Studies on the efficacy of novel disinfectant and therapeutic agents against *Acanthamoeba*

Thesis submitted for the degree of

Doctor of Philosophy

At the University of Leicester

By

Reanne Hughes, BSc.

Department of Microbiology and Immunology

May, 2004.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Statement

The work in this thesis was carried out by the author during the period October 1999 to September 2002, under the supervision of Dr. S. Kilvington in the Department of Microbiology and Immunology, University of Leicester. This thesis has been submitted for the degree of Doctor of Philosophy at the University of Leicester, and has not been submitted in full or part for any other degree.

This thesis may be made available for consultation, photocopying and for use through other lending libraries, either directly or through the British lending Library.

R. Hughes

Abstract

The efficacy of current commercial contact lens solutions was studied against the resistant cyst form of the opportunistic corneal pathogen *Acanthamoeba*. The cysticidal activity of hydrogen peroxide was enhanced against *Acanthamoeba* by optimising a peroxide-peroxidase-halide system. This led to the investigation of enhancing the activity of hydrogen peroxide against *Acanthamoeba* and other ocular pathogens in a self-neutralising system to provide an antimicrobial system for the cleaning of contact lenses.

The effects of cyst production on the susceptibility of contact lens disinfectants and current therapies was also studied, this highlighted the need for standardised and reproducible methods for evaluating contact lens disinfectants and therapeutic agents against *Acanthamoeba*. Investigation of current multipurpose contact lens solutions led to the investigation of a component (MAPD) of one of these solutions and it's potential therapeutic use for acanthamoeba keratitis patients. The antimicrobial activity of MAPD was investigated and found to be active against *Acanthamoeba* cysts and other ocular pathogens providing a potential compound in the treatment of acanthamoeba keratitis and, other forms of microbial keratitis.

As the rate of treatment failures with acanthamoeba keratitis is high, the possibility of exposure of *Acanthamoeba* to therapeutic compounds causing the development or selection of a resistant population was investigated over a 4 week period, it was found that in culture, the organism lost any increased resistance that it may have gained. Finally, other methods were investigated to measure the viability of *Acanthamoeba*, to replace the current culture method. It was found that the results differed significantly from those obtained by flow cytometry. Measuring ATP activity proved to be ineffective as the levels of ATP obtained were too low to measure.

Acknowledgements

Completion of this thesis would not have been possible without the assistance of the following individuals, to whom I would like to pass on my gratitude:

Dr. Simon Kilvington for his supervision, enthusiasm, help and advice throughout the project.

Professor Peter Andrew for his ideas and advice throughout the project.

Natalie Allcock and Mr. Stefan Hyman for their help with the EM work.

All of my friends and colleagues in the department of Microbiology and Immunology past and present; especially Helen Rees, Dan Hayward, Pat Spink, Wayne Heaselgrave and Pippa Bonny/ Orton for their support, advice and Friday night beers.

Finally I would like to thank my parents, family and Glen for their support and encouragement over the years.

This work was funded by Alcon Laboratories, Fort Worth, USA.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AK	acanthamoeba keratitis
ANOVA	One way analysis of variance
ATCC	American type culture collection
CLX	chlorhexidine
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	computersied tomography
DAPI	4', 6-diamindino-2-phenylindole
GAE	granulomatous amoebic encephalitis
H_2O_2	hydrogen peroxide
HOI	hyperiodous acid
HOCl	hyperochlorous acid
HRP	horseradish peroxidase
KI	potassium iodide
LB	Luria Bertani
MAPD	myristamidopropyl dimethylamine
MCC	minimum cysticidal concentration
$MgCl_2$	magnesium chloride
MPN	most probable number
MPO	myeloperoxidase
MPS	multipurpose solution
MTAC	minimum trohozoite amoebicidal concentration
NCTC	National Culture Type Collection
NNA	non-nutrient agar
OD	optical density
PAM	primary amoebic meningitis
PBS	phosphate buffered saline
PHMB	polyhexamethylene biguanide
PI	propidium iodide
RTF	resistance transfer factor
SBP	soyabean peroxidase
SDA	sabouraud dextrose agar
SEM	scanning electron microscopy
SOD	superoxide dismutase
TEM	transmission electron microscopy
TSA	tryptone soya agar
V/v	Volume per volume
W/v	Weight per volume

1 General Introduction

1.1 History

Parasitic amoebae were first described in the late 19th century. The relationship between *Entamoeba histolytica* and chronic dysentery in a 24-year-old Russian farmer was first reported in 1875 (Lösch 1875). Fifteen years later, a sluglike amoeba was discovered that could feed off dead and decaying matter, and named *Hartmanella limax* (E. Penard 1890). Subsequently, an amoebo-flagellate was isolated from a patient with dysentery in 1899 and named *Amoeba gruberi* (Schardinger 1899). In 1905, Vahlkampf described the *Amoeba limax* (Vahlkampf 1905) and Näegler described the non-pathogenic amoebo-flagellate *Naegleria gruberi* in 1909 (Näegler 1909). The morphology of *Entamoeba histolytica* was described in 1910 by Hartmann (Hartmann 1910). A *vahlkampfia* spp. amoeba was isolated from a patient with chronic diarrhoea by Chatton and Lalung-Bonnaire in 1912. In 1930, Castellanii discovered a contaminant of a yeast culture of *Cryptococcus pararoseus*, later that year it was placed in the genus *Hartmanella*, naming it *Hartmanella castellanii*, but was later re-classified as *Acanthamoeba castellanii* (Castellani 1930; Douglas 1930).

In 1958, Culbertson and colleagues discovered a contamination of a monkey kidney cell line whilst developing a polio vaccine. The presence of plaques on these cells led the group to believe that the contaminant was viral. However, when the culture was inoculated into monkeys and mice they developed an incapacitating disease and died (Culbertson, Smith *et al.*, 1958). Post mortem examination revealed "unusual cells", identified upon microscopic examination as *Acanthamoeba* spp. However, due to the confusion at that time over the taxonomy and nomenclature of *Acanthamoeba* and *Hartmanella*, he named the isolate as an H-A amoeba (belonging to the *Harmanella-Acanthamoeba* group). The species containing the isolate has since been named *Acanthamoeba culbertsonii* (Singh and Das 1970). The knowledge that *Acanthamoeba* spp. can cause disease in animals suggested that they could also be pathogenic to humans. The first recorded case of human infection associated with *Acanthamoeba* spp. was in a patient with Hodgkin's disease who developed granulomatous amoebic encephalitis (GAE) (Jager and Stamm 1972). A year later a Texan farmer became the first recorded case of acanthamoeba keratitis (AK) after a trauma to his eye (Jones, Visvesvara *et al.*, 1975; Visvesvara, Jones *et al.*, 1975). The interest in *Acanthamoeba* spp.

Increases during the 1980's due to the rapid increase in AK associated with contact lens wear.

1.2 Taxonomy

The genus *Acanthamoeba* belonging to the order Amoebida, suborder Acanthapodina, and family Acanthamoebidae are free-living amoebae found ubiquitously in the environment. The family Acanthamoebidae was first proposed in 1975 to include the spiny or threadlike pseudopod-forming sluglike amoebas that can form wrinkled or smooth spherical cysts (Sawyer and Griffen 1975).

1.2.1 Classification of *Acanthamoeba*

Kingdom:	Protista
Subkingdom:	Protozoa
Phylum:	Sarcomastigophora
Subphylum:	Sarcodina
Superclass:	Rhizopoda
Class:	Lobosea
Subclass:	Gymnamoeba
Order:	**Amoebida**
Suborder:	**Acanthopodina**
Family:	**Acanthamoebidae**
Genus:	*Acanthamoeba*

The taxonomic classification of *Acanthamoeba* is largely derived from morphological observations of the trophozoite and cyst stages. By 1976, seven species had been identified (Page 1967; Page 1976). The following year, observations in the variation of the cyst morphology led Pussard and Pons to conclude that 18 species existed (Pussard and Pons 1977). The species were assigned to 3 distinct morphological groups labelled I-III (Table 1 & Figure 1).

Table 1 : Morphological Groups of *Acanthamoeba*

	Species	Morphological Observations
Group I	*A. astronyxi*[a], *A. comandoni*, *A. echinulata*, *A. tubiashi*	Ectocyst smooth or gently wrinkled, thicker than the inner wall. The two walls are well separated except at opercula. Endocyst usually stellate.
Group II	*A. castellanii*[a,b], *A. divionensis*, *A. griffini*, *A. hatchetti*[b], *A. lugdunensis*, *A. mauritaniensis*, *A. polyphaga*[a,b], *A. quina*[b], *A. rhysodes*[a,b], *A. stevensoni*, *A. terricola*, *A. triangularis*	Intermediate observations of groups I and III. The walls are neither thick nor thin.
Group III	*A. culbertsoni*[a,b], *A. healyi*, *A. jacobsi*, *A. lenticulata*, *A. royreba*, *A. palestinensis*[a], *A. pustulosa*	The two walls are close to one another; the ectocyst is thin.

[a] Isolated from human central nervous system (CNS)

[b] Isolated from human cornea

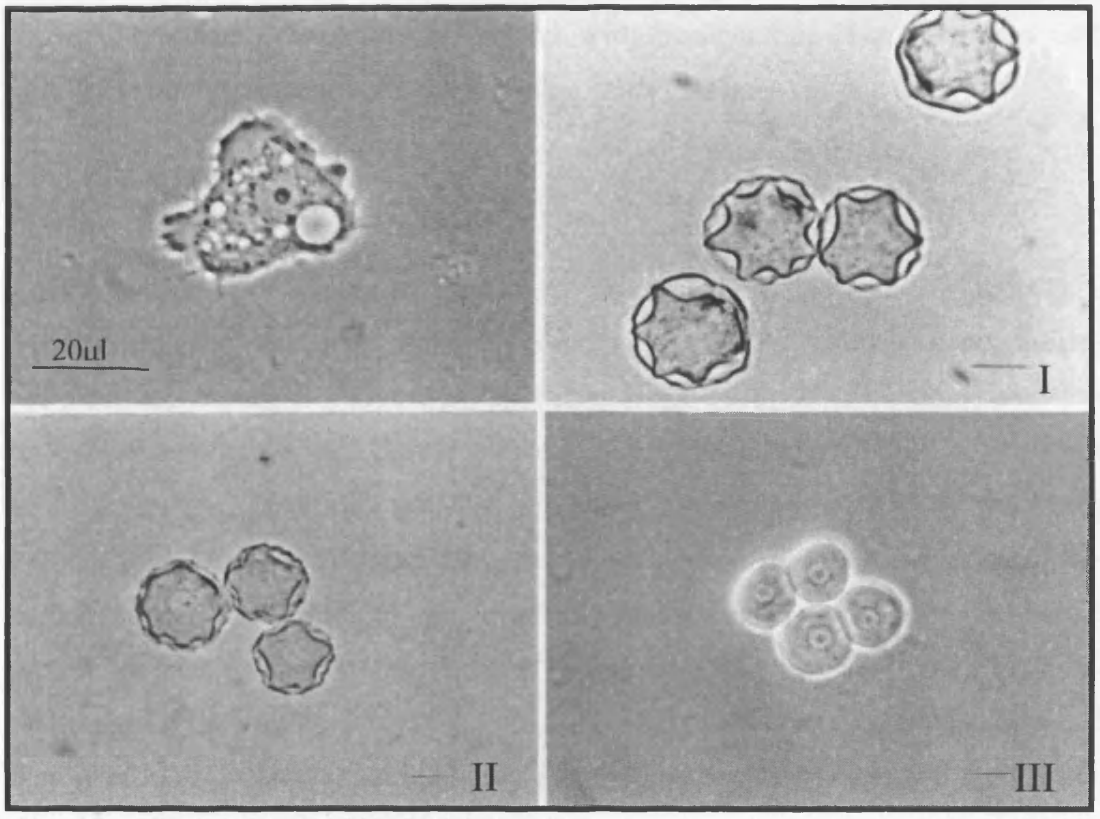

Figure 1: Taxonomy of *Acanthamoeba* Based on Morphology

Clockwise from top left: *Acanthamoeba* trophozoite; Group I cyst showing separate cyst walls; Group II cyst with wrinkled ectocyst which meets the endocyst at regular intervals; Group III cyst with two walls close to one another.

Although the general appearance of the cysts enables the differentiation of groups, a large intraspecies variation occurs, even within clonal cultures making the identification of species highly subjective (Stratford and Griffiths 1978). A large variation in strains within a species was discovered using isoenzyme analysis and mitochondrial DNA analysis (Costas and Griffiths 1985).

Although classification is still largely based on morphology (Visvesvara 1991), the introduction of DNA typing has enabled the characterisation of individual isolates. Phylogenetic analysis of the nuclear 18s ribosomal RNA gene (18s rDNA) has identified genetic clades and been useful in taxonomic studies of this genus, identifying RnT4 as the clade containing most of the keratitis isolates (Stothard, Schroeder-Diedrich *et al.,* 1998; Walochnik, Obwaller *et al.,* 2000; Schroeder, Booton *et al.,* 2001). More recently, mitochondrial 16s rDNA sequences of clinical isolates have been studied and have been

shown to be suitable for identifying isolates, with the advantage of being smaller sequences with fewer variable insertions (Ledee, Booton *et al.,* 2003).

1.3 Biology

Acanthamoeba is a genus of free-living amoeba found almost ubiquitously in the environment. They are characterised by a motile feeding and replicating trophozoite stage and a dormant double-walled resistant cyst stage (Page 1988). Both the trophozoite form and the cyst form contain a large central nucleolus within the nucleus (Figure 2). The trophozoite is 25-40μm long and also contains a central contractile vacuole in the cytoplasm. Pseudopodia (false feet) extend from the large hyaline zone and are used in the slow polydirectional movement of the trophozoites. Acanthapodia, miniature needle-like projections from the body of the trophozoites, can be observed (Page 1988). The growth and replication of the trophozoites under favourable conditions occurs by binary fission (Byers, Kim *et al.,* 1991). During the mitotic division, the nuclear membrane and the nucleolus are thought to disappear (Singh 1975).

Under adverse conditions, *Acanthamoeba* trophozoites form resistant cysts. These are smaller, usually less than 25μm in diameter. There are three stages of encystment; pre-encystment, cyst initiation and cyst wall synthesis (Byers, Kim *et al.,* 1991; Khunkitti, Lloyd *et al.,* 1998), governed by the interaction of various enzymes within the trophozoite cell. Adenylate cyclase is thought to be activated either during the stationary phase or during encystment in non-nutrient medium, causing an increase in the rate of production of cyclic AMP (cAMP) within the trophozoite, this occurs by 6-8 hours of the cell cycle, and is known as pre-encystment. Cyst initiation occurs by about 12 hours when cyclic AMP protein kinase (cAMP-PK) is activated. The enzyme cellulose synthase is then phosphorylated and altered covalently, resulting in its activation. At about 24-48 hours cellulose is secreted from the cell and covers the trophozoite leading to the formation of a resistant mature cyst (Achar 1991). The two cyst walls are joined at intervals giving rise to a polygonal arrangement. Ostioles, or pores, can be seen at the intersection of the walls. These are sealed by the operculum or plug. The ostioles are thought to be the recognition site for the excystment of the amoeba (Matsugo, Kasahara *et al.,* 1998).

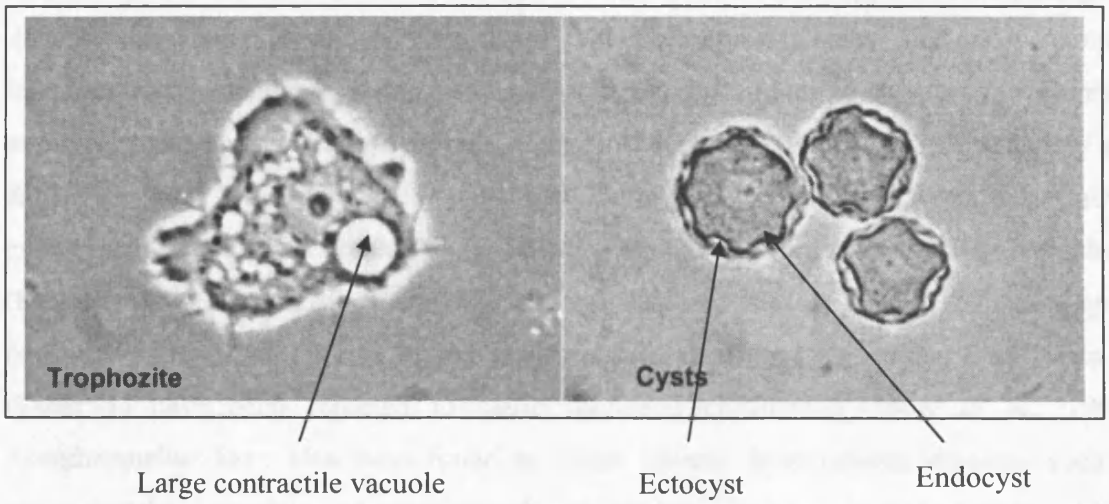

Figure 2: Morphology of *Acanthamoeba* cyst and trophozoite.

1.4 Ecology

Acanthamoeba cysts are resistant to desiccation (Kingston and Warhurst 1969), extreme temperatures (Biddick, Rogers *et al.,* 1984) and most contact lens disinfection solutions (Seal, Stapleton *et al.,* 1992). Cysts have been found in many adverse environments including water-cooling towers, and chlorinated swimming pools (De Jonckheere 1991). Viable cysts have been isolated from temperatures ranging from -20°C to 56°C (Biddick, Rogers *et al.,* 1984). Under favourable conditions, excystment occurs and the trophozoites feed on bacteria, preferably Gram negative rods, for example *Escherichia coli* (Weisman 1976). However, the two most common bacterial commensals in the eye are *Staphylococcus epidermidis* and *Micrococcus* spp., which are both Gram positive and can support the growth of *Acanthamoeba* equally well (Larkin and Easty 1991).

1.5 Pathogenesis

Until the discovery that *Acanthamoeba* spp. when delivered intranasally could invade the olfactory mucosa, migrate to the brain and cause a fatal meningoencephalitis (Culbertson, Smith *et al.,* 1958), all known species were considered harmless. *Acanthamoeba* spp. are not

true parasites as they do not rely on the infection of a host organism to complete their life cycle, they are therefore referred to as "free-living". However, as opportunistic pathogens, *Acanthamoeba* are the causative agents of two diseases of humans. The most common infection is acanthamoeba keratitis, a painful and potentially blinding infection of the cornea usually in previously healthy people (Jones, Visvesvara *et al.,* 1975; Nagington and Richards 1976). *Acanthamoeba* is also known to cause a rare but fatal encephalitis, granulomatous amoebic encephalitis (GAE), predominantly in immunodeficient individuals (Kenney 1971). Trophozoites are the infective stage of the organism. The majority of pathogenic strains belong to morphological group II, although some strains from group III (Table 1) have been reported to cause disease (Schaumberg, Snow *et al.,* 1998). Acanthamoebae have also been found to cause several disseminating diseases such as acanthamoeba dermatitis and acanthamoeba pneumonitis in the immunocompromised host (Martinez and Visvesvara 1997).

1.5.1 Granulomatous Amoebic Encephalitis

The first reported fatal encephalitis caused by *Acanthamoeba* spp. in humans was reported in 1972 (Jager and Stamm 1972). The term granulomatous amoebic encephalitis (GAE) was proposed 8 years later by Martinez to describe the histopathological features of the disease (Martinez, Garcia *et al.,* 1980). To date, over 100 cases have been reported worldwide (Martinez and Visvesvara 1997); (Walochnik, Duchene *et al.,* 2002), however, the true number is likely to be much higher as the disease is usually diagnosed postmortem.

Granulomatous amoebic encephalitis is a rare subacute necrotising infection most commonly caused by *A. castellanii, A. culbertsoni,* or *A. polyphaga.* Predisposing factors of GAE include chemotherapy, steroid treatment, alcoholism, radiation therapy, systemic lupus, AIDS, and haematologic disorders (Martinez and Visvesvara 1997). Infection is thought to occur from primary infection of the skin or lungs and is carried through the bloodstream to the CNS. The incubation period for GAE is not known but is thought to be at least 10 days. Clinical symptoms are initially characterised by insidious headaches, mood swings, lethargy, confusion and a low grade fever (Ma, Visvesvara *et al.,* 1990). Often these symptoms are accompanied by seizures, focal neurological defects such as hemiparesis, a stiff neck, and nausea (Ma, Visvesvara *et al.,* 1990). Associated disorders of GAE include skin ulcers, liver

disease, pneumonitis, diabetes mellitus, renal failure, rhinitis, pharyngitis, and tuberculosis. Usually the cause of death is bronchopneumonia.

Computerised tomography (CT) scans may show bilateral low-density areas in the cerebral cortex and subcortical white matter (Sell, Rupp *et al.,* 1997). The most effective diagnosis is the isolation and identification of *Acanthamoeba* trophozoites and cysts from the CNS to exclude other treatable lesions, such as herpes simplex meningoencephalitis (Martinez 1993). It is important to perform a biopsy early because the chance of successful therapy decreases rapidly with the onset of clinical symptoms. A positive diagnosis may be found within a few minutes of obtaining the specimen by examining frozen sections stained with eosin and haemolyxin (Ma, Visvesvara *et al.,* 1990). Amoebae are rarely found in the CSF, although it usually shows a lymphocytic pleocytosis with mildly elevated protein and normal glucose. However, a lumbar puncture may be contraindicated because of signs of increased intracranial pressure (Calore, Cavaliere *et al.,* 1997; Martinez and Visvesvara 1997).

The macroscopic examination of tissues shows oedema and softening of the cerebral hemispheres with abscesses, haemorrhages and necrosis (Martinez and Visvesvara 1997). The meninges overlying this area may be cloudy and there may be uncal or cerebellar tonsillar herniation. Lesions may be found in the cerebral hemispheres, brain stem, basal ganglia and the cerebellum (Ma, Visvesvara *et al.,* 1990). Microscopically, a chronic and granulomatous reaction with lymphocytes, macrophages and multinucleated cells with the infective organisms cause the majority of the lesions. Trophozoites and cysts are found throughout the lesions but are mainly found invading blood vessel walls. There may be thrombosis of the blood vessels associated with haemorrhaging and necrosis (Ma, Visvesvara *et al.,* 1990).

1.5.1.1 Treatment of GAE

To date there is no effective treatment for GAE as most cases have been diagnosed postmortem. Several antimicrobial agents have been shown to be effective against *Acanthamoeba* trophozoites *in vitro*. These include propamidine and pentamidine isethionate, ketoconazole, clotrimazole, neomycin, polymyxin E and paromomycin (Casemore 1970; Duma and Finley 1976; Nagington and Richards 1976; Ferrante, Rowan-Kelly *et al.,* 1984); (Wright, Warhurst *et al.,* 1985); (Driebe, Stern *et al.,* 1988). Due to the

immunosupressed status of the patients prognosis is poor and patients may acquire other microbial infections. Sulphadiazine has been reported to protect mice from experimental GAE infection (Culbertson, Holmes *et al.,* 1965). Of the few reported successful treatments of GAE, one involves a previously healthy 7 year old girl who was successfully treated with ketoconazole chemotherapy and total excision of the infected area (Ofori-Kwakye, Sidebottom *et al.,* 1986), another involves a case of AIDS-associated GAE which was successfully treated with fluconazole and sulphadiazine combined with excision of the infected area (Seijo Martinez, Gonzalez-Mediero *et al.,* 2000). The combination of the immunosuppressed state of GAE patients and low level of activity of antiamoebic drugs as well as the inability to deliver these drugs in high concentrations due to the blood brain barrier results in a grave prognosis for patients with this disease.

1.5.2 Acanthamoeba Keratitis

Acanthamoeba keratitis (AK) is a severe vision threatening chronic inflammation of the cornea (Jones, Visvesvara *et al.,* 1975; Beattie, Slomovic *et al.,* 1990; Kilvington and White 1994). The first reported case of acanthamoeba keratitis occurred in 1973, in a Texan farmer who scratched his eye on some straw and rinsed the eye with water (Jones, Visvesvara *et al.,* 1975). The disease was extremely rare until the mid 1980's when there was a sharp increase in infection correlating with the increased use of contact lenses. Subsequently, incidences fell, partly due to the intense media attention that highlighted the risk factors for the soft contact lens related AK (Radford, Minassian *et al.,* 2002).

It has been suggested that the use of disposable contact lenses increases the risk of infection due to poor lens hygiene and over night wearing of lenses (Matthews, Frazer *et al.,* 1992); (Schein, Buehler *et al.,* 1994). Patients may see less need for care with disposable lenses, which may account for the increase in acanthamoeba keratitis infection in disposable lens wearers. Contact lenses agitate the cornea and disrupt the tear film, leading to hypoxia, or decreased oxygen, to the corneal epithelium. This has been shown to enable the adherence of micro-organisms to the surface of the eye (Cao, Jefferson *et al.,* 1998). Bacteria such as *S. epidermidis* and *Micrococcus* spp. are found in high numbers in the lens cases of infected individuals. These bacteria may play a role in the poorly understood pathogenesis of acanthamoeba keratitis as well as supporting the growth of the trophozoites (Larkin and Easty 1991).

Infection is usually associated with poor lens hygiene, such as rinsing the lenses under tap water, and using non-sterile saline solutions (Moore, McCulley *et al.,* 1985); (Stehr-Green, Bailey *et al.,* 1989). It is now estimated that 92 % of acanthamoeba keratitis infections occur in contact lens wearers, the remaining cases are caused by accidental trauma to the epithelium. (Radford, Bacon *et al.,* 1995; Radford, Minassian *et al.,* 1998). A case of an infection resulting from corneal surgery with no other identified risk factors has been documented (Parrish and O'Day 1991).

Pathogenic strains of *Acanthamoeba* will all grow at 26°C - 37°C with an optimum growth temperature of 30°C. Ocular temperature is lower than the rest of the body at about 34°C, this may account for the ability of *Acanthamoeba* to grow successfully on the eye. At least 5 different species of *Acanthamoeba* have been found to cause corneal infection: *A. castellanii, A. culbertsoni, A. hatchetti, A. polyphaga* and *A. rhysodes* (Ma, Visvesvara *et al.,* 1990; Dart, Stapleton *et al.,* 1991), although the two most common pathogenic strains isolated from the eye are, *A. castellanii,* and *A. polyphaga* (Brandt, Ware *et al.,* 1989). Pathogenic trophozoites have been isolated from the lens storage cases of asymptomatic contact lens wearers, suggesting that previous trauma to the epithelium may be a contributory factor to infection (Larkin, Kilvington *et al.,* 1990).

Patients typically have inflammation of the eye and are in considerable pain (Figure 3), which is worsened by exposure to bright light (Cheng, Ling *et al.,* 2000). Initial infection is on the corneal surface, but later ulceration may occur. An alternative clinical appearance is of microscopic abscesses, which may be in a circular pattern or as greyish white infiltrate patches (Cheng, Ling *et al.,* 2000). If *Acanthamoeba* is not identified quickly as the cause of infection, the disease will worsen. The protozoa can penetrate into the area behind the cornea, causing deep abscesses, and the cornea may be perforated, leading to loss of vision.

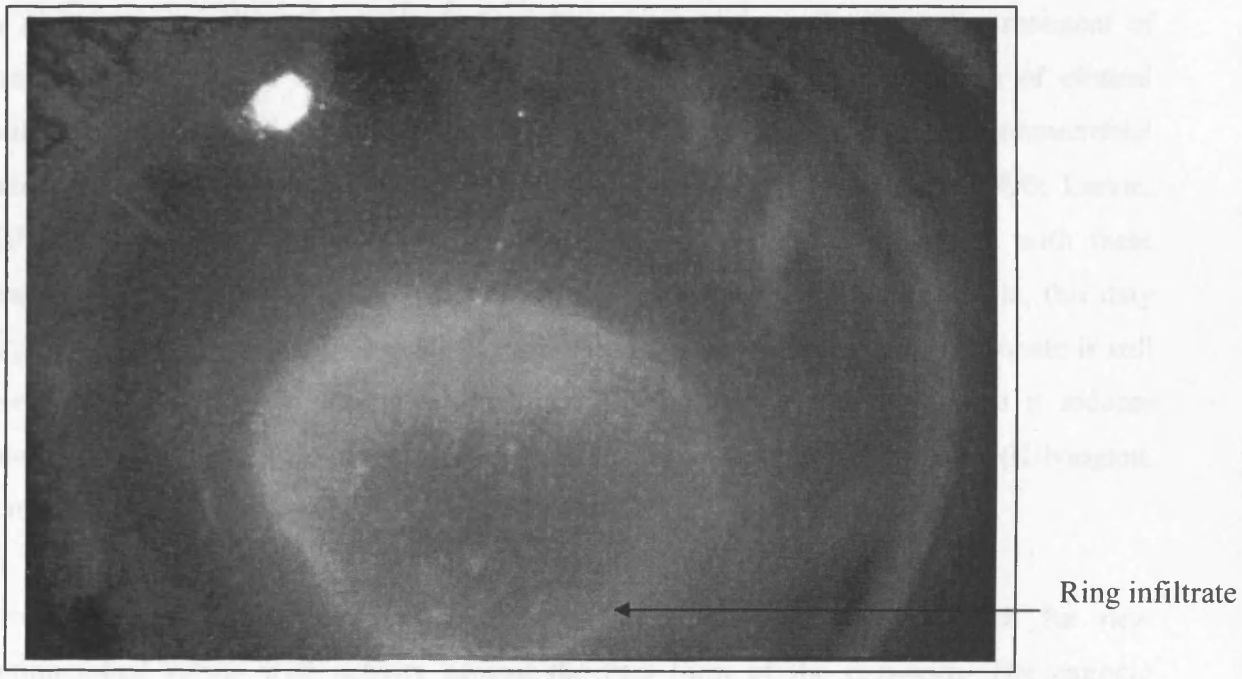

Figure 3: Patient with advanced stage of AK. The white ring infiltrate can be seen within the iris.

1.5.2.1 Treatment of AK

Successful treatment of acanthamoeba keratitis requires rapid diagnosis and aggressive medical therapy. However, at concentrations that can be tolerated by the cornea most antimicrobial agents do not provide any cysticidal activity. This makes acanthamoeba keratitis one of the most difficult ocular infections to manage successfully (Turner, Russell *et al.,* 1999). Prolonged medical treatment, often a year or more, is required to eliminate viable organisms from the cornea (Larkin, Kilvington *et al.,* 1992; Dart 1996). Treatment failures are still common, the reasons for which are unclear but may be due to inherent or acquired resistance or failure to achieve therapeutic levels within the cornea at a level that is not toxic to the cornea.

Wright and colleagues reported the first successful medical treatment of acanthamoeba keratitis in 1985 using topical application of the aromatic diamidine propamidine isethionate,

sold in the UK as Brolene® (Wright, Warhurst *et al.,* 1985). Further successes have been reported using propamidine isethionate alone or in combination with the aminoglycoside neomycin and with imadazole derivatives miconazole, clotrimazole, ketoconazole and itraconazole (Ficker, Seal *et al.,* 1990; Ishibashi, Matsumoto *et al.,* 1990; Elder, Kilvington *et al.,* 1994). However, the rationale for using many of these agents in the treatment of acanthamoeba keratitis is not clear. *In vitro* antimicrobial sensitivity testing of clinical isolates has shown that whilst aminoglycosides and imidazole agents provide antimicrobial activity against trophozoites, cysts are resistant (Kilvington, Larkin *et al.,* 1990; Larkin, Kilvington *et al.,* 1992; Kilvington and White 1994). Therefore, treatment with these reagents serves only to eradicate the trophozoites whilst the cysts remain viable, this may explain the need for prolonged medical treatment. Although propamidine isethionate is still used in treatment for acanthamoeba keratitis, at sub-inhibitory concentrations it induces encystment. In addition to this, acquired resistance may occur during therapy (Kilvington, Larkin *et al.,* 1990; Ficker, Seal *et al.,* 1990).

Problems with the treatment of acanthamoeba keratitis led to the search for new antimicrobial agents with activity against the cyst form of the organism. The cationic antiseptic, polyhexamethylene biguanide (PHMB) has been successful in treating cases of acanthamoeba keratitis that were unresponsive to conventional therapy of propamidine isethionate and antifungal agents (Larkin, Kilvington *et al.,* 1992). Polyhexamethylene biguanide is also used as a swimming pool disinfectant and as a cosmetic preservative, working by inhibiting the membrane function of organisms. The biguinide chlorhexidine (CLX) has also been shown to exert cysticidal activity and has been used in the successful treatment of acanthamoeba keratitis (Seal, Hay *et al.,* 1996).

Typically, combined treatment of PHMB and propamidine isethionate is used. These must be applied every hour, day and night for the first 2 days, then hourly during the day only for the next 3 days, this is then reduced to 6 times a day, tapering to 4 times a day (Duguid, Dart *et al.,* 1997; Radford, Lehmann *et al.,* 1998). However, even with such intense therapy, it is difficult to achieve sufficient concentrations in the cornea to achieve cysticidal activity. Chlorhexidine has also been used in conjunction with propamidine isethionate successfully (Hay, Kirkness *et al.,* 1994), and more recently CLX by itself (Kosrirukvongs, Wanachiwanawin *et al.,* 1999).

The treatment period usually lasts for 2 to 12 months. Those that are unresponsive to chemotherapy require penetrating keratoplasty (corneal grafting).

1.5.2.2 Progression of Infection

The infective process begins when the trophozoites bind to mannose receptors on the corneal epithelium (Panjwani, Zhao *et al.*, 1997). It has been shown that the trophozoites are able to separate the intracellular junctions of the epithelium and so can penetrate the intact corneal button (Moore, Ubelaker *et al.*, 1991). The resultant rapid sloughing of the cells is achieved by the infective trophozoites in 3 ways:

a) Direct cytolysis, which is dependent on the calcium ionophore A23187 (Taylor, Pidherney *et al.*, 1995).
b) Phagocytosis; *Acanthamoeba* trophozoites are able to directly ingest cells (Pettit, Williamson *et al.*, 1996).
c) Apoptosis; this is achieved by the secretion of proteases from the trophozoite inducing host cell death (Yang, Cao *et al.*, 1997; Leher, Silvany *et al.*, 1998).

1.5.3 Other Implications of *Acanthamoeba* Infections

The trophozoites and cysts of *Acanthamoeba* have also been found to support the growth and replication of the bacterium *Legionella pneumophila*, the cause of Legionaire's disease in humans (Barbaree, Fields *et al.*, 1986). The cyst form of the amoebae is able to protect the bacterium from chlorine disinfection and, therefore, may play an important role in the prevalence of this pathogen in the environment (Kilvington and Price 1990).

1.5.4 Contamination of Storage Cases of Asymptomatic Wearers

In a study of 101 contact lens storage cases, 81% of cases were found to be contaminated with micro-organisms (Gray, Cursons *et al.*, 1995). Of these, 8% contained *Acanthamoeba* spp. The participants in this study did not use home-made saline solutions, a known source of contamination. 75% of these storage cases were disinfected with hydrogen peroxide, the majority of these using the one-step peroxide system. These systems involve the simultaneous neutralisation of hydrogen peroxide during disinfection. The organisms isolated were all found to contain catalase that decomposes hydrogen peroxide to water and

oxygen. It has been suggested, that the use of hydrogen peroxide for an extended period of time has selected for resistant populations of micro-organisms.

1.5.5 Current Methods of Contact Lens Disinfection

As most cases of acanthamoeba keratitis are associated with lack of care of lenses, it is necessary to use a disinfecting solution that not only destroys the resistant cyst form, but also is easy to use. Three main types of contact lens disinfection systems are currently available, most of which have little cysticidal activity against *Acanthamoeba* spp. These are moist heat systems, cold chemical disinfection, and hydrogen peroxide.

1.5.5.1 Heat Disinfection

Treatment of contact lenses with the moist heat disinfection systems at 65°C and 70°C resulted in a 4-log reduction of cysts after 15 minutes and 2 minutes respectively (Kilvington 1989). However, cysts have been isolated from heat-treated storage cases of patients (Ludwig, Meisler *et al.,* 1986). This indicates that the systems may not be able to raise the temperature high enough for long enough to kill the cysts. Also, there is no continuous antimicrobial activity after the heat treatment for continued protection. As disinfection serves only to reduce the number of viable organisms, when the disinfectant is removed, the organisms are able to replicate. Heat treatment can only be used with soft contact lenses, with higher water content, as the heat may distort other types. Protein deposits on the surface of the lenses are denatured by the heat and baked onto the lens. The disadvantages of these types of systems outweigh the advantages; therefore heat systems are now rarely used.

1.5.5.2 Multi-Purpose Solutions

Multi-purpose solutions (MPS) represent a single solution for the cleaning, disinfection and storage of lenses and, therefore, provide continued disinfection during lens storage. The majority of these solutions contain polyhexanide, a biguanide eg. PHMB, which is an eye irritant at concentrations great enough to exert cysticidal activity against *Acanthamoeba* spp. (Silvany, Dougherty *et al.,* 1991). However, as these solutions are easy to use, they are popular amongst contact lens wearers and may result in increased patient compliance.

1.5.5.3 Peroxide Based Solutions

Hydrogen peroxide is an effective microbial disinfectant, destroying pathogens by oxidation. It is also effective against the cyst form of *Acanthamoeba.* However, hydrogen peroxide is also toxic to the cornea, therefore must be neutralised before the lenses can be worn (Spector 1990).

Table 2: Summary of Advantages and Disadvantages of Soft Contact Lens Disinfection Systems

Method of Disinfection	Advantages	Disadvantages
Heat	• Kills wide range of organisms • Easy to use • Inexpensive • No chemicals or preservatives	• Damages lenses with high H_2O content • May decrease life of lenses with low H_2O content • Can bake on deposits • Cannot judge whether system is working
Multi-purpose	• Easy to use • Can be used with all soft lenses • Preservation continued after disinfection complete	• Kills organisms slowly • Ineffective against *Acanthamoeba* and fungi • Requires long soak time • Chemical residues may cause eye irritation
Hydrogen peroxide	• Rapid kill of micro-organisms • No preservatives, therefore, no irritation • Effective against Acanthamoeba after two hours	• Neutralisation of peroxide required • No continued anti-microbial activity after neutralisation

Adapted from Levey & Cohen, 1996. pp 247

The details of contact lens disinfection systems currently available are discussed in more detail in later chapters, this table covers the principle advantages and disadvantages of each system.

1.6 Aims

Although there has been much work published on the efficacies of disinfectants against *Acanthamoeba,* to date there has been no consistent comprehensive study of commercially available solutions. Moreover, results are difficult to interpret as a variety of methods have been used. This study initially involved determining a reliable and reproducible method for testing the amoebicidal and cysticidal activity of disinfectants. In addition to this, alternative systems were investigated, including enhancing the cysticidal activity of hydrogen peroxide and investigating the efficacy of a novel amidoamine compound in the treatment for acanthamoeba keratitis.

2 Materials & Methods

2.1 Introduction

Disinfection is fundamental to safe contact lens use and hence the prevention of infection. Although the ISO stipulates methods for testing of bacteria and fungi no standard protocol exists for *Acanthamoeba* contact lens disinfectant efficacy testing (International Organization for Standardization 2000). As a consequence, a variety of strains, methods for cyst production and experimental protocols have been employed (Anger, Rupp *et al.,* 1991; Buck, Rosenthal *et al.,* 1998); (Hughes and Kilvington 2001); (Hugo, McLaughlin *et al.,* 1991); (Kilvington, Hughes *et al.,* 2002); (Noble, Ahearn *et al.,* 2002); (Silvany, Dougherty *et al.,* 1991), often with contradictory findings (Khunkitti, Lloyd *et al.,* 1998; Borazjani, May *et al.,* 2000; Buck, Rosenthal *et al.,* 2000; Hiti, Walochnik *et al.,* 2002). Many protocols also involve different methods of viability determination, therefore the results of these varied methods are not comparable (Buck, Rosenthal *et al.,* 2000).

In a review of methods used to evaluate the effectiveness of contact lens solutions against *Acanthamoeba* it was found that of the studies reviewed, 30% did not use a quantitative method, merely reporting the presence or absence of viable amoebae (Buck, Rosenthal *et al.,* 2000). Others have used quantitative methods, such as direct counting with a haemocytometer (Connor, Hopkins *et al.,* 1991; Buck and Rosenthal 1996), standard plaque assay (Hugo, McLaughlin *et al.,* 1991; Khunkitti, Lloyd *et al.,* 1996), a quantitative microtitre method (Buck and Rosenthal 1996), and enumeration of track-forming units developed on nonnutrient agar with a bacterial overlay (Kilvington 1998) and the most probable number technique (Perrine, Chenu *et al.,* 1995; Beattie, Seal *et al.,* 2003).

2.2 Methods

2.2.1 Test organisms

The *A polyphaga* Ros strain that was used in this study was originally isolated from an unpublished case of Acanthamoeba keratitis in the U.K. in 1994. The fungal and bacterial strains used were obtained from either NCTC (Colindale, UK) or ATCC (Manassas, USA) culture collections (Table 3).

Table 3: Strains and cultures used in this study

Organism	Strain	Source	Date isolated
Acanthamoeba polyphaga	Ros	AK*	1991
Acanthamoeba quina	L1a		
Acanthamoeba culbertsoni	30171	ATCC[1]	1957
Acanthamoeba palestinensis	1547/1	CCAP[3]	1967
Acanthamoeba polyphaga	AK-1	AK*	1998
Acanthamoeba polyphaga	AK-2	AK*	1998
Acanthamoeba polyphaga	AK-3	AK*	1999
Acanthamoeba polyphaga	AK-4	AK*	1999
Pseudomonas aeruginosa	9027	ATCC[1]	
Staphylococcus aureus	6538	ATCC	
Candida albicans	10231	ATCC	
Fusarium solani	36031	ATCC	
Bacillus subtilis	laboratory strain	Prof. Peter Andrew[2]	
Escherichis coli	JM101	Prof. Peter Andrew[2]	

* Acanthamoeba Keratitis case from Moorfields Eye Hospital, London, UK.

[1] American Type Culture Collection, Rockville, USA

[2] Professor Peter Andrew, Department of Microbiology and Immunology, University of Leicester. UK.

[3] Culture Collection of Algae and Protozoa, Windermere, UK.

2.2.2 Chemicals

All chemicals were obtained from Sigma Chemical Company (Poole, UK) or BDH (Poole, UK) unless otherwise stated. All chemicals were sterilised before use, either by autoclaving at 120°C for 15 minutes, or by passage through a 0.2 µm filter (Nalgene, Rochester, USA).

