Identification and Drug Sensitivities of *Acanthamoeba* Species Causing Keratitis

A Thesis Presented in Partial Fulfillment of the
Requirements for the Degree of
Master of Science in Microbiology
at
Massey University,
Palmerston North,
New Zealand.

Sally Vanessa Johnston
1997

Abstract:

Acanthamoebic keratitis is a distinct, vision-threatening ophthalmological condition, the incidence of which is increasing with increased usage of contact lenses. Diagnosis can be difficult and chemotherapeutic treatment is prolonged and often ineffective.
It is therefore desirable to discover a quick and accurate means of diagnosing acanthamoebic keratitis, and to gain knowledge regarding which chemotherapeutic agents are most effective against acanthamoebic keratitis.

The first goal of this thesis was to examine ten DNA extraction procedures and determine their effectiveness in extracting DNA from *Acanthamoeba* cells.
Of these ten methods, four (2, 7, 8, 10) could be performed in less than one day and showed consistent results in PCR reactions.

The second goal of this thesis involved the application of arbitrarily primed polymerase chain reaction (AP-PCR) in an attempt to type and group strains of *Acanthamoeba* species.

Examination of 16 isolates with primers APO1, APO2, AM1, AM2, P1 and P2, showed each of the banding patterns, resulting from AP-PCR analysis, were unique to the isolate tested. Further, there were few bands which occurred in more than one isolate, with insufficient similarities to form groupings of isolates.

Two chemotherapeutic agents were selected for a preliminary study into drug sensitivities in *Acanthamoeba* species and strains.
The first of these was Baquacil (20% polyhexamethylene biguanide (PHMB)), and the second was Brolene (0.1% propamidine isethionate).

Within 48 hours 97% of all isolates tested reached zero viability at a concentration of 0.05% PHMB, and 100% of isolates tested reached zero viability at 0.1% PHMB. The results of this study would suggest that the concentration of PHMB be at least 0.05% when used to treat acanthamoebic keratitis.

Within 48 hours of exposure to 0.1% propamidine isethionate (Brolene), only 30% of all isolates tested reached zero viability. However, 60% of isolates tested showed at least 80% reduction in viability within 48 hours of exposure to 0.1% propamidine isethionate.

The ultimate goal of this thesis was to form groups of isolates using PCR and drug sensitivities and to discover any correlation between these groups. The results of AP-PCR analysis however suggests a high genetic heterogeneity within the *Acanthamoeba* genus, thus preventing any correlation with drug sensitivity tests.

Acknowledgements:

This thesis was not a solo effort, a lot of people helped in many different ways, so I'd just like to take this opportunity to say a BIG thanks to all of them.

The Lab Rats:

- The Department of Microbiology and Genetics, Massey University, for providing the opportunity and facilities for this study.
- My Supervisors, **Dr Ray Cursons** for all his help, positive encouraging manner and for arranging funding for this project, and **Professor Tim Brown** for taking a chance on me and for providing motivation whenever I was almost ready to give up.
- The staff within the Protozoa Research Unit, especially Kirsty Farrant and Trish McLenachan for teaching me all about PCR and other useful stuff.
- All the staff and students within the Department who have lent a hand or just made me laugh - it's been a fun few years!
- ICI Chemicals for kindly donating the Baquacil used in these studies.

The 33'ers and The Hot Shots

I'm sorry, there's just not enough room to mention you all but thanks to each of you for listening to my ravings, making me laugh - lots, and for always understanding why I didn't go out, write or shoot, as often as we all would have liked. I don't know what I would have done without you!
I especially want to say a huge thanks to Debs and Manda for being the bestest friends I could ever wish for.

My Family

Mum, Dad and Helen. I think I must have the best family ever! Thanks for all your love and support, for always standing by me and believing in me - even, or especially, when I didn't believe in myself.
This Masterate belongs as much to you as it does to me. I love you.

And finally, I will acknowledge my own efforts. I didn't give up, when that seemed the only course, and now that I'm finished this part of my life I find myself secure in the knowledge that, whatever comes next, I can do it.
More than just science, this degree has taught me many skills I can use in all facets of my life. I guess the SGHS motto makes sense after all - Non Scholae et Vitae Discimus, Not for School but for Life we are Learning.

Contents:

List of Tables:

List of Figures:

CHAPTER ONE: Introduction

1.1 PATHOGENIC FREE-LIVING AMOEBAE

In the late nineteenth century amoebae were implicated in cases of dysentery and of organ and tissue abscesses (Wilhelmus, 1991). This was the first time amoebae were identified in human disease. These amoebae were parasitic, that is, needing a host to survive e.g. *Entamoeba histolytica*. Since then normally free-living (without need of a host) amoebae have also been recognised as opportunistic agents of human disease.
Of all free-living amoebae only those of the genera *Naegleria*, *Acanthamoeba* and *Balamuthia* have been shown to cause disease in humans and other animals. (Visvesvara *et al*, 1993)
These amoebae are able to live free in the environment, and as endoparasites. Normally they do not cause disease but live as phagotrophs, found in large numbers in soil, water and air, feeding on bacteria. (Anon., 1988 & John, 1993) As opportunists they may produce serious infection of the central nervous system (CNS) or the eye. (John, 1993)
Naegleria fowleri, Balamuthia mandrillaris and several species of *Acanthamoeba* are able to cause an often fatal infection involving the CNS, amoebic meningoencephalitis. (Visvesvara *et al*, 1993)
Additionally, some species of *Acanthamoeba* are able to produce a chronic, vision-threatening infection of the cornea, known as acanthamoebic keratitis. (John, 1993)

1.2 HISTORY OF *ACANTHAMOEBA* (John, 1993 & Wilhelmus, 1991)

1930	Castellani mentioned the presence of a special amoeba in cultures of *Cryptococcus pararoseus* and this was classified in the subgroup *Hartmannella-Acanthamoeba*.(John, 1993)
1957-58	Two scientists, Culbertson and Jahnes, detected *Acanthamoeba* species as contaminants of tissue cultures causing cytopathic effects in Monkey kidney cell cultures. (John, 1993 & Wilhelmus, 1991)
1958-59	It was proposed that these amoebae have pathogenic potential. This was demonstrated when *A. culbertsoni* was inoculated intracerebrally and intravenously into monkeys and mice causing meningoencephalitis. (John, 1993)
1971-73	The first clearly identified *Acanthamoeba* infections of the CNS in humans occurred. Prior to this date these amoebae were occasionally referred to as *Hartmanella* species, although it is now commonly accepted there are no (known) pathogenic *Hartmannella* species. (John, 1993)
1974	The first two cases of acanthamoebic keratitis were reported from Great Britain. (John, 1993)

1975	Amoebae cultured from both (above) British patients were identified as *Acanthamoeba* species. (John, 1993) The first United States cases of acanthamoebic keratitis were reported. (John, 1993)
1980	The term granulomatous amoebic encephalitis (GAE) was proposed to describe the CNS infection caused by *Acanthamoeba* species. (John, 1993)
1983	*Acanthamoeba* species were isolated and cultured from human tissue samples for the first time. (John, 1993)
1985-95	Dramatic increase in numbers of acanthamoebic keratitis cases reported. This increase has been linked to the wearing of contact lenses, especially soft contact lenses. (John, 1993 & Wilhelmus, 1991)
1996	To date there have been six cases of acanthamoebic keratitis reported in New Zealand. (Cursons, *pers comm.*)

1.3 BIOLOGY

1.3.1 Morphology:

Acanthamoeba is found in two morphologically distinct forms; an environmentally resistant cyst and a feeding trophozoite or amoeba. It is the amoeba which is infective but both forms have been found in human tissue. (Anon, 1988 & John, 1993)
The trophozoites are uninucleate, with characteristic fine cytoplasmic projections (acanthapodia) and the cysts are also uninucleate having a resistant stellate double wall. Both forms are resistant to most antimicrobial agents. (Anon, 1988) The nuclei of *Acanthamoeba* are characterised by having a large central nucleolus, or karyosome, and a nuclear membrane without chromatin granules. (John, 1993)

1.3.1.a. Trophozoite:

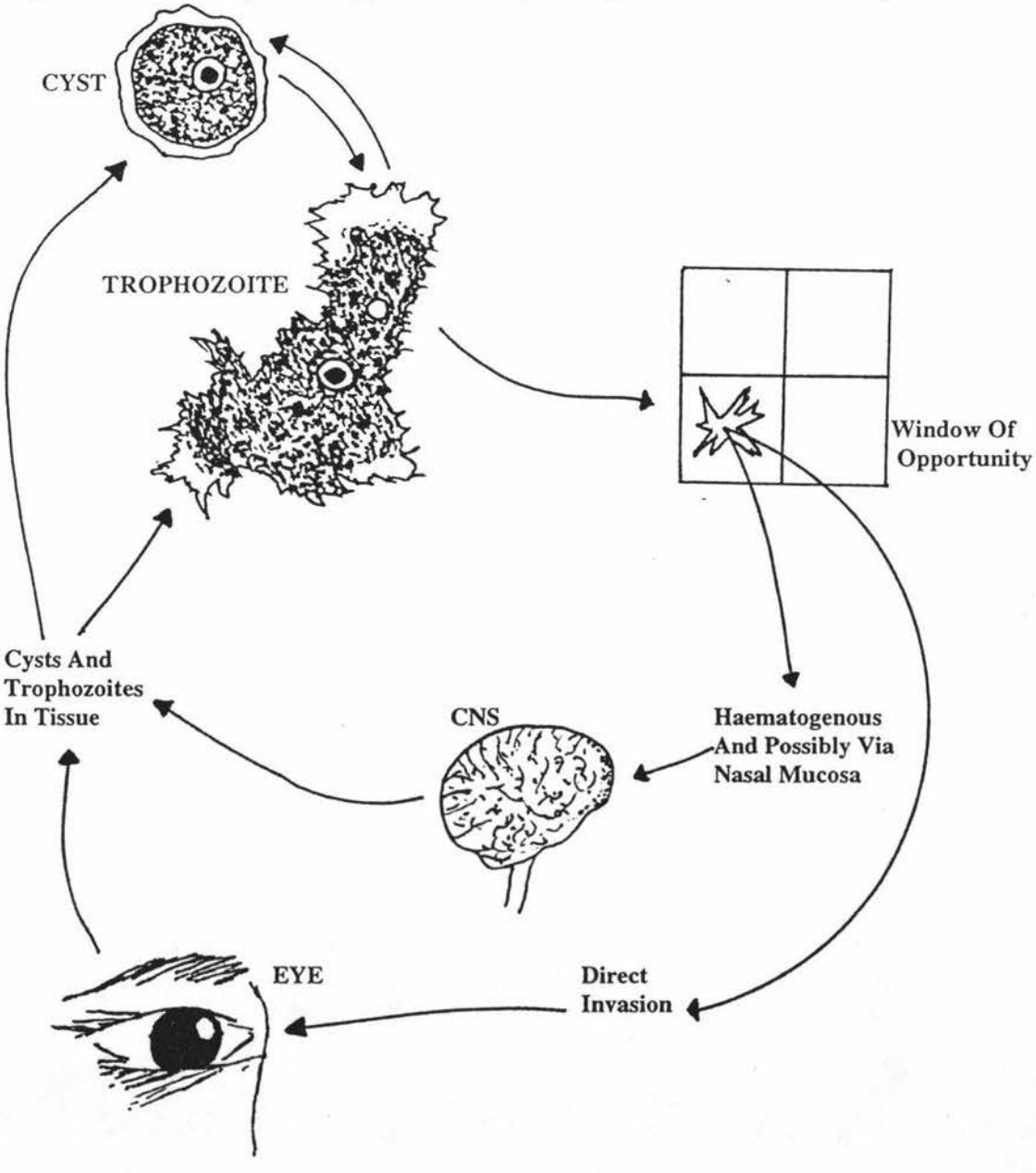
A distinguishing feature of the *Acanthamoeba* genera is the presence of acanthapodia, tapering spike-like pseudopodia. *Acanthamoeba* moves slowly on a broad front without direction and trophozoites average about 24-56μm in length. Nuclear division is metamitotic, where the nucleolus and the nuclear membrane disintegrate during early karyokinesis. (John, 1993)
The cytoplasm consists of an amorphous hyaloplasm in the periphery emerging gradually with the granular endoplasm and its array of organella, mitochondria, ribosomes, phagocytic and pinocytic vacuoles, the pulsative complex, the centrosphere (essential for mitosis) and the nucleus. The numerous mitochondria usually have club-like or ovoid shape. (Rondanelli and Scaglia, 1987)

