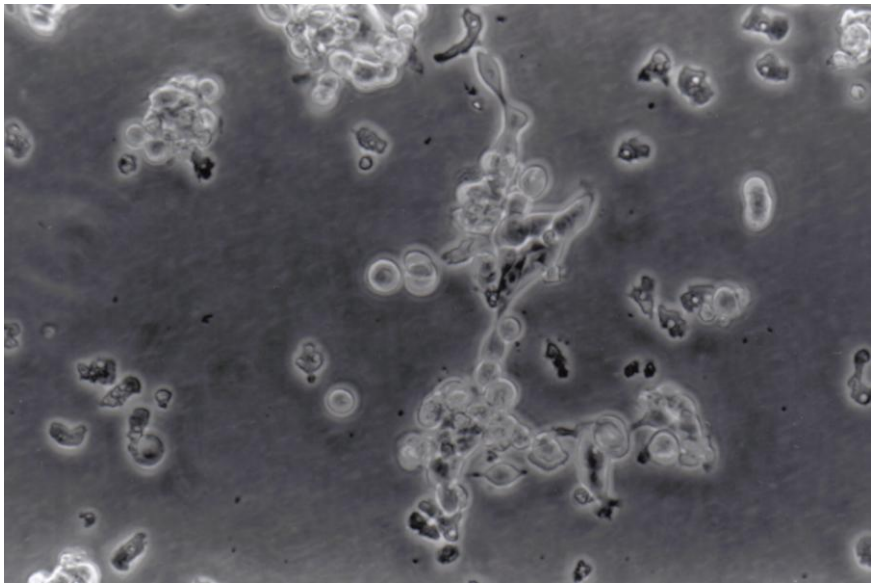
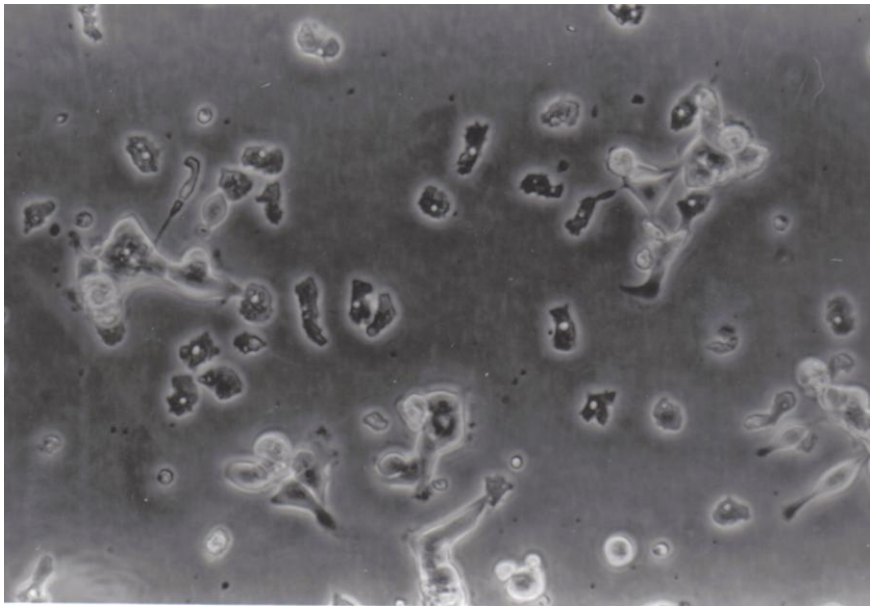


Figure 10.13. Destruction of human oesophageal squamous cell carcinoma (SNO) cells by *Acanthamoeba* trophozoites of strain Ac/PHL/9. The cells became clumped, rounded up and detached from the substrate. Furthermore, debris was seen to accumulate in the culture. (x 220)





Figures 10.14 and 10.15. Destruction of human oesophageal squamous cell carcinoma (SNO) cells by *Acanthamoeba* trophozoites of strain ATCC 50676. The cells became clumped, rounded up and detached from the substrate. Furthermore, debris was seen to accumulate in the culture. (x 220)

Table 10.6. Effect of conditioned medium from 90 000 normal rat kidney (NRK) cells on 60 000 amoebae of different strains.

	Days							
Strain of amoeba	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Ac/PHL/9	+++	+++	+++	++	++	++	+	+
Ac/PHL/9 control	+++	+++	+++	++	++	++	+	+
Ac/PHL/17	+++	++	++	++	+	+	-	-
Ac/PHL/17 control	+++	++	++	++	+	+	-	-
ATCC 30868	+++	+++	++	++	++	+	+	-
ATCC 30868 control	+++	+++	++	++	++	+	+	-
ATCC 50676	+++	+++	+++	++	+	+	+	-
ATCC 50676 control	+++	+++	+++	++	+	+	+	-
ATCC 50677	+++	+++	+++	++	++	++	++	+
ATCC 50677 control	+++	+++	+++	++	++	++	++	+
ATCC 50684	+++	+++	+++	+++	++	+	+	-
ATCC 50684 control	+++	+++	+++	+++	++	+	+	-
ATCC 50686	+++	+++	+++	+++	+++	++	++	+
ATCC 50686 control	+++	+++	+++	+++	+++	++	++	+

Key:

+ = few trophozoites with many cysts.
++ = trophozoites present with a few cysts.
+++ = all trophozoites present.
- = all trophozoites encysted.

10.4 Discussion

10.4.1 *Isolates used*

In relation to the primary objective of observing the interaction between *Acanthamoeba* and cultured MCs, the precise characterisation and geographical origin of the *Acanthamoeba* strains tested were not considered to be important. Therefore, the various clinical and environmental isolates were chosen largely at random.

No attempt was made to give subgeneric taxonomic designations for all the *Acanthamoeba* strains used. Results based on biochemical data and morphological criteria for conventional specific taxa do not always correlate, a phenomenon also observed by other researchers (Page, 1988; Badenoch *et al*., 1995). With time and advances in knowledge, many specific identifications given in the literature may in retrospect prove to be meaningless, particularly in such equivocal cases as are referred to above. However, the isolates tested in this study have been deposited in the American Type Culture Collection (ATCC) so that they are readily available to other workers.

10.4.2 *CPE determinants*

Various factors have been thought to determine or at least influence the CPE of *Acanthamoeba* (da Rocha-Azevedo & e Silva-Filho, 2007; Cao *et al.*, 2008; Saravanan *et al.*, 2008; da Rocha-Azevedo *et al.*, 2009; Köhsler *et al.*, 2009). These include incubation temperature, the virulence of the amoebae, the concentration and age of the inoculum (Stevens & O'Dell, 1974), incubation time (Larkin *et al*., 1991), and the susceptibility of the type of cell monolayer. More specifically, variations in growth response and differences in probability of encystment under similar conditions have been suggested as reasons for the

occurrence of differences in virulence between pathogenic and non-pathogenic strains (Culbertson, 1971). CPE studies using *Naegleria* have shown that differences between the axenic media used can result in slower growth of amoebae, target cell destruction by piecemeal ingestion rather than by contact-dependent lysis, and less resistance to complement-mediated lysis (Marciano-Cabral & Toney, 1994).

De Jonckheere (1980) is of the opinion that pathogenicity testing should be performed as soon after isolation as possible. The environmental strains isolated by the present researcher (ATCC 50685, ATCC 50686 and ATCC 50687) were kept in axenic culture for seven years and, as has clearly been shown above, are highly cytopathogenic *in vitro*. Furthermore, it is of interest to note that these sewage isolates had not been passaged *in vivo* or *in vitro* in association with MCs, but are nonetheless highly cytopathic. Similarly, none of the corneal strains used had been passaged through a mammalian system. Regardless of when they were originally isolated, the majority of strains showed CPE within the first few hours of co-culture with MCs. However, reported variations in CPE are sufficient to make obligatory the routine monitoring of pathogenic amoebae maintained under culture conditions (Stevens & O'Dell, 1974; John & John, 1994; Gupta & Das, 1999). This requires a screening system that is fast and reproducible. Both of these criteria are satisfied by the system used in the experiments described in this chapter.

10.4.3 *Direct/indirect cytopathic effect*

There has been some discussion regarding the precise method of destruction of mammalian cells by amoebae, with evidence pointing toward there being both an enzymatic and a mechanical component (Dunnebacke & Schuster, 1977; Cursons

& Brown, 1978; Cursons *et al*., 1978; Marciano-Cabral *et al*., 1982; Dunnebacke & Schuster, 1985; Dunnebacke & Dixon, 1989; He *et al*., 1990; McLaughlin *et al*., 1991; Sissons *et* al., 2005; Martin-Navarro *et al.,* 2010c). The results of the experiments performed in this study with CM from both MCs and amoebic cells seem to indicate that unless amoebae secrete an enzyme or enzymes only in the presence of MCs, it is mainly mechanical destruction that is occurring. Alternatively, the *Acanthamoeba* trophozoites may not be secreting sufficient cytopathic material into the medium to have an effect. These findings are in agreement with those of Dunnebacke & Schuster (1977), who reported that the presence of intracellular cytopathic material is specific for isolates of *Naegleria*, but not for those of *Acanthamoeba*.

However, John & John (1989), working with *N. fowleri*, found no evidence that excreted cytolytic products were involved in the destruction of MC cultures. That is, CPE appeared to be the result of direct contact between amoebae and MC. Stopak *et al*. (1991) showed that acanthamoebic growth is supported by confluent layers of human corneal epithelial cells and stromal keratocytes, but not stromal homogenates. Their results suggest that *Acanthamoeba* trophozoites depend on the cellular component of the cornea as a substrate for growth. They postulate that *Acanthamoeba* feeds directly on the dense epithelial cell layer, causing disruption that eventually provides access to the corneal stroma for the organisms. Cursons *et al*. (1978) consider it likely that the secretion of an enzyme constitutes the initial step whereby host tissue is prepared for endocytosis by these amoebae. Fulford & Marciano-Cabral (1986) have suggested that phospholipases A and C, as well as other as yet unidentified cytolytic factors, may be responsible for producing 51Cr release from target cells by the soluble fraction of *N. fowleri* extracts. In addition, filtrate from axenically cultured *Acanthamoeba* was shown

to produce a CPE on Vero cells, an effect attributed to phospholipase (Visvesvara & Balamuth, 1975). The studies of He *et al*. (1990) and Mitro *et al*. (1994) strongly suggest a role for parasite-released proteinases that degrade collagen in acanthamoebic keratitis.

In contrast, Mitro *et al*. (1994) point out that the possibility that neutrophil-associated proteinases contribute to or cause pathology, has not yet been excluded. Cao *et al.* (1998) have shown that the lectin-mediated adhesion of *Acanthamoeba* to host cells is a prerequisite for the amoeba-induced cytolysis of target cells, and have implicated a contact-dependent metalloproteinase in the cytopathogenic mechanisms of *Acanthamoeba*. *N*-acetyl-D-glucosamine (GlcNAc), which does not inhibit adhesion of amoebae to host cells, is also an inhibitor of amoeba-induced CPE (Cao *et al*., 1998). It appears that GlcNAc inhibits CPE indirectly by influencing the expression and/or secretion of the molecules involved in cytopathogenic mechanisms of *Acanthamoeba* (Cao *et al*., 1998). Niederkorn *et al*. (1999b) state that the pathogenesis of acanthamoebic keratitis involves parasite-mediated cytolysis and phagocytosis of corneal epithelial cells, and induction of programmed cell death. *Acanthamoeba* spp. secrete a variety of proteases which may facilitate cytolysis of the corneal epithelium, invasion of the extracellular matrix, and dissolution of the corneal stromal matrix (Niederkorn *et al*., 1999b).

Leher *et al.* (1998) have shown that oral immunisation of mice with aqueous *Acanthamoeba* antigen, mixed with cholera toxin, induced the production of parasite-specific IgA in mucosal secretions, and prevented corneal infection. Leher *et al*. (1999) showed that monoclonal anti-*Acanthamoeba* IgA antibodies can protect against acanthamoebic keratitis, and suggest that this occurs by

inhibiting adhesion of the parasite to the corneal epithelium. It seems that the immune system is incapable of mounting an effective defence against *Acanthamoeba* trophozoites once they have attached to the corneal surface (Leher *et al*., 1998).

Being aware of the tendency of substrate-dependent MCs to detach themselves in unfavourable conditions, especially those cells susceptible to contact inhibition of growth, the researcher was careful to divorce the problems associated with mammalian cell density from acanthamoebic CPE. CPE is directly related to the amoeba:mammalian cell ratio, in that the more amoebae inoculated, the more rapidly CPE is produced (John & John, 1989). Data reported by Larkin *et al*. (1991) indicate that there is a threshold concentration of *Acanthamoeba* necessary for induction of CPE in corneal cell cultures. Although the amoeba:cell ratio directly affects CPE (John & John, 1989; Larkin *et al*., 1991), a standardised ratio cannot be universally applied, especially when dealing with mammalian cells of disparate sizes. In fact, CPE may be related only indirectly to the amoeba:cell ratio, in that the amount of space between attached epithelial cells may be of greater importance than the total number of cells present. Differences in susceptibility between MC types may, therefore, be nothing more than a reflection of the ease with which amoebae can gain access to the edges of adherent cells. In this respect, SNO cells are much smaller than NRK cells, and when equivalent numbers of these two lines were used, SNO cells were destroyed faster than NRK cells. Tyndall *et al*. (1979) found that multiplication of amoebae was stimulated concomitantly with destruction of mammalian cells. This happened when the number of amoebae grown in a designated MC culture increased from 2:1 to 3:1, compared with those grown in the same culture medium in the absence of mammalian cells. The most cytopathogenic of the *Acanthamoeba* strains tested in

this study destroyed confluent plates of both NRK and SNO cells equally successfully. Interestingly, destruction by trophozoites of NRK and SNO cells occurred within the same time-span.

In conclusion, the use of MC culture is an accurate, rapid and useful means of distinguishing cytopathic from non-cytopathic strains of *Acanthamoeba*. The experiments recorded in this chapter demonstrated that not all strains of *Acanthamoeba* lose cytopathogenicity after they have been cultivated axenically over extended periods of time. The need to assess the MC lines used by taking into account differences in cell size (which affects the amount of space between cells) as well as the number of cells plated out and their state of confluence, has also been emphasised. Finally, the study results suggest that destruction of MC cultures may be mechanical rather than enzymatic in nature.

CHAPTER ELEVEN – CYTOPATHOGENICITY OF A STRAIN OF *MASTIGINA* FOR TWO MAMMALIAN CELL LINES

11.1 Determination of the cytopathogenicity of *Mastigina*

The isolation of a strain of *Mastigina* (identified by T.A. Nerad of the American Type Culture Collection, Rockville, Maryland, USA) from a contact lens case, a soft contact lens and a bottle kept for storage of saline for use with contact lenses, implicated this protist as the possible causative agent in a case of ocular infection in a South African patient (Niszl & Markus, 1991).

In the case of *Acanthamoeba*, determination of the cytopathic effect (CPE) in cell culture is reported to have become the most reliable means of ascertaining whether or not a strain is pathogenic (Cursons & Brown, 1978; De Jonckheere, 1980). Hitherto, the choice of cell line has not appeared to be important (Cursons & Brown, 1978; Larkin *et al*., 1991), although HeLa cells are possibly unsuitable because both pathogenic and non-pathogenic amoebae have a CPE on this cell line (Chang, 1971).

The researcher wanted to see how different densities of a South African isolate of *Mastigina* sp. interacted with two different mammalian cells lines *in vitro*, following her cytopathogenicity research that was done on *Acanthamoeba*. A secondary consideration in carrying out the study, was that no information on the cytopathogenicity of a *Mastigina* from southern Africa or, to the writer's knowledge, from anywhere else in the world, had hitherto been obtained experimentally.

11.2 Materials and methods

11.2.1 Mastigina *organisms*

Mastigina was cloned in the same way as *Acanthamoeba* sp. (Chapter 2).

Organisms were adapted to axenic culture in peptone-yeast extract glucose broth (PYG)[25] (Lasman & Feinstein, 1986) at 37°C. The growth of trophozoites (amoeboid and flagellated forms are referred to together in this thesis as "trophozoites", as these stages are rapidly interchangeable) in PYG medium was very poor. Exponentially growing organisms were harvested from axenic cultures by low speed centrifugation (740 g for five minutes), washed three times in saline and suspended in Dulbecco's modification of Eagle's medium (DME) containing one per cent foetal bovine serum (FBS). Trophozoite counts were performed using a haemocytometer.

11.2.2 *Cell culture*

Two quite different mammalian cell (MC) lines were used in the cytopathogenicity experiments. These were a normal rat kidney (NRK) cell line (Highveld Biological, Johannesburg, South Africa), which is an indicator line widely used for assaying various factors in medium conditioned by other cells (Rizzino, 1987), and a human oesophageal squamous cell carcinoma (SNO) line as an example of a human keratinocyte (Bey *et al*., 1976). Both MC lines were maintained at 37°C in DME supplemented with five per cent FBS. Log phase

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25. See Appendix II.

cultures were used in all experiments without the addition of antibiotics; 90 x 10^3 cells in DME containing one per cent FBS were seeded into a 3 cm tissue culture dish and allowed to attach, forming a subconfluent layer. At no stage were the cell layers allowed to become confluent. The FBS was reduced to the minimum level that still supported NRK and SNO cell growth, that is, one per cent, thus limiting nutrients in the medium that the *Mastigina* sp. might feed on. Cell numbers were determined using a haemocytometer.

Routinely, a total volume of 3 ml per dish was used in all experiments, and all tests were performed at 37°C. Each experiment was run in duplicate.

11.2.3 *Controls*

Control dishes of both MC lines and *Mastigina* organisms were subjected to precisely the same experimental conditions. Inoculated experimental dishes and non-inoculated controls were examined daily, and the CPE was scored. The experiment was allowed to proceed until the control MC cultures became contact-inhibited (usually after seven to eight days) or the trophozoites encysted (after about seven days).

11.3 Results

11.3.1 *Cytopathic effect on normal rat kidney cells*

Compared to the controls, the NRK cells became spindle-like and contained numerous cytoplasmic granules (Figs 11.1 – 11.3). It became apparent that at low *Mastigina*:MC ratios, this strain was able to destroy some of the cells, but the rate of proliferation of *Mastigina* was low (Table 11.1). This permitted the MC cultures to outgrow the trophozoites and eventually restore the monolayer. This

strain of *Mastigina* had a clear CPE on NRK cells, even though they were unable to destroy the monolayer within seven days (Table 11.1).

11.3.2 *Verification of cytopathic effect on human epithelial cell line*

Because of the variations in CPE reported for HeLa and Vero cells lines (Chang, 1971; John & John, 1989), the SNO cell line was used to confirm the above results (Table 11.1). SNO is a squamous cell carcinoma line that retains most of its differentiated characteristics (Bey *et al*., 1976).

A comparison between NRK and SNO cells shows that trophozoites of this strain of *Mastigina* were able to destroy SNO cells more rapidly than the same number of NRK cells. CPE caused the SNO cells to clump, round up and detach from the surface of the dish. In general, trophozoites caused SNO cells to detach from the substrate more easily than NRK cells, which displayed the ability to remain firmly attached until almost completely destroyed.

11.3.3 *Trophozoite:tissue culture cell ratio*

The concentrations of trophozoites used, represent *Mastigina*:cell ratios of 1:1, 2:1 and 3:1, respectively. Experiments using these three different concentrations of *Mastigina* organisms (Table 11.1) showed clearly that the time to destruction of 90×10^3 NRK and SNO cells was dependent on the number of trophozoites inoculated into the culture; in other words, the *Mastigina*:cell ratio.

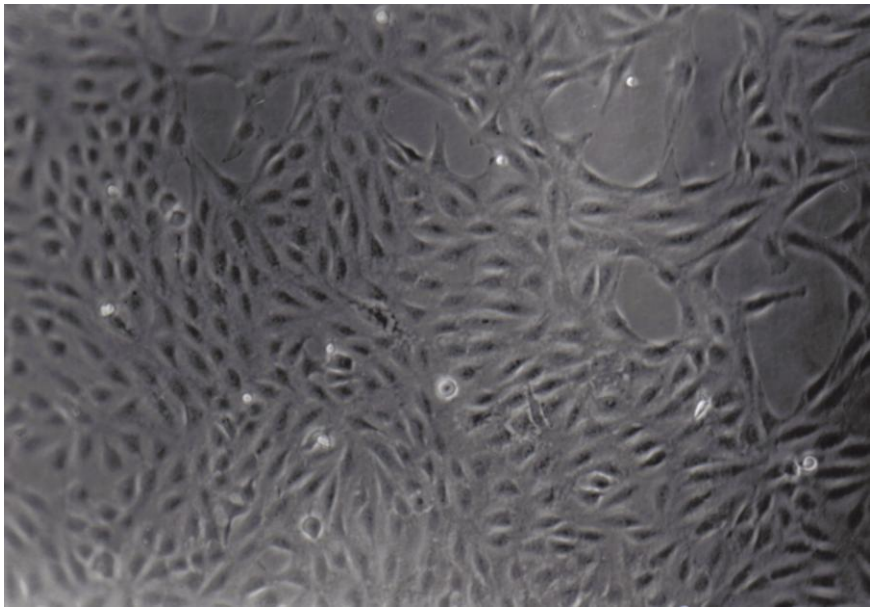
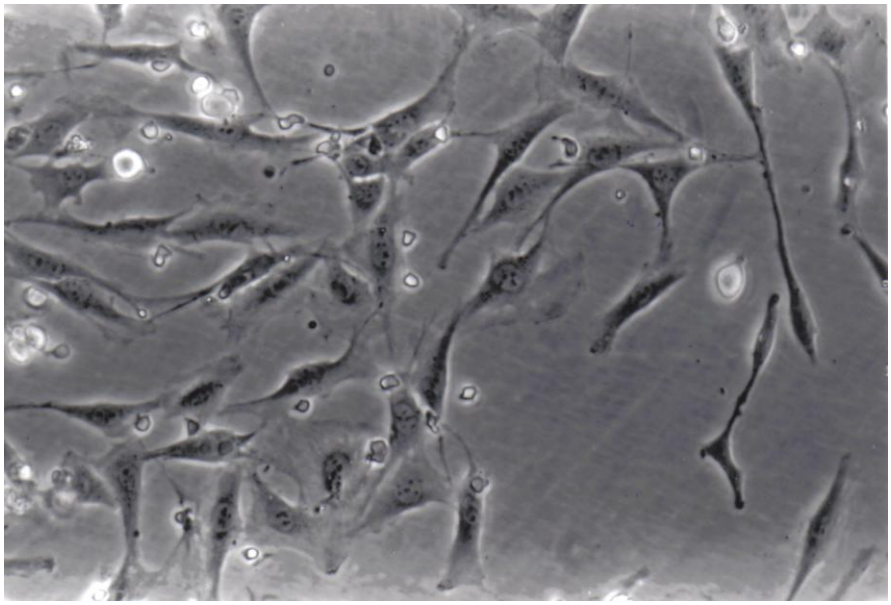
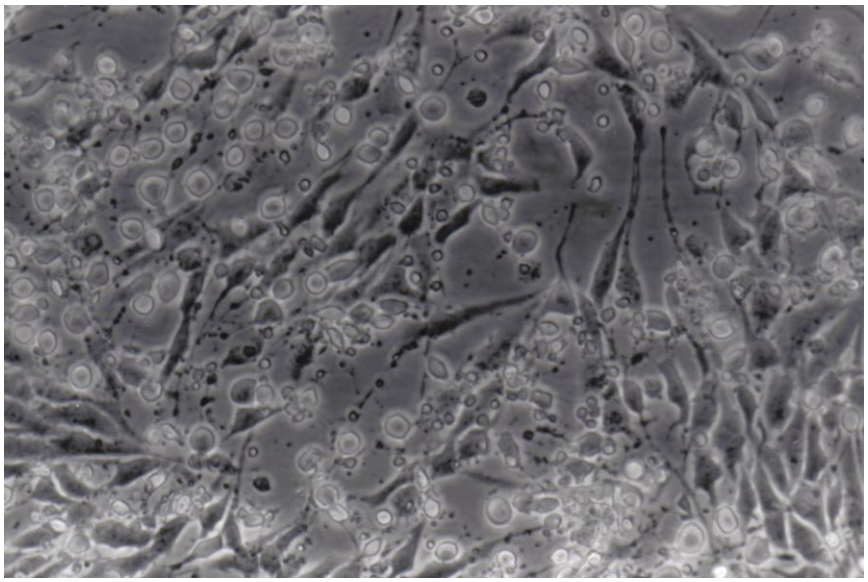


Figure 11.1. Control normal rat kidney (NRK) cells. (x 110)





Figures 11.2 and 11.3. Normal rat kidney (NRK) cells, showing the cytopathic effect (CPE) of *Mastigina* strain SAWL 91/2. The reaction of NRK cells to the presence of trophozoites was invariably that the cells became spindle-shaped and developed a granular cytoplasm. Note also how the cells rounded up and disintegrated; and the resultant accumulation of debris in the culture. (x 220)

Table 11.1. Cytopathic effect of different densities of *Mastigina* strain SAWL 91/2 on 90 000 normal rat kidney (NRK) cells and on 90 000 squamous cell carcinoma (SNO) cells.

	Days						
Density of *Mastigina*	**1**	**2**	**3**	**4**	**5**	**6**	**7**
30 000/ml on NRK	0	0	0	1+	1+	0	0
30 000/ml on SNO	0	1+	1+	2+	2+	2+	1+
60 000/ml on NRK	0	0	1+	2+	2+	2+	2+
60 000/ml on SNO	0	2+	2+	2+	3+	3+	3+
90 000/ml on NRK	0	2+	2+	2+	2+	3+	3+
90 000/ml on SNO	2+	2+	2+	4+	4+	5+	5+

Unless specifically indicated, all control organisms remained in good condition throughout the seven-day experimental period.

Key:

0 = no CPE (normal cell monolayer).
1+ = slight CPE with few detached cells and cells looking “spindly".
2+ = 25% of cells destroyed by *Mastigina* organisms or no longer adhering to base of dish.
3+ = 50% of cells destroyed by *Mastigina* organisms or no longer adhering to base of dish.
4+ = 75% of cells destroyed by *Mastigina* organisms or no longer adhering to base of dish.
5+ = small patches of cells remain.

11.4 Discussion

CPE is directly related to the amoeba:mammalian cell ratio in that the more amoebae inoculated, the more rapidly CPE is produced (John & John, 1989). The work done with *Mastigina* in this study shows that this observation also applies to the protostelid strain used. Data reported by Larkin *et al.* (1991) indicate that there is a threshold concentration of *Acanthamoeba* necessary for induction of CPE in corneal cell cultures. CPE may be only indirectly related to the trophozoite:cell ratio in that the amount of space between attached epithelial cells might be of greater importance than the total number of cells. Differences in susceptibility between MC types may, therefore, be nothing more than a reflection of the ease with which trophozoites can gain access to the edges of adherent cells. In this respect, SNO cells are much smaller than NRK cells; when equivalent numbers of these two lines were used for *Mastigina*, SNO cells were destroyed faster than NRK cells. Tyndall *et al*. (1979) found that multiplication of amoebae was stimulated concomitantly with destruction of mammalian cells. This happened when the number of amoebae grown in a designated MC culture increased from 2:1 to 3:1 compared to those grown in the same culture medium in the absence of mammalian cells.

In conclusion, the use of MC culture has given an indication of the cytopathogenicity of the strain of *Mastigina* tested. The need to assess MC lines used by taking into account differences in cell size (which affects the amount of space between cells) has also been emphasised.

CHAPTER TWELVE – CELLULOSE ACETATE AND POLYACRYLAMIDE GEL ELECTROPHORESIS ON STRAINS OF *ACANTHAMOEBA* AND A STRAIN OF *MASTIGINA*

12.1 The need for rapid identification of strains of *Acanthamoeba* in cases of ocular infection

In view of the serious consequences of ocular infection with *Acanthamoeba*, a rapid means of identification to the specific level is required so that the most effective drug combination for the causative species can be used timeously. Isoenzymatic typing of protozoa, especially *Acanthamoeba* (De Jonckheere, 1983; Moura *et al*., 1992), *Naegleria* (Warhurst & Thomas, 1978; De Jonckheere, 1982; Pernin *et al*., 1985; Moss *et al*., 1988; Pernin & Grelaud, 1989), *Giardia* (Moss *et al*., 1992), *Vahlkampfia* (Daggett & Nerad, 1983) and *Entamoeba* (Mathews *et al*., 1983; Moss & Mathews, 1987), using several enzymes, has proved to be a highly discriminating method for identification to the species level. Isoenzyme analysis is also useful for comparison of protozoa in clinical and phylogenetic studies (Weekers & De Jonckheere, 1997). However, De Jonckheere (1983) cautions that different zymodemes may exist within a species of *Acanthamoeba*, as is the case for the genus *Naegleria*.

Identification of *Acanthamoeba* by isoenzymes does not always correspond with the traditional morphological species designation (Tyndall *et al*., 1979; Daggett *et al*., 1980; Costas & Griffiths, 1984). De Jonckheere & Michel (1988) found, using agarose isoelectric focusing, that the identification by isoenzymes of 19 strains of *Acanthamoeba* differed from that obtained by cystic morphology, but that it correlated with growth at 40°C.

Moura *et al*. (1992) mention that *Acanthamoeba* of groups I and III can easily be differentiated from one another, based only on hexokinase (HK), acid phosphatase (ACP) and esterase (EST) profiles; which can reduce the investigator's time input, effort expended, materials used and expenses. They suggest that if numerical analysis of data is planned or other strains are isolated that cannot be differentiated by these three enzymes, then other enzymes such as phosphoglucomutase (PGM), malic enzyme (ME) and glucose-6-phosphate dehydrogenase (G6PD) may be used.