Horseradish peroxidase (HRP: EC 1.11.1.7) was dissolved in nanopure water, filter sterilised and stored at –20 °C. The KI stock solution was stored at room temperature in the dark.

2.2.3 Storage of H_2O_2

The H_2O_2 stock solution was stored in the dark at 4°C and the concentration measured before use, using a molar extinction coefficient of 80 at 230 nm.

2.2.4 Growth and maintenance of *Acanthamoeba* cultures

All cultures of *Acanthamoeba* were recovered from liquid nitrogen storage unless otherwise stated. *Acanthamoeba* trophozoites were maintained in tissue culture flasks (Nunc, Rochester, USA) at 32°C in a semi defined axenic culture medium (#6 Base) at 30 °C and subcultured every 3 days. Subculturing involved shaking the flask gently to remove some from the side of the flask and replacing the entire medium. When needed for an experiment, the trophozoites were removed from the flask after 2 days this ensured that they were in log phase of growth. The cells were washed X 3 in ¼ Ringer's solution and adjusted to 10^6 per ml unless otherwise stated.

2.2.5 Axenic culture media (#6 Base)

Acanthamoeba strains were maintained in axenic culture before use. The medium comprised of 20 g Biosate peptone (Beckton Dickinson), 5 g glucose, 0.3 g potassium dihydrogen orthophosphate (KH_2PO_4), 10mg vitamin B_{12}, and 15 mg L-methionine per 900ml of double distilled water. If required the pH of the medium was adjusted 6.5-6.6 with 1 M sodium hydroxide (NaOH) and then aliquoted into 225 ml portions before autoclaving.

Prior to use penicillin/streptomycin to a final concentraion of 150 U/ml was added to the medium that was then made up to 250 ml with 10% heat inactivated foetal calf serum

(Gibco), or sterile distilled water depending on strain of *Acathamoeba* used. The complete medium was stored at 4°C and used within one month.

2.2.6 Cryopreservation of Acanthamoeba

Log phase cultures of axenic trophozoites were harvested by centrifugation at 1000 x g for 5 minutes at room temperature. The cell pellet was resuspended in fresh #6 Base to approximately 1 x 10^6 trophozoites ml^{-1} and 0.5 ml volumes placed into 1.2 ml screw capped polypropylene ampoules (Nunc, Hereford, UK). An equal volume of #6 Base containing 10 % dimethylsulphoxide (DMSO) (Koch-Light Ltd., Suffolk, UK) was added to each ampoule and the contents carefully mixed by inversion. The ampoules were placed into a cell-freezing box (Nalgene, Hereford, UK.) for a further 60 minutes, and then placed at –80 °C for 4 hours before being transferred to liquid nitrogen for long-term storage.

2.2.7 Recovery from cryopreservation

Trophozoites were recovered by removing an ampoule from the liquid nitrogen and placing into an enclosed container at room temperature under an operating Class 2 safety cabinet. This safety precaution is necessary as liquid nitrogen can penetrate the washer sealing between the ampoule cap and the tube on warming, producing an explosion. After 3 minutes, the ampoule was placed in a 37 °C water bath for rapid thawing. The ampoule contents were inoculated into culture tubes containing 2 ml of fresh #6 Base that had been pre-warmed to 32 °C. The tubes were then incubated at 32 °C for an hour, during which time the viable trophozoites adhere to the sides of the tubes. The culture medium was then gently poured off and replaced with 3 ml of fresh medium and the tubes re-incubated at 32 °C to establish axenic growth.

2.2.8 Preparation of cysts

Unless otherwise stated, cysts were produced using Neff's defined encystment medium. *Acanthamoeba* trophozoites were pelleted by centrifugation (1000 x g for 10mins) and then approximately 10^7 trophozoites were added to 100 ml of the encystment medium in a 175cm^2 tissue culture flask. The flask was then placed in a shaking incubator at 32 °C for 7 days for the encystment process to complete. Mature cysts, as judged by phase contrast microscopy, were recovered and washed three times by centrifugation at 1000 x g in ¼

Ringer's solution then the cysts were sonicated before with three pulses at 50 % amplitude each for 5 seconds (Ultrasonic Engineering Ltd, London, England). Previous experiments had shown that this removed cyst clumps without affecting viability (results not shown). Cell counts were performed with a haemocytometer, adjusted to a concentration of 5 x 10^6 cysts per ml and stored at 4 °C for use within 14 days. Before use, the cells were examined under a microscope to ensure >90% were mature cysts.

2.2.8.1 Neff's encystment medium

The medium comprised of 0.1 M potassium chloride (KCl), 0.008 M magnesium sulphate ($MgSO_4$), 0.0004 M calcium chloride ($CaCl_2$), 0.001 M sodium hydrogen carbonate ($NaHCO_3$) and 0.02M Tris buffer. The components were dissolved in nanopure water (nH_2O) and adjusted to a pH of 8.9-9.0 before autoclaving (Neff, S. A. Ray *et al.,* 1964; Hughes and Kilvington 2001).

2.2.8.2 NNA cysts

Log phase trophozoites were washed three times in ¼ Ringer's solution at 1000 X g and inoculated onto 2% non-nutrient agar (Oxoid Ltd, Basingstoke, England) seeded with live *Escherichia coli* in air at 32°C for 7 days. The cysts were recovered from the agar by adding ¼ Ringer's solution and gently rubbing with a cell scraper. The liquid was then carefully pipetted off and centrifuged at 1000 x g for 10 minutes. The resulting cyst pellet was washed three times in ¼ Ringer's solution by centrifugation and thereafter stored at 4 °C for testing within 14 days.

2.2.8.3 Taurine encystment medium

This medium comprised of ¼ Ringer's solution containing 20 mM taurine and 15 mM magnesium chloride ($MgCl_2$).

2.2.8.4 # 6-Mg encystment medium

Trophozoites were inoculated at a concentration of 2 x 10^5 ml^{-1} into the semi-defined axenic medium (#6-Base) supplemented after autoclaving with filter sterilised 50 mM magnesium

chloride $MgCl_2$ in a 175 cm^2 tissue culture flask. This was incubated in a shaking incubator at 32 °C for 7 days and harvested as above.

2.2.9 Preparation of *E.coli* stocks

Escherichia coli (JM101 strain) was inoculated onto a Luria Bertani (LB) agar (Difco, Maryland, USA) plate and incubated overnight at 37 °C to allow for growth and colony formation. Single colonies of *E. coli* were then picked using a sterile loop and placed in 100 ml of LB Broth (Difco, Maryland, USA) in a 175 cm^2 tissue culture flask and incubated overnight at 37 °C in a shaking incubator. The *E. coli* suspension was aseptically transferred to two 50 ml polypropylene tubes and centrifuged at room temperature at 2000 x g for 30mins. The supernatant was gently decanted and the pellet was washed three times by re-suspending in ¼ Ringer's and centrifuging at 2000 x g for 30 mins. The final *E. coli* pellet was re-suspended in 10 ml of ¼ Ringer's solution and stored at 4 °C for use within a week.

2.2.10 Bacterial and fungal culture

Pseudomonas aeruginosa (ATCC 9027), *Staphylococcus aureus* (ATCC 6538), *Candida albicans* (ATCC 10231) and *Fusarium solani* (ATCC 36031) were all obtained from the American Type Culture Collection, Rockville, USA.

F. solani was cultured on potato dextrose agar (Difco, Maryland, USA) in the dark at 25°C for 4 days, then exposed to daylight for a further 10-14 days, allowing conidia formation. The cells were scraped off the plates in Dulbecco's phosphate buffered saline with 0.05 % (w/v) Tween 80. Cells were then vortexed for 10 seconds in the presence of a few 2 mm glass beads before being filtered through sterile glass wool to separate conidia from the hyphae. The conidia were pelleted at 2000 x g for 10 minutes and re-suspended in ¼ Ringer's solution to give a final concentration of 1×10^7–1×10^8 $ml^{-1}$and stored at 4 °C for testing within 7 days.

C. albicans was grown on sabouraud dextrose agar (Difco, Maryland, USA) at 32 °C for 24-36 hours, cells were counted using a haemocytometer and adjusted to 1×10^7 – 1×10^8 cells ml^{-1} for use the same day.

Bacterial cultures were grown on tryptone soya agar (Difco, Maryland, USA) and incubated for 2 days at 32 °C Cells were resuspended in ¼ Ringer;s solution and adjusted to 1 x 10^7–1 x 10^8 cells ml^{-1} before use.

2.2.11 Microscopy

2.2.11.1 Scanning Electron Microscopy

One ml samples of 10^7 cysts were washed and stored in 0.1 M Na Cacodylate (pH 7.4) with 2 % Gluteraldehyde (Sigma Chemical Company, Dorset, England) and stored overnight at 4 °C. The sample was centrifuged for 4 minutes at 1000 x g, the supernatant removed and the sample re-suspended in 0.1 M Na Cacodylate and left to infiltrate for 10 minutes, this process was repeated 3 times to remove all traces of Gluteraldehyde. After repeating centrifugation, the sample was re-suspended in 1 % osmium (Sigma Chemical Company, Dorset, England) in 0.1 M Na Cacodylate and left for 1 $^1/_2$ hours. The samples were then washed 3 times by centrifugation in distilled water to remove any excess Osmium, then placed into glass fibre filters and dehydrated through a series of Acetone concentrations (30 %, 50 %, 70 %, 90 % and 2 washes of 100 %) for 30 minutes each.

The samples were then critical point dried in a Balzers Critical Point Drier CPD 030, giving four 10 min exchanges through liquid CO_2, before critical point drying. Finally, the glass fibre filters were mounted on aluminium stubs and sputter coated in a Polaron SC7640 Sputter coater for 90 seconds. The samples were then viewed in the Hitachi S-3000H Scanning Electron Microscope.

2.2.11.2 Transmission electron microscopy

A batch of Ros-91 #6-Mg and Neff cysts were examined by transmission electron microscopy. Briefly, 1 ml samples of 10^7 cysts were washed and stored in 0.1 M Na Cacodylate (pH 7.4) (Agar Scientific Ltd., Essex, England) with 2 % Gluteraldehyde (Agar Scientific Ltd., Essex, England) and stored overnight at 4 °C. The sample was centrifuged for 3 minutes at 1000 x g, the supernatant removed and the sample re-suspended in 0.1 M Na Cacodylate (pH 7.4) and left to infiltrate for 10 minutes, this process was repeated 3 times to remove all traces of Gluteraldehyde. After repeating centrifugation, the sample was re-suspended in 1 % osmium tetroxide (EM scope Laboratories Ltd., Kent, England) in 0.1 M

Na Cacodylate and left for 1 $^{1}/_{2}$ hours. The samples were then washed 3 times by centrifugation in distilled water to remove any excess osmium tetroxide. The samples were then suspended in 2 % agar and centrifuged leaving a pellet in the agar. Once set, the agar containing the sample was diced into small cubes approx 0.5 mm^2. The sample was then dehydrated through a series of ethanol concentrations (30%, 50%, 70%, 90% and 2 washes of 100%) for 30 minutes each. The samples were washed in Propylene oxide (Fisher chemicals, Leicester, England) twice, left for 10 minutes between each wash; this was replaced with a solution of 1 part Propylene oxide to one part Spurr's resin (Agar Scientific Ltd., Essex, England) (Spurr, 1969) and incubated at room temperature covered with foil for 4 hours. The lid was pierced and the sample left over night to allow the propylene oxide to evaporate. Fresh Spurr's resin was added to the samples they were incubated on a rotary mixer for 5 hours at room temperature. The samples were then embedded and polymerised at 60 °C for 16 hours. Samples were then sectioned on a Reichert Ultracut S Microtome to produce silver gold sections. Grids were counter-stained in 2 % Aqueous uranyl acetate (Agar Scientific Ltd, Essex, England) for 20 minutes, followed by 3% Lead citrate for 4 minutes. The samples were viewed under the JEOL 100CX TEM (RHC & RHD) and Seimens 102 TEM (RHA & RHB).

2.2.12 Minimum cysticidal concentration (MCC) assay

This assay is used to determine the minimum concentration of a solution that can kill 100 cysts in 24 hours and is used to screen compounds for cysticidal activity. The assay relies on the ability of surviving cysts to hatch and grow after disinfection when a suitable food source is available. A 1000 µg ml^{-1} solution of the test compound was prepared in a suitable diluent and then 200 µl was added to column 1 rows A-H of a 96 well plate (Helena Biosciences, Sunderland, UK) with the remainder of the wells filled with 100 µl of ¼ Ringer's solution (See Figure 4).

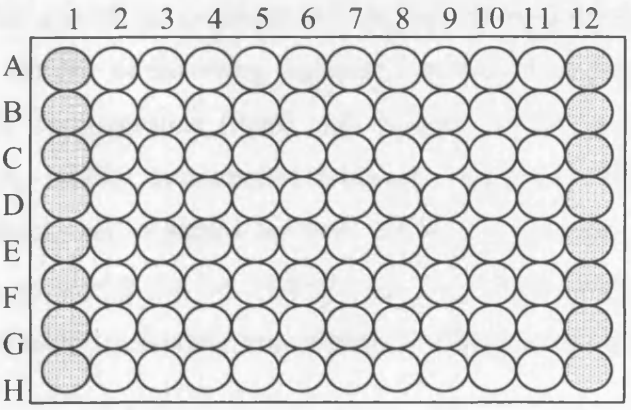

Figure 4: Layout of microtitre plate in determining the MCC

Serial two-fold dilutions were then performed across the plate from column 1-11, leaving column 12 as a control, containing only ¼ Ringer's. Fifty µl of cysts at a concentration of 2 x 10^4 ml^{-1} were added to each well and the plate was sealed and incubated at 32 ºC for 24 hours.

The solutions in the wells were then removed and replaced with 200 µl of ¼ Ringer's and left at room temperature for 15 minutes for cysts to adhere to the bottom of the wells. The washing procedure was repeated twice more to ensure complete removal of the test solution before finally filling the wells with 100 µl of ¼ Ringer's containing live *E. coli* at an $O.D._{540}$ of 0.2. The plates were then sealed and incubated at 32 °C for up to 7 days. The minimum cysticidal concentration (MCC) was defined as the lowest concentration of test compound that resulted in no excystment and trophozoite replication.

2.2.13 Time kill assay

One hundred µl of organisms were inoculated into 10 ml of test solution in a 50 ml polypropelene tube (Becton Dickinson, Oxfordshire, England) to give a final concentration of $1x10^5$ cysts per ml. At time intervals of 0, 1, 2, 4, 6, 8 and 24 hours, 20 µl was removed in quadruplet and placed into 180 µl of the appropriate neutraliser in a 96 well microtitre plate. The test solution was allowed to neutralise for 2 minutes before it was serially diluted 10 fold across the wells of the microtitre plate to 10^{-4}. After each time point was completed, 25 µl of *Escherichia coli* (JM101: OD_{600} 0.4) was added to each well and the plate sealed

and incubated at 32 °C for up to 7 days. The plates were inspected daily for 7 days for the presence of amoebal growth (excystment and trophozoite replication) at the various dilutions in the wells. The number of surviving organisms at each time point was determined using Reed and Muench computations (Reed and Muench 1938) as previously described for *Acanthamoeba* cyst viability enumeration (Buck and Rosenthal 1996). All experiments were performed in triplicate and repeated on three separate occasions. The reduction in viable cysts was plotted as change in log viability for each time point compared to zero time viability. Statistical analysis was performed using one-way analysis of variance (ANOVA).

2.2.14 Data analysis

The reductions in viable cells were plotted as delta logs using the standard error of the mean for each time point using the formula:

$$\log T_n - \log T_0$$

Where T_n is the viable cell count at any given experimental time point and T_0 is the initial viable cell count. A template was constructed in Excel (Microsoft software) to carry out the delta log calculation from which scatter plots were then produced.

Error bars represent standard error of the mean from triplicate experiments. In control experiments using only ¼ Ringer's solution <0.5 log reduction in cyst viability occurred (data not shown) unless otherwise stated.

2.2.15 Time kill assay of bacterial and fungal pathogens

The method used for bacterial and fungal pathogens was as stated in ISO/DIS 14729 which is an international standard method developed to evaluate the efficacy of contact lens solutions against bacterial and fungal pathogens. The number of surviving organisms were counted using the Miles and Misra technique (Miles and Misra 1938). Briefly, the bacterial suspensions were serially diluted in ¼ Ringer's solution and 20 µl volumes were spotted in triplicate onto the appropriate agar and incubated at 30 °C for 24 hours before enumeration. The reduction in viable organisms was plotted as the change in log viability for each time

point compared with time zero. Statistical analysis was performed using one-way analysis of variance (ANOVA).

2.2.16 Comparison of ATP kits

2.2.16.1 Generation of standard curves of ATP kits

In order to determine the possibilities of alternative viability assays, two different ATP kits investigated. The kits were compared against one another; firstly the standard curves were made for cysts as explained below.

Kit 1. Celsis Ltd, (Landgraaf, The Netherlands).

To make the standard curve, stock solutions of $1x10^6$ cysts were used. Serial 10-fold dilutions of 50 µl were made in ¼ Ringer's solution down to 1 x 10^1 cysts in a black 96-well microtitre plate (Bibby Sterilin Ltd., Staffordshire, UK). As a negative control, 10^6 heat-killed cysts (at 85 °C for 10 minutes) were used. The ATP standard supplied was diluted 10 fold to create a reference of ATP concentration.

A Luminoscan RS (Thermo Labsystems, Finland) with two automatic injectors was used to analyse the light generated by the reaction. Firstly, all of the injector tubing was flushed with deionised H_2O then sterilised with 70 % ethanol. The injector tubing was then rinsed with sterile deionised water to remove any traces of alcohol. The luminometer was programmed to dispense 150 µl of extraction buffer and after a delay of 10 seconds dispense 100 µl of the Luminate enzyme reagent into the relevant wells. Finally, the luminometer measured the light emission immediately using a 10 second integration time.

Kit 2. ENLITEN® Hygeine monitering reagent set (Promega, USA).

To make the standard curve, stock solutions of $1x10^6$ cysts were used. Serial 10-fold dilutions of 100 µl were made in ¼ Ringer's solution down to 1 x 10^1 cysts in a black 96-well microtitre plate (Bibby Sterilin Ltd., Staffordshire, UK). As a negative control, 10^6 heat-killed cysts (at 85 °C for 10 minutes) were used. The ATP standard supplied was diluted 10 fold to create a reference of ATP concentration.

A Luminoscan RS (Thermo Labsystems, Finland) with two automatic injectors was again used. The tubing was sterilised as above and programmed to dispense 100 µl of extraction buffer and after a delay of 1 second dispense 100 µl of the enzyme reagent into the relevant wells. Finally, the luminometer measured the light emission immediately using a 10 second integration time.

2.2.16.2 ATP Measurement

At each time point during a time kill assay, 500 µl of solution was removed for ATP measurement. This was neutralised in 5 ml of the appropriate neutraliser and left for 5 minutes before vortexing at 1000 x g for 5 min. The supernatant was then carefully removed and the remaining cysts washed again by centrifugation in ¼ Ringer's solution. The cysts were finally suspended in 500 µl ¼ Ringer's solution. The samples were added in triplicate to a black 96-well microtitre plate and the ATP activity was measured using the two kits as explained above. The results were compared against the standard curves previously created to estimate the number of surviving cells at each time point.

2.2.17 Flow Cytometry

One ml of test solution was removed at each time point during a time kill assay. This was neutralised in 5 ml of the appropriate neutraliser and left for 5 minutes before vortexing at 1000 x g for 5 min. The supernatant was then carefully removed and the remaining cysts washed again by centrifugation in ¼ Ringer's solution. The cysts were finally suspended in 90 µl ¼ Ringer's solution and stained with PI and SYTO 9. Briefly, 5 µl of 40 µg ml^{-1} solution of propidium iodide and 5 µl of 40 µg ml^{-1} solution of SYTO 9 was added to the cells and vortexed and incubated in the dark at room temperature for 15 minutes. After vortexing, 1 ml of nH_2O was added to the stained cysts. The samples were then analysed with a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany) and analysed using CellQuest® Pro v. 4.0.1 (Becton Dickinson, Heidelberg, Germany).

2.2.17.1 Preparation of Stains

Live and heat-killed cysts were also examined under a fluorescent microscope. The Fluorescent dyes used are listed in Table 4. All of the dyes were obtained from Molecular Probes (Oregon, USA) or Sigma Chemical Company (Dorset, England). Stock solutions of

the stains were prepared in DMSO to give the concentrations shown in Table 5 and stored at –20 °C. Working solutions of the stains were prepared using filter sterilised nano-pure H_2O for each day of use from completely thawed stocks (Table 4).

Table 4: Fluorescent dyes used

	Fluorophore characteristics[1]		Filter set used
	Excitation maximum (nm)	Emission maximum (nm)	
Acridine Orange	502	526	B 2-A
Bis Benzimide (Hoechst 33258)	346	460	UV 2-A
Calcofluor™ white M2R	440	500-520	G 2-A
4', 6-diamindino-2-phenylindole (DAPI)	358	461	UV-2A
Fluorescein Diacetate	488	530	B 2-A
Propidium iodide (PI)	535	617	G-2A
SYTO 9	354	470	UV 2-A

[1]Spectral characteristics obtained from Molecular Probes Inc, Eugene Oregon, USA (http://www.probes.com)

Table 5: Stock and working concentrations of stains

Stain	Stock Concentration (mg ml^{-1})	Working Concentration (µg ml^{-1})
Acridine Orange	10	1000
Hoechst 33258	1	100
Calcofluor™ white	10	1000
DAPI	10	500
FDA	1	20
PI	1	20
SYTO 9	1	20

3 Efficacy of Commercial Hydrogen peroxide Systems

3.1 Introduction

3.1.1 Hydrogen peroxide disinfection

In 1818, Thenard synthesised hydrogen peroxide (H_2O_2) by reacting dilute acid with barium dioxide to yield a relatively unstable 3 – 4% solution. By the mid 19th century the disinfection properties of H_2O_2 were recognised and it was used in medicine and surgery as well as in the treatment of milk and sewage. It is now known that H_2O_2 is a powerful antimicrobial agent, destroying pathogens by oxidation resulting in protein denaturation (Jackett, Aber *et al.,* 1978). As such, it finds wide application for disinfection processes in the food, water and contact lens industries.

3.1.2 Contact lens disinfection

The use of H_2O_2 solutions (3%) for the disinfection of soft contact lenses began during the 1970's (Penley, Llabres *et al.,* 1985); (Holden 1990) and gained popularity during the 1980's when the use of soft contact lenses dramatically increased (Holden 1990; Janoff 1990).

Hydrogen peroxide is bactericidal (Holden 1990). It is also active against the resistant cyst form of *Acanthamoeba* when used at a concentration of 3% providing an exposure time of at least 4 to 6 hours is employed (Davies 1990; Kilvington and White 1994); (Hughes and Kilvington 2001). However, H_2O_2 is toxic to the cornea and must be neutralised before lens wear to avoid pronounced stinging, lacrimation, hyperaemia, and possible corneal damage (Holden 1990). Concentrations above 30 ppm have been shown to be toxic to the cornea (Holden 1990).

Two-step peroxide systems employ a separate neutralisation step through the addition of a catalase or sodium pyruvate solution after a designated disinfection time. These solutions catalyse the decomposition of H_2O_2 to water and oxygen. Two-step systems have been shown to exert cysticidal activity against *Acanthamoeba* providing the recommended disinfection time is greater than 4 hours (Kilvington 1998).

One-step hydrogen peroxide systems are available which do not require a separate neutralisation step. These have the convenience of a single step and guaranteed neutralisation of the peroxide which increases patient compliance, an important risk factor. Here, neutralisation is achieved in the storage case during disinfection by using a platinum-coated disk or soluble catalase tablet. However, lenses cannot be stored in these solutions for longer than 24 hours as they contain no preservatives once the H_2O_2 has been neutralised allowing any surviving organisms to grow (Pitts and Krachmer 1979; Rosenthal, Stein *et al.*, 1995).

3.2 Aims

Although previous studies have demonstrated the activity of hydrogen peroxide-based contact lens disinfectants against *Acanthamoeba,* none have evaluated the relative efficacy of commercially available systems against this organism (Kilvington and Anger 2001). To this end, the activities of one-step and two-step hydrogen peroxide contact lens disinfection systems, their respective solutions, native 3% hydrogen peroxide, the peroxigen sodium perborate and the lens protein remover subtilisin A were compared against the trophozoites and cysts of *A. polyphaga.*

3.3 Materials & Methods

3.3.1 Acanthamoeba strains

A. polyphaga (Ros strain) was used throughout the study. Log phase trophozoites were harvested immediately before testing. Cysts were prepared by 7 days incubation with Neff's encystment medium and sonicated before use (Chapter 2).

3.3.2 Test solutions.

Seven commercially available hydrogen peroxide based contact lens disinfection solutions were studied (Table 6). Chemical solutions of 3% hydrogen peroxide (BDH, Poole, England) and 0.06% - 6 % of the peroxigen sodium perborate (Sigma Chemical Company, England and Fluka, Germany) were also investigated. The serine protease subtilisin A, from the

bacterium *Bacillus subtilis*, is used for the enzymatic removal of protein from contact lenses (Ultrazyme®, Allergan) in the presence of hydrogen peroxide in the Oxysept®1 system. Experiments were conducted to determine the cysticidal activity of subtilisin A, and whether it enhanced the efficacy of the hydrogen peroxide.

All commercial contact lens disinfection systems were tested according to the manufacturers' recommendations. In separate experiments the efficacy of the hydrogen peroxide solutions used in the one- and two-step systems was also compared. Testing of the one-step systems was conducted in the contact lens storage cases supplied by the manufacturer. The two-step systems and solutions were tested in sterile 50 ml sterile polypropylene tubes (Becton Dickinson, Oxfordshire, England). All experiments were performed in triplicate and repeated on three separate occasions. Control experiments used ¼ Ringer's solution in place of the test solution.

Prior to testing, the pH of all peroxide solutions was measured using a pH meter (Jenway 3310: Jenway Ltd, Essex, England). The concentration of hydrogen peroxide during the assays was determined using Peroxid® test strips (BDH Darmstadt, Germany).

3.3.3 Contact lens solution efficacy testing.

3.3.3.1 One-step systems.

Eighty micro litres of organisms were inoculated into 8 ml of contact lens disinfection solution to give a final concentration of $1x10^5$ cysts per ml. Immediately the appropriate neutraliser was added (Table 6). The time kill study described in chapter 2 was performed at time intervals of 0, 1, 2, 4, 6, 8 and 24 hours. The number of surviving organisms at each time point was determined using Reed and Muench computations (Reed and Muench 1938) as previously described for *Acanthamoeba* cyst viability enumeration (Buck and Rosenthal 1996). All experiments were performed in triplicate and repeated on three separate occasions. The reduction in viable cysts was plotted as change in log viability for each time point compared to zero time viability. Statistical analysis was performed using one-way analysis of variance (ANOVA).

3.3.3.2 Two-step systems, hydrogen peroxide solutions and sodium perborate.

Eight ml of test solution was challenged with 80 µl of cysts or trophozoites to give a final concentration of 1 x 10^5 ml^{-1}. Assays were performed as described for the one-step systems. Subtilisin A (Ultrazyme®) was tested either dissolved in 8 ml of ¼ Ringer's solution, Oxysept®1Step or the Oxysept®1Step system. The minimum cysticidal concentration (MCC) of sodium perborate was studied (Chapter 2). Two-fold dilutions in ¼ Ringer's solution (100 µl) of 12% sodium perborate or 0.12% buffered sodium perborate were made across the rows of a microtitre plate (Triple Red Laboratory Technology, Oxfordshire, England). An equal volume of 2 x 10^3 cysts was then added to each well and the plate sealed and incubated at room temperature overnight. Using a multi-channel pipette the solutions in the wells were removed and replaced with 200 µl of ¼ Ringer's and left at room temperature for 15 minute. The washing procedure was repeated twice more before finally filling the wells with 100 µl of ¼ Ringer's containing live *E. coli* at an $O.D._{540}$ of 0.2. The plates were then sealed and incubated at 32°C for up to 7 days. The minimum cysticidal concentration (MCC) was defined as the lowest concentration of sodium perborate solution that resulted in no excystment and trophozoite replication.

3.3.4 Lens cleaning studies.

Multi® is reported to have cleaning as well as disinfection properties. Studies were conducted to determine whether the system could remove *Acanthamoeba* from contact lenses. Briefly, 10 µl aliquots of 1 x 10^4 cysts or trophozoites in ¼ Ringer's solution were inoculated onto to both sides of low water content group I (Sauflon 38: 62% poly [2-hydroxyethyl methacrylate], 38 % water) or high water content group IV lenses (Sauflon 55: 45 % ocufilcon D, 55 % water) and left to adhere for 5-10 minutes. The lenses were then disinfected with Multi® according to the manufacturer's instructions and cultured for survivors in ¼ Ringer's solution with *E. coli* at 32 °C for 7 days. All studies were performed in triplicate.

Table 6: Hydrogen peroxide solutions and systems studied.

System	Neutralisation	Exposure time*
One-step		
Concerto® (Essilor)	Platinum disc	6 h
OxySept 1Step® (Allergan)	Catalase tablet (0.1 mg)	6 h
Multi® (Sauflon)	Platinum disc	6 h
AOSept® 1-Step (Ciba Vision)	Platinum disc	6 h
Blue Sept® (Ciba Vision)	Catalase tablet (0.3 mg)	6 h
Two- step		
OxySept® 1(Allergan)	Catalase solution (260 U ml^{-1})	20 min - overnight
10-10® (Ciba Vision)	Sodium pyruvate solution (0.5%)	10 min - overnight
Other		
Hydrogen peroxide (3%: BDH)	0.02% catalase	-
Sodium perborate (6%: Fluka)	0.02% catalase	-
Sodium perborate, phosphate-citrate buffered (0.06%: Sigma)	0.02% catalase	-
Subtilisin A (0.4 mg: Ultrazyme®, Allergan)	-	6 h

*Manufacturer's product data sheet

3.4 Results

3.4.1 Trophozoites.

All the one and two-step peroxide systems were active against the *Acanthamoeba* trophozoites giving at least a 3-log reduction (99.9 % kill) within the first time sample point of 1 hour (results not shown).

3.4.2 One-Step systems.

The activities of the commercial one-step peroxide disinfection systems, used according to the manufacturers' recommendations, against *Acanthamoeba* cysts are shown in Figures 5 and 6. With the exception of AOSept® and Blue Sept ®, the systems gave a less than 1-log reduction after 8 hours contact time. 10-10 gave only a 0.25 ± 0.124 log reduction after 6 hours contact time. AOSept® gave a 1.28 ± 0.41 log reduction in viability after the manufacturer's recommended disinfection time of 6 hours compared with 1.61 ± 0.50 for Blue Sept®. By 8 hours the log reduction in viable cysts for AOSept® and Blue Sept® were 1.48 ± 0.29 and 1.96 ± 0.23 respectively (Figures 5 and 6). However, this observation was not statistically significant ($p>0.05$). No further reduction in cyst viability occurred between 8 hours and 24 hours contact time for all of the systems (results not shown).

The pH of the hydrogen peroxide solutions and the concentrations of hydrogen peroxide throughout the study are shown in Table 7. Initially, all of the solutions had a hydrogen peroxide concentration of 3%. This had decomposed to 0% within 1 hour for OxySept 1 Step and 10-10, 2 hours for Blue Sept® and within 4 hours for AOSept 1 Step. Both Concerto and Multi showed residual hydrogen peroxide of 0.0002% and 0.005% respectively after 6 hours. The pH of the peroxide solutions ranged from 3.25 to 6.45, however, this had no effect on the efficacies of the systems ($p>0.05$).

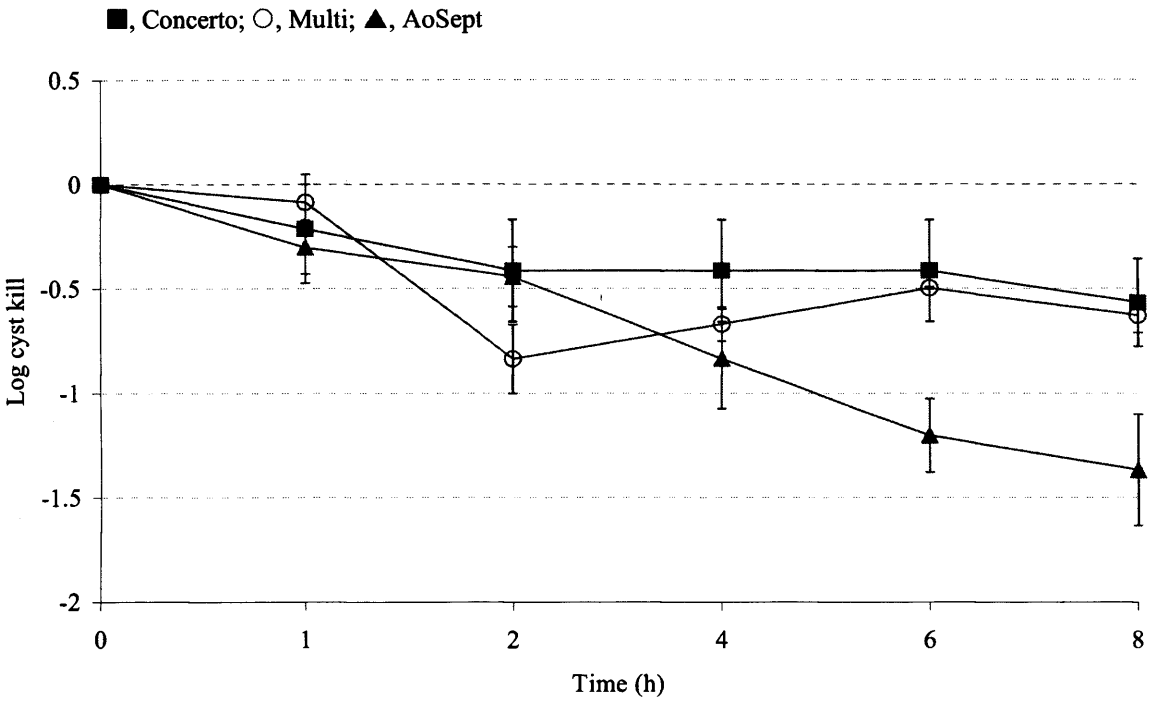

Figure 5: Activities of one-step hydrogen peroxide contact lens disinfection systems against *A. polyphaga* cysts. By 6 h AoSept has achieved over a log kill, wheras both Concerto and Multi have achieved under 0.5 log kill.

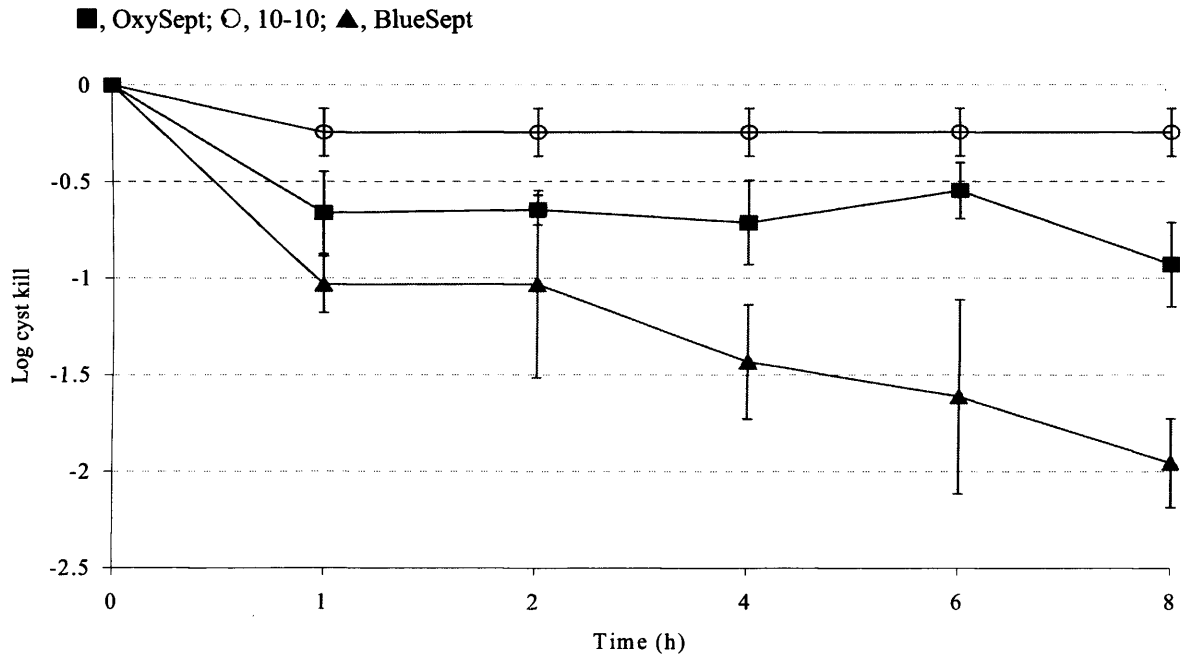

Figure 6: Activities of one-step hydrogen peroxide contact lens disinfection systems against *A. polyphaga* cysts. 10-10 achieved very little reduction in viable cysts after 8 hours compared with OxySept and BlueSept.

Table 7: Hydrogen peroxide concentrations during one- and two-step disinfection and log cyst kill

System	pH	% Hydrogen peroxide over time (h)						Log kill at 6 h[a]
		0	1	2	4	6	8	
One-Step								
Concerto®	5.85	3.0	0.02	0.01	0.0005	0.0002	0.00005	0.43 ± 0.23
OxySept 1 Step®	3.25	3.0	0	0	0	0	0	0.55 ± 0.14
Multi®	6.30	3.0	0.2	0.05	0.005	0.0005	0.0002	0.50 ± 0.005
AOSept® 1 Step	6.45	3.0	0.02	0.002	0	0	0	1.28 ± 0.41
Blue Sept®	6.24	3.0	2	0.00002	0	0	0	1.612 ± 0.51
Two-Step								
10-10®	3.66	3.0	0	0	0	0	0	3.41 ± 0. 21[b]
Oxysept 1	3.35	3.0	3.0	3.0	3.0	3.0	3.0	3.85 ± 0.26[b]
Other								
Hydrogen peroxide (3%)	5.20							4.25 ± 0.00
Sodium perborate (6%)	9.50							0.00 ± 0.00[c]
Sodium perborate, phosphate-citrate-buffered (0.06%)	5.00							0.00 ± 0.00[c]

[a]Manufacturer's recommended disinfection times for all one-step systems.

[b] Manufacturer's recommended disinfection times are between 10 or 20 min and overnight

[c]Exposure, 24 hours

3.4.3 Hydrogen peroxide solutions.

The cysticidal activity of the individual hydrogen peroxide solutions used in the one- and two-step systems are shown in Figures 7 & 8. All solutions were active against *Acanthamoeba* cysts, giving at least a 3-log reduction in viability after 4 to 6 hours contact time. However, variation in the rate of killing between the solutions was observed after 4 hours exposure. At this time all of the solutions achieved at least a 3-log kill with the exception of AOSept and Concerto, which gave a 2.09 ± 0.09 and 1.76 ± 0.04- log kill respectively. This is statistically significant ($p < 0.05$). By 6 hours, all the solutions gave at least a 3-log reduction in viability; there was no significant difference in cyst kill at this time ($p > 0.05$). No significant difference was found between the efficacies of the commercial contact lens peroxide solutions and that of the BDH chemical solution, which gave log reductions of 2.38 ± 0.08 after 4 hours and 4.25 ± 0.00 after 6 hours (Figure 7).

Concentrations of up to 6% sodium perborate and 0.06% phosphate-citrate-buffered perborate, yielding 1% and 0.02% hydrogen peroxide respectively, were not cysticidal after 24 hours of exposure (Table 7). In all experiments, the ¼ Ringer's controls showed no reduction in viable cysts after 8 hours (results not shown).

3.4.4 Lens cleaning studies

No *Acanthamoeba* were cultured from lenses following disinfection with Sauflon Multi™.

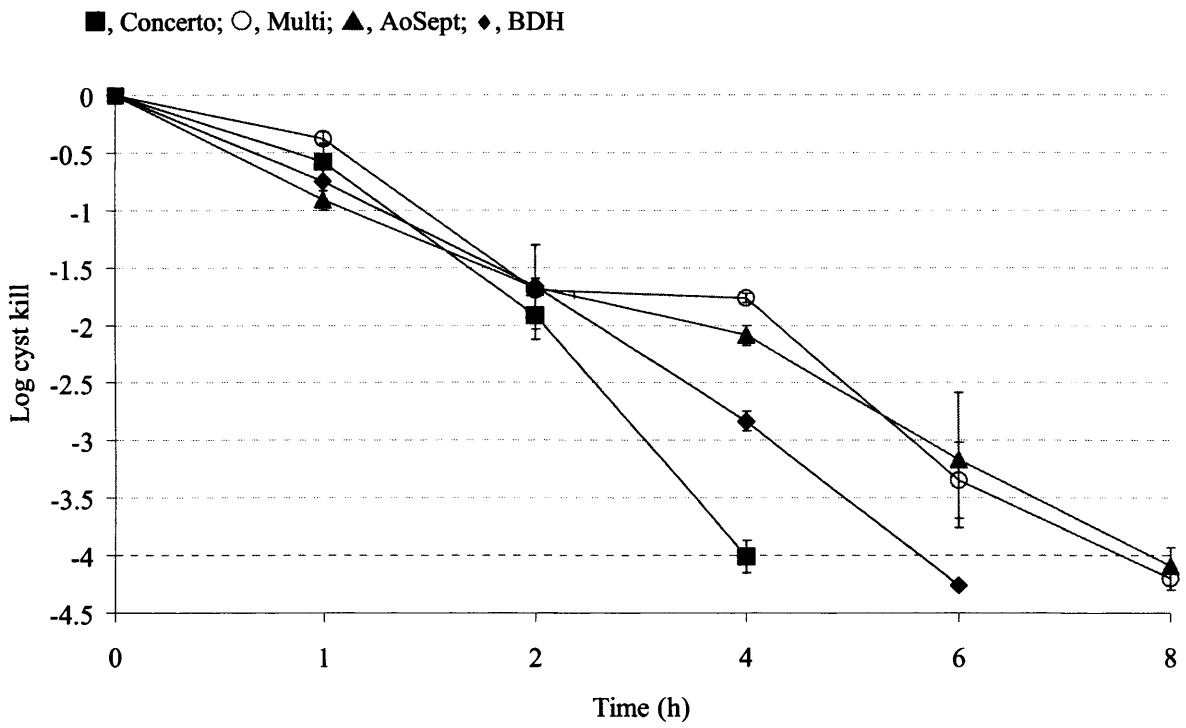

Figure 7: Activities of hydrogen peroxides from contact lens disinfectant kits against *A. polyphaga* cysts. All of the hydrogen peroxide solutions achieved over a 4 log kill by 8 hours.