1.3.1.b. Cyst:

The cystic stage is preceded by several morphologic and functional changes among the cytoplasmic organella contained in the trophozoite. (Rondanelli and Scaglia, 1987)
Considerable variation in cyst morphology occurs among the different species and this has been used in the naming of species. (John, 1993)
Cysts are double walled and, therefore, quite resistant in the environment. The cyst wall is made up of an outer wrinkled, or rippled, ectocyst and an inner endocyst. (John, 1993)
The two membranes appear separate along most of the cell perimeter and join only in areas corresponding to the tips of the polygonal limbs, where they form pores. A metacystic trophozoite is believed to break through these to leave the cyst. (Rondanelli and Scaglia, 1987)
The karyosome in the cyst is smaller than that of the trophozoite and the granular cytoplasm contains many empty vacuoles and liquid droplets which, at times, line the whole inner surface of the cellular membrane. (Rondanelli and Scaglia, 1987)

1.3.2 Life Cycle:

Figure 1.3.2: Life Cycle of Opportunistically Pathogenic *Acanthamoeba* Species.



Invasion of the CNS appears to be by way of the circulation, with amoebae originating from a primary focus elsewhere in the body, possibly the respiratory tract, or ulcers of the skin or mucosa, or other wounds.(Ma *et al*, 1990 & John, 1993)
GAE tends to occur in persons who are debilitated, chronically ill, or immunocompromised. In contrast acanthamoebic keratitis usually occurs in healthy individuals, and infection is by direct invasion of the cornea through trauma to the eye or the wearing of contaminated contact lenses.(John, 1993)

1.4 CLASSIFICATION

The genus *Acanthamoeba* was established in 1931. Since then *Acanthamoeba* species have been variously referred to as *Acanthamoeba, Hartmannella*, and *Mayorella*.

In 1930 Castellani discovered an amoeba in a culture of the fungus *Cryptoccocus pararoseus*. Douglas placed this amoeba in the genus *Hartmanella* that same year.
Later, the genus *Hartmanella* was considered an artificial assemblage of unrelated amoebae and was subdivided into three genera, one of which was *Acanthamoeba*.
These amoebae are characterised by the appearance of pointed spindles at mitosis and have double walled cysts with ostioles and an irregular outer layer.
Between the years 1952 and 1979 there were continuing debates regarding the validity of the genus *Acanthamoeba*. These culminated in the acceptance of 17 species in the genus *Acanthamoeba*. Seven of these have been shown to be pathogenic to humans with a further three species pathogenic to mice. The currently accepted position of *Acanthamoeba* in the taxonomic scheme of the Society of Protozoologists is shown in Table 1.4.1 (Visvesvara, 1991).

An additional classification system that is generally accepted is that of Pussard and Pons (1977) which uses cyst morphology to divide the genus into three groups.
Strains isolated from human infection fall into groups II and III with the majority of corneal isolates falling into group II.

Although the genus *Acanthamoeba* is easily recognised because of its distinctive acanthapodia and cyst structure, species identification is rather difficult.
Traditional taxonomic criteria have been based on cyst morphology, nuclear divisions, temperature tolerance, and pathogenicity. The more recent approaches to *Acanthamoeba* classification have used isoenzyme analysis, restriction enzyme analysis of mitochondrial DNA and analysis of random fragment length polymorphism of total cellular DNA. (Cursons, 1978 & Ma *et al*, 1990). However, the results of these analyses often have not correlated with species identification based on the more traditional criteria.
In the end, the best classification scheme will be one that relies on many different characteristics, including morphological, physiological and biochemical ones. (John, 1993)

Table 1.4.1: Currently Accepted Taxonomy (Visvesvara, 1991 & Ma *et al*, 1990)

Kingdom:	Protista	
Subkingdom:	Protozoa	
Phylum:	Sarcomastigophora	
Subphylum:	Sarcodina	
Superclass:	Rhizopodia	(locomotion by lobopodia, filopodia, rectilopodia, or protoplasmic flow without production of discrete pseudopodia)
Class:	Lobosea	
Subclass:	Gymnamoebia	
Order:	Amoebida	(typically uninucleate; mitochondria present; no flagellate stage)
Suborder:	Acanthopodina	(more or less finely tipped, sometimes filiform, often furcate hyaline subpseudopodia produced from a broad hyaline lobe not regularly discoid; cyst usually formed; nuclear division mesomitotic or metamitotic).
Family:	Acanthamoebidae	
Genus:	*Acanthamoeba*	

Species: Isolated from human infections at indicated site(s):

A. astronyxis	(CNS)
A. castellani	(CNS, eye)
A. culbertsoni	(CNS, eye)
A. hatchetti	(eye)
A. palestinensis	(CNS)
A. polyphaga	(CNS, eye)
A. rysodes	(CNS, eye)
A. lugdunensis	(eye)

Not isolated from human infections:

A. comandoni
A. griffini
*A. lenticulata**
*A. royreba**
A. tubiashi
A. enchinulata
A. divionensis
A. triangularis
A. mauritaniensis

* shown to be pathogenic for mice

1.5 EPIDEMIOLOGY

1.5.1 Occurrence/distribution of *Acanthamoeba* species:

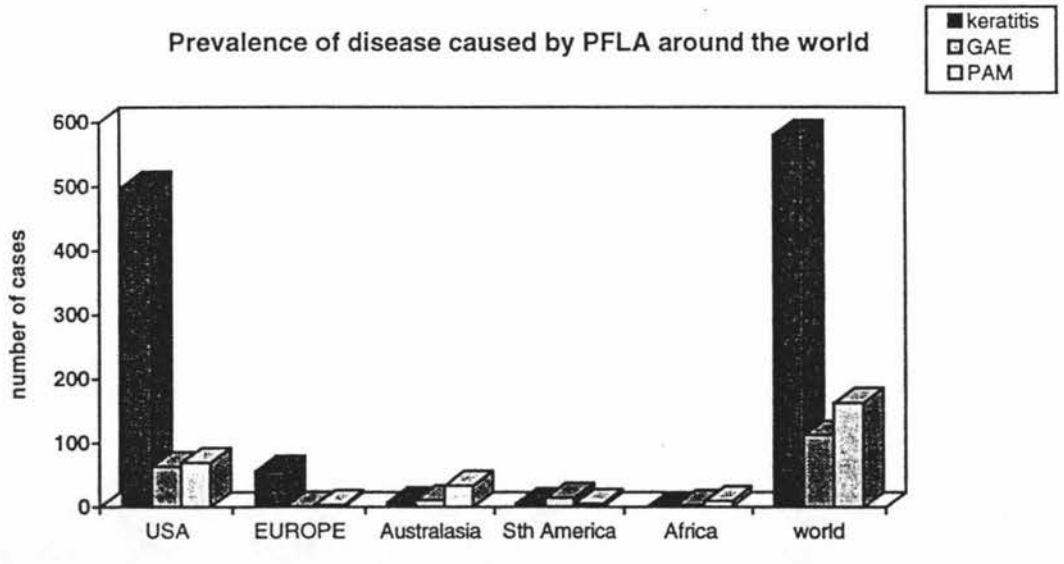
Acanthamoebae appear to be truly ubiquitous organisms with isolations recorded from: freshwater, bottled mineral water, frozen swimming water, distribution (i.e. tap) water, chlorinated swimming pools, physiotherapy pools and medicinal pools, hot tubs, brackish and sea water as well as ocean sediments, soil, compost, sewage, mushrooms and vegetables, dust in air, cooling towers of electric and nuclear power plants, heating, ventilating and air conditioning units, bacterial, fungal and mammalian cell cultures, fish, reptiles, birds, mammals (intestines, cerebral tissue, lung tissue, skin wounds, corneas). In humans they have been found in the nose and throats of people with respiratory illness as well as from healthy persons, and in bronchial secretions, ear discharge, stool samples of patients with diarrhoea, intrauterine contraceptive devices, dialysis units, gastrointestinal washings, gastric-lavage tubing, dental units, contact lenses and contact lens solutions. (De Jonckheere *et al*, 1991 & Visvesvara *et al*, 1990)

This apparently ubiquitous distribution is no doubt due to the possession of resistant cysts enabling these pathogenic free-living amoebae to withstand unfavourable conditions and to take advantage of the intermittent occurrence of favourable conditions. (Cursons, 1978)
The distribution of pathogenic species to non-pathogenic ones is still unknown. In general pathogenic species are found in conditions where temperatures are above ambient, and closer to body temperature. (Cursons, 1978)

1.5.2 Prevalence of disease caused by pathogenic free-living amoebae

The following graph provides a comparison of the numbers of Primary Amoebic Meningitis, Granulomatous Amoebic Meningoencephalitis and acanthamoebic keratitis in several areas of the world (Visvesvara, *pers comm.*).

Figure 1.5.1



In most countries acanthamoebic keratitis is not a notifiable disease, therefore the true incidence is not known. A study in the USA revealed that where clinicians were familiar with acanthamoebic keratitis the numbers of cases reported were higher.
The following graph compares reported numbers of acanthamoebic keratitis with those of GAE and PAM in the USA (Visvesvara and Stehr-Green, 1990).