Cellulose acetate electrophoresis (CAE) has been successfully employed for biochemical identification of *Leishmania* (Kreutzer & Christensen, 1980) and for characterisation of enzymes of *Trypanosoma cruzi* (Lanham *et al*., 1981). The relative results of starch-gel electrophoresis (SGE) and CAE of 18 enzymes for three stocks of zymodemes of *Trypanosoma cruzi* were compared by Lanham *et al*. (1981), who found that the CAE zymograms of 16 enzymes were as good as, and in some cases better than, those of SGE, although two enzymes gave diffuse or weak enzyme patterns. There was increased CAE resolution for ME and G6PD, which enabled all three zymodemes to be distinguished (Lanham *et al*., 1981). These researchers found that single CAE bands replaced double SGE bands in some cases and vice versa, without affecting the zymodeme classification. They reached the conclusion that both CAE and SGE are suitable for isoenzyme characterisation, and are complementary to each other (Lanham *et al*., 1981).

Moss & Matthews (1987) were able to separate invasive and non-invasive *Entamoeba histolytica* (now *E. histolytica* and *E. dispar*, respectively) and *E. histolytica*-like organisms by CAE. Working with *Entamoeba*, these researchers

found that all but one of the CAE profiles in their study were similar to those obtained using the polyacrylamide gel electrophoresis (PAGE) isoenzyme method. The exception occurred with the glucose phosphate isomerase (GPI) zymogram. The authors surmise that this can possibly be ascribed to the fact that GPI from these strains is a small, highly-charged molecule that encounters minimal resistance in cellulose acetate, but increased resistance in polyacrylamide. Thus, some *Entamoeba* strains may not yield the same coenzyme profiles on CAE as on PAGE. CAE was nevertheless found to be a good means of separating *E. histolytica*-like organisms and what had formerly been regarded as invasive and non-invasive *Entamoeba histolytica* (Moss & Matthews, 1987).

CAE has also been shown to distinguish clearly and rapidly between *Naegleria fowleri* and *N. lovaniensis*, using the enzyme aspartate aminotransferase (Kilvington *et al*., 1984).

The writer chose CAE to examine the enzyme profiles of 17 *Acanthamoeba* isolates and a strain of *Mastigina*. Reasons were the small amount of sample required (involving less time and expense than is needed for culturing large numbers of organisms), the availability of a commercial matrix, speed, simplicity, sensitivity and economy. It is, to the author's knowledge, the first time that CAE has been used for *Acanthamoeba* identification.

High resolution polyacrylamide gel electrophoresis (PAGE) has been used successfully for separating proteins with different molecular conformations (Moss *et al*., 1988). With this technique, protein zones are resolved by a molecular sieving effect that forces mobile proteins to stack in progressively smaller gel pores. Proteins are separated by their molecular conformation rather than their

ionic charge or isoelectric points. In addition, the protein zones in gradient gels do not diffuse and cause obscurity in isoenzyme profiles as readily as they do in homogeneous gels (Moss *et al*., 1988).

PAGE was conducted on ten strains of *Acanthamoeba* so that results using this method could be compared with those obtained by CAE.

12.2 Materials and methods

12.2.1 *Strains*

CAE was done on the following 17 *Acanthamoeba* isolates and one strain of *Mastigina*: Ac/PHL/4; Ac/PHL/9; Ac/PHL/17; Ac/PHL/22; Ac/PHL/23; ATCC 30868; ATCC 30873; ATCC 50676; ATCC 50677; ATCC 50682; ATCC 50683; ATCC 50685; ATCC 50686; ATCC 50687; RYD; SAWL 91/2; 435/89; 452/89.[26]

PAGE was carried out on ten strains of *Acanthamoeba*, namely Ac/PHL/4; Ac/PHL/9; Ac/PHL/17; Ac/PHL/22; Ac/PHL/23; ATCC 30868; ATCC 30873; RYD; 435/89; and 452/89.[27]

12.2.2 *Enzymes*

CAE was done using the following 11 enzymes:[28] ACP; EST; G6PD; GPI; HK; lactate dehydrogenase (LDH); malate dehydrogenase (MDH); ME; mannose-phosphate isomerase (MPI); 6-phosphogluconate dehydrogenase (6PGD); and PGM.

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26. See Appendix IV.

27. See Appendix IV.

28. See Appendix VIII.

PAGE was done using the following ten enzymes:[29] aspartate amino transferase (AAT); ACP; EST; G6PD; GPI; HK; isocitrate dehydrogenase (IDH); ME; octanol dehydrogenase (ODH); and superoxide dimutase (SODS).

12.2.3 *Cloning*

Cloning of isolates of *Acanthamoeba* was carried out as described in Chapter 2.

12.2.4 *Preparation of extracts for CAE and PAGE*

Amoebae were axenically cultured at 30°C in 500 ml antibiotic-free peptone-yeast extract glucose broth (PYG) (Lasman & Feinstein, 1986) in 5 litre flasks on a shaker set at 100 rpm for 48 hours. Early exponentially growing trophozoites were harvested from axenic cultures by centrifugation in their medium at 2 000 rpm for 5 minutes. The medium was then decanted to leave a pellet of trophozoites. Harvested organisms were washed three times in amoeba saline, and pelleted as above after each wash. After the final wash, amoebae were transferred into Eppendorf tubes and centrifuged at 3 000 rpm for one minute. Excess fluid was decanted. A volume of solution equal to that of the remaining pellet of organisms, containing 2 mM dithiothreitol, 2 mM 6-amino caproic acid and 2 mM disodium ethylene-diamine tetracetic acid (EDTA),[30] was added to the pellet and mixed thoroughly. Lysis was achieved by freezing in liquid nitrogen and thawing at laboratory temperature. The suspension was centrifuged at 5 000 rpm for 15 minutes. The supernatant was removed and stored in liquid nitrogen in the form of beads made by dropping this liquid from a Pasteur pipette into liquid

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29. See Appendix VIII.

30. See Appendix VIII.

nitrogen.

12.2.5 *CAE*

12.2.5.1 Conducting CAE

Electrophoresis was carried out using cellulose acetate membranes (Sartorius GmbH) with the Sartophor system. Beads of extracts were thawed on ice and used immediately. Cellulose acetate paper was soaked in cold buffer, and all buffers were kept cold for the duration of the run.

The electrophoretic conditions are given in Appendix VIII. Sucrose was included at five per cent in the running buffer in order to reduce the current. Cellulose acetate membranes that had been pre-soaked for 20 minutes were removed from the membrane buffer,[31] blotted and aligned on the aligning base. An aliquot of 10 μl was
taken from the specimen sample with a microdispenser, and placed in the well plate. About 0.5 μl of the aliquot was transferred from the well plate to the membrane with an applicator. Most of the enzymes in this study required a triple application of sample. Additional applications were made from the original aliquot, and three or four enzyme systems were studied from one 10 μl aliquot. A maximum of ten samples was run on each cellulose acetate membrane. The reproducibility in appearance and position of the isoenzyme bands was ascertained, using different extracts.

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31. See Appendix VIII.

12.2.5.2 Developing solutions for CAE

After electrophoresis, the membranes were removed from the cell and placed, enzyme side down, on previously-prepared stain. The developing solutions (according to Richardson *et al*., 1986) are listed in Appendix VIII. Sucrose at ten per cent was included in the stain buffer for better resolution. All enzymes were developed in the dark at 37°C until the bands indicating enzyme activity were dark enough to be observed (ten to 15 minutes for most enzymes in this study). The membranes were then removed from the reaction mixture, placed in five per cent acetic acid to stop the reaction, washed in tap water, blotted, and allowed to air dry. The zymograms were photographed and photocopied, and band positions were marked on the mylar side of the membranes with a pen.

In the case of the fluorescent stain done for ACP and EST, the membranes were counterstained with a solution of pH 10 and viewed under ultraviolet light (UV). Membranes were blotted dry with clean blotting paper, and bands were marked with a waterproof marker under UV light.

The position of the bands was recorded in relation to five reference strains (see section 12.2.5.3 below). Each strain of *Acanthamoeba* was electrophoresed for each enzyme at least four times.

12.2.5.3 Controls for CAE

Extracts of the following strains of *Acanthamoeba* were electrophoresed alongside the other samples, and served as a reference for all comparisons: Ac/PHL/4, Ac/PHL/9, Ac/PHL/17, Ac/PHL/22 and Ac/PHL/23. Substrate control gels were immersed in the same developing solution as the experimental gels, but with substrate omitted.

12.2.6 *PAGE*

12.2.6.1 Conducting PAGE

Horizontal slab gel electrophoresis was carried out using the methods of Harris & Hopkinson (1976) and Coetzee *et al*. (1993). The gel-casting method of Coetzee *et al*. (1993) allowed four enzyme systems to be visualised for individual specimens, using horizontal PAGE. A 7.5% polyacrylamide gel with a five per cent stacking polyacrylamide was used.[32] Gels measured 9.5 x 14.5 cm and were 1.8 mm thick. Gels in TEB buffer were run at 500 V (90-100 mA) for three to four hours, and at 150–200 V (90–100 mA) for five to six hours, when the tris citrate buffer was used. The plates were maintained at 1°C by circulating cooled water in the base plate of the apparatus.

Beads of extracts were thawed on ice and used immediately. The samples (15 μl of extract) were applied to the stacker gels on inserts made from small Whatmann No. 1 (9.0 cm) pieces of paper.

A maximum of 20 samples was electrophoresed on each stack of gels. A marker consisting of whole blood, distilled water and bromophenol blue was used to indicate how far the samples had moved in the gel. Gels were allowed to run until the haemoglobin had moved 2.5 cm into the 7.5% polyacrylamide gel. (The electrophoretic conditions are described in Appendix VIII.)

12.2.6.2 Developing solutions for PAGE

The developing solutions are specified in Appendix VIII. The gels were removed after electrophoresis and placed in stain that had been prepared previously. All

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32. See Appendix VIII.

enzymes were developed in the dark at 37°C, except for SOD, which was developed in the light until the bands indicating enzyme activity were dark enough to be observed. The gels were then removed from the reaction mixture, washed in tap water and placed in ten per cent acetic acid to stop the reaction. Gels were photographed and then stored either in acetic acid in sealed plastic bags, or dried and mounted on cardboard for permanent storage.

12.3 Results

12.3.1 *CAE*

Diagrammatic representations of the enzyme bands obtained using CAE for ACP, EST, G6PD, GPI, HK, LDH, MDH, ME, MPI, 6PGD and PGM are given in Figures 12.1 – 12.2. Photographs are presented in Figures 12.3 – 12.11.

The following strains of *Acanthamoeba* have either been morphologically identified as *A. polyphaga* or are strains that are morphologically similar to this species (Nagington *et al*., 1974; Warhurst & Thomas, 1975; Kilvington, 1989; Kilvington *et al*., 1991a): Ac/PHL/4; Ac/PHL/9; Ac/PHL/17; Ac/PHL/22; Ac/PHL/23; ATCC 30873; 435/89; RYD. Zymodemes of these strains were similar for some enzymes, but were not always identical (Figs. 12.1; 12.2). However, all eight strains showed identical zymodemes for MDH and, with the exception of ATCC 30873, were identical for 6PGD. Strains Ac/PHL/17 and Ac/PHL/23 displayed similar patterns for all 11 enzymes studied (Figs. 12.1 – 12.4; 12.9 – 12.11).

Strains identified as *A. mauritaniensis* (ATCC 50676; ATCC 50677; ATCC 50682; ATCC 50683) (Schroeder *et al*., 2001) showed similar zymodemes for several enzymes, but differed from each other for other enzymes (Figs. 12.1;

12.2).

A. culbertsoni (452/89) demonstrated similarities to other strains for certain enzymes, but showed unique zymodemes for GPI and MDH (Figs. 12.1; 12.2; 12.10).

A. castellanii (ATCC 30868) gave no bands at all for six repeat runs of HK. This strain could be distinguished clearly from all other strains by means of its G6PD, GPI, MDH, ME and PGM bands (Figs 12.1; 12.2; 12.9; 12.10).

Strains of *Acanthamoeba lenticulata* (ATCC 50685, ATCC 50686 and ATCC 50687) displayed similar bands for all of the different enzymes studied (Figs 12.1; 12.2; 12.4; 12.5; 12.7; 12.11 – 12.15).

Mastigina (SAWL 91/2) showed a similar banding pattern to some strains of *Acanthamoeba* for the enzymes MPI and 6PGD, but the banding patterns of this protostelid were unique for all of the other enzymes (Figs 12.1; 12.2). No bands were visible after six repeated runs with different batches of enzymes for ACP or EST (Figs 12.1; 12.4).

Results obtained for the strains of *Acanthamoeba* studied were similar when using different buffers for ACP 1, 2 and 3 and for EST 1 and 2.[33]

No visible enzymatic activity was detected in the substrate control gels, which involved unloaded gels being subjected to the same electrophoretic conditions as the samples.

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33. See Appendix VIII.

12.3.2 *PAGE*

Diagrammatic representations of the enzyme bands obtained using PAGE for AAT, IDH and SODS are given in Figure 12.12. Photographs are presented in Figures 12.13 – 12.18. In most cases, the electrophoretic bands obtained using PAGE were similar to those for CAE for the following enzymes: ACP, EST, G6PD, GPI, HK and ME. The strains that are morphologically similar to *A. polyphaga* (see section 12.3.1) had corresponding bands for the enzymes AAT, IDH and SODS (Fig. 12.12). Strains identified as *A. mauritaniensis* (Schroeder *et al*., 2001), namely ATCC 50676, 50677, 50678, 50679, 50680 and 50684, had similar zymodemes for AAT. Likeness also occurred in some of the strains for IDH and SODS. There were similar electrophoretic bands for strains of *A. lenticulata* (ATCC 50685, 50686 and 50687) for all of the enzymes studied (Figs 12.12 – 12.18).

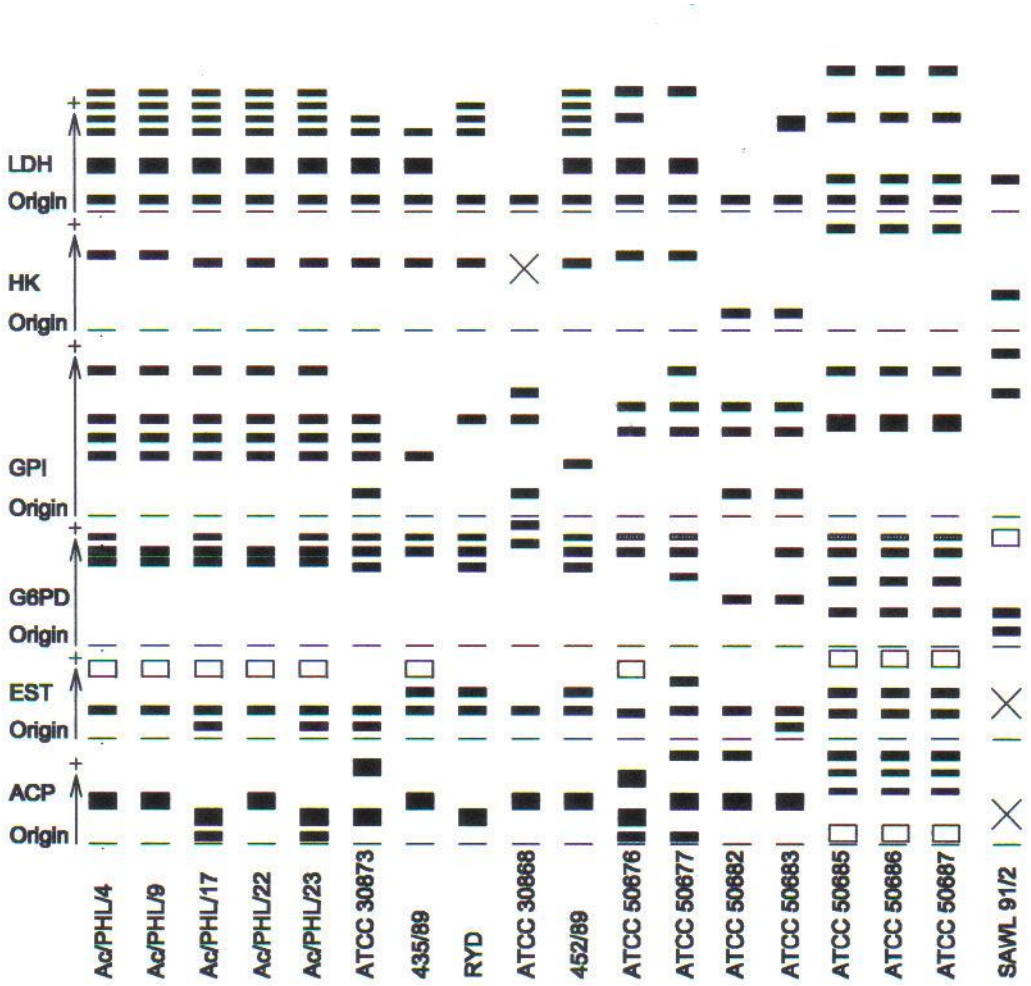


Figure 12.1. Diagrammatic representation of the enzyme bands obtained using cellulose acetate electrophoresis (CAE) for 17 *Acanthamoeba* isolates and one strain of *Mastigina*.

Key:

ACP	=	acid phosphatase.
EST	=	esterases.
G6PD	=	glucose-6-phosphate dehydrogenase.
GPI	=	glucose-phosphate isomerase.
HK	=	hexokinase.
LDH	=	lactate dehydrogenase.

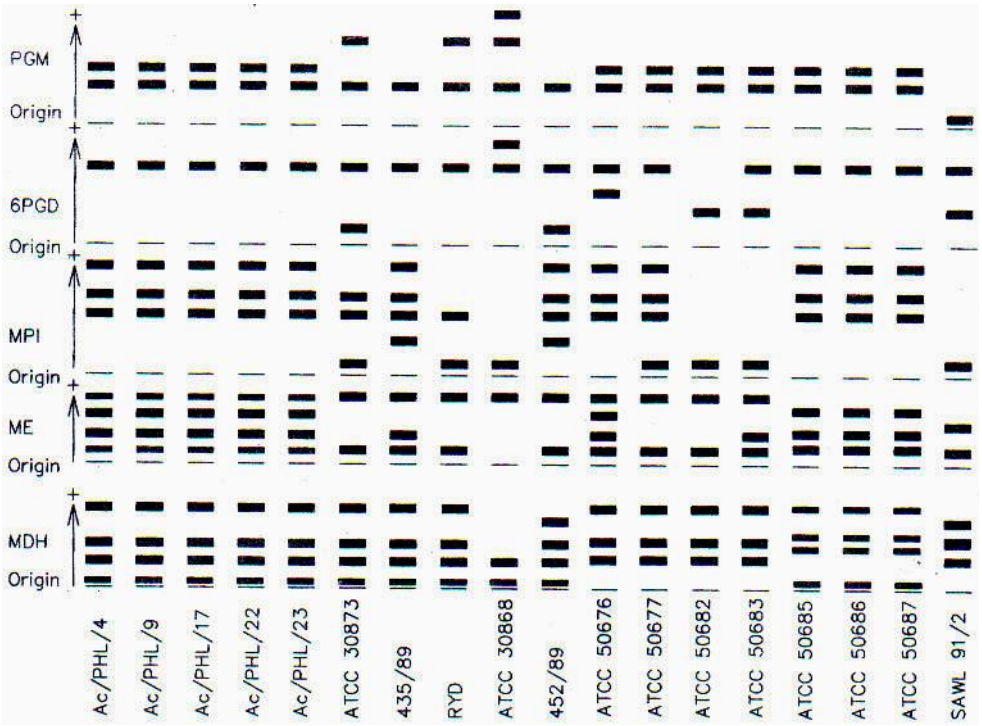
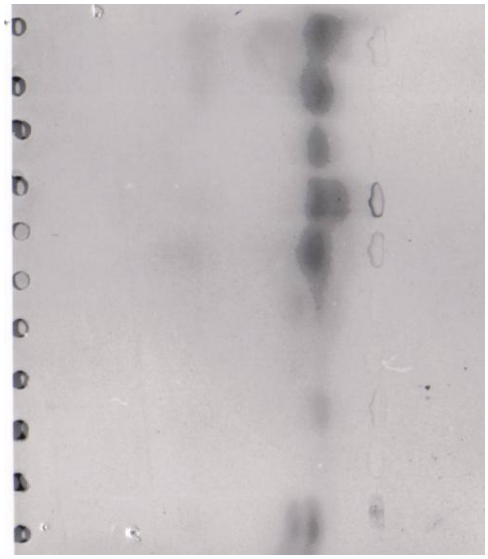


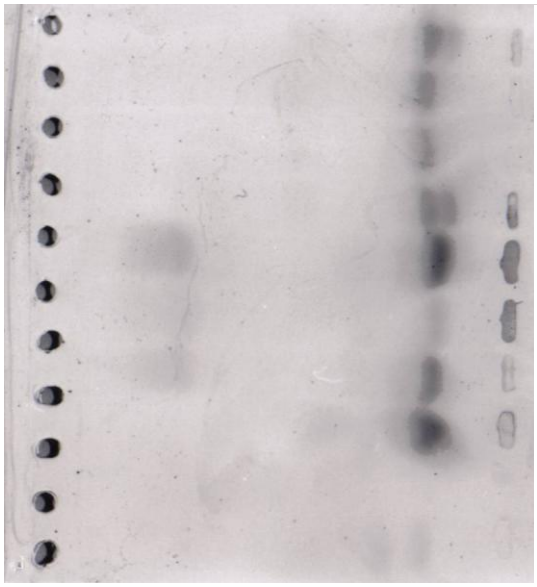
Figure 12.2. Diagrammatic representation of the enzyme bands obtained using cellulose acetate electrophoresis (CAE) for 17 *Acanthamoeba* isolates and one strain of *Mastigina*.

Key:

MDH	=	malate dehydrogenase.
ME	=	malic enzyme.
MPI	=	mannose-phosphate isomerase.
6PGD	=	6-phosphogluconate dehydrogenase.
PGM	=	phosphoglucomutase.



Ac/PHL/23
Ac/PHL/9
Ac/PHL/22
Ac/PHL/17
Ac/PHL/4
452/89
RYD
ATCC 30873
ATCC 30868
435/89



Ac/PHL/23
Ac/PHL/4
Ac/PHL/22
Ac/PHL/17
ATCC 50686
ATCC 50687
ATCC 50685
ATCC 50676
SAWL 91/2
ATCC 50677

Figures 12.3 and 12.4. Photographs of enzyme bands obtained using cellulose acetate electrophoresis (CAE) for esterases (EST) on ten strains of *Acanthamoeba.*

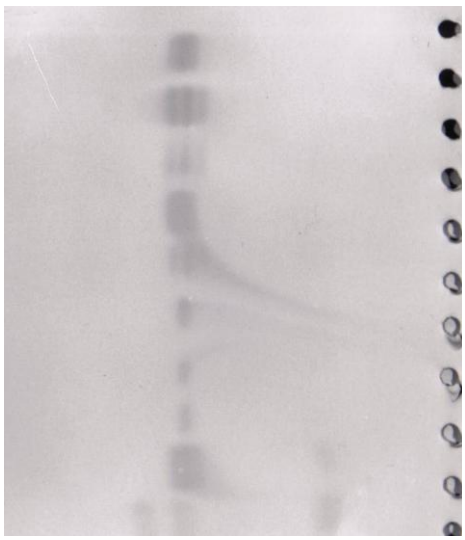


Figure 12.5. Photograph of enzyme bands obtained using cellulose acetate electrophoresis (CAE) for glucose phosphate isomerase (GPI) on ten strains of *Acanthamoeba.*

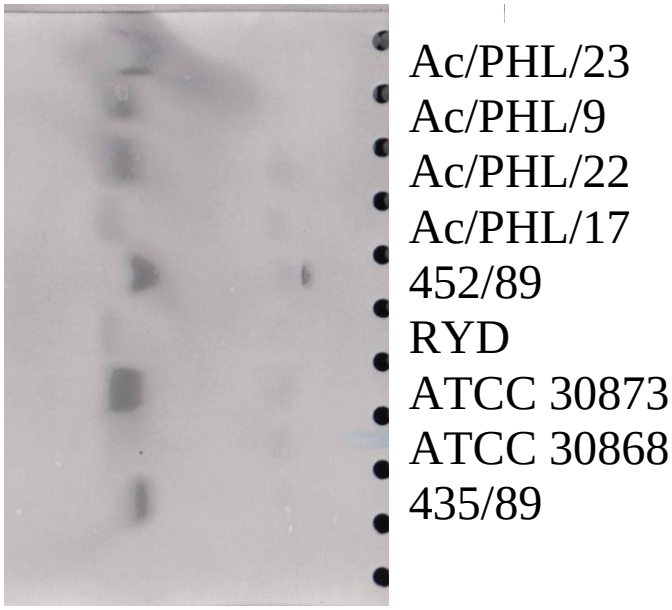


Figure 12.6. Photograph of enzyme bands obtained using cellulose acetate electrophoresis (CAE) for glucose phosphate isomerase (GPI) on nine strains of *Acanthamoeba.*

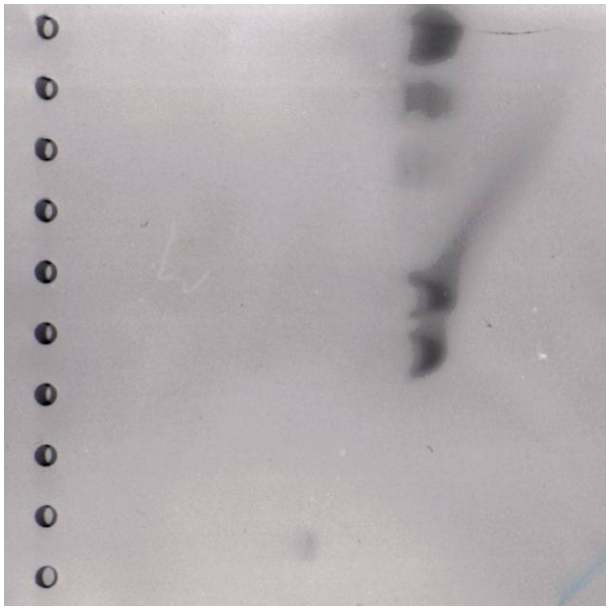
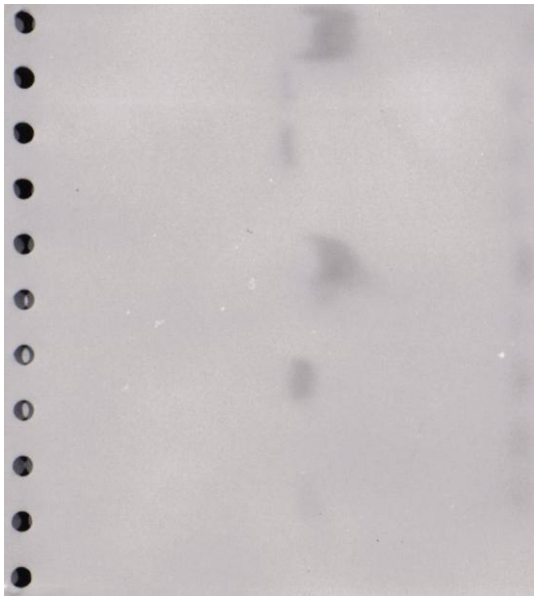
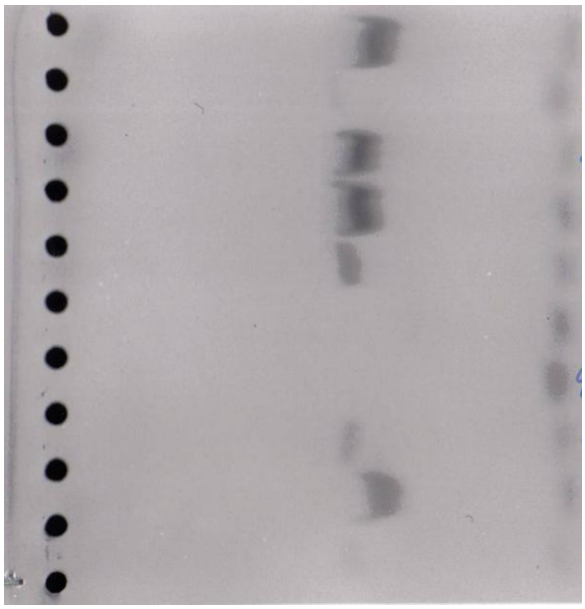


Figure 12.7. Photograph of enzyme bands obtained using cellulose acetate electrophoresis (CAE) for glucose phosphate isomerase (GPI) on five strains of *Acanthamoeba* and a strain of *Mastigina.*



Ac/PHL/23
Ac/PHL/9
Ac/PHL/22
Ac/PHL/17
452/89
RYD
ATCC 30873
ATCC 30868
435/89



Ac/PHL/23
Ac/PHL/9
Ac/PHL/22
Ac/PHL/17
Ac/PHL/4
SAWL 91/2
ATCC 30868
RYD
452/89
435/89

Figures 12.8 and 12.9. Photographs of enzyme bands obtained using cellulose acetate electrophoresis (CAE) for hexokinase (HK) on ten strains of *Acanthamoeba.*

Figure 12.10. Photograph of enzyme bands obtained using cellulose acetate electrophoresis (CAE) for malate dehydrogenase (MDH) on ten strains of *Acanthamoeba.*

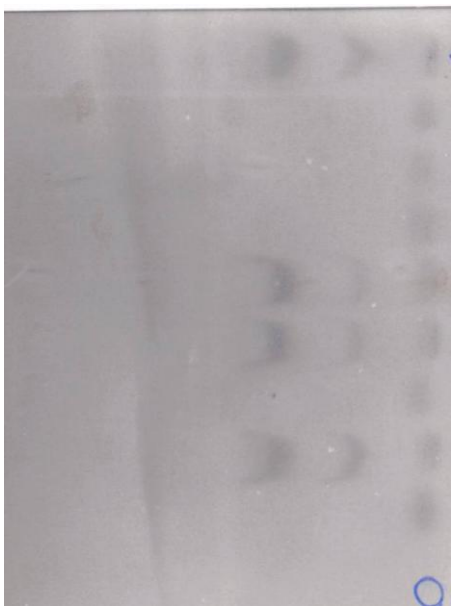


Figure 12.11. Photograph of enzyme bands obtained using cellulose acetate electrophoresis (CAE) for malic enzyme (ME) on eight strains of *Acanthamoeba* and a strain of *Mastigina.*

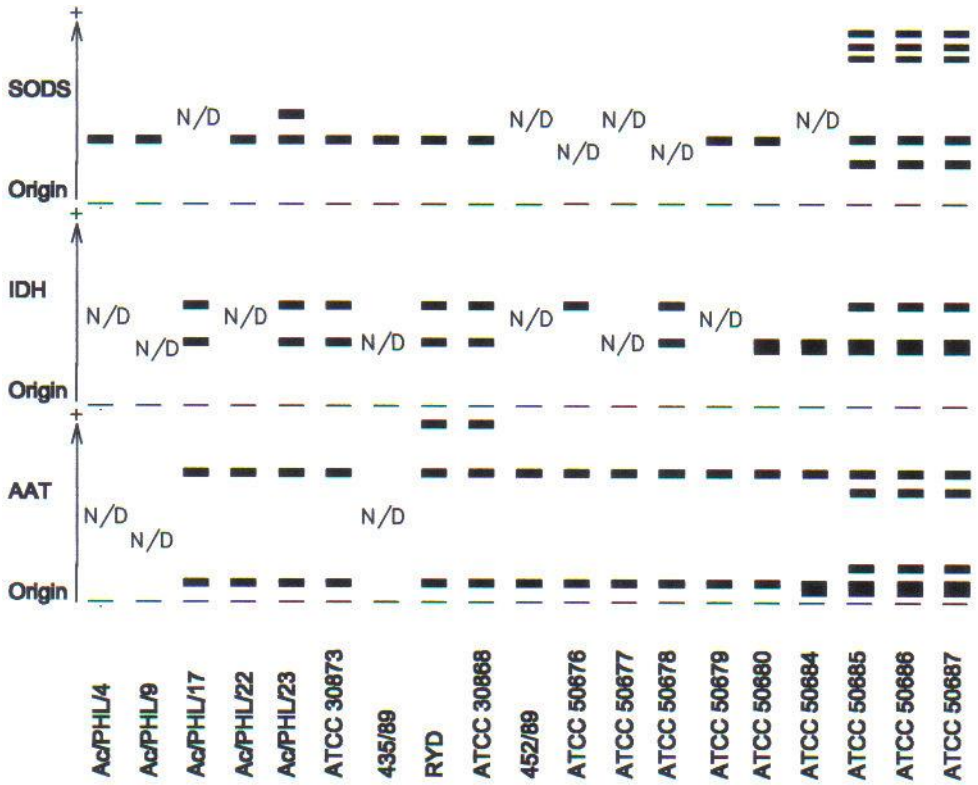


Figure 12.12. Diagrammatic representation of the enzyme bands obtained using polyacrylamide gel electrophoresis (PAGE) on *Acanthamoeba* isolates.