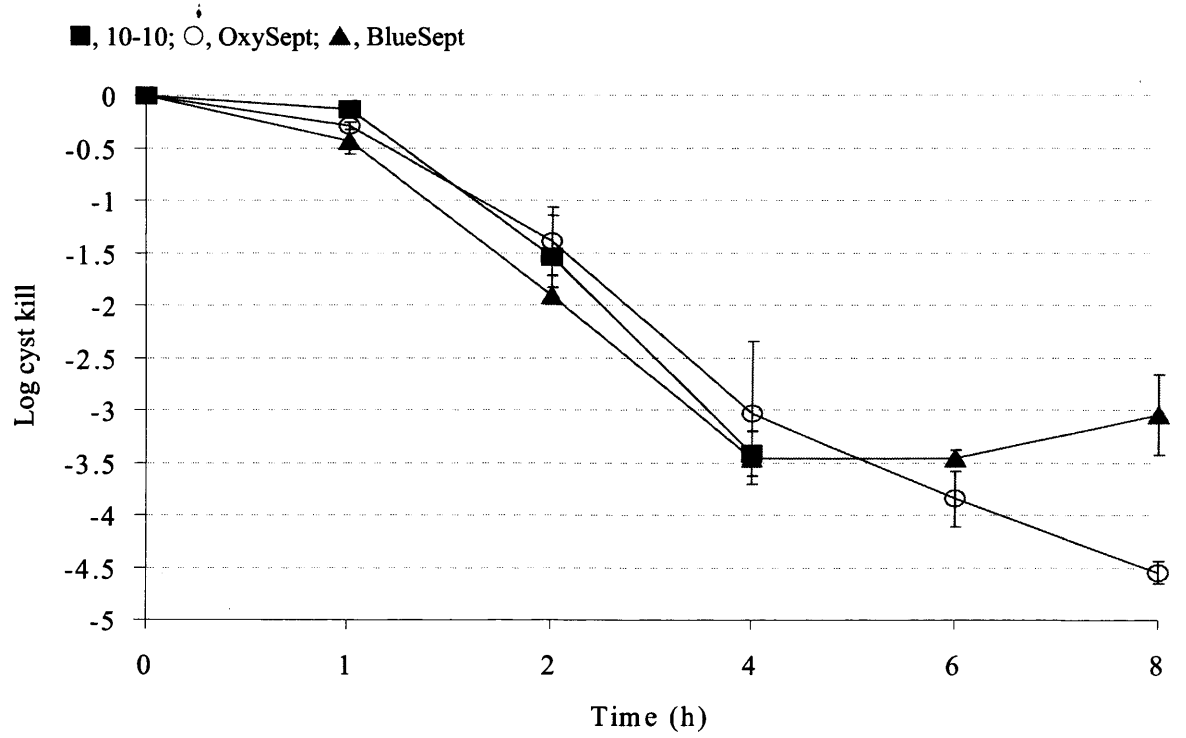

Figure 8: Activities of hydrogen peroxides from contact lens disinfectant kits against *A. polyphaga* cysts. All of the solutions achieve a 3 log kill after 8 h disinfection.

3.4.5 OxySept 1 Step with subtilisin A.

Subtilisin A (Ultrazyme) at 50 μg ml^{-1} in ¼ Ringer's solution showed no cysticidal activity after 24 hours exposure to *Acanthamoeba* cysts (Figure 9). Addition of subtilisin A to OxySept 1 did not result in an enhanced cysticidal activity, giving a 3.55 ± 0.41-log kill after 4 hours compared with a 3.68 ± 0.050-log kill with the peroxide solution alone (Figure 9). Use of the OxySept 1 Step system with subtilisin A as recommended by the manufacturer (○, OxySept + catalase + Subtilisin A) did not result in cysticidal activity, achieving only a 0.41 ± 0.13–log kill in 8 hours (Figure 9).

■, Subtilisin A; ○, OxySept + catalase + Subtilisin A; ▲, OxySept + catalase; ♦, OxySept + Subtilisin A; ●, OxySept.

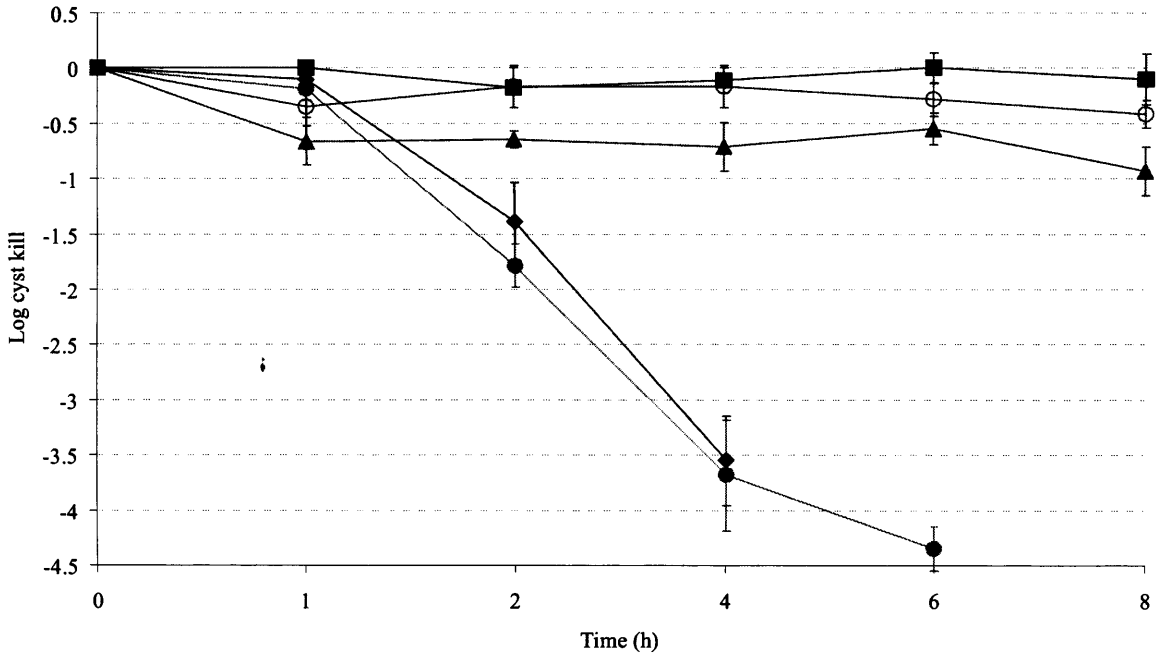

Figure 9: Effect of Subtilisin A on the OxySept 1 Step hydrogen peroxide contact lens disinfection system against *A. polyphaga* cysts. The two solutions that contain the OxySept without the catalase both achieved a 3.5 log kill after 4 h. However, the solutions containing OxySept and catalase did not achieve a 2 log kill in 8 h. The Subtilisin A on it's own showed no cysticidal activity after 8 h achieving less than 1 log kill.

3.5 Discussion

The findings of this study demonstrate that 3% hydrogen peroxide-based contact lens disinfection systems are effective against *Acanthamoeba* cysts providing an adequate disinfectant concentration and exposure time are maintained. This requirement is not met by one-step systems that rapidly neutralise the peroxide resulting in no cysticidal activity (Rosenthal, Stein *et al.,* 1995; Kilvington and Anger 2001). The possible exceptions were found with use of the AOSept 1-Step system and the Blue Sept system, which resulted in log reductions of 1.28 ± 0.41 and 1.61 ± 0.50 respectively after the manufacturer's recommended contact time of 6 hours. These compared with less than 1-log reduction in cyst viability for the other systems. 10-10 was neutralised after 10 minutes as suggested by the manufacturer, this resulted in no antimicrobial activity after this time, therefore this solution only achieved a 0.24 ± 0.12 log kill after 8 hours. However, this observation was not found to be statistically significant ($p > 0.05$). The reasons for the apparent greater activity of AOSept and BlueSept are not clear as neither the pH nor the rate of peroxide neutralisation had a statistically significant effect on the degree of cyst killing. Nor was the peroxide solution of the system any more potent when tested alone against cysts (see below). Possibly, other components of the AOSept and Blue Sept peroxide solutions, such as the stabilisers, may interact with the platinum and catalase catalysts producing an additive effect.

The pH of the peroxide solutions ranged from 3.25 to 6.45 although this had no effect on the disinfectant activity ($p > 0.05$), supporting the previous observation with testing against bacteria and fungi (Lowe, Vallas *et al.,* 1992). The OxySept 1 step system achieved complete neutralisation within an hour, compared with 4 hours for the Blue Sept system, despite the presence of 3 times as much catalase in the Blue Sept system as in the OxySept 1 step system. However, the slow neutralisation may have been due to a protective coating. The slower neutralising Blue Sept System achieved a log cyst kill of 1.61 ± 0.50 after 6 hours compared with 0.55 ± 0.14 by the OxySept 1 step system, although this was not significant ($p > 0.05$).

Two of the seven systems (Concerto and Multi) failed to achieve complete neutralisation after the recommended disinfection time of 6 hours. However, the residual peroxide levels at this time of 0.0002 to 0.0005% (2 to 5 ppm) are not likely to cause irritation to the eye as

30 ppm has been reported to induce cytotoxicity and 100 ppm has been reported to cause noticeable discomfort (Holden 1990).

In contrast, both the two-step systems, 10-10 and OxySept 1, were cysticidal, resulting in at least a 3-log kill after 4 hours of contact time and complete kill after 6 hours. This observation is in accord with previous studies for two-step systems (Davies 1990; Kilvington and Anger 2001). As expected, the hydrogen peroxide solutions used in the one-step systems were also cysticidal. However, variation in the rate of cyst killing was observed at 4 hours with Concerto and AOSept, which gave a 1.8- to 2.1-log kill compared with a 3.0- to 4.0-log kill for the remainder ($p < 0.05$). Reasons for the difference in activity between the solutions are unclear but would not appear to be due to pH. The pHs of Concerto and AOSept were 5.85 and 6.45, respectively, compared with 3.25, 6.3, 3.66 and 6.25 for OxySept 1 Step, Multi, 10-10 and Blue Sept, all of which gave at least a 3-log kill in 4 hours. Native 3% hydrogen peroxide (BDH) gave comparable cyst killing to the contact lens disinfecting solutions. However, use of homemade hydrogen peroxide contact lens disinfectant is not recommended as it can contain stabilizers such as phosphoric acid, acetanilide, phenacetin, and sodium stanate.

This study has confirmed the efficacy of two-step commercial hydrogen peroxide contact lens disinfection systems against *Acanthamoeba* cysts providing a contact time of at least 4 hours is used before neutralisation (Brandt, Ware *et al.,* 1989). The potential disadvantage of hydrogen peroxide disinfection is that the lenses cannot be stored in the solution (Rosenthal, Stein *et al.,* 1995). Therefore, once neutralisation is complete there is no residual disinfectant activity for continued antimicrobial protection to prevent contamination by surviving organisms or those introduced from the environment if the case is opened (Rosenthal, Stein *et al.,* 1995). One-step systems offer the convenience of a single disinfection-neutralisation process and prevent the painful consequence of inserting non-neutralised lenses into the eye. However, one-step systems may offer less protection against AK, as peroxide neutralisation occurs too rapidly to allow cyst killing to occur. To this end, the development of a one-step solution where the neutralisation process is slowed to provide cysticidal activity would be a valuable addition to contact lens disinfection. It would also be of benefit to enhance the efficacy of hydrogen peroxide for use in the disinfection of lenses. This would allow rapid antimicrobial activity and therefore would encourage users to wait for the full disinfection time before wearing the lenses.

4 Enhanced Peroxide Killing

4.1 Introduction

4.1.1 The Myeloperoxidase System *in vivo*

In the mammalian body, stimulated phagocytes produce hydrogen peroxide by dismutation of the superoxide anion during the respiratory burst (Iyer, Nair *et al.,* 1961). This occurs spontaneously, optimally at pH 4.8, or may be catalysed by superoxide dismutase (SOD). The antimicrobial activity of H_2O_2 is well documented, although is low compared with other toxic products produced by phagocytes. The killing of phagocytosed bacteria is further enhanced by the production of hyperhalous acids.

Myeloperoxidase (MPO) is released from cytoplasmic granules by degranulation into the phagosome and plays a crucial role in the killing of phagocytosed bacteria in neutrophils. This occurs by the reaction of MPO with H_2O_2, forming an enzyme-substrate complex that oxidises halide to produce even more toxic agents, primarily hyperhalous acid. The halides found in the mammalian body are bromide (Br^-), chloride (Cl^-) and iodide (I^-). The most abundant halide is Cl^-, thus producing hyperchlorous acid (HOCl). Hyperhalous acid is 1000,000 times more effective than H_2O_2 at killing *Pseudomonas aeruginosa* and 10,000 fold more potent against *Candida albicans* (Agner 1972). The combined action of HOCl with H_2O_2 provides a potent antimicrobial combination destroying phagocytosed bacteria by halogenation and oxidation of cell surface components (Schultz 1980); (Klebanoff 1980); (Klebanoff and Rosen 1978).

4.1.2 Mechanisms of myeloperoxidase system mediated killing.

4.1.2.1 Halogenation.

The halide is oxidised by the MPO and the H_2O_2 and binds covalently to components of the organism. In the case of iodide, iodination of tyrosine residues on the organism form mono- and di-iodotyrosine (Thomas and Aune 1978). Other components that have been shown to be iodinated include unsaturated fatty acids (Turk, Henderson *et al.,* 1983) and sulphydryl groups (Thomas and Aune 1978).

4.1.2.2 Oxidation.

Powerful oxidants, such as HOCl produced in this system can attack cells in a variety of ways through reactions with a variety of chemical groups:

i) Free sulphydral groups

Some enzymes require free sulphydral groups for optimal activity, these may be oxidized by the peroxidase system leading to toxicity of the cell. Sulphydral groups of *Escherichia coli* are readily oxidised by the peroxidase-H_2O_2-iodide system and by iodine, one of the products (Thomas and Aune 1978).

ii) The iron-sulphur centres

The peroxidase-H_2O_2-halide system is also able to oxidise the iron-sulphur centres of the non-haem bound iron of cells such as *E. coli* (Rosen and Klebanoff 1985). This interferes with the electron transport chain in the cytoplasmic membrane, which in turn disrupts the metabolism of the organism. However, this has not been observed with I^- as the halide. Iodide-derived oxidants appear to oxidise the microbial iron-sulphur centres without releasing iron. Although this mechanism is not yet understood, it has been suggested that the iodide-derived oxidants damage the microbial iron sulphur to a point where they are catalytically inactive but still retain their iron (Rosen and Klebanoff 1985). Alternatively, the chelator required for the solubilisation of the iron may be unable to reach the site, or transfer it to a point where it can be measured.

iii) Heme proteins.

The MPO-H_2O_2–halide system can readily oxidise porphyrins, haemes and haem proteins (Rosen and Klebanoff 1985). Myeloperoxidase, a haem protein, is inactivated by H_2O_2 and halide (Naskalaski, 1977).

iv) Sulphur-ether groups

The sulphur-ether groups in the target organism of both free and peptide bound methionine can be oxidised to the corresponding sulphoxide, which may contribute to cell death (Tsan and Chen 1980).

v) Oxidative decarboxylation and deamination

Both free and peptide-linked amino acids may be decarboxylated or deaminated with associated peptide in the phagosome and cytoplasm respectively (Selvaraj, Paul *et al.,* 1974). The peptide-associated amino acids are decarboxylated by the peroxidase system, although the soluble free amino acids are decarboxylated by the noperoxidase, cellular amino acid oxidative pathway.

vi) Lipid peroxidation.

Following the ingestion of the particle by the phagocyte, polyunsaturated fatty acids are oxidised leading to membrane damage. This can be initiated by a number of the reactive oxygen species produced in the peroxidase- H_2O_2-halide system and is propagated by radical species that are formed (Venge and Olsson 1975; Venge, Olsson *et al.,* 1975). A loss of cell viability occurs when the cell is oxidised at chemical sites on or close to the surface of the organism by HOCl. Within milliseconds HOCl is able to prevent *E. coli* replication (Shafer, Martin *et al.,* 1986).

vii) Oxidation

Hyperchlorous acid is responsible for oxidising several chemical sites on or close to the surface of the organism leading to the loss of cell viability. Within milliseconds HOCl is able to prevent *E. coli* replication (Shafer, Martin *et al.,* 1986).

4.1.3 Antimicrobial activity of the peroxidase-H_2O_2-halide system

This innate "myeloperoxidase-mediated antimicrobial system" was first described by Klebanoff in 1966 (Klebanoff, Clem *et al.,* 1966). Subsequently, it was demonstrated that this mechanism can be reproduced in vitro to obtain enhanced hydrogen peroxide killing of a variety of pathogenic microbes including bacteria (Klebanoff 1968); (Jackett, Aber *et al.,* 1978); (Klebanoff and Shepard 1984); (Tenovuo, Makinen *et al.,* 1985; Yang and Anderson 1999) fungi (Yang and Anderson 1999), the virus HIV-1 (Klebanoff and Kazazi 1995) and the parasitic helminth *Schistoma mansoni* (Jong, Chi *et al.,* 1984).

Peroxidases other than MPO have been used, including eosinophil peroxidase (EP) (Turk, Henderson *et al.,* 1983; Klebanoff and Shepard 1984), lactoperoxidase (LP), horseradish peroxidase (HRP) and also catalase (Jackett, Aber *et al.,* 1978); (Jong, Chi *et al.,* 1984;

Tenovuo, Makinen *et al.,* 1985). In addition, bromide and iodide have been shown to be capable of replacing chloride in the reaction (Yang and Anderson 1999).

4.2 Aims

To date the effects of this potent antimicrobial system on protozoa have not been reported. Previous studies have shown that H_2O_2 is effective against the cyst form of *Acanthamoeba* giving a 3 log reduction in viable cysts after 6 hours of contact time (Hughes and Kilvington 2001) this study was undertaken to determine whether the peroxide-peroxidase-halide system could be employed to enhance the cysticidal activity of hydrogen peroxide.

4.3 Materials & Methods

4.3.1 *Acanthamoeba* strains and culture.

A. polyphaga (Ros strain) was used in all developmental experiments. The pathogenic species *A. quina* (L1a) and *A. culbertsoni* (ATCC 30171) and four strains from recent keratitis cases (Ak 1-4) were also tested. Trophozoites were maintained in a semi-defined axenic broth medium (Chapter 2). Cysts were prepared from the trophozoite cultures using Neff's encystment medium (Chapter 2). Mature cysts were sonicated for 5 s at 50% amplitude 3 times to break clumps before being adjusted to 5 $x10^6$ cysts ml^{-1} and stored at 4 °C for use within 14 days.

4.3.2 Test solutions.

All chemicals and enzymes used in the study were obtained from the Sigma Chemical Company (Poole, UK) or BDH (Poole, UK) unless otherwise stated. Catalase (EC 1.11.1.6), horseradish peroxidase (HRP: EC 1.11.1.7) and soybean peroxidase (SBP: EC 1.11.1.7) were dissolved in nanopure water, filter sterilised and stored at –20 °C. The halide stock solutions were stored at room temperature in the dark. The H_2O_2 stock solution was stored in the dark at 4 °C and the concentration measured before use, using a molar extinction coefficient of 80 at 230 nm.

4.3.3 Cysticidal assay.

The assay method used to determine cyst killing was as described previously (Hughes and Kilvington 2001; Chapter 2). The time intervals measured were 0, 1, 2, 4 and 6 h and 0.02 % w/v bovine liver catalase was used as the neutraliser.

The test reactions were kept in the dark between each time point measurement. The concentration of H_2O_2 at each time point was determined using Peroxid® test strips (BDH Darmstadt, Germany). The pH of the solutions was measured at the beginning and end of the experiments.

4.3.4 Enhanced peroxide system.

A chequerboard experimental design was used to determine the cysticidal effect of varying combined concentrations of HRP (50 - 300 U ml^{-1}) and KI (50 µM - 200 µM) with H_2O_2 (0.125 – 3 % v/v) in ¼ Ringer's solution. Control experiments used H_2O_2 alone in the assays. The combination of HRP-H_2O_2-KI showing greatest enhanced cysticidal efficacy was then tested on three separate occasions. Controls omitting the individual components of the system were included.

Assays were conducted in either ¼ Ringer's solution (pH 4.31 - 5.62), or phosphate buffered saline (PBS: pH 6.55 - 7.13) to determine the effect of pH on the activity of the system. Assays were also performed in which KI was replaced with KCl (50 µM – 200 µM), HRP with SBP (soyabean peroxidase) (150 U-200 U ml^{-1}) or catalase (100 U – 200 U ml^{-1}). In addition, H_2O_2 was replaced with the peroxide generating chemical sodium perborate (1 % - 3 % w/v) and tested using HRP from 5 U – 50 U ml^{-1} and KI from 20 - 50 µM.

The HRP-H_2O_2 -KI combination giving the greatest enhancement of cysticidal killing for *A. polyphaga* (Ac-Ros) was then tested against the additional *Acanthamoeba* species and strains.

4.3.5 Data analysis.

The number of surviving organisms in the microtitre plates at each time point was determined using Reed and Muench computations (Chapter 2). All experiments were

performed in triplicate and repeated on three separate occasions. The reduction in viable cysts was plotted as change in log viability for each time point compared to zero time viability. Statistical analysis was performed using one-way analysis of variance (ANOVA).

4.4 Results

4.4.1 Hydrogen peroxide killing.

The cysticidal activity of H_2O_2 alone in ¼ Ringer's solution (pH 4.31 -5.62) against *A. polyphaga* (Ac-Ros) is shown in Figure 10. Hydrogen peroxide at <1% showed little cysticidal activity, giving <1 log kill in 6 hours (results not shown). At 1%, 2% and 3%, H_2O_2 gave log cyst killing after 4 hours exposure of 1.10 ± 0.31, 2.35 ± 0.49 and 2.75 ± 0.00 respectively. By 6 hours the respective values were 1.67 ± 0.38, 3.46 ± 0.08 and 4.33 ± 0.08 (Figure 10). Similar results were obtained with H_2O_2 tested in PBS (pH 6.55 - 7.13) with no statistical difference in the cysticidal activity compared with ¼ Ringer's solution ($p>0.05$, results not shown).

4.4.2 Enhanced hydrogen peroxide killing.

Addition of KI and HRP enhanced the cysticidal activity of H_2O_2 (Figure 10). The chequerboard experiments showed that maximum activity was achieved with 50 µM KI and 50 U ml^{-1} HRP for 1 % H_2O_2, 150 U ml^{-1} HRP for 2 % H_2O_2 and 200U HRP ml^{-1} for 3% H_2O_2 (Figure 10). Omission of either the KI or HRP from the reaction resulted in no enhanced cysticidal activity (results not shown). KI, HRP or KI-HRP alone showed no cysticidal activity (results not shown).

With 1% H_2O_2 enhancement in cysticidal activity was observed at all time points and by 6 hours the kill was increased from 1.7 ± 0.30 log in H_2O_2 alone to 2.76 ± 0.27 log ($p<0.05$). For 2 % H_2O_2 significant enhancement was observed at all time points ($p<0.05$) and total kill of the cyst inoculum occurred at 4 hours compared with 6 hours for the peroxide alone. Significant cysticidal activity was also observed with 3 % H_2O_2 at all time points ($p<0.05$) and resulted in total kill at 4 hours compared with 6 hours for the peroxide solution alone.

□, 1% H_2O_2; ■, 1% H_2O_2, 50 U ml^{-1} HRP and 50 μM KI; △, 2% H_2O_2; ▲, 2% H_2O_2, 150 U ml^{-1} HRP and 50 μM KI; ○, 3% H_2O_2; ●, 3% H_2O_2, 200 U ml^{-1} HRP and 50 μM KI.

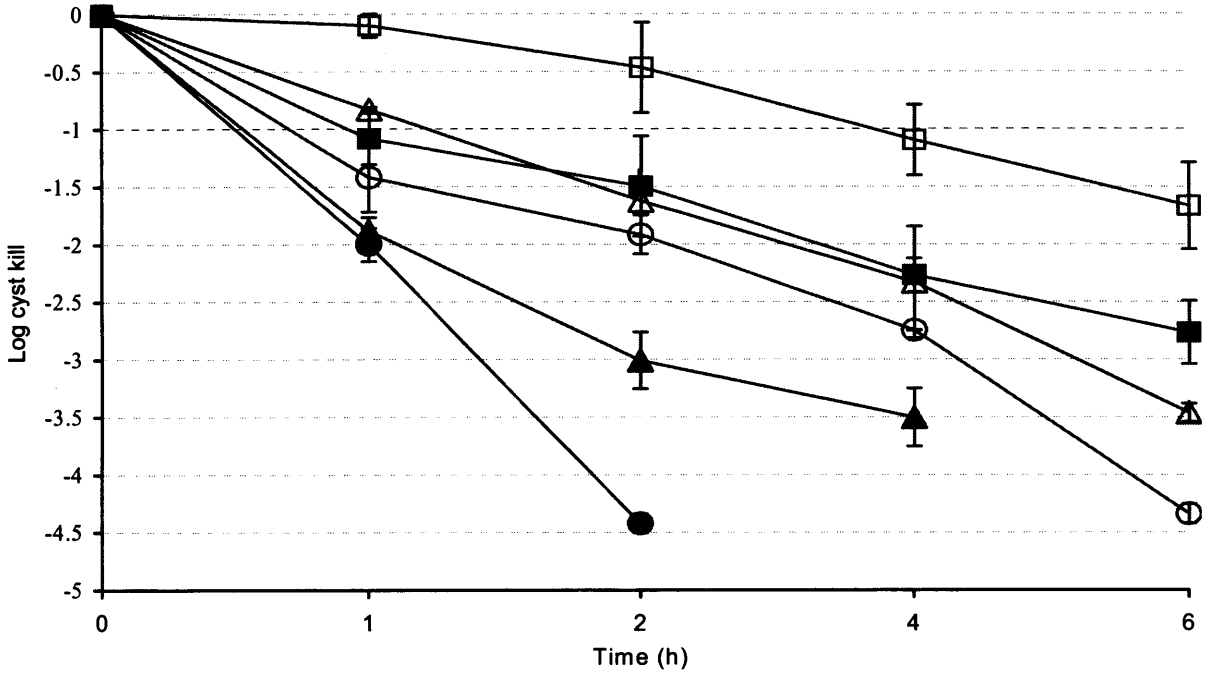

Figure 10: Enhanced killing of *A. polyphaga* cysts with peroxidase-hydrogen peroxide-halide system. With 1% hydrogen peroxide over 1.5 log reduction in cyst viability is achieved in 6 h, however with the addition of the HRP and KI, a reduction over 2.5 log reduction is achieved within this time. With 2% hydrogen peroxide total cyst kill is achieved in 6 h which is shortened to 4 h using the enhanced peroxide system. Likewise, by adding the HRP and KI to 3% peroxide total cyst kill can be achieved in 2 h instead of 6 h using just 3% hydrogen peroxide.

4.4.3 Modifications to the enhanced hydrogen peroxide system.

Replacing KI with the 50μM - 200μM KCl resulted in no enhancement of hydrogen peroxide cysticidal activity (results not shown). Similarly, increasing the amounts of HRP to 200U did not give significant enhancement (results not shown).

Replacing HRP with SBP resulted in a statistically significant ($p<0.05$) enhanced cysticidal activity that was comparable ($p>0.05$) with the HRP system (Figure 11). For example 2 % v/v hydrogen peroxide with 150U HRP and 50 μM KI gave 3.50 ± 0.25 log kill after 4 hours and 3.89 ± 0.45 with 150U SBP and 50 μM KI. These values compared with log 2.33 ± 0.49 kill with 2 % hydrogen peroxide alone at this time point.

■, 2 % H_2O_2; ▲, 2% H_2O_2, 150 U ml^{-1} HRP and 50 μM KI; ●, 2% H_2O_2, 150 U ml^{-1} SBP and 50 μM KI.

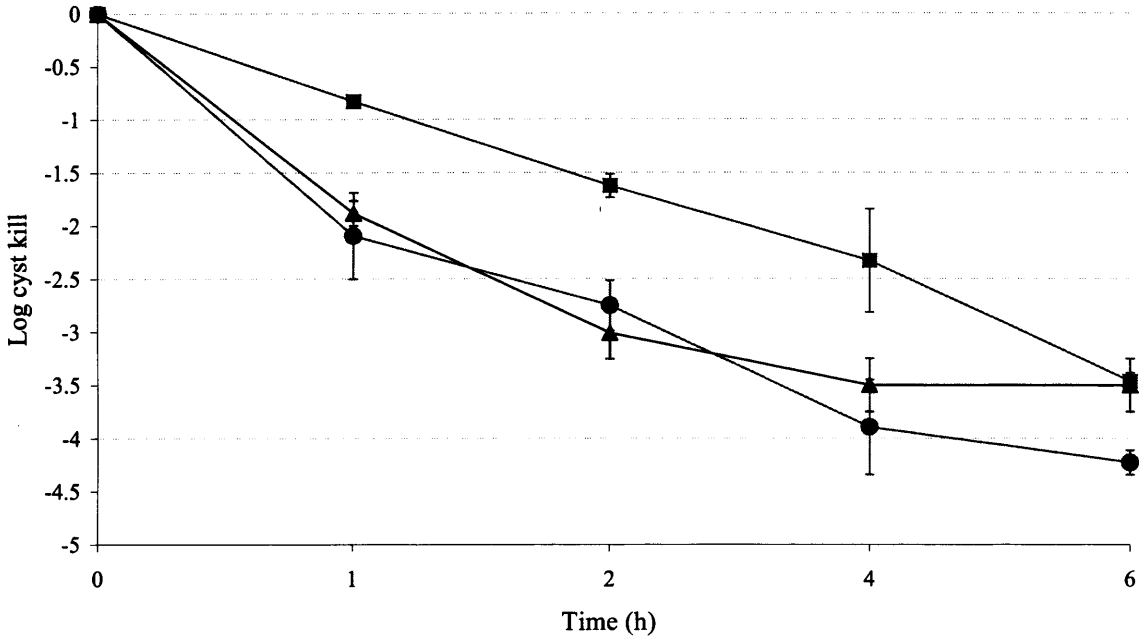

Figure 11: Enhanced killing of *A. polyphaga* cysts with the peroxidase-hydrogen peroxide-halide system using HRP and SBP. The addition of HRP and KI to the 2% hydrogen peroxide increases the cyst kill in 4 h from under 2.5 log to 3.5 log. Replacing the HRP with SBP further increases the kill within this time to nearly 4 log.

Substituting catalase (100 U – 200 U) for HRP decreased the efficacy of the system. For example, 3 % hydrogen peroxide with 100U catalase and 50 μM KI gave log 0.51 ± 0.27 kill after 4 hours disinfection compared with 2.75 ± 0.00-log with 3 % peroxide alone at that time. Similarly, 2 % hydrogen peroxide with 100U catalase and 50 μM KI gave log 0.20 ± 0.36 kill at 4 hours compared with log 2.33 ± 0.49 with a 2 % peroxide solution alone at that time (results not shown).

The addition of KCl (50 μM – 200 μM) and 50 – 200 U catalase to 2 % hydrogen peroxide also significantly reduced the cysticidal activity of the peroxide. For example a 2 % peroxide solution with 50 μM KCl and 150U catalase resulted in a 0.58 ± 0.19 log reduction in viable cysts after 4 hours respectively (results not shown).

Sodium perborate (1 % w/v – 3 % w/v) was not cysticidal after 8 hours contact time (<1 log kill). Addition of HRP (5U-20U and 150 U) and KI or KCl (50 μM) did not result in increased cysticidal activity (results not shown).

When the enhanced systems were retested using PBS in place of ¼ Ringer's solution, no significant difference in cyst killing was observed for the 1% hydrogen peroxide and 2 % hydrogen peroxide enhanced systems ($p>0.05$). However, with the 3 % hydrogen peroxide system with ¼ Ringer's solution at 2 hours the log cyst kill was 4.42 ± 0.08 compared with 2.84 ± 0.10 for PBS ($p<0.05$) (figures 10 & 12).

□, 1% H_2O_2; ■, 1% H_2O_2, 50 U ml^{-1} HRP and 50 µM KI; △, 2% H_2O_2; ▲, 2% H_2O_2, 150 U ml^{-1} HRP and 50 µM KI; ○, 3% H_2O_2; ●, 3% H_2O_2, 200 U ml^{-1} HRP and 50 µM KI.

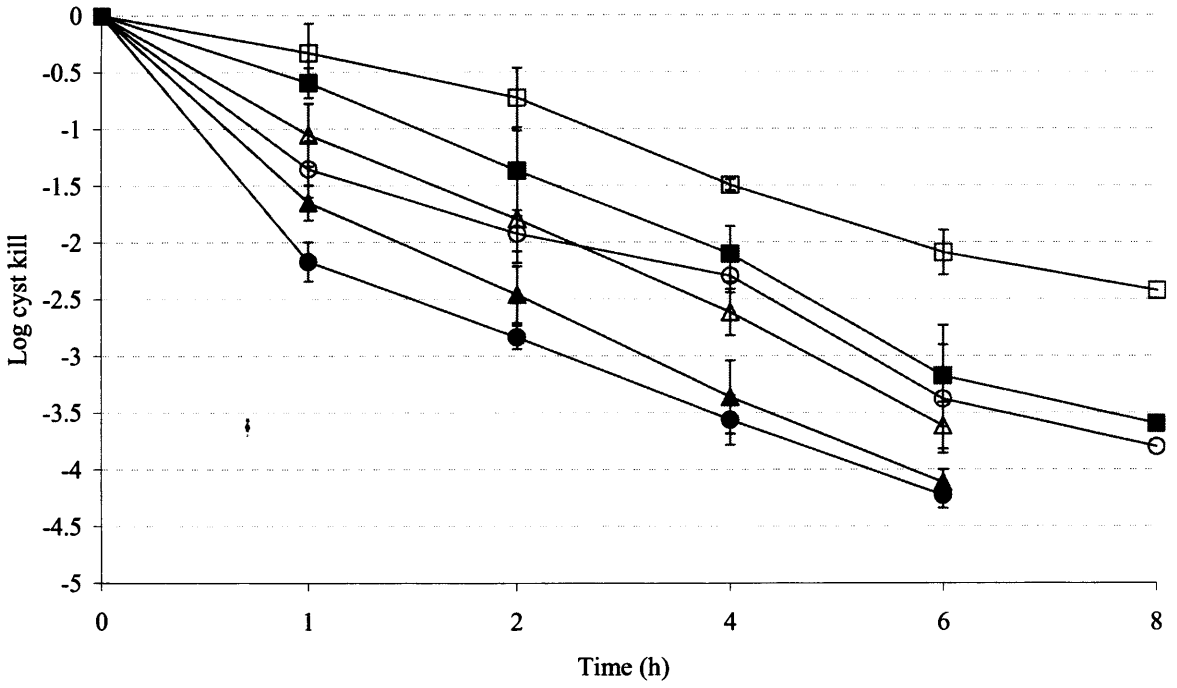

Figure 12: Enhanced killing of *A. polyphaga* cysts with peroxidase-hydrogen peroxide-halide system in PBS. 1% hydrogen peroxide achieved nearly a 2.5 log kill after 8 h. When the HRP and KI are added, 1% hydrogen peroxide achieved over 3.5 log kill in 8 h. 2% hydrogen peroxide achieved over 3.5 log cyst kill in 6 h. With the addition of HRP and KI 2 % hydrogen peroxide achieved over 4 log kill. 3% hydrogen peroxide on its own achieved nearly a 3.5 log reduction in 6h which was increased to over a 4 logg cyst reduction in the same time with the addition of HRP and KI.

The replacement of peroxidase with catalase resulted in rapid neutralisation of the hydrogen peroxide. Replacing horseradish peroxidase with soybean peroxidase did not alter the rate of neutralisation of the hydrogen peroxide (Table 8). The native peroxide solutions maintained

the original concentration of hydrogen peroxide throughout the full time course of the experiment.

Table 8: Concentrations of Hydrogen peroxide throughout the assay

Time	Hydrogen peroxide concentration (%)						
(h)	1%+50U HRP+50µ MKI	2%+150U HRP+50µ MKI	3%+200U HRP+50µ MKI	2%+150U HRP+50µ MKCl	2%+150U catalase+50µ MKI	2%+150U catalase+50µ MKCl	2%+150U SBP
0	1	2	3	2	2	2	2
1	1	2	3	2	0.2	0.2	2
2	1	2	3	2	0.2	0.2	2
4	1	2	3	2	0.2	0.2	2
6	1	2	3	2	0.2	0.2	2
8	0.5	2	2.5	2	0.2	0.2	2

4.5 Enhanced activity against other *Acanthamoeba* species and strains.

When tested in ¼ Ringer's solution, all additional *Acanthamoeba* species and strains showed enhanced killing with 2% H_2O_2 + 150U HRP + 50 µM KI (Figure 13). This was statistically significant at 4 hours compared to H_2O_2 alone ($p<0.05$) for strains Ak-3, Ak-Ros, Ak-4, *A. castellanii* (Neff) and *A. palestinensis* (CCAP 1547/1). By 6 hours the enhanced system had achieved total kill (>3 log) for all species and strains studied compared with < 3 log for the 2% H_2O_2 alone (results not shown).

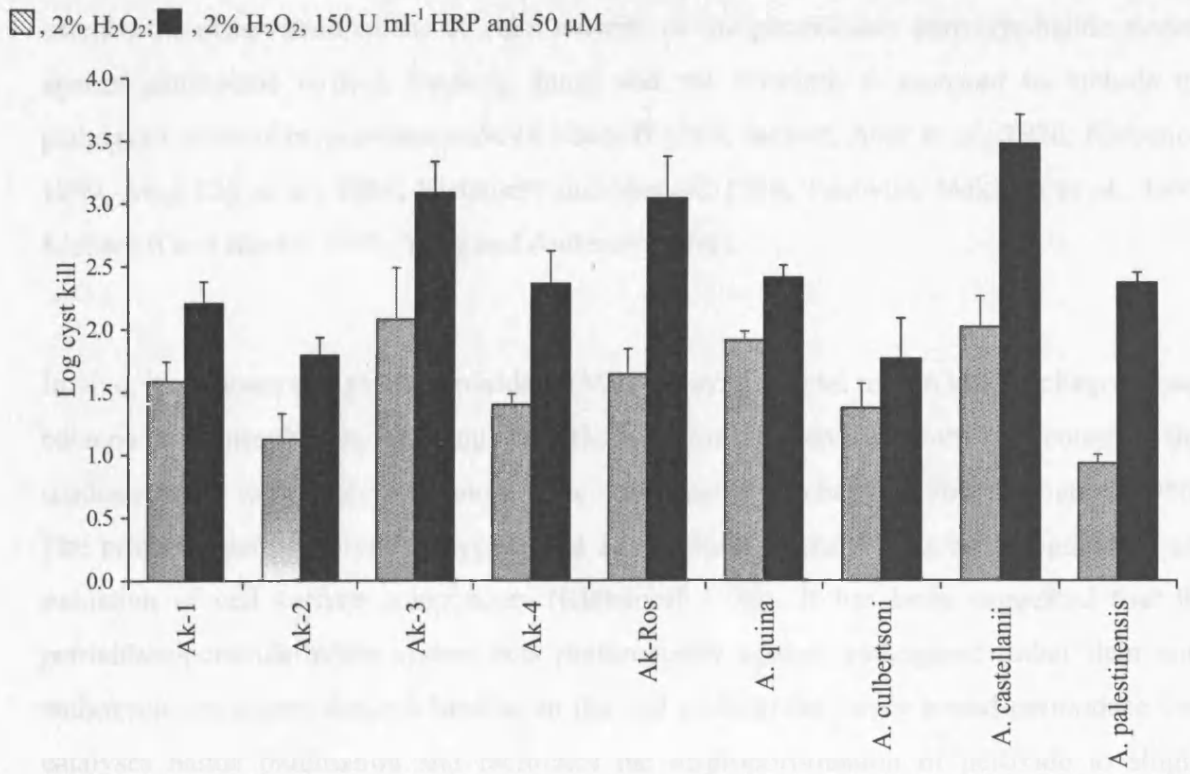

Figure 13: Enhanced cyst killing of Acanthamoeba species and strains with peroxidase-hydrogen peroxide-halide after 4 hours exposure. All of the strains show an increased cyst kill after 4 h with the addition of HRP and KI to the hydrogen peroxide compared with the 2% hydrogen peroxide by itself.

In control experiments using only ¼ Ringer's solution <0.6 log reduction in cyst viability occurred (data not shown).

4.6 Discussion

Hydrogen peroxide (3 %) is an effective disinfectant against *Acanthamoeba* cysts, giving at least a 3-log reduction in viability provided an exposure time of at least 4-6 hours is used (Hughes and Kilvington 2001). This study demonstrates that the activity of H_2O_2 against *Acanthamoeba* cysts can be enhanced through the addition of the halide I^- and a plant derived peroxidase. This resulted in an increased rate of cysticidal activity, giving total kill of the cyst inocula by 2 hours with 3 % H_2O_2 and 4 hours with 2 % H_2O_2. The system also enabled sub-lethal 1 % H_2O_2 to produce a 3 log kill within 6 hours. Furthermore, this

extends the observation of the *in vitro* activity of the peroxidase- peroxide-halide system against pathogenic viruses, bacteria, fungi and the helminth *S. mansoni* to include the pathogenic protozoan *Acanthamoeba* (Klebanoff 1968; Jackett, Aber *et al.,* 1978; Klebanoff 1980; Jong, Chi *et al.,* 1984; Klebanoff and Shepard 1984; Tenovuo, Makinen *et al.,* 1985; Klebanoff and Kazazi 1995; Yang and Anderson 1999).

In vivo, it is known that myeloperoxidase (MPO) plays a crucial role in killing phagocytosed bacteria in neutrophils by reacting with H_2O_2 to form an enzyme substrate complex that oxidises halide to produce even more toxic components (Klebanoff 1968; Klebanoff 1980). The primary agent involved is hypohalous acid, which destroys cells by halogenation and oxidation of cell surface components (Klebanoff 1980). It has been suggested that the peroxidase-peroxide-halide system acts preferentially against pathogenic rather than non-pathogenic organisms through binding to the cell surface; the target-bound peroxidase then catalyses halide oxidination and facilitates the disproportionation of peroxide to singlet molecular oxygen at the surface of the target microbe (Agner 1972). As the lifetime of singlet molecular oxygen is short lived and its diffusion potential is proportionally limited, the target organism is killed with minimum damage to any other non-pathogenic microbes or host cells (Agner 1972).