Figure 1.5.2

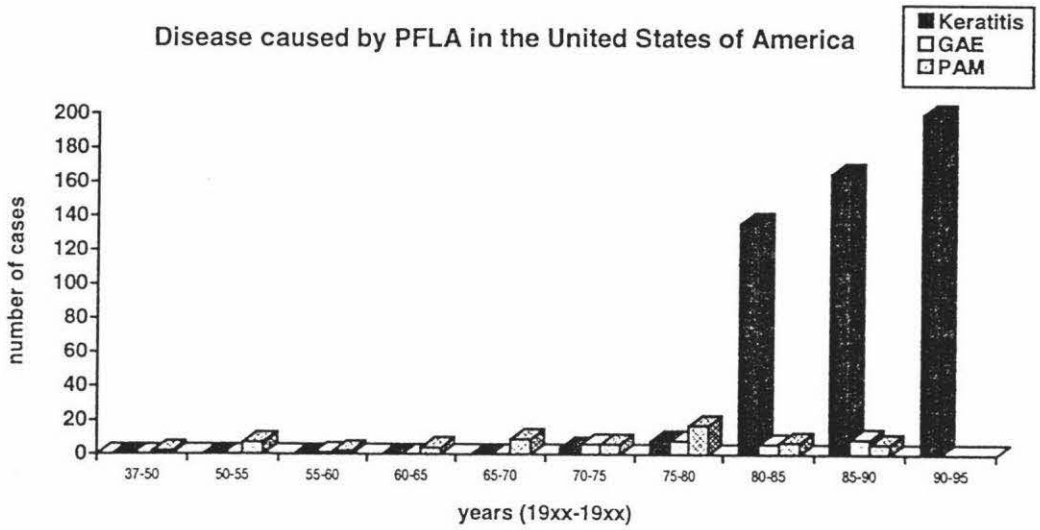


Figure 1.5.2 shows a dramatic increase in cases of acanthamoebic keratitis since 1980, while numbers of cases of PAM and GAE remain approximately static in occurrence. This increase correlates with increased usage of contact lenses. It has been suggested that contact lens use for purely cosmetic reasons is high in the United States of America. Therefore it would not be unreasonable to expect an increase in acanthamoebic keratitis in those areas of the world, shown in Figure 1.5.1, which currently have low contact lens usage, as contact lenses become more popular. It is also likely that although PAM and GAE appear to be of more importance in some areas, these diseases are not being reported at a higher frequency and acanthamoebic keratitis has the potential to overtake these statistics in a very short amount of time.
For example, in New Zealand the majority of reported PAM cases occurred in a short period of time in the early 1970's. Since that time perhaps 1 or 2 further cases have occurred. In comparison, 6 cases of acanthamoebic keratitis have been reported within the last 5 years (Cursons, *pers comm.*).

1.6 DISEASES PRODUCED

1.6.1 Granulomatous Amoebic Encephalitis (GAE):

GAE usually occurs in debilitated or chronically ill, or immunocompromised individuals, although some cases have been reported in otherwise healthy people.
This disease is not well defined with the course of infection being subacute or chronic and lasting from weeks to months, or maybe even years. Onset is insidious with a prolonged clinical course during which single or multiple space-occupying focal granulomatous lesions develop in the brain.

An altered mental state is a prominent feature in GAE, with headache, seizures and neck stiffness occurring in about half of the cases. Nausea and vomiting may be present in some cases.
Acanthamoeba infection probably occurs through the lower respiratory tract or through ulcers of the skin or mucosa. Invasion of the CNS occurs by haematogenous spread from the primary focus of infection. As the brain has no lymphatic channels the invasion must take place via the bloodstream. (John, 1993)

1.6.2 Acanthamoebic keratitis:

A chronic infection of the cornea caused by direct contact of the cornea with amoebae. This may occur through minor corneal trauma or exposure to contaminated water or contact lenses. (John, 1993)
Acanthamoebic keratitis usually develops over a period of weeks to months.
Characteristics of the disease include severe ocular pain, often out of proportion to the degree of inflammation, affected vision, and a paracentral corneal ring infiltrate.
If not correctly treated this infection can lead to loss of vision and, in extreme cases, removal of the infected eye may be necessary. This disease on occasion is mistakenly diagnosed as *Herpes simplex* keratitis, with the only major difference in symptoms being the severity of ocular pain occurring in acanthamoebic infections.(John, 1993).
This disease is further discussed later in this introduction.

1.7 IMMUNITY

Because of the frequency and ease of isolation of pathogenic free-living amoebae (PFLA) from the environment, many authors have been puzzled regarding the low incidence of infection by PFLA in the human population. This has led many authors to speculate upon the existence of probable host-related susceptibility factors although these remain undefined.
Antibodies to PFLA have been detected in normal human sera by indirect fluorescent-antibody testing. These have been to both pathogenic and non-pathogenic free-living amoebae (Cursons *et al.*, 1980 & John, 1993)

Predisposition to infection by contact lens wearers may be because,
1. the lens holds the amoebae in contact with the cornea,
2. the lens causes minor trauma, allowing the amoebae to enter through the epithelial layers,
3. the lens protects the amoebae from removal by blinking or tear secretions, and/or
4. levels of antibodies and complement are low in the eye.

1.8 ACANTHAMOEBIC KERATITIS

In 1973 Visvesvara reported the first case of acanthamoebic infection of the eye. (Asbell, 1993) Sporadic reports of ocular acanthamoebic infection followed; in 1982, not a single case of acanthamoebic keratitis was found in a 30-year review of 700 patients with corneal ulcers, all of whom had undergone laboratory evaluation. (Asbell, 1993) It was not until about 1985, after contact lens use, in particular soft and disposable (extended wear) contact lens use, had dramatically increased, that the

numbers of acanthamoebic keratitis cases being reported showed a marked increase and a direct association between contact lens wear and acanthamoebic keratitis was recognised. (Asbell, 1993) Between 1980 and 1985 there was a 59% increase in numbers of people wearing contact lenses in the United States of America. (John, 1993)
Acanthamoebic keratitis is a distinct, vision-threatening ophthalmological condition, causing serious concern in many countries. It is more common in economically developed countries where the wearing of contact lenses, for cosmetic and sight corrective purposes, is both fashionable and affordable. (John, 1993)
Corneal trauma often precedes infection but this may not be a necessity. (Moore, 1990). Even minor trauma caused during the insertion, removal, or normal wearing of contact lenses can provide an avenue for entry of the micro-organisms. (Asbell, 1993)
Because the clinical signs of acanthamoebic keratitis resemble herpetic, fungal or bacterial keratitis, diagnosis and treatment may be erroneous. (Horne *et al*, 1994)

1.8.1 Diagnosis:

Common clinical signs of ocular acanthamoebic infection are breakdown of the corneal epithelium, the presence of satellite lesions, and iritis, sometimes with hypopyon. Often, after an initial period, the infection progresses slowly. Infection waxes and wanes, with corneal healing followed by recurrent epithelial deterioration. This can present a confusing picture to the clinician in practice. There may be elevated intraocular pressure and scleral inflammation. (Asbell, 1993)
The primary sign of infection that has penetrated deep into the cornea is the combination of a central or paracentral ring infiltrate and remarkably intense pain. (Asbell, 1993)
The initial method of diagnosis is to take corneal scrapings for culture on non-nutrient agar seeded with bacteria, and subsequent microscopic examination. It is often useful to examine contact lenses, storage cases and care solutions (where appropriate) as well as corneal tissue for the presence of acanthamoebic contamination. In some cases, biopsy may be necessary to obtain a firm diagnosis. (Horne *et al*, 1994)
It is important that specimens be examined by someone who is thoroughly familiar with the appearance of *Acanthamoebae* because these micro-organisms may be confused with degenerated epithelium or macrophages. (Anon., 1988)
Species identification may be determined using a variety of staining techniques including indirect immunofluorescent antibody, Giemsa, and calcofluor white. Identification in histopathological specimens may be achieved by employing the haematoxylin and eosin procedure or a more specialised staining method e.g., Heidenhain's haematoxylin, Gomori's chromium haematoxylin, periodic acid-Schiff, Bauer chromic acid-Schiff and silver methanamine. (John, 1993)
Herpes simplex keratitis is the most common misdiagnosis. The single most consistent clinical symptom is severe ocular pain, which is not characteristic of infection limited to the cornea and generally not present in *Herpes simplex* infection. Additional distinguishing features include a history of direct exposure to soil or water, wearing contact lenses, scleritis, and failure of cultures from the inflamed eye to reveal bacteria, fungi or viruses. (John, 1993)

1.8.2 Pathology:

The pathology of acanthamoebic keratitis is characterised by chronic progressive keratitis (John, 1993). In the early period of infection pseudodentritic figures in the

epithelium or just beneath the epithelium, in the anterior stroma, are typical pathological features. (John, 1993)
This period is thus characterised by limbitis, perineural infiltrates and superficial epithelial changes, with few ring infiltrates and little anterior uveitis. (Bacon *et al*, 1993)
As the infection progresses into the active period pathology includes frank ulceration, ring infiltrates and anterior uveitis often with hypopyon. Sometimes endothelial plaques causing corneal oedema occur. Perineural infiltrates are less common in early disease. (Bacon *et al*, 1993) In advanced cases there may be marked stromal infiltrate and necrosis. The whitish inflammatory infiltrate, often appearing ring-shaped around the corneal ulcer, consists mainly of polymorphonuclear leukocytes and macrophages with a few lymphocytes. (John, 1993) Evolution of severe features of the disease include ring ulcers, abscesses, scleritis, glaucoma, cataracts and secondary microbial infection. (Bacon *et al*, 1993).
Although granulomatous inflammation has been described in acanthamoebic keratitis (John, 1993), in most of the reports, neutrophils, and not lymphocytes, are the predominant infiltrating cells. Corneal ulceration may progress to perforation (John, 1993). Acanthamoebic ocular infections have been described variously as conjunctivitis, iritis, scleritis, and uveitis. (John, 1993).
Acanthamoeba trophozoites and cysts occur within infected tissue. (John, 1993)

1.8.3 Treatment:

Initially most patients required surgical treatment, ranging from surgical debridement of the affected cornea to corneal transplantation or removal of the infected eyeball. (Asbell, 1993 & John, 1993)
The first successful chemotherapeutic treatment used a combination of dibromopropamidine and propamidine isethionate. Treatment was prolonged but curative. (Asbell, 1993)
Current data suggest that cure is possible if acanthamoebic keratitis is diagnosed early, and appropriate medical treatment is initiated quickly and administered for a prolonged period i.e., six to twelve months.(Asbell, 1993)

1.8.3.1 Pharmacology:

The following drugs with varying efficacy have been used to treat cases of acanthamoebic keratitis.
Topical propamidine, topical miconazole, systemic ketonazole, neosporin topical clotrimazole, oral itraconazole, neomycin., polyhexamethylene biguanide (PHMB), paromomycin, acriflavine, polymyxin B, colistin, crystal violet, methyl benzlkonium chloride, an experimental drug (R11/29) and brilliant green.
In some patients a combination of between three and twelve of these drugs were used, sometimes in combination with surgical debridement of lesions. (Asbell, 1993, John, 1993, Bacon *et al*, 1993, Horne *et al*, 1994)
The most efficacious amoebistatic agents at present are pentamidine isethionate, propamidine isethionate, stilbamidine isethionate, clotrimazole and ketonazole. Ethanol and methanol have also showed activity against amoebae (Tomlinson, 1991).