Key:

AAT = aspartate amino transferase.
IDH = isocitrate dehydrogenase.
ND = not done.
SODS = superoxide dimutase.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

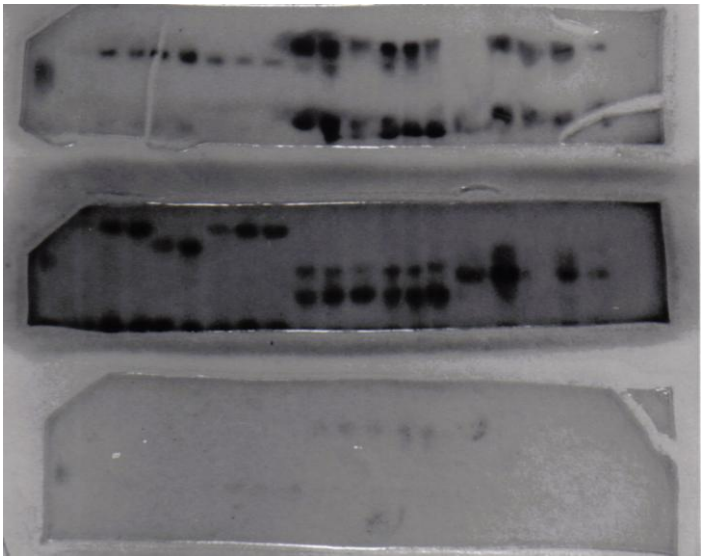


Figure 12.13. Photograph of enzyme bands obtained using polyacrylamide gel electrophoresis (PAGE) for aspartate amino transferase (AAT), isocitrate dehydrogenase (IDH) and malic enzyme (ME) on seven strains of *Acanthamoeba.*

Lanes (from left): 1 = marker; 2-9 mosquitoes; 10 = ATCC 50686; 11 = ATCC 50686; 12 = ATCC 50685; 13 = ATCC 50685; 14 = ATCC 50687; 15 = ATCC 50687; 16 = ATCC 50676; 17 = ATCC 50676; 18 = ATCC 30873; 19 = Ac/PHL/23; 20 = Ac/PKL/22.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

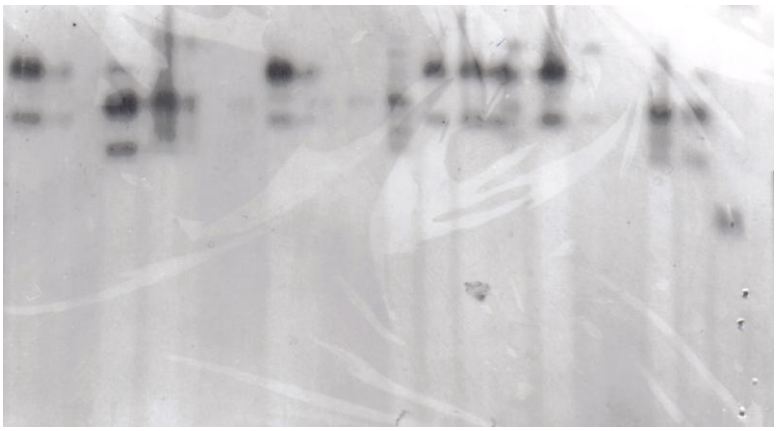


Figure 12.14. Photograph of enzyme bands obtained using polyacrylamide gel electrophoresis (PAGE) for aspartate amino transferase (AAT) for different strains of *Acanthamoeba.*

Lanes (from left): 1 = ATCC 50678; 2 = ATCC 50677; 3 = Ac/PHL/22; 4 = ATCC 30868; 5 = Ac/PHL/23; 6 = 435/89; 7 = ATCC 30873; 8 = Ac/PHL/17; 9 = Ac/PHL/9; 10 = Ac/PHL/4; 11 = ATCC 50684; 12 = ATCC 50685; 13 = ATCC 50687; 14 = ATCC 50680; 15 = ATCC 50686; 16 = 452/89; 17 = ATCC 50679; 18 = ATCC 50676; 19 = RYD; 20 = MARKER.

Figure 12.15. Photograph of enzyme bands obtained using polyacrylamide gel electrophoresis (PAGE) for aspartate amino transferase (AAT) for different strains of *Acanthamoeba.* Lanes (from left): 1 = ATCC 30873; 2 = 435/89; 3 = RYD; 4 = Ac/PHL/23; 5 = Ac/PHL/17; 6 = Ac/PHL/9; 7 = Ac/PHL/22; 8 = 452/89; 9 = Ac/PHL/4; 10 = ATCC 30868; 11 = ATCC 50678; 12 = ATCC 50677; 13 = ATCC 50680; 14 = ATCC 50679; 15 = ATCC 50684; 16 = ATCC 50676; 17 = ATCC 50686; 18 = ATCC 50685; 19 = ATCC 50687; 20 = MARKER.

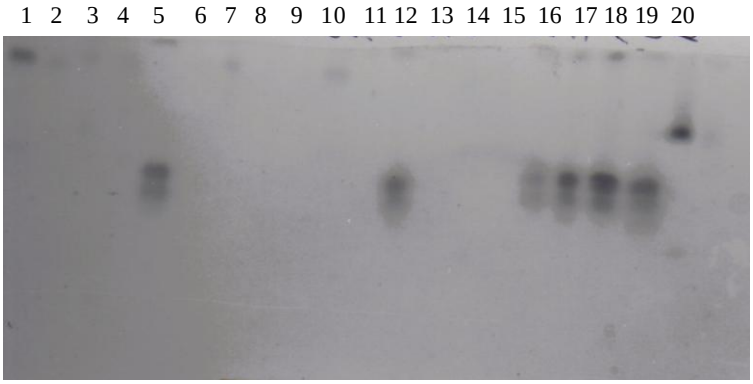


Figure 12.16. Photograph of enzyme bands obtained using polyacrylamide gel electrophoresis (PAGE) for esterases (EST) for different strains of *Acanthamoeba.* Lanes (from left): 1 = ATCC 30873; 2 = 435/89; 3 = RYD; 4 = Ac/PHL/23; 5 = Ac/PHL/17; 6 = Ac/PHL/9; 7 = Ac/PHL/22; 8 = 452/89; 9 = Ac/PHL/4; 10 = ATCC 30868; 11 = ATCC 50678; 12 = ATCC 50677; 13 = ATCC 50680; 14 = ATCC 50679; 15 = ATCC 50684; 16 = ATCC 50676; 17 = ATCC 50686; 18 = ATCC 50685; 19 = ATCC 50687; 20 = MARKER.

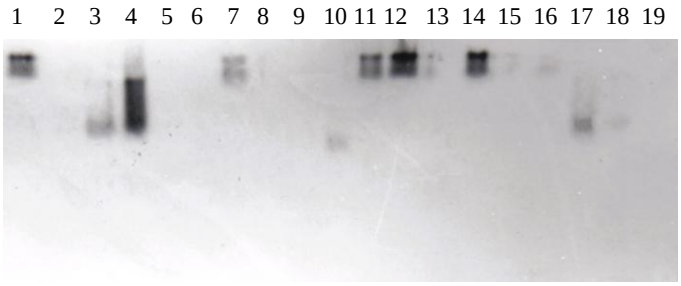


Figure 12.17. Photograph of enzyme bands obtained using polyacrylamide gel electrophoresis (PAGE) for glucose-6-phosphate dehydrogenase (G6PD) for different strains of *Acanthamoeba.* Lanes (from left): 1 = ATCC 50678; 2 = ATCC 50677; 3 = Ac/PHL/22; 4 = ATCC 30868; 5 = Ac/PHL/23; 6 = 435/89; 7 = ATCC 30873; 8 = Ac/PHL/17; 9 = Ac/PHL/9; 10 = Ac/PHL/4; 11 = ATCC 50684; 12 = ATCC 50685; 13 = ATCC 50687; 14 = ATCC 50680; 15 = ATCC 50686; 16 = 452/89; 17 = ATCC 50679; 18 = ATCC 50676; 19 = RYD.

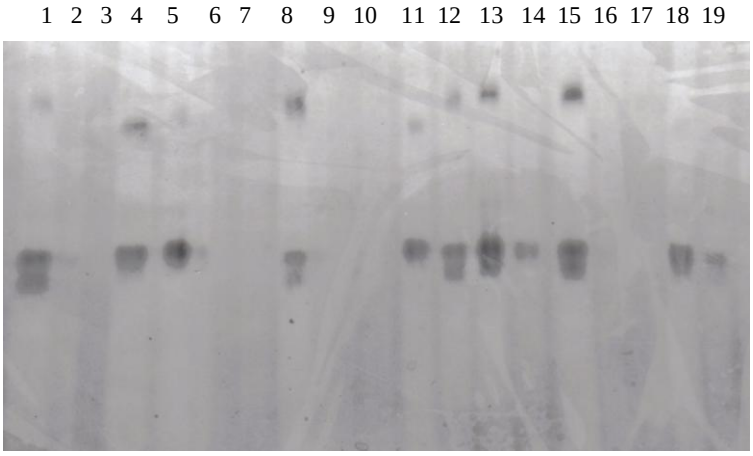


Figure 12.18. Photograph of enzyme bands obtained using polyacrylamide gel electrophoresis (PAGE) for isocitrate dehydrogenase (IDH) for different strains of *Acanthamoeba.* Lanes (from left): 1 = ATCC 50678; 2 = ATCC 50677; 3 = Ac/PHL/22; 4 = ATCC 30868; 5 = Ac/PHL/23; 6 = 435/89; 7 = ATCC 30873; 8 = Ac/PHL/17; 9 = Ac/PHL/9; 10 = Ac/PHL/4; 11 = ATCC 50684; 12 = ATCC 50685; 13 = ATCC 50687; 14 = ATCC 50680; 15 = ATCC 50686; 16 = 452/89; 17 = ATCC 50679; 18 = ATCC 50676; 19 = RYD.

12.3 Discussion

There appears to be no correlation between isoenzyme patterns and morphological types for certain species in the genus *Acanthamoeba* (Visvesvara, 1991). Many strains that had been assigned to the same species using morphological criteria, have shown different enzyme patterns (Tyndall *et al*., 1979; Costas & Griffiths, 1980; Daggett *et al*., 1980; De Jonckheere, 1983; Visvesvara *et al*., 1983; Costas & Griffiths, 1984; De Jonckheere, 1987a; 1987c; De Jonckheere & Michel, 1988).

Non-morphological characters are useful in delimiting genera of amoebae, as there is no confusion between zymograms of *Acanthamoeba* and those of other genera. Certain species within the genus *Acanthamoeba* (*A. astronyxis, A. culbertsoni* and *A. pustulosa*) have distinctive enzyme patterns (Costas & Griffiths, 1984).

Isoenzyme analysis may be a strong aid in assigning *Acanthamoeba* strains to species, but different zymodemes may exist within a species, as is the case for the genus *Naegleria* (De Jonckheere, 1983). In some strains of *Acanthamoeba*, not all isoenzymes correspond to the pattern of the type strain to which they have been assigned (Daggett *et al*., 1982; De Jonckheere & Michel, 1988). De Jonckheere (1983) and Costas & Griffiths (1984) have found that isoenzyme pattern variations occur with *A. polyphaga* in particular. Some strains of *A. polyphaga* have been found to exhibit similar electrophoretic patterns to strains of *A. castel-lanii* (De Jonckheere, 1983). Differences in isoenzyme profiles have been noticed in strains of *A. castellanii*, and isoenzyme profiles of *A. mauritaniensis* seem to resemble those of *A. castellanii* (De Jonckheere, 1987c). Three reference strains of *A. mauritaniensis* investigated by De Jonckheere (1983) showed differences in their zymograms except for MDH, and minor variations appeared in their protein

banding patterns, but the overall similarities were striking. An *A. lenticulata* isolate studied by De Jonckheere (1983) had large differences in banding compared to other *A. lenticulata* strains. In addition, several isoenzyme types have been found that do not fit into any species pattern. This implies that species exist which have not yet been described and/or that different zymodemes of the same species are involved (De Jonckheere, 1987c). The same researcher found that when the pattern of only one enzyme is identical to that of a reference strain, the morphology usually confirms the identification (De Jonckheere, 1987c). A combination of isoenzyme analysis and morphological examination therefore appears to be a promising means of identifying an *Acanthamoeba* isolate to the specific level.

Electrophoretic results obtained by different researchers for the same strains of *Acanthamoeba* do not always correlate. Costas & Griffiths (1980), using starch gel electrophoresis, identified the Neff strain of *A. castellanii* (ATCC 30010) and ATCC 30869 as belonging to a single group in their study. Daggett *et al*. (1982), also using starch gel electrophoresis, reported that these two strains were not in the same group.

The present researcher found certain similarities and some diversity in the isoenzyme patterns for certain enzymes within strains assigned to the same species. Strains ATCC 30873 and Ac/PHL/23 had previously been identified as *A. polyphaga* (Nagington *et al*., 1974; Warhurst & Thomas, 1975; Kilvington, 1989). Kilvington *et al*. (1991a) described strains Ac/PHL/4, Ac/PHL/17, Ac/PHL/22, Ac/PHL/23 and ATCC 30873 as being morphologically similar. The results of Kilvington *et al*. (1991a) showed that strains Ac/PHL/17 and Ac/PHL/23 had similar zymodemes for all 11 enzymes used, but there was slight

variation for strains Ac/PHL/4, Ac/PHL/22 and ATCC 30873 (CCAP 1501/3d (Gachon *et al.*, 2007)) for some enzymes. Kilvington *et al*. (1991a) studied 33 strains that were morphologically similar, when viewed by light microscopy, to *A. polyphaga* or possibly *A. castellanii* (including the five strains that the present author studied), using restriction enzyme analysis of DNA. Even though Ac/PHL/4, Ac/PHL/17, Ac/PHL/22, Ac/PHL/23 and ATCC 30873 are morphologically similar, only strains Ac/PHL/17 and Ac/PHL/23 showed identical restriction fragment length polymorphisms (RFLPs) when whole-cell DNA of the samples was digested by three different enzymes (Kilvington *et al*., 1991a). These researchers assigned the 33 morphologically similar strains to seven multiple-strain and three single-strain groups on the basis of their whole-cell DNA results, but reported that it remains unclear whether *Acanthamoeba* mitochondrial DNA RFLPs indicate intra-or interspecies differences. Restriction fragments of mitochondrial DNA showed that a strain of *A. castellanii* and one of *A. polyphaga* gave identical patterns (Costas *et al*. 1983), confirming the close similarity which has also been found using both enzyme electrophoresis of esterases and acid phosphatases, and immunoelectrophoresis.

The southern African strains of *A. mauritaniensis* that were identified using subgenic 18S rDNA PCR showed zymogram differences for several enzymes, but all MDH and PGM zymograms were identical. Some of the *A. mauritaniensis* strains showed enzyme patterns similar to those of isolates designated as *A. polyphaga*. Zymodemes of *A. castellanii* (ATCC 30868) differed from those of the other species for most enzymes. All three of the southern African *A. lenticulata* isolates had similar zymograms for all the enzymes studied.

De Jonckheere (1983) found that only a few strains of *Acanthamoeba* differed from the general GPI pattern and that all the bands were at the same point for PGM, so no useful information was obtained from these enzymes. Twelve of the 18 strains examined in this research had identical PGM bands, but only five of the 18 strains had identical GPI bands.

The three isozyme bands obtained in this study on the anodal side for the enzyme ACP for the southern African strains of *A. lenticulata* (ATCC 50685, ATCC 50686 and ATCC 50687) were similar to those obtained by Daggett *et al*. (1982) for a strain of *A. lenticulata* and two strains of *A. culbertsoni*. They found that two of these three bands showed variable expression, and also detected a band on the cathodal side that this researcher did not.

Using agarose isoelectric focusing, De Jonckheere (1983) found that the enzymes ACP, MDH and propionyl esterase gave the best separation of bands for strains of *Acanthamoeba*. The present writer was able to separate certain strains with the ACP bands obtained. The MDH results were similar for most of the strains studied, with differences found only for three strains. Tyndall *et al*. (1979) postulated that because of the reproducibility and individuality of profiles of esterase activity of *Acanthamoeba*, esterase analysis might be a useful tool for speciating *Acanthamoeba*. The esterase results in this study were not as useful in separating the strains of *Acanthamoeba* as those of the other enzymes examined by this researcher.

Richardson *et al*. (1986) state that the fluorescence staining method for ACP is usually more sensitive than the azo dye method. In the writer's experience, however, the results were similar for both methods when used for ACP and for

EST, with the fluorescent staining method giving good confirmation of the other results. On the other hand, the results of the fluorescent technique are more difficult to record as the fluorescent bands fade quickly, and they must be marked immediately after staining with a waterproof marker.

De Jonckheere (1982) found that although small isoenzyme differences could be detected amongst strains of *N. lovaniensis* from Europe, no comparable differences were seen in the restriction fragment patterns of the repetitive DNA. He also reported (De Jonckheere, 1987c) that when the pattern of only one enzyme is identical to that for the reference strain, then the morphology often conforms to the reference strain. De Jonckheere & Michel (1988) strongly advised that, in the absence of any refined biochemical identification method, a virulence test in mice should be carried out. Even such a test, coupled with morphological examination, does not guarantee a species identification, as some of the strains of *A. lenticulata* identified by isoenzymes were non-virulent (Molet *et al*., 1981; De Jonckheere & Michel, 1988). Moreover, one of the isolates that showed isoenzymes and virulence compatible with *A. lenticulata*, did not show the typical morphology of this species (De Jonckheere & Michel, 1988).

Isoenzyme differences have been used to distinguish between a pathogenic and a non-pathogenic strain of *A. castellanii* by means of electrophoresis using polyacrylamide gels (Visvesvara *et al*., 1983). These strains had been identified as *A. castellanii*, because they were morphologically identical to strains previously described as *A. castellanii*. Also, these strains reacted with the rabbit anti-*A. castellanii* serum in the indirect immunofluorescence test to the same end-point dilution as the homologous system. The pathogenic stain had distinct isoenzyme profiles, and consistently differed from the other strain for all the

enzymes tested, both in the number of bands produced and in the mobility of the individual bands (Visvesvara *et al*., 1983). De Jonckheere (1983), using agarose isoelectric focusing, found differences between the zymograms for the enzyme ACP for the Neff strain of *A. castellanii* and for two strains of *A. castellanii* isolated from pools in France. Seven strains that were assigned to *A. castellanii* on the basis of morphological criteria could be placed in four groups on the basis of their enzyme patterns (Costas & Griffiths, 1980).

Cariou & Pernin (1987) studied electrophoretic variation for 15 enzyme-coding genes in various *Naegleria* species. They found that every enzyme that they analysed was variable in the *Naegleria* species surveyed, and concluded that such broad electrophoretic variability can be attributed largely, if not entirely, to genetic variation. Possibly, the same applies to *Acanthamoeba*. Electrophoretic variation is perhaps explicable in terms of allelic variation. The enzymatic survey (Cariou & Pernin, 1987) also suggested that *Naegleria* may be diploid. At several *Naegleria* enzymatic loci, heterozygotes and both corresponding homozygotes occurred within a given species. Some of the enzymes showed three genotypes. As seven of the nine paired combinations theoretically possible were observed, these look like randomly assorted genotypes. When the genotypic structures of the strains were compared at each of the eight polymorphic loci, they differed, on average, at 4.2 loci (Cariou & Pernin, 1987).

When the present researcher compared her CAE zymograms for strains ATCC 30873 and ATCC 30868 for the enzymes ACP and EST with those obtained using SGE (Costas & Griffiths, 1984), fewer bands were present (with CAE). Isoelectric focusing on agarose gels using 30 strains of *Acanthamoeba* also yielded several bands for ACP and MDH, but few bands for GPI (De Jonckheere,

1983). Likewise, isoelectric focusing on polyacrylamide gels yielded several bands for ACP and EST (Matias *et al*., 1991). Few bands were, however, obtained with PAGE for PGM, ME, MDH and HK (Visvesvara *et al*., 1983).

The results obtained by the current author with PAGE confirm the CAE results. A possible disadvantage of CAE is the staining intensity obtained for some isozymes, because CAE uses a smaller amount of sample than other media. There is, therefore, less enzyme on the gel, but this is only a problem for weak isozymes (Richardson *et al*., 1986). In this study, overloading of some gels was tried to see if more bands would be apparent, but this resulted in the existing bands becoming diffuse and very intense instead of more bands being revealed. The researcher does not, however, see this as a problem, because it remained possible to distinguish between the different strains. Both the speed with which isolates can be electrophoresed (due to the small number of organisms required to obtain the small quantities of sample needed) and the speed with which results can be obtained, are of great advantage.

Electrophoresis appears to be a stable method to use, as no variation has been found for enzyme patterns during log growth in batch culture (Costas & Griffiths, 1980). The different batches of lysates used in the present study gave similar results. However, differences in typical isoenzyme patterns between lysates of axenically and monoxenically-grown amoebae have been found (Weekers & De Jonckheere, 1997). In most cases, the intensity of the bands for lysates from monoxenic cultures was strongly reduced; bands were even absent. Therefore, Weekers & De Jonckheere (1997) warn that isoenzyme analysis for the identification of amoebae should be used with caution when different growth conditions are used to culture the amoebae. For the purpose of strain

identification or phylogenetic analysis based on isoenzyme patterns, Weekers & De Jonckheere (1997) recommend the use of axenically grown amoebae.

The variation in zymodemes found within species of *Acanthamoeba* indicates that caution is needed in using isoenzyme electrophoresis to identify species of *Acanthamoeba* (Daggett *et al*., 1982). Differing isoenzyme patterns must not be used as the sole criterion to establish new species. Nor should a morphological analysis alone be used. A variety of criteria should be considered before a new species is described.

CHAPTER THIRTEEN – CHARACTERISATION OF *ACANTHAMOEBA* BY DNA TYPING

13.1 The identification of *Acanthamoeba* species by means of non-morphological criteria

Species identification of *Acanthamoeba* based on morphological features alone is difficult, especially for amoebae in groups II and III (Visvesvara, 1991). This is because specific features, such as size and subtle differences in the morphology of the cyst stage, are not as clear as those for the genus (Visvesvara, 1991).[34] Difficulties could be experienced in describing the characters objectively. In addition, differentiation of species based on morphology alone may not always be correct, since the morphology of cysts within a species may vary according to cultural conditions, with resultant pleomorphism of the endocysts within the population (Page, 1988), as has been shown for *Acanthamoeba castellanii* by Stratford & Griffiths (1978). In addition, variation in the morphology of trophozoites and cysts seen within a clone is a problem (Badenoch *et al*., 1995). The most detailed study of cyst morphology is that of Pussard & Pons (1977). However, they used only one strain each of the majority of species investigated, so that interclonal variation, which does occur, could not always be taken into account (Page, 1988).

Due to the unreliability of morphological criteria, non-morphological criteria such as optimal growth temperature; antigenic relationships; isoenzyme electrophoresis; banding patterns of electrophoretically separated proteins; and

34. See Appendix X.

restriction fragment length polymorphisms (RFLPs) of the mitochondrial or genomic DNA, may have to be used (Visvesvara, 1991).

Restriction endonuclease digestion of whole-cell DNA has shown differences in banding patterns between various species of *Naegleria*, a species of *Didascalus*, and one of *Willaertia* (De Jonckheere *et al*., 1987a). Significant differences were observed between strains of *N. fowleri* according to the continent of origin, with a strain of *N. fowleri* isolated from one of the very few cured human infections showing the most distinct pattern within the species (De Jonckheere, 1987a).

Comparisons of restriction endonuclease digest fragment patterns ("fingerprints") of mitochondrial (mtDNA) and whole cell DNA have been used as an aid to establishing the taxonomy of *Acanthamoeba* and for their potential use in characterising pathogenic and non-pathogenic *Acanthamoeba* spp. (Bogler *et al*., 1983; Costas *et al*., 1983; Byers *et al*., 1990; Kilvington *et al*., 1990; Yagita & Endo, 1990; Kilvington *et al*., 1991a). In addition, RFLP analysis of whole cell DNA has been evaluated as a method for tracing possible sources of infection (Kilvington *et al*., 1990), while analysis of mtDNA was judged to be an epidemiological tool for identifying potential reservoirs of *Acanthamoeba* infection (Gautom *et al*., 1994). The most common clinical isolates have counterparts readily recoverable from the surrounding environment, and some of these appear to be geographically widespread, according to Gautom *et al*. (1994). This has been confirmed by restriction endonuclease analysis of *Acanthamoeba* whole-cell DNA, as banding patterns of strains isolated from a patient's cornea, contact lens storage container, saline rinsing solution, and kitchen cold water tap, were identical (Kilvington *et al*., 1990).

Strains of *Acanthamoeba* that are morphologically similar do not necessarily have the same banding patterns of restricted DNA (Kilvington *et al.*, 1991a). Bearing in mind the unreliability of morphological criteria, the present researcher used DNA typing on nine different enzymes to compare RFLPs of some of the southern African isolates with known strains from overseas. RFLP studies were also done to see whether these findings confirmed the isoenzyme electrophoresis results. To the writer's knowledge, no southern African strains have been typed before.

13.2 Materials and methods

13.2.1 *Organisms*

The following strains of *Acanthamoeba* were used: Ac/PHL/9; Ac/PHL/17; ATCC 30873; ATCC 50676; ATCC 50685; ATCC 50686; and ATCC 50687.[35]

Cloning was carried out as described in Chapter 2. Amoebae were axenically cultured at 30°C in antibiotic-free peptone-yeast extract glucose broth (Lasman & Feinstein, 1986)[36] in screw-cap 80 cm^2 (260 ml) Nunc tissue culture flasks. Sub-cultures of *Acanthamoeba* trophozoites were grown in 500 ml broth in 5 litre flasks on a shaker set at 100 rpm.