The precise mode of action of the peroxidase-hydrogen peroxide-halide system described here against *Acanthamoeba* cysts is unclear but it appears equally effective against both pathogenic and non-pathogenic species (*A. castellanii* Neff and *A. palestinensis* CCAP 1547/1). However, it seems probable that under the reaction conditions described, this results in the formation of HOI (hyperiodous acid) that enhances the cysticidal activity of the H_2O_2. Whether this reaction is localised through primary binding of the peroxidase on the cyst wall surface or occurs within the cyst has yet to be elucidated.

Replacing the KI with KCl in the system did not result in enhanced cysticidal activity. This is in accordance with studies on other micro-organisms, which have shown that I^- is the most effective halide, followed by Br^- and then Cl^- (Klebanoff 1968; Jong, Chi *et al.,* 1984; Klebanoff and Shepard 1984). Furthermore, the enzyme SBP can replace HRP in the system to give comparable cysticidal enhancing activity. Enhanced activity was also found with

bovine lactoperoxidase although this was not as efficacious as the plant peroxidases. Replacing these peroxidases with bovine liver catalase resulted in no cysticidal activity as the H_2O_2 was rapidly neutralised within 1 hour. Attempts to use lower levels of catalase and replacing KI with KCl did not produce enhanced H_2O_2 activity. This is in contrast to the findings for SBP or HRP when the H_2O_2 levels remained constant through out the time course of the experiments. Attempts to replace the H_2O_2 with sodium perborate (3 % w/v yielding 0.5 % H_2O_2) in the system showed no cysticidal activity presumably due to the low peroxide generating capacity of this chemical (Hughes and Kilvington 2001).

In light of the findings that the activity of H_2O_2 against *Acanthamoeba* cysts can be enhanced through the addition of the halide I^- and a plant derived peroxidase, the next step is to determine whether this system can also enhance the cysticidal activity of current commercial One-Step hydrogen peroxide based contact lens disinfection systems.

5 Enhanced One-Step Disinfection

5.1 Introduction

Hydrogen peroxide is an effective microbial disinfectant, and at a concentration of 3% it is commonly used as a contact lens disinfectant. However, hydrogen peroxide is toxic to the cornea and must be neutralised before lens wear to avoid pronounced stinging, lacrimation, hyperaemia and possible corneal damage. (Gyulai, Dziabo *et al.*, 1987). This is achieved through the addition of a neutraliser, such as catalase or sodium pyruvate solution after the disinfection period, to catalyse the decomposition of hydrogen peroxide to water and oxygen (Hughes and Kilvington 2001). To increase convenience and reduce the painful consequence of inserting non-neutralised lenses into the eye, one-step peroxide systems are available which do not require a separate neutralisation step. Neutralisation is achieved in the storage case during disinfection using a platinum-coated disc or a soluble catalase tablet (Hughes and Kilvington 2001). However, in one-step systems this neutralisation process occurs too rapidly for any cysticidal activity against *Acanthamoeba* to occur (Hughes and Kilvington 2001).

Chapter 4 shows the innate peroxidase-H_2O_2-halide system found in neutrophils can be reproduced *in vitro* to provide a potent antimicrobial combination, capable of killing 10^4 cysts in 2 hours (Hughes, Andrew *et al.*, 2003). It has been shown that this mechanism can be reproduced *in vitro* to obtain enhanced hydrogen peroxide killing of a variety of pathogenic microbes including bacteria (Jackett, Aber *et al.*, 1978; Klebanoff and Shepard 1984; Tenovuo, Makinen *et al.*, 1985), fungi, (Yang and Anderson 1999), the virus HIV (Klebanoff and Kazazi 1995) and the parasitic helminth *Schistoma mansoni* (Jong, Chi *et al.*, 1984).

5.2 Aims.

In light of the finding that the peroxidase-H_2O_2-halide system results in the significant enhancement of *Acanthamoeba* cyst killing, this chapter looks at the possibilities of applying this potent antimicrobial disinfectant system to enhance the efficacy of commercial One-Step systems using a platinum neutralising disc.

5.3 Methods

5.3.1 Test organisms and culture.

A. polyphaga (Ros strain) was used in all experiments (See chapter 2). Other ocular pathogens studied were *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538), *Candida albicans* (ATCC 10231) and *Fusarium solani* (ATCC 36031) all obtained from the from the American Type Culture Collection, Rockville, USA. All strains were cultured on appropriate agar media (Chapter 2). *Bacillus subtilis* (laboratory strain) spores were also tested (Chapter 2).

5.3.2 Test solutions.

All chemicals and enzymes were obtained from the Sigma Chemical Company (Poole, Dorset, UK) or BDH (Poole, Dorset, UK) unless otherwise stated. Horseradish peroxidase (HRP: EC 1.11.1.7) was dissolved in nanopure water, filter sterilised and stored at –20 °C. The KI stock solution was stored at room temperature in the dark. A commercial one-step hydrogen peroxide contact lens disinfection system, employing a platinum disc neutralising disc, was obtained from the manufacturer (AoSept Plus, Ciba Vision, Atlanta, USA). The AoSept Plus H_2O_2 solution (3 % v/v) of the one-step system was tested in 50 ml polypropylene tubes (Becton Dickinson, Oxfordshire, UK) with and without the presence of the platinum neutralising disc.

5.3.3 Challenge test assays.

The method used to determine *Acanthamoeba* cyst killing is described in Chapter 2. The log kill was measured at time points of 0, 1, 2, 4 and 6 hours and 0.02 % w/v bovine liver catalase was used as the neutraliser (Chapter 2).

The bacterial and fungal strains were tested against the commercial one-step system in accordance with the internationally approved method used for the evaluation of the efficacy of contact lens solutions against ocular pathogens (International Organization for Standardization 2000).

5.3.4 Peroxidase-hydrogen peroxide-halide system with platinum neutralisation.

Eight ml of AoSept Plus H_2O_2 (3 %), 50 µM KI and 200 U ml^{-1} HRP were combined in a 50 ml polypropylene tube. A platinum coated disc (Aodisc®, Ciba Vision, Atlanta, USA), used in the neutralisation of hydrogen peroxide based contact lens disinfectant systems was added. The system was immediately challenged with *A. polyphaga* (Ros strain) cysts and viability assays performed over a 6 hour period. Control experiments in which the H_2O_2 alone and H_2O_2 with the platinum coated disc were also conducted. The test reactions were kept in the dark between each time point measurement. At each time point the concentration of H_2O_2 was determined using Peroxid ® test strips (BDH Darmstadt, Germany). The pH of the solutions was measured at the beginning and end of each experiment.

5.3.5 Data analysis.

The number of surviving *Acanthamoeba* cysts in the microtitre plates at each time point was determined using Reed and Muench computations as described previously (Reed and Muench 1938; Hughes and Kilvington 2001). Viable bacterial and fungal numbers were determined using the Miles and Misra technique (Miles and Misra 1938). Briefly, the bacterial suspensions were serially diluted in ¼ Ringer's solution and 20 µl volumes were spotted in triplicate onto the appropriate agar and incubated at 30 °C for 24 hours before enumeration. The reduction in viable organisms was plotted as the change in log viability for each time point compared with time zero. Statistical analysis was performed using one-way analysis of variance (ANOVA).

5.4 Results

5.4.1 Commercial one-step peroxide system.

The efficacy of the commercial one-step peroxide disinfection system against the bacteria, fungi and *A. polyphaga* cysts is shown in Figure 14. The system gave a 6.4 and 4.4 log kill of *Ps. aeruginosa* and *S. aureus* after 0.5 and 1 hour respectively and a 2.5 log kill of *C. albicans* by 4 hours or 5.5 log after 6 hours. After 4-6 hours of disinfection the log kill of *F. solani* conidia was 1.8 log, *B. subtilis* spores 0.9 log and *A. polyphaga* cysts 1.0 log.

Peroxide concentrations during disinfection by the one-step system were 0.05 % after 1 hour, 0.01 % by 2 hours, 0.005 % by 4 hours and 0 % by 6 hours (Table 9).

□, *A. polyphaga*; ●, *B. subtilis*; △, *F. solani;* ■, *C. albicans*; △, *Ps. aeruginosa*; ○, *S. aureus.*

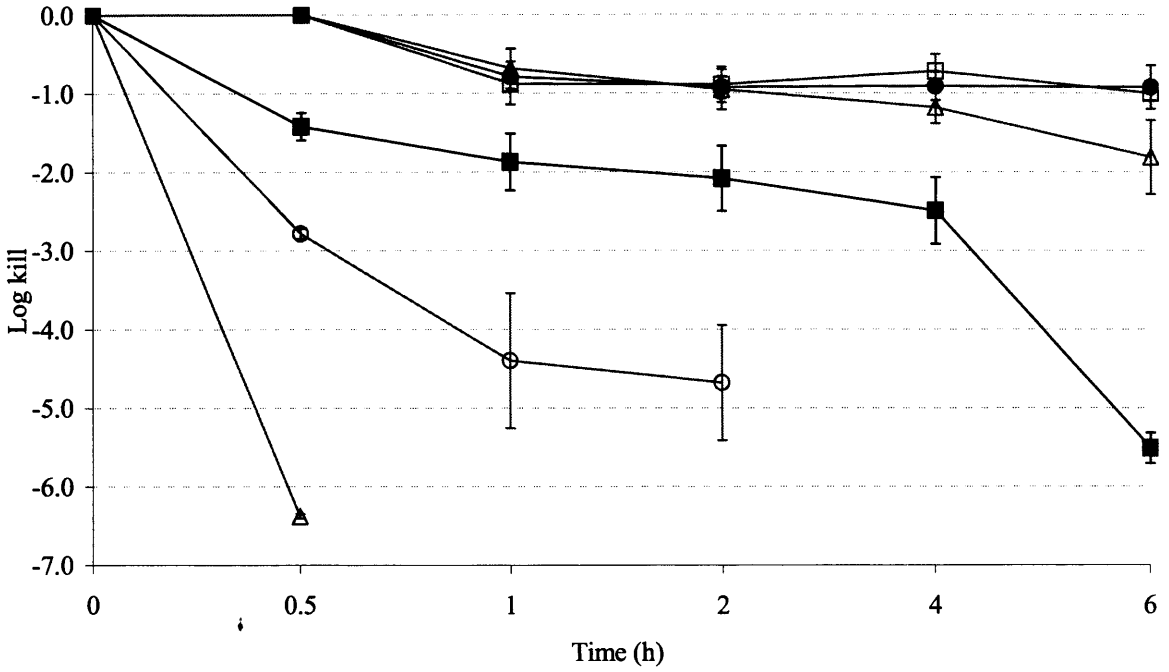

Figure 14: Efficacy of the AoSept System against corneal pathogens. After 6 h exposure the system achieved less than 2 log kill for *F. solani, B. subtilis* and *A. polyphaga.* However, it achieved more than 2 log kill for *C. albicans, S. aureus* and *Ps. Aeruginosa* in this time.

In the absence of the platinum neutralising disc, the 3 % H_2O_2 alone gave a 3.5 log kill and 4.45 log kill in 4 hours and 6 hours respectively for *A. polyphaga* cysts. Addition of 200 U ml^{-1} HRP and 50 μM KI to the 3 % H_2O_2 (peroxidase-halide-peroxide) significantly enhanced the cysticidal efficacy giving a 4.5 log kill at 4 hours ($p<0.05$). The efficacy of the solution, with or with out HRP or KI, against the other organisms was not tested.

5.4.2 Peroxidase-halide-peroxide-platinum-one-step system.

Addition of the peroxidase-halide combination to the commercial one-step peroxide system produced enhanced microbial killing in most cases (Figure 15). No enhancement was detectable with *Ps. aeruginosa* because total kill (6.4 log) occurred by the first time point of

0.5 hour. With *S. aureus* there was a significant increase with a 6.1 log kill within 0.5 hour ($p < 0.001$). Similarly, *C. albicans* showed a 5.9 log kill after 4 hours ($p < 0.01$), *B. subtilis* spores a 2.3 log kill at 4 hours ($p < 0.01$) and *A. polyphaga* cysts a 2.4 log kill at 6 hours ($p < 0.01$). The enhanced one-step system did not increase the extent of killing with *F. solani* that had a 2 log kill after 6 hours ($p > 0.05$). The H_2O_2 levels with the peroxidase-halide-peroxide alone (without the platinum neutralising disc) remained constant over a 6 hour period. However, in the presence of the platinum neutralising disc the levels declined to 2%, 0.5 %, 0.0025 % and 0 % after 1, 2, 4 and 6 hours respectively (Table 9).

Table 9: Efficacies of AoSept system and Enhanced Aosept system against ocular pathogens

Time	Solution[b]	$[H_2O_2]$ (%)[c]	Organism and Log kill with SEM[a]					
			B. subtilis	*C. albicans*	*F. solani*	*A. polyphaga*	*Ps. aeruginosa*	*S. aureus*
0	AoSept	3	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
	Enhanced	3	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
1	AoSept	0.05	0.8 ±0.4	1.9 ±0.3	0.7 ±0.1	0.9 ±0.1	6.4 ±0.0*	4.4 ±0.9
	Enhanced	2	1.2 ±0.5	1.7 ±0.1	1.2 ±0.3	1.3 ±0.2	6.4 ±0.2*	6.1 ±0.3*
2	AoSept	0.01	0.9 ±0.1	2.0 ±0.4	1.0 ±0.3	0.9 ±0.2		4.7 ±0.8*
	Enhanced	0.5	1.4 ±0.4	2.5 ±0.1	1.3 ±0.4	1.6 ±0.2		
4	AoSept	0.005	0.9 ±0.2	2.5 ±0.4	1.2 ±0.2	0.7 ±0.2		
	Enhanced	0.0025	2.3 ±0.4	4.0 ±0.5	1.7 ±0.2	2.0 ±0.1		
6	AoSept	0	0.9 ±0.3	5.5 ±0.2*	1.8 ±0.5	1.0 ±0.1		
	Enhanced	0	2.4 ±0.4	5.9 ±0.0*	2.0 ±0.4	2.4 ±0.2		

[a] Log cyst kill with standard error of the mean (SEM) from experiments conducted in triplicate

[b] AoSept, AoSept system; Enhanced, AoSept system with (3%), 50 μM KI and 200 U ml^{-1} HRP

[c] Concentration of hydrogen peroxide (%)

* Total kill has been achieved

□, *A. polyphaga*; ●, *B. subtilis*; ▲, *F. solani;* ■, *C. albicans*; △, *Ps. aeruginosa*; ○, *S. aureus.*

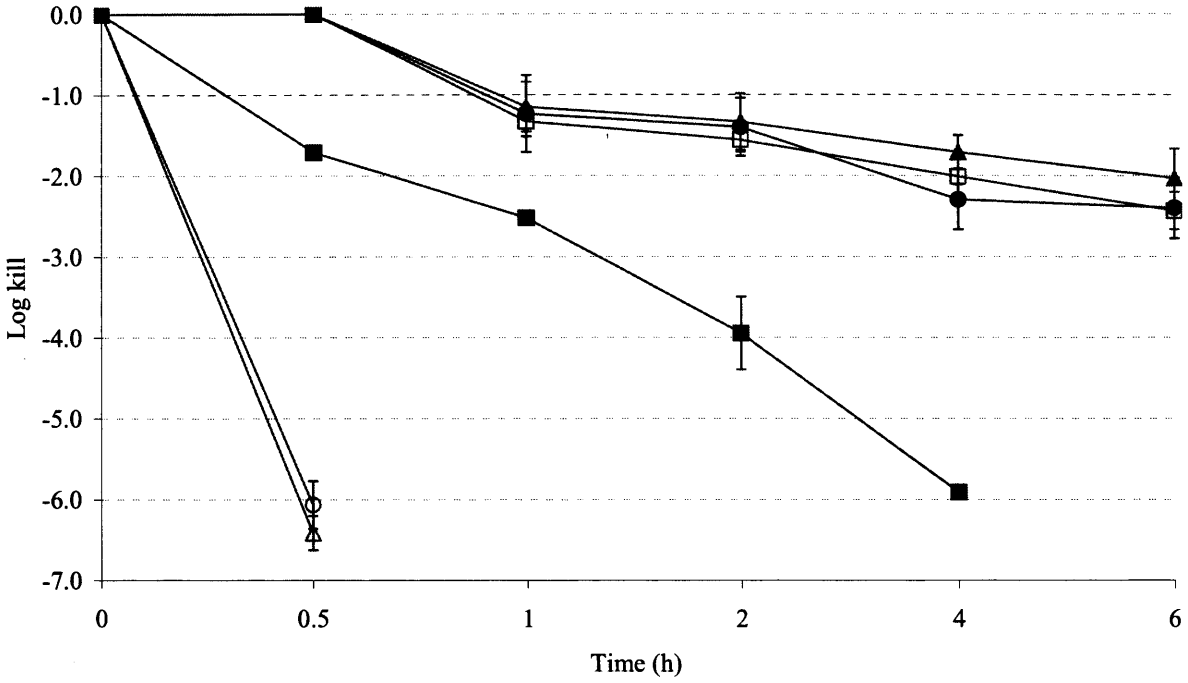

Figure 15: Enhanced one-step killing of corneal pathogens using the AoSept System. With the addition of the HRP and KI, over 6 log kill was achieved against *S. aureus* and *Ps. Aeruginosa* in 30 minutes. Total kill of *C. albicans* was achieved in 4 h. More than 2 log kill was achieved by the enhanced system against *F. solani, A. polyphaga* and *B. subtilis.*

The microbiocidal efficacy of the commercial one-step peroxide and the peroxide-peroxidase-halide systems after 4 hours and 6 hours exposure are compared in Figure 16. Addition of KI or HRP alone to the one-step system did not result in increased microbiocidal activity when tested against *A. polyphaga* cysts (results not shown). Attempts to obtain enhanced microbial killing in a different one-step peroxide system that used a catalase tablet for neutralisation were unsuccessful due to the rapid neutralisation of the H_2O_2 (results not shown).

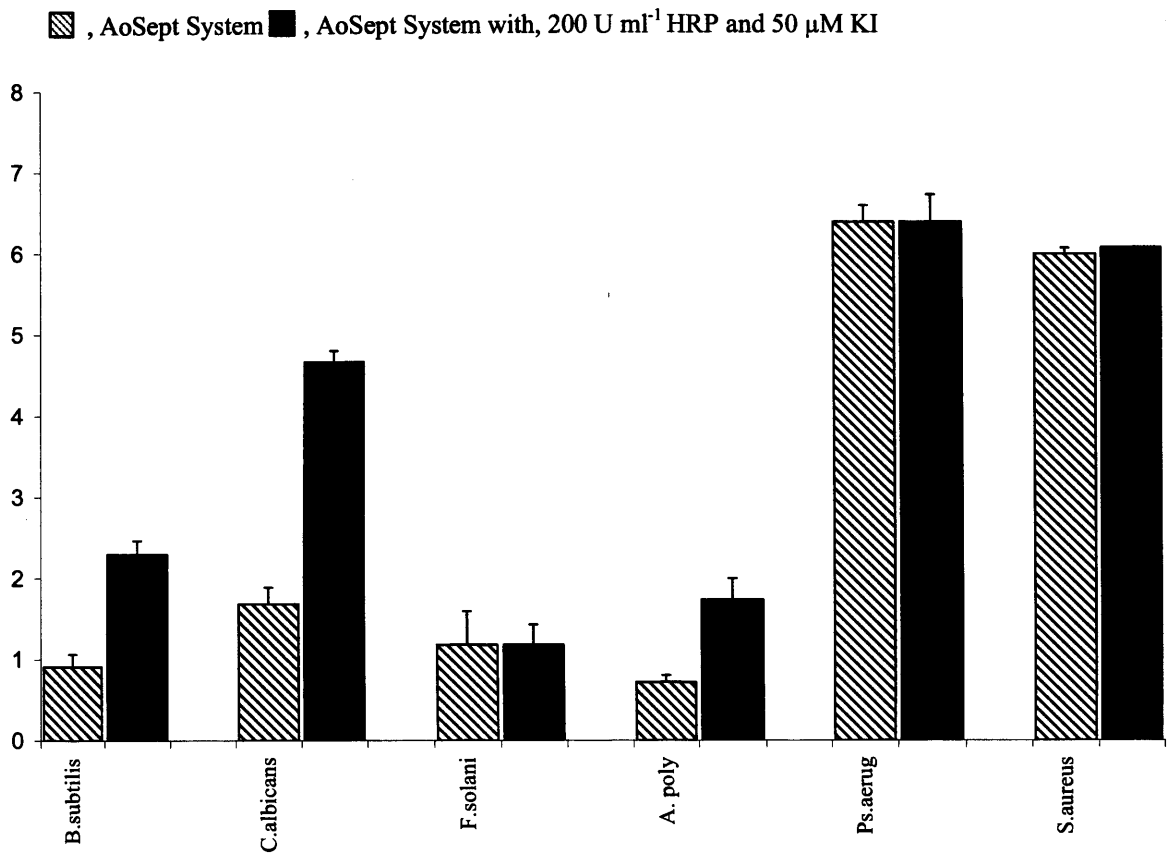

Figure 16: Enhanced one-step killing of ocular pathogens with peroxidase-hydrogen peroxide-halide after 4 hours exposure. The antimicrobial activity against *B. subtilis*, *C.albicans* and *A.polyphaga* was increased by at least a log with the addition of the HRP and KI compared with the AoSept system alone. However, *Ps. Aeruginosa F.solani* and *S. aureus* all achieved comparable results with and without the addition of HRP and KI.

5.5 Discussion

Hydrogen peroxide is commonly used in the disinfection of contact lenses although it must be neutralised before the lenses are worn to avoid corneal damage (Gyulai, Dziabo *et al.*, 1987). One-step hydrogen peroxide systems are available which do not require a separate neutralisation step; this is achieved during disinfection in the storage case using a platinum-coated disc or soluble catalase tablet which catalyses the decomposition of hydrogen peroxide. However as shown here, in one-step systems this neutralisation process occurs too

rapidly for cysticidal activity to occur (Hughes and Kilvington 2001). The peroxidase-hydrogen peroxide-halide system developed in Chapter 4, whilst demonstrating enhanced cysticidal activity, does not result in the neutralisation of the H_2O_2 during the reaction. However, when the reaction was conducted in the presence of a platinum disc, not only was the enhanced killing maintained but the peroxide was neutralised to <0.5 ppm by 4 hours. Levels of 2-5 ppm H_2O_2 are not likely to cause irritation to the eye for which 30 ppm has been reported to induce cytotoxicity and 100 ppm noticeable discomfort (Holden 1990).

The peroxidase-halide-peroxide system in the presence of a platinum neutralising disc resulted in the significant increase in the extent of killing for *C. albicans*, *B. subtilis* spores and *A. polyphaga* cysts. Although catalase has also been shown to enhance the killing of *Mycobacterium tuberculosis* in the presence of a halide and H_2O_2 (Jacket *et al.,,* 1980), attempts to reproduce this enhanced antimicrobial killing in a one-step peroxide system that uses a catalase tablet for neutralisation were unsuccessful due to the rapid neutralisation of the H_2O_2 . The concentrations of HRP and KI used were optimised for the killing of *Acanthamoeba* cysts and may not be optimal for other organisms, as was seen with the lack of significant enhanced killing with *F. solani* conidia.

The peroxidase-halide-peroxide system does not result in the neutralisation of the H_2O_2. However, when the reaction is performed in the presence of a platinum disc the microbiocidal activity is retained and neutralisation of the H_2O_2 results. This enhanced activity produced in a 2.4 log kill of *A. polyphaga* cysts compared with only 1 log with the one-step system. Whilst this is not as efficacious as a two-step peroxide system that gives at least a 3 log kill in 4-6 hours, it represents a significant enhancement in the activity of a one-step peroxide disinfection system (Hughes and Kilvington 2001). When the reaction is performed in the presence of a platinum disc the cysticidal activity is retained and the complete neutralisation of the H_2O_2 is obtained. This may provide a significant improvement to the efficacy of one-step H_2O_2 systems against *Acanthamoeba* cysts and, possibly, other contact lens related ocular pathogens.

6 Effect of Cyst Production on Susceptibility to Disinfectants

6.1 Introduction

Contact lens wearers are most at risk from *Acanthamoeba* infection and account for some 90% of all reported cases of AK in the western hemisphere (Radford, Minassian *et al.,* 1998; Radford, Minassian *et al.,* 2002). Failure to comply with recommended lens cleaning and disinfection instructions and the rinsing or storing lenses in non-sterile saline solutions or tap water are recognised risk factors (Stehr-Green, Bailey *et al.,* 1989; Radford, Bacon *et al.,* 1995).

Disinfection is fundamental to safe contact lens use and hence the prevention of infection. This is commonly achieved through multi-purpose solutions in which a single solution is used for disinfecting, cleaning and storing lenses, or with hydrogen peroxide based systems (Rosenthal, Stein *et al.,* 1995; Buck, Rosenthal *et al.,* 1998; Hughes and Kilvington 2001). Unlike the requirements for bacteria and fungi no standard protocol exists for *Acanthamoeba* contact lens disinfectant efficacy testing (ISO/CD 14729, 2000). As a consequence, a variety of strains, methods for cyst production and experimental protocols have been employed (Anger, Rupp *et al.,* 1991; Hugo, McLaughlin *et al.,* 1991; Silvany, Dougherty *et al.,* 1991; Hughes and Kilvington 2001; Kilvington, Hughes *et al.,* 2002; Noble, Ahearn *et al.,* 2002), often with contradictory findings (Khunkitti, Lloyd *et al.,* 1998; Borazjani, May *et al.,* 2000; Buck, Rosenthal *et al.,* 2000; Hiti, Walochnik *et al.,* 2002).

The relative ease with which most *Acanthamoeba* species and strains can be adapted and maintained in axenic culture has made the organism an ideal model for cellular and molecular biological studies, notably the process of differentiation in to the cyst stage (Neff, S. A. Ray *et al.,* 1964; Byers, Kim *et al.,* 1991; Lloyd, Turner *et al.,* 2001). In the laboratory, encystment can be induced by starvation through depletion of the bacterial food source from prolonged culture of trophozoites on nonnutrient agar seeded with *E. coli* (NNA- *E. coli*) or in axenic media, often for up to 6 weeks or more (Page 1988; Buck and Rosenthal 1996; Buck, Rosenthal *et al.,* 1998). In an attempt to control the process under defined conditions, media have been developed that promote rapid and synchronous encystment. These include Neff's constant pH encystment medium containing the divalent cations Mg^{2+}, $Ca^{2+}$and amine buffer at a pH of 8.9-9.0 or through the addition of 50 mM $MgCl_2$ to axenically growing trophozoite cultures (Neff, S. A. Ray *et al.,*

1964; Khunkitti, Lloyd *et al.,* 1998). Incubation with the amino acid taurine and Mg^{2+} has also been shown to induce encystment (Singh 1975).

6.2 Aims

In an attempt to develop standardised and reproducible methods for evaluating contact lens disinfectants and therapeutic agents against *Acanthamoeba* the effect of age in culture and method of cyst production on the efficacy of contact lens disinfectants and experimental agents on a strain of *A. polyphaga* were compared. Some of the most popular multipurpose disinfection solutions were also compared with one another to determine their efficacy against the resistant pathogen *Acanthamoeba.*

6.3 Materials and Methods

6.3.1 *A. polyphaga* strains, culture and cyst preparation

A. polyphaga strain Ros-02 was cryopreserved at the time of isolation in 1991 from a patient with Acanthamoeba keratitis (Kilvington, Beeching *et al.,* 1991). Strain Ros-91 was the same isolate but had been maintained in continuous axenic broth culture since 1991. Trophozoites were adapted and maintained in a semi-defined axenic broth (#6 medium) as previously described (Hughes and Kilvington 2001); Chapter 2). Ros-02 was passaged no more than 5 times before being used for cyst production.

Cysts of both strains were prepared from the trophozoites in the following manner (See chapter 2):

(i) Culture on 2 % non-nutrient agar in ¼ Ringer's solution seeded with live *Escherichia coli* (NNA cysts) in air at 32 °C for 7 days.

(ii) With Neff's constant pH encystment medium (Neff cysts) as described previously (Neff, S. A. Ray *et al.,* 1964; Hughes and Kilvington 2001).

(iii) In ¼ Ringer's solution containing 20 mM taurine and 15 mM $MgCl_2$ under the same conditions described for the preparation of the Neff cysts (Taurine cysts).

(iv) By growing trophozoites in the semi-defined axenic medium supplemented after autoclaving with filter sterilised 50 mM $MgCl_2$ (#6-Mg), again with the same incubation

conditions described previously (Khunkitti, Lloyd *et al.*, 1998; Hughes and Kilvington 2001).

The cysts were harvested and washed three times with ¼ Ringer's solution by centrifugation at 1000 x g for 5 minutes and stored at 4 °C for testing within 7 days.

A single batch of each cyst preparation was used in all the experimental studies. To determine the reproducibility of the cyst preparations, four different batches of Ros-91 #6-Mg cysts were prepared and tested against MPS-1 on separate occasions. In addition, different batches of Ros-91 #6-Mg, Neff and NNA cysts were also made and retested against MPS-2. To assess the effect of storage on cyst susceptibility, a batch of Ros-91 #6-Mg cysts was prepared and stored at 4 °C for testing with MPS-1 over an 8 week period.

6.3.2 Flow cytometry

Unstained cyst preparations were compared with a FACSCalibur flow cytometer (BD Bioscience, CA, USA). At least 2000 events were collected and the forward scatter plotted to compare the cell size of the preparations. The findings were analysed using CellQuest® Pro. V. 4.0.1. Software. The unstained cysts were examined using phase contrast microscopy.

6.3.3 Transmission electron microscopy

A batch of Ros-91 #6-Mg and Neff cysts were examined by transmission electron microscopy using the method described in chapter 2.

6.3.4 Test solutions

The following contact lens disinfectants and experimental agents were studied: 3% hydrogen peroxide (contact lens disinfection solution), polyhexamethylene biguanide (PHMB) at 3 $\mu g\ ml^{-1}$ in H_2O (Bausch & Lomb, Rochester, USA), chlorhexidine digluconate (CLX) at 30 $\mu g\ ml^{-1}$ in H_2O (Sigma Chemical Company, Dorset, UK), the amidoamine myristamidopropyl dimethylamine (MAPD) at 20 $\mu g\ ml^{-1}$ in 2 mM Tris.HCl, pH 7.2 (Alcon Laboratories Inc., Fort Worth, USA), multi-purpose contact lens disinfection solutions (MPS-1 and MPS-2) both

containing 1 μg ml^{-1} PHMB. In control experiments, ¼ Ringer's solution was used in place of the test solutions.

6.3.5 Cysticidal assay

The method used to determine the kinetics of cyst killing was as described previously (Hughes and Kilvington 2001; Kilvington, Hughes *et al.,* 2002), using *A. polyphaga* (Ros) cysts (Chapter 2). For all test solutions except hydrogen peroxide the tubes were "aged" overnight by filling with 10 ml of the test solution, which was then discarded and replaced with fresh solution for testing. The time intervals tested were 0, 1, 2, 4 and 6 hours and 4 mg ml^{-1} bovine liver catalase (Sigma Chemical Company, Dorset, England) was used for the neutralisation of hydrogen peroxide and 0.1% Tween 80 (Sigma Chemical Company, Dorset, England) for the other disinfectants.

6.3.6 Data analysis

The number of surviving cysts in the time-kill studies was determined using Reed and Muench computations (Chapter 2).

6.4 Results

The efficacy of the disinfectants against the various cyst preparations of *A. polyphaga* Ros-91 and Ros-02 is shown in Table 10, which gives the log cyst kill with SEM from triplicate experiments. Only the findings after 4 and 6 hours exposure are shown as little significant kill occurred before these time points. All control experiments using ¼ Ringer's solution gave ≤0.3 log kill (Table 10). Comparative statistical analysis (ANOVA) of the experimental findings are summarised in Table 11. Although the patterns of resistance varied between the test solutions, significant differences in susceptibility occurred depending on the cyst preparation and age of the test strain (Tables 10-11).

For example, with PHMB (3 μg ml^{-1}) Ros-91 showed a 3-4 log kill at 6 hours with #6-Mg, Neff and NNA cysts yet only a 0.4 log kill with Taurine cysts ($p<0.001$). With Ros-02, a 3 log kill was also obtained with #6-Mg cysts yet the values for the Neff, Taurine and NNA cysts were 0.3, 0.2 and 1.2 respectively ($p<0.01$).

MAPD (20 μg ml^{-1}) was active against all the cyst types of Ros-91 giving a 2.8-4 log kill at 6 hours although the value of 2.8 log for the NNA cyst was significantly lower than the other types ($p<0.01$). With Ros-02, the values ranged from 2.7-4.5 log kill at 6 hours with the decreased values of 2.9 and 2.7 log for the Taurine and NNA cysts being significant with respect to #6-Mg and Neff cysts ($p<0.05$ and $p<0.01$ respectively).

The multi-purpose contact lens disinfectants MPS-1 and MPS-2, both based on 1 μg ml^{-1} PHMB, varied in their cysticidal efficacy. With MPS-1 and Ros-91, only a 1.7 log kill occurred at 6 hours with NNA cysts compared to a 4 log kill for the rest ($p<0.01$). With Ros-02, the #6-Mg and Neff cysts gave a 3.8 and 3.4 log kill compared with 1.5 and 1.7 log kill for Taurine and NNA cysts ($p<0.01$).

With MPS-2, Ros-91 gave a 3.3-3.7 and log kill at 6 hours with Taurine and #6-Mg cysts but only 1 log kill with Neff and NNA ($p<0.001$). In contrast, Ros-02 #6-Mg cysts a 2.4 log kill at 6 hours compared to 1.0-1.8 log kill for the other cyst preparations ($p<0.05$). The greater sensitivity of the Ros-91 #6-Mg cysts compared to those of Ros-02 was also significant ($p<0.05$).

CLX (30 μg ml^{-1}) had only low cysticidal activity giving 0.8 – 1.8 log kill after 6 hours for the Ros-91 cyst preparations and 0.1-1.1 log with Ros-02; none of the findings at 6 hours showed significant differences ($p>0.05$). H_2O_2 (3%) showed greater consistency of activity against all cyst types and strain age, giving at least a 2.7 – 4.3 log kill after 6 hours exposure with no significant differences were observed between the cyst types or strains at 6 hours.

The four separate batches of Ros-91 #6-Mg cysts and tested against MPS-1 gave log cyst kills after 4 hours exposure of 2.6 (±0,7), 3.1 (±0.4), 3.0 (±0.6) and 1.8 (±0.5). No significance in susceptibility was found between the cyst batches ($p>0.05$). Different batches of Ros-91 #6-Mg, Neff and NNA cysts tested against MPS-2 gave 6 hours log kills that were consistent with the results obtained with other batches of these cyst preparations when tested with MPS-2 (Table 10).

The batch of Ros-91 #6-Mg cysts stored at 4°C for testing over an 8 week period against MPS-1 showed a progressively increasing susceptibility to disinfection although there was no loss in viability during storage ($p>0.05$). With the freshly prepared cysts, a 1.4 (±0.3) log kill was obtained after 4 hours exposure. However, after 1, 2, 4 and 8 weeks storage the values were 2.0

(±0.0), 3.6 (±0.1), 3.9 (±0.2) and 3.2 (±0.3) log kill respectively. This increasing susceptibility to disinfection of the cysts on storage was significant for weeks 2-8 ($p<0.01$).

FACS analysis of the preparations showed differences in relative size ($p< 0.001$) with the exception of Ros-91 NNA and Ros-91 Neff. Overall the differences in size between Ros-02 and Ros-91 cysts were significantly different ($p<0.001$) although these differences did not correlate with susceptibility or resistance to disinfection (Tables 10-11). Phase contrast microscopy of the various cyst preparations showed them to be of similar morphology with >90 % in the mature form (results not shown). Transmission electron microscopy of the Ros-91 #6-Mg and Neff cysts showed a slight thickening of the ectocyst wall in the Neff prepared cysts compared with the #6-Mg prepared cysts (Figure 17).

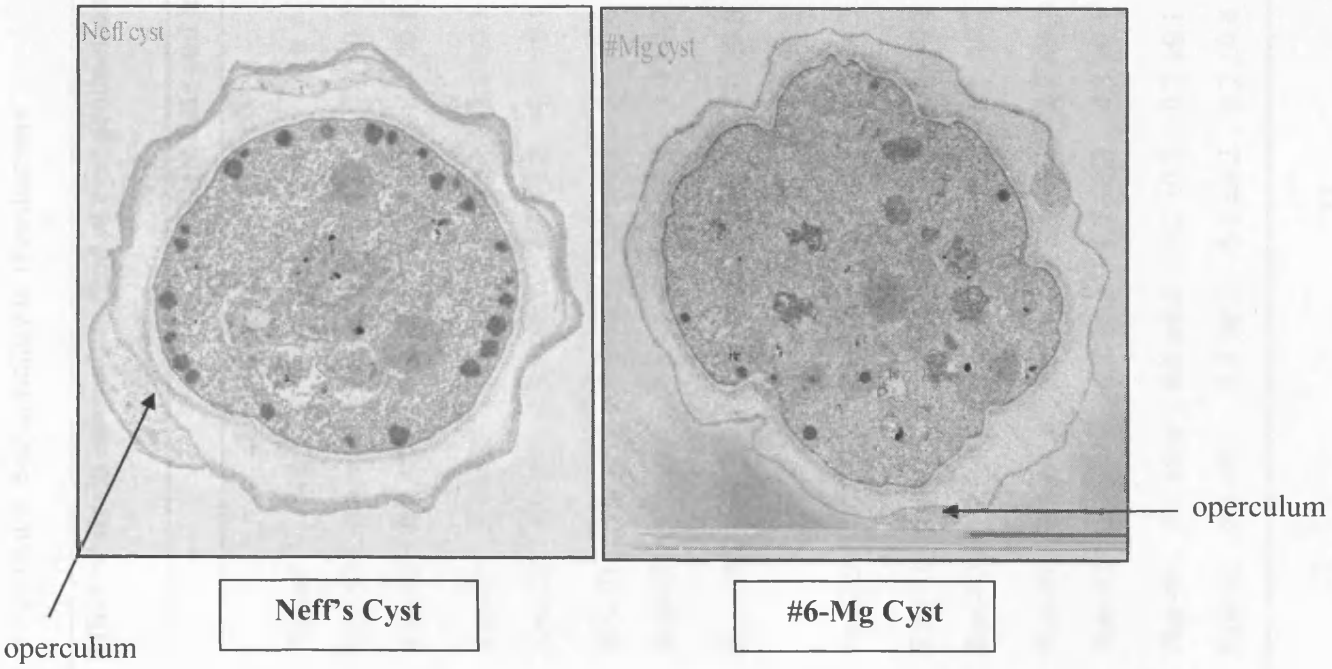

Figure 17: Transmission Electron Microscopy images of Neff prepared cyst and a #Mg prepared cyst. The Neffs cyst appears to have a thicker ectocyst and a more uniform shape.

Table 10: Effect of strain age and method of cyst production on A. polyphaga disinfection.

		Cyst type and log kill with SEM[a]							
		#6-Mg		Neff		Taurine		NNA	
Drug[b]	Strain[c]	4 h	6 h	4 h	6 h	4 h	6 h	4 h	6 h
PHMB (3 μg ml^{-1})	Ros-91	4.0 ±0.2	4.0 ±0.2	1.7±0.7	3.1 ±0.2	0.4 ±0.3	0.4 ±0.3	2.6 ±0.4	3.3 ±0.2
	Ros-02	0.9 ±0.4	3.4 ±0.6	0.2 ±0.1	0.3 ±0.1	0.2 ±0.1	0.2 ±0.1	0.8 ±0.1	1.2 ±0.3
MAPD (20 μg ml^{-1})	Ros-91	3.9 ±0.1	3.9 ±0.1	3.7 ±0.1	3.7 ±0.1	2.9 ±0.4	3.8 ±0.1	1.7 ±0.2	2.8 ±0.3
	Ros-02	3.9 ±0.1	3.9 ±0.1	2.1 ±0.2	4. 5 ±0.1	1.8 ±0.0	2.9 ±0.3	1.7 ±0.2	2.7 ±0.2
MPS-1 (1 μg ml^{-1})	Ros-91	3.6 ±0.4	4.3 ±0.1	2.8 ±0.2	3.9 ±0.6	2.8 ±0.1	3.7 ±0.4	1.6 ±0.3	1.7 ±0.3
	Ros-02	3.0 ±0.4	3.8 ±0.1	1.9 ±0.5	3.4 ±0.2	1.2 ±0.3	1.5 ±0.3	0.9 ±0.2	1.7 ±0.4
MPS-2 (1 μg ml^{-1})	Ros-91	1.8 ±0.4	3.7 ±0.4	0.8 ±0.1	1.0 ±0.0	2.4 ±0.3	3.3 ±0.4	0.4 ±0.2	1.0 ±0.2
			2.9 ±0.5[d]		1.4 ±0.2				1.1 ±0.3
	Ros-02	1.7 ±0.2	2.4 ±0.1	1.2 ±0.2	1.8 ±0.3	1.1 ±0.1	1.3 ±0.0	0.2 ±0.1	1.0 ±0.1
CLX (30 μg ml^{-1})	Ros-91	0.8 ±0.1	1.0 ±0.3	1.7 ±0.3	1.8 ±0.3	0.5 ±0.1	0.8 ±0.2	0.8 ±0.1	1.5 ±0.5
	Ros-02	0.7 ±0.2	1.2 ±0.5	0.6 ±0.4	1.1 ±0.2	0.1 ±0.1	0.4 ±0.1	0.2 ±0.3	0.2 ±0.3
Peroxide (3%)	Ros-91	2.9 ±0.3	3.8 ±0.1	2.4 ±0.1	2.7 ±0.1	2.1 ±0.2	3.5 ±0.5	2.5 ±0.2	3.9 ±0.5
	Ros-02	3.3 ±0.6	4.2 ±0.2	1.7 ±0.3	4.3 ±0.1	2.5 ±0.2	3.4 ±0.6	0.9 ±0.3	2.8 ±0.5
¼ Ringer's (control)	Ros-91	0.1 ±0.0	0.0 ±0.0	0.2 ±0.3	0.2 ±0.1	0.1 ±0.1	0.2 ±0.2	0.2 ±0.2	0.0 ±0.1
	Ros-02	0.2 ±0.1	0.0 ±0.2	0.3 ±0.2	0.2 ±0.4	0.0 ±0.0	0.0 ±0.1	0.1 ±0.1	0.1 ±0.2

[a] Log cyst kill after 4 and 6 hours with standard error of the mean (SEM) from experiments conducted in triplicate

[b] PHMB, polyhexamethylene biguanide; CLX, chlorhexidine digluconate; MAPD, myristamidopropyl dimethylamine; MPS-1 and MPS-2 multi-purpose contact lens disinfection solutions (both containing 1 μg ml^{-1} PHMB); Peroxide, hydrogen peroxide contact lens disinfectant solution.