Studies suggest that inhibitors of polyamine synthesis, e.g. diminazene aceturate, pentamidine and methylglyoxal bis(guanylhydrazone)(mitoguazone) could be developed to aid in chemotherapy for acanthamoebic infections. (Shukla, 1991)
Magainins from the skin of a south African toad were shown to be inhibitory to trophozoite growth and, although not cysticidal, may be useful in the management of acanthamoebic keratitis. (Feldman *et al*, 1991)
Local corticosteroids may be detrimental to the host in the presence of acanthamoebic keratitis. These were commonly used in early cases where prognosis was likely keratoplasty in order to limit inflammation and increase successful surgery. (John *et al*, 1991b)

1.8.4 Risk factors:

Risk factors involved in contraction of acanthamoebic keratitis include wearing contact lenses, use of nonpreserved ophthalmologic products (e.g., home-made saline), exposure of eyes to nonsterile water sources (e.g., well, tap, sea and surface water), contamination of contact-lens storage cases and of storage and rinsing solutions, trauma to the eye, compromised host defence mechanisms and corneal surgery. (Horne *et al*, 1994)
Eighty percent of reported cases of acanthamoebic keratitis have occurred among contact lens wearers, 75% of whom wore daily-wear or extended-wear soft hydrogel lenses.
There is also evidence to suggest that a great many lens wearers remove lenses, usually because of dust, dirt or ocular irritation, and reinsert them later after a rinse in tap water or saline, without disinfection. (Asbell, 1993)

The risk factors associated with contact lens wear include:

Lens materials. All modern contact lenses contain a certain amount of water which serves as a medium for oxygen influx. In proportion to their water content, lenses absorb material, including potential pathogens, from cleaning solutions, lens cases, and patients hands. Thus, lens material itself can be a risk factor for acanthamoebic infection. (Asbell, 1993)

Lens Cleaning and Disinfection. Preservatives in solutions were major culprits in discomfort. This led practitioners to recommend the use of unpreserved, home-made, solutions. At this time it was not known that *Acanthamoeba* was ubiquitous in the environment, and particularly in tapwater. The marked increase in acanthamoebic infections during the 1980s certainly reflected, to a degree, widespread use of non-sterile, unpreserved solutions. This practice is now actively discouraged. (Asbell, 1993)
Other sources of lens contamination result from the patient not following disinfection regimes properly or rinsing lenses in tap water to remove disinfecting solution or dust particles. (Asbell, 1993)

Lens Storage Cases. In studies involving 102 asymptomatic lens wearers and their lens cases it was discovered that about half of the lens cases contained significant amounts of bacteria and seven cases contained *Acanthamoeba.* In general *Acanthamoeba* do not occur alone and it is possible a synergistic or symbiotic relationship with other bacteria allows *Acanthamoeba* to grow to levels where there becomes a significant risk of pathogenic infection.

At the moment there is no obvious solution to this problem (Asbell, 1993), although one suggestion is that the contact lens case be replaced with the purchase of disinfection systems.

Extended Wear. A study in 1989 showed that soft-lens wearers who slept with lenses in were 10-15 times more likely to develop ulcerative keratitis than those who wore lenses only during waking hours. (Asbell, 1993)

Lens Degradation. Contact lenses, over a period of time, eventually develop deposits. This is unavoidable and are in fact part of a biofilm. This biofilm is produced in response to a foreign body (the lens) enabling the body to recognise the lens and not react adversely to them. However it is also a source of contamination, adding to the risk of infection. (Asbell, 1993)

1.9 SUMMARY AND GOALS

There are few free-living amoebae that are also opportunistic agents of human disease. One genera of amoebae that is capable of this is *Acanthamoeba*.
Some strains of *Acanthamoeba* are able to produce one or both of two distinct disease states in humans. The first of these is Granulomatous Amoebic Encephalitis (GAE), an infection of the central nervous system that can be compared to amoebic meningitis (or primary amoebic meningoencephalitis - PAM). PAM is caused by another pathogenic free-living amoeba, *Naegleria fowleri*.
The second disease state caused by Acanthamoebae is a chronic infection of the cornea known as acanthamoebic keratitis.
All diseases caused by pathogenic free-living amoebae occur infrequently, however the incidence of acanthamoebic keratitis is increasing. This increase has been linked with the increased usage of contact lenses, and in particular, soft contact lenses. Once diagnosed, chemotherapeutic treatment is prolonged and often ineffective. This can render necessary surgical means of controlling the disease. Surgical options include debridement of lesions, keratoplasty or, in extreme cases, removal of the infected eyeball.
It is therefore desirable to discover a quick and accurate means of diagnosing acanthamoebic keratitis, and to gain knowledge regarding which chemotherapeutic agents are most effective against acanthamoebic keratitis.

The goals of this thesis are:

1. To compare methods of extracting DNA from *Acanthamoeba* species in order to

2. Evaluate the polymerase chain reaction as a means of diagnosis of acanthamoebic keratitis and differentiation of *Acanthamoeba* species and strains.

3. To conduct preliminary *in vitro* drug sensitivity trials.

4. To determine whether any correlation exists between drug sensitivities and banding patterns revealed by PCR.

CHAPTER TWO: Investigation Of Techniques For DNA Extraction From *Acanthamoeba* Species

2.0 INTRODUCTION

For many organisms, there is detailed information for the DNA extraction process and thus there are published protocols of the best way to extract the various nucleic acids from their cells. This is not the case for *Acanthamoeba* species.
Therefore the first goal of this thesis was to examine several DNA extraction procedures and determine their effectiveness in extracting DNA from *Acanthamoeba* cells.
There are several factors which were considered when classifying the usefulness of each method.
1. The DNA should be genomic DNA because the primers being used in subsequent PCR reactions are random (i.e. arbitrarily primed). Genomic DNA will give the greatest number of sites for priming and therefore a greater likelihood of differences being seen.
2. The DNA must react consistently in the arbitrarily primed PCR reaction.
3. DNA yield must be sufficient, and free of inhibitors, to be useful for PCR reactions
4. The number of steps in the extraction procedure where the DNA could be lost should be minimised.
5. The time required to perform the extraction should be as short as possible
These factors have been identified as being important for this thesis and when developing a procedure to be used in a diagnostic laboratory. Other factors may be more important when the DNA is to be used for different procedures.

2.1 MATERIALS AND METHODS

Acanthamoeba isolates were provided in axenic culture by Dr. R.T.M. Cursons, Waikato Hospital, Hamilton, New Zealand.

Isolate identification is shown in *Appendix 1*.

2.1.1 Propagation of *Acanthamoeba* Isolates for DNA extraction.

i. Peptone-Yeast Extract-Glucose (PYG) Medium for axenic culture of *Acanthamoeba*

Proteose Peptone	20.00g
Yeast Extract	2.00g
$MgSO_4$	0.98g
$CaCl_2$	0.059g
Sodium Citrate.2 H_2O	1.00g
$Fe(NH_4)_2(SO_4)_2.6\ H_2O$	0.02g
KH_2PO_4	0.34g
$Na_2HPO_4.\ 7\ H_2O$	0.355g
Glucose	18.00g
Distilled water to	1000.0ml

All ingredients, except $CaCl_2$ were dissolved in 900ml of distilled water. The $CaCl_2$ was added while the solution was being stirred.
The pH was adjusted to pH 6.5 ± 0.2 and distilled water was added to reach the final volume of 1000ml.
The media was autoclaved for 15 minutes at 121°C.
It is recommended that Difco products be used.

Prior to inoculation with *Acanthamoeba* the following antibiotics were added to the media:

ii. Antibiotic cocktail

Gentamycin to give a final concentration in media of	100µg/ml
Vancomycin to give a final concentration in media of	100µg/ml
Amphotericin B to give a final concentration in media of	5µg/ml

To each 10ml aliquot of PYG media (+ antibiotics) a 0.5ml inoculum of *Acanthamoeba* was added. The cultures were propagated at 30°C on a shaker for three to five days.

iii. Page's Amoeba Saline (PAS) Agar

NaCl	0.12g
$MgSO_4.7\ H_2O$	0.004g
$CaCl_2.2\ H_2O$	0.004g
Na_2HPO_4	0.142g
KH_2PO_4	0.136g
Davis Agar	15.00g
Distilled water to	1000.0ml

Ingredients were added to approximately 900ml distilled water and the pH adjusted to pH 6.8 before the volume was brought to 1000.0ml with distilled water.
The media was autoclaved at 121°C for 15 minutes before being dispensed into sterile petri dishes.

2.1.2 Preparation of cells for DNA Extraction

Three millilitres of Acanthamoebae cultured in PYG media were centrifuged in a Falcon tube at 4000g for 10 minutes and the supernatent discarded. The pellet was resuspended in 1ml PBS, centrifuged at 4000g for 10 minutes and the supernatent discarded. The pellet was used for DNA Extraction.

2.1.3 DNA Extraction

2.1.3.1 *Method 1.*

i. Phosphate buffered Saline (PBS) pH 7.2

NaCl	8.5g
$Na_2HPO_4.\ 12\ H_2O$	2.7g
$NaH_2HPO_4\ .\ 2\ H_2O$	0.39g

Distilled water to 1000.0ml

Autoclave for 15 minutes at 121°C and store at room temperature

ii. Tris - EDTA (TE) Buffer

10mM Tris HCl (pH 7.5)
1mM disodium salt EDTA (pH 7.2)

iii. Sodium Dodecyl Sulphate (SDS) 10% (w/v)

Stored at room temperature without autoclaving

iv. Pronase Type XIV 10mg/ml

This solution was preincubated at 37°C for three hours to self-digest contaminants, especially DNase activity. This was filtered through a 0.2μm filter and stored at -20°C.

v. RNase (Ribonuclease 1) 2mg/ml

This solution was preincubated at 90°C for 10 minutes to destroy DNase activity. It was filtered through a 0.2μm filter and stored at -20°C.

vi. 5M Sodium Perchlorate

vii. Phenol/chloroform/isoamyl alcohol

A 25:24:1 solution, respectively, was prepared.

viii. Saline Tris-EDTA (STE) Buffer (x10)

1.0 M NaCl
0.5M Tris-HCl (pH 7.5)
10mM EDTA (disodium salt) (pH 7.2)

ix. 5M NaCl

x. Absolute Ethanol

xi. 70% Ethanol

The pellet of cells containing the Acanthamoebae was resuspended in 1.0ml Tris-EDTA Solution, 0.1ml SDS (10% w/v) and 0.1ml Pronase Type XIV (10mg/ml) and incubated at 50°C overnight to ensure all cells were lysed and proteins digested. 0.1ml RNase was added to the lysate and incubated for a further 60 minutes at 50°C to digest the RNA. Sodium perchlorate (5M) was added to give a final concentration of 1M and incubated at 50°C for 60 minutes.