Late-log-phase cultures were concentrated by centrifugation in their medium at 740 x g for five minutes. The medium was decanted to leave a pellet of trophozoites. The harvested organisms were washed three times in amoeba saline and centrifuged at 740 x g for five minutes after each wash. Trophozoites were used immediately.

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35. See Appendix IV.

36. See Appendix II.

Four different methods for DNA extraction were tried.

13.2.2 ***Mitochondrial DNA extraction*** *(Hettiarachchy & Jones, 1974, with a few modifications)*

- All work carried out at room temperature (R.T.), unless stated otherwise.
- Harvest cultures by centrifugation at 500 x g for ten minutes.
- Conduct all subsequent operations at 2–4°C (on ice).
- Wash cells 1x in amoeba saline.
- Resuspend in extraction buffer consisting of 0.3M sucrose containing 10 mM Tris; 0.2M EDTA and 0.1% (w/v) BSA adjusted to pH 7.4 with HCl.
- Centrifuge at 500 x g for ten minutes.
- Resuspend the washed cells in 5 x volume of extraction buffer and homogenise by passing through a glass homogeniser.
- Centrifuge homogenate at 500 x g for ten minutes (keep supernatant on ice).
- Resuspend pellet of unbroken cells in 5 x volume of extraction buffer and homogenise again.
- Centrifuge at 500 x g for ten minutes and combine supernatants.
- Centrifuge combined supernatants at 5500 x g for ten minutes.
- Resuspend crude mitochondrial pellet gently in buffer.
- Centrifuge at 500 x g for ten minutes to remove remaining cell debris (keep supernatant).
- Wash mitochondria once by centrifugation at 5500 x g for ten minutes.
- Incubate mitochondria with 50 μg of DNase/ml for 30 minutes at 37°C in a buffer consisting of 0.3 M sucrose; 10 mM Tris HCl; 5 mM $MgCl_2$; pH 7.4, to remove contaminating nuclear DNA.
- Wash mitochondria three times by centrifugation in the extraction buffer (5500 x g for ten minutes).

- Use only siliconised glassware hereafter to prevent the DNA from adhering to the sides of the glass.
- Split mitochondria in a lysis buffer consisting of 0.15 M NaCl; 0.015 M EDTA; 0.5% SDS. Lysis occurs immediately.
- Shake suspension a few times and then allow it to stand for about ten minutes at R.T.

13.2.2.1 Phenol method of extraction of mitochondrial DNA

- Add volume of phenol equal to that of suspension.
- Shake well for ten minutes.
- Add volume of chloroform (i.e. 24:1 chloroform: isoamyl alcohol) equal to that of suspension and shake vigorously for ten minutes.
- Separate phases by centrifugation at 12 000 x g (five minutes).
- Remove top layer (containing buffer and nucleic acids) and discard the rest.
- Repeat the procedure on the top layer by first adding phenol followed by chloroform, until the "white fuzz" layer has disappeared.
- Store the top aqueous layer at -20°C after adding 2x amount of ice-cold 100% ethanol.

13.2.2.2 Cesium chloride method of extraction of mitochondrial DNA

- Add 10 g/10 ml CsCl to suspension (see 13.2.2) to obtain a density of 1.393.
- Centrifuge at 4 000 rpm, add extra buffer to the top protein layer and process the two layers separately.
- Add 20 μl/ml ethidium bromide (stock 200 μg/ml).
- Run overnight at 36 000 rpm in a Beckman ultracentrifuge at 21°C.
- Remove CsCl with several washes of isobutanol, keeping the bottom layer and discarding the top, which is pinkish because of the ethidium bromide.

13.2.3 *Restriction endonuclease digestion of* **Acanthamoeba** *whole cell DNA (De Jonckheere, 1987a)*

- Grow amoebae for 48–72 hours at 30°C.
- Shake vessel to dislodge amoebae.
- Pellet trophozoites and suspend in 2 x amount of suspension buffer, that is 100 mM EDTA; 100 mM NaCl; 10 mM Tris; pH 7.8.
- Add 15 μg/ml proteinase K.
- Lyse cells in 1% Sarkosyl.
- Mix gently by inversion and incubate at 65°C for 20 minutes.
- Chill on ice for five minutes.
- Extract 2 x by gentle mixing for five minutes with phenol, and then for five minutes with chloroform-isoamyl alcohol (25:24:1), using equal volumes of phenol-chloroform mixture and suspension.
- Extract 1 x with equal volume of chloroform-isoamyl alcohol (24:1).
- Separate at 2000 x g for ten minutes at room temperature after each extraction.
- Keep upper aqueous phase.
- Precipitate nucleic acids with 2 x volume of 100% ethanol at -20°C for one hour to overnight.
- Centrifuge at 2000 x g for ten minutes at room temperature.
- Dissolve sample in 2 ml TE buffer, that is 10 mM Tris; 0.1 mM EDTA; pH 7.5.
- Add RNase to a final concentration of 50 μg/ml for 30 minutes at 37°C.
- Add proteinase K to a final concentration of 50 μg/ml and 0.1% SDS for 30 minutes at 37°C.
- Extract 1 x with equal volume of phenol-chloroform-isoamyl alcohol (25:24:1).

- Precipitate DNA with 0.1 volume 3M sodium acetate (pH 5.2) (i.e. 200 μl in 2 ml) and 2 x volume 100% ethanol.
- Store at -20°C for one hour to overnight.
- Pellet by centrifugation at 2000 x g for ten minutes.
- Dissolve DNA in 0.5 ml TE buffer.
- Check DNA and protein concentration on a spectrophotometer.
- Dilute so that DNA concentration is 2 μg/ml.
- Aliquot 50 μl of sample per Eppendorf tube.
- Add 1/10 volume sodium acetate, then 2 x volume of 100% ethanol and store at -20°C.

13.2.4 *Alkaline lysis method of isolation of* **Acanthamoeba** *mitochondrial DNA* *(Yagita & Endo, 1990)*

- Grow amoebae for 48–72 hours at 30°C.
- Shake vessel to dislodge amoebae.
- Pellet trophozoites from a 180 cm^2 tissue culture flask, that is 10^7-10^8 amoebae.
- Wash twice with phosphate-buffered saline pH 7.2 (*1).
- Transfer pellet to an Eppendorf tube and wash twice with TES buffer (*2).
- Resuspend pellet in 200 μl of ice-cold TES.
- Add 400 μl of freshly prepared 1% SDS in 0.2M NaOH (*3).
- Mix gently but thoroughly, and place on ice for five minutes.
- Add 300 μl of ice-cold 3M potassium acetate buffer (pH 6.0) (*4).
- Mix gently but thoroughly and place on ice for 30 minutes.
- Microfuge at 12 000 g for ten minutes at 0°C.
- Transfer supernatant to fresh Eppendorf tube, extract with phenol-chloroform-isoamyl alcohol and centrifuge at 12 000 x g for ten minutes.

- Transfer supernatant to a fresh Eppendorf tube and add 1/10th the volume of 3 M sodium acetate (pH 5.2) and 2 x volume of 100% ethanol, then leave at -20°C for 30 minutes.
- Microfuge at 12 000 x g for five minutes.
- Remove all supernatant.
- Dry pellet in a vacuum desiccator and redissolve pellet in 60 μl of TE buffer.
- Store at -20°C until used.

**1 Phosphate Buffered Saline (pH 7.2)*

NaCl	8 g/l
KCl	0.2 g/l
Na_2HPO_4 (anhydrous)	1.15 g/l
KH_2PO_4	0.2 g/l

This is normally at pH 7.4. Use concentrated HCl to obtain the correct pH.

**2 TES Buffer*

Tris-HCl (pH 8.0)	50mM (6.06 g/l)
EDTA (pH 8.0)	10mM (3.72 g/l)
Sucrose	50mM (17.12 g/l)

**3 NaOH/SDS*

NaOH	0.2 M
SDS	1%

Prepare fresh from separate stock solutions of 1M NaOH (40 g/l) and 10% SDS.

For a total of 1 ml use:

0.2 ml	1M NaOH
0.1 ml	10% SDS
0.7 ml	H_2O

**4 Potassium Acetate Buffer (pH 6.0)*

60 ml of 5 M (490.75 g/l) potassium acetate
11.5 ml glacial acetic acid
28.5 ml H_2O
Adjust to pH 6.0.

13.2.5 *Restrictions* *(Sambrook et al., 1989)*

Nine restriction endonucleases with their appropriate buffers, namely *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Pvu*I, *Sal*I and *Xba*I, all supplied by Boehringer Mannheim, were used.

- Centrifuge DNA sample in Eppendorf (12 000 x g).
- Re-dissolve DNA in TE buffer.
- Add buffer and enzyme to DNA sample, using 10–20 U enzyme/μg DNA (the ratio of enzyme to total volume must not be greater than 1:10 due to inhibition of the restriction by glycerol present in the enzyme solution).
- Include RNase (5 μl/ml of 10 mg/ml stock solution) at the restriction stage or after the first phenol extraction; if the latter, precipitate DNA, redissolve in 2 ml TE buffer and add RNase; incubate for two to three hours and then do phenol extraction (13.2.2.1) and precipitation.
- Incubate at 37°C for three hours.
- Boil at 65°C for five minutes to stop the enzyme activity.
- Remove samples from water bath and add loading buffer for gel, that is 10 μl loading buffer for each 50 μl sample (1:5 loading buffer:sample).
- Include DNA standards consisting of fragments from cleavage of lambda-DNA with restriction endonuclease *Hin*dIII (DNA molecular weight marker II) (Boehringer Mannheim) or with *Eco*RI and *Hin*dIII (DNA molecular weight marker III) (Boehringer Mannheim) as size markers.
- To prepare markers for loading on to gel: 3 μl loading buffer; 3 μl molecular weight marker by heating at 65°C for five minutes before use.
- Add markers and samples to wells.
- Make up agarose gels in TAE buffer (see below).
- Run unrestricted DNA on a 0.5% agarose gel and restricted DNA on a 0.8%–1% agarose gel.
- Cover with Gladwrap and run for 18–20 hours at no more than 5 V/cm.
- Soak the gel in 0.5 μg/ml ethidium bromide made up in buffer or distilled water for 30–45 minutes to stain: to destain for smaller bands, soak stained

gel in water or 1mM $MgSO_4$ for 20 minutes to one hour at room temperature, or incorporate ethidium bromide into the gel (see below).

- View and photograph under UV light.

13.2.6 ***Preparation of a large agarose gel*** *(Quantities for 2 gels)*

- Take 500 ml distilled water and 10 ml TAE buffer (50 x concentrated).
- Mark level of fluid and add 5 g agarose.
- Microwave on "High", using cotton wool stopper, until clear.
- When cool, add 25 μl ethidium bromide (from 10 mg/ml stock).
- Pour into mould and add slots.

Tank buffer

40 ml TAE buffer (50 x concentrated) made up to 2,000 ml with distilled water.

TAE Buffer (Concentrated stock solution (50 x))

Per litre:

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

13.3 Results

Large yields of amoebae were required to obtain DNA bands using the mtDNA CsCl and phenol methods (13.2.2.1 and 13.2.2.2) for isolation of DNA. Restrictions with enzymes from three hours to overnight gave either no cutting of DNA or smearing. Whole-cell DNA extraction (13.2.3) worked well, and results were initially obtained using this method. However, the researcher found that the alkaline lysis technique of isolating mtDNA (13.2.4) was superior, and therefore preferred that method.

Results are summarised in Figures 13.1 – 13.7 and these bands were obtained from an average of at least 5 gel runs. The figures are representations of the gel runs. The scale for the kBP was calculated according to the distances travelled by DNA molecular weight marker III (Boehringer Mannheim).

Strains ATCC 50685, ATCC 50686 and ATCC 50687 gave the same RFLPs with enzymes *Bam*HI, *Eco*RI and *Hin*dIII (Figs. 13.1; 13.3; 13.4; 13.8). All the other strains showed unique RFLPs, and this was consistent for each restriction endonuclease used (Figs. 13.1 – 13.10). The sums of the sizes (in kilobases) of the RFLPs detected with the different enzymes, as well as average sizes, are shown in Table 13.1.

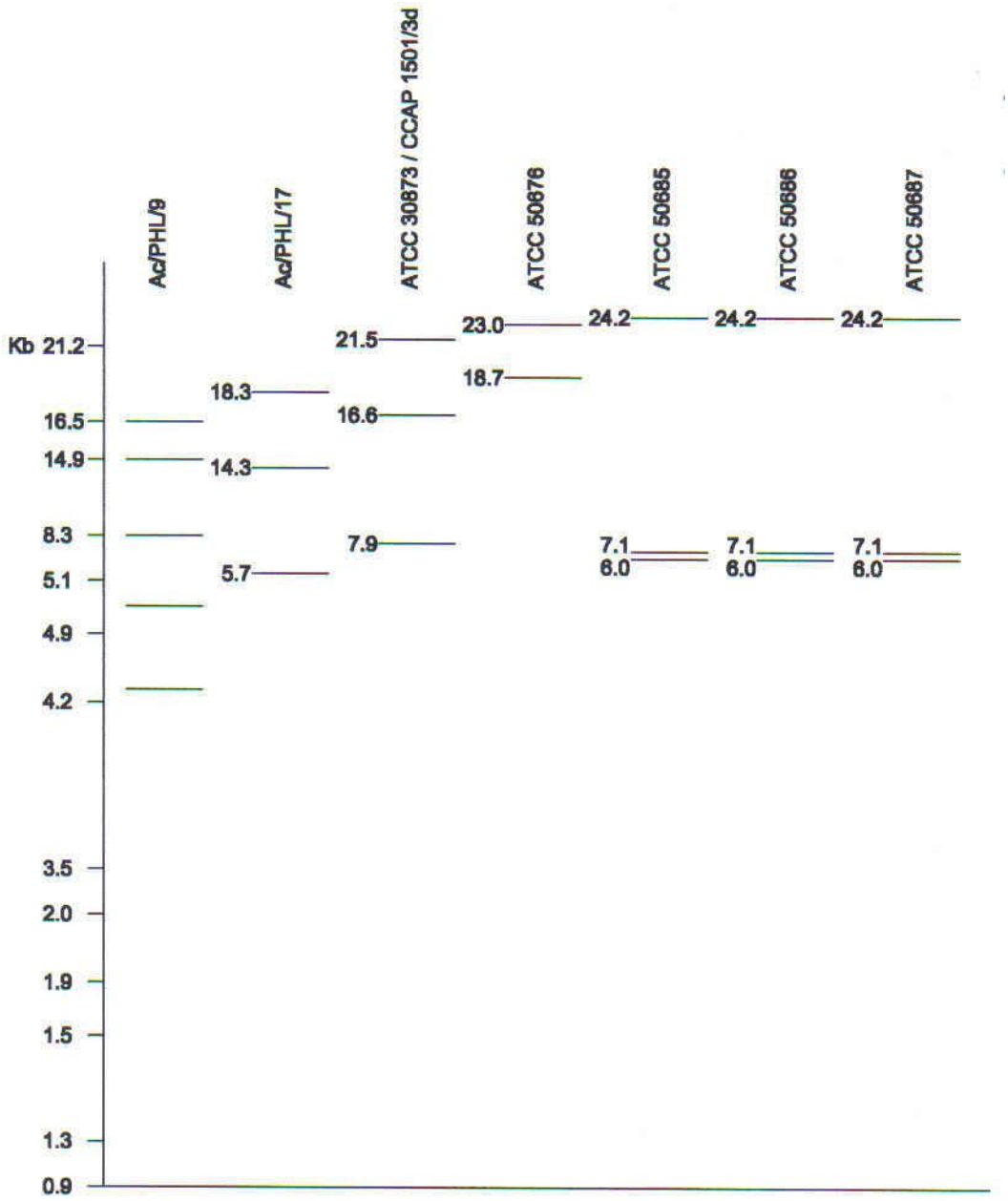


Figure 13.1. Diagrammatic representation of differentiation between strains of various *Acanthamoeba* species (see Appendix IV) by *Bam*HI restriction fragment length polymorphism (RFLP) groups.

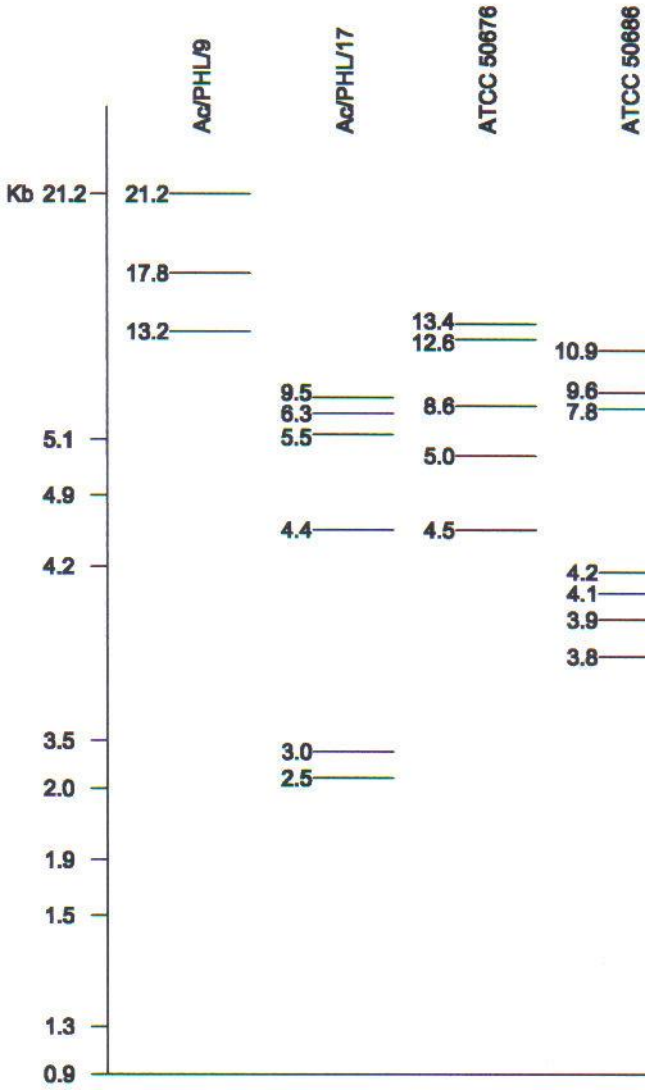


Figure 13.2. Diagrammatic representation of differentiation between strains of various *Acanthamoeba* species (see Appendix IV) by *Bgl*II restriction fragment length polymorphism (RFLP) groups.

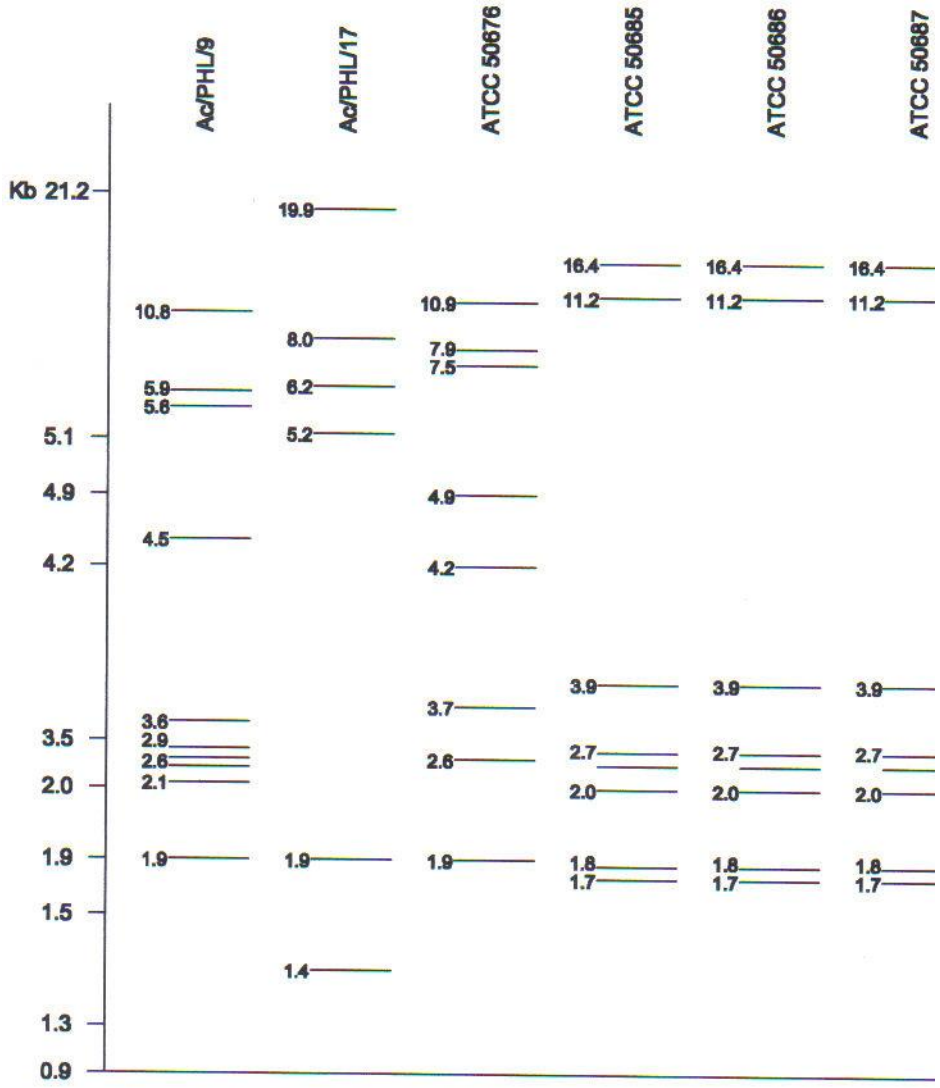


Figure 13.3. Diagrammatic representation of differentiation between strains of various *Acanthamoeba* species (see Appendix IV) by *Eco*RI restriction fragment length polymorphism (RFLP) groups.

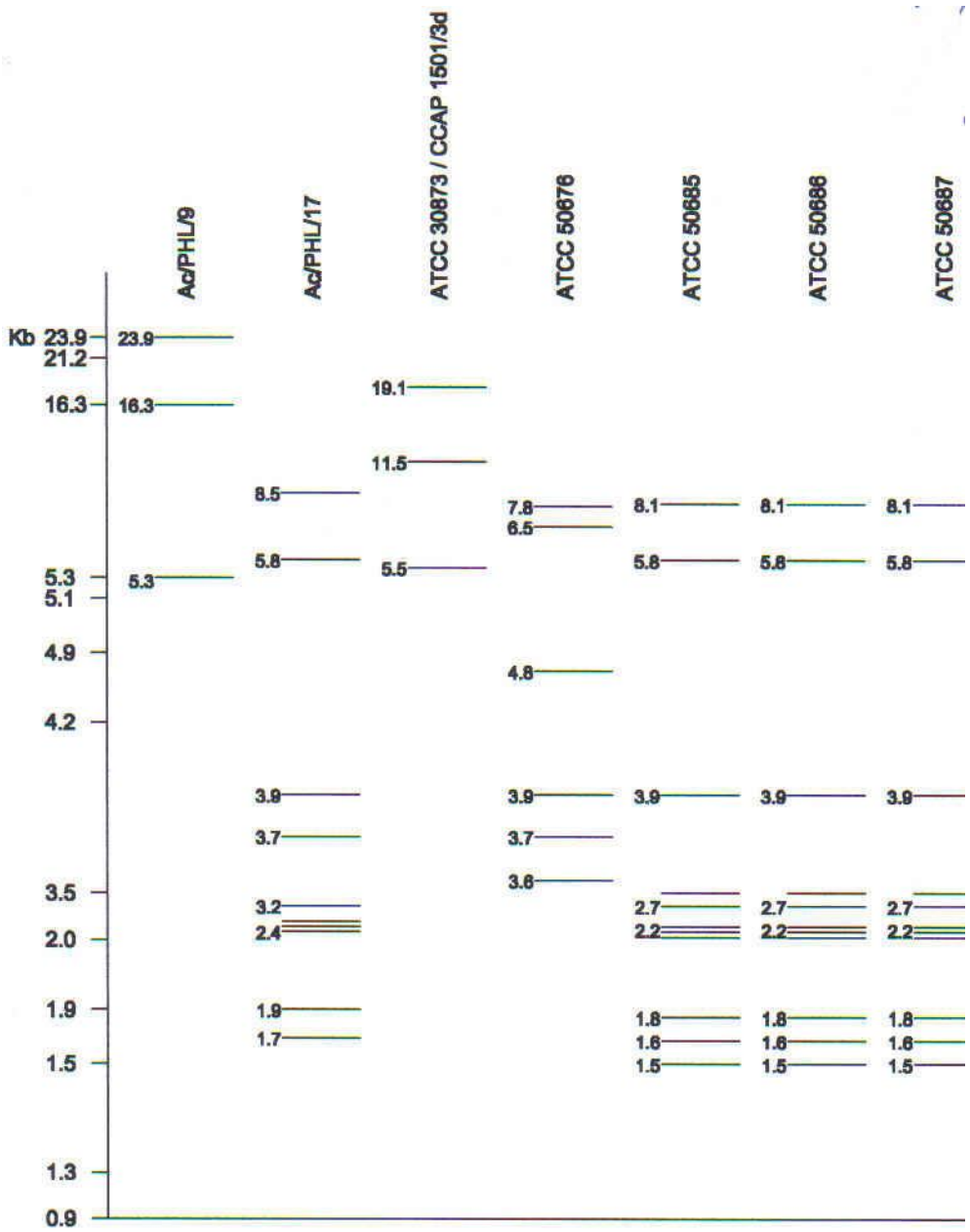


Figure 13.4. Diagrammatic representation of differentiation between strains of various *Acanthamoeba* species (see Appendix IV) by *Hin*dIII restriction fragment length polymorphism (RFLP) groups.

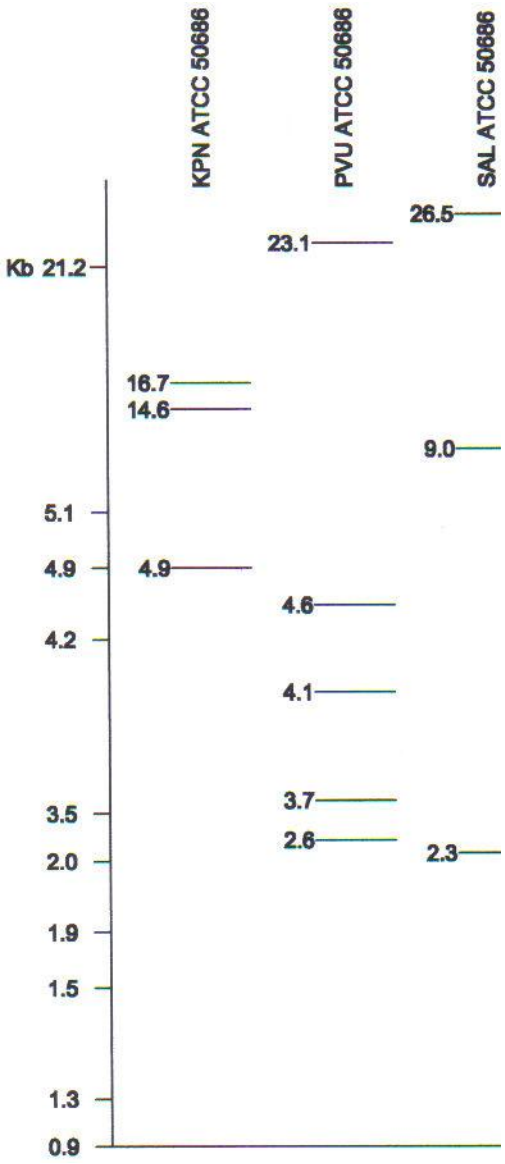


Figure 13.5. Diagrammatic representation of *Kpn*I, *Pvu*I and *Sal*I restriction fragment length polymorphism (RFLP) groups of *Acanthamoeba lenticulata* strain ATCC 50686.

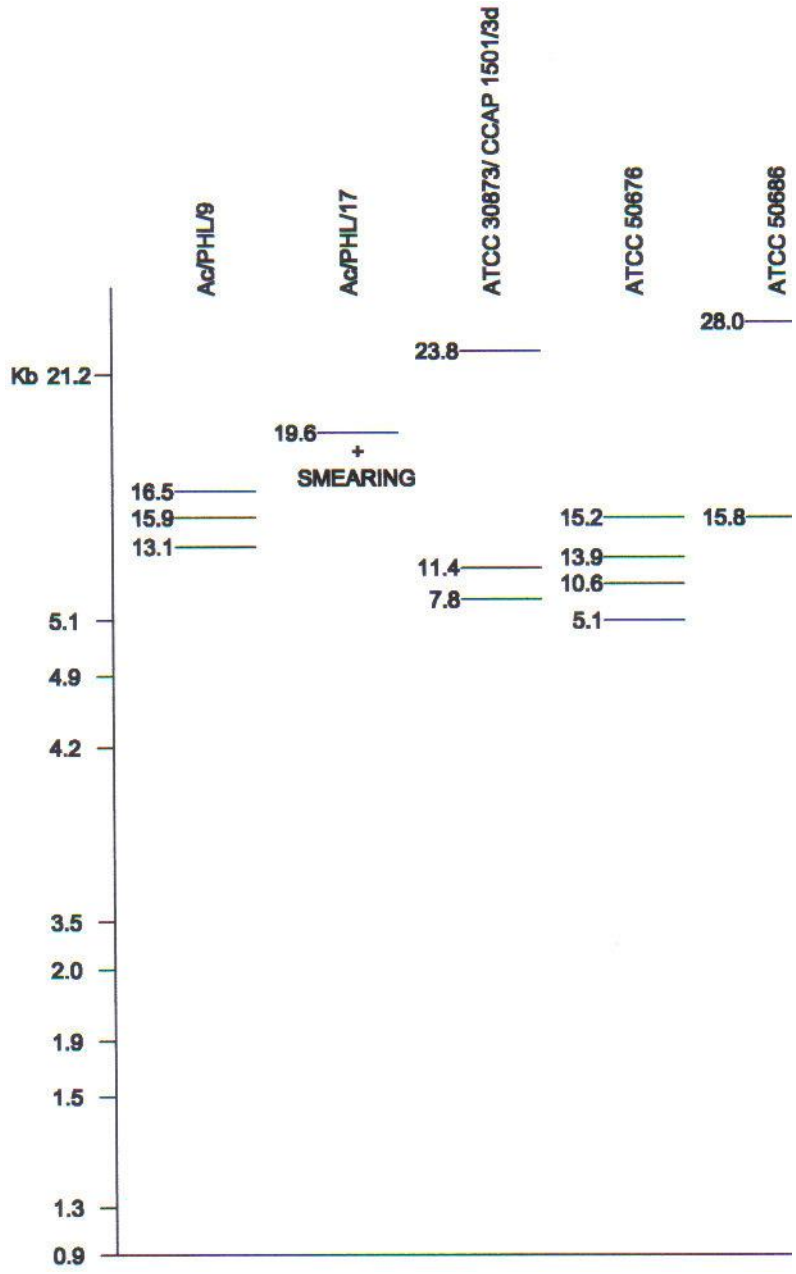


Figure 13.6. Diagrammatic representation of differentiation between strains of various *Acanthamoeba* species (see Appendix IV) by *Pst*I restriction fragment length polymorphism (RFLP) groups.