[c] *A. polyphaga* strain Ros-02 was cryopreserved shortly after isolation in 1991 and recovered for this study; Ros-91 has been in continuous axenic culture since this time.

[d] Kill obtained with additional batches of Ros-91 cysts tested on a separate occasion.

Table 11: Statistical comparison (ANOVA) of A. polyphaga strains (Ros-91 and Ros-02) and different cyst preparations against disinfectant agents.

	Polyhexamethylene biguanide (3 µg ml^{-1})							Myristamidopropyl dimethylamine (20 µg ml^{-1})						
Strain	Mg-91[a]	Mg-02	Neff-91	Neff-02	Taur-91	Taur-02	NNA-91	Mg-91	Mg-02	Neff-91	Neff-02	Taur-91	Taur-02	NNA-91
Mg-02	***/ns[b]							ns/ns						
Neff-91	**/ns	ns/ns						ns/ns	ns/ns					
Neff-02	***/***	ns/***	ns/***					***/ns	***/ns	***/*				
Taur-91	***/***	ns/***	ns/***	ns/ns				*/ns	*/ns	ns/ns	ns/ns			
Taur-02	***/***	ns /***	ns/***	ns/ns	ns/ns			***/*	***/*	***/ns	ns/***	*/*		
NNA-91	ns/ns	ns/ns	ns/ns	**/***	**/***	**/***		***/**	***/**	***/*	ns/***	**/*	ns/ns	
NNA-02	***/***	ns/**	ns/**	ns/ns	ns/ns	ns/ns	*/**	***/**	***/**	***/*	ns/***	*/**	ns/ns	ns/ns
	Multi-purpose solution-1							**Multi-purpose solution-2**						
Strain	Mg-91[a]	Mg-02	Neff-91	Neff-02	Taur-91	Taur-02	NNA-91	Mg-91	Mg-02	Neff-91	Neff-02	Taur-91	Taur-02	NNA-91
Mg-02	ns/ns							ns/*						
Neff-91	ns/ns	ns/ns						ns/***	ns/*					
Neff-02	*/ns	ns/ns	ns/ns					ns/**	ns/ns	ns/ns				
Taur-91	ns/ns	ns/ns	ns/ns	ns/ns				ns/ns	ns/ns	**/***	ns/*			
Taur-02	**/***	*/**	*/**	ns/*	*/**			ns/***	ns/*	ns/ns	ns/ns	*/***		
NNA-91	**/**	ns/**	ns/**	ns/*	ns/*	ns/ns		*/***	*/ns	ns/ns	ns/ns	***/***	ns/ns	
NNA-02	***/**	**/**	*/**	ns/*	*/*	ns/ns	ns/ns	**/***	**/*	ns/ns	ns/ns	***/***	ns/ns	ns/ns

	Chlorhexidine digluconate (30 µg ml^{-1})							Hydrogen peroxide (3%)						
Strain	Mg-91[a]	Mg-02	Neff-91	Neff-02	Taur-91	Taur-02	NNA-91	Mg-91	Mg-02	Neff-91	Neff-02	Taur-91	Taur-02	NNA-91
Mg-02	ns/ns							ns/ns						
Neff-91	ns/ns	ns/ns						ns/ns	ns/ns					
Neff-02	ns/ns	ns/ns	*/ns					ns/ns	*/ns	ns/ns				
Taur-91	ns/ns	ns/ns	*/ns	ns/ns				ns/ns	ns/ns	ns/ns	ns/ns			
Taur-02	ns/ns	ns/ns	**/ns	ns/ns	ns/ns			ns/ns	ns/ns	ns/ns	ns/ns	ns/ns		
NNA-91	ns/ns	ns/ns	ns/ns	ns/ns	ns/ns	ns/ns		ns/ns	ns/ns	ns/ns	ns/ns	ns/ns	ns/ns	
NNA-02	ns/ns	ns/ns	**/ns	ns/ns	ns/ns	ns/ns	ns/ns	**/ns	**/ns	ns/ns	ns/ns	ns/ns	*/ns	*/ns

[a]Cyst type and strain: 91, Ros-91; 02, Ros-02; Mg, #6 Mg medium; Neff, Neff's medium; Taur, Taurine-Mg medium; NNA, Non-nutrient agar-*E. coli* medium

[b]Statistical significance at 4 and 6 h; ns= $p>0.05$; *= $p<0.05$; **= $p<0.01$; ***= $p<0.001$

6.5 Discussion

Unlike efficacy testing of contact lens disinfectants against bacteria and fungi, no such requirement or standard protocol exists for *Acanthamoeba* (International Organization for Standardization 2000). As a consequence, a variety of species, strains, methods of cyst preparation cysts and experimental protocols have been employed, frequently with contradictory findings, as reviewed by Buck and colleagues (Buck, Rosenthal *et al.,* 2000). In an attempt to develop a standardised, reproducible, method for assessing the cysticidal efficacy of therapeutic agents and contact lens solutions we compared the affect of age in culture and method of cyst production with *A. polyphaga.* The demonstration that these variables can significantly affect the susceptibility of *Acanthamoeba* cysts to disinfection has important implications for the testing and interpretation of cysticidal efficacy of disinfectant and therapeutic agents.

As compared in this study, a variety of methods have been described for the production of *Acanthamoeba* cysts (Buck, Rosenthal *et al.,* 2000). Axenic trophozoite culture in semi-defined media supplemented with up to 50 mM Mg^{2+} is commonly used for this purpose. However, the findings of this study indicate that cysts derived from this approach are more susceptible to killing to PHMB and, to a lesser degree, MAPD. Both these agents are used in multi-purpose contact lens solutions and this may, in part, explain the discrepancies in the reported cysticidal efficacy of such disinfectants when different methods of cyst production are used (Buck, Rosenthal *et al.,* 1998; Van Duzee and Schlech 1999; Buck, Rosenthal *et al.,* 2000; Kilvington and Anger 2001). The reasons for the increased susceptibility of the Mg^{2+} derived cyst preparation are unclear. All appeared to be of similar, mature cyst, morphology under light microscopy. However, under the electron microscope, the ectocyst wall appeared to be thicker in the Neff prepared cysts. The sizes of the cysts appeared the same in both groups under the electron microscope, although flow cytometry analysis suggested differences in relative size between preparations. The thickness of the wall may account for some of the differences in susceptibility and resistance to disinfection. It is also possible that encystment induced from the presence of Mg^{2+} ions results in a different chemical composition of the cyst wall that allows greater penetration of these agents.

It is also apparent that cysts derived from a laboratory strain that has been in prolonged axenic culture are more susceptible to disinfection than those produced from the original isolate. This variable is addressed in the standard protocol for assessing the efficacy of contact lens disinfectant solutions against bacteria and fungi, requiring that the test strains be passaged no more that five times from the original culture prior to testing (International Organization for Standardization 2000). Laboratory culture is known to induce biological changes in *Acanthamoeba* such as loss of virulence and decrease in cellular enzyme activity (Mazur and Hadas 1994; Mazur, Hadas *et al.,* 1995). Such changes may also be reflected in the biochemical composition of the cyst wall resulting in a greater susceptibility to disinfection.

Other factors also influence the susceptibility of *Acanthamoeba* cysts to disinfection. Here it was found that Ros-91 #6-Mg cysts displayed increasing susceptibility to disinfection by MPS-1 during storage at 4°C although the culture viability of the cysts remained unchanged during this period. *Acanthamoeba* cysts have been shown to remain viable at 4°C for at least 24 years (Mazur, Hadas *et al.,* 1995). The storage times for *Acanthamoeba* cysts prior to use in disinfection assays is not always stated but has ranged from 1–6 weeks and highlights another experimental variable that may significantly affect disinfectant efficacy findings (Hugo, McLaughlin *et al.,* 1991; Hughes and Kilvington 2001). Although the cyst preparations used here were of the mature form, it has also been demonstrated that immature cysts are more susceptible to disinfection and also stresses the importance of using homogeneous cyst populations of known maturity when conducting such assays (Kilvington and Anger 2001);(Turner, Harris *et al.,* 2000).

Acanthamoeba encystment is a physiological response to adverse environmental changes such as depletion of the bacterial food source, increasing anaerobiasis, osmolarity and presence of sublethal concentrations of certain therapeutic and biocidal agents (Page 1988; Kilvington, Larkin *et al.,* 1990; Byers, Kim *et al.,* 1991; Lloyd, Turner *et al.,* 2001). In vitro, the process is characterised by discrete stages of trophozoite rounding, early cyst wall synthesis of the immature cyst and mature cyst formation (Neff, S. A. Ray *et al.,* 1964; Byers, Kim *et al.,* 1991; Visvesvara 1991). The composition of the *Acanthamoeba* cyst wall has not been studied extensively but the ectocyst is believed to comprise an acid insoluble protein containing material, with the endocyst containing approximately 33% cellulose (Singh 1975; Turner, Harris *et al.,* 2000; Lloyd, Turner *et al.,* 2001). A greater understanding of the

molecular and biochemical factors controlling the *Acanthamoeba* encystment process and composition of the cyst wall is clearly required. This would also aid the development of improved therapeutic and disinfectant agents in both the treatment and prevention of Acanthamoeba keratitis.

This study has demonstrated some fundamental factors that can influence the observed efficacy of disinfectant and therapeutic agents against *Acanthamoeba* cysts. In view of the variety of experimental approaches and differing outcomes, standardisation for *Acanthamoeba* disinfectant efficacy testing, as required for bacteria and fungi, is clearly needed (Buck, Rosenthal *et al.,* 2000). This should take into account the *Acanthamoeba* species and strain used, the time in laboratory culture, method of cyst preparation and storage period of the cysts. Whilst encystment methods such as trophozoite culture in the presence of Mg^{2+} provides large numbers of cysts that are usually required for molecular, biochemical and differentiation analysis their use in disinfection studies should be interpreted cautiously as they are more susceptible to killing by biguanide and amidoamines.

7 Efficacy of Multipurpose Solutions

7.1 Introduction

Although it was first reported that *Acanthamoeba* spp. could cause ocular infections in 1975 (Jones, Visvesvara *et al.,* 1975), it was not until the mid-1980s that a connection between contact lens wear and disease was established (Moore, McCulley *et al.,* 1985). It is now known that the storage case may become contaminated from rinsing with contaminated tap water. The organisms can then attach to the lens, which acts as a vector, transmitting the amoebae onto the corneal surface, where invasion and subsequent infection can occur. In 1996, it was shown by matching DNA isolates of *A. griffini* from the corneal scraping, the lens storage case, and the bathroom water supply of an infected individual (Ledee, Hay *et al.,* 1996). Infection is usually associated with poor lens hygiene, such as rinsing the lenses under tap water, and using non-sterile saline solutions (Moore, McCulley *et al.,* 1985; Stehr-Green, Bailey *et al.,* 1989). It is now estimated that 92 % of acanthamoeba keratitis infections occur in contact lens wearers, the remaining cases are caused by accidental trauma to the epithelium (Radford, Bacon *et al.,* 1995; Radford, Minassian *et al.,* 1998).

The prevalence of *Acanthamoeba* infection in contact lens wearers became apparent in 1996 when the eyes of 217 patients with keratitis were screened using tandem scanning confocal microscopy for the presence of *Acanthamoeba* (Mathers, Sutphin *et al.,* 1996). The organism was suspected in 51 patients, and the presence of the organism was confirmed by cytology in 43 of them. Surveys in England and Wales found the incidence of acanthamoeba keratitis dropping from 1 case in 39,370 contact lens wearers in 1992 to 1 case in 55,555 contact lens wearers in 1999 (Radford, Lehmann *et al.,* 1998; Radford, Minassian *et al.,* 2002). However, it was found that there was under reporting in this survey and the data for 1998 and 1999 were in fact 1 in 32,260 and 1 in 37,040, respectively (Seal 2003; Seal, Beattie *et al.,* 2003). It is thought that the introduction of multipurpose cleaning and disinfecting solutions has been a major factor contributing to the reduction in the frequency of this disease (Stevenson and Seal 1998).

The increased recognition of contact lens-associated AK during the late 1980s and early 1990s resulted in several investigations into the amoebicidal effects of contact lens solutions. Due to the frequent failures of medical and surgical intervention, disinfecting solutions

effective at killing *Acanthamoeba* are important in preventing corneal infection (Zanetti, Fiori *et al.,* 1995). However, the experimental findings of these studies vary due to different methodology and different susceptibilities of strains used (Ludwig, Meisler *et al.,* 1986; Lindquist, Doughman *et al.,* 1988; Brandt, Ware *et al.,* 1989). Standardisation of testing of the efficacy of contact lens solutions is needed (Meisler and Rutherford 1991).

In a review of methods used to evaluate the effectiveness of contact lens solutions against *Acanthamoeba* it was found that of the studies reviewed, 30% did not use a quantitative method, merely reporting the presence or absence of viable amoebae (Buck, Rosenthal *et al.,* 2000). Others have used quantitative methods, such as direct counting with a haemocytometer (Connor, Hopkins *et al.,* 1991; Buck and Rosenthal 1996), standard plaque assay (Hugo, McLaughlin *et al.,* 1991; Khunkitti, Lloyd *et al.,* 1996), a quantitative microtitre method (Buck and Rosenthal 1996), and enumeration of track-forming units developed on nonnutrient agar with a bacterial overlay (Kilvington 1998) and the most probable number technique (Perrine, Chenu *et al.,* 1995; Beattie, Seal *et al.,* 2003).

7.2 Aims

Although previous studies have demonstrated the activity of multipurpose solutions against *Acanthamoeba,* the data is contradictory. To this end, using a reliable, reproducible assay method and known pathogenic strains of *Acanthamoeba,* the activities of commercially available multipurpose solutions were compared against the trophozoites and cysts of *A. polyphaga.*

7.3 Materials and Methods

7.3.1 *Acanthamoeba* strains

A. polyphaga (Ros strain and Cole strain), and *A. culbertsoni* (ATCC 30171) were used throughout the study. Log phase trophozoites were harvested immediately before testing. Cysts were prepared by 7 days incubation with Neff's encystment medium, stored at 4 °C for a maximum of 7 days and sonicated before use, as described earlier (Chapter 2).

7.3.2 Test solutions

Eight commercially available multipurpose contact lens disinfection solutions were studied (Table 12).

7.3.3 Cysticidal assay

The method used to determine the kinetics of cyst killing was as described previously (Chapter 2). For all test solutions the tubes were "aged" overnight by filling the tubes with the appropriate solution and leaving at room temperature for 12 hours. Optifree Express was tested in glass universal bottles as it has been reported that one of the active ingredients in Optifree Express, MAPD adheres to certain types of plastics (Van Duzee and Schlech 1999). All solutions were tested at time intervals of 0, 1, 2, 4 and 6 hours.

All commercial contact lens disinfection systems were tested according to the manufacturers' recommendations. All experiments were performed in triplicate and repeated on three separate occasions. Control experiments used ¼ Ringer's solution in place of the test solution. After 24 hours, the tubes were centrifuged at 1000 x g any remaining organisms washed 3 times in ¼ Ringer's by centrifugation and observed by scanning electron microscopy.

7.3.4 Data analysis

The number of surviving cysts in the time-kill studies was determined using Reed and Muench computations as previously described for *Acanthamoeba* (Reed and Muench 1938; Buck and Rosenthal 1996).

Table 12: Commercial multipurpose solutions tested in this study.

Solution	Manufacturer	Active Ingredient	Concentration (%)	Exposure Time (h)*
Quattro	Abatron	PHMB[a]	0.0001	4
Focus	Ciba Vision	PHMB	0.0001	4
Focus Plus	Ciba Vision	PHMB	0.0001	10 min
Renu Multi	Bausch & Lomb	PHMB	0.0001	4
Concerto Soft	Lunelle	PHMB	0.0005	4
		Hexetidine	0.00025	
Complete	Allergan	PHMB	0.0001	6
Optifree Express	Alcon	Polyquad[b]	0.001	6
		MAPD[c]	0.0005	

*Manufacturer's product data sheet

[a] PHMB is polyhexamethylene biguanide

[b] polyquad is polidronium chloride.

[c] MAPD is myrstamidopropyldimethylamine

7.3.5 Scanning Electron Microscopy

Cysts were investigated using scanning microscopy (Chpater 2).

7.4 Results

7.4.1 Trophozoites.

All the multipurpose solutions were active against the *Acanthamoeba* trophozoites giving at least a 3 log reduction (99.9 % kill) within 4 hours (results not shown).

7.4.2 Activity of multipurpose solutions against Ros cysts

The activities of the commercial multipurpose solutions at 4 and 6 hours used according to the manufacturers' recommendations, against Ros cysts are shown in Figure 18.

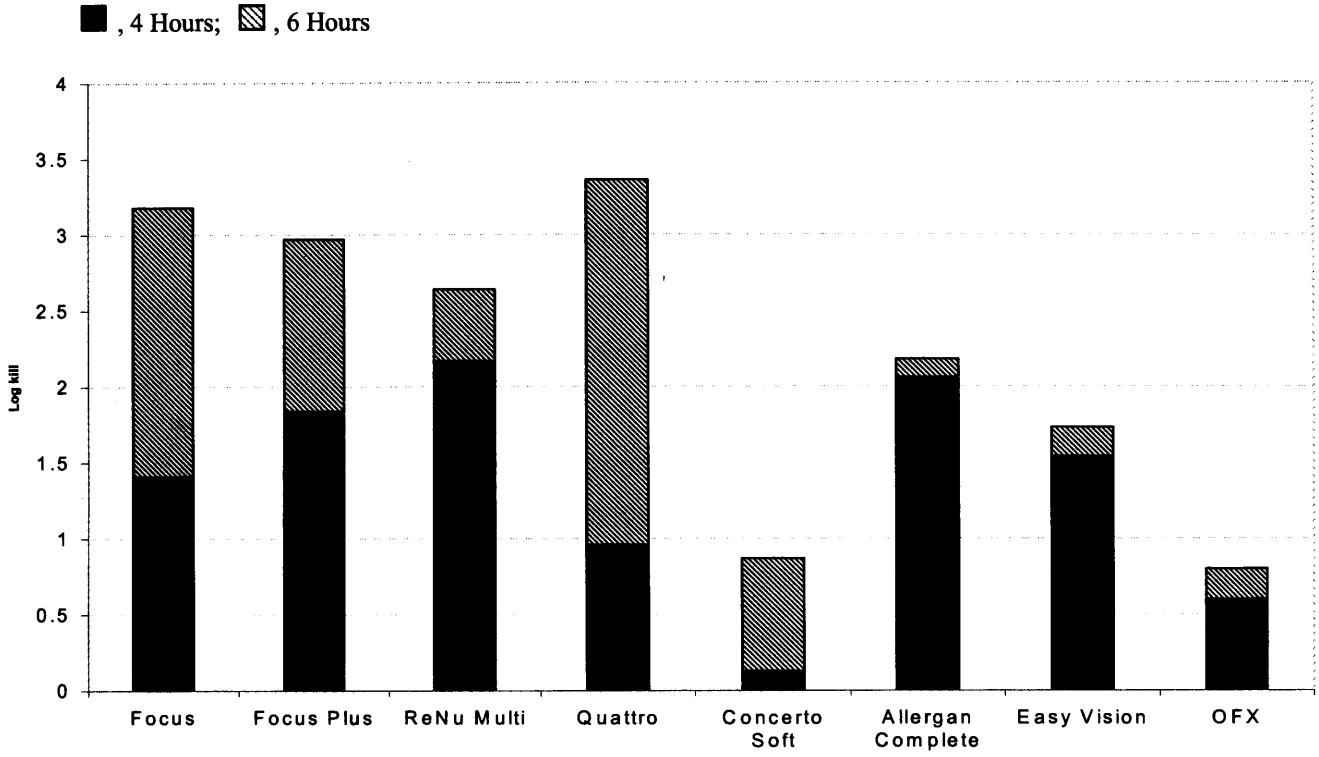

Figure 18: Activities of commercial multipurpose solutions against Ros cysts. After 4 h all of the solutions achieved greater than 1 log kill against Cole cysts with the exceptions of OFX and Concerto Soft. By 6 h all MPS solutions except OFX and Concerto Soft had achieved greater than 1.5 log kill.

The manufacturer's minimum recommended disinfection time is 4 hours for all of the solutions with the exception of Focus Plus, Allergan and Optifree Express® which are 10 minutes, 6 hours and 6 hours respectively (Table 12). Only 1 of the solutions achieved a 2 log kill by the manufacturer's minimum recommended disinfection time, ReNu Multi® achieved a log kill of 2.17 ± 0.1 by 4 hours.

The 0.13 ± 0.1 log kill achieved by Concerto Soft and the 0.6 ± 0.15 log kill achieved by Optifree Express after 4 hours was statistically significant from Focus, Focus Plus and ReNu ($p<0.05$) at this time, which achieved a 1.41 ± 0.41, 1.84 ± 0.29 and 2.17 ± 0.1 log kill respectively. Quattro achieved a 0.97 ± 0.12 log kill after 4 hours, which was also statistically significant from the 2.17 ± 0.1 log kill achieved by ReNu Multi.

With the exception of Concerto soft and Optifree Express, the solutions gave at least a 2 log (99 %) reduction after 6 hours contact time. Quattro achieved a 3.36 ± 0.56 log kill by this time which was total kill of the innoculum.

7.4.3 Activity of multipurpose solutions against Cole cysts

The activities of the commercial multipurpose solutions at 4 and 6 hours against Cole cysts, used according to the manufacturers' recommendations, are shown in Figure 19.

With this strain, 3 of the solutions achieved a 2 log kill by the manufacturer's minimum recommended disinfection time: ReNu Multi®, Focus and Allergan Complete gave a of 3.00 ± 0.51, 3.18 ± 0.25 and 3.28 ± 0.16 log kill respectively. Focus Plus achieved a log kill of 2.12 ± 0.75 by 4 hours, however, by 10 minutes, the recommended minimum disinfection time, the log kill achieved was 1.22 ± 0.15 (results not shown).

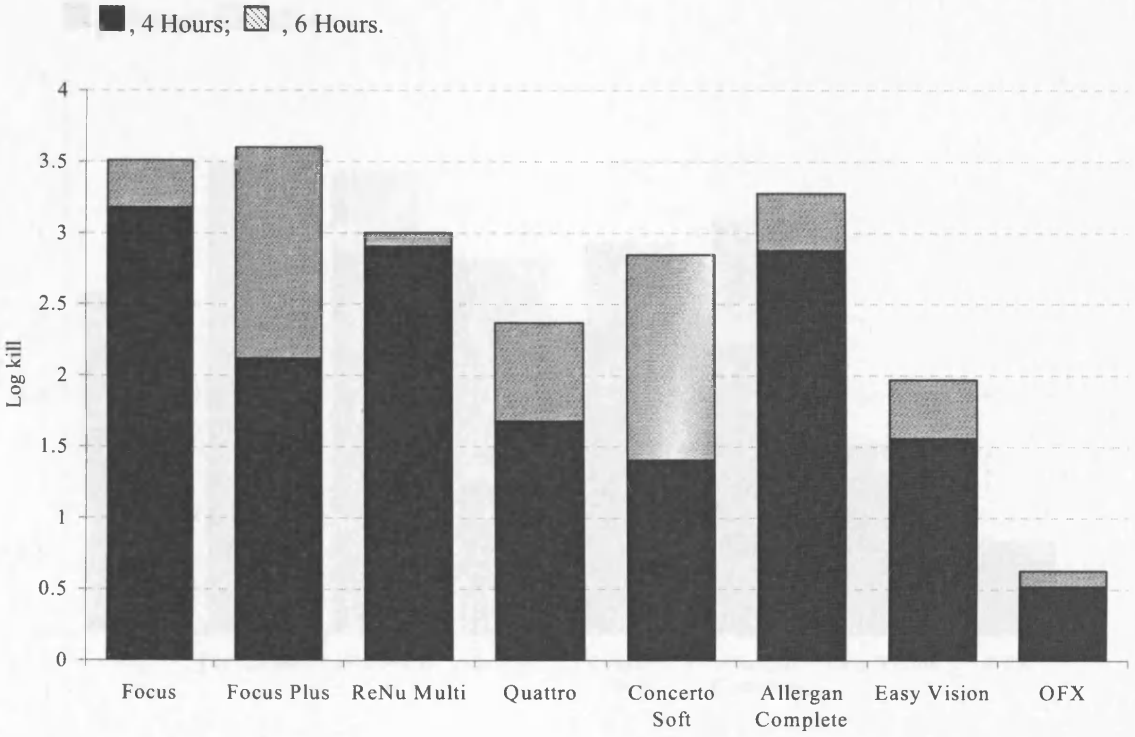

Figure 19: Activities of commercial multipurpose solutions against Cole cysts. After 4 h all of the solutions achieved greater than 1 log kill against Cole cysts except OFX. By 6 h all MPS solutions with the exception of OFX had achieved greater than 1.5 log kill.

The 3.18 ± 0.25 log kill achieved by Focus after 4 hours was statistically significant from Concerto soft and Optifree Express ® ($p<0.05$), which achieved 1.41 ± 0.15 and 0.52 ± 0.18 log kill respectively.

By 6 hours, all of the solutions had achieved a 2 log kill except for Easy vision and Optifree Express which achieved a 1.97 ± 0.12 and 0.63 ± 0.07 log kill respectively. The kill achieved by Optifree Express is significantly less than Allergan Complete, Focus and Focus Plus at this time ($p<0.05$).

7.4.4 Activity of multipurpose solutions against *A. culbertsoni* cysts

The activities of the commercial multipurpose solutions at 4 and 6 hours against *A. culbertsoni* cysts, used according to the manufacturers' recommendations, are shown in Figure 20.

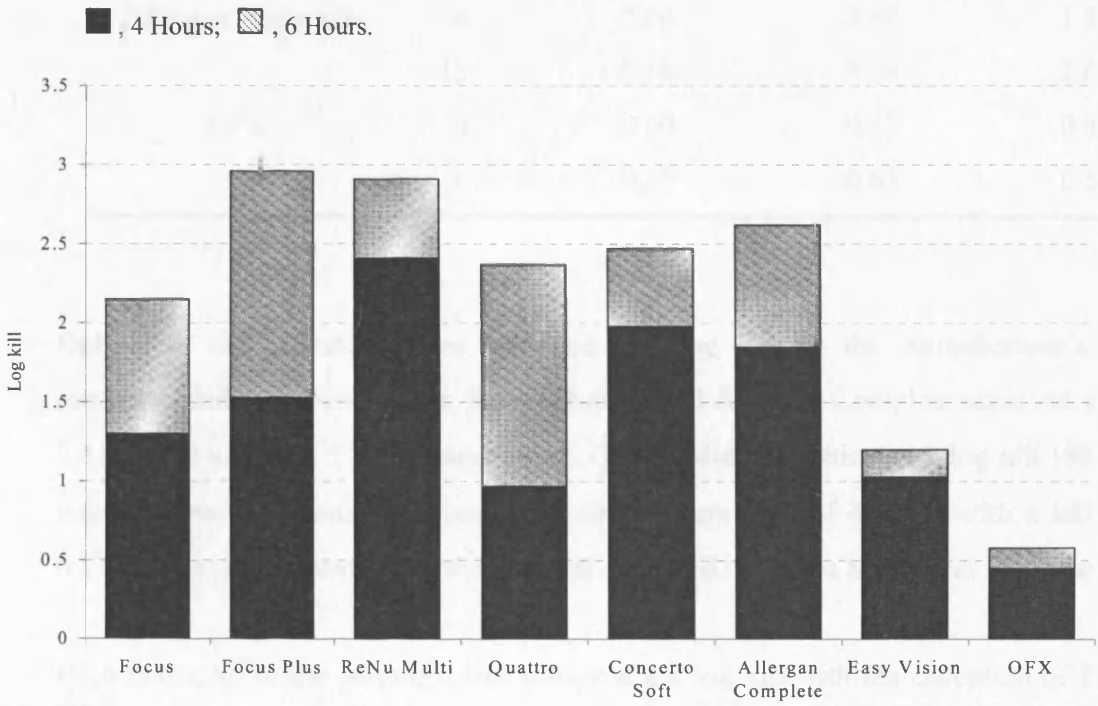

Figure 20: Activities of commercial multipurpose solutions against *A. culbertsoni* cysts. By 4 h all of the MPS achieved over 1 log kill apart from Quattro and OFX. By 6 h with the exception of OFX and Easy Vision all of the solutions achieved greater than 2 log kill.

Table 13: Mean log kill of multipurpose solutions against 3 *Acanthamoeba* strains

Solution	Hours	Mean log cyst kill		
		Ros	Cole	*Culbertsoni*
Focus	4	1.41	3.18	1.30
	6	3.18	3.51	2.15
Focus Plus	4	1.84	2.12	1.53
	6	2.97	3.60	2.96
ReNu Multi	4	2.17	2.91	2.41
	6	2.64	3.00	2.91
Quattro	4	0.96	1.68	0.97
	6	3.36	2.37	2.37
Concerto Soft	4	0.13	1.41	1.98
	6	0.87	2.85	2.47
Allergan Complete	4	2.06	2.88	1.85
	6	2.18	3.28	2.62
OFX	4	0.60	0.52	0.45
	6	0.80	0.63	0.58

Only 2 of the solutions tested achieved a 2 log kill by the manufacturer's minimum recommended disinfection time, ReNu Multi®, and Allergan Complete achieved a log kill of 2.41 ± 0.30 and 2.62 ± 0.20 respectively. Quattro did not achieve a 1 log kill (90 %) by the manufacturer's minimum recommended disinfection time of 4 hours with a kill of 0.97 ± 0.13 this was significantly less than the kill achieved by ReNu Multi ® at this time ($p< 0.05$).

By 6 hours, all of the solutions had achieved a 2 log kill with the exception of Easy vision and Optifree Express which achieved a 1.20 ± 0.37 and 0.58 ± 0.04 log kill respectively. The kills achieved by Easy vision and Optifree Express are significantly less than ReNu Multi, Concerto soft, Allergan Complete and Focus Plus ($p<0.05$) which achieved log kills of 2.80 ± 0.1, 2.4 ± 0.27, 2.62 ± 0.20 and 2.96 ± 0.37 respectively.

7.4.5 Scanning electron microscopy

Approximately twenty cysts were examined by electron microscopy. Figure 21 shows a viable mature Ros cyst before disinfection. The cyst is approximately 15 μm in diameter. Figure 22 shows a cyst after disinfection with ReNu Multi for 4 hours, there are holes perforated in the wall and the cyst appears hollow where the trophozoite within has been killed.

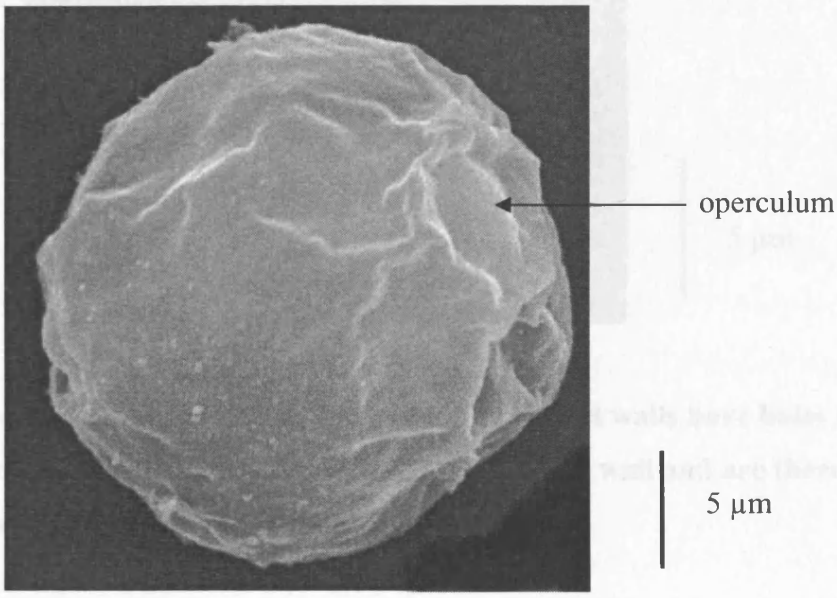

Figure 21: Mature Ros cyst before disinfection. An operculum can be seen on the right hand side of picture. The ectocyst is in tact.

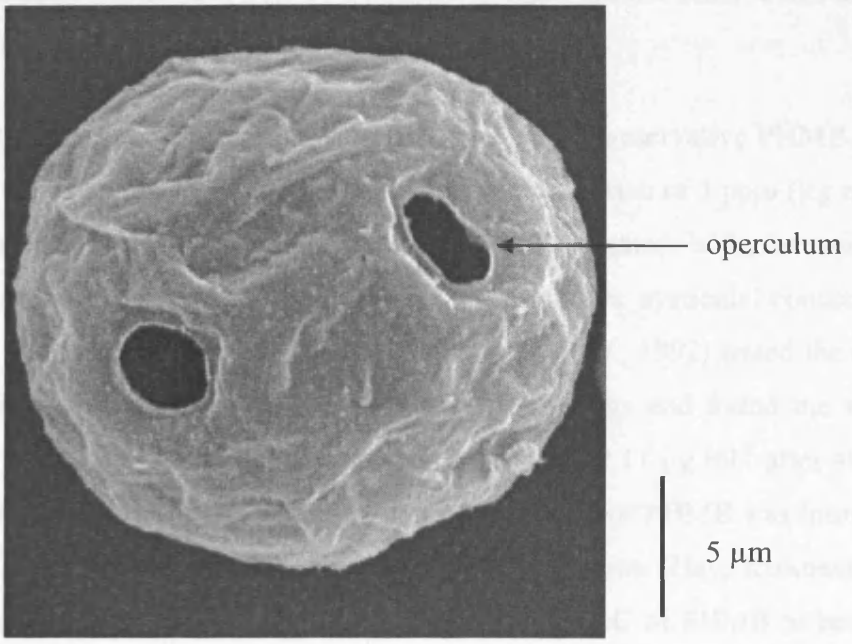

Figure 22: Non-viable Ros cyst after disinfection. The ectocst walls have holes in them where the opercula were. These are the thinnest parts of the wall and are therefore likely to be the site of action of disinfectants.

7.5 Discussion

Currently contact lens disinfecting solutions are required to achieve a 3 log reduction with each of three species of bacteria and a 1 log reduction with each of two species of fungi by the manufacturers' minimum recommended disinfection time (International Organization for Standardization 2000). However, it does not require solutions to be tested against *Acanthamoeba*.

Of the multipurpose solutions tested, none were able to achieve a 3 log reduction for all of the strains of cysts within the manufacturers' minimum recommended disinfection time. All of the solutions however, did achieve a 3 log kill against trophozoites by 4 hours. As cysts are more resistant to disinfection than trophozoites (Larkin, Kilvington *et al.,* 1992; Elder, Kilvington *et al.,* 1994; Hay and Seal 1994) this result is of less relevance. ReNu Multi achieved a 3 log kill against the Ros strain in the manufacturers' minimum recommended disinfection time of 4 hours, but did not achieve this with the other 2 strains. Allergan

Complete achieved a greater than 3 log kill against the Cole strain, in the manufacturers' minimum recommended disinfection time of 6 hours, but again did not achieve this kill with the Ros and *A. culbertsoni* strains.

With the exception of Optifree Express, all solutions contain the preservative PHMB, with a concentration of 5 ppm ($\mu g\ ml^{-1}$) in Concerto Soft and a concentration of 1 ppm ($\mu g\ ml^{-1}$) in each of the remaining solutions. Several studies have investigated both the minimum trophozoite amoebicidal concentration (MTAC) and the minimum cysticidal concentration (MCC) of PHMB. Larkin and colleagues (Larkin, Kilvington *et al.,* 1992) tested the *in vitro* sensitivities of corneal isolates of *Acanthamoeba* to various drugs and found the average MTAC of PHMB to be 0.87 $\mu g\ ml^{-1}$ and the average MCC to be 2.11 $\mu g\ ml^{-1}$ after 48 hours of exposure. In a later study, also with corneal isolates, the MTAC of PHMB was found to be 1 $\mu g\ ml^{-1}$ and the MCC to be 3 $\mu g\ ml^{-1}$ after 48 hours of exposure (Hay, Kirkness *et al.,* 1994). Another study published in the same year found the MTAC of PHMB to be 1.3 $\mu g\ ml^{-1}$ and the MCC to be 2.2 $\mu g\ ml^{-1}$ after 48 hours of exposure (Elder, Kilvington *et al.,* 1994).

It would therefore be expected that Concerto Soft which contains 5 $\mu g\ ml^{-1}$ of PHMB (5 ppm), would be the most cysticidal. However, the solution did not achieve a 3 log kill after 6 hours exposure with any of the strains tested. Concerto Soft also contains hexetidine, which is an antifungal agent. Perhaps the buffering agents in this solution do not enhance the antimicrobial activity of the PHMB, or actively work against it. The remaining solutions containing 1 μg of PHMB per ml (1 ppm) would have been expected to demonstrate less cysticidal activity against *Acanthamoeba.* In fact both ReNu Multi and Focus were able to produce a log reductions of 3.00 ± 0.51 and 3.18 ± 0.25 respectively within 4 hours against Cole. Allergan Complete also achieved a 3 log reduction within the manufacturers' minimum recommended disinfection time with a 3.28 ± 0.16 log kill in 6 hours.

A successful cysticidal disinfectant must gain access to the trophozoite within the cyst. The most likely route would be through the ostioles in the double cell wall, which allow the amoeba to communicate with its environment. Ostioles are plugged with mucopolysaccharide (Matsugo, Kasahara *et al.,* 1998), which would have to be weakened before allowing penetration of the disinfectant. It is though that PHMB, a highly positively charged molecule, binds to the mucopolysaccharide, resulting in penetration and permanent

damage to the cell membrane and the trophozoite within. Initially the damage caused by the PHMB is associated with the leakage of calcium ions from the plasma membrane (Beattie, Seal *et al.,* 2003). It has been suggested that the chelating agent hydranate, present in ReNu Multiplus potentiates this leakage, which would explain the enhanced cysticidal activity of this solution compared to the other solutions containing 1 ppm PHMB. Eventually the rapid chelation of the calcium ions and further leakage leads to the cellular protein disruption and irreversible damage. It has also been shown that the sodium borate and boric acid in ReNu Multiplus, can potentiate the activity of PHMB by twofold (Khunkitti, Lloyd *et al.,* 1996). However, EDTA, which is also present in ReNu Multiplus at 0.1%, has been found to inhibit activity fourfold at this concentration (Khunkitti, Lloyd *et al.,* 1996).

Several studies have been carried out on the amoebicidal activity of Optifree Express, which contains MAPD and Polyquad (Rosenthal, Buck *et al.,* 1999; Rosenthal, McAnally *et al.,* 2000). After an exposure time of 6 hours, Optifree Express was found to produce log reductions in viability of 1.3 to 4.8 for trophozoites and 2 to 3.2 for cysts of various species and strains of *Acanthamoeba.* In another study using different strains of *Acanthamoeba,* including *A. castellanii* and *A. polyphaga,* Optifree Express achieved log reductions kills of approximately 4 for trophozoites and 2 to 3 for cysts after the manufacturers' minimum recommended disinfection time (Kilvington 1998).

The efficacy of Optifree Express against cysts of *A. polyphaga* produced by different methods; Neff's constant pH encystment medium and NNA plates seeded with *Escherichia coli,* and also at different stages of maturity found that a log reduction of only 0.5 was achieved in 6 h with the mature cysts (Kilvington and Anger 2001). Mowrey-McKee (Mowery-McKee, 2002) also demonstrated that Optifree Express had a limited amoebicidal effect after the 6 hours when it was tested against a strain of *A. castellanii,* giving log reductions of 2.5 and 0.5 for trophozoites and cysts, respectively. The results in this study correlate with these findings, with log kills ranging from 0.58-0.63 after 6 h of disinfection.

However, studies in our lab of the cysticidal activity of Optifree Express with only Polyquad as the antimicrobial agent show that the amoebicidal activity is greatly reduced (results not shown). This implies that the majority of the cysticidal activity of this solution is due to the addition of the MAPD. As the introduction of PHMB, either alone or in combination therapy, has dramatically improved the prognosis for acanthamoeba keratitis, MAPD may have a potential for use as a therapeutic agent for acanthamoeba keratitis sufferers.

8 Antimicrobial Activity of MAPD

8.1 Introduction

The first successful medical treatment of acanthamoeba keratitis was reported by Wright and colleagues in 1985 at Moorfields Eye Hospital, London using topical application of the diamidine compound propamidine isethionate (Wright, Warhurst *et al.,* 1985). Subsequent, successes have been reported using this agent alone or in combination with the aminoglycoside neomycin (Moore & Mc Culley, 1989) and with imidazole derivatives miconazole (Lindquist, Doughman *et al.,* 1988; Ishibashi, Matsumoto *et al.,* 1990), clotrimazole (Ficker, Seal *et al.,* 1990), ketoconazole (Cohen, Parlato *et al.,* 1987) and itraconazole (Ishibashi, Matsumoto *et al.,* 1990). However, the rationale for the use of many of these agents is not supported by *Acanthamoeba* sensitivity testing, which has shown that whilst aminoglycoside and imidazole agents are active against the trophozoites, the cysts are markedly resistant (Wright, Warhurst *et al.,* 1985; Kilvington, Larkin *et al.,* 1990; Larkin, Kilvington *et al.,* 1992; Lim, Coster *et al.,* 2000). Therapy with these agents may serve to eradicate trophozoites but the cysts may remain viable and so account for the grumbling nature of the disease and the need for prolonged medical treatment (Larkin, Kilvington *et al.,* 1992; Elder, Kilvington *et al.,* 1994).