The phenol/chloroform/iso-amyl alcohol (25:24:1 v/v/v) solution was mixed with one tenth its volume of STE Buffer. Air was bubbled through the preparation to facilitate mixing. This buffered phenol/chloroform/iso-amyl alcohol solution was stored at room temperature and used to deproteinise the lysate.

An equal volume of buffered phenol/chloroform/iso-amyl alcohol solution was added to the lysate, mixed by repetitively inverting (approximately 30 times) the tube followed by a settling time of five minutes during which the interface formed. This mixture was centrifuged at 950-1400g for 10 minutes at room temperature following which the upper aqueous layer, containing the DNA, was removed using a 5ml serological pipette and placed in another Falcon tube. The DNA was re-extracted twice more with the phenol/chloroform/iso-amyl alcohol solution.

A one twentieth volume of 5M NaCl was added followed by two times the total volume of -20°C absolute ethanol. The tube was left at -20°C overnight before being centrifuged at 4000g for 25 minutes. The supernatent was removed and 70% ethanol poured onto the pellet. This was centrifuged at 4000g for 10 minutes and the supernatent discarded. The pellet was dried at 37°C following which it was resuspended in 500µl Tris-EDTA Solution and transferred to an eppendorf 1.5ml tube for easier handling.

2.1.3.2 ***Method 2.***

i. Lysis Buffer L6

GuSCN	120.00g
0.1M Tris HCl (pH 6.4)	100.00ml
0.2M EDTA	22.00ml
Triton X100	2.60g

ii. Diatom Suspension

Diatoms	10.0g
32% (wt/vol) HCl	500µl
Distilled water to	50ml

Dispense in 1ml aliquots to small bottles and autoclave for 15 minutes at 121°C

iii. Washing Buffer L2

GuSCN	120.00g
0.1M Tris HCl (pH 6.4)	100.00ml

iv. 70% ethanol

v. Acetone

vi. 0.1M Tris-HCl

Trizma base	1.211g
Distilled water to	100.0ml
Adjust to pH 6.4 with HCl	

vii. 0.2 M EDTA

EDTA	6.5448g
Distilled water to	100.0ml
Adjust to pH 8.0 with NaOH	

viii. TE Buffer (100X)

Trizma base	12.11g
EDTA (disodium salt)	3.7224g
Distilled water to	100.0ml

Adjust to pH 8.0 with HCl and, as required, dilute 1:99 to give 10mM Tris - 1mM EDTA pH 8.0

The pellet containing the *Acanthamoeba* cells was resuspended in 200µl TE Buffer and transferred to a 1.5ml eppendorf tube. In a separate 1.5ml eppendorf tube 900µl L6 Lysis Buffer and 40µl Diatom suspension were added and mix by vortex to homogeneity.
The lysis buffer lyses cells also inactivating nucleases while, in the presence of high concentrations of GuSCN, diatoms provide a structure to which nucleic acids adhere whilst other cellular components are washed away. Diatoms are fossilised cell walls of unicellular algae and consist almost entirely of silica but are much larger than pure silica particles.
To the Diatom/Lysis solution 50µl cell suspension was added. This mixture was immediately mixed by vortex for 5 seconds before sitting at room temperature for 10 minutes. The tube was centrifuged at 14 500g for 15 seconds and the supernatent discarded.

The pellet was washed* twice with L2 Washing Buffer, washed* twice with 70% ethanol and finally washed* once with acetone.
The pellet was then dried at 56°C in a heat block for 10 minutes before being resuspended in 50µl TE Buffer. The suspension was incubated at 56°C for 10 minutes, mixed by vortex for 5 seconds and centrifuged at 14 500g for 2 minutes.
The supernatent, containing DNA and RNA was removed, placed in a fresh tube and stored at -20°C.

* Add 1ml wash liquid, vortex until pellet resuspended, centrifuge at 14 500g for 15 seconds, dispose of supernatent by suction.

2.1.3.3 *Method 3*

i. Vacuum grease

ii. Sterile deionised water

iii. Lysis Buffer L6

As for *Method 2*

iv. Phenol/ chloroform/ isoamyl alcohol

A 25:24:1 solution, respectively.

v. Isopropanol

vi. TE Buffer

As for *Method 2*

The pellet of cells containing the Acanthamoebae was resuspended in 100µl deionised water in a 1.5ml eppendorf tube. To this 50µl vacuum grease and 400µl L6 Lysis Buffer was added. The Vacuum grease provides silica to which the DNA adheres while other cellular components are removed. The tube was slowly inverted and rotated for 10 minutes at room temperature. 500µl of phenol/chloroform/iso-amyl alcohol (25:24:1 v/v/v) was added to deproteinise the lysate and the tube mixed by vortex for 5 seconds before being centrifuged at 14 500g for 10 minutes.
The supernatent was removed and placed in a new tube and precipitated with an equal volume of isopropanol overnight at -20°C.

The following day the mixture was centrifuged at 14 500g for 30 minutes, the supernatent discarded and the pellet was dried at 37°C before being resuspended in 50µl TE Buffer.

2.1.3.4 Method 4

i. TE Buffer

As for *Method 2*

ii. Sarkosyl (10% w/v)

iii. 6M Guanidine thiocyanate

iv. Isopropanol

v. 5M NaCl

vi. CTAB/NaCl Solution (10% CTAB in 0.7% NaCl)

CTAB (hexadecyltrimethylammonium bromide)	10g
NaCl	4.1g
Distilled water to	100.0ml

Dissolve NaCl in 80ml water; slowly add CTAB while heating and stirring; it may be necessary to heat to 65°C to dissolve into solution; adjust to a final volume of 100ml.

vii. Chloroform

viii. Absolute Ethanol

ix. 70% Ethanol

The pellet containing Acanthamoebae cells was resuspended in 250µl TE Buffer and 300µl Sarkosyl (10% w/v) added along with 2.45ml GuSCN (6M). This mixture was incubated at 65°C for 10 minutes to lyse the cells. Precipitation of the DNA was achieved by adding 0.6 volumes of isopropanol and the tube left at -20°C overnight.

The tube was centrifuged at 4000g for 30 minutes, the supernatent discarded and the pellet resuspended in 600µl and 100µl NaCl (5M). To this 80µl CTAB/NaCl solution was added and the mixture incubated for 10 minutes at 65°C. The CTAB/NaCl solution removes polysaccharides present in the lysate, thus increasing available binding sites for primer binding in PCR reactions. An equal volume of chloroform was added and vortexed through the mixture prior to the tube being centrifuged at 14 500g for 10 minutes.

The upper aqueous layer was removed and placed in a new tube with an equal volume of absolute ethanol at -20°C to precipitate the DNA. The tube was centrifuged at 14 500g for 30 minutes and the supernatent was discarded. The pellet was washed with 70% ethanol and centrifuged at 14 500g for a further 3 minutes. The supernatent was discarded, the pellet dried at 37°C and finally resuspended in 50µl TE Buffer.

2.1.3.5 *Method 5*

i. L6 Lysis Buffer

As for *Method 2*

ii. Phenol/chloroform/iso-amyl alcohol

A 25:24:1 solution, respectively.

iii. Saline Tris-EDTA (STE) Buffer (x10)

As for *Method 1*

iv. 3M Sodium Acetate

v. Isopropanol

vi. Absolute Ethanol

vii. 70% Ethanol

viii. CTAB in deionised H_2O (2% w/v)

ix. High Salt TE Buffer

10mM Tris-HCl pH 8.0
0.1mM EDTA pH 8.0
1M NaCl

x. Chloroform

Pelleted cells were resuspended in 5ml L6 Lysis solution and incubated at 65°C for 45 minutes. An equal volume of phenol/chloroform/iso-amyl alcohol/STE (see Method 1) was added to deproteinise the lysate and the tube centrifuged for 10 minutes at 2500g. The upper aqueous was removed and placed in a new tube where it was gently mixed with one tenth its volume of sodium acetate (3M) to aid precipitation of the DNA. A 0.6 volume of isopropanol was added to further ensure DNA precipitation and the mixture centrifuged for 10 minutes at 2500g. The supernatent was removed to a new tube while the pelleted DNA was resuspended in 600µl sterile distilled water. A thin glass rod was used to spool any DNA remaining in the supernatent and this DNA was added to that resuspended in water.

The resuspended DNA was mixed gently with an equal volume of phenol/chloroform/iso-amyl alcohol/STE to ensure the DNA was fully deproteinised and centrifuged at 11500rpm for 2 minutes. The upper aqueous layer, containing the DNA, was transferred to a new tube. This process was repeated once.
The DNA was precipitated from this extract by adding one tenth volume sodium acetate and 2.5 volumes of absolute ethanol. This mixture left at -20°C for two hours followed by centrifugation (11 500rpm) for 15 minutes at 4°C.
The supernatent was discarded, 1ml 70% ethanol added and the tube centrifuged (11 500rpm) for 15 minutes at room temperature. The supernatent was discarded and the pellet resuspended in 700µl sterile distilled water.

The DNA was incubated for 10 minutes at room temperature with 500µl of 2% CTAB (in deionised water) Solution to remove any polysaccharide contamination. This incubation was followed by a 10 minute centrifugation at 11 500rpm. The supernatent was discarded and the pellet resuspended overnight in 200µl high salt buffer.
The solution was diluted 1:1 with sterile distilled water and equal volume of chloroform added. The solution was centrifuged for 5 minutes at 11 500rpm and the upper aqueous layer transferred to a new tube.
An equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1 v/v/v) was mixed gently through the solution, centrifuged for 5 minutes and the upper aqueous layer transferred to a new tube. This process was repeated once.

An equal volume of absolute ethanol was added and the mixture centrifuged for 30 minutes at 11 500rpm. The supernatent was discarded, absolute ethanol added to the pellet and centrifuged for a further 10 minutes.
The supernatent was discarded, the pellet dried at 37°C and resuspended in 300µl TE Buffer.