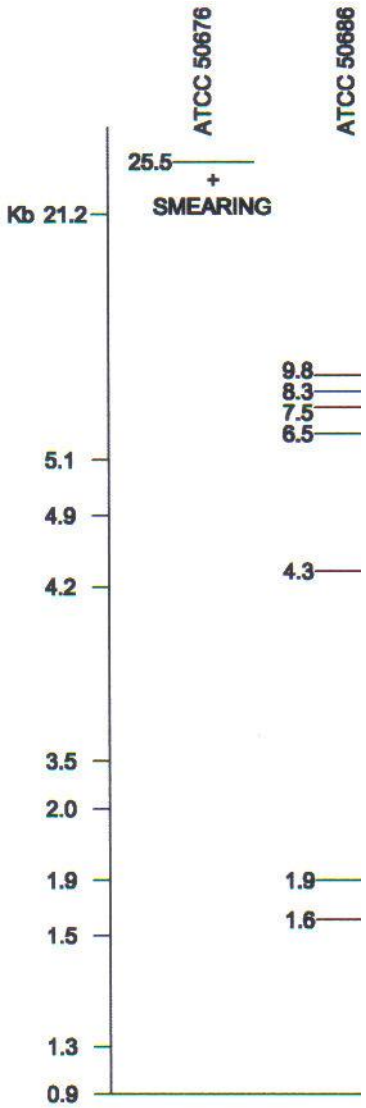


Figure 13.7. Diagrammatic representation of differentiation between strains of various *Acanthamoeba* species (see Appendix IV) by *Xba*I restriction fragment length polymorphism (RFLP) groups.

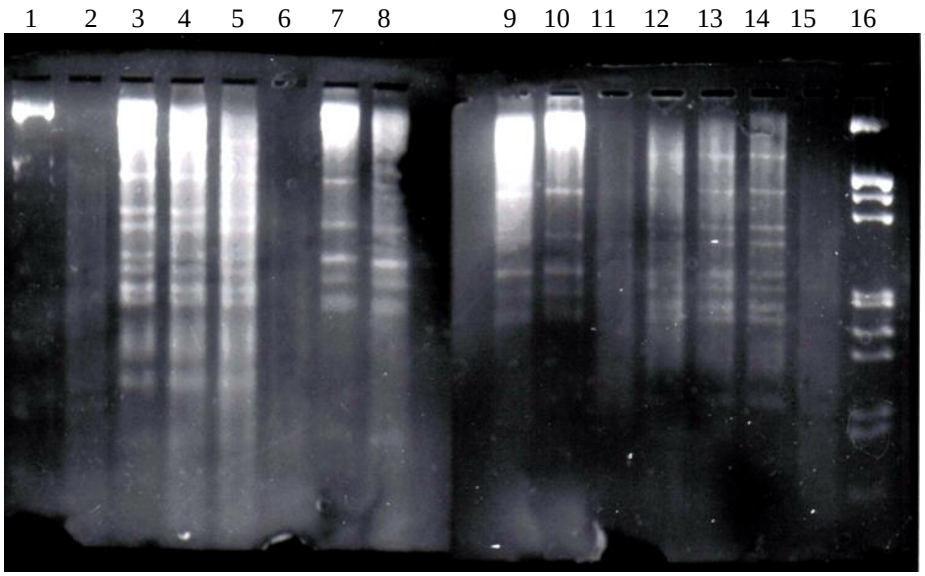


Figure 13.8. Photograph of agarose-gel electrophoresis of *Eco*RI and *Hin*dIII digests of *Acanthamoeba* whole-cell DNA.

Key:

Lane 1 = *Hin*dIII-digested lambda molecular size standard.
Lane 2 = *Hin*dIII digest of ATCC 50676.
Lane 3 = *Hin*dIII digest of ATCC 50685.
Lane 4 = *Hin*dIII digest of ATCC 50686.
Lane 5 = *Hin*dIII digest of ATCC 50687.
Lane 6 = *Eco*RI digest of ATCC 50676.
Lane 7 = *Eco*RI digest of ATCC 50686.
Lane 8 = *Eco*RI digest of ATCC 50685.
Lane 9 = *Eco*RI digest of ATCC 50685.
Lane 10 = *Eco*RI digest of ATCC 50686.
Lane 11 = *Eco*RI digest of ATCC 50676.
Lane 12 = *Hin*dIII digest of ATCC 50687.
Lane 13 = *Hin*dIII digest of ATCC 50685.
Lane 14 = *Hin*dIII digest of ATCC 50686.
Lane 15 = *Hin*dIII digest of ATCC 50676.
Lane 16 = *Hin*dIII-digested lambda molecular size standard.

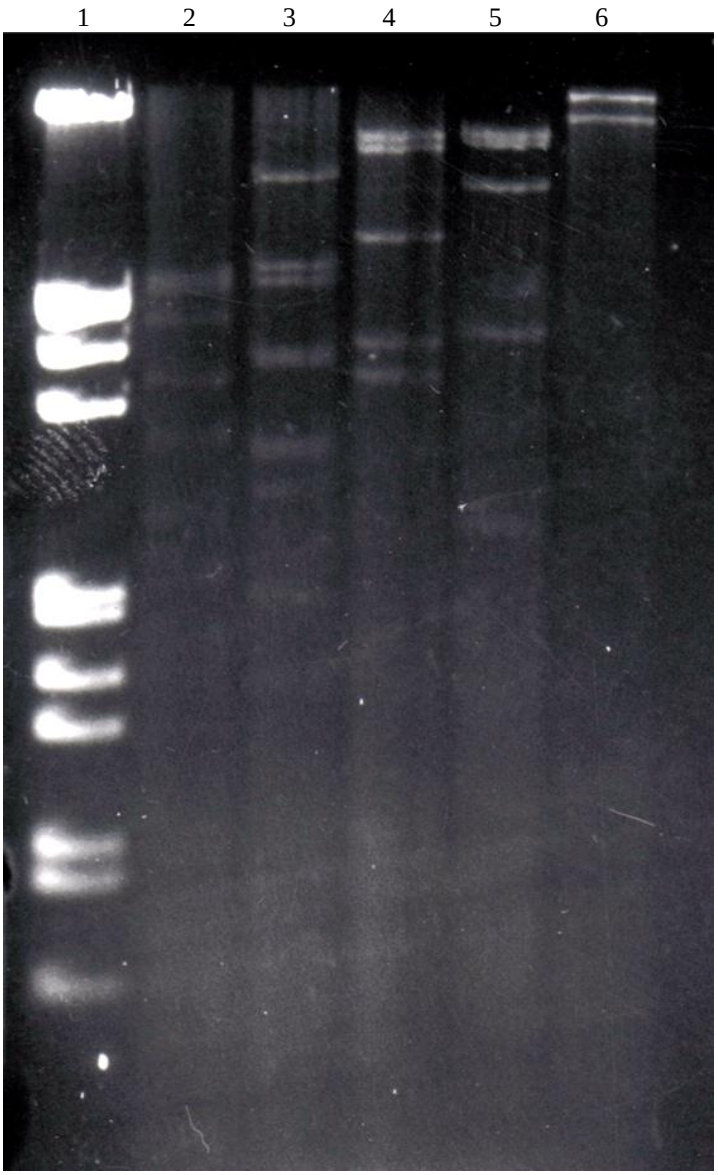


Figure 13.9. Photograph of agarose-gel electrophoresis of *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII and *Pst*I digests of *Acanthamoeba mauritaniensis* strain ATCC 50676 whole-cell DNA.

Key:

Lane 1 = *Hin*dIII-digested lambda molecular size standard.
Lane 2 = *Hin*dIII digest of ATCC 50676.
Lane 3 = *Eco*RI digest of ATCC 50676.
Lane 4 = *Bgl*II digest of ATCC 50687.
Lane 5 = *Pst*I digest of ATCC 50676.
Lane 6 = *Bam*HI digest of ATCC 50676

1 2 3 4 5 6 7 8 9 10 11 12 13

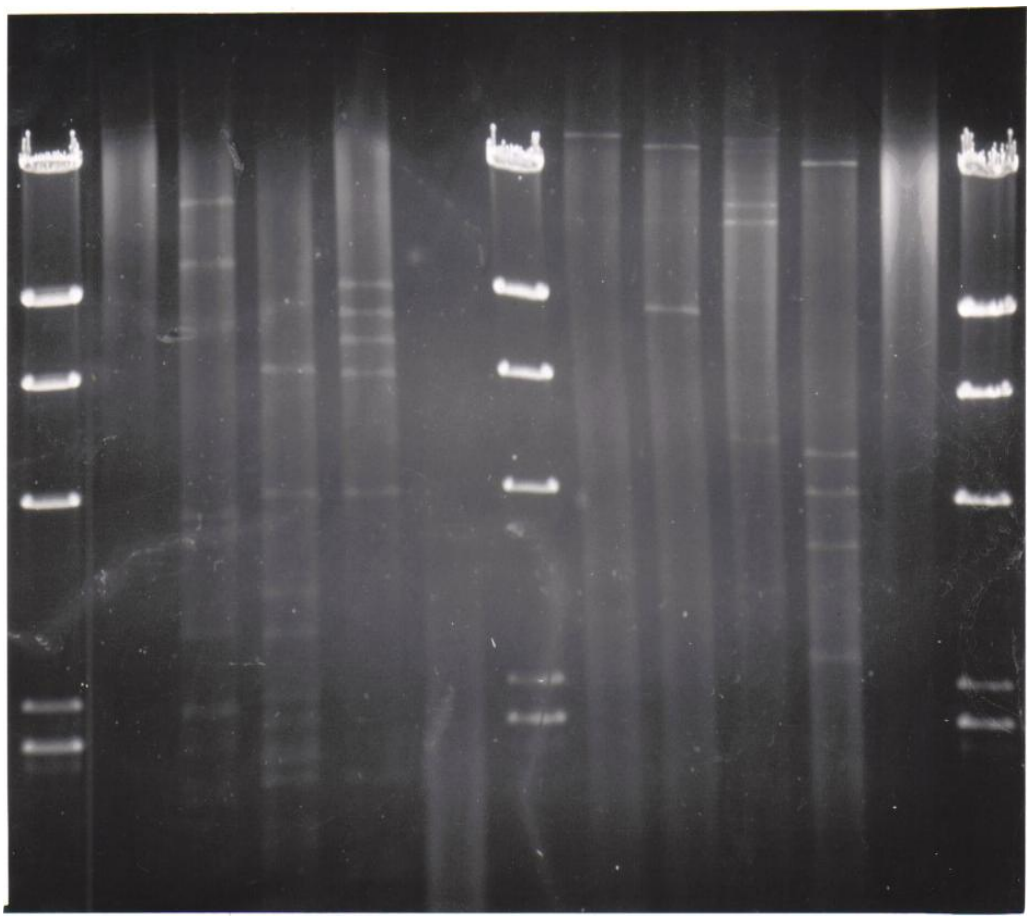


Figure 13.10. Photograph of agarose-gel electrophoresis of *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Pvu*I, *Sal*I and *Xba*I digests of *Acanthamoeba lenticulata* strain ATCC 50686 whole-cell DNA.

Key:

Lanes 1, 7, 13	= *Hin*dII-digested lambda molecular size standard.
Lane 2	= Whole DNA
Lane 3	= *Eco*RI digest of ATCC 50686.
Lane 4	= *Hin*dIII digest of ATCC 50686.
Lane 5	= *Xba*I digest of ATCC 50686.
Lane 6	= *Bam*I digest of ATCC 50686.
Lane 8	= *Pst*I digest of ATCC 50686.
Lane 9	= *Sal*I digest of ATCC 50686.
Lane 10	= *Kpn*I digest of ATCC 50686.
Lane 11	= *Pvu*I digest of ATCC 50686.
Lane 12	= *Asp*700 digest of ATCC 50686.

Table 13.1. Total sizes of *Acanthamoeba* restriction fragment length polymorphisms (RFLPs).

RFLP (kb) sizes after cutting with restriction endonucleases

***Acanth-amoeba* Strain**	***Bam* HI**	***Bgl*II**	***Eco* RI**	***Hin*d III**	***Kpn*I**	***Pst*I**	***Pvu*I**	***Sal*I**	***Xba*I**	**Ave**
Ac/PHL/9	49	52.2	42.3	45.5		45.5				46.9
Ac/PHL/17	38.3	31.2	42.6	37.0		*				37.3
ATCC 30873	46			36.1		43.0				41.7
ATCC 50676	41.7	44.1	43.6	30.3		44.8			*	40.9
ATCC 50685	37.3		42.0	36.1						38.5
ATCC 50686	37.3	44.3	42.0	36.1	36.2	43.8	38.1	37.8	39.9	39.5
ATCC 50687	37.3		42.0	36.1						38.5
										40.5

*Only one discrete band occurred with smearing underneath; therefore, total size cannot be given.

13.4 Discussion

Using restriction endonuclease digestion of *Acanthamoeba* whole-cell DNA, the writer was able to detect RFLPs that could be used to separate the seven strains examined into five groups. The three strains (ATCC 50685, ATCC 50686 and ATCC 50687) that gave similar RFLPs with enzymes *Bam*HI, *Eco*RI and *Hin*dIII, were also morphologically similar. These strains have been identified by 18S rDNA polymerase chain reaction (PCR) as *A. lenticulata* (Schroeder *et al*., 2001). Strain ATCC 30873 has been classified on morphological grounds as *A. polyphaga*, while strain Ac/PHL/17 formed cysts typical of group II *Acanthamoeba* spp. and resembled *A. polyphaga* or possibly *A. castellanii* (Kilvington *et al*. 1991a). Even though these strains were morphologically similar, neither of them showed similar RFLP patterns when digested with *Bam*HI, *Hin*dIII or *Pst*I. The number and positions of the bands for strain Ac/PHL/17, when using the enzymes *Bgl*II, *Eco*RI and *Hin*dIII, were similar to those obtained for the same strain by Kilvington *et al*. (1991a). The RFLP patterns for a corneal isolate (ATCC 50676), identified as *A. mauritaniensis* by 18S rDNA PCR (Schroeder *et al*., 2001), differed from those for all the other isolates.

The existence of differences between RFLPs for strains that are morphologically similar, have been confirmed by other authors (Bogler *et al*., 1983; Costas *et al*., 1983; Byers *et al*., 1990; Yagita & Endo, 1990; Kilvington *et al*., 1991a). Enzyme electrophoresis of esterases and acid-phosphatases (Costas & Griffiths, 1980) as well as immunoelectrophoresis (Willaert, 1976) have shown a close similarity between two strains that had been identified as *A. castellanii* and *A. polyphaga*, respectively. These results have been confirmed by the finding of identical mtDNA restriction patterns for both of these strains with the enzyme

*Hha*I (Costas *et al*., 1983). The restriction patterns of the two strains differed markedly from those of *A. rhysodes* and *A. griffini*, with few common bands being found (Costas *et al*., 1983). In addition, mtDNAs of two non-pathogenic strains that were classified as different species, namely *A. castellanii* and *A. polyphaga*, were identical when cut with five different enzymes (Byers *et al*., 1990). Yagita & Endo (1990) have found that three strains morphologically classified as *A. polyphaga* share a single RFLP phenotype with the Ma strain of *A. castellanii*.

Kilvington *et al*. (1991a) used restriction endonuclease digestion of *Acanthamoeba* whole-cell DNA to study the relationship between 33 morphologically identical strains, all forming cysts typical of group II *Acanthamoeba* spp. and resembling *A. polyphaga* or possibly *A. castellanii*. By comparing RFLPs, these researchers assigned the strains to seven multiple-strain and three single-strain groups. In light of their results, Kilvington *et al*. (1991a) state that it appears to be unclear whether *Acanthamoeba* mtDNA RFLPs indicate intra- or inter-species differences.

The results obtained by Bogler *et al*. (1983) suggest that extensive nucleotide sequence diversity occurs among strains of a single species of *Acanthamoeba*, but that sub-groups of strains with similar sequences occur. Both the Lewin and Bos phenotypes are shared by two species, *A. castellanii* and *A. polyphaga*. Bogler *et al*. (1983) ascribe this result to difficulties in distinguishing between these two species. Byers *et al*. (1990) have found that restriction fragment length profile studies of mtDNA reveal relatively high levels of overall sequence diversity. Bogler *et al*. (1983) concluded that restriction enzyme analysis can identify clusters of strains and may be a useful approach to classification in the genus.

The average size of the DNA for the seven strains of *Acanthamoeba* for which research results are reported in this thesis, is 40.5 kb. This is similar to the values found for mtDNA for this genus by Bogler *et al*. (1983) and Kilvington *et al*. (1991a). Bogler *et al*. (1983) found that mtDNA for 15 *Acanthamoeba* strains was circular, averaging 41.6 +/- 1.5 kilobase pairs. Bogler *et al*. (1983) estimated that the size of the mtDNA genome for *Acanthamoeba* strain Ac/PHL/29 (CCAP 1501/1a, Neff strain) is 40.6 kb, which is similar to the value of 40.1 kb found for whole-cell DNA of this strain by Kilvington *et al*. (1991a). The molecular weights of *A. griffini* were low (31.1 kb) when compared with *A. castellanii*/*A. polyphaga* (36.7 kb) and two different strains of *A. rhysodes* (36.0 and 40.7kb) (Costas *et al*. (1983). Bogler *et al*. (1983) found that electrophoretic patterns of fragments were identical for mtDNA from whole cells or from purified mitochondria. Kilvington *et al*. (1991a) have concluded that restriction endonuclease digestion of *Acanthamoeba* whole-cell DNA gives rise to RFLPs originating from mtDNA.

Kilvington *et al*. (1991a) found that digestion of *Acanthamoeba* whole-cell DNA with the restriction endonucleases B*am*HI, *Kpn*I and *Pst*I resulted in only a smearing of DNA after agarose gel electrophoresis and staining with ethidium bromide. This effect was not altered by increasing the restriction endonuclease concentration and incubation time. Kilvington *et al*. (1991a) ascribe this failure of the restriction endonucleases to cleave the mtDNA in *Acanthamoeba* whole-cell DNA to the absence of endonuclease sequence recognition sites on the mtDNA or to methylation of the sites, which would render them resistant to digestion (Nelson & McClelland, 1989). The DNA samples of Ac/PHL/17 (one of the strains worked on by Kilvington *et al*., 1991a) as well as strains Ac/PHL/9, ATCC 30873, ATCC 50676, ATCC 50685, ATCC 50686 and ATCC 50687, which were

examined by the present researcher, produced discrete bands of DNA when digested with the restriction endonuclease *Bam*HI, which enabled specific RFLPs to be detected. Kilvington *et al*. (1991a) had smearing with restriction endonuclease *Pst*I. A similar result with this enzyme was obtained in this study for strains Ac/PHL/17 and ATCC 50686. However, one discrete band was found in each case. During this investigation, distinct bands were seen for the other strains of *Acanthamoeba* for restrictions with *Pst*I, and for restrictions done with the enzyme *Kpn*I for whole DNA of strain ATCC 50686. McLaughlin *et al*. (1988) found that resolution of more intense and distinct bands depended on gentle mixing during DNA isolation; there being sufficient restriction enzyme; low voltage (below 10 V/cm) usage during electrophoresis; and omission of ethidium bromide during the gel run.

The relationship between *Acanthamoeba* strains from different continents was studied by restriction endonuclease digestion of purified mtDNA (Byers *et al*., 1983). Five strains of *Acanthamoeba* from Europe, North America and New Zealand were found by Byers *et al*. (1983) to have identical digestion phenotypes with five enzymes. Consequently, very similar nucleotide sequences are predicted. All of the strains were pathogenic to humans or mice. The mtDNA sequences of the eight remaining strains are judged to differ from this cluster and, in most cases, from each other (Byers *et al*., 1983).

The results of the DNA studies reported in this thesis confirm the groupings of the southern African *Acanthamoeba* strains obtained by cellular acetate isoenzyme electrophoresis (Chapter 12). More work needs to be done on southern African isolates of *Acanthamoeba* to determine whether local strains have similar RFLPs to those of overseas isolates.

Restriction endonuclease analysis of whole-cell DNA appears to be a valuable technique for detecting mtDNA RFLPs in the differentiation of morphologically identical *Acanthamoeba* strains (McLaughlin *et al*., 1988). This technique may, therefore, prove useful in resolving the complex taxonomy of the genus, which has hitherto been founded on subjective morphological criteria (Kilvington *et al*., 1991a). Once the RFLP patterns for both pathogenic and non-pathogenic strains have been obtained and recorded, it might be possible to identify potential pathogens according to their RFLP patterns.

CHAPTER FOURTEEN – GENERAL CONSIDERATIONS

Martinez & Visvesvara (1997) found that none of the cases of primary invasion of the human eyeball described up until that time provided evidence for the occurrence of accompanying invasion of other organs. Mazur *et al*. (1999), on the other hand, found that *Acanthamoeba* infections do not appear to be organ specific, necessarily. According to the latter researchers, we might expect that some of the eye invasions described in humans, especially those of undetermined route of infection, could represent secondary infections preceded by undetected invasion of the CNS or other organs. However, the fact that eye infections with *Acanthamoeba* are generally not accompanied by serological positivity indicates that most eye infections are not secondary, since most systemic infections are accompanied by serological positivity. Future work could perhaps entail checking for signs of acanthamoebic infection elsewhere in the body to ascertain whether the invasion of the eye is a secondary infection, as was found experimentally by Mazur *et al*. (1999).

The medical importance of *Acanthamoeba* extends beyond its ability to infect the human body. Long ago, the survival of *Legionella pneumophila* within cysts of *A. polyphaga* was suggested as a possible mechanism by which the organism evades disinfection and spreads to colonise new environments (Kilvington & Price, 1990). *Acanthamoeba* cysts produced from infected trophozoites were shown to protect the legionellas from at least 50mg/l free chlorine (Kilvington & Price, 1990). The activities of biocides against *L. pneumophila* grown in amoebae were

greatly reduced (Barker *et al*., 1992). Two *Acanthamoeba* species fed *L. pneumophila* at three different temperatures, expelled vesicles containing living *L. pneumophila* cells. Theoretically, these vesicles could contain several hundred bacteria (Berk *et al*., 1998). Clusters of bacteria in vesicles were not dispersed by freeze-thawing and sonication, and such vesicles might be agents for the transmission of legionellosis associated with cooling towers (Berk *et al*., 1998).

An outbreak of Legionnaire's Disease at a South African clinic in the 1990s led the authorities to attempt to isolate *Legionella* from the clinic's water tanks. It was not cultured from the tanks. Consequently, the Water Board decided that the water was free of *Legionella*. However, direct fluorescence of water showed the presence of *Legionella*, and the present author isolated amoebae from the water tanks. The amoebae were possibly harbouring the bacteria. This might explain why *Legionella* could not be cultured directly from the water, despite the fact that the organisms could be detected by direct fluorescence. Because the water tanks at South African hospitals can contain pathogenic organisms harboured by amoebae, a thorough survey should be carried out for amoebae as well as bacteria; particularly in tanks at hospitals where certain diseases have broken out. It is important to find ways in which amoebae harbouring bacteria can be eliminated from places in which patients (particularly those who are immunosuppressed) are at high risk of contracting infections.

Members of the taxonomically diverse *Burkholderia cepacia* complex have become a major health risk for patients with cystic fibrosis. *Acanthamoeba* might be a reservoir for *B. cepacia*, and possibly a vehicle for transmission (Marolda *et al*., 1999). Tomov *et al*. (1999) described a mechanism involving *Acanthamoeba* spp. for the spread, replication and persistence of obligately anaerobic bacteria in

the environment, and new possible sources, reservoirs and transfer mechanisms of infections caused by obligate anaerobe bacteria.

Thom *et al*. (1992) found that *Vibrio cholerae* might have an intra-cellular/amoebal habitat. The survival of *V. cholerae* within cysts of certain species of amoebae for long periods would provide a protected niche under unfavourable conditions as well as a means of dispersal. An alteration in conditions could result in the emergence of the trophozoite from the cyst, the intra-amoebal multiplication of the *Vibrio*, and its subsequent release into the environment (Thom *et al*., 1992).

Ben Salah and Drancourt (2010) used microscopic analyses to demonstrate the engulfment and replication of species of *Myobacterium* in vacuoles of *A. polyphaga* trophozoites. The mycobacteria have been found to survive within the amoebal exocyst and this particular location preserves mycobacteria from an adverse environment and allows them to escape rapidly from the cyst (Ben Salah & Drancourt, 2010).

Campylobacter jejuni, harboured inside *Acanthamoeba castellanii*, has been found to colonise broilers (Snelling *et al*., 2008). *Campylobacter* is an important human pathogen, acknowledged as the most common bacterial agent of paediatric diarrhoea. Broilers are recognised to be the human food source most frequently infected by *Campylobacter*.

Stothard *et al*. (1999) described the first genus- and subgenus-specific fluorescent oligonucleotide probes for *in situ* staining of *Acanthamoeba*. Sequences of these phylogeny-based probes complement the 18S rRNA and the gene encoding it (18S

rDNA). The use of a genus-specific probe with cultured trophozoites and cysts from corneal scrapings illustrated the suitability of using fluorescent oligonucleotide probes for identification of the genus *Acanthamoeba* in both environmental and clinical samples (Stothard *et al*., 1999). In addition, the use of a sequence type T4-specific probe with cultured amoebae indicated the potential of oligonucleotide probes for use in subgeneric classification (Stothard *et al.*, 1999). As has been explained elsewhere in this thesis, molecular determination of the genotypes of isolates of *Acanthamoeba* is starting to become routine. This should also be done in South Africa. See the comments in Appendix IV re classification of isolates of *Acanthamoeba*.

At this time, no effective treatment is available for acanthamoebic infections of the CNS. It is reassuring to see (from literature cited elsewhere in this thesis) that various drugs, etc., are being tested on clinical isolates of *Acanthamoeba.* Different agents or combinations thereof are being tested for their potential in the treatment of acanthamoebic keratitis so that alternatives can become available, should strains develop resistance to the drugs that are effective at present; or in the event that new strains are unresponsive to the present treatment regimens.

Contact lens disinfection systems need to be developed for hard and soft lenses. These systems should be effective against *Acanthamoeba* within a shorter period than that required by the solutions used at present. This aside, the author's results indicate that solutions effective abroad would be effective against southern African isolates and *vice versa.*

Acanthamoeba is ubiquitous in nature, and is able to withstand adverse conditions. Therefore, eradication of its pathogenic strains from the environment is not a

feasible concept. Exposure to *Acanthamoeba* must be common, because 50–100% of the normal population possess antibodies against acanthamoebic antigens (Niederkorn *et al.*, 1999a). There has been be an increase in cases of acanthamoebic infection in various sites of the human body due to the association of this organism with immunocompromised hosts (Chandrasekar *et al*., 1997; Migueles & Kumar, 1998); the number of patients infected has increased with the spread of AIDS. Owing to the fact that over 80% of the patients diagnosed with acanthamoebic keratitis wear contact lenses, and that soft contact lenses account for approximately 75% of the cases (Niederkorn *et al.*, 1999a), the increase in the number of acanthamoebic keratitis cases referred to elsewhere in this thesis has probably followed, in part, more frequent use of contact lenses. A greater awareness of both the danger posed by *Acanthamoeba* and the means of preventing infection is needed amongst health care workers and the general population, to assist in reducing the number of infections caused by this potential pathogen.

C O N C L U S I O N

In general, results presented in this thesis have substantiated and extended the work of other investigators. Also, a number of new features have emerged.

- Potentially pathogenic strains of *Acanthamoeba* were found to be widely distributed in the environment in Gauteng, South Africa.
- A number of strains of *Acanthamoeba* were isolated from corneal scrapings and/or contact lenses or contact lens solutions in suspected cases of acanthamoebic keratitis. They are now reference isolates.
- These strains were cultured and several of them were characterised by light microscopy, electron microscopy, isoenzyme electrophoresis, restriction endonuclease digestion of whole-cell DNA and subgeneric 18S rDNA PCR.
- The effects of contact lens solutions and drugs on several South African isolates of *Acanthamoeba* were determined (controls were strains from overseas), as no information had hitherto been available for local isolates.
- A strain of *Mastigina* that was apparently associated with the infected eye of a patient was isolated (*Mastigina* not having previously been implicated in ocular disease).
- The susceptibility of this South African *Mastigina* isolate to contact lens solutions and drugs was studied.
- The cytopathogenicity of *Acanthamoeba* isolates and the strain of *Mastigina* were evaluated on two different cell lines.