Although propamidine isethionate can be effective in the medical cure of acanthamoeba keratitis, failures are frequent because of its variable cysticidal activity, development of resistance during therapy (Ficker, Seal *et al.,* 1990; Elder, Kilvington *et al.,* 1994; Hay, Kirkness *et al.,* 1994), stimulation of trophozoite encystment, (Kilvington, Larkin *et al.,* 1990) and epithelial toxicity (International Organization for Standardization 2000). A significant advance in the medical treatment of acanthamoeba keratitis came as the result of the observed cysticidal activity of the polymeric biguanide, polyhexamethylene biguanide (PHMB). Topical administration of a 0.02 % solution of PHMB was first used by Larkin and colleagues in 1992 to cure 5 cases of infection that were unresponsive to conventional therapy based on propamidine isethionate or antifungal agents (Larkin, Kilvington *et al.,* 1992).

The introduction of PHMB, either alone or in combination therapy, has dramatically improved the prognosis for acanthamoeba keratitis and several studies have confirmed its *in vitro* and *in vivo* superiority over other treatments (Larkin, Kilvington *et al.,* 1992; Burger, Franco *et al.,* 1994; Gray, Gross *et al.,* 1994; Duguid, Dart *et al.,* 1997; Lim, Coster *et al.,* 2000). More

recently, topical chlorhexidine digluconate (0.02 %) has been shown to also have good cysticidal activity and has been used in the successful treatment of the infection (Radford, Minassian *et al.,* 1998). Other diamidine homologues of propamidine have been studied. Hexamidine di-isethionate has been shown to have greater cysticidal activity than propamidine and has been used alone and in combination therapy with PHMB or chlorhexidine to treat acanthamoeba keratitis (Brasseur, Favennec *et al.,* 1994; Perrine, Chenu *et al.,* 1995).

Despite these advances in the treatment of acanthamoeba keratitis, failures still occur necessitating surgical intervention and prolonged and intensive medical therapy (Larkin, Kilvington *et al.,* 1992; Bacon, Frazer *et al.,* 1993; Murdoch, Gray *et al.,* 1998). This can culminate in permanent loss of visual acuity or, ultimately, enucleation of the eye (Bacon, Frazer *et al.,* 1993; Duguid, Dart *et al.,* 1997).

8.2 Aims

Previous results have shown that Opti-Free® Express® exerts some cysticidal activity against *A. polyphaga* (Buck, Rosenthal *et al.,* 2000; Kilvington, Hughes *et al.,* 2002). This is due to the addition of a new antimicrobial agent (myristamidopropyl dimethylamine, MAPD). To address the need for improved therapeutic agents work in this chapter concentrates on the cysticidal activity of the cationic amidoamine MAPD against *Acanthamoeba* strains recalcitrant to conventional medical therapy. Once the formula was optimised against *A. polyphaga*, the activity against fungi and bacteria was also investigated.

8.3 Materials and Methods

8.3.1 Strains and culture.

Nine clinical isolates of *Acanthamoeba*, eight of which showed poor response to conventional medical therapy, were studied. The strains were isolated and grown on nonnutrient agar seeded with *Escherichia* coli (NNA-*E. coli*) in air at 32 °C for 7 days (Kilvington and White 1994). Trophozoites were then adapted and maintained in a semi-defined axenic broth medium as previously described (Hughes and Kilvington 2001).

For the minimum cysticidal (MCC) studies, cysts were obtained from prolonged incubation of the trophozoites on NNA-*E. coli* in air at 32 °C for 7 days (Kilvington and White 1994). In the time-kill studies with MAPD, cysts were obtained by growing trophozoites in the semi-defined axenic medium supplemented after autoclaving with filter sterilised 50 mM $MgCl_2$ (#6-Mg medium).

Other ocular pathogens studied were *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538), *Candida albicans* (ATCC 10231) and *Fusarium solani* (ATCC 36031) all obtained from the from the American Type Culture Collection, Rockville, USA. All strains were cultured on appropriate agar media (Chapter 2).

8.3.2 Antimicrobial agents.

The following therapeutic and experimental agents were studied: propamidine isethionate 0.1 % (Brolene® Rhone-Poulenc Rorer, England), hexamidine di-isethionate 0.1% (Desomedine®, Chauvin Laboratory, Montpellier, France), PHMB 0.02 % (Moorfields Eye Hospital Pharmacy, London, England), chlorhexidine digluconate 0.02 % (Moorfields Eye Hospital Pharmacy, London, England) and MAPD 0.01% (Alcon Laboratories Inc., Fort Worth, USA).

8.3.3 Minimum cysticidal concentration (MCC) assay.

This was investigated for the *Acanthamoeba* cysts (Chapter 2).

8.3.4 Challenge test assays.

The activity of MAPD against *A. polyphaga* (Ros) #6-Mg cysts was studied in detail. The method used to determine the kinetics of cyst killing was as described previously (Hughes & Kilvington, 2001). MAPD was dissolved and diluted in 2 mM tris.HCl, pH 7.2 for testing at 5-100 $\mu g\ ml^{-1}$. Tris.HCl was chosen as the diluent as it was found that MAPD was difficult to dissolve in deionised water or ¼ Ringer's solution. The assay was carried out in 20 ml glass universal bottles (Bibby-Sterilin, Staffordshire, England) and measured at time intervals of 0, 1, 2, 4,6 and 8 hours.

In additional studies, the effect of test bottle material on MAPD activity against *A. polyphaga* (Ros) was investigated in glass (Bibby-Sterilin UC/30), polypropylene (Nunc 373687) and

polystyrene (Bibby-Sterilin 128C). The activity of MAPD in glass at 50 μg ml^{-1} was then studied against the cysts of all *Acanthamoeba* strains.

The bacterial and fungi strains were tested against the MAPD solution in accordance with the internationally approved method used for the evaluation of the efficacy of contact lens solutions against ocular pathogens (International Organization for Standardization, 2000). Viable bacterial and fungal numbers were determined by making serial 10-fold dilutions of organism in triplicate across the rows of a microtitre plate. Fifty microlitres of each dilution was then inoculated as three drops, in triplicate, on appropriate agar culture media. After allowing the liquid to absorb, the plates were incubated in air at 32 °C for 48 hours.

8.3.5 Data Analysis

The number of surviving cysts in the time-kill studies was determined using Reed and Muench computations (Chapter 2)

8.4 Results

8.4.1 Minimum cysticidal concentration (MCC)

The activity of the therapeutic agents and MAPD against the nine acanthamoeba keratitis isolates is shown in Figure 23. MCCs for MAPD were 6.25 – 25 μg ml^{-1} (16.0 ±2.4 μg ml^{-1}). Those for PHMB were 1.6 – 6.75 μg ml^{-1} (3.2 ±0.47 μg ml^{-1}) and chlorhexidine 15.6 – 31.25 μg ml^{-1} (26.7 ±1.7 μg/ml). The values for propamidine were ≥500 μg ml^{-1} (5 strains 500 μg ml^{-1}) and hexamidine 250-500 μg ml^{-1} (361 ±44 μg ml^{-1}). The difference in activity between propamidine and hexamidine for the strains was statistically significant ($p<0.001$). No statistical differences were found between PHMB, chlorhexidine and MAPD ($p>0.05$).

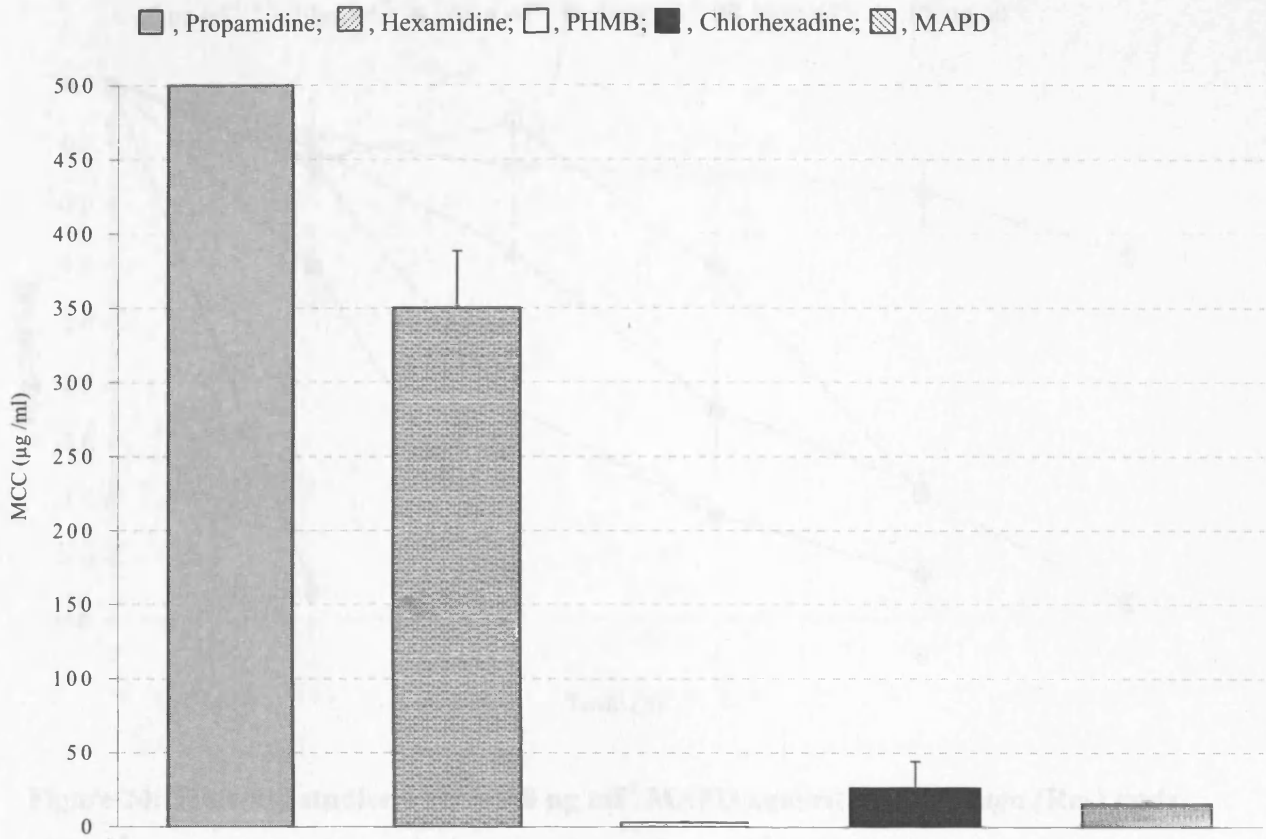

Figure 23: Average minimum cysticidal concentration (MCC) of therapeutic agents and MAPD against nine acanthamoeba keratitis isolates. The results for Hexamidine and Propamidine are very high, 350 µg/ml and 500 µg/ml respectively. The MCCs for PHMB, MAPD and CLX were all less than 50 µg/ml.

8.4.2 MAPD cysticidal assays

The kinetics of MAPD killing of *A. polyphaga* (Ros) cysts with 5-100 µg ml^{-1} is shown in Figure 24. Maximum log kill with 5 µg ml^{-1} was 1.44 ± 0.17 log at 8 hours; 10 µg ml^{-1} 4.35 ±0.09 at 8 hours; 20 µg ml^{-1} 3.38 ± 0.08 at 6 hours; 30 µg 4.3 ±0.11 at 6 hours or 3.62 ±0.56 at 4 hours; 50 µg ml^{-1} 4.08 ±0.34 at 2 hours; and 100 µg ml^{-1} 4.23 ±0.11 at 1 hour.

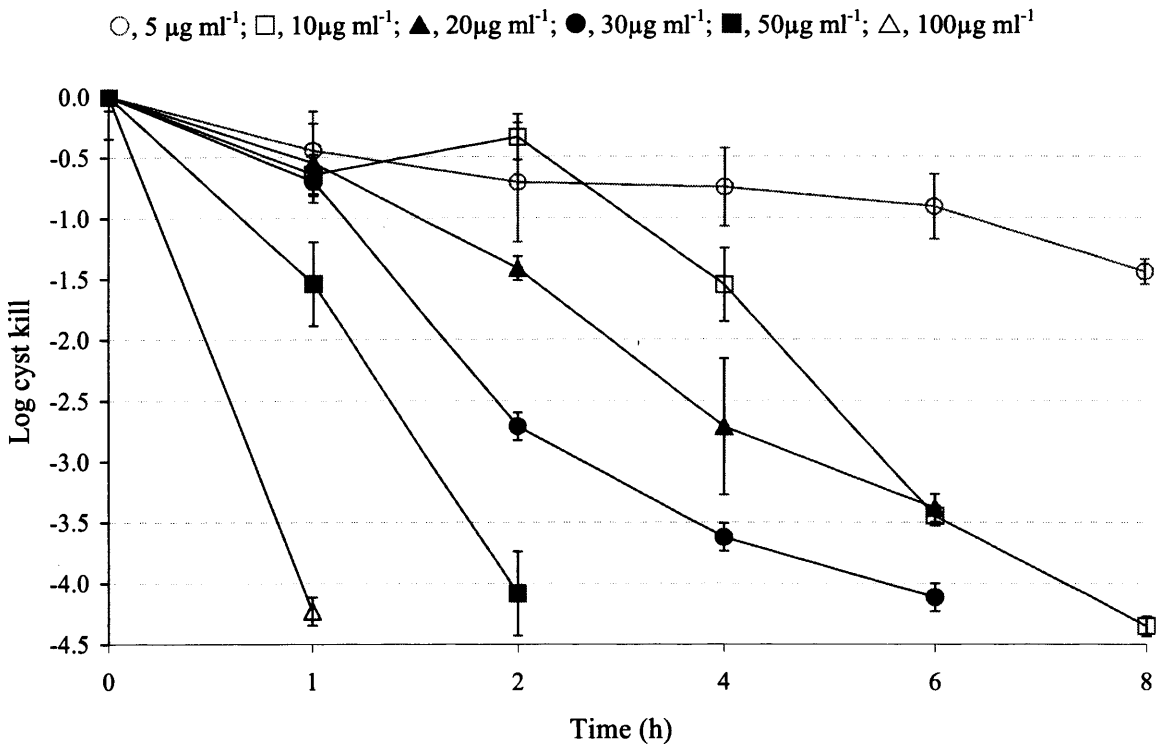

Figure 24: Time-kill studies with 5-100 μg ml^{-1} MAPD against *A. polyphaga* (Ros) cysts. AlThe time kill shows that with the exception of 5 μg ml^{-1} MAPD, all of the solutions achieved greater than 3 log kill in 6 hours.

Loss of MAPD activity was observed when assays were performed in polypropylene and polystyrene tubes (Figure 25). In glass, 10 μg ml^{-1} gave 3.44 ±0.48 log kill of *A. polyphaga* (Ros) at 6 hours compared to 1.57 ±0.37 in polypropylene and 1.96 ±0.14 in polystyrene. The decreased activity in polypropylene and polystyrene compared to glass is significant at this time point and also for polypropylene but not polystyrene at 8 hours ($p<0.05$).

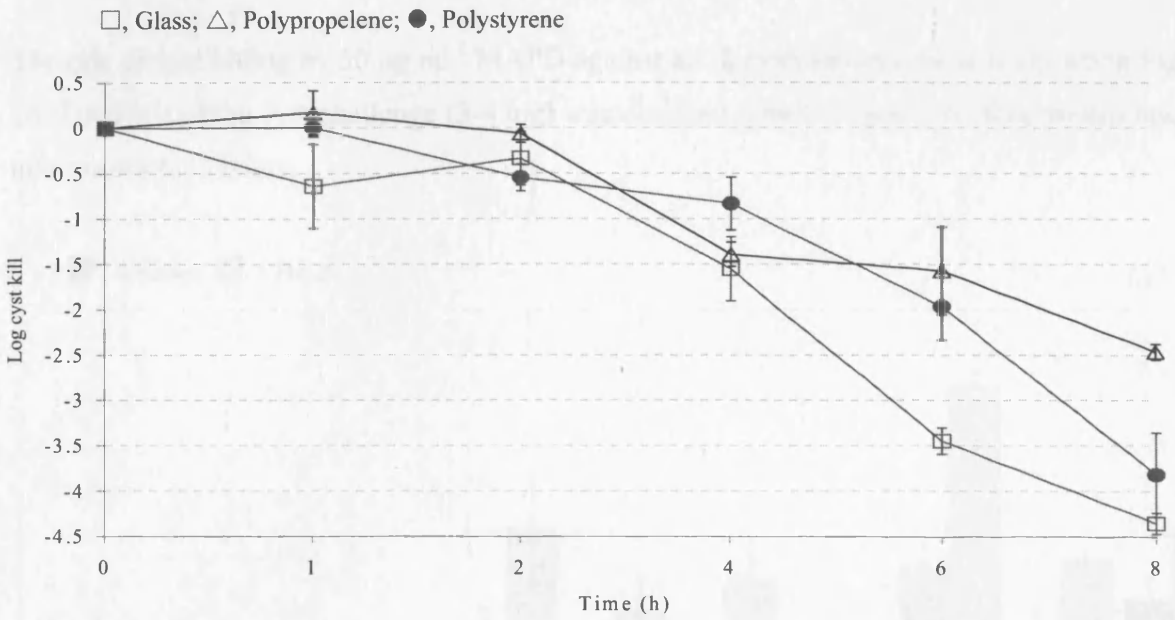

Figure 25: Comparison of MAPD cysticidal activity at 10 µg ml^{-1} against *A. polyphaga* (Ros) tested in glass, polypropylene and polystyrene bottles. MAPD in the glass bottles gave 3.5 log kill after 6 h exposure. In polypropelene tubes the least kill was seen with approximately 1.5 log kill in 6 h, compared with nearly 2 log kill in polystyrene tubes.

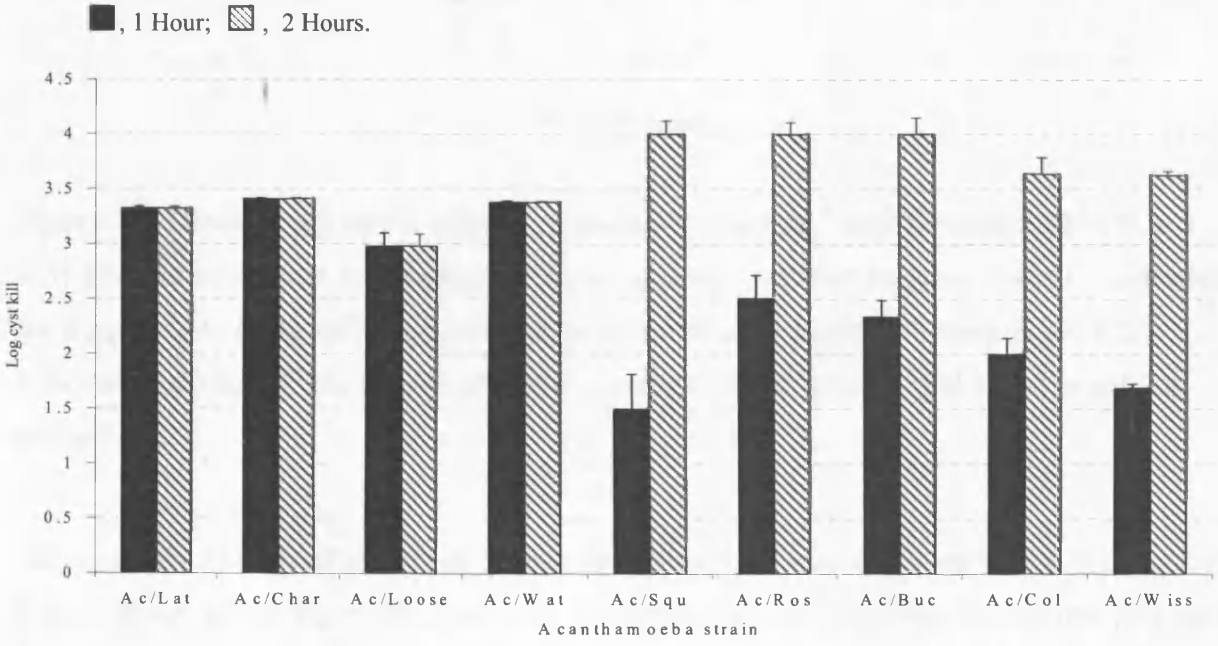

Figure 26: Log cyst kill for nine acanthamoeba keratitis strains exposure to 50 µg ml^{-1} MAPD. Cyst kill after 1 h exposure ranged from 1.5 log tonearly 3.5 log. After 2 h disinfection cyst kill varied from 3 log to 4 log.

The rate of cyst killing by 50 μg ml^{-1} MAPD against all *Acanthamoeba* stains is shown in Figure 26. Total kill of the cyst challenge (3-4 log) was obtained within 1 hour with four strains and all nine strains by 2 hours.

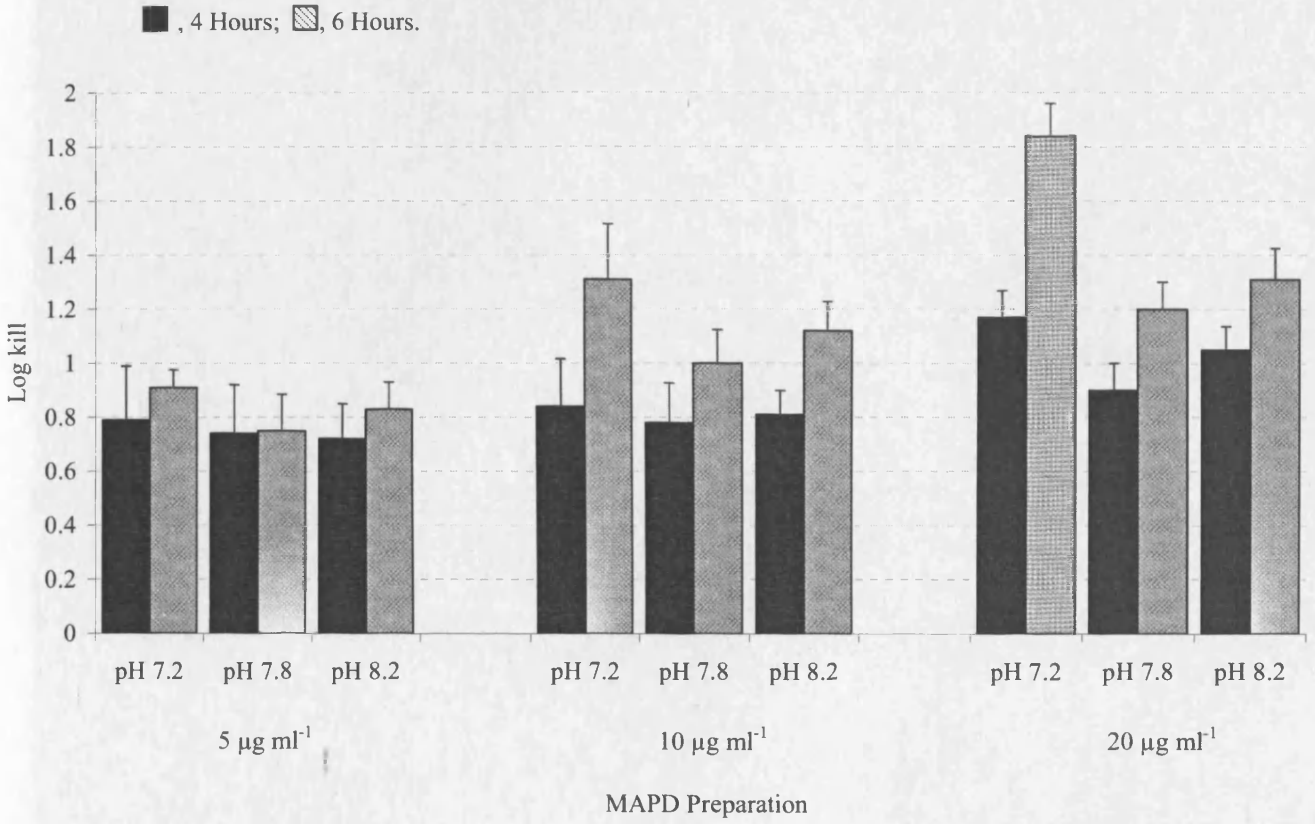

Figure 27: Effects of pH on the efficacy of 5μg ml^{-1}, 10 μg ml^{-1} and 20 μg ml^{-1} MAPD in 2 mM Tris buffer against *A. polyphaga*. There was little variation between the pHs measured for 5 μg ml^{-1}. At 10 μg ml^{-1} more kill was achieved at pH 7.2 after 6 h than at pH 8.2. Likewise at 20 μg ml^{-1} the log kill after 6 h exposure was greater for pH 7.2 than pH 7.8 and pH 8.2.

The cysticidal activity of MAPD at concentrations of 5, 10 and 20 μg ml^{-1} were compared at three different pHs in figure 27. At 4 hours, the difference in kill between the different pHs were not significantly different for any of the concentrations tested ($p>0.05$). At 6 hours there was no significant difference in kill with the 5 μg ml^{-1} or 10 μg ml^{-1} MAPD solutions between the different pHs ($p>0.05$). At a concentration of 20 μg ml^{-1} there was significantly greater kill at pH 7.2 than at 7.8 or 8.2 ($p<0.05$).

■, *P. aeruginosa*; △, *S. aureus*; ●, *C. albicans*; □, *F. solani.*

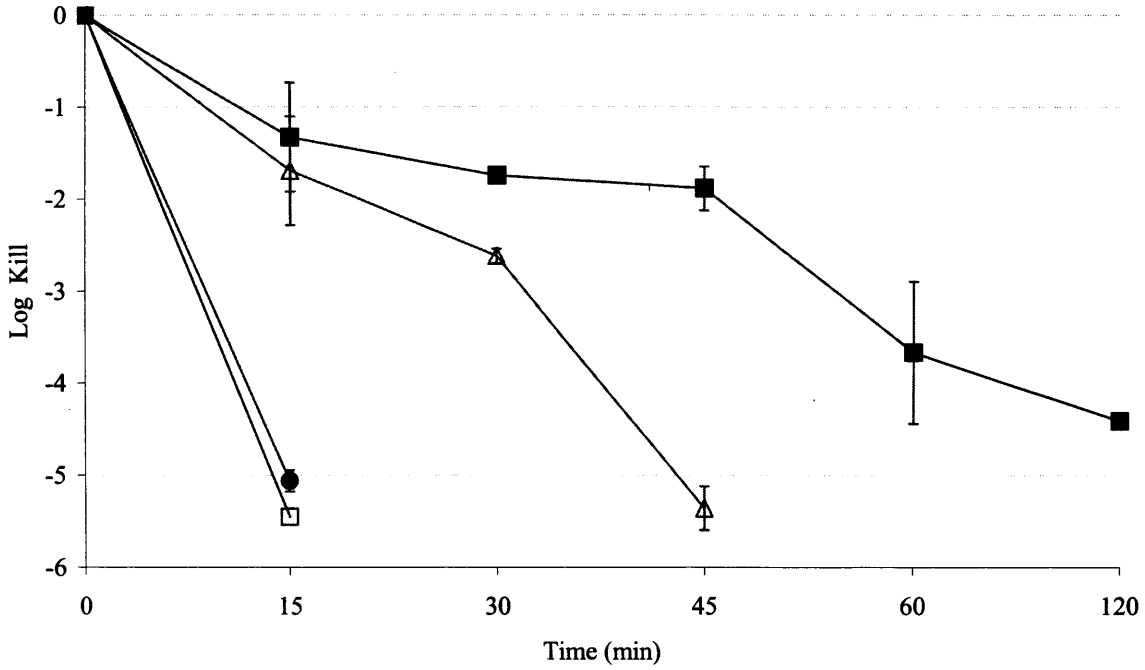

Figure 28: Activity of 50 µg ml^{-1} MAPD at pH 7.2 against bacterial and fungal pathogens. Total kill was achieved against *F. solani* and *C. albicans* by 15 min and for *S. aureus* by 45 min. A total log kill of *P. aeruginosa* was achieved by 2 h.

The activity of MAPD against the test organisms is shown in Figure 28. MAPD at 50 mg/l gave a 3.67 ±0.77 log kill of *P. aeruginosa* after 60 minutes and 4.41 ±0.08 log after 120 minutes. With *S. aureus*, a 5.36 ±0.08 log reduction occurred by 45 min. For *C. albicans* and *F. solani*, a 5.05 ±0.12 and 5.45 ±0.07 log kill respectively, resulted by the first time point of 15 minutes. No decline in viability was found in the control experiments using one quarter Ringer's solution (results not shown).

8.5 Discussion

Acanthamoeba keratitis is a rare but potentially devastating corneal infection. Increased awareness of the condition, improvements in diagnostic techniques and the advent of modern effective anti-acanthamoebal therapy have greatly improved the prognosis for the infection (Larkin, Kilvington *et al.,* 1992; Bacon, Frazer *et al.,* 1993; Seal, Hay *et al.,* 1996). However, a number of cases show a protracted clinical course with corneal and scleral inflammation that culminates in severe visual loss in some patients (Bacon, Frazer *et al.,* 1993; Duguid, Dart *et al.,* 1997). Penetrating keratoplasty is often required to rehabilitate the sight but reinfection can occur in up to 50 % of grafts (Bacon, Frazer *et al.,* 1993). In severe cases, the only recourse is enucleation of the eye. Although the introduction of PHMB and chlorhexidine has dramatically improved the treatment of acanthamoeba keratitis, relapse with continued culture positive isolation of *Acanthamoeba* occurs in up to 10% of patients (Elder, Kilvington *et al.,* 1994; Duguid, Dart *et al.,* 1997; Murdoch, Gray *et al.,* 1998). Therefore the search for improved therapeutic agents for acanthamoeba keratitis continues. A new antimicrobial agent (myristamidopropyl dimethylamine, MAPD) in Opti-Free® Express® Multi-Purpose Disinfecting Solution multi-purpose disinfecting solution for contact lenses has been shown to exhibit *Acanthamoeba* cysticidal activity and was, therefore, evaluated here as a potential therapeutic agent (Buck, Rosenthal *et al.,* 1998).

In this study, the *in vitro* sensitivity of nine acanthamoeba keratitis isolates was compared, eight of which were from separate cases considered to be medical failures following prolonged treatment with PHMB or chlorhexidine digluconate in combination with propamidine isethionate and hexamidine di-isethionate. In vitro drug sensitivity testing showed the strains to be resistant to propamidine isethionate with MCCs of ≥500 $\mu g\ ml^{-1}$ and, to a lesser extent, hexamidine di-isethionate with a MCC of 250-500 $\mu g\ ml^{-1}$ (361 ±44 $\mu g\ ml^{-1}$). The greater cysticidal activity of hexamidine di-isethionate over propamidine isethionate has been noted previously and used in the successful treatment of the infection (Brasseur, Favennec *et al.,* 1994; Perrine, Chenu *et al.,* 1995). However, all strains were sensitive to PHMB 1.6 – 6.75 $\mu g\ ml^{-1}$ (3.2 ±0.47 $\mu g\ ml^{-1}$), chlorhexidine digluconate 15.6 - 31.25 $\mu g\ ml^{-1}$ (26.6 ±1.7 $\mu g\ ml^{-1}$) and MAPD 6.25 – 25 $\mu g\ ml^{-1}$ (16.0 ±2.4 $\mu g\ ml^{-1}$).

Accordingly, there is a poor correlation between in vitro MCC findings and patient response to these therapeutic agents as has been observed previously (Elder and Dart 1995). Reasons for the poor prognosis in patients infected with *Acanthamoeba* that appear sensitive *in vitro* to therapeutic agents are unclear. However, poor penetration of the drugs into the cornea, differences in host immune response and *Acanthamoeba* strain virulence are possibilities. In previous studies where *Acanthamoeba* isolates have been tested against therapeutic and experimental agents, conflicting findings particularly with respect to propamidine isethionate have been found (Kilvington, Larkin *et al.,* 1990; Lim, Coster *et al.,* 2000). In one study, the MCCs for 10 strains against propamidine isethionate were <10 μg ml^{-1} (Lim, Coster *et al.,* 2000) compared to 1.9 – 500 μg ml^{-1} (mean 46 μg ml^{-1}) for 23 strains (Elder, Kilvington *et al.,* 1994) and <6.25 – 50 μg ml^{-1} for 13 strains (Hay, Kirkness *et al.,* 1994). This is in contrast to the findings reported here where all strains showed a propamidine isethionate MCC of ≥500 μg ml^{-1}. One possible explanation for these observed differences is that 8 of these strains were unusual in that they were clinically resistant, as shown by repeatedly positive corneal cultures after weeks of treatment with both biguanides and diamidines; diamidine resistance could be a characteristic of this resistance pattern. Another possible explanation is that the cysts in this study were exposed to the drug for 24 hours compared to 2 or 7 days in the other studies (Elder, Kilvington *et al.,* 1994; Hay, Kirkness *et al.,* 1994; Lim, Coster *et al.,* 2000). In contrast to the findings of this study, Lim and colleagues found the MCCs for PHMB and chlorhexidine digluconate ranged from 16 - 256 μg ml^{-1} after 7 days exposure to the drugs (Lim, Coster *et al.,* 2000). The reasons for this difference are unclear but our findings are in accord with those reported in previous studies with PHMB and chlorhexidine digluconate (Larkin, Kilvington *et al.,* 1992; Elder, Kilvington *et al.,* 1994; Hay, Kirkness *et al.,* 1994).

MAPD is a cationic amidoamine known also as stearamidopropyl dimethylamine, N-[3-(dimethylamino) propyl] octadecanamide or N,N-dimethyl-N'-tetradecanoyl-1,3-propylenediamine. It is present at 5 μg ml^{-1} in the contact lens disinfecting system Opti-Free® Express® Multi-Purpose Disinfecting Solution (Alcon Laboratories, Inc.). The *Acanthamoeba* cysticidal activity of the disinfection system has been reported previously and this was the rationale behind the selection of MAPD for detailed investigation in this study (Buck and Rosenthal 1996). Here, MAPD in 2 mM tris.HCl, pH 7.2 showed a MCC of 6.25 - 25 μg ml^{-1} (16.0 ±2.4 μg ml^{-1}) for the strains using a test inoculum of 1 x 10^4 cysts in the microtitre plate assays and an exposure time of 24 hours. At 50 μg ml^{-1} MAPD gave total cyst kill within 1-2

hours following a challenge inoculum of 3-4 log organisms in the time-kill studies. The efficacy of MAPD against bacteria and fungi associated with keratitis was also demonstrated, with a greater than 4 log kill achieved within 2 hours.

Unlike the requirements for efficacy testing of contact lens disinfectants against bacteria and fungi, no standard protocol exists for *Acanthamoeba* cysts (International Organization for Standardization 2000). The activity of MAPD appeared decreased when testing was conducted in polypropylene and polystyrene compared to glass. MAPD is known to adsorb to certain plastics resulting in a rapid loss of activity from the solution (Van Duzee and Schlech 1999). Accordingly, the type of material in which the assays are conducted is an important consideration when determining the cysticidal activity of MAPD. Factors such as test material, organism strain and method of cyst production may have significant influence on the results obtained for efficacy testing of therapeutic and disinfectant agents against *Acanthamoeba* and further studies are required to standardise such assays for this organism (Buck, Rosenthal *et al.*, 2000).

The mode of action of MAPD and therapeutic agents against *Acanthamoeba* is not known. In bacteria, PHMB and chlorhexidine cause cytoplasmic membrane damage resulting in an irreversible loss of essential cellular components following binding to the cell wall (Chawner and Gilbert 1989). The alternative chemical structure and low molecular weight of MAPD (mol wt 300) compared to PHMB (average mol wt 2340) and chlorhexidine digluconate (mol wt 898) might permit better penetration into the cornea to achieve therapeutic levels. The findings of this study indicate that MAPD is an effective *Acanthamoeba* cysticidal compound and may represent an improved agent in the treatment of acanthamoeba keratitis and, other forms of microbial keratitis.

9 Development of Resistance

9.1 Introduction

Resistance of pathogens to chemotherapy first appeared when antibiotics were introduced into clinical practice, as early as 1917 (Fleming 1929). The ability of bacteria to survive and multiply in the presence of drug concentrations at therapeutic dosage leads to the presence of resistant populations against therapies.

Resistance typically occurs through a spontaneous mutation in the bacterial chromosome, which imparts resistance to a member of the bacterial population. In the selective environment of the antibiotic, the wild type (non mutants) are killed and the resistant mutant is allowed to grow and flourish. As the mutation rate for most bacterial genes is approximately 10^8, if a bacterial population doubles from 10^8 cells to 2×10^8 cells, there is likely to be a mutant present for any given gene. Since bacteria grow to reach population densities far in excess of 10^9 cells, such a mutant could develop from a single generation during 15 minutes of growth. Resistance acquired through mutation will be confined to the mutant clone and emergence and spread will depend on the clone's ability to multiply and infect new hosts, it is known as vertical transmission.

Resistance can also be passed from one organism to another; facilitated by transduction, transformation, and conjugation (Garcia-Castellanos, Mallorqui-Fernandez *et al.,* 2004), this is known as horizontal transmission. Conjugation involves cell-to-cell contact as DNA crosses a sex pilus from donor to recipient. During transduction, a virus transfers the genes between mating bacteria. In transformation, DNA is acquired directly from the environment, having been released from another cell. Genetic recombination can follow the transfer of DNA from one cell to another leading to the emergence of a new genotype (recombinant). Often DNA is transferred as plasmids between mating bacteria. Since bacteria usually develop their genes for drug resistance on plasmids (resistance transfer factors, or RTFs), they are able to spread drug resistance to other strains and species during genetic exchange processes.

As pathogens increasingly acquire resistance, against antibiotics via plasmids, transposons, and bacteriophages, the problem has now grown to such an extent that the World Health

Organisation has targeted antibiotic resistance as one of the major emerging public health problems (WHO. 2001).

Resistance to therapy has been observed in several protozoa including; *Plasmodium* spp. (Vathsala, Pramanik *et al.,* 2004), *Leishmania* spp. (Thakur, Kanyok *et al.,* 2000), *Giardia* spp. (Wright, Dunn *et al.,* 2003) and *Toxoplasma* spp. (McFadden, Camps *et al.,* 2001). There have also been reported cases of resistance of *Acanthamoebba* developing during therapy (Ficker, Seal *et al.,* 1990; Ledee, Seal *et al.,* 1998). It has been shown that a single *Acanthamoeba* strain developed propamidine resistance during therapy and that the resistance wasn't due to a second strain as only one strain was isolated from the infection. Although a drug-resistant mutant or subpopulation may have been present at the time of infection the significant increase in propamidine resistance was a response to therapy (Ledee, Seal *et al.,* 1998). However, it is not known by what mechanism the amoebae develop resistance or whether this resistance is a stage of encystment of the amoebae in the eye.

9.2 Aims

As treatment failures regularly occur in patients with strains that have previously been shown *in vitro* to be susceptible to treatment, this chapter looks at the development of resistance in *Acanthamoeba.*

9.3 Materials and Methods

9.3.1 Acanthamoeba strains

A. polyphaga (Ros-02 strain) was used throughout the study. Log phase trophozoites were harvested immediately before testing. Cysts were prepared by 7 days incubation at 30 °C in the semi-defined axenic medium supplemented after autoclaving with filter sterilised 50 mM $MgCl_2$ (#6-Mg) and sonicated before use (Chapter 2).

9.3.2 Test solutions

The following experimental agents were studied: 1.5 % hydrogen peroxide (Sigma Chemical Company, Dorset, England), polyhexamethylene biguanide (PHMB) at 2 μg ml^{-1} in H_2O (Bausch & Lomb, Rochester, USA), chlorhexidine digluconate (CLX) at 15 μg ml^{-1} in H_2O (Sigma Chemical Company, Dorset, UK), the amidoamine myristamidopropyl dimethylamine (MAPD) at 10 μg ml^{-1} in 2 mM Tris.HCl, pH 7.2 (Alcon Laboratories Inc., Fort Worth, USA). In control experiments, ¼ Ringer's solution was used in place of the test solutions.

9.3.3 Cysticidal assay

The method used to determine the kinetics of cyst killing is described in Chaptter 2. For all test solutions the tubes were "aged" overnight by filling with 10 ml of the test solution, which was then discarded and replaced with fresh solution for testing. MAPD was tested in glass universal bottles as it has been reported that it adheres to certain types of plastics (Van Duzee & Schlech, 1999). The time intervals measured were 0, 1, 2, 4 and 6 hours. The neutraliser used was 0.1% Tween 80 (Sigma Chemical Company, Dorset, England) for all of the solutions except H_2O_2, which was neutralized with 4 mg ml^{-1} bovine liver catalase (Sigma Chemical Company, Dorset, England).

9.3.4 Selection of resistant organisms

After 24 hours of disinfection, the tubes were centrifuged at 1000 x g and any remaining organisms washed by centrifugation once in the appropriate neutraliser, twice in ¼ Ringer's and once more in the semi-defined axenic broth (#6 medium). The cells were then re-suspended in # 6 Medium in a tissue culture flask (Nunc, Rochester, USA) and incubated at 32°C to allow excystment of any surviving organisms. The excysted trophozoites were sub-cultered twice a week (Chapter 2). At the end of each week for the subsequent four weeks the cells were harvested and cysts were prepared using #6Mg as above for re-testing, against the same disinfectant they were originally exposed to.

9.4 Results

9.4.1 Resistance against H_2O_2

The H_2O_2 (1.5 %) achieved a 2.67 ± 1.17 kill against *A. polyphaga* by 6 hours, and a total kill of the innoculum of 3.97 ± 1.02 by 24 hours. After the initial time kill assay against 1.5 % H_2O_2, no survivors could be cultured (results not shown).

9.4.2 Resistance against PHMB

Figure 29 shows the efficacy of 2 µg PHMB against *A. polyphaga* cysts. Originally after an exposure time of 4 hours, the PHMB achieved a 1.40 ± 0.15 kill and by 6 hours achieved a 2.89 ± 0.35 log kill.