2.1.3.6 *Method 6*

i. TES Buffer

2mM Tris-HCl
10mM EDTA
50mM Sucrose
Adjust to pH 8.0

ii. Sodium Dodecyl Sulphate (1% in 0.2N NaOH)

iii. 3M Potassium Acetate Buffer

5M Potassium Acetate	60.0ml
Glacial Acetic Acid	11.5ml
Distilled Water	28.5ml
Adjust to pH 6.0	

iv. Phenol/chloroform/iso-amyl alcohol

v. TE Buffer

As for *Method 2*

vi. 3M Sodium Acetate

vii. Absolute Ethanol

viii. 70% Ethanol

Pelleted cells were resuspended in 100µl TES solution and 200µl SDS (1% in 0.2N NaOH) was added in order to lyse the cells. The tube was inverted gently until the contents mixed before being incubated on ice for 3 minutes.
150µl chilled potassium acetate buffer (3M) was added, the solution mixed by inversion and incubated on ice for 60 minutes.
The tube was centrifuged for 10 minutes at 4°C and the supernatent transferred to a new tube where it was gently mixed with an equal volume of phenol/chloroform/iso-amyl alcohol which had been saturated with TE Buffer (pH 8.0).
The tube was centrifuged for 10 minutes and the upper aqueous layer placed in a new tube where the DNA was precipitated with 0.1 volume sodium acetate (3M) and 2 volumes absolute ethanol at -20°C. This was incubated at -20°C overnight.

Centrifugation for 20 minutes at 4°C was carried out the next morning. The supernatent was discarded, 70% ethanol added and a further centrifugation of 5 minutes at room temperature was carried out.
The supernatent discarded, the pellet was dried at 37°C and resuspended in 30µl TE Buffer.

2.1.3.7 *Method 7*

i. BIORAD Instagene Purification Matrix

ii. Chloroform/ isoamyl alcohol

This solution was prepared to a 24:1 (v/v) ratio, respectively.

Pelleted cells in an eppendorf tube were resuspended in 200µl Biorad Instagene Purification Matrix and incubated at 56°C for 30 minutes. Following this incubation the tube was vortexed for 10 seconds and incubated for 8 minutes at 100°C. The tube was again vortexed for 10 seconds and 200µl chloroform/isoamyl alcohol (24:1 v/v) added. After mixing the chloroform/isoamyl alcohol with the Instagene solution the tube was centrifuged at 11 500rpm for 3 minutes.
The supernatent is then used in PCR reactions with any remainder being stored at -20°C. When using supernatent which has been frozen, allow to thaw, vortex for 10 seconds and centrifuge at 11 500rpm for 3 minutes.

2.1.3.8 *Method 8*

i. Chelex - 100 Solution

Chelex - 100 (Biorad, Richmond, CA)	10% w/v
SDS	0.1% w/v
Nonidet P40 (NP40)	1% v/v
Tween 20 in aqueous solution	1% v/v

Pellet was resuspended in five volumes of Chelex - 100 Solution and incubated for 30 minutes at 100°C. After centrifugation (5 minutes, 14 500g) the supernatent was collected for use in PCR reactions.

2.1.3.9 *Method 9*

i. Promega Buffer (I used Promega Buffer K)

ii. Proteinase K (20 mg/ml)

Pellet resuspended in 100µl Promega Buffer and 10µl Proteinase K. This preparation was incubated for 1 hour at 50°C then transferred to 100°C for ten minutes. After centrifugation (5 minutes, 14 500g) the supernatent was collected for use in PCR reactions.

2.1.3.10 *Method 10*

i. Phosphate Buffered Saline (PBS) pH 7.2

As for *Method 1*

ii. 1M Tris pH 9

iii. Sodium Dodecyl Sulphate (10% w/v)

iv. Proteinase K (20 mg/ml)

v. Benzyl chloride (50% v/v)

vi. 3M Sodium Acetate

vii. Isopropanol

viii. 70% Ethanol

ix. Resuspension Buffer

Promega PCR Buffer (10x)	150µl
$MgCl_2$ (25mM)	150µl
Distilled water	1.2ml

The cell pellet was washed by resuspension in 1.5ml PBS and centrifugation at 4000g for 5 minutes. The PBS was discarded and the pellet resuspended in 400µl 1M Tris pH9. This suspension was transferred to a 1.5ml eppendorf tube and 80µl 10% SDS was added and gently mixed into the solution. Then 10µl Proteinase K (20mg/ml) was added and the preparation was incubated at 56°C for at least 1 hour. Following incubation 240µl Benzyl Chloride was added.

After completion of a 30 minute incubation at 50°C, 240µl 3M Sodium Acetate pH 5.2 was added and the preparation placed at -20°C for 30 minutes. This incubation was followed by centrifugation at 14 500g for 5 minutes after which the supernatent was transferred to a fresh 1.5ml eppendorf tube where an equal volume of isopropanol was mixed through. The preparation was left at room temperature for 15 minutes before centrifugation at 14 500g for 15 minutes. The supernatent was discarded and the pellet was resuspended in 1ml 70% Ethanol followed by centrifugation at 14 500g for 5 minutes. The supernatent was removed by suction and the pellet was dried at room temperature.

Finally the pellet was resuspended in 50µl Resuspension Buffer.

2.2 RESULTS

Methods 1 to 6 were performed with 4.5×10^5 cells of NZHM3 culture.
Following extraction 10µl of the product in 990µl TE Buffer was measured by spectrophotometry at wavelengths λ258, λ260, λ270, λ280, λ300.

Table 2.2.1: Spectrophotometric Analysis of Methods 1-6

	λ258	λ260	λ270	λ280	λ300
Method 1	0.028	0.021	0.009	0.007	0.004
Method 2	0.042	0.035	0.023	0.019	0.011
Method 3	0.796	0.913	1.148	0.804	0.029
Method 4	0.033	0.029	0.016	0.009	0
Method 5	0.074	0.071	0.061	0.053	0.040
Method 6	0.149	0.147	0.121	0.076	0.015

From these readings can be calculated the amount of nucleic acids present and the relative purity of the sample.
The concentration of Nucleic Acids (NA) present was determined as described by Ionas (1989).
i.e.

AMOUNT NA PRESENT = (λ258 - λ300)/20 x 100

Method 1: (0.028 - 0.004)/20 x 100 = 0.120 mg/ml

Method 2: (0.042 - 0.011)/20 x 100 = 0.155 mg/ml

Method 3: (0.796 - 0.029)/20 x 100 = 3.835 mg/ml

Method 4: (0.033 - 0.000)/20 x 100 = 0.165 mg/ml

Method 5: (0.074 - 0.040)/20 x 100 = 0.170 mg/ml

Method 6: (0.149 - 0.015)/20 x 100 = 0.670 mg/ml

λ270 > λ260 indicates phenol contamination

Purity of the sample is indicated by λ260/λ280. Where the result of this calculation lies between the numbers 1.8 - 2.0 the sample is considered pure.

Method 1: 0.021/0.007 = 3.00

Method 2: 0.035/0.019 = **1.84**

Method 3: 0.913/0.804 = 1.14

Method 4: 0.029/0.009 = 3.20

Method 5: 0.071/0,053 = 1.34

Method 6: 0.147/0.076 = **1.93**

The samples were used in PCR reactions.

1. 2µl of each sample was used, neat, in a PCR reaction (See 3.1.1.b).
   No bands were seen

2. 2ul of each sample diluted 1/10 was used.
   Samples 2, 3 and 6 gave a PCR product.

Following completion of this comparison two other methods were found which seemed promising. A further comparison series was performed with Methods 2, 6, and the new methods labeled Method 7 and Method 8.
Methods 2 and 6 were chosen because their purity was within "acceptable" limits.

Methods were performed with 4 x 10^5 cells of NZHM3 culture.
Following extraction 10µl of the product in 990µl TE Buffer was measured by spectrophotometry at wavelengths λ258, λ260, λ270, λ280, λ300.

Table 2.2.2: Spectrophotometric Analysis of Methods 2, 6, 7, 8.

	λ258	λ260	λ270	λ280	λ300
Method 2	0.018	0.019	0.014	0.014	0.006
Method 6	0.088	0.085	0.071	0.040	0.005
Method 7	0.106	0.097	0.084	0.058	0.015
Method 8	0.095	0.093	0.086	0.060	0.017

AMOUNT NA PRESENT = (λ258 - λ300)/20 x 100

Method 2: (0.018 - 0.014)/20 x 100 = 0.060 mg/ml

Method 6: (0.088 - 0.005)/20 x 100 = 0.415 mg/ml

Method 7: (0.106 - 0.015)/20 x 100 = 0.455 mg/ml

Method 8: (0.095 - 0.017)/20 x 100 = 0.390 mg/ml

Purity of the sample is indicated by λ260/λ280. Where the result of this calculation lies between the numbers 1.8 - 2.0 the sample is considered pure.

Method 2: 0.019/0.014 = 1.36

Method 6: 0.085/0.040 = 2.125

Method 7: 0.097/0.058 = 1.67

Method 8: 0.093/0.060 = 1.55

DNA from each of these methods was used in a series of PCR reactions. Results were consistent using DNA from methods 7 and 8, while DNA from methods 2 and 6 did not perform consistently.

Two methods used for extraction of DNA from *Acanthamoeba* and other protozoan were forwarded for comparison by Dr. Ray Cursons, Waikato Hospital, New Zealand. These methods were compared with Method 7, which had previously given high DNA yield and consistent reaction in PCR experiments.

Table 2.2.3: Spectrophotometric Analysis of Methods 7, 9, 10.

	λ258	λ260	λ270	λ280	λ300
Method 7	0.019	0.018	0.018	0.012	0.004
Method 9	0.008	0.015	0.035	0.033	0.000
Method 10	0.596	0.595	0.483	0.296	0.035

AMOUNT NA PRESENT = (λ258 - λ300)/20 x 100

Method 7: (0.019 - 0.004)/20 x 100 = 0.75 mg/ml

Method 9: (0.008 - 0.000)/20 x 100 = 0.04 mg/ml

Method 10: (0.596 - 0.035)/20 x 100 = 2.81mg/ml

PURITY:

Method 7: 0.018/0.012 = 1.5

Method 9: 0.015/0.033 = 0.45

Method 10: 0.595/0.296 = 2.01

In subsequent PCR reactions only methods 7 and 10 yielded product.

Following this series of comparisons Methods 2, 7, 8 and 10 were used to extract DNA from three additional *Acanthamoeba* isolates. Each of the methods were able to extract DNA from each of the Isolates with sufficient yield and purity to perform consistently in PCR reactions.

2.3 CONCLUSIONS

Methods 1, 4,5, and 9 failed to yield products in PCR reactions and Methods 3 and 6 were inconsistent in PCR reactions. These methods were removed from further comparison.