APPENDIX I

Summary of Classification

The taxonomic system employed in this key is derived from a modified classification of the Rhizopoda (Page, 1987; Page, 1988). Taxa outside the scope of the key, including marine gymnamoebae, are omitted.

Phylum RHIZOPODA

Heterotrophic protists with lobopodia, filopodia, or reticulopodia.

Class HETEROLOBOSEA
- Order SCHIZOPYRENIDA
  - Family VAHLKAMPFIIDAE
    - *Adelphamoeba, Naegleria, Paratetramitus, Tetramastigamoeba, Tetramitus, Vahlkampfia, Willaertia*
  - Family GRUBERELLIDAE
    - *Stachyamoeba*
- Order ACRASIDA
  - Family ACRASIDAE
    - *Acrasis, Pocheina*
  - Family GUTTULINOPSIDAE
    - *Guttulinopsis*

Class CARYOBLASTEA
- Order PELOBIONTIDA
  - Family PELOMYXIDAE
    - *Pelomyxa*

Class LOBOSEA
- Subclass GYNMAMOEBIA
  - Order EUAMOEBIDA
    - Family AMOEBIDAE
      - *Amoeba, Chaos, Deuteramoeba, Hydramoeba, Polychaos, Trichamoeba*
    - Family THECAMOEBIDAE
      - *Dermamoeba, Pseudothecamoeba, Sappinia, Thecamoeba, Thecochaos*
    - Family HARTMANNELLIDAE
      - *Cashia, Glaeseria, Hartmannella, Saccamoeba*
    - Family VANNELLIDAE
      - *Platyamoeba, Vannella*
    - Family PARAMOEBIDAE
      - *Dactylamoeba, Mayorella*
    - Family VEXILLIFERIDAE
      - *Vexillifera*
  - Order LEPTOMYXIDA*
    - Suborder RHIZOFLABELLINA

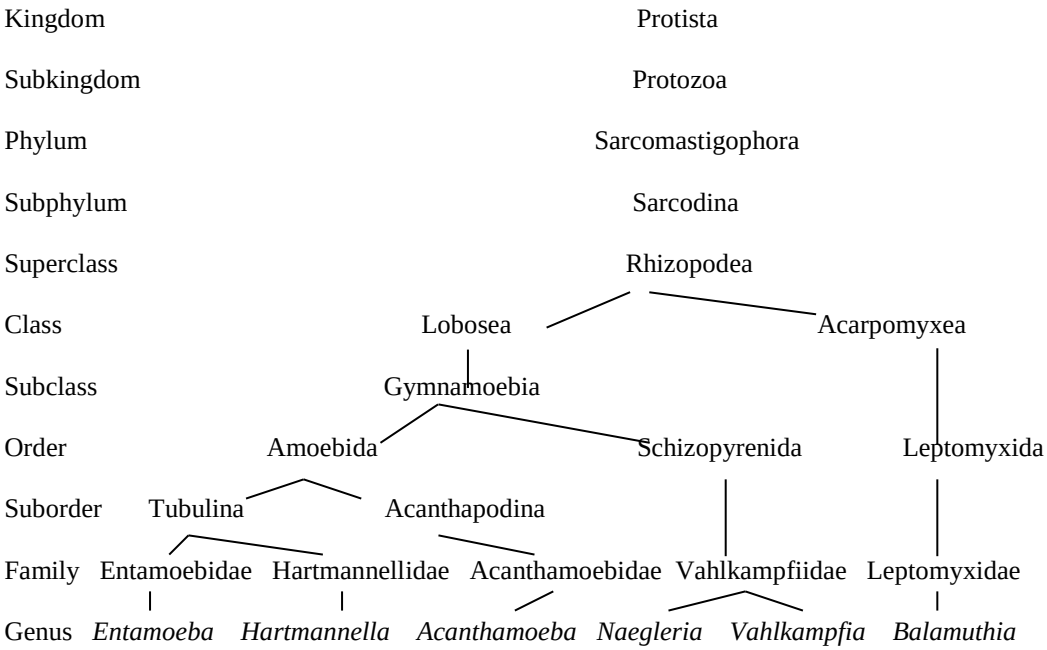
Family FLABELLULIDAE
Paraflabellula
Family LEPTOMYXIDAE
Leptomyxa, Rhizamoeba
Suborder LEPTORAMOSINA
Family GEPHYRAMOEBIDAE
Gephyramoeba
Order ACANTHOPODIDA
Family ACANTHAMOEBIDAE
Acanthamoeba, Protacanthamoeba

Gymnamoebia *incertae sedis*:
Families of uncertain ordinal relationships
HYALODISCIDAE (*Flamella, Hyalodiscus*)
ECHINAMOEBIDAE (*Comandonia, Echinamoeba, Filamoeba*)
ENTAMOEBIDAE (*Entamoeba*)
Genera of uncertain familial relationshipa
Dinamoeba
Phreatamoeba
Subclass TESTACEALOBOSIA
Order HIMATISMENIDA
Family COCHLIOPODIIDAE
Cochliopodium, Gocevia, Paragocevia

**Balamuthia mandrillaris*, n.g., n.sp., has been included in the Order Leptomyxida, Family Leptomyxidae (Visvesvara *et al*., 1993).

Martinez & Visvesvara (1997) have presented the taxonomic scheme as follows:

Position of *Naegleria, Acanthamoeba* and *Balamuthia* in the taxonomic scheme



APPENDIX II

Culturing of amoebae

A) Agar slope

8 g nutrient broth/500 ml distilled water.

6 g agar/500 ml broth.

Store bacterial cultures on agar slopes in the refrigerator. Bacterial cultures must be subcultured every three months.

B) Non-nutrient agar (NNA)

12 g agar/litre distilled water.

Autoclave for 15 minutes at 120°C and pour into Petri dishes.

Allow to cool.

Can be stored in plastic bags in the refrigerator to prevent desiccation.

C) Nutrient broth

8 g nutrient broth (Biolab Chemicals or Difco)/500 ml distilled water.

Shake up and it dissolves readily.

Autoclave for 15 minutes at 120°C.

Grow bacteria in broth at 37°C overnight and then spread over NNA the next day.

D) Peptose-yeast extract glucose (PYG) Medium (Lasman & Feinstein, 1986)

0.1% NaCl.

0.01% K_2HPO_4.

0.01% $MgSO_4$.

0.01% $CaCl_2$.

1% glucose.

0.75% proteose-peptone (Difco).

0.25% yeast extract.

1 litre distilled water.

(All wt/vol.).

Autoclave together: A) NaCl, $CaCl_2$, $MgSO_4$.

B) Glucose, K_2HPO_4, proteose-peptone, yeast extract.

Combine A and B after autoclaving.

E) Chang's serum-casein-glucose-yeast extract medium (SCGYEM)
(De Jonckheere, 1977)

Solution 1:

10 g	Isoelectric or purified casein.
1.325 g	Na_2HPO_4.
800 ml	Distilled water.

Solution 2:

2.5 g	Glucose.
0.8 g	KH_2PO_4.
80 ml	Distilled water.

Autoclave solutions 1 and 2 for 15 minutes at 120°C.

Solution 3:

5.0 g Yeast extract.

200 μg/ml Streptomycin sulphate.

200 U/ml Penicillin.

Sterilise solution 3 by Seitz filtration.

Solution 4:

100 ml Sterile foetal calf serum (inactivated).

Combine the four solutions and dispense 3 ml amounts in sterile, screw-capped tubes for tissue culture.

F) Ampicillin (Sigma)

25 mg/ml in distilled water (stock solution).

Sterilise by filtration and store in aliquots at -20°C.

Working solution: 35 - 50 μg/ml.

Use 20 μl of stock solution for 10 ml of medium.

G) Amoeba saline (De Jonckheere *et al*., 1974)

0.12 g NaCl.

0.004 g $MgSO_4.7H_2O$.

0.004 g $CaCl_2.2H_2O$.

0.142 g Na_2HPO_4.

0.136 g KH_2PO_4.

1 000 ml Distilled water.

H) Siliconising glassware

Leave glassware in 0.5 N NaOH (that is, 20 g NaOH/litre distilled water) overnight.

Rinse in distilled water.

Dip into 5% silica (5 ml dichlordimethylsilan + 95 ml chloroform) and leave for a few minutes.

Bake for four hours at 160°C.

I) Storing amoebae in liquid nitrogen and subsequent reactivation of isolates

Loosen amoebae from sides of container by agitation.

Centrifuge at 400 x g for five minutes.

Use a pipette to remove growth medium and discard it.

Place drop of amoebae into haemocytometer and count amoebae.

Add PYG medium to pellet to give approximately 10 000 amoebae/ml.

Store amoebae in a 1 ml Nunc vial: 0.15 ml glycerol + 0.85 ml PYG medium containing amoebae.

Expose amoebae to fumes of liquid nitrogen overnight, then transfer the closed vial directly into liquid nitrogen the next day.

To reactivate an isolate, heat quickly in water bath at 37°C to thaw amoebae.

Centrifuge to remove glycerine and add fresh medium.

J) Encystment medium (Neff *et al.*, 1964b)

0.1 M KCl (7.456 g).

0.02 M Tris (2.4228 g).

0.008 M $MgSO_4$ (1.972 g).

0.0004 M $CaCl_2$ (0.059 g).

0.001 M $NaHCO_3$ (0.084 g).

1 litre distilled water.

pH 8.9 – 9.00, using concentrated HCl.

Adjust to correct pH before autoclaving: no readjustment is required. Maximum concentration of cells for 1 litre of this medium is 5×10^5 cells/ml.

Check the pH of the medium regularly if more amoebae are used. It takes 20 hours for the amoebae to encyst, and 90% encystment is safely achieved within 48 hours.

APPENDIX III

Acanthamoebic keratitis cases

Various doctors and laboratories approached this researcher to test for acanthamoebic keratitis when their cultures were negative for other organisms or when the patient's symptoms were suggestive of an infection by *Acanthamoeba*. The amount and quality of information regarding the patient's history, medical treatment, diagnosis, etc., varied, depending on the source of the sample.

1) Patient: Mr. C. Source: Dr. C.

Corneal scrapings were obtained on 1998/02/01 and sent to the author for testing. These samples, and two nasal swabs taken from the patient at the same time, were inoculated on to non-nutrient (NNA) agar plates seeded with *E. coli*. Plates were kept at 28°C and at 35°C. *Acanthamoeba* was not found on microscope slides upon examination of fresh corneal scrapings. No growth of *Acanthamoeba* occurred on the NNA plates for any of the samples within 14 days.

2) Patient: Ms. S. (17 years old.) Source: A General Practitioner and Dr. C.

The patient experienced sudden sharp pain in her left eye at the end of July 1990. This pain continued. Her mother administered EyeGene, but no relief was obtained. There was no previous history of swimming or of trauma or damage to the eye. A corneal ulcer developed, and a corneal scraping was taken on 1990/08/15 for this researcher. Fresh preparations showed no amoebae, and calcofluor white staining was done. One cyst was seen, but this was not typical of *Acanthamoeba*. Cultures of the corneal scraping were done on NNA plates seeded with *E. coli*, and these were negative for *Acanthamoeba*. The patient was taken to an ophthalmologist, Dr. C., who diagnosed a viral ulcer and prescribed Zovirax. Dr. C. said that the ulcer was too recent in origin to be acanthamoebic,

and that the structure of the ulcer was typically that found when there is viral aetiology. The ulcer was already very deep and vision was limited to light perception at this stage.

3) Patient: Mr. X. Source: Dr. H.

The patient was a soft (extended wear) contact lenses wearer who used hydrogen peroxide disinfection: he swam and bathed with his lenses inserted. Other solutions used by the patient were Hydrocare Sorbiclean daily lens cleaner; LC 65 daily lens cleaner; and Fizzy Protein removing tablets.

The patient's symptoms started on approximately 1991/01/10. The patient had been treated by another doctor for six weeks and then referred to Dr. H. He suspected *Acanthamoeba* because of the patient's severe pain and the presence of a corneal ulcer that was not typically fungal or bacterial in nature. There was no stromal ring infiltrate.

The researcher (I.A. Niszl) saw the patient on 1991/03/15, when the condition of his eye had already much improved. A corneal scraping to check for *Acanthamoeba* was done. All cultures from the corneal scrapings tested negative for *Acanthamoeba*, but one of the slides stained with calcofluor white showed an apple-green cyst that had the distinct double wall which is typical of *Acanthamoeba*. The patient's contact lenses, contact lens case and solutions were all negative for *Acanthamoeba* upon culture.

The patient's drug regimen at the time when the corneal scrapings were taken, was as follows (the periods given in brackets refer to the length of time since the patient had started using the drug): Brolene (three weeks); Neomycin (2.5 weeks,

stopped four days before the corneal scraping was done); Natacyn (five days: this drug was not well tolerated by the patient, as it is in suspension, and the "gritty pieces" irritated the patient's eye); ketoconazole, orally (eight days). Dr. H. was intending to include Miconazole cream nocte; Miconazole 2.5 mg/ml three-hourly; Clotrimazole two to three-hourly.

4) Patient: Mrs. G. (35 years old.) Source: Dr. A.

The patient wore soft contact lenses and used chemical sterilisation (Bausch and Lomb soaking solution; LC 65 cleaner; and protein tablets made up with Tedro distilled water). She had been reacting badly to the contact lens solutions she was using. Mrs. G.'s first symptoms started in September 1989, with twitching in the lower lid of the right eye. Her optometrist told her that she might be suffering from depression and advised her to see a neurosurgeon. She suffered from continual eye infections, and her optometrist suggested that she should use disposable contact lenses (Johnson & Johnson), which she acquired in November 1989. The patient used home-made saline made from Tedro non-sterile distilled water and salt tablets, which she mixed in a used Barnes and Hind container without chemical or heat disinfection, for the disposable contact lenses. The patient was advised by her optometrist to remove her contact lenses nightly, but that the same pair could be kept for three weeks. Mrs. G. occasionally slept while wearing her contact lenses.

The patient went to a spa (hot water spring) in Rehoboth, Namibia, from 19−21 March 1990. Recycled water is used in the pool at Rehoboth. Mrs. G did not put her head under water and wore her contact lenses when she was in the pool, but some water could have splashed into her eyes. She also went into a pool in Upington, South Africa. She returned from the spa on 1990/03/25. In April, she

had a discharge from her right eye in the mornings, but had no other problems with her eyes.

On 1990/05/02, the patient's right eye became very red and painful, and she experienced severe pain from her head to her neck. She consulted her optometrist on several occasions. He eventually diagnosed a corneal ulcer and referred her to an ophthalmologist, Dr. S.

At the time corneal scrapings were taken by a local laboratory, the patient was using the following drugs: Zovirax, Tobrex, Spersadex Comp., Maxitrol, Atropine, Diamox Tablets and Erymax. When she was referred to Dr. A, who saw her on 1990/05/21, she had a corneal ulcer and a small corneal infiltrate to the nasal side of the ulcer. The diagnosis was *Herpes* with *Candida* infection (that is, suppurative herpes), and Dr. A. treated the patient with 5-fluorocytosine (Alcobon). On 1990/05/21, a Garamycin injection was given into the conjunctiva (0,3cc). On 1990/05/22, Dr. A. added Natacyn two-hourly to the regimen because he felt sure the ulcer was fungal in origin. There was a slight improvement in the condition of the right eye.

On 1992/06/01, Mrs. G. was put on Garasone. She started complaining of severe pain and on 1990/06/05, was given a bandage contact lens for the ulcer. Dr. S. saw her for the next few weeks and there was a slight improvement in the condition of her eye. On 1990/06/27, Dr. S. said that the patient had developed a hypopyon and the ulcer was worse. A stromal ring infiltrate was seen, as well as disciform keratitis. Dr. S. removed the bandage lens.

On 1990/06/28, surgery was carried out, at which time corneal scrapings were examined as a fresh preparation by L. Laboratories in Johannesburg. In addition, an anterior chamber tap (including the hypopyon) was done, and cryosurgery (-5°C to -10°C), which changes the permeability to allow Daktarin to penetrate the anterior chamber, was performed. L. Laboratories kept samples from the anterior chamber tap on sterile swabs for culturing of *Acanthamoeba.* Acanthamoebic keratitis was suspected, as trophozoites were visible on a microscope slide made from the corneal scrapings. Treatment with Daktarin was started hourly, and Nizoral was administered twice daily. The eye started improving. Dr. S. added Neosporin and Brolene to the regimen.

Culturing for *Acanthamoeba* was started on 1990/07/03 by this researcher. The right and left sides of the patient's contact lens case and the Barnes and Hind container used by the patient to mix her home-made saline were dry, so the author used sterile amoeba saline to rinse them out. This was plated onto NNA seeded with *E. coli.* Drops of Mrs. G.'s Tedro distilled water as well as the "sediment" obtained from centrifuging the remaining water were applied directly onto NNA plates seeded with *E. coli.* Two of the patient's salt tablets were mixed with sterile amoeba saline (5 ml) and plated as above. The results are given in tabular form, as seven different elements were tested.

Results

Anterior chamber tap	+
Left contact lens case	+
Right contact lens case	+
Salt tablets (both plates)	-
Distilled water (all 4 plates)	-
Amoeba saline control	-
Swab taken from patient's nose	-

Key:

+ = growth of organisms

- = no growth of organisms.

NNA plates were incubated on 1990/07/03. By 1990/07/05, positive growth (on plates where amoebae were present) was visible. The isolate of *Acanthamoeba* was given the number **ATCC 50676.**

By 1990/07/06, the infection had spread into the deep layers of the cornea and the patient's condition had deteriorated. A corneal scraping was done, from which *Acanthamoeba* was cultured by the researcher. Treatment with Diflucan (300 mg/day), Nizoral tablets (2x daily) and Daktarin (she used an ointment initially, but continued with an oral gel, as this was tolerated better) was initiated. Other drugs being used by the patient were Isopto-atropine, Phenylephrine eyedrops, Neosporin and Tenston (two-hourly, for pain).

After two weeks of using Diflucan (by 1990/07/20), there was still a lot of activity in the right eye, but the patient was feeling better. She still had the same degree of light perception and could see shadows, and her photophobia was severe.

5) Patient: Mrs. B. Source: Dr. D.

The patient was a soft contact lens wearer who disinfected her lenses by boiling them regularly in a standard soft contact lens boiler. She used a "Rosewater Boots" 1987 bottle as the stock bottle. Saline was poured from this bottle into a plastic squeeze bottle. She also used deproteining tablets.

In October 1990, the patient developed a series of chronic ulcers in her right eye. She was seen by a general practitioner in Nelspruit and diagnosed as having herpes simplex infection. She was treated with corticosteroids, which had no effect. When Dr. D. (ophthalmologist) saw her, he did not agree with the initial diagnosis, so he took the patient off Zovirax, and gave her topical steroids and antibiotics and told her not to wear her contact lenses. In November 1990, Mrs. B. used Brolene. The condition of the eye improved. She then acquired disposable contact lenses. She developed another corneal ulcer in January 1991, but this cleared with Brolene.

On 1991/03/27, a corneal lesion and epithelial erosions and underlying peripheral infiltrate appeared. A corneal scraping was done, and samples were sent to the researcher, who also tested the patient's water bottle, her soft and disposable contact lenses, and solutions from both the disposable and soft contact lens cases. No organisms grew from the corneal scraping, but *Mastigina* grew from all the other samples. This was designated as **SAWL 91/2**.

Dr. D. treated Mrs. B. with antibiotics, to which she responded fairly well. The condition of her eye improved when she started using Brolene on 1991/04/27.

A second sample of her soft contact lenses and fluid from the contact lens case was received from Mrs. B. on 1991/07/31, four months after treatment with Brolene had begun. All sampling was negative for *Acanthamoeba* and *Mastigina*. Strain No.: SAWL 91/2.

6) Patient: Mr. Y. Source: Dr. M.

Samples from a contact lens case, contact lenses and solutions used by the patient were sent to the researcher, who plated them on to NNA seeded with *E. coli* on 1991/07/24. All tested negative for *Acanthamoeba* culturing.

Results

Soft contact lenses, both right and left	-
Bausch and Lomb Soflens soaking solution	-
Bausch and Lomb preserved saline solution	-
Bausch and Lomb daily cleaner for soft contact lenses	-
Solutions from right and left contact lens case	-

Key:

- = no growth of organisms.

7) Patient: Mrs. S. (28 years old.) Source: Dr. M.

The patient developed a corneal ulcer in her right eye. The author received corneal scrapings and fluid from her soft contact lens container on 1991/07/29 to culture for *Acanthamoeba*. The eye scrapings were negative for *Acanthamoeba*.

Fluid from the contact lens case was found to be positive for strain **ATCC 50682** on 1991/07/31.

Swabs and scrapings were done again on 1991/08/02. Corneal scrapings and the contact lens cases were checked once more. Right and left corneal scrapings and the patient's contact lens case were all negative for *Acanthamoeba*.

8) Patient: Mrs. H. Source: Dr. A.

Fluid from the patient's contact lens case and contact lens solutions was plated out on 1991/08/07. (No contact lenses were available for culturing.) The following results were obtained on looking for *Acanthamoeba*:

LC 65 daily lens cleaner	-
Hydrocare Sorbiclean daily contact lens cleaner	-
Contact lens case with solutions	+ (1/5 plates)

Key:

+ = growth of organisms

- = no growth of organisms.

The strain was designated as **ATCC 50683**.

9) Patient: Mr. L. Source: Dr. H.

The patient had worn disposable soft contact lenses for five years. He used Poly-cleaner, Polytab and saline to clean his lenses. In 1991, he developed black spots on a pair of his contact lenses that looked like welding marks, but the patient was asymptomatic. The author attempted to culture the patient's contact lenses and solutions in January 1992 but all the cultures were negative for amoebae. The

black spots looked distinctly fungal, as hyphae were visible, so the contact lenses, cases and solutions were sent back to L. Laboratories for fungal culture. The results were positive.

10) Patient: Mrs. P. (29 years old.) Source: Dr. M.
The researcher received a right eye swab in jelly on 1992/04/01. An attempt to culture this for *Acanthamoeba* yielded a negative result.

11) Patient: Mrs. D. Source: Dr. M.
The researcher received a left eye swab in jelly on 1992/05/21. No growth of *Acanthamoeba* occurred in this sample.

12) Patient: Mrs. M. (51 years old.) Source: Dr. D.
The patient had used soft contact lenses five years before (in 1987), developing a painful right eye. She stopped wearing the contact lenses in 1987. This was the only potential predisposing factor to acanthamoebic keratitis that was apparent, as the patient recalled no incident of trauma to the eye.

Mrs. M.'s first eye problem started in 1989, at which time she was treated with Betnesol. She had had three episodes of corneal ulceration after 1989, leaving her with poor vision in her right eye. No organisms were ever cultured from corneal scrapings from her eyes. During this time, the patient had been in London, where she had been treated with the following drugs: Amoxil in 1988; Betnesol, Clotrimazole cream (Canesten), Neomycin and Brolene in 1989; Opticrom, Zovirax, Predsol and Erythromycin in 1990; and Lacrilube Eye Drops and Cyclogyl in 1991.

The patient visited Dr. D. on 1992/07/24, complaining of poor vision in the right eye. At this stage, she had been using artificial tears for ten days, containing benzalkonium chloride as preservative. Dr. D. advised her to stop using the artificial tears. The author received a tear sample from the patient's right eye on a sterile ear bud on 1992/07/29. Cultures tested negative for *Acanthamoeba*.

On 1992/08/10, a corneal scraping was done. The researcher obtained both epithelial and stromal samples for culture. All cultures were negative for *Acanthamoeba.*

13) Patient: Mr. K. (57 years old.) Source: Dr. M.
A corneal swab (taken on 1992/07/31) and the fluid in which the swab was kept, were cultured for *Acanthamoeba* by the researcher and found to be negative.

14) Patient: Mr. W. Source: Dr. R.
The researcher received a swab from the patient's cornea on 1992/08/03. Samples cultured on the *E. coli* plates were negative for *Acanthamoeba,* but the samples grown in PYG medium were positive. The strain was designated as **ATCC 50677**.

15) Patient: Mrs. D. Source: Dr. G.
The researcher received a corneal scraping on 1992/11/11 that had been taken on 1992/11/04. The sample tested negative for *Acanthamoeba* on both *E. coli* plates and in PYG medium.

16) Patient: Mrs. D. Source: Dr. R. (via pathologists D.).

A corneal scraping was done on 1993/03/10. Culturing proved negative for *Acanthamoeba*.

17) Patient: Mr. W. Source: Dr. P.
Eye swabs and corneal scrapings were taken on 1993/01/26. All cultures from the eye swabs and corneal scrapings were negative for *Acanthamoeba*.

18) Mrs. W. (24 years old.) Source: E. Clinic and L. Laboratories.
A corneal scraping was done in theatre on 1993/01/28. It was negative for *Acanthamoeba*.

19) Patient: Mrs. S. (29 years old.) Source: D. Laboratories.
The patient used disposable contact lenses with Bausch & Lomb daily cleaner and sterile saline. She never rinsed her lenses in non-sterile water. The researcher cultured from the patient's contact lens on a NNA plate on 1993/07/20. The result was positive for a mixed culture (apparent from the cyst shapes) of *Acanthamoeba*. The strain was designated as **ATCC 50684**.

20) Patient: Mrs. N. (28 years old.) Source: Dr. C.
The patient used disposable Johnston and Johnston contact lenses that were inserted and used for one month and then discarded. During this time, the contact lenses were removed daily and placed in sterile saline, but no method of disinfection was employed. Two weeks prior to the start of infection, Mrs. N. spent a weekend with a friend and used saline from the friend's saline drip, decanting it into a plastic saline bottle with a hole in the lid. No tap water was ever used on the contact lenses and lenses were not worn whilst swimming, but remained inserted while the patient was bathing and washing her hair.

The patient's symptoms started in mid-November 1993. Her right eye initially felt scratchy on insertion of the lens, but this cleared during the day. These symptoms continued for approximately two weeks. The right eye then became itchy, swollen and red. The patient stopped using contact lenses and went to a doctor, who advised her to steam her eye and to use Maxitrol. The left eye remained without symptoms. The patient's right eye was constantly painful, as were her sinuses and head. Mrs. N. was then told to use Chloromycetin and Sofradex, and to take Pred Forte every two hours. She subsequently saw another doctor who prescribed Tobradex, but this hurt her eye, so she stopped using it. The patient, who was in the Western Cape province by that time, then saw Dr. R. who gave her FML (a sulphur drug) and Zovirax cream as well as high doses of vitamins, particularly vitamin A. Dr. R. then went on leave, so the patient was seen by Dr. B., who prescribed an antibiotic hourly initially and then three hourly. She was also given Pondocillin and Atropine.

The infection had lasted for approximately seven weeks by the time that Dr. C. saw the patient. She was in great pain and was hospitalised on 1993/12/26. Dr. C. then put Mrs. N. on the following medication: Nizoral one b.d.; Neosporin six-hourly; Spersadex four-hourly; and Brolene one-hourly. A typical (for *Acanthamoeba* infection) white stromal ring could be seen in the cornea, but this decreased in size after the treatment described above. Dr. C. also commented on the appearance of broken vessels in the cornea, indicative of acanthamoebic infection. The patient had enlarged lymph glands: the doctors were investigating what else might be wrong with her.

Corneal scrapings were done on 1993/12/29, two to three days after medical treatment had been initiated. The researcher received samples from the scrapings. The patient's right and left contact lenses were also cut up and cultured on NNA plates seeded with *E. coli*. When the contact lenses were placed on the agar, a large number of cysts could be seen on both the right and left contact lenses. These were left in culture overnight, along with fluid from the contact lens case. All came up positive for *Acanthamoeba* within 24 hours. Various shapes of cysts were apparent, possibly indicative of a multiple species infection. On 1993/12/30, Dr. C. said that the patient was already looking a lot better. The corneal scrapings grown in PYG medium and on NNA (strain **ATCC 50678**) were positive for *Acanthamoeba* on 1994/01/01. Large numbers of fat trophozoites were present. Dr. C. said that he would try to cure the patient by continued medical treatment before considering a corneal graft.

On 1994/02/10 Dr. C. said that the patient was much improved. Clotrimazole was included in her regimen. Dr. C. wanted to do a corneal transplant, so a second corneal scraping was requested to check whether viable cysts were still present. One month later all corneal scrapings were still negative for *Acanthamoeba*.

21) Patient: Mrs D. Source: Dr. P.

The patient used soft contact lenses initially, with a Bausch and Lomb "All in One" cleaner and disinfectant. In September 1993, she developed an infection in the right eye which made her eye red and painful. Dr. L. gave her eye drops, and she was advised to boil her contact lenses until the infection had cleared up. She boiled them daily for three to four days until her eye felt better.

On 1993/12/26, the patient went to Margate on a hike. Whilst on the trip, she used disposable contact lenses which she removed nightly, cleaned with Bausch and Lomb daily cleaner and stored in sterile saline. The contact lenses were kept for two weeks and then discarded. On 1994/01/01, an infection started in the patient's right eye. She had washed her contact lens holder out with tap water and had swum in the sea while wearing her contact lenses. As soon as the infection started, she stopped wearing her right contact lens. A doctor in Margate diagnosed the infection as a foreign body in the eye.