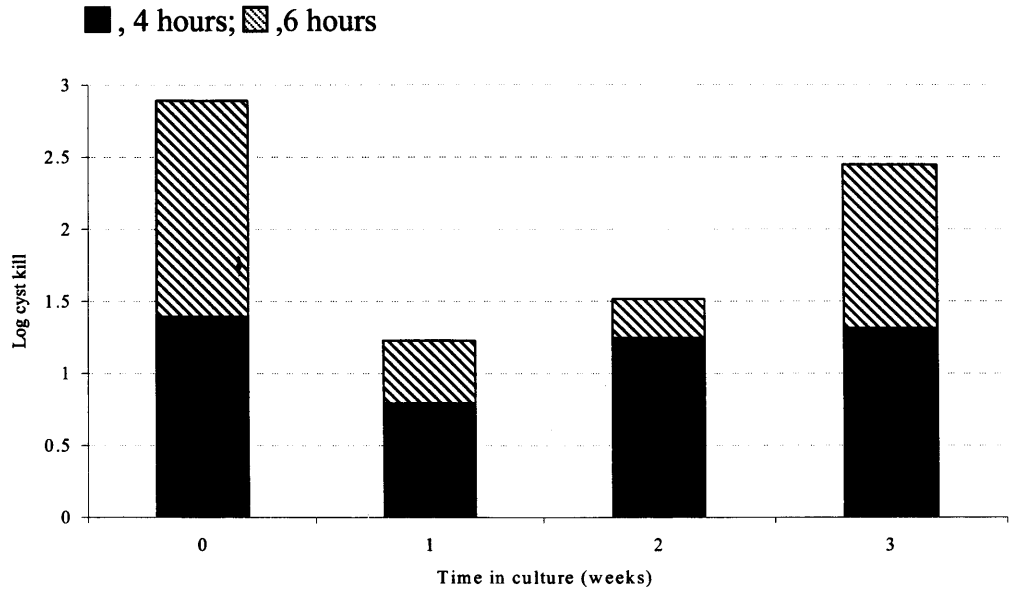

Figure 29: Activity of 2 µg ml-1 PHMB against *A. polyphaga* cysts after previous exposure to 2 µg ml-1 PHMB. The passaged culture at one week showed a marked increase in resistance to PHMB, this resistance was lessened after 2 and 3 weeks.

After the survivors of this experiment had been cultured for a week and were re-tested, the same concentration of PHMB achieved a 0.79 ± 0.12 and 1.23 ± 0.27 log kill after 4 and 6 hours respectively. This difference was significant at 6 hours ($p<0.01$). After further culture for a second week, the cysts were still noticeable more resistant, with log kills of 1.24 ± 0.26 and 1.51

± 0.12 after 4 and 6 hours exposure. Again at 6 hours, this was significantly different from the original culture ($p<0.05$). After 3 weeks of culture after the original selection of surviving organisms, the PHMB produced a 1.31 ± 0.11 log kill by 4 hours and 2.45 ± 0.18 log kill by 6 hours, this was statistically significant from the 1 week culture at 6 hours ($p<0.05$). After 4 weeks culture, the log kill achieved with the PHMB was the same as with the original cyst population (results not shown).

9.4.3 Resistance against CLX

Figure 30 shows the efficacy of CLX against *A. polyphaga* cysts. Twenty μg ml^{-1} CLX achieved log kills of 1.31 ± 0.18, 1.95 ± 0.23 and 3.38 ± 0.31 after 2, 4 and 6 hours respectively against the original culture. However, after initial exposure to the CLX and selection of the survivors, the kill achieved by the CLX against the1 week culture was 0.37 ± 0.20, 0.90 ± 0.20, and 2.00 ± 0.17 at 2, 4 and 6 hours respectively. These results were significantly different for all of these time points ($p<0.05$).

After a second week of culture after the initial exposure to the CLX, the disinfection with 20 μg ml^{-1} CLX achieved a 0.27 ± 0.23, 1.30 ± 0.20, and 2.13 ± 0.13 log kills after 2, 4 and 6 hours exposure respectively. These were statistically different from the original culture at 2 and 6 hours ($p<0.05$). By the third week of culture, the CLX achieved log reductions of 0.47 ± 0.23, 1.23 ± 0.14 and 2.95 ± 0.38 after 2, 4 and 6 hours exposure respectively. This is significantly different from the original culture at 2 hours ($p<0.05$). After 4 weeks culture, the log kill achieved with the CLX was the same as with the original cyst population (results not shown).

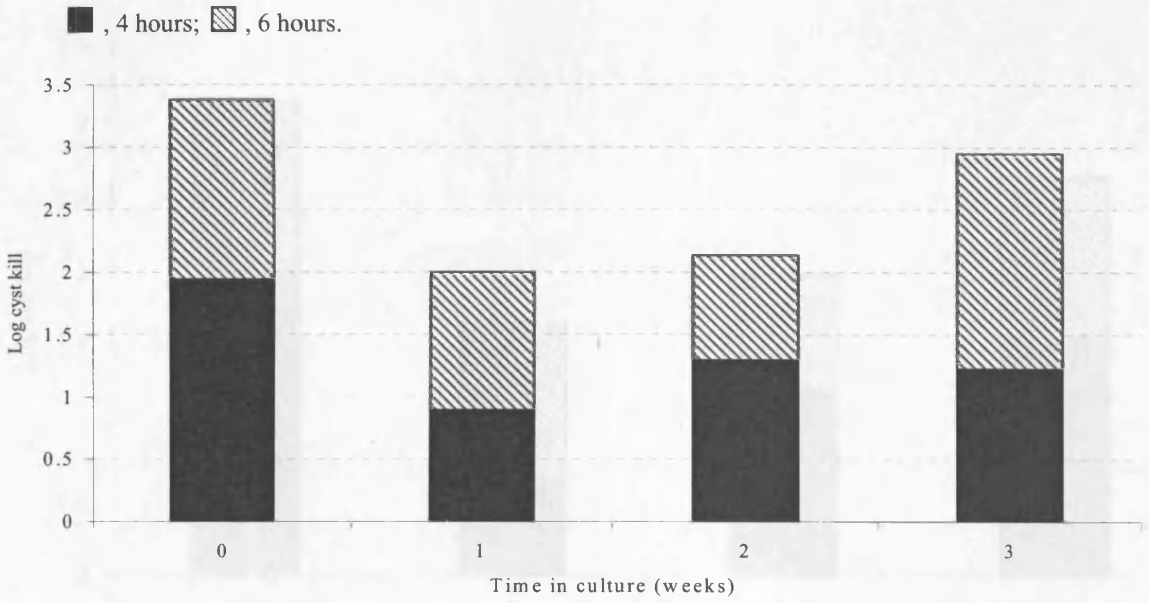

Figure 30: Activity of 20 µg ml-1 CLX against *A. polyphaga* cysts after previous exposure to 20 µg ml-1 CLX. After exposure to MAPD the culture becomes more resistant, with a log kill of 2 after 6 h, compared with over 3 at the same time point for the original culture. However, by 3 weeks most of this resistance is lost with a log kill after 6 h exposure of nearly 3.

9.4.4 Resistance against MAPD

Figure 31 shows the efficacy of MAPD against *A. polyphaga* cysts. MAPD (10 µg ml^{-1}) achieved log kills of 1.15 ± 0.15, 1.79 ± 0.10 and 3.13 ± 0.29 after 2, 4 and 6 hours respectively against the original culture. After initial exposure to the MAPD and selection of the survivors, the kill achieved by the MAPD against the1 week culture was 0.33 ± 0.10, 0.64 ± 0.06, and 1.66 ± 0.22 at 2, 4 and 6 hours respectively. These results were significantly different at all of these time points ($p<0.01$).

■ , 4 hours; ▨ , 6 hours.

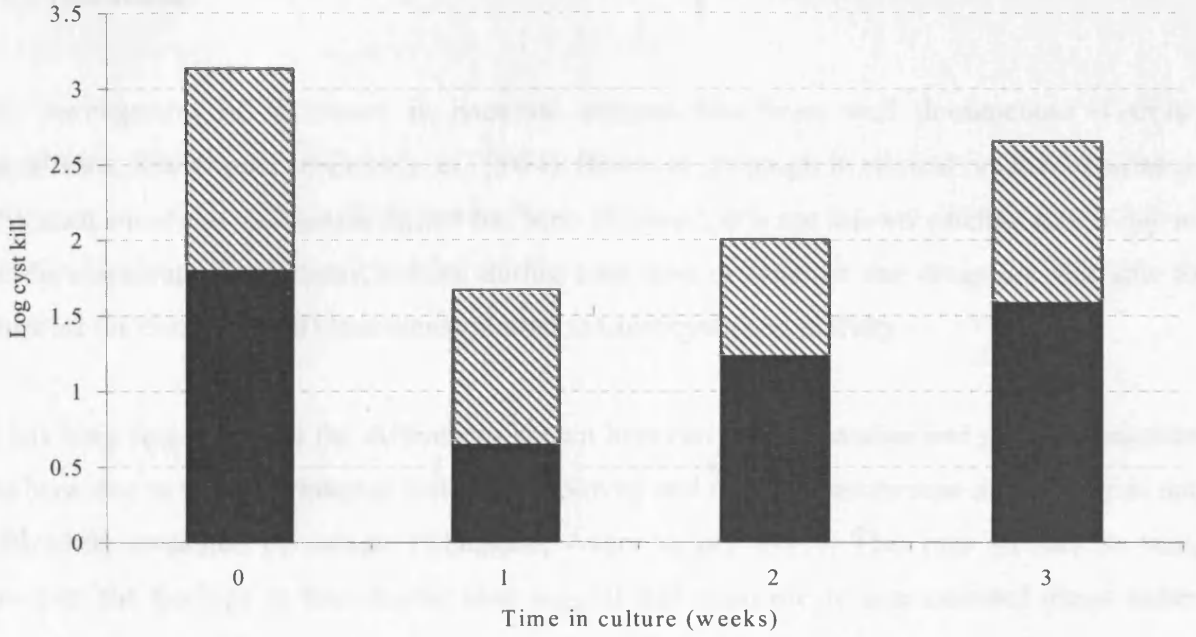

Figure 31: Activity of 10 µg ml-1 MAPD against *A. polyphaga* cysts after previous exposure to 10 µg ml-1 MAPD. Initially the MAPD gives a log kill of over 3 at 6 h, however, after being passaged through the disinfectant, the culture is more resistant to the drug and a kill of over 1.5 log is achieved in 6 h. However, the resistance is lost and by week 3, the log kill at 6 h is over 2.5.

After a second week of culture after the initial exposure to the MAPD, the disinfection with 10 µg ml^{-1} MAPD achieved a 0.59 ± 0.10, 1.23 ± 0.11, and 2.00 ± 0.17 log kills after 2, 4 and 6 hours exposure respectively. These were statistically different from the original culture at 2 and 6 hours ($p<0.05$). By the third week of culture, the MAPD achieved log reductions of 0.71 ± 0.16, 1.58 ± 0.211 and 2.65 ± 0.18 after 2, 4 and 6 hours exposure respectively. This is significantly different from the one week culture at 4 and 6 hours ($p<0.05$). After 4 weeks culture, the log kill achieved with the MAPD was the same as with the original cyst population (results not shown).

9.5 Discussion

The development of resistance in bacterial cultures has been well documented (Garcia-Castellanos, Mallorqui-Fernandez *et al.*, 2004). However, although in clinical practice resistance of *Acanthamoeba* to therapeutic agents has been observed, it is not known whether this is due to the development of a resistant culture during treatment or whether the drugs are not able to penetrate the cornea at sufficient concentration to exert cysticidal activity.

It has been suspected that the differences shown between *in vitro* studies and patient treatment has been due to the experimental methods employed and that a measurement of viability is not sufficiently measured by culture (Khunkitti, Avery *et al.*, 1997). This may in fact be true, however, the findings in this chapter also suggest that exposure to anti-amoebal drugs either selects out an already resistant population within the culture or prime the organisms which develop mechanisms of resistance during disinfection. As the culture is a clonal population, for there to be a resistant sub-population, a degree of mutation within the culture must be occurring.

The results in this study show that a selected resistant population of cysts can be isolated after disinfection and re-cultured to provide a more resistant population. As the cultures are grown in the trophozoite form then re-encysted, this suggests that there some genetic differences have occurred in this population and when exposed to the encystment medium they are able to synthesise a stronger cyst wall. Observation under a light microscope showed no differences between the resistant population and the original culture, but perhaps, they are able to secrete more cellulose, thus leading to a more mature cyst.

It would be of interest determine whether these resistant populations are specifically resistant to the drug they have been exposed to, or whether the culture originally isolated from the PHMB time kill experiment would also show a marked resistance to CLX.

The resistant populations also appear to lose their increased resistance after prolonged culture. After the resistant culture had been maintained in culture for 4 weeks, they were as susceptible to therapeutic agents as the original cultures. This pattern has also been noted when *Acanthamoeba* trophozoites are passaged through tissue culture, initially they develop a greater

resistance against therapeutic agents, however, after a few weeks in culture they have been shown to lose this resistance (per comm. S. Kilvington).

As mentioned above, the difference between *in vitro* drug efficacy studies and patient responses has been attributed to the assay methods employed. The results here suggest that during treatment either a resistant sub-population of amoebae are present in infections, or the continual exposure of drugs to the cysts allow the organisms to develop a resistance during therapy.

10 Alternative Viability Assays

10.1 Introduction

The viability of *Acanthamoeba* spp. have usually been enumerated by one of two means. The first is the standard plaque assay where the samples of *Acanthamoeba* are spread onto agar plates after disinfection and overlaid with a non-nutrient agar, which contains live *E. coli*. The plates are then incubated and plaques counted up to 10 days afterwards (Anger, Rupp *et al.*, 1991; Hugo, McLaughlin *et al.*, 1991). The second is the microtitre plate method (Hughes and Kilvington 2001; Kilvington, Hughes *et al.*, 2002). Both of these methods rely upon the ability of trophozoites to hatch out from the cysts, and either phagocytose a bacterial lawn (Anger *et al.*,, 1991; Hugo *et al.*,, 1991), or divide by binary fission to be observed under a light microscope (Hughes and Kilvington 2001; Kilvington, Hughes *et al.*, 2002).

These methods both take several days, as the extent of phagocytosis is time dependent (Chambers and Thompson 1976). Also these techniques do not provide real-time information on the physiological status of the organism *in situ*. These techniques also do not allow for the possibility that the drug-treated cysts may be viable but non-culturable. Therefore, a rapid high throughput and accurate methods of *Acanthamoeba* cysts detection is crucial.

Fluorescent dyes have been used to stain *Acanthamoeba* cysts as indicators of viability and the cells sorted through a flow cytometer (Khunkitti, Avery *et al.*, 1997). Khunkitti and colleagues used Fluorescein diacetate (FDA) and propidium iodide (PI) to stain live and dead cysts respectively. They found that comparing this method to the plaque assay method highlighted a large number of cysts that were not capable of hatching and phagocytosing *E. coli* but were metabolically active (Khunkitti, Avery *et al.*, 1997). They concluded that the cysts that were staining live were in fact dying and therefore unable to phagocytose bacteria.

The LIVE/DEAD® *Bac*Light™ bacterial viability kits (Molecular Probes, Leiden The Netherlands) allow the examination of nucleic acid stained bacteria under a fluorescent microscope. This kit provides a sensitive, single-step, two-colour fluorescence-based assay for bacterial viability. The *Bac*Light™ assays can quantitatively distinguish live and dead bacteria in minutes and are well suited for use with a fluorescence microscope.

The assay involves the use of two nucleic acid stains, the green fluorescent SYTO® 9 stain and the red fluorescent propidium iodide (PI). These stains differ in their spectral characteristics and their ability to penetrate healthy bacterial cells. SYTO® 9 stain can label both live and dead bacteria. However, if membrane integrity is lost, PI can enter and reduce the SYTO® 9 fluorescence. Therefore, live bacteria stain green and dead ones red. These kits have successfully been used successfully with a variety of bacteria including; *Escherichia coli* (Maurer and Dougherty 2001) and *Streptococcus pyogenes* (Decker 2001).

Another method that has been used to determine bacterial viability is the measurement of ATP in cells (Fukui, Kato *et al.,* 1988; Lehtinen, Virta *et al.,* 2003; Pericone, Park *et al.,* 2003). This is measured using firefly luciferase. Firefly luciferases catalyses the following reaction:

$$\mathbf{ATP + D\text{-}luciferin + O_2 \leftrightarrow AMP + PP_i + oxyluciferin + H_2O + CO_2 + light}$$

PP_i =(inorganic pyrophosphate)

D-Luciferin passes through cell membranes, and the reaction is initiated without disruption of the cells (Wood and DeLuca 1987). Because only catabolically active cells produce ATP, the bioluminescence emission is directly proportional to activity of cells. This makes bioluminescence a good indicator of the number of viable bacteria (Virta, Karp *et al.,* 1994).

10.2 Aims

To date these kits have not been used against amoebae, therefore, it would be interesting to see whether these kits are able to penetrate the cyst walls to provide a reliable method of live dead staining of *Acanthamoeba.* The aim of this chapter is to determine whether the results obtained with the culture method are comparable with those obtained from flow cytometry and those obtained with measuring ATP activity. This would produce a fast reliable, reproducible method of determining the number of live *Acanthamoeba* cells.

10.3 Materials and Methods

10.3.1 Acanthamoeba strains

A. polyphaga (Ros strain), was used throughout the study. Log phase trophozoites were harvested immediately before testing. Cysts were prepared by 7 days incubation with #Mg encystment medium and sonicated before use (Chapter 2).

10.3.2 Preparation of cells

Cell suspensions of 10^7 cysts / ml were prepared and stored in the fridge for use within 1 month. Heat killed cysts were also prepared by Immersing the 10^7 suspension of cells in a water bath at 80 °C for 15 minutes. Previous experiments have shown that this is sufficient to kill the cysts (results not shown). A 50:50 live/dead suspension of cysts was made and stored at 4 °C.

10.3.3 Antimicrobial agents.

The following therapeutic and experimental agents were studied: polyhexamethylene biguanide (PHMB) at 2 μg ml^{-1} in H_2O (Bausch & Lomb, Rochester, USA), myristamidopropyl dimethylamine (MAPD) at 10 μg ml^{-1} in 2 mM Tris.HCl, pH 7.2 (Alcon Laboratories Inc., Fort Worth, USA), and 3% H_2O_2 (Sigma Chemical Company, Dorset, England). In control experiments, ¼ Ringer's solution was used in place of the test solutions.

10.3.4 Generation of standard curves of ATP kits

The kits were compared against one another; firstly the standard curves were made for cysts as explained in chapter 2.

Kit 1. Celsis Ltd, (Landgraaf, The Netherlands).

Serial 10-fold dilutions of 50 μl of cysts were made from 1 x 10^6 cysts down to 1 x 10^1 cysts in ¼ Ringer's solution in a black 96-well microtitre plate (Bibby Sterilin Ltd., Staffordshire, UK). As a negative control, 10^6 heat-killed cysts (at 85 °C for 10 minutes) were used. The ATP standard supplied was diluted 10 fold to create a reference of ATP concentration.

A Luminoscan RS (Thermo Labsystems, Finland) with two automatic injectors was used to analyse the light generated by the reaction. The luminometer was programmed to dispense 150 µl of extraction buffer and after a delay of 10 seconds dispense 100 µl of the Luminate enzyme reagent into the relevant wells. Finally, the luminometer measured the light emission immediately using a 10 second integration time.

Kit 2. ENLITEN® Hygiene monitoring reagent set (Promega, USA).

Serial 10-fold dilutions of 50 µl of cysts were made from 1 x 10^6 cysts down to 1 x 10^1 cysts in ¼ Ringer's solution in a black 96-well microtitre plate (Bibby Sterilin Ltd., Staffordshire, UK). As a negative control, 10^6 heat-killed cysts (at 85 °C for 10 minutes) were used. The ATP standard supplied was diluted 10 fold to create a reference of ATP concentration.

A Luminoscan RS (Thermo Labsystems, Finland) measured the light emission immediately using a 10 second integration time.

10.3.5 Cysticidal assay

The method used to determine the kinetics of cyst killing is described in Chapter 2. However, 20ml of test solution was used in this experiment at times of 0, 1, 2, 4, 6 and 24 hours. 0.1 % Tween 80 (Sigma Chemical Company, Dorset, England) was used to neutralise the didnfectant during the assay. At each time point an extra 1500 µl of solution was removed and neutralised for use with the other viability tests described. All experiments were performed in triplicate and repeated on three separate occasions. Control experiments used ¼ Ringer's solution in place of the test solution.

10.3.6 ATP Measurement

At each time point during the experiment above (10.2.4), 500 µl of solution was removed for ATP measurement. This was neutralised in 5 ml of the appropriate neutraliser and left for 5 minutes before vortexing at 1000 X g for 5 min. The supernatant was then carefully removed and the remaining cysts washed again by centrifugation in ¼ Ringer's solution. The cysts were finally suspended in 500 µl ¼ Ringer's solution. The samples were added in triplicate to a black 96-well microtitre plate and the ATP activity was measured using the two kits as explained

above. The results were compared against the standard curves previously created to estimate the number of surviving cells at each time point.

10.3.7 Flow Cytometry

During the above experiment, at each time point an extra 1 ml of test solution was removed at each time point. This was neutralised in 5 ml of the appropriate neutraliser and left for 5 minutes before vortexing at 1000 x g for 5 min. The supernatant was then carefully removed and the remaining cysts washed again by centrifugation in ¼ Ringer's solution. The cysts were finally suspended in 90 µl ¼ Ringer's solution and stained with PI and SYTO 9. Briefly, 5 µl of 40 µg ml^{-1} solution of PI and 5 µl of 40 µg ml^{-1} solution of SYTO 9 was added to the cells and vortexed and incubated in the dark at room temperature for 15 minutes. After vortexing, 1 ml of nH_2O was added to the stained cysts. The samples were then analysed with a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany) and analysed using CellQuest® Pro v. 4.0.1 (Becton Dickinson, Heidelberg, Germany).

10.3.8 Fluorescent Staining

Live and heat-killed cysts were also examined under a fluorescent microscope. The Fluorescent dyes used are listed in Table 4. The cysts were examined under the microscope after being stanined using the fluorescent dyes (Chapter 2).

10.4 Results

10.4.1 Activity of therapeutic agents as measured by the culture method

The kinetics of killing of *A. polyphaga* (Ros) cysts with 2 µg ml^{-1} PHMB, 10 µg ml^{-1} MAPD and 3 % H_2O_2 are shown in figure 32. By 4 hours, PHMB had achieved a 0.73 ± 0.25 log kill, and by 24 hours a 1.83 ± 0.43 log kill. The H_2O_2 achieved a 3.5 ± 0.10 log kill by 4 hours and a total kill of 3.96 ± 0.23 log by 24 hours. MAPD achieved a total kill of the innoculum (3.41 ± 0.21 log) by 2 hours.

●, PHMB (2 μg ml^{-1}); ■, H_2O_2 (3 %); ▲, MAPD (10 μg ml^{-1}).

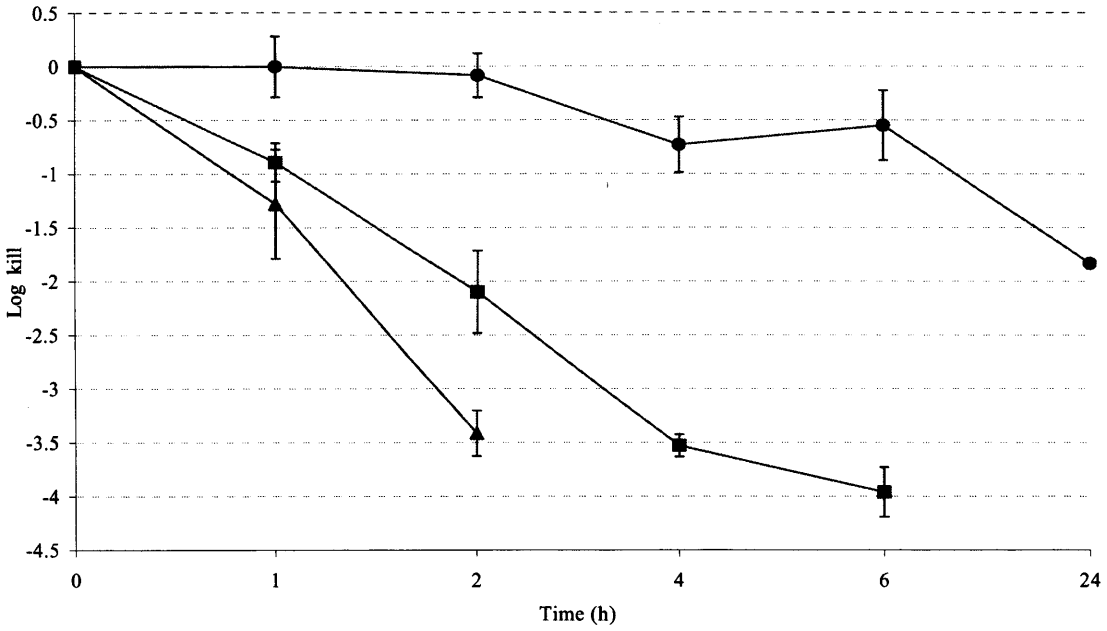

Figure 32: Time kill of *Acanthamoeba* cysts by therapeutic agents as measured by the culture method. 2 μg ml^{-1} PHMB achieved over 0.5 log kill in 6 h, 3 % hydrogen peroxide achieved nearly 4 log kill in the same time and 10 μg ml^{-1} MAPD achieved total kill after this time.

10.4.2 Activity of therapeutic agents as measured by flow cytometry

The kinetics of killing of *A. polyphaga* (Ros) cysts with 2 μg ml^{-1} PHMB, 10 μg ml^{-1} MAPD and 3 % H_2O_2 are shown in figure 33. By 4 hours, PHMB had achieved a 2.51 ± 0.26 % kill, and by 24 hours a 12.47 ± 4.77 % kill. The H_2O_2 achieved a 1.22 ± 1.14 % kill by 4 hours and a kill of 11.15 ± 0.2.7 % by 24 hours. MAPD achieved kills of 17.91 ± 0.68 % and 20.31 ± 3.11 % by 4 and 24 hours respectively.

●, PHMB (2 μg ml^{-1}); ■, H_2O_2 (3 %); ▲, MAPD (10 μg ml^{-1}).

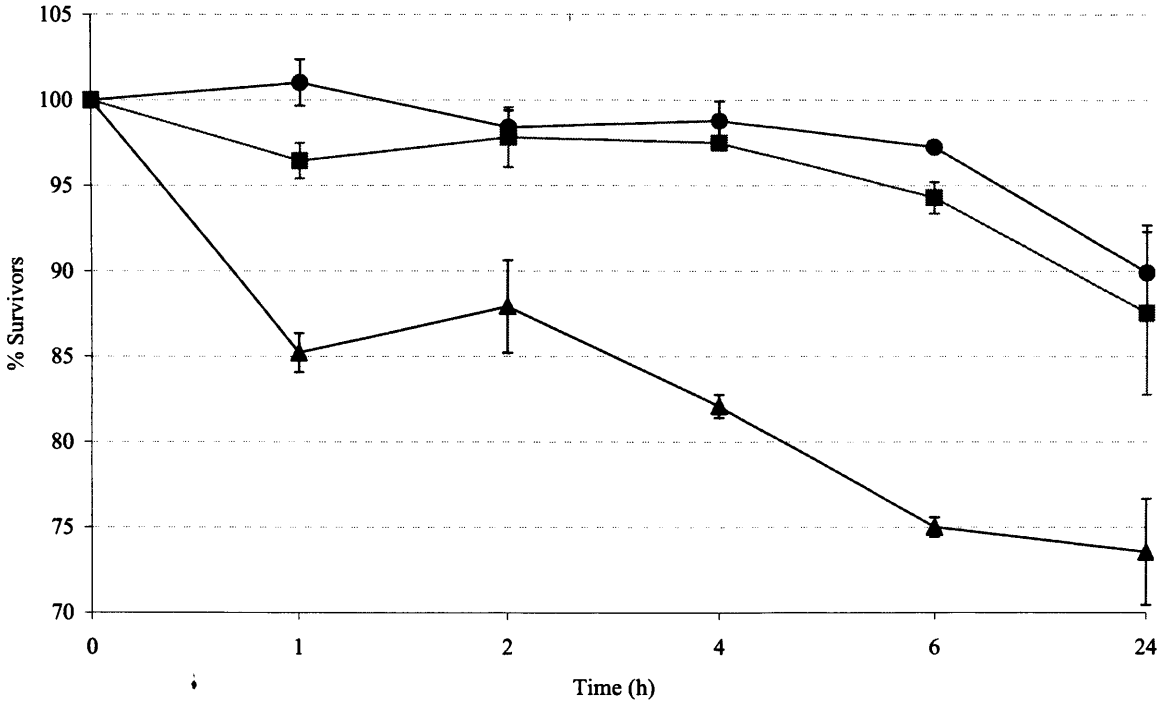

Figure 33: Time kill of *Acanthamoeba* by therapeutic agents as measured by flow cytometry. After 6 h exposure time, 2 μg ml^{-1} PHMB achieved over 10 % kill, 3 % hydrogen peroxide achieved 12 % kill and 10μg ml^{-1} MAPD achieved over 25% kill.

10.4.3 ATP measurements

Both of the kits studied were able to detect levels of ATP down to 0.01 mM (results not shown). When compared against known concentrations of live cysts, the kits were unable to detect levels below 1 X 10^5 cysts (results not shown). Therefore, the kits were not suitable in the time kill assays.

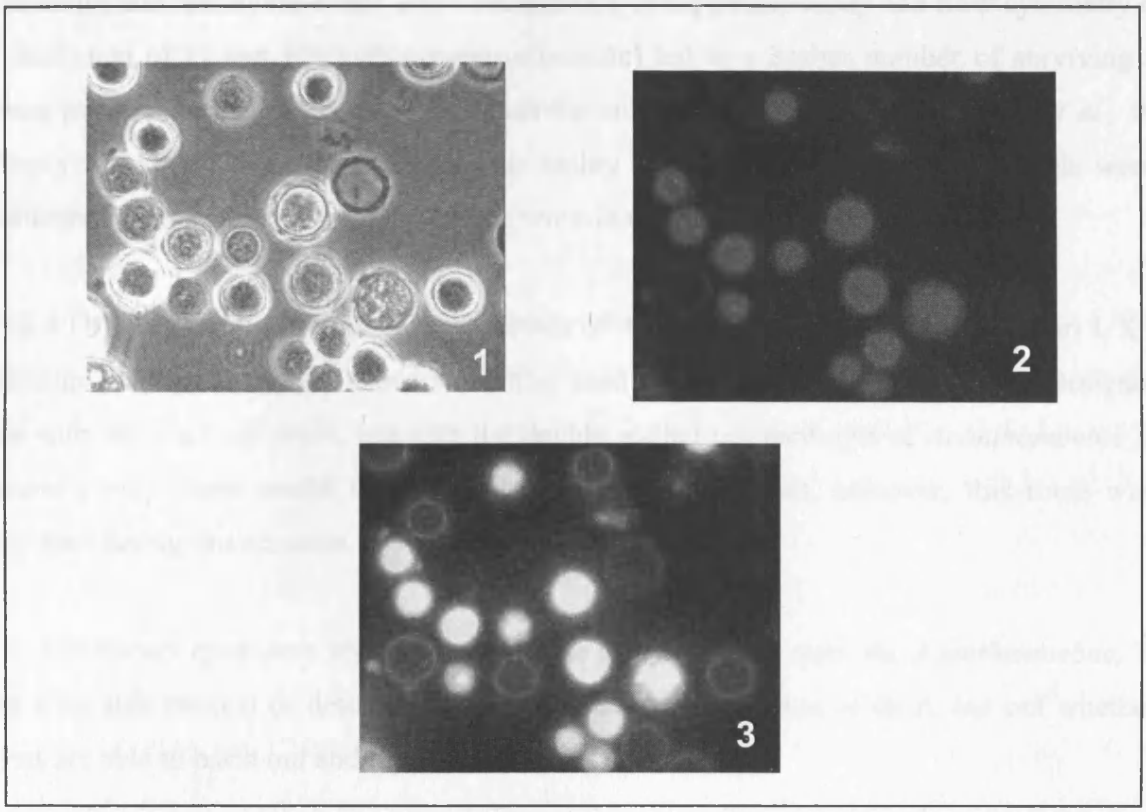

Figure 34: Fluorescent Staining of Cells; (1) under light microscopy, (2) Stained with PI, (3) Dual stained with DAPI and PI. Staining with PI results in the staining of the dead cysts (2), however, when the preparations are dual stained, the live cells can also be seen.

10.5 Discussion

The results of the flow cytometry with the vital stain and the culture method vary greatly. The culture method shows there to be cysticidal activity with all of the solutions within 24 hours, with all of the solutions giving greater than one log kill within this time (90 % kill). However, the flow cytometry results indicate that the cysticidal activity is in fact far less, with only the MAPD achieving greater than 75 % kill in 24 hours. The other two solutions did not achieve even a 15 % kill within this time. This could explain the correlation between the sensitivities of *Acanthamoeba* cysts *in vitro,* with patient responses to therapeutic agents, which have been observed previously (Elder and Dart 1995). It is possible that the viability tests performed on *Acanthamoeba* cysts using the traditional culture methods are not accurate as many cysts are alive but not culturable, however, further investigation is necessary to verify this.

Khunkitti and colleagues found that a comparison of the plaque assay and flow cytometry using a duel stain of PI and FDA (fluorescein diacetate) led to a higher number of surviving cysts being measured by the flow cytometry than the culture method (Khunkitti, Avery *et al.*, 1997). They propsed that dying cells retain their ability to hydrolyse FDA, yet these cells were not culturable. This theory is supported by the work in this chapter.

The ATP kits were unable to detect the levels of ATP in a concentration of less than 1 X 10^5 / ml. This may be due to the extraction buffer used with the kits, these buffers are designed for use with bacterial cell walls, not with the double walled resistant cyst of *Acanthamoeba.* In the future it may prove useful to try different extraction methods, however, this route was not explored during this research.

The fluorescent dyes were able to penetrate the cyst walls and stain the *Acanthamoebae.* These are a reliable method of determining whether the cysts are alive or dead, but not whether the cysts are able to hatch out and reproduce.

Currently the methods used for determining cyst viability are slow and appear to undestimate the number of cysts that are alive. It remains to be seen whether in fact these viable but non-culturable organisms are responsible for the treatment failures in strains that appear to be sensitive to therapeutic agents *in vitro*, or whether the non-culturable organisms pose no threat to patients. It does seem that the fluorescence based detection methods such as flow cytometry would provide a rapid method of screening potential therapeutic agents and may be a more stringent indicator of the efficacy of disinfectants.

11 Conclusions

Acanthamoeba keratitis is a potentially sight threatening infection most commonly seen among contact lens wearers (Radford, Lehmann *et al.,* 1998; Radford, Minassian *et al.,* 2002). Unlike efficacy testing of contact lens disinfectants against bacteria and fungi, no such requirement or standard protocol exists for *Acanthamoeba* (International Organization for Standardization 2000). As a consequence, a variety of species, strains, methods of cyst preparation cysts and experimental protocols have been employed, frequently with contradictory findings, as reviewed by Buck and colleagues (Buck, Rosenthal *et al.,* 2000). Work during this study has aimed to develop a reliable and reproducible method of testing the efficacy of disinfectants against the ocular pathogen *Acanthamoeba.* This allowed a comprehensive study of the efficacy of the current contact lens solutions available to be carried out using a standardised technique.

Hydrogen peroxide (3 %) is an effective disinfectant against *Acanthamoeba* cysts, giving at least a 3-log reduction in viability provided an exposure time of at least 4-6 hours is used (Hughes and Kilvington 2001); Chapter 3). Hydrogen peroxide is commonly used in the disinfection of contact lenses although it must be neutralised before the lenses are worn to avoid corneal damage (Gyulai, Dziabo *et al.,* 1987). One-step hydrogen peroxide systems are available which do not require a separate neutralisation step; this is achieved during disinfection in the storage case using a platinum-coated disc or soluble catalase tablet. However as shown in chapter 3, in one-step systems this neutralisation process occurs too rapidly for cysticidal activity to occur (Hughes and Kilvington 2001). The peroxidase-hydrogen peroxide-halide system developed in Chapter 4, whilst demonstrating enhanced cysticidal activity, does not result in the neutralisation of the H_2O_2 during the reaction. However, when the reaction was conducted in the presence of a platinum disc, not only was the enhanced killing maintained but the peroxide was neutralised to <0.5 ppm by 4 hours (Chapter 5). Levels of 2-5 ppm H_2O_2 are not likely to cause irritation to the eye for which 30 ppm has been reported to induce cytotoxicity and 100 ppm noticeable discomfort (Holden 1990).

The peroxidase-halide-peroxide system in the presence of a platinum neutralising disc resulted in the significant increase in the extent of killing for *C. albicans*, *B. subtilis* spores and *A. polyphaga* cysts. Although catalase has also been shown to enhance the killing of *Mycobacterium tuberculosis* in the presence of a halide and H_2O_2 (Jackett, Aber *et al.,* 1978), attempts to reproduce this enhanced antimicrobial killing in a one-step peroxide system that uses

a catalase tablet for neutralisation were unsuccessful due to the rapid neutralisation of the H_2O_2. The concentrations of HRP and KI used were optimised for the killing of *Acanthamoeba* cysts and may not be optimal for other organisms, as was seen with the lack of significant enhanced killing with *F. solani* conidia.

Of the multipurpose solutions tested, none were able to achieve a 3 log reduction for all of the strains of cysts within the manufacturers' minimum recommended disinfection time, although they did achieve a 3 log kill against trophozoites by 4 hours. As cysts are more resistant to disinfection than trophozoites (Elder, Kilvington *et al.,* 1994) this result is of less relevance. Both ReNu Multi and Allergan Complete achieved a 3 log kill against one strain out of three in the manufacturers' minimum recommended disinfection time of 4 hours and 6 hours respectively.

With the exception of Optifree Express, all solutions contain the preservative PHMB. Concerto Soft which contains 5 $\mu g\ ml^{-1}$ of PHMB, did not achieve a 3 log kill after 6 hours exposure with any of the strains tested. It is possible that the buffering agents in this solution inhibit the antimicrobial activity of the PHMB. The remaining solutions containing 1 μg of PHMB per ml ReNu Multi, Allergan Complete and Focus were all able to produce log reductions of 3 or more within the manufacturers' minimum recommended disinfection time against Cole.

Several studies have been carried out on the amoebicidal activity of Optifree Express, which contains MAPD and Polyquad (Buck, Rosenthal *et al.,* 1998; Rosenthal, Buck *et al.,* 1999; Rosenthal, McAnally *et al.,* 2000). After an exposure time of 6 hours, Optifree Express was found to produce log reductions in viability of 1.3 to 4.8 for trophozoites and 2 to 3.2 for cysts of various species and strains of *Acanthamoeba.* An independent study again, with various species of *Acanthamoeba*, including *A. castellanii* and *A. polyphaga*, Optifree Express achieved log reductions in viability of approximately 4 for trophozoites and 2 to 3 for cysts after the manufacturers' minimum recommended disinfection time (Kilvington 1998). The differences in results are thought to be due to the materials used and the method of cyst preparation. The efficacy of Optifree Express against cysts of *A. polyphaga* produced by different methods; Neff's constant pH encystment medium and NNA plates seeded with *Escherichia coli,* and also at different stages of maturity found that a log reduction of only 0.5 was achieved in 6 hours with the mature cysts (Kilvington and Anger 2001).

However, studies in our lab of the cysticidal activity of Optifree Express with only Polyquad as the antimicrobial agent show that the amoebicidal activity is greatly reduced (results not shown). This implies that the majority of the cysticidal activity of this solution is due to the addition of the MAPD. As the introduction of PHMB, either alone or in combination therapy, has dramatically improved the prognosis for acanthamoeba keratitis, MAPD may have a potential for use as a therapeutic agent for acanthamoeba keratitis sufferers. It would be interesting to investigate the joint efficacy of PHMB and MAPD as a potential for treatment of AK, or for use within contact lens multipurpose disinfectants.

As compared in this study, a variety of methods have been described for the production of *Acanthamoeba* cysts (Buck, Rosenthal *et al.,* 2000). Axenic trophozoite culture in semi-defined media supplemented with up to 50 mM Mg^{2+} is commonly used for this purpose. However, the findings of this study indicate that cysts derived from this approach are more susceptible to killing to PHMB and, to a lesser degree, MAPD. Both these agents are used in multi-purpose contact lens solutions and this may, in part, explain the discrepancies in the reported cysticidal efficacy of such disinfectants when different methods of cyst production are used (Buck, Rosenthal *et al.,* 1998; Buck, Rosenthal *et al.,* 2000; Kilvington and Anger 2001). The reasons for the increased susceptibility of the Mg^{2+} derived cyst preparation are unclear. All appeared to be of similar, mature cyst, morphology under light microscopy. However, under the electron microscope, the ectocyst wall appeared to be thicker in the Neff's prepared cysts. The sizes of the cysts appeared the same in both groups under the electron microscope, although flow cytometry analysis suggested differences in relative size between preparations. The thickness of the wall may account for some of the differences in susceptibility and resistance to disinfection. It is also possible that encystment induced from the presence of Mg^{2+} ions results in a different chemical composition of the cyst wall that allows greater penetration of these agents. It would be interesting to investigate the chemical composition of the wall, for example, does the NNA produced cyst contain more cellulose than a #6-Mg produced cyst?

Also, cysts derived from a laboratory strain that has been in prolonged axenic culture are more susceptible to disinfection than those produced from the original isolate. This variable is addressed in the standard protocol for assessing the efficacy of contact lens disinfectant solutions against bacteria and fungi, requiring that the test strains be passaged no more that five times from the original culture prior to testing (International Organization for Standardization 2000). Laboratory culture is known to induce biological changes in *Acanthamoeba* such as loss

of virulence and decrease in cellular enzyme activity (Mazur and Hadas 1994; Mazur, Hadas *et al.,* 1995). Such changes may also be reflected in the biochemical composition of the cyst wall resulting in a greater susceptibility to disinfection, this could also be investigated if more time was available.

This study has demonstrated some fundamental factors that can influence the observed efficacy of disinfectant and therapeutic agents against *Acanthamoeba* cysts. In view of the variety of experimental approaches and differing outcomes, standardisation for *Acanthamoeba* disinfectant efficacy testing, as required for bacteria and fungi, is clearly needed (International Organization for Standardization 2000) Buck, Rosenthal *et al.,* 2000). This should take into account the *Acanthamoeba* species and strain used, the time in laboratory culture, method of cyst preparation and storage period of the cysts. Whilst encystment methods such as trophozoite culture in the presence of Mg^{2+} provides large numbers of cysts that are usually required for molecular, biochemical and differentiation analysis their use in disinfection studies should be interpreted cautiously as they are more susceptible to killing by biguanide and amidoamines.

The efficacies of current treatments for AK were also examined in this study *in vitro*. The *in vitro* sensitivity of nine acanthamoeba keratitis isolates was compared, eight of which were from separate cases considered to be medical failures following prolonged treatment with PHMB or chlorhexidine digluconate in combination with propamidine isethionate and hexamidine di-isethionate. In vitro drug sensitivity testing showed the strains to be resistant to propamidine isethionate and, to a lesser extent, hexamidine di-isethionate. The greater cysticidal activity of hexamidine di-isethionate over propamidine isethionate has been noted previously and used in the successful treatment of the infection (Brasseur, Favennec *et al.,* 1994). However, all strains were sensitive to PHMB, chlorhexidine digluconate and MAPD.