Methods 2, 7, 8 and 10 could all be performed in less than one day and consistently performed in PCR reactions. Methods 2, 7 and 8 were approximately equal in efficiency of DNA extraction and purity of the resulting DNA. Method 2, however, resulted in only about a third of the amount of supernatent, so diluted to give a comparable supernatent yielded markedly less DNA. Method 10 had the highest consistent yield of DNA.

It was decided that Method 10 would be used for the remainder of the experiments for this thesis. This decision was based on two main points. Firstly, the DNA yield was higher in similar volumes of supernatent than any of the other methods. This allows for dilution giving a large stock of DNA and reduces the need for further extractions.

Secondly, the aliquot of supernatent required for PCR was smaller, providing a bigger allowance for adjusting other ingredients in the reaction mixture.

Any of these four methods (2, 7, 8, 10) could be used with success in similar experiments, and choosing which one to use will come down to individual requirements and personal preference.

CHAPTER THREE: Investigation Into The Use Of Arbitrarily Primed Polymerase Chain Reaction (PCR) To Distinguish Species And Strains Of *Acanthamoeba*.

3.0 INTRODUCTION

There are six recognised species of *Acanthamoeba* currently known to infect the eye. It is thought that within these species there are also several infective strains, which may react differently to chemotherapeutic agents. It would be useful for a clinician to identify the different strains of *Acanthamoeba* species, particularly if these strains show varying susceptibility or resistance to common treatments for the infection.

We are concerned here with the application of the polymerase chain reaction (PCR) in an attempt to type and group strains of *Acanthamoeba* species. It is hoped that the results will correlate with the groups of isolates gained from drug sensitivity tests performed and reported in Chapter Four.

Recent literature describes success in identifying *Acanthamoeba* at the generic and specific levels using PCR (Vodkin et al, 1992), however there are no reports of this technique being used to distinguish strains of the organism.
A PCR-based technique, called random amplification of polymorphic DNA (RAPD) was described by Williams et al (1990), and called arbitrary primed PCR (AP-PCR) fingerprinting by Welsh and McClelland (1990). With this technique, single arbitrary primers are used to amplify random sequences of DNA. A variation of this technique, using primers designed for other purposes than RAPD analysis (Welsh and McClelland, 1990) was used to attempt to identify differences in 16 isolates of *Acanthamoeba* species.

By using a PCR cycle with low stringency (low annealing temperature) at the primer annealing step, a single primer with unknown homology to the DNA being examined may anneal to a number of sites. Providing the sites occur less than several kilobases (kb) apart and in the correct orientation, the region between two such sites is amplified. The products of this amplification are then examined by agarose gel electrophoresis.

As any one primer may be unsuitable for the purpose we used six different primers (see materials and methods section) to examine the DNA extractions. The first two primers used, Apo1 and Apo2 are those designed by Vodkin *et al* (1992) and have been used as a pair to identify the genus and species of *Acanthamoeba* isolates. Primers P1 and P2 were used by Lai *et al* (1994) for detection of the *Acanthamoeba* genera. Primers AM1 and AM2 were also designed as a pair to detect the genus *Acanthamoeba*. In this thesis these primers are used individually in the manner a random primer is used.

3.1 MATERIALS AND METHODS

3.1.1 For PCR Analysis

i. Sterile distilled water

ii. dNTP's (1.25mM)

3. DNA Taq Polymerase Buffer (x10)

As supplied by manufacturer (GibcoBRL)

4. DNA from *Acanthamoeba* isolates

See *Appendix 1*

5. $MgCl_2$ (25mM)

As supplied by manufacturer (Epicentre Technologies)

6. Primer

Supplied as dry pellet by Oligos Etc. Inc., and diluted with sterile distilled water to a concentration of 1nmol/µl.
A further 1/10 dilution was made to produce working solutions of these primers.

The primer sequences are as follows:

Apo1	5' GAATTCCGCTCGGAAGCC	3' (18-mer)
Apo2	5' AACGCTGGTCTGACGCC	3' (17-mer)
Am1	5' CTGTTGTCGACGTTTATCCA	3' (20-mer)
Am2	5' GATCACCAGTGGAGGGTGTC	3' (20-mer)
P1	5' GGAGCTCCCACGGGAGGCC	3' (19-mer)
P2	5' TGGACCGCGTGAGGCTGCGGCT	3' (22-mer)

7. Taq Polymerase (5units/µl)

As supplied by manufacturer (GibcoBRL)

3.1.1a Optimisation of PCR Conditions for Amplification.

Before beginning the PCR analysis of the Acanthamoeba isolates, it was first necessary to optimise the PCR conditions. A number of PCR runs were carried out where one reagent or physical parameter was altered. The optimum condition was then selected and used in subsequent reactions. The annealing temperature used in all the reactions was 45°C.

3.1.1b PCR Analysis

The standard amplification reaction contained 2µl DNA, 5µl 10x Taq polymerase buffer, 32.5µl water, 8.75µl $MgCl_2$, 1µl dNTP's, 1unit Taq polymerase and 0.5µl primer to give a final volume of 50µl.

The Amplification was performed in a Perkin Elmer Cetus DNA Thermal Cycler (9600), programmed for a 1 cycle of: 2 minutes at 93°C, 1 minute at 45°C, 2 minutes at 71°C; 45 cycles of: 1 minute at 93°C, 1 minute at 45°C, 2 minutes at 71°C; and 1 cycle of: 1 minute at 93°C, 1 minute at 45°C, 5 minutes at 71°C, using the fastest available transitions between each temperature.

3.1.2 For Gel Electrophoresis

1. Biorad Ultrapure DNA grade agarose (1.6% in 1x E-buffer)

2. E-buffer (10x)

trizma base	96.88g
EDTA (disodium salt)	7.44g
sodium acetate	8.20g

pH to 7.8 with glacial acetic acid, make up to 2000ml with distilled water.
Store at 4°C and dilute 1/10 with distilled water when required

3. Bromophenol blue dye (0.1% bromophenol blue w:v, 80% glycerol v:v)

4. Ethidium bromide (5µg/ml)

5. BRL 1kb Ladder
See Appendix 2

The amplification products were size fractionated by electrophoresis through a horizontal 1.6% agarose gel matrix in an Horizon 11.14 gel electrophoresis apparatus.
A 5µl aliquot of each sample was mixed with 5µl of bromophenol blue dye and loaded. The agarose gels were run at 100 volts for 2.5 hours and DNA detected by staining with ethidium bromide for 10 minutes, destaining in distilled water for 10 minutes and viewing on a short wave transilluminator.
The BRL 1kb ladder was included in the gels in lanes adjacent to the samples as size markers.

3.1.3 Gel Photography:

Images of Gels were recorded using the computer based digital imaging system IS-1000 Version 2.0.
It was decided to use this system, in preference to traditional gel photography, based on the following advantages:

- Differing exposures between gels can be recognised instantly, eliminating the need for several photographs with different exposures to be taken.
- Without altering results, small imperfections can be minimised in gel photographs, using several enhancement features.
- Although not used in this thesis, this computer package can be used to determine sizes of bands in PCR, annotate gel features and perform many other useful tasks with a wide range of application.

- The quality of the resulting photograph is very good, and cheaper and easier to replicate.
- The resulting image can be saved to disk and used with other software packages.

3.2 RESULTS

The PCR reaction was optimised by individually varying the PCR reagents the annealing temperature, and the cycle number. In this context, optimisation implies the production of clear bands, small enough in number to be readily resolvable, but large enough in number to allow isolates to be differentiated.
The optimisation experiments were carried out by Dr. Ray Cursons at Waikato Hospital, New Zealand. The results are not illustrated in this thesis however, it was concluded that for our purposes (attempting to distinguish isolates), the optimal PCR conditions are as described in 3.1.1b.

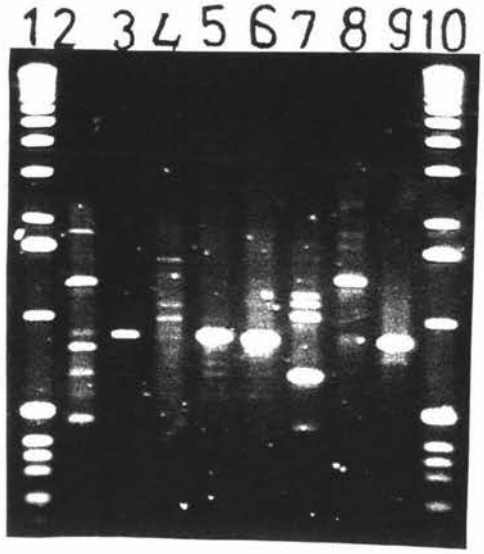
Using the optimum conditions and each of the six primers described above, 16 *Acanthamoeba* isolates were analysed using AP-PCR.
The results are displayed in figures 3.2.1 - 3.2.12.

FIGURE 3.2.1: Examination of the heterogeneity of 8 isolates of Acanthamoeba by RAPD analysis with the primer APO1.

The samples (left to right) are: Lane 1, 1kb ladder; Lane 2, Isolate 9; Lane 3, Isolate 19; Lane 4, Isolate 23; Lane 5, Isolate 24; Lane 6, Isolate 25; Lane 7, Isolate 63*; Lane 8, Isolate AU; Lane 9, Isolate HM2; Lane 10, 1kb ladder.

FIGURE 3.2.2: Examination of the heterogeneity of 8 isolates of Acanthamoeba by RAPD analysis with the primer APO1.

The samples (left to right) are: Lane 1, 1kb ladder; Lane 2, Isolate 10; Lane 3, Isolate 15; Lane 4, Isolate 17; Lane 5, Isolate 10*; Lane 6, Isolate 27*; Lane 7, Isolate 75*; Lane 8, Isolate 90*; Lane 9, Isolate A1; Lane 10, 1kb ladder.