Having returned from Margate, Mrs. D. consulted Dr. L. who diagnosed viral herpes and prescribed Zovirax, Oxylin drops, Tobrex and Virobis tablets. The patient used Oxysept overnight as well as neutralising tablets for her left contact lens, but she could not wear her right contact lens because of the infection.

By 1993/03/11, the patient was using a second prescription of Zovirax. The pain was getting worse, with only occasional slight improvement. The patient was taking painkillers daily, and was wearing a patch over the infected eye.

A corneal scraping was done on 1994/03/11, suspended in 1 ml sterile saline, and brought to the researcher within an hour for immediate culturing on *E. coli* plates. By 1994/03/14, the plates were still negative, but several fungal patches were visible. Dr. P. mentioned that he had removed the epithelium over the entire affected area. The epithelium was rather brittle and it broke into fragments in the saline. Thickened nerve endings and blood vessels over the limbus were also visible.

The patient was given Brolene on 1994/03/10. On 1994/03/12, Neosporin was included in the regimen. The fact that the patient was using Brolene might account for the negative result on the initial corneal scraping. Alternatively, the reason might be that because the infection was well established, *Acanthamoeba* had already migrated deeper into the eye (Fickler, 1993). According to Dr. P., the appearance of the eye was typical of acanthamoebic keratitis and not of herpes. There was a large, round central lesion with full thickening of the epithelium that came away, only to grow again. There were satellites around it and intermittent, severe pain.

By 1994/03/14, the patient was already much improved. The effect of treatment with Brolene and Neosporin was to lessen the redness and soreness of her eye. By 1994/03/22 the lesion was 50% smaller, but the central portion remained the same.

A corneal biopsy was done on 1994/03/23. Both the milky fluid in the corneal scraping taken and the sample from the corneal biopsy tested positive for *Acanthamoeba*. The strain was designated as **ATCC 50679**. Dr. P. added a 20% solution of Baquacil (polyhexamethylene biguanide) to the drug regimen.

22) Patient: Mrs. H. (71 years old.) Source: Dr. C.
A corneal scraping was taken on 1994/03/24 and sent to the researcher although Dr. C. did not think that the infection in the patient's eye looked typical of *Acanthamoeba*. Although the corneal biopsy sample had been stored in saline, it had already dried out by the time the author received it. The scraping tested negative for *Acanthamoeba*.

23) Patient: Mrs. K. (53 years old.) Source: Dr. M.

The patient's eye problems started after a bout of influenza. She had a history of repeated corneal ulcers in both eyes that were diagnosed as being of viral origin. The ulcers would improve and then deteriorate again. In addition, she had a two-week history of severe conjunctivitis and keratitis, possibly caused by wearing disposable contact lenses. She had left her contact lenses in solution for two weeks before wearing them again. She then kept her contact lenses inserted because they acted as bandage lenses and "soothed" her eyes.

The entire corneal epithelium came away when the biopsy was being done on 1994/05/25. The corneal material was stored in sterile saline and was brought to the researcher immediately after the scrapings had been done. The corneal biopsy samples were plated out on 1994/05/25. A film of oil could be seen around the samples on the *E. coli* plates. The patient had apparently been using some sort of ointment (not one for treating *Acanthamoeba*). The samples proved negative for *Acanthamoeba*.

24) Patient: Mr. B. (10 years old.) Source: Dr. C.

On 1994/06/06, the patient had a painful eye, which he washed out with tap water. His mother obtained drops "for an eye infection" from the chemist for him. On 1994/06/09, there was a white spot in the right eye. The patient was taken to a general practitioner on 1994/06/10 and was given chloramphenicol (Spersanicol) and Tobrex hourly. He was referred to Dr. C., who noticed an ulcer with corrugated margins and with positive corneal sensation, surrounded by corneal epithelial oedema and inflammatory cells. Corneal scrapings were done, which the laboratory said were positive for Gram staining and positive for Cocci. Later

the laboratory said that this was just debris. There was no history of damage to cornea.

On 1994/06/12, the patient woke up and complained of severe pain in the eye, for which he was given painkillers. Up to this point the pain had not been severe. The patient had to keep his eye closed because of light sensitivity. The corneal ulcer grew rapidly (within 24 hours). The patient was put on Zovirax on 1994/06/13. When the researcher saw the patient on 1994/06/14, he was reported to be much improved. The ulcer no longer had such a jagged edge. Dr. C. said that the appearance of this ulcer was not typical of viral or fungal ulcers, so a test for *Acanthamoeba* was required. A corneal scraping from the patient's right eye was done on 1994/06/14. The result was negative for *Acanthamoeba*.

25) Patient: Mr. S. (33 years old.) Source: Dr. R.
The patient was a soft contact lens wearer. He wore his contact lenses daily, but cleaned and sterilised them with Bausch & Lomb Multi-Purpose solution only every one to two weeks. When Dr. R. first saw Mr. S., he had dendritic ulcers on the cornea of his right eye. The ulcers responded to treatment, but the patient unfortunately stopped treatment immediately his condition improved. The patient contacted Dr. R. on 1994/06/27 to say that he had a recurrence of the condition. The patient had stopped wearing his soft contact lenses when the corneal ulcer reappeared. Mr. S. had corneal infiltrates, which Dr. R. was sure were secondary. When Dr. R. saw him on 1994/07/04, the corneal problem seemed to have cleared up. The ulcer responded well to Zovirax, but then flared up again. The patient was hospitalised for schizophrenia. The researcher was called to the hospital to do a corneal scraping on 1994/07/28. At this stage, the ulcer looked dendritic. The eye was "stinging" and not all that painful, but both Drs. R. and D. thought

the appearance of the ulcer indicated a possible acanthamoebic infection. The patient was being treated with Tobradex and Zovirax at this stage. The cultures for A*canthamoeba* were negative.

26) Patient: Mr. B. (18 years old.) Source: Dr. T.

The patient, a resident of Botswana, wore disposable contact lenses for a week at a time. His lenses were removed nightly and stored in sterile saline. No form of disinfection or cleaning was used. When Dr. T. saw the patient on 1994/05/19, he had two corneal abscesses in his left eye which were thought to be fungal. The patient was treated with Brolene bd, Neosporin, Baquacil bd. and Spersadexoline 6x/d. On 1994/06/09, Drs R. and partners cultured *Acanthamoeba* trophozoites from corneal scrapings of the eye. On 1994/07/12, the patient was given Baquacil q2h, Brolene q2h, Spersadexoline q4h and Pred G q6h. By 1994/07/25, his eye appeared much better. On 1994/08/03, the eye showed abscesses in a ring. Natacyn was added to his drug regimen. The researcher was provided with a corneal scraping on 1994/08/09, which was positive for *Acanthamoeba* on 1994/08/11. The isolate is **ATCC 50680**.

27) Patient: Mrs. K. (53 years old.) Source: M. Hospital.

A corneal scraping in sterile saline from the patient's right eye was sent to the author on 1994/08/23. The culture tested negative for *Acanthamoeba*.

28) Patient: Mr Y. Source: Dr. R.

The patient wore disposable, soft contact lenses for three weeks at a time, and did not disinfect in-between. On 1995/04/19, the patient's eye became scratchy and he was treated with an antibacterial agent (Exocin, a 4-quinoline). The patient then developed a round ocular lesion. This initial reaction was not painful. One

week later, the eye had become very painful. Bacterial cultures produced negative results. Dr. R. felt that the period within which the lesion had developed was too short for it to be acanthamoebic, but that the organism should nevertheless be tested for. Corneal scrapings cultured by the author yielded negative results for *Acanthamoeba.* The patient was being treated with Baquacil drops (polyhexamethylene biguanide solution 1 in 2000, hourly).

29) Patient: Mrs. P. (43 years old.) Source: Dr. C.
The patient wore disposable contact lenses for three weeks at a time, without sterilising or cleaning them, before throwing them away. She swam a great deal, always wearing her contact lenses. After Mrs P. had swum in Hartebeespoort Dam (near Pretoria), her right eye became scratchy and painful, but she continued with the habit of swimming while wearing her contact lenses. The next day her eye was very painful. Dr. C. said that it was very red, with generalised corneal oedema. The patient was treated with Zovirax & Tobradex. A corneal scraping was done on 1995/02/16, and on 1995/02/17, tested positive for *Acanthamoeba*. The strain has been designated as **ATCC 50681**.

30) Patient: Mrs. V. (approximately 30 years old.) Source: Dr. K.
The patient changed doctors, so no information concerning initial symptoms, diagnosis or treatment is available. She did not wear contact lenses. A corneal scraping was taken on a swab in theatre and placed in sterile saline and sent to D. Laboratories, Pretoria. A non-nutrient agar plate seeded with *E. coli* was sent to the researcher from that laboratory on 1995/09/01. The isolate was cloned, cultured and identified morphologically as *A. polyphaga*. It was given the number **SAWE 95/7**.

31) Patient: Mr. M. (20 years old.) Source: Dr. P.

The patient had worn disposable contact lenses for long periods, without any method of sterilisation. He lived overseas in 1995. His ocular infection started in January 1996, when he was back in South Africa. The patient had a ring ulcer in the right eye. He saw numerous ophthalmologists, who diagnosed a herpes or fungal infection clinically. N. Laboratories received a corneal scraping from the patient's right eye on 1996/02/29 and reported that it was negative for *Acantha-moeba*. That laboratory also checked the sample for bacteria, fungi and herpes virus. Dr. P. said that the patient's eye looked like a typical case of acanthamoebic infection, which is why he had pursued the matter of diagnosis. Another corneal biopsy was taken from the patient's right eye at the P. Institute on 1996/03/07, and sent to the researcher. This sample was positive for *Acanthamoeba*, strain number **SAWE 96/8**, on 1996/03/08.

32) Patient: Mrs. H. (29 years old.) Source: L. Laboratories.

The patient wore disposable contact lenses. Both eyes became infected. Dr. A. saw the patient initially on 1996/04/05 as an emergency case. She had a history of severe eye pain, which had been associated with a drop in visual acuity in the left eye for one week. She had been seeing Dr. E., who had treated her with Kefzol, Daktarin and Cyclogyl topically, with minimal effect. Examination showed her to have severe inflammation in the left eye, with a spreading intra-corneal abscess, multiple keratic precipitates and surrounding spread through a halo of corneal fibres. There was early evidence of an infiltrate in the right eye as well.

Dr. A. said that the picture in the right eye was almost certainly that of acanthamoebic keratitis caused by contaminated contact lens use, and resulting in severe ocular pain. One of patient's disposable lenses was sent to L. Laboratories

on Friday 1996/04/05 for acanthamoebic culturing. The researcher received the contact lens on 1996/04/09 and plated it on to NNA that day. The contact lens was folded up, but dried out in a cage-like container that was open to the air. The result was negative for *Acanthamoeba*.

Dr. A. immediately commenced with Natacyn hourly to both eyes, Brolene in the form of drops and ointment, and Neosporin. The patient continued the earlier regime of Cyclogyl and Kefzol, but stopped the Daktarin because of the severe pain it caused. When the patient was seen again by Dr. A. on 1996/04/09, there had been a dramatic improvement, with the visual acuity improved to 20/25 in the right eye and 20/60 in the left eye, taking into account her spectacle correction. Dr. A. was intending to prescribe Diflucan, which the patient was to use daily for a couple of months.

33) Patient: Mrs. K. Source: South African Institute of Medical Research (SAIMR).

A corneal swab was taken from the patient on 1996/04/02 by the SAIMR. No organisms were cultured from this sample. However, the present researcher found that the solution from the patient's contact lens case sent by her to the SAIMR was positive for *Acanthamoeba*. The strain (**SAIMR 96/9**) looked morphologically like *A. polyphaga*. Dr. S. Kilvington confirmed this finding.

34) Patient: Miss M. (15 years old.) Source: Dr. P.

Miss M. was in Port Elizabeth in December 1996 and swam in the sea and in lagoons while her soft contact lenses were inserted. When the patient visited Dr. P. on 1997/01/28, a corneal ring infiltrate could be seen in her left eye. A corneal scraping and biopsy were done in theatre on 1997/01/30 and sent to the

researcher. By 1997/01/31, all three plates had given positive results for *Acanthamoeba* (strain **SAWE 97/10**).

Covomycin D had been prescribed for the patient. On positive confirmation of the presence of *Acanthamoeba*, Dr. P. started her on Baquacil 1x hourly for the first week, then 1x two-hourly, as well as Neosporin 1x three times daily and Brolene 1x three times per day. By 1997/02/04, the patient's eye had improved slightly.

35) Patient: Mrs. B. (48 yrs old.) Source: Dr. P.
The patient was a wearer of disposable contact lenses. She developed a corneal ulcer in her left eye, and was treated with antibiotics for two weeks with no improvement before she saw Dr. P. on 1997/03/10. The patient was in great pain, and fainted when Dr. P. tried to open her eye. All the vessels around the cornea were very red. A corneal scraping and biopsy were done under general anaesthetic on 1997/03/13. Both the scraping and biopsy were tested by the researcher and found negative for *Acanthamoeba*. The patient telephoned Dr. P. on 1997/03/14 and said that she did not know what the doctor had done, but that her eye was feeling much better.

36) Patient: Mrs. M. (53 years old.) Source: Dr. A.
The patient presented in February 1997 with a corneal ulcer on the right temporal side. There was evidence of vascularisation and infiltration. She was initially treated with Tobradex, and Zovirax was added later. There was a slight improvement in the condition by April 1997, although the scarring and ulcer remained visible. Treatment was discontinued.

The patient was seen again as an emergency case on 1997/05/13. Her condition had suddenly flared up. Examination showed that the ulcer was spreading. It was extremely painful. A culture was taken 1997/05/17 and tested negative for fungal growth in a local laboratory.

The patient was put on the following drug regimen: Diflucan 50mgs od; Guttae Natacyn two hourly; Guttae Homatropine 2% two hourly; Brolene t.d.s.; and Guttae Diflucan suspension 150 mg/ml saline twice daily.

Cultures were taken in theatre on 1997/05/29. There was an early positive result for fungal growth, but cultures tested by the author were negative for *Acanthamoeba.* Baquacil (in tears) was added to the patient's drug regimen.

37) Patient: Mr. H. Source: Dr. S.

The patient was referred to Dr. S. on 1998/01/29 for a painful red eye following a corneal foreign body (cast iron) removal. On examination, he was found to have extensive corneal abrasion and a small rust ring. There was a large epithelial defect (10.0 mm), Descemets folds, and dilation of the scleral vessels. The rust ring was removed, and the eye washed out with water. Ocular Chloromycetin and a pad were applied. The patient subsequently developed a very painful eye. Dr. S. treated him with mydriatics (Mydriacyl) and Exocin, with no response. He then tried Brolene and of Zovirax, but still without success. The ulcer eventually epithelialised. Ciloxan did not reduce the patient's pain, so Dr. S. prescribed Spersadexoline.

The patient was still in pain and taking Exocin when a corneal scraping was done on 1998/01/29 and samples sent to the author for testing. Vessels in the iris had

developed and the circular lesion was unchanged. The eye had not responded to medication. The researcher's cultures were negative for *Acanthamoeba.*

38) Patient: Miss L. (24 years old.) Source: Dr. M.
The sample from a corneal scraping taken on 1998/06/04 was sent to the author via Dr. H. The patient had a corneal ulcer in her right eye, which was sore and very sensitive. She had been on Covosan and was using Cyloxan (one drop every 15 minutes) at the time. A corneal scraping was done, and samples from the cornea and the soft contact lenses worn by Miss L., as well as their fluid, were cultured for *Acanthamoeba*. All the cultures were negative for *Acanthamoeba*.

39) Patient: Mrs. M. (57 years old.) Source: Dr. D.
The patient presented with a film over both of her eyes, which were very red. She had had a corneal scraping done six years previously, but this had tested negative for *Acanthamoeba*. The patient had not been wearing contact lenses since her earlier infection six years before. A scraping was done on the 1998/09/30 and sent to the researcher for testing. The attempted culturing for *Acanthamoeba* proved negative.

40) Patient: Mrs. Z. (45 years old.) Source: Dr. K.
The patient had a superficial infection in both eyes. Corneal scrapings of both eyes were negative for *Acanthamoeba*.

ADDITIONAL SPECIMENS EXAMINED

A) Culturing cerebrospinal fluid (CSF) of a patient for amoebae

Patient: Mr. D. Source: Dr. H.

The CSF of the patient had tested negative in all bacterial and viral tests carried out by a commercial laboratory. On 1997/07/28, the researcher dotted 0.5 ml of CSF on to NNA plates seeded with *E. coli*. Plates were incubated at 30°C and at 37°C. The plates were negative for amoebae 14 days later. In addition, slides were made and stained with May Grunwald and Giemsa, but no amoebae were visible.

B) Water from M. Clinic

The researcher received three containers of water (1 litre each in sterile containers) on 1991/03/11 from different tanks used by the M. Clinic.

Tank 1: Central plant room.

Tank 2: Cooling tower.

Tank 3: Penthouse.

Culturing was done from these samples. Amoebae were isolated from tanks 1 and 2, but not from tank 3. Two different kinds of amoebae were present in both tanks 1 and 2, one kind considerably smaller than the other.

Direct fluorescence of water showed the presence of *Legionella*. However, *Legionella* could not be cultured from this water, so the Water Board decided that the water was safe to use. *Acetobacter* was grown from the water in the tanks. It is possible that the amoebae were harbouring the bacteria, which prevented their being cultured from the water, despite the fact that the bacteria could be detected by direct fluorescence.

APPENDIX IV

List of *Acanthamoeba* strains

Isolate	Source	Geographic origin	Date isolated	Designation
Ac/PHL/4*	AK	England		Morphologically similar to *A. polyphaga* (see Kilvington *et al.*, 1991a)
Ac/PHL/9*	AK	England		
Ac/PHL/17*	AK	England		Morphologically similar to *A. polyphaga* (see Kilvington *et al.*, 1991a)
Ac/PHL/22*	AK	England		Morphologically similar to *A. polyphaga* (see Kilvington *et al.*, 1991a)
Ac/PHL/23*	AK	England		Morphologically similar to *A. polyphaga* (see Kilvington, 1989)
ATCC 30868/CCAP 1501/2g$^{\alpha}$ (Nagington *et al.*, 1974; Gachon *et al.*, 2007)	AK	England	74/9/20	*A. castellanii* (see Warhurst & Thomas, 1975)
ATCC 30873/CCAP 1501/3d$^{\alpha}$ (Nagington *et al.*, 1974; Gachon *et al.*, 2007)	AK	England	74/5/10	*A. polyphaga* (see Warhurst & Thomas, 1975)
ATCC 50676$^{\beta}$	AK	Namibia or South Africa	90/7/6	*A. mauritaniensis* (see Schroeder *et al.*, 2001)
ATCC 50677$^{\beta}$	AK	South Africa	92/8/3	*A. mauritaniensis* (see Schroeder *et al.*, 2001)

ATCC 50678[β]	AK	South Africa	93/12/29	*A. mauritaniensis* (see Schroeder *et al.*, 2001)
ATCC 50679[β]	AK	South Africa	94/3/23	*A. mauritaniensis* (see Schroeder *et al.*, 2001)
ATCC 50680[β]	AK	Botswana or South Africa	94/8/9	*A. mauritaniensis* (see Schroeder *et al.*, 2001)
ATCC 50681[β]	AK	South Africa	95/2/17	*A. mauritaniensis* (see Schroeder *et al.*, 2001)
ATCC 50682[β]	L and R sides of contact lens case	South Africa	91/7/29	*A. mauritaniensis* (see Schroeder *et al.*, 2001)
ATCC 50683[+]	L and R sides of contact lens case	South Africa	91/8/7	*A. mauritaniensis* (see Schroeder *et al.*, 2001)
ATCC 50684[β]	Contact lens	South Africa	93/7/20	*A. mauritaniensis* (see Schroeder *et al.*, 2001)
ATCC 50685[β]	Sewage sludge	South Africa	87/4/26	*A. lenticulata* (see Schroeder *et al.*, 2001)
ATCC 50686[β]	Sewage sludge	South Africa	87/4/26	*A. lenticulata* (see Schroeder *et al.*, 2001)
ATCC 50687[β]	Sewage sludge	South Africa	87/4/30	*A. lenticulata* (see Schroeder *et al.*, 2001)
RYD[α]	AK	England	90/06	*A. polyphaga*
SAIMR 96/9[**]	Contact lens	South Africa	96/04/02	*A. polyphaga* (see Dini *et al.*, 2000)
SAWE 95/7[β]	AK	South Africa	95/9/1	
SAWE 96/8[β]	AK	South Africa	96/3/8	
SAWE 97/10[β]	AK	South Africa	97/1/30	
SAWL 91/2[β]	Contact lens and solutions	South Africa	91/5/27	*Mastigina* sp. (see Niszl & Markus, 1991; Niszl *et al.*, 1995)

435/89[δ]	AK	South India		*A. polyphaga*
452/89[δ]	AK	India		*A. culbertsoni*

Key:

[*] Donated by S. Kilvington

[α] Donated by D.C. Warhurst

[**] The initial isolation was made at the South African Institute for Medical Research. Records held by I.A. Niszl

[β] Records held by I.A. Niszl

[δ] From P. Thomas (donated by D.C. Warhurst)

AK = Acanthamoebic keratitis.

Provisional classification of isolates

Species	Strain	ATCC Number
A. mauritaniensis	SAWE 90/1	ATCC 50676*
A. mauritaniensis	SAWE 92/2	ATCC 50677*
A. mauritaniensis	SAWE 93/3	ATCC 50678*
A. mauritaniensis	SAWE 94/4	ATCC 50679*
A. mauritaniensis	SAWE 94/5	ATCC 50680*
A. mauritaniensis	SAWE 95/6	ATCC 50681*
A. mauritaniensis	SAWL 91/3	ATCC 50682*
A. mauritaniensis	SAWL 91/4	ATCC 50683*
A. mauritaniensis	SAWL 93/1	ATCC 50684*
A. lenticulata	SAWS 87/1	ATCC 50685*
A. lenticulata	SAWS 87/2	ATCC 50686*
A. lenticulata	SAWS 87/3	ATCC 50687*
A. polyphaga	ATCC 30873	
A. polyphaga	Ac/PHL/23	
A. polyphaga	SAIMR 96/9	
A. castellanii	ATCC 30868	
Mastigina	SAWL 91/2	ATCC 50687

*Ledee *et al*. (2003

Note: The genotyping analysis of Ledee *et al*. (2003) differs in terms of groupings from the electrophoretic and RFLP results reported in this thesis in that the genotyping reflects less variation. Therefore, further molecular work needs to be carried out (as a "control"), but in South Africa – using the reference isolate organisms that are available locally, as opposed to using amoebae from the corresponding isolates that were deposited in the ATCC.

APPENDIX V

Inactivation of amoebae by contact lens solutions

Inactivation of cysts of *Acanthamoeba* strain Ac/PHL/23*

	Contact Time				
Solution	**2 h**	**4 h**	**8 h**	**3 d**	**7 d**
Bausch and Lomb Multipurpose	+	+	+	+	+
Complete	+	+	+	+	+
Duracare	+	+	+	-	-
Hydrocare	+	+	+	-	-
Optifree	+	+	+	+	+
Optisoak	+	+	+	+	+
Oxysept 1	+	+	-	-	-
Oxysept complete	+				
Total	+	+	+	-	-
Transoak	+	-	-	-	-
Transol	+	+	+	+	+
Control (amoeba saline + cysts)	+	+	+	+	+
Control (amoeba saline)	-	-	-	-	-

Key:

*Experiments were conducted in triplicate and results were the same for all three trials.

+ = growth of organisms,

- = no growth of organisms.

Inactivation of cysts of *Acanthamoeba* strain ATCC 30868/CCAP 1501/2g*

Solution	**2 h**	**4 h**	**8 h**	**3 d**	**7 d**
Bausch and Lomb Multipurpose	+	+	+	+	+
Complete	+	+	+	+	+
Duracare	+	+	-	-	-
Hydrocare	+	+	+	-	-
Optifree	+	+	+	+	+
Optisoak	+	+	+	+	+
Oxysept 1	+	+	-	-	-
Oxysept complete	+				
Total	+	+	-	-	-
Transoak	+	-	-	-	-
Transol	+	+	+	+	+
Control (amoeba saline + cysts)	+	+	+	+	+
Control (amoeba saline)	-	-	-	-	-

Key:

*Experiments were conducted in triplicate and results were the same for all three trials.

+ = growth of organisms,

- = no growth of organisms.

Inactivation of cysts of *Acanthamoeba* strain ATCC 30873/CCAP 1501/3d*

Solution	**2 h**	**4 h**	**8 h**	**3 d**	**7 d**
Bausch and Lomb Multipurpose	+	+	+	+	+
Complete	+	+	+	+	+
Duracare	+	+	+	+	-
Hydrocare	+	+	+	+	+
Optifree	+	+	+	+	+
Optisoak	+	+	+	+	+
Oxysept 1	+	-	-	-	-
Oxysept complete	+				
Total	+	+	+	-	-
Transoak	+	-	-	-	-
Transol	+	+	+	+	+
Control (amoeba saline + cysts)	+	+	+	+	+
Control (amoeba saline)	-	-	-	-	-

Key:

*Experiments were conducted in triplicate and results were the same for all three trials.

+ = growth of organisms,

- = no growth of organisms.

Inactivation of cysts of *Acanthamoeba* strain ATCC 50676*

Solution	**2 h**	**4 h**	**8 h**	**3 d**	**7 d**
Bausch and Lomb Multipurpose	+	+	+	+	+
Complete	+	+	+	+	+
Duracare	+	+	-	-	-
Hydrocare	+	+	+	+	+
Optifree	+	+	+	+	+
Optisoak	+	+	+	+	+
Oxysept 1	+	+	-	-	-
Oxysept complete	+				
Total	+	+	+	+	-
Transoak	+	-	-	-	-
Transol	+	+	+	+	+
Control (amoeba saline + cysts)	+	+	+	+	+
Control (amoeba saline)	-	-	-	-	-

Key:

*Experiments were conducted in triplicate and results were the same for all three trials.

+ = growth of organisms,

- = no growth of organisms.

Inactivation of cysts of *Acanthamoeba* strain ATCC 50677*

Solution	**2 h**	**4 h**	**8 h**	**3 d**	**7 d**
Bausch and Lomb Multipurpose	+	+	+	+	+
Complete	+	+	+	+	+
Duracare	+	+	+	-	-
Hydrocare	+	+	+	+	-
Optifree	+	+	+	+	+
Optisoak	+	+	+	+	+
Oxysept 1	+	-	-	-	-
Oxysept complete	+				
Total	+	+	+	-	-
Transoak	+	-	-	-	-
Transol	+	+	+	+	+
Control (amoeba saline + cysts)	+	+	+	+	+
Control (amoeba saline)	-	-	-	-	-

Key:

*Experiments were conducted in triplicate and results were the same for all three trials.

+ = growth of organisms,

- = no growth of organisms.

Inactivation of cysts of *Acanthamoeba* strain ATCC 50678*

Solution	**2 h**	**4 h**	**8 h**	**3 d**	**7 d**
Bausch and Lomb Multipurpose	+	+	+	+	+
Complete	+	+	+	+	+
Duracare	+	+	+	-	-
Hydrocare	+	+	+	-	-
Optifree	+	+	+	+	+
Optisoak	+	+	+	+	+
Oxysept 1	+	+	+	-	-
Oxysept complete	+				
Total	+	+	+	-	-
Transoak	+	-	-	-	-
Transol	+	+	+	+	+
Control (amoeba saline + cysts)	+	+	+	+	+
Control (amoeba saline)	-	-	-	-	-

Key:

*Experiments were conducted in triplicate and results were the same for all three trials.

+ = growth of organisms,

- = no growth of organisms.

Inactivation of cysts of *Acanthamoeba* strain ATCC 50679*

Solution	**2 h**	**4 h**	**8 h**	**3 d**	**7 d**
Bausch and Lomb Multipurpose	+	+	+	+	+
Complete	+	+	+	+	+
Duracare	+	+	+	-	-
Hydrocare	+	+	+	+	+
Optifree	+	+	+	+	+
Optisoak	+	+	+	+	+
Oxysept 1	+	-	-	-	-
Oxysept complete	+				
Total	+	+	-	-	-
Transoak	+	-	-	-	-
Transol	+	+	+	-	-
Control (amoeba saline + cysts)	+	+	+	+	+
Control (amoeba saline)	-	-	-	-	-

Key:

*Experiments were conducted in triplicate and results were the same for all three trials.

+ = growth of organisms,

- = no growth of organisms.

Inactivation of cysts of *Acanthamoeba* strain ATCC 50680*

Solution	**2 h**	**4 h**	**8 h**	**3 d**	**7 d**
Bausch and Lomb Multipurpose	+	+	+	+	+
Complete	+	+	+	+	+
Duracare	+	+	+	-	-
Hydrocare	+	+	+	+	-
Optifree	+	+	+	+	+
Optisoak	+	+	+	+	+
Oxysept 1	+	+	-	-	-
Oxysept complete	+				
Total	+	+	+	-	-
Transoak	+	-	-	-	-
Transol	+	+	+	+	+
Control (amoeba saline + cysts)	+	+	+	+	+
Control (amoeba saline)	-	-	-	-	-

Key:

*Experiments were conducted in triplicate and results were the same for all three trials.

+ = growth of organisms,

- = no growth of organisms.