Accordingly, there is a poor correlation between in vitro MCC findings and patient response to these therapeutic agents as has been observed previously (Elder and Dart 1995). Reasons for the poor prognosis in patients infected with *Acanthamoeba* that appear sensitive *in vitro* to therapeutic agents are unclear. However, poor penetration of the drugs into the cornea, differences in host immune response and *Acanthamoeba* strain virulence are possibilities. It is also possible that the *in vitro* resuts include viable but non-culturable organisms which are able to grow and infect the cornea in patients.

MAPD in 2 mM Tris.HCl, pH 7.2 showed good cysticidal activity against *Acanthamoeba* in both the time kill assays and the MCC experiments. It was also found to have microbial activity against bacteria and fungi associated with keratitis. The mode of action of MAPD and therapeutic agents against *Acanthamoeba* is not known. In bacteria, PHMB and chlorhexidine cause cytoplasmic membrane damage resulting in an irreversible loss of essential cellular components following binding to the cell wall (Chawner and Gilbert 1989). The alternative chemical structure and low molecular weight of MAPD (mol wt 300) compared to PHMB (average mol wt 2340) and chlorhexidine digluconate (mol wt 898) might permit better penetration into the cornea to achieve therapeutic levels. The findings of this study indicate that MAPD is an effective *Acanthamoeba* cysticidal compound and may represent an improved agent in the treatment of acanthamoeba keratitis and, other forms of microbial keratitis.

The activity of MAPD appeared decreased when testing was conducted in polypropylene and polystyrene compared to glass. MAPD is known to adsorb to certain plastics resulting in a rapid loss of activity from the solution (Van Duzee and Schlech 1999). Accordingly, the type of material in which the assays are conducted is an important consideration when determining the cysticidal activity of MAPD. Factors such as test material, organism strain and method of cyst production and age of culture may have a significant influence on the results obtained for efficacy testing of therapeutic and disinfectant agents against *Acanthamoeba* and further studies are required to standardise such assays for this organism (Buck, Rosenthal *et al.,* 2000).

The development of resistance of *Acanthamoeba* to therapeutic agents has been observed in clinical practice. It has largely been suspected that the differences shown between *in vitro* studies and patient treatment has been due to the experimental methods employed and that a measurement of viability is not sufficiently measured by culture (Khunkitti, Avery *et al.,* 1997). This may in fact be true, however, the findings in this study also suggest that exposure to anti-amoebal drugs either selects out an already resistant population within the culture or prime the organisms which develop mechanisms of resistance during disinfection. As the culture is a clonal population, for there to be a resistant sub-population, a degree of mutation within the culture must be occurring.

The results in this study show that a selected resistant population of cysts can be isolated after disinfection and re-cultured to provide a more resistant population. As the cultures are grown in the trophozoite form then re-encysted, this suggests that some genetic differences have occurred

in this population and when exposed to the encystment medium they are able to synthesise a stronger cyst wall. Observation under a light microscope showed no differences between the resistant population and the original culture, but perhaps, they are able to secrete more cellulose, thus leading to a more mature cyst.

The resistant populations also appear to lose their increased resistance after prolonged culture. After the resistant culture had been maintained in culture for 4 weeks, they were equally susceptible to therapeutic agents as the original cultures. This pattern has also been noted when *Acanthamoeba* trophozoites are passaged through tissue culture, initially they develop a greater resistance against therapeutic agents, however, after a few weeks in culture they have been shown to lose this resistance (per comm. S. Kilvington).

As mentioned above, the difference between *in vitro* drug efficacy studies and patient responses has been attributed to the assay methods employed. The results here suggest that during treatment either a resistant sub-population of amoebae are present in infections, or the continual exposure of cysts to the drugs allow the organisms to develop a resistance during therapy.

Another suggested reason for treatment failures, are the inability of the drug to penetrate the cornea at sufficient concentrations to exert cysticidal activity. It has also been posed that the methods used to determine cysticidal activity are not accurate as they rely on the disinfected cysts to be able to excyst and grow. It is possible that some of these organisms are viable but not culturable. Therefore, during this study it was attempted to develop a method using vital stains and compare this with the standard culture assay.

The results of the flow cytometry with the vital stain and the culture method vary greatly. The culture method shows there to be cysticidal activity with all of the solutions within 24 hours, with all of the solutions giving greater than one log kill within this time (90 % kill). However, the flow cytometry results indicate that the cysticidal activity is in fact far less, with only the MAPD achieving greater than 75 % kill in 24 hours. The other two solutions did not achieve even a 15 % kill within this time. This could explain the correlation between the sensitivities of *Acanthamoeba* cysts *in vitro,* with patient responses to therapeutic agents, which have been observed previously (Elder and Dart 1995). It is possible that the viability tests performed on *Acanthamoeba* cysts using the traditional culture methods are not accurate as cysts may be alive but non-culturable. However, although these methods may not strictly be accurate as they are

underestimating the number of live cells, the results may be more relevant as non-viable cells should pose no problem to the patient. This in fact probably is true in the contact lens storage case, however, could explain treatment failures cysts remaining in the eye after treatment, thought to be dead, may in fact be dormant and hatch out at a later stage causing re-infection.

The ATP kits were unable to detect the levels of ATP in a concentration of less than 1×10^5 ml^{-1}. This may be due to the extraction buffer used with the kits, these buffers are designed for use with bacterial cell walls, not with the double walled resistant cyst of *Acanthamoeba*. In the future it may prove useful to try different extraction methods, however, this route was not explored during this research.

Currently the methods used for determining cyst viability are slow and appear to undestimate the number of cysts that are alive. It remains to be seen whether in fact these viable but non-culturable organisms are responsible for the treatment failures in strains that appear to be sensitive to therapeutic agents *in vitro*, or whether the non-culturable organisms pose no threat to patients. It does seem that the fluorescence based detection methods such as flow cytometry would provide a rapid method of screening potential therapeutic agents and may be a more stringent indicator of the efficacy of disinfectants.

11.1 Further Work

This thesis has opened further questions and more work is necessary in this area of research. The investigations into MAPD in chapter 8 show that has a good activity against *Acanthamoeba* cysts, but it would be interesting to know whether it could be used in conjunction with PHMB or CLX to provide increased disinfection. Also the results in Chapter 9 on the development of resistance could be further investigated to see whether the resistance would confer to the disinfectants to which the cultures have not been subjected to. For example, would the cysts that had been passaged through PHMB also be more resistant to CLX? Also it would be interesting to see whether it would be possible to repeadedly passage the cysts through disinfectants to create an even more resistant strain. The viability assays in Chapter 10 also warrant further investigation. For example, the extraction method of ATP could be improved which could allow another rapid viability assay to be compared with the flow cytometry.

Bibliography

Achar, S. B. (1991). "Identification of cyclic AMP-dependent protein kinase activity in Acanthamoeba." Rev Infect Dis **13 Suppl 5**: S393.

Agner, K. (1972). Biological effects of hyperchlorous acid formed by "MPO" peroxidation in the presence of chloride ions. Structure and function of oxidation-reduction enzymes. I. A. A. a. A. E. (ed.). New York, Pergamon Press. **18:** 329-335.

Anger, C., D. Rupp, *et al.,* (1991). "A quantitative bacterial plaque assay for the enumeration of viable Acanthamoeba cells." Rev Infect Dis **13 Suppl 5**: S396.

Bacon, A. S., D. G. Frazer, *et al.,* (1993). "A review of 72 consecutive cases of Acanthamoeba keratitis, 1984-1992." Eye **7 (Pt 6)**: 719-25.

Barbaree, J. M., B. S. Fields, *et al.,* (1986). "Isolation of protozoa from water associated with a legionellosis outbreak and demonstration of intracellular multiplication of Legionella pneumophila." Appl Environ Microbiol **51**(2): 422-4.

Beattie, A. M., A. R. Slomovic, *et al.,* (1990). "Acanthamoeba keratitis with two species of Acanthamoeba." Can J Ophthalmol **25**(5): 260-2.

Beattie, T. K., D. V. Seal, *et al.,* (2003). "Determination of amoebicidal activities of multipurpose contact lens solutions by using a most probable number enumeration technique." J Clin Microbiol **41**(7): 2992-3000.

Biddick, C. J., L. H. Rogers, *et al.,* (1984). "Viability of pathogenic and nonpathogenic free-living amoebae in long-term storage at a range of temperatures." Appl Environ Microbiol **48**(4): 859-60.

Borazjani, R. N., L. L. May, *et al.,* (2000). "Flow cytometry for determination of the efficacy of contact lens disinfecting solutions against Acanthamoeba spp." Appl Environ Microbiol **66**(3): 1057-61.

Brandt, F. H., D. A. Ware, *et al.,* (1989). "Viability of Acanthamoeba cysts in ophthalmic solutions." Appl Environ Microbiol **55**(5): 1144-6.

Brasseur, G., L. Favennec, *et al.,* (1994). "Successful treatment of Acanthamoeba keratitis by hexamidine." Cornea **13**(5): 459-62.

Buck, S., R. Rosenthal, *et al.,* (1998). "Amoebicidal activity of a preserved contact lens multipurpose disinfecting solution compared with a disinfection/ neutralization peroxide system." Contact Lens and Anterior Eye **21**: 81-84.

Buck, S. L. and R. A. Rosenthal (1996). "A quantitative method to evaluate neutralizer toxicity against Acanthamoeba castellanii." Appl Environ Microbiol **62**(9): 3521-6.

Buck, S. L., R. A. Rosenthal, *et al.,* (2000). "Methods used to evaluate the effectiveness of contact lens care solutions and other compounds against Acanthamoeba: a review of the literature." Clao J **26**(2): 72-84.

Burger, R. M., R. J. Franco, *et al.,* (1994). "Killing acanthamoebae with polyaminopropyl biguanide: quantitation and kinetics." Antimicrob Agents Chemother **38**(4): 886-8.

Byers, T. J., B. G. Kim, *et al.,* (1991). "Molecular aspects of the cell cycle and encystment of Acanthamoeba." Rev Infect Dis **13 Suppl 5**: S373-84.

Calore, E. E., M. J. Cavaliere, *et al.,* (1997). "Cerebral amebiasis in the acquired immunodeficiency syndrome." Acta Neurol Belg **97**(4): 248-50.

Cao, Z., D. M. Jefferson, *et al.,* (1998). "Role of carbohydrate-mediated adherence in cytopathogenic mechanisms of Acanthamoeba." J Biol Chem **273**(25): 15838-45.

Casemore, D. P. (1970). "Sensitivity of Hartmannella (Acanthamoeba) to 5-fluorocytosine, hydroxystilbamidine, and other substances." J Clin Pathol **23**(8): 649-52.

Castellani, A. (1930). "an amoeba found in culture of yeast." J Trop Med Hyg **2**: 160.

Chambers, J. A. and J. E. Thompson (1976). "Phagocytosis and pinocytosis in Acanthamoeba castellanii." J Gen Microbiol **92**(2): 246-50.

Chawner, J. A. and P. Gilbert (1989). "A comparative study of the bactericidal and growth inhibitory activities of the bisbiguanides alexidine and chlorhexidine." J Appl Bacteriol **66**(3): 243-52.

Cheng, C. L., M. L. Ling, *et al.*, (2000). "A case series of acanthamoeba keratitis in Singapore." Singapore Med J **41**(11): 550-3.

Cohen, E. J., C. J. Parlato, *et al.*, (1987). "Medical and surgical treatment of Acanthamoeba keratitis." Am J Ophthalmol **103**(5): 615-25.

Connor, C. G., S. L. Hopkins, *et al.*, (1991). "Effectivity of contact lens disinfection systems against Acanthamoeba culbertsoni." Optom Vis Sci **68**(2): 138-41.

Costas, M. and A. J. Griffiths (1985). "Enzyme composition and the taxonomy of Acanthamoeba." J Protozool **32**(4): 604-7.

Culbertson, C. G., D. H. Holmes, *et al.*, (1965). "Hartmanella Castellani (Acanthamoeba Sp): Preliminary Report on Experimental Chemotherapy." Am J Clin Pathol **43**: 361-4.

Culbertson, C. G., J. W. Smith, *et al.*, (1958). "Acanthamoeba: observations on animal pathogenicity." Science **127**(3313): 1506.

Dart, J. (1996). Contact lens and prosthesis infections. Duane's foundations of clinical opthalmology. W. Tasman and E. Jaeger. New York, Lipincott-Raven.

Dart, J. K., F. Stapleton, *et al.*, (1991). "Contact lenses and other risk factors in microbial keratitis." Lancet **338**(8768): 650-3.

Davies, D., Anthony,JG., Meakin, BJ., Kilvington, S. (1990). "Evaluation of the anti-acanthamoebal acitivity of five contact lens disinfectants." ICLC **17**: 14-20.

De Jonckheere, J. F. (1991). "Ecology of Acanthamoeba." Rev Infect Dis **13 Suppl 5**: S385-7.

Decker, E. M. (2001). "The ability of direct fluorescence-based, two-colour assays to detect different physiological states of oral streptococci." Lett Appl Microbiol **33**(3): 188-92.

Douglas, M. (1930). "Notes on the classification of the amoeba found by Castellani on culture of a yeast-like fungus." J Trop Med Hyg **33**: 258-59.

Driebe, W. T., Jr., G. A. Stern, *et al.,* (1988). "Acanthamoeba keratitis. Potential role for topical clotrimazole in combination chemotherapy." Arch Ophthalmol **106**(9): 1196-201.

Duguid, I. G., J. K. Dart, *et al.,* (1997). "Outcome of acanthamoeba keratitis treated with polyhexamethyl biguanide and propamidine." Ophthalmology **104**(10): 1587-92.

Duma, R. J. and R. Finley (1976). "In vitro susceptibility of pathogenic Naegleria and Acanthamoeba speicies to a variety of therapeutic agents." Antimicrob Agents Chemother **10**(2): 370-6.

E. Penard (1890). "Les Rhizopodes de la faune profonde dans le Lac Leman." Mem Soc Phys Hist Nat. Geneve. **31**: 1-142.

Elder, M. J. and J. K. Dart (1995). "Chemotherapy for acanthamoeba keratitis." Lancet **345**(8952): 791-2; author reply 792-3.

Elder, M. J., S. Kilvington, *et al.,* (1994). "A clinicopathologic study of in vitro sensitivity testing and Acanthamoeba keratitis." Invest Ophthalmol Vis Sci **35**(3): 1059-64.

Ferrante, A., B. Rowan-Kelly, *et al.,* (1984). "In vitro sensitivity of virulent Acanthamoeba culbertsoni to a variety of drugs and antibiotics." Int J Parasitol **14**(1): 53-6.

Ficker, L., D. Seal, *et al.,* (1990). "Acanthamoeba keratitis--resistance to medical therapy." Eye **4 (Pt 6)**: 835-8.

Fukui, K., K. Kato, *et al.,* (1988). "Kinetic study of a change in intracellular ATP level associated with aerobic catabolism of ethanol by Streptococcus mutans." J Bacteriol **170**(10): 4589-93.

Garcia-Castellanos, R., G. Mallorqui-Fernandez, *et al.,* (2004). "On the transcriptional regulation of methicillin resistance: MecI repressor in complex with its operator." J Biol Chem **279**(17): 17888-96.

Gray, T. B., R. T. Cursons, *et al.,* (1995). "Acanthamoeba, bacterial, and fungal contamination of contact lens storage cases." Br J Ophthalmol **79**(6): 601-5.

Gray, T. B., K. A. Gross, *et al.,* (1994). "Acanthamoeba keratitis: a sobering case and a promising new treatment." Aust N Z J Ophthalmol **22**(1): 73-6.

Gyulai, P., A. Dziabo, *et al.,* (1987). "Relative neutralization ability of six hydrogen peroxide disinfection systems." Contact Lens Spectrum **2**: 61-65.

Hartmann, M. (1910). "Untersuchungen uber parasitische amoeben." Arch Protistenk **18**: 207-20.

Hay, J., C. M. Kirkness, *et al.,* (1994). "Drug resistance and Acanthamoeba keratitis: the quest for alternative antiprotozoal chemotherapy." Eye **8 (Pt 5)**: 555-63.

Hay, J. and D. V. Seal (1994). "Contact lens wear by hospital health care staff: is there cause for concern?" Br J Hosp Med **51**(10): 538-41.

Hiti, K., J. Walochnik, *et al.,* (2002). "Viability of Acanthamoeba after exposure to a multipurpose disinfecting contact lens solution and two hydrogen peroxide systems." Br J Ophthalmol **86**(2): 144-6.

Holden, B. (1990). "A report card on hydrogen peroxide for contact lens disinfection." Clao J **16**(1 Suppl): S61-4.

Hughes, R., P. W. Andrew, *et al.,* (2003). "Enhanced killing of Acanthamoeba cysts with a plant peroxidase-hydrogen peroxide-halide antimicrobial system." Appl Environ Microbiol **69**(5): 2563-7.

Hughes, R. and S. Kilvington (2001). "Comparison of hydrogen peroxide contact lens disinfection systems and solutions against Acanthamoeba polyphaga." Antimicrob Agents Chemother **45**(7): 2038-43.

Hugo, E. R., W. R. McLaughlin, *et al.*, (1991). "Quantitative enumeration of acanthamoeba for evaluation of cyst inactivation in contact lens care solutions." Invest Ophthalmol Vis Sci **32**(3): 655-7.

International Organization for Standardization (2000). ISO/CD 14729. Ophthalmic optics—contact lens care products—microbiological requirements and test methods for products and regimens for hygienic management of contact lenses. Geneva, Switzerland., International Organization for Standardization,.

Ishibashi, Y., Y. Matsumoto, *et al.*, (1990). "Oral itraconazole and topical miconazole with debridement for Acanthamoeba keratitis." Am J Ophthalmol **109**(2): 121-6.

Iyer, G. Y., M. S. Nair, *et al.*, (1961). "Metabolic effects of alpha-oxobutyric acid: effect on respiration of rat-liver homogenates." Biochem J **78**: 766-9.

Jackett, P. S., V. R. Aber, *et al.*, (1978). "Virulence and resistance to superoxide, low pH and hydrogen peroxide among strains of Mycobacterium tuberculosis." J Gen Microbiol **104**(1): 37-45.

Jackett, P. S., V. R. Aber, *et al.*, (1978). "Virulence of Mycobacterium tuberculosis and susceptibility to peroxidative killing systems." J Gen Microbiol **107**(2): 273-8.

Jager, B. V. and W. P. Stamm (1972). "Brain abscesses caused by free-living amoeba probably of the genus Hartmannella in a patient with Hodgkin's disease." Lancet **2**(7791): 1343-5.

Janoff, L. E. (1990). "Origin and development of hydrogen peroxide disinfection systems." Clao J **16**(1 Suppl): S36-42.

Jones, D. B., G. S. Visvesvara, *et al.*, (1975). "Acanthamoeba polyphaga keratitis and Acenthamoeba uveitis associated with fatal meningoencephalitis." Trans Ophthalmol Soc U K **95**(2): 221-32.

Jong, E. C., E. Y. Chi, *et al.,* (1984). "Human neutrophil-mediated killing of schistosomula of Schistosoma mansoni: augmentation by schistosomal binding of eosinophil peroxidase." Am J Trop Med Hyg **33**(1): 104-15.

Kenney, M. (1971). "The Micro-Kolmer complement fixation test in routine screening for soil amoeba infection." Health Lab Sci **8**: 5-10.

Khunkitti, W., S. V. Avery, *et al.,* (1997). "Effects of biocides on Acanthamoeba castellanii as measured by flow cytometry and plaque assay." J Antimicrob Chemother **40**(2): 227-33.

Khunkitti, W., D. Lloyd, *et al.,* (1996). "The lethal effects of biguanides on cysts and trophozoites of Acanthamoeba castellanii." J Appl Bacteriol **81**(1): 73-7.

Khunkitti, W., D. Lloyd, *et al.,* (1998). "Acanthamoeba castellanii: growth, encystment, excystment and biocide susceptibility." J Infect **36**(1): 43-8.

Kilvington, S. (1989). "Moist-heat disinfection of pathogenic *Acanthamoeba* cysts." Letters in Applied Microbiology **9**: 187-189.

Kilvington, S. (1998). "Activity of contact lens disinfectants against Acanthamoeba species." Optometry Today **38**(19): 40-41.

Kilvington, S. and C. Anger (2001). "A comparison of cyst age and assay method of the efficacy of contact lens disinfectants against Acanthamoeba." Br J Ophthalmol **85**(3): 336-40.

Kilvington, S., J. R. Beeching, *et al.,* (1991). "Differentiation of Acanthamoeba strains from infected corneas and the environment by using restriction endonuclease digestion of whole-cell DNA." J Clin Microbiol **29**(2): 310-4.

Kilvington, S., R. Hughes, *et al.,* (2002). "Activities of therapeutic agents and myristamidopropyl dimethylamine against Acanthamoeba isolates." Antimicrob Agents Chemother **46**(6): 2007-9.

Kilvington, S., D. F. Larkin, *et al.,* (1990). "Laboratory investigation of Acanthamoeba keratitis." J Clin Microbiol **28**(12): 2722-5.

Kilvington, S. and J. Price (1990). "Survival of Legionella pneumophila within cysts of Acanthamoeba polyphaga following chlorine exposure." J Appl Bacteriol **68**(5): 519-25.

Kilvington, S. and D. White (1994). "Acanthamoeba: biology, ecology and human disease." Reveiws in Medical Microbiology **5**: 12-20.

Kingston, D. and D. C. Warhurst (1969). "Isolation of amoebae from the air." J Med Microbiol **2**(1): 27-36.

Klebanoff, S. J. (1968). "Myeloperoxidase-halide-hydrogen peroxide antibacterial system." J Bacteriol **95**(6): 2131-8.

Klebanoff, S. J. (1980). "Oxygen metabolism and the toxic properties of phagocytes." Ann Intern Med **93**(3): 480-9.

Klebanoff, S. J., W. H. Clem, *et al.,* (1966). "The peroxidase-thiocyanate-hydrogen peroxide antimicrobial system." Biochim Biophys Acta **117**(1): 63-72.

Klebanoff, S. J. and F. Kazazi (1995). "Inactivation of human immunodeficiency virus type 1 by the amine oxidase-peroxidase system." J Clin Microbiol **33**(8): 2054-7.

Klebanoff, S. J. and H. Rosen (1978). "The role of myeloperoxidase in the microbicidal activity of polymorphonuclear leukocytes." Ciba Found Symp(65): 263-84.

Klebanoff, S. J. and C. C. Shepard (1984). "Toxic effect of the peroxidase-hydrogen peroxide-halide antimicrobial system on Mycobacterium leprae." Infect Immun **44**(2): 534-6.

Kosrirukvongs, P., D. Wanachiwanawin, *et al.,* (1999). "Treatment of acanthamoeba keratitis with chlorhexidine." Ophthalmology **106**(4): 798-802.

Larkin, D. F. and D. L. Easty (1991). "The relationship of Acanthamoeba to the external eye flora." Rev Infect Dis **13 Suppl 5**: S394-5.

Larkin, D. F., S. Kilvington, *et al.,* (1992). "Treatment of Acanthamoeba keratitis with polyhexamethylene biguanide." Ophthalmology **99**(2): 185-91.

Larkin, D. F., S. Kilvington, *et al.,* (1990). "Contamination of contact lens storage cases by Acanthamoeba and bacteria." Br J Ophthalmol **74**(3): 133-5.

Ledee, D. R., G. C. Booton, *et al.,* (2003). "Advantages of using mitochondrial 16S rDNA sequences to classify clinical isolates of Acanthamoeba." Invest Ophthalmol Vis Sci **44**(3): 1142-9.

Ledee, D. R., J. Hay, *et al.,* (1996). "Acanthamoeba griffini. Molecular characterization of a new corneal pathogen." Invest Ophthalmol Vis Sci **37**(4): 544-50.

Ledee, D. R., D. V. Seal, *et al.,* (1998). "Confirmatory evidence from 18S rRNA gene analysis for in vivo development of propamidine resistance in a temporal series of Acanthamoeba ocular isolates from a patient." Antimicrob Agents Chemother **42**(8): 2144-5.

Leher, H., R. Silvany, *et al.,* (1998). "Mannose induces the release of cytopathic factors from Acanthamoeba castellanii." Infect Immun **66**(1): 5-10.

Lehtinen, J., M. Virta, *et al.,* (2003). "Fluoro-luminometric real-time measurement of bacterial viability and killing." J Microbiol Methods **55**(1): 173-86.

Lim, L., D. J. Coster, *et al.,* (2000). "Antimicrobial susceptibility of 19 Australian corneal isolates of Acanthamoeba." Clin Experiment Ophthalmol **28**(2): 119-24.

Lindquist, T. D., D. J. Doughman, *et al.,* (1988). "Acanthamoeba-contaminated hydrogel contact lenses. Susceptibility to disinfection." Cornea **7**(4): 300-3.

Lloyd, D., N. A. Turner, *et al.,* (2001). "Encystation in Acanthamoeba castellanii: development of biocide resistance." J Eukaryot Microbiol **48**(1): 11-6.

Lösch, F. A. (1875). "Massenhafte Entwickelung von möben in dickdarn." Virchows Arch Pathol Anat Klin led **65**: 196-211.

Lowe, R., V. Vallas, *et al.,* (1992). "Comparative efficacy of contact lens disinfection solutions." Clao J **18**(1): 34-40.

Ludwig, I. H., D. M. Meisler, *et al.,* (1986). "Susceptibility of Acanthamoeba to soft contact lens disinfection systems." Invest Ophthalmol Vis Sci **27**(4): 626-8.

Ma, P., G. S. Visvesvara, *et al.,* (1990). "Naegleria and Acanthamoeba infections: review." Rev Infect Dis **12**(3): 490-513.

Martinez, A. J. (1993). "Free-living amebas: infection of the central nervous system." Mt Sinai J Med **60**(4): 271-8.

Martinez, A. J., C. A. Garcia, *et al.,* (1980). "Granulomatous amebic encephalitis presenting as a cerebral mass lesion." Acta Neuropathol (Berl) **51**(2): 85-91.

Martinez, A. J. and G. S. Visvesvara (1997). "Free-living, amphizoic and opportunistic amebas." Brain Pathol **7**(1): 583-98.

Mathers, W. D., J. E. Sutphin, *et al.,* (1996). "Outbreak of keratitis presumed to be caused by Acanthamoeba." Am J Ophthalmol **121**(2): 129-42.

Matsugo, S., S. Kasahara, *et al.,* (1998). "Morphological and molecular analysis of the cyst on *Acanthamoeba* spp." Molecular Biology of the Cell **9**: 468.

Matthews, T. D., D. G. Frazer, *et al.,* (1992). "Risks of keratitis and patterns of use with disposable contact lenses." Arch Ophthalmol **110**(11): 1559-62.

Maurer, J. A. and D. A. Dougherty (2001). "A high-throughput screen for MscL channel activity and mutational phenotyping." Biochim Biophys Acta **1514**(2): 165-9.

Mazur, T. and E. Hadas (1994). "The effect of the passages of Acanthamoeba strains through mice tissues on their virulence and its biochemical markers." Parasitol Res **80**(5): 431-4.

Mazur, T., E. Hadas, *et al.,* (1995). "The duration of the cyst stage and the viability and virulence of Acanthamoeba isolates." Trop Med Parasitol **46**(2): 106-8.

McFadden, D. C., M. Camps, *et al.,* (2001). "Resistance as a tool in the study of old and new drug targets in Toxoplasma." Drug Resist Updat **4**(2): 79-84.

Meisler, D. M. and I. Rutherford (1991). "Acanthamoeba and disinfection of soft contact lenses." Rev Infect Dis **13 Suppl 5**: S410-2.

Miles, A. and S. Misra (1938). "The estimation of the bactericidal power of the blood." J. Hyg **38**: 732-748.

Moore, M. B., J. P. McCulley, *et al.,* (1985). "Acanthamoeba keratitis associated with soft contact lenses." Am J Ophthalmol **100**(3): 396-403.

Moore, M. B., J. E. Ubelaker, *et al.,* (1991). "In vitro penetration of human corneal epithelium by Acanthamoeba castellanii: a scanning and transmission electron microscopy study." Cornea **10**(4): 291-8.

Murdoch, D., T. B. Gray, *et al.,* (1998). "Acanthamoeba keratitis in New Zealand, including two cases with in vivo resistance to polyhexamethylene biguanide." Aust N Z J Ophthalmol **26**(3): 231-6.

Näegler, K. (1909). "Entwicklungsgeschliche Studien uber Amoben." Arch Protistenk. **15**: 1-53.

Nagington, J. and J. E. Richards (1976). "Chemotherapeutic compounds and Acanthamoebae from eye infections." J Clin Pathol **29**(7): 648-51.

Neff, R. J., S. A. Ray, *et al.,* (1964). "Induction of synchronous encystment (differentiation) in Acanthamoeba sp." Methods Cell Physiol **1**: 55-83.

Noble, J. A., D. G. Ahearn, *et al.,* (2002). "Phagocytosis affects biguanide sensitivity of Acanthamoeba spp." Antimicrob Agents Chemother **46**(7): 2069-76.

Ofori-Kwakye, S. K., D. G. Sidebottom, *et al.,* (1986). "Granulomatous brain tumor caused by Acanthamoeba. Case report." J Neurosurg **64**(3): 505-9.

Page, F. (1976). An illustrated key to freshwater and soil amoebae. Ambleside, Freshwater Biology Association.

Page, F. (1988). A new key to freshwater and soil gymnamoebae, Inst.Terr.Ecol.

Page, F. C. (1967). "Re-definition of the genus Acanthamoeba with descriptions of three species." J Protozool **14**(4): 709-24.

Panjwani, N., Z. Zhao, *et al.,* (1997). "Acanthamoebae bind to rabbit corneal epithelium in vitro." Invest Ophthalmol Vis Sci **38**(9): 1858-64.

Parrish, C. M. and D. M. O'Day (1991). "Acanthamoeba keratitis following penetrating keratoplasty in a patient without other identifiable risk factors." Rev Infect Dis **13 Suppl 5**: S430.

Penley, C. A., C. Llabres, *et al.,* (1985). "Efficacy of hydrogen peroxide disinfection systems for soft contact lenses contaminated with fungi." Clao J **11**(1): 65-8.

Pericone, C. D., S. Park, *et al.,* (2003). "Factors contributing to hydrogen peroxide resistance in Streptococcus pneumoniae include pyruvate oxidase (SpxB) and avoidance of the toxic effects of the fenton reaction." J Bacteriol **185**(23): 6815-25.

Perrine, D., J. P. Chenu, *et al.,* (1995). "Amoebicidal efficiencies of various diamidines against two strains of Acanthamoeba polyphaga." Antimicrob Agents Chemother **39**(2): 339-42.

Pettit, D. A., J. Williamson, *et al.,* (1996). "In vitro destruction of nerve cell cultures by Acanthamoeba spp.: a transmission and scanning electron microscopy study." J Parasitol **82**(5): 769-77.

Pitts, R. E. and J. H. Krachmer (1979). "Evaluation of soft contact lens disinfection in the home environment." Arch Ophthalmol **97**(3): 470-2.

Pussard, M. and R. Pons (1977). "Morphology de la paroi kystique et taxonomie du genre Acanthamoeba (Protozoa, Amoebida)." Protistologica **8**: 557-598.

Radford, C. F., A. S. Bacon, *et al.,* (1995). "Risk factors for acanthamoeba keratitis in contact lens users: a case-control study." Bmj **310**(6994): 1567-70.

Radford, C. F., O. J. Lehmann, *et al.,* (1998). "Acanthamoeba keratitis: multicentre survey in England 1992-6. National Acanthamoeba Keratitis Study Group." Br J Ophthalmol **82**(12): 1387-92.

Radford, C. F., D. C. Minassian, *et al.,* (1998). "Disposable contact lens use as a risk factor for microbial keratitis." Br J Ophthalmol **82**(11): 1272-5.

Radford, C. F., D. C. Minassian, *et al.,* (2002). "Acanthamoeba keratitis in England and Wales: incidence, outcome, and risk factors." Br J Ophthalmol **86**(5): 536-42.

Radford, D. J., J. M. Lord, *et al.,* (1999). "The activation of the neutrophil respiratory burst by anti-neutrophil cytoplasm autoantibody (ANCA) from patients with systemic vasculitis requires tyrosine kinases and protein kinase C activation." Clin Exp Immunol **118**(1): 171-9.

Reed, L. J. and H. Muench (1938). "A simple method for estimating fifty percent endpoints." Am. J. Hyg **27**: 493-497.

Rosen, H. and S. J. Klebanoff (1985). "Oxidation of microbial iron-sulfur centers by the myeloperoxidase-H2O2-halide antimicrobial system." Infect Immun **47**(3): 613-8.

Rosenthal, R. A., S. Buck, *et al.,* (1999). "Antimicrobial comparison of a new multi-purpose disinfecting solution to a 3% hydrogen peroxide system." Clao J **25**(4): 213-7.

Rosenthal, R. A., C. L. McAnally, *et al.,* (2000). "Broad spectrum antimicrobial activity of a new multi-purpose disinfecting solution." Clao J **26**(3): 120-6.

Rosenthal, R. A., J. M. Stein, *et al.,* (1995). "A comparative study of the microbiologic effectiveness of chemical disinfectants and peroxide-neutralizer systems." Clao J **21**(2): 99-110.

Sawyer, T. and J. Griffen (1975). "Aproposed new family. Acanthamoebidae n. fam. (order Amoebida), for certain cyst-forming ilose amoebae." Trans Am. Microsc. Soc. **94**: 93-98.

Schardinger, F. (1899). "Entwicklungskreis einer Amoeba lobosa (Gymnamoeba): Amoeba gruberi." Sitzb Kaiserl Akad Wiss Wien. **108**: 713-734.

Schaumberg, D. A., K. K. Snow, *et al.,* (1998). "The epidemic of Acanthamoeba keratitis: where do we stand?" Cornea **17**(1): 3-10.

Schein, O. D., P. O. Buehler, *et al.,* (1994). "The impact of overnight wear on the risk of contact lens-associated ulcerative keratitis." Arch Ophthalmol **112**(2): 186-90.

Schroeder, J. M., G. C. Booton, *et al.,* (2001). "Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of acanthamoebae from humans with keratitis and from sewage sludge." J Clin Microbiol **39**(5): 1903-11.

Schultz, D. R. (1980). "Complement in host defense." Scand J Infect Dis Suppl **Suppl 24**: 22-9.

Seal, D., J. Hay, *et al.,* (1996). "Successful medical therapy of Acanthamoeba keratitis with topical chlorhexidine and propamidine." Eye **10 (Pt 4)**: 413-21.

Seal, D., F. Stapleton, *et al.,* (1992). "Possible environmental sources of Acanthamoeba spp in contact lens wearers." Br J Ophthalmol **76**(7): 424-7.

Seal, D. V. (2003). "Acanthamoeba keratitis update-incidence, molecular epidemiology and new drugs for treatment." Eye **17**(8): 893-905.

Seal, D. V., T. K. Beattie, *et al.,* (2003). "Acanthamoeba keratitis." Br J Ophthalmol **87**(4): 516-7.

Seijo Martinez, M., G. Gonzalez-Mediero, *et al.,* (2000). "Granulomatous amebic encephalitis in a patient with AIDS: isolation of acanthamoeba sp. Group II from brain tissue and successful treatment with sulfadiazine and fluconazole." J Clin Microbiol **38**(10): 3892-5.

Sell, J. J., F. W. Rupp, *et al.,* (1997). "Granulomatous amebic encephalitis caused by acanthamoeba." Neuroradiology **39**(6): 434-6.

Selvaraj, R. J., B. B. Paul, *et al.,* (1974). "Oxidative peptide cleavage and decarboxylation by the MPO-H2O2-Cl- antimicrobial system." Infect Immun **9**(2): 255-60.

Shafer, W. M., L. E. Martin, *et al.,* (1986). "Late intraphagosomal hydrogen ion concentration favors the in vitro antimicrobial capacity of a 37-kilodalton cationic granule protein of human neutrophil granulocytes." Infect Immun **53**(3): 651-5.

Silvany, R. E., J. M. Dougherty, *et al.,* (1991). "Effect of contact lens preservatives on Acanthamoeba." Ophthalmology **98**(6): 854-7.

Singh, B. (1975). Pathogenic and non-pathogenic amoebae. London, Macmillan.

Singh, B. N. and S. R. Das (1970). "Studies on pathogenic and non-pathogenic small free-living amoebae and the bearing of nuclear division on the classification of the order amoebida." Philos Trans R Soc Lond B Biol Sci **259**(832): 435-76.

Spector, A. (1990). "Oxidation and aspects of ocular opthalmology." Clao J **16**: 8-10.

Spurr, A. R. (1969). "A low-viscosity epoxy resin embedding medium for electron microscopy." J Ultrastruct Res **26**(1): 31-43.

Stehr-Green, J. K., T. M. Bailey, *et al.,* (1989). "The epidemiology of Acanthamoeba keratitis in the United States." Am J Ophthalmol **107**(4): 331-6.

Stevenson, R. and D. Seal (1998). "Has the introduction of multi-purpose solutions contributed to a reduced incidence of Acanthamoeba keratitis in contact lens wearers?" Contact Lens Ant Eye **21**: 89–92.

Stothard, D. R., J. M. Schroeder-Diedrich, *et al.,* (1998). "The evolutionary history of the genus Acanthamoeba and the identification of eight new 18S rRNA gene sequence types." J Eukaryot Microbiol **45**(1): 45-54.

Stratford, M. P. and A. J. Griffiths (1978). "Variations in the properties and morphology of cysts of Acanthamoeba castellanii." Journal of General Microbiology **108**: 33–7.

Taylor, W. M., M. S. Pidherney, *et al.,* (1995). "In vitro characterization of Acanthamoeba castellanii cytopathic effect." J Parasitol **81**(4): 603-9.

Tenovuo, J., K. K. Makinen, *et al.,* (1985). "Antibacterial effect of lactoperoxidase and myeloperoxidase against Bacillus cereus." Antimicrob Agents Chemother **27**(1): 96-101.

Thakur, C. P., T. P. Kanyok, *et al.,* (2000). "A prospective randomized, comparative, open-label trial of the safety and efficacy of paromomycin (aminosidine) plus sodium stibogluconate versus sodium stibogluconate alone for the treatment of visceral leishmaniasis." Trans R Soc Trop Med Hyg **94**(4): 429-31.

Thomas, E. L. and T. M. Aune (1978). "Cofactor role of iodide in peroxidase antimicrobial action against Escherichia coli." Antimicrob Agents Chemother **13**(6): 1000-5.

Tsan, M. F. and J. W. Chen (1980). "Oxidation of methionine by human polymorphonuclear leukocytes." J Clin Invest **65**(5): 1041-50.

Turk, J., W. R. Henderson, *et al.,* (1983). "Iodination of arachidonic acid mediated by eosinophil peroxidase, myeloperoxidase and lactoperoxidase. Identification and comparison of products." Biochim Biophys Acta **751**(2): 189-200.

Turner, N. A., J. Harris, *et al.,* (2000). "Microbial differentiation and changes in susceptibility to antimicrobial agents." J Appl Microbiol **89**(5): 751-9.

Turner, N. A., A. D. Russell, *et al.,* (1999). "Acanthamoeba spp., antimicrobial agents and contact lenses." Sci Prog **82 (Pt 1)**: 1-8.

Vahlkampf, E. (1905). "Beiträge zur Biologie und Entwicklungsgeschlichte von Amoeba limax einschlieblich der Züchtung auf künstlichen Nährböden." Arch Protistenk **5**: 167-220.

Van Duzee, B. and B. Schlech (1999). "The activity of a multi-purpose solution against Acanthamoeba." Optician **217**: 34-35.

Vathsala, P. G., A. Pramanik, *et al.,* (2004). "Widespread occurrence of the Plasmodium falciparum chloroquine resistance transporter (Pfcrt) gene haplotype SVMNT in P. falciparum malaria in India." Am J Trop Med Hyg **70**(3): 256-9.

Venge, P. and I. Olsson (1975). "Cationic proteins of human granulocytes. VI. Effects on the complement system and mediation of chemotactic activity." J Immunol **115**(6): 1505-8.

Venge, P., I. Olsson, *et al.,* (1975). "Cationic proteins of human granulocytes. V. Interaction with plasma protease inhibitors." Scand J Clin Lab Invest **35**(8): 737-44.

Virta, M., M. Karp, *et al.,* (1994). "Nitric oxide donor-mediated killing of bioluminescent Escherichia coli." Antimicrob Agents Chemother **38**(12): 2775-9.

Visvesvara, G. S. (1991). "Classification of Acanthamoeba." Rev Infect Dis **13 Suppl 5**: S369-72.

Visvesvara, G. S., D. B. Jones, *et al.,* (1975). "Isolation, identification, and biological characterization of Acanthamoeba polyphaga from a human eye." Am J Trop Med Hyg **24**(5): 784-90.

Walochnik, J., M. Duchene, *et al.,* (2002). "Cytotoxic activities of alkylphosphocholines against clinical isolates of Acanthamoeba spp." Antimicrob Agents Chemother **46**(3): 695-701.

Walochnik, J., A. Obwaller, *et al.,* (2000). "Correlations between morphological, molecular biological, and physiological characteristics in clinical and nonclinical isolates of Acanthamoeba spp." Appl Environ Microbiol **66**(10): 4408-13.

Weisman, R. A. (1976). "Differentiation in Acanthamoeba castellanii." Annu Rev Microbiol **30**: 189-219.

Wood, K. V. and M. DeLuca (1987). "Photographic detection of luminescence in Escherichia coli containing the gene for firefly luciferase." Anal Biochem **161**(2): 501-7.

Wright, J. M., L. A. Dunn, *et al.,* (2003). "Efficacy of antigiardial drugs." Expert Opin Drug Saf **2**(6): 529-41.

Wright, P., D. Warhurst, *et al.,* (1985). "Acanthamoeba keratitis successfully treated medically." Br J Ophthalmol **69**(10): 778-82.

Yang, Y. and E. Anderson (1999). "Antimicrobial activity of a porcine myeloperoxidase against plant pathogenic bacteria and fungi." Journal of Applied Microbiology **86**: 211-220.

Yang, Z., Z. Cao, *et al.,* (1997). "Pathogenesis of Acanthamoeba keratitis: carbohydrate-mediated host-parasite interactions." Infect Immun **65**(2): 439-45.

Zanetti, S., P. L. Fiori, *et al.,* (1995). "Susceptibility of Acanthamoeba castellanii to contact lens disinfecting solutions." Antimicrob Agents Chemother **39**(7): 1596-8.