Inactivation of cysts of *Acanthamoeba* strain ATCC 50684*

Solution	**2 h**	**4 h**	**8 h**	**3 d**	**7 d**
Bausch and Lomb Multipurpose	+	+	+	+	+
Complete	+	+	+	+	+
Duracare	+	+	+	+	+
Hydrocare	+	+	+	+	+
Optifree	+	+	+	+	+
Optisoak	+	+	+	+	+
Oxysept 1	+	+	-	-	-
Oxysept complete	+				
Total	+	+	+	-	-
Transoak	+	-	-	-	
Transol	+	+	+	+	+
Control (amoeba saline + cysts)	+	+	+	+	+
Control (amoeba saline)	-	-	-	-	-

Key:

*Experiments were conducted in triplicate and results were the same for all three trials.

+ = growth of organisms,

- = no growth of organisms.

Inactivation of cysts of *Acanthamoeba* strain ATCC 50686*

Solution	**2 h**	**4 h**	**8 h**	**3 d**	**7 d**
Bausch and Lomb Multipurpose	+	+	+	+	+
Complete	+	+	+	+	+
Duracare	+	+	+	+	-
Hydrocare	+	+	+	-	-
Optifree	+	+	+	+	+
Optisoak	+	+	+	+	+
Oxysept 1	+	-	-	-	-
Oxysept complete	+				
Total	+	+	+	-	-
Transoak	+	-	-	-	-
Transol	+	+	+	+	+
Control (amoeba saline + cysts)	+	+	+	+	+
Control (amoeba saline)	-	-	-	-	-

Key:

*Experiments were conducted in triplicate and results were the same for all three trials.

+ = growth of organisms,

- = no growth of organisms.

Statistical Analysis of Results

Strain of *Acanthamoeba*	**Test at 5% level**
Ac/PHL/23 vs ATCC 30868 (A vs B)	Not significantly different
Ac/PHL/23 vs ATCC 30873 (A vs C)	Significantly different
Ac/PHL/23 vs ATCC 50676 (A vs D)	Not significantly different
Ac/PHL/23 vs ATCC 50677 (A vs E)	Not significantly different
Ac/PHL/23 vs ATCC 50678 (A vs F)	Not significantly different
Ac/PHL/23 vs ATCC 50679 (A vs G)	Not significantly different
Ac/PHL/23 vs ATCC 50680 (A vs H)	Not significantly different
Ac/PHL/23 vs ATCC 50684 (A vs I)	Not significantly different
Ac/PHL/23 vs ATCC 50686 (A vs J)	Not significantly different
ATCC 30868 vs ATCC 30873 (B vs C)	Significantly different
ATCC 30868 vs ATCC 50675 (B vs D)	Not significantly different
ATCC 30868 vs ATCC 50677 (B vs E)	Significantly different
ATCC 30868 vs ATCC 50678 (B vs F)	Not significantly different
ATCC 30868 vs ATCC 50679 (B vs G)	Not significantly different
ATCC 30868 vs ATCC 50680 (B vs H)	Significantly different
ATCC 30868 vs ATCC 50684 (B vs I)	Not significantly different
ATCC 30868 vs ATCC 50686 (B vs J)	Significantly different
ATCC 30873 vs ATCC 50676 (C vs D)	Not significantly different
ATCC 30873 vs ATCC 50677 (C vs E)	Not significantly different
ATCC 30873 vs ATCC 50678 (C vs F)	Not significantly different
ATCC 30873 vs ATCC 50679 (C vs G)	Not significantly different
ATCC 30873 vs ATCC 50680 (C vs H)	Not significantly different
ATCC 30873 vs ATCC 50684 (C vs I)	Not significantly different
ATCC 30873 vs ATCC 50686 (C vs J)	Not significantly different
ATCC 50676 vs ATCC 50677 (D vs E)	Not significantly different
ATCC 50676 vs ATCC 50678 (D vs F)	Not significantly different
ATCC 50676 vs ATCC 50679 (D vs G)	Not significantly different
ATCC 50676 vs ATCC 50680 (D vs H)	Not significantly different
ATCC 50676 vs ATCC 50684 (D vs I)	Not significantly different
ATCC 50676 vs ATCC 50686 (D vs J)	Not significantly different
ATCC 50677 vs ATCC 50678 (E vs F)	Not significantly different
ATCC 50677 vs ATCC 50679 (E vs G)	Not significantly different
ATCC 50677 vs ATCC 50680 (E vs H)	Not significantly different
ATCC 50677 vs ATCC 50684 (E vs I)	Not significantly different
ATCC 50677 vs ATCC 50686 (E vs J)	Not significantly different

ATCC 50678 vs ATCC 50679 (F vs G)	Not significantly different
ATCC 50678 vs ATCC 50680 (F vs H)	Not significantly different
ATCC 50678 vs ATCC 50684 (F vs I)	Not significantly different
ATCC 50678 vs ATCC 50686 (F vs J)	Not significantly different
ATCC 50679 vs ATCC 50680 (G vs H)	Not significantly different
ATCC 50679 vs ATCC 50684 (G vs I)	Not significantly different
ATCC 50679 vs ATCC 50686 (G vs J)	Not significantly different
ATCC 50680 vs ATCC 50684 (H vs I)	Not significantly different
ATCC 50680 vs ATCC 50686 (H vs J)	Not significantly different
ATCC 50684 vs ATCC 50686 (I vs J)	Not significantly different

Contact lens solution	**Test at 5% level**
Duracare vs Hydrocare (5 vs 6)	Not significantly different
Duracare vs Oxysept 1 (5 vs 7)	Significantly different
Duracare vs Total (5 vs 8)	Not significantly different
Hydrocare vs Oxysept 1 (6 vs 7)	Significantly different
Hydrocare vs Total (6 vs 8)	Significantly different
Oxysept 1 vs Total (7 vs 8)	Significantly different.

APPENDIX VI

Fixation and staining of amoebae for light microscopy

Slides of corneal scrapings

Use a sterile spatula for obtaining corneal scrapings and for smearing the inoculum on to various media.

Touch areas of the glass slide quickly with the epithelium-laden spatula.

Do not air-dry slides for examination of amoebae because this causes rupture of the trophozoites; although not of the cysts.

Fix slides in methyl alcohol for three to five minutes.

Examine these slides directly or when they have been stained.

Note that cysts are easier to recognise than trophozoites because the cytoplasm is surrounded by a clear space created by the shrinkage of the organism within the cyst.

I Haematoxylin and Eosin Stain

A Modified Mayer's Haematoxylin

4.0 g	haematoxylin.
1 000 ml	distilled water.
0.3 g	sodium iodate.
50.0 g	ammonium or potassium alum.
1.5 g	citric acid.
75.0 g	chloral hydrate.

Dissolve alum in water (unheated). Add haematoxylin, iodate, citric acid and chloral hydrate, in that order. Filter haematoxylin solution through coarse filter paper.

B Eosin Y

5 g eosin in 500 ml distilled water.

2.5 g phloxine in 250 ml distilled water.

Add 750 ml distilled water.

Stain in Mayer's haematoxylin (A) for five minutes after fixation.

Wash well in running tap water until sections "blue" – five minutes or less.

Differentiate in 1% acid alcohol (1% HCl in 70% alcohol) for five seconds.

Wash well in tap water until sections "blue" again – five minutes or less.

Stain in 1% eosin Y (B) for five minutes.

Wash in running tap water for five minutes.

Place in 95% alcohol for one minute.

Transfer to 100% alcohol for 2 x 1 minute.

Mount in Entellan.

II Trichome Stain

Fix smear.

Immerse in iodine alcohol for one minute.

Transfer to 70% ethanol for one minute.

Transfer to 70% ethanol for one minute.

Differentiate in 95% ethanol containing one drop glacial acetic acid in 10 ml for 10–20 seconds.

Wash in 95% ethanol (two changes) for two minutes each.

Dehydrate in absolute ethanol (two changes) for two minutes each.

Clear in xylol and mount in DPX.

III Calcofluor White Staining (Wilhelmus *et al*., 1986)

Add several drops of solution containing 0.1% calcofluor white (Cellufluor) and 0.1% Evans blue (dissolved in distilled water) after fixation.

Remove excess stain after five minutes and apply coverslip.

Examine with fluorescence microscope for chemofluorescence of amoebic cysts. Calcofluor white stains cysts with a brilliant white colour under the correct UV conditions (excitation 365 nm, emission 420 nm). Other cells, including amoebic trophozoites, stain red-brown.

IV Weigert's Iron Haematoxylin

A Haematoxylin Solution

1 g	haematoxylin.
100 ml	absolute alcohol.

This is allowed to ripen naturally for 4 weeks before use.

B Iron Solution

4 ml	30% aqueous ferric chloride (anhydrous).
1 ml	hydrochloric acid (concentrated).
95 ml	distilled water.

This solution is filtered and added to an equal volume of the haematoxylin solution immediately before the stain is used. The mixture should be violet-black in colour; it must be discarded if it is brown.

Stain for 20 minutes.

APPENDIX VII

Fixing and processing of amoebae for transmission electron microscopy

A Glutaraldehyde Fixative

5 ml glutaraldehyde (25%).

15 ml cacodylate buffer (as in (B)).

20 ml distilled water.

Use within 24 hours.

B 0.2M Cacodylate Buffer

42.8g Sodium cacodylate ($Na(CH_3)_2AsO_2.3H_2O$).

6.9 ml 1 N. HCl (80.4 ml conc. HCl and add distilled water up to 1 litre).

Add water up to 1 000 ml.

C 1% Osmium Tetroxide

2 ml OsO_4 (4%).

6 ml 0.2M cacodylate buffer.

1) Grow amoebae in PYG medium and centrifuge at 740 x g for ten minutes to form a pellet.
2) Fix in glutaraldehyde (A) for ten minutes at 0°C.
3) Wash in 0.2M cacodylate buffer (B) for ten minutes at 4°C.
4) Transfer to Eppendorf tube.
5) Wash for 2 x 10 minutes in 0.2M cacodylate buffer.
6) Add 40% bovine serum albumin (BSA) made up in 0.2M cacodylate buffer, mix well and centrifuge.
7) Pipette off excess BSA.

8) Add one drop of pure (25%) glutaraldehyde to gel BSA.
9) Use syringe filled with cacodylate buffer to expel gelled BSA and amoebae from Eppendorf tube.
10) Cut BSA into blocks approximately 2 mm^3 for processing. Examine cubes under a microscope at this stage to ascertain whether amoebae are present, and discard blocks with no amoebae. Blocks can be treated as bits of tissue.
11) Post-fix for one hour at 4°C in 1% osmium tetroxide (OsO_4) (C).
12) Wash 3 x 10 minutes at 4°C in cacodylate buffer.
13) Place in 30% ethyl alcohol for five minutes at 4°C.
14) Transfer to 50% ethyl alcohol for five minutes at 4°C.
15) Leave in 70% ethyl alcohol/1% uranyl acetate for one hour at 4°C.
16) Transfer to 95% ethyl alcohol for ten minutes at room temperature (RT).
17) Place in 100% ethyl alcohol for 3 x 10 minutes at RT.
18) Rotate in LR White (Agar Scientific Limited) in fridge overnight.
19) Soak for 2 x 30 minutes in LR White.
20) Embed in gelatine capsules and allow to polymerise for 23 hours at 60°C (temperature is critical within one to two degrees).
21) Soak gelatine capsules off in water.

Staining of sections for electron microscopy

Staining of semi-thin sections for electron microscopy

Solution A

1% pyronin in distilled water.

Solution B

1% toluidine blue in 1% borax.

Mix one part of Solution A and four parts of Solution B well, and filter before use.

Staining of ultrathin sections for electron microscopy

Solution A (saturated uranyl acetate)

1 small spatula uranyl acetate.

50% ethanol (or distilled water).

Solution B (lead citrate)

1.33 g lead nitrate.

1.76 g sodium citrate.

Place in 30 ml distilled water to dissolve.

Add 8.0 ml N.NaOH after 30 minutes.

Make up to 50 ml with distilled water.

1) Centrifuge both uranyl acetate (A) and lead citrate (B) solutions at 3,650 rpm for three minutes.
2) Stain grids in Solution A for ten minutes.
3) Rinse grids in three beakers of 50% ethanol.
4) Stain grids in Solution B for ten minutes (staining dish contains NaOH pellets).
5) Rinse grids three times in distilled water, adding seven drops of N.NaOH to the first container of distilled water.

APPENDIX VIII

Preparation of Extracts for Electrophoresis

Stock Cocktail (200 mM)

0.031 g dithiothreitol.

0.026 g caproic acid/hexanoic acid.

Dissolve in 1 ml 200 mM EDTA (pH 7.0).

This stock solution keeps for one month in the fridge.

Working solution (2 mM)

Make up 0.1 ml stock and to 10 ml with distilled water.

This working solution keeps for two weeks in the fridge.

Table 1. Electrophoretic conditions for revealing isoenzymes of amoebae on cellulose acetate gels.

Enzyme	Tank buffer	Running time (min.)	Volts across gel	Origin
ACP (1) (3.1.3.2)*	A	90	200	Cathode
ACP (2)	C	90	200	Cathode
ACP (3)	A	90	200	Cathode
EST (1) (3.1.1.1)	A	90	200	Cathode
EST (2)	A	90	200	Cathode
G6PD (1.1.1.49)	B	45	200	Cathode
GPI (5.3.1.9)	A	45	200	Cathode
HK (2.7.1.1)	A	45	200	Cathode
LDH (1.1.1.27)	A	45	200	Cathode
MDH (1.1.1.37)	A	45	200	Cathode
ME (1.1.1.40)	A	45	200	Cathode
MPI (5.3.1.8)	B	45	200	Cathode
6PGD	B	45	200	Cathode
PGM (2.7.5.1)	A	45	200	Cathode

*Enzyme Commission number (Commission on Biochemical Nomenclature, 1973).

Recipe

Buffer	**Final molarity of each buffer constituent**	**Quantities for 1 litre of buffer**
A: 0.05 M Tris-Maleate pH 7.8	50 mM Tris 20 mM Maleic Acid	6.06 g 2.32 g
B: 0.02 M Phosphate pH 7.0	11.6 mM Na_2HPO_4	4.15 g $Na_2HPO_4.12H_2O$ 1.31 g $NaH_2PO_4.2H_2O$
C: 0.015 M Tris-EDTA-Borate $MgCl_2$ pH 7.8	15 mM Tris 5 mM Na_2EDTA 10 mM $MgCl_2$ 5.5 mM Boric Acid	1.82 g 1.82 g 0.95 g anhydrous $MgCl_2$ 0.34 g

Preparation of cellulose acetate paper and buffers

Soak cellulose acetate paper in buffer.

Keep all buffers cold for the run.

Include 5% sucrose in running buffer and 10% sucrose in stain buffer for better resolution.

Table 2. Developmental conditions for revealing isoenzymes of amoebae on cellulose acetate gels.

Enzyme	Substrate	Co-enzyme	Linking enzyme	Other additions	Buffer
ACP (1)	α-Napthyl acid phosphate, 5 mg			Fast Garnet GBC, 6 mg	1
ACP (2)	α-Napthyl acid phosphate, 5 mg			Fast Garnet GBC, 6 mg	1
ACP (3)	Methylum-belliferyl phosphate, 0.5 mg		See note below*		1
EST (1)	α-Napthyl acetate, 8 mg (dissolve in 0.2 ml acetone)			Fast Garnet GBC, 6 mg	2
EST (2)	Methylum-belliferyl acetate, 0.5 mg (dissolve in 0.2 ml acetone)		See note below*		2
G6PD	Glucose-6-phosphate, 6 mg	NADP, 0.1 ml		1 M $MgCl_2$, MTT, PMS (0.1 ml each)	3
GPI	Fructose-6 phosphate, 5 mg	NADP, 0.1 ml	Glucose-6-phosphate dehydrogenase (2 I.U.)	1 M $MgCl_2$, MTT, PMS (0.1 ml each)	3

Enzyme	**Substrate**	**Co-enzyme**	**Linking enzyme**	**Other additions**	**Buffer**
HK	Glucose, 2 mg ATP, 6 mg	NADP, 0.1 ml	Glucose-6-phosphate dehydrogenase (2 I.U)	1 M $MgCl_2$, MTT, PMS (0.1 ml each)	4
LDH	Lactate, 0.1 ml	NAD, 0.1 ml		MTT, PMS (0.1 ml each)	3
MDH	Malate, 0.2 ml	NAD, 0.1 ml		MTT, PMS (0.1 ml each)	3
PGM	Glucose-1-phosphate, 10 mg	NADP, 0.1 ml	Glucose-6-phosphate dehydrogenase (2 I.U.)	1 M $MgCl_2$, MTT, PMS (0.1 ml each)	3

*__Note:__ The stains must be viewed under ultraviolet light.

The intensity of isozyme bands can be enhanced by counterstaining for ten seconds with 3 ml of a high pH solution at the end of staining.

Buffers used

1) 0.05 M Citric acid pH 4.0 (2.5 ml).
2) 0.1 M Tris-maleate pH 6.5 (2 ml).
3) 0.1 M Tris-HCl pH 8.0 (2 ml).
4) 0.1 M Tris-HCl pH 8.6 (2 ml).
5) 0.1 M Tris-HCl pH 7.4 (2 ml).

Stock solutions used (all stored at $4^{o}C$)

Reagent	**Molarity**	**Quantity**
MTT	14.5 mM	6 mg/ml
PMS	6.5 mM	2 mg/ml
NADP	25 mM	20 mg/ml
NAD	40 mM	25 mg/ml
0.2 M $MgCl_2$	0.2 mM	19 mg/ml
1 M $MgCl_2$	1 mM	95 mg/ml

Table 3. Electrophoretic conditions for revealing isoenzymes of amoebae on polyacrylamide gels.

Enzyme	Tank buffer	Running time	Volts across gel	Origin
AAT/GOT (2.6.1.1)*	A (TC) B (TEB)	5 h 3-3.5 h	200 500	Cathode
ACP (3.1.3.2)	A (TC)	5 h	200	Cathode
EST (3.1.1.1)	A (TC)	5 h	200	Cathode
G6PD (1.1.1.49)	A (TC)	5 h	200	Cathode
GPI (5.3.1.9)	B (TEB)	3-3.5 h	500	Cathode
HK (2.7.1.1)	A (TC)	5 h	200	Cathode
IDH (1.1.1.42)	B (TEB)	3-3.5 h	500	Cathode
ME (1.1.1.40)	A (TC)	5 h	200	Cathode
ODH (1.1.1.73)	B (TEB)	3-3.5 h	500	Cathode
SOD (1.15.1.1)	B (TEB)	3-3.5 h	500	Cathode

*Enzyme Commission number (Commission on Biochemical Nomenclature, 1973).

Recipe

Electrode Buffer (quantities for 1litre of buffer)

A: Tris-Citrate pH 8.6
79.92g Tris.
17.44g Citric Acid.

B: TEB pH 8.6
40.0g Tris.
12.4g Boric Acid.
4.0g EDTA.

7.5% Acrylamide (4 x 1.8 mm thick gels)

A	**B**	**C**
9.0 g acrylamide 0.27 g meth-bis-acryl 60 ml water	3.0 g sucrose 0.09 ml temed 30 ml TEB buffer (or TC buffer)	0.09 g ammonium persulphate 30 ml water

Mix A, B, C separately; then mix B and C together and add them to A.

5% Acrylamide (stacking gel for above sandwich)

A	**B**	**C**
2.5 g acrylamide 0.07 g meth-bis-acryl 25 ml water	0.83 g sucrose 0.025 ml temed 12.5 ml TEB buffer (or TC buffer)	0.025 g ammonium persulphate 12.5 ml water

Mix as for 7.5% acrylamide.

Table 4. Developmental conditions for revealing isoenzymes of amoebae on polyacrylamide gels.

Enzyme	Substrate	Co-enzyme	Linking enzyme	Other additions	Buffer
AAT	Cysteine sulphonic acid, 50 mg α-ketoglutaric acid, 50 mg	NAD, 20 mg	Glutamate dehydrogenase, 32 units	NBT, 20 mg PMS, 1 mg (add after 15 minutes)	1

Incubate at 37°C in the dark. Add PMS after 15 minutes.

Enzyme	Substrate	Co-enzyme	Linking enzyme	Other additions	Buffer
ACP	α-Napthyl acid phosphate, 100mg			Fast Garnet GBC, 120 mg	2

Incubate at 37° in the dark.

Enzyme	Substrate	Co-enzyme	Linking enzyme	Other additions	Buffer
EST	1% napthyl acetate in acetone, 4 ml			Fast Blue RR, 50 mg (made to paste)	3

Pour half on gel and incubate at room temperature for 15 minutes. Add Fast Blue RR (made to paste with remaining solution) and incubate at room temperature.

Enzyme	Substrate	Co-enzyme	Linking enzyme	Other additions	Buffer
G6PD	Glucose-6 phosphate (monosodium salt), 100 mg	NADP, 20 mg		NBT, 12 mg PMS, 1 mg	4

Incubate at 37°C in the dark until the bands are fully developed.

Enzyme	Substrate	Co-enzyme	Linking enzyme	Other additions	Buffer
GPI	Fructose-6-phosphate di-Na salt, 35 mg	NADP, 20 mg	Glucose-6-phosphate dehydroge-nase (50 units), 0.072 ml	1 M $MgCl_2$, 2.5 ml NBT, 12 mg PMS, 1 mg	5

Incubate at 37°C and wait for bands to form.

Enzyme	Substrate	Co-enzyme	Linking enzyme	Other additions	Buffer
HK	D, L-glucose, 45 mg ATP, 10 mg	NADP, 20 mg	Glucose-6-phosphate dehydro-genase (50 units)	10% $MgCl_2$, 5 drops NBT, 12 mg PMS, 1 mg	5

Incubate at 37°C in the dark until the bands are fully developed.

Enzyme	Substrate	Co-enzyme	Linking enzyme	Other additions	Buffer
IDH	Isocitric acid, 75 mg	NADP, 20 mg		10% $MgCl_2$, 10 drops NBT, 12 mg PMS, 1 mg	5

Incubate at 37°C in the dark until bands are fully developed.

Enzyme	Substrate	Co-enzyme	Linking enzyme	Other additions	Buffer
ME	2 M neutralised malic acid, 2.5 ml	NADP, 20 mg		1 M $MgCl_2$, 10 drops	5

Incubate at 37°C in the dark until bands are fully developed.

Enzyme	Substrate	Co-enzyme	Linking enzyme	Other additions	Buffer
ODH SOD	Ethanol, 1 ml Octan-2-ol, 0.2 ml	NAD, 25 mg		NBT, 20 mg PMS, 5 mg	5

Leave for one hour in 37°C waterbath with lid on to allow ODH bands to develop in the dark and then for one hour in 37°C waterbath, with lid off to allow SOD bands to develop in the light.

Buffers

1) 50 ml 1 M Tris-HCl pH 8.0 (5 ml stock + 45 ml distilled water).
2) 50 ml 0.05 M Citric acid pH 4.0 with Tris.
3) 50 ml water with 0.66 g $NaPO_4$ (monobasic) and 0.27 g $NaPO_4$ (dibasic).
4) 50 ml 0.05M Tris-HCl buffer pH 7.1.
5) 50 ml 0.05 M Tris-HCl pH 8.5 (1.25 ml stock + 45 ml distilled water)

Stock

2M Tris (242.2 g/l) pH 8.5 with HCl.

APPENDIX IX

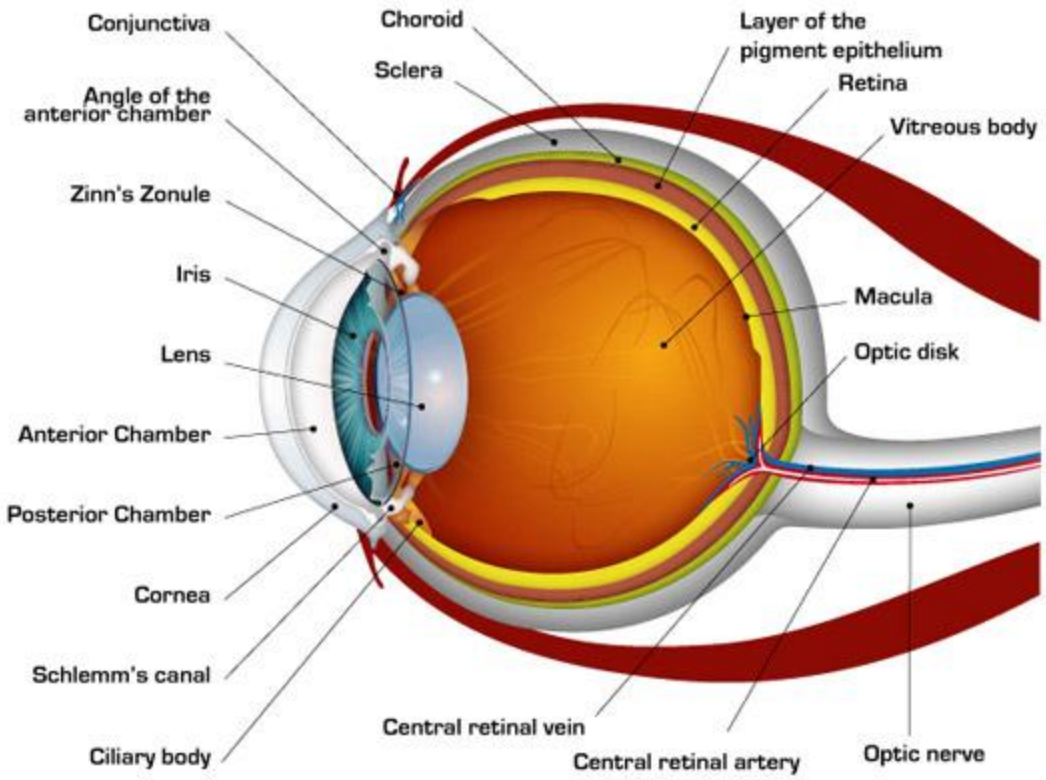


Diagram of the human eye. (http://www.nikonlenswear.ca/en/Eye_Chart.php)

APPENDIX X

Classification of *Acanthamoeba* (Page, 1988; Visvesvara, 1991).

Species	Mean diam. of cysts	Distinguishing features	Figure number
GROUP 1			
A. astronyxis	19.2 μm	Endocyst has 5-7 rays or arms that touch endocyst in same plane	
A. comandoni	25.6 μm	Endocyst has 6-10 rays or arms that touch endocyst in different planes	
A. echinulata	~ 25 μm	Endocyst has 12-14 rays or arms that are situated in different planes	
A. tubiashi	22.6 μm	Endocyst has 3-5 rays or arms	
GROUP 2			
A. castellanii	14-16 μm	More or less spherical endocyst connected to ectocyst by little conical arms	7.11; 7.12;
A. polyphaga	14 μm	Endocyst very irregular, practically never stellate; mean no. of endocyst corners 5-6	7.1; 7.2; 7.5; 7.6; 7.7; 7.8; 7.9; 7.10; 7.13; 7.14; 7.38; 7.39; 7.53
A. rhysodes	16-18 μm	Endocyst often with conical or tubular arms; a minority stellate; mean no. of endocyst arms 7-7.5	
A. griffini	14 μm	Endocyst variable; mean no. of endocyst arms 6	
A. hatchetti	13 μm	3 or 4 endocyst arms	
A. triangularis	13 μm	Mean number of endocyst arms fewer than 4	
A. divionensis	13 μm	Endocyst spherical or ovoid; mean number of endocyst arms 5-6	
A. mauritaniensis	14 μm	Endocyst spherical or ovoid, rarely polyhedral; distinct arms rarely formed	2.1; 2.2; 2.5; 2.6; 7.15-7.30; 8.16-8.19
A. lugdunensis	14 μm	Endocyst polyhedral with tendency to be spherical; any arms broad, not prominent	
A. quina	12 μm	Endocyst spherical or ovoid; mean number of endocyst arms 5-6	
GROUP 3			
A. culbertsoni	<18 μm	Endocyst usually round but may have 3-5 "gentle" corners	7.54
A. palestinensis	<18 μm	Endocyst spherical or ovoid, rarely with a few conical protuberances	
A. royreba	<18 μm	Mean number of cyst pores 13-14	
A. lenticulata	<18 μm	Endocyst circular, ectocyst sometimes thin and slightly undulated, sometimes thick and folded	2.7; 7.31-7.37; 8.20-8.32

APPENDIX XI

Species of *Acanthamoeba* implicated in corneal infections*

Species	Age of Patient	Sex of Patient	Predisposing conditions	Publication
A. castellanii	13 years	Female	Daily wear soft contact lenses; contact lens solutions made with salt tablets and distilled water	Moore *et al*., 1985
A. culbertsoni	38 years	Male	Daily wear soft contact lens user	Newton *et al*., 1986
A. hatchetti	22 years	Male	Daily wear soft contact lens user	Newton *et al*., 1986
A. griffini	32 years	Male	Disposable soft contact lenses; washing & storing lenses in water from bathroom tap; no chemical disinfection of lenses	Ledee *et al*., 1996
A. lugdunensis	17 years	Male	Daily wear soft contact lens user	Yu *et al*., 2004
A. polyphaga	59 years	Male	Rinsing his eye with tap water after he had been struck in the eye with straw fragments	Jones *et al*., 1975
A. quina	?	?	?	Simitzis-Le Flohic *et al*., 1989
A. rhysodes	23 years	Female	Daily wear soft contact lens user	Newton *et al*., 1986

*Numerous cases of *Acanthamoeba* keratitis have been reported in the literature. At least eight species of *Acanthamoeba* have been implicated (those listed in the table). The information provided here for patients is merely reflective of examples of cases for the species of *Acanthamoeba* listed.

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