1 2 3 4 5 6 7 8 9 10

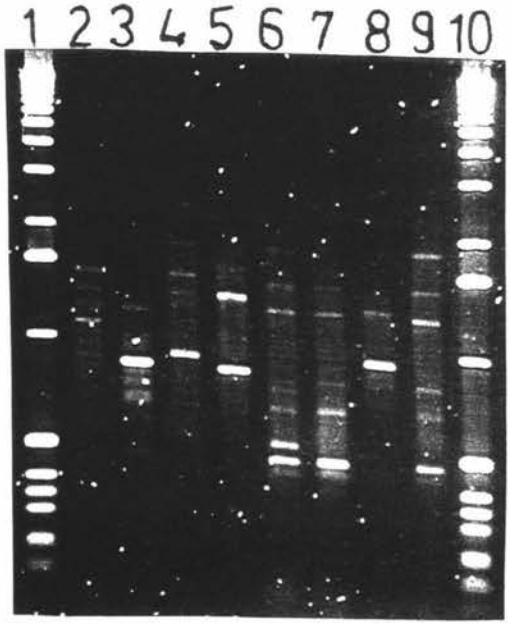
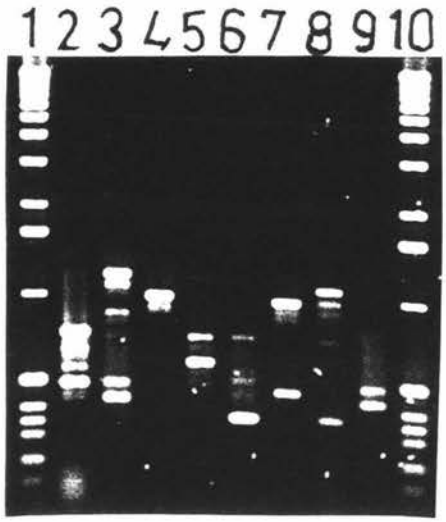

1 2 3 4 5 6 7 8 9 10

FIGURE 3.2.3: Examination of the heterogeneity of 8 isolates of Acanthamoeba by RAPD analysis with the primer APO2.

The samples (left to right) are: Lane 1, 1kb ladder; Lane 2, Isolate 9; Lane 3, Isolate 19; Lane 4, Isolate 23; Lane 5, Isolate 24; Lane 6, Isolate 25; Lane 7, Isolate 63*; Lane 8, Isolate AU; Lane 9, Isolate HM2; Lane 10, 1kb ladder.

FIGURE 3.2.4: Examination of the heterogeneity of 8 isolates of Acanthamoeba by RAPD analysis with the primer APO2.

The samples (left to right) are: Lane 1, 1kb ladder; Lane 2, Isolate 10; Lane 3, Isolate 15; Lane 4, Isolate 17; Lane 5, Isolate 10*; Lane 6, Isolate 27*; Lane 7, Isolate 75*; Lane 8, Isolate 90*; Lane 9, Isolate A1; Lane 10, 1kb ladder.


1 2 3 4 5 6 7 8 9 10

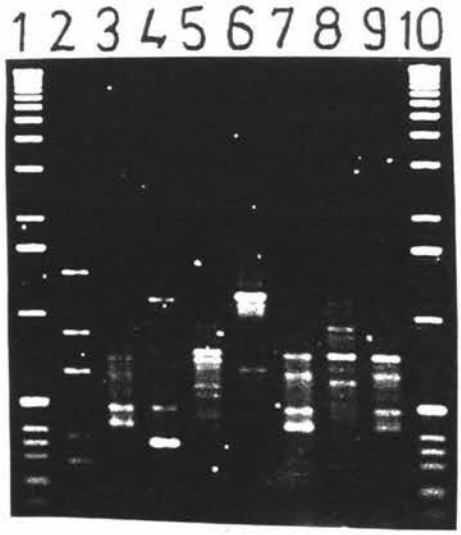
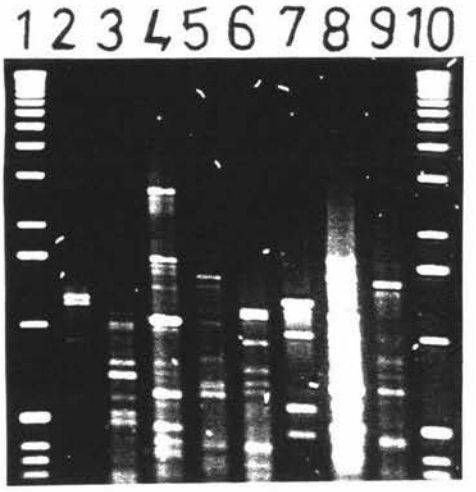

1 2 3 4 5 6 7 8 9 10

FIGURE 3.2.5: Examination of the heterogeneity of 8 isolates of Acanthamoeba by RAPD analysis with the primer AM1.

The samples (left to right) are: Lane 1, 1kb ladder; Lane 2, Isolate 9; Lane 3, Isolate 19; Lane 4, Isolate 23; Lane 5, Isolate 24; Lane 6, Isolate 25; Lane 7, Isolate 63*; Lane 8, Isolate AU; Lane 9, Isolate HM2; Lane 10, 1kb ladder.

FIGURE 3.2.6: Examination of the heterogeneity of 8 isolates of Acanthamoeba by RAPD analysis with the primer AM1.

The samples (left to right) are: Lane 1, 1kb ladder; Lane 2, Isolate 10; Lane 3, Isolate 15; Lane 4, Isolate 17; Lane 5, Isolate 10*; Lane 6, Isolate 27*; Lane 7, Isolate 75*; Lane 8, Isolate 90*; Lane 9, Isolate A1; Lane 10, 1kb ladder.


1 2 3 4 5 6 7 8 9 10

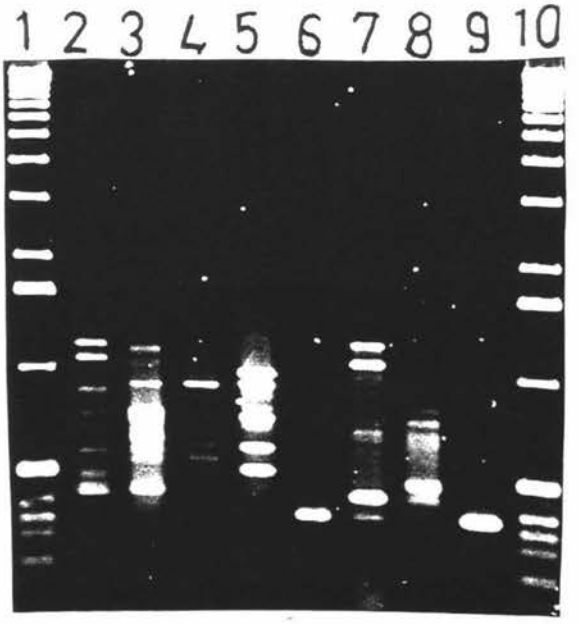
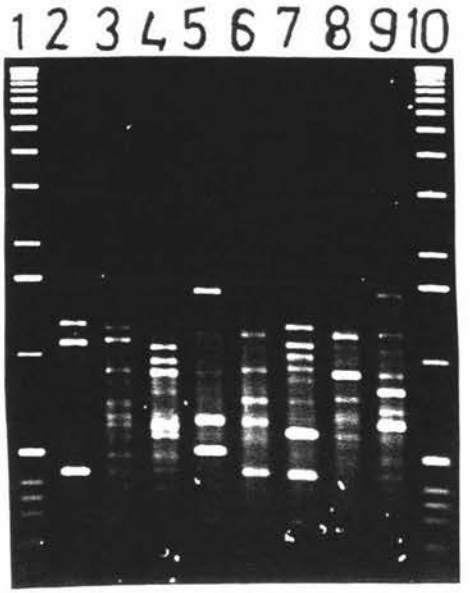

1 2 3 4 5 6 7 8 9 10

FIGURE 3.2.7: Examination of the heterogeneity of 8 isolates of Acanthamoeba by RAPD analysis with the primer AM2.

The samples (left to right) are: Lane 1, 1kb ladder; Lane 2, Isolate 9; Lane 3, Isolate 19; Lane 4, Isolate 23; Lane 5, Isolate 24; Lane 6, Isolate 25; Lane 7, Isolate 63*; Lane 8, Isolate AU; Lane 9, Isolate HM2; Lane 10, 1kb ladder.

FIGURE 3.2.8: Examination of the heterogeneity of 8 isolates of Acanthamoeba by RAPD analysis with the primer AM2.

The samples (left to right) are: Lane 1, 1kb ladder; Lane 2, Isolate 10; Lane 3, Isolate 15; Lane 4, Isolate 17; Lane 5, Isolate 10*; Lane 6, Isolate 27*; Lane 7, Isolate 75*; Lane 8, Isolate 90*; Lane 9, Isolate A1; Lane 10, 1kb ladder.


1 2 3 4 5 6 7 8 9 10

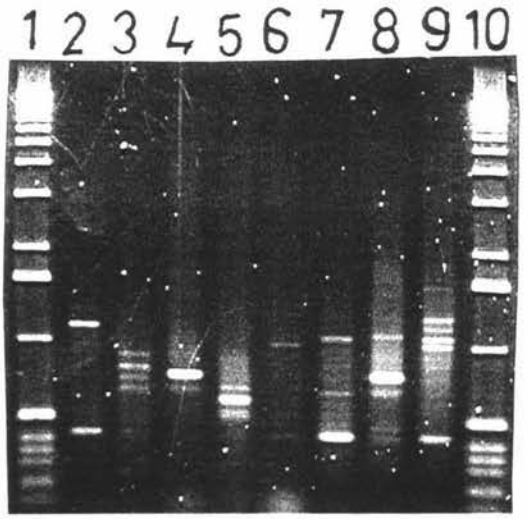
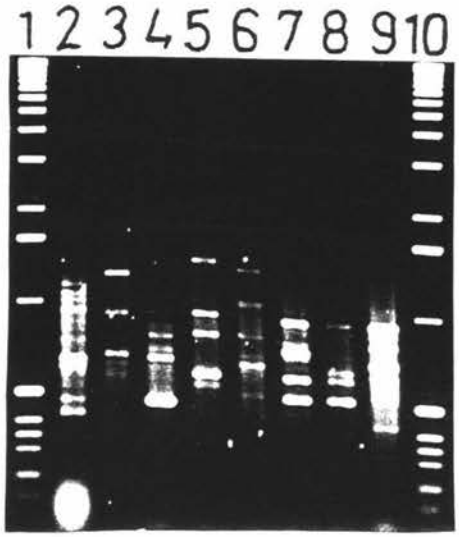

1 2 3 4 5 6 7 8 9 10

FIGURE 3.2.9: Examination of the heterogeneity of 8 isolates of Acanthamoeba by RAPD analysis with the primer P1.

The samples (left to right) are: Lane 1, 1kb ladder; Lane 2, Isolate 9; Lane 3, Isolate 19; Lane 4, Isolate 23; Lane 5, Isolate 24; Lane 6, Isolate 25; Lane 7, Isolate 63*; Lane 8, Isolate AU; Lane 9, Isolate HM2; Lane 10, 1kb ladder.

FIGURE 3.2.10: Examination of the heterogeneity of 8 isolates of Acanthamoeba by RAPD analysis with the primer P1.

The samples (left to right) are: Lane 1, 1kb ladder; Lane 2, Isolate 10; Lane 3, Isolate 15; Lane 4, Isolate 17; Lane 5, Isolate 10*; Lane 6, Isolate 27*; Lane 7, Isolate 75*; Lane 8, Isolate 90*; Lane 9, Isolate A1; Lane 10, 1kb ladder.


1 2 3 4 5 6 7 8 9 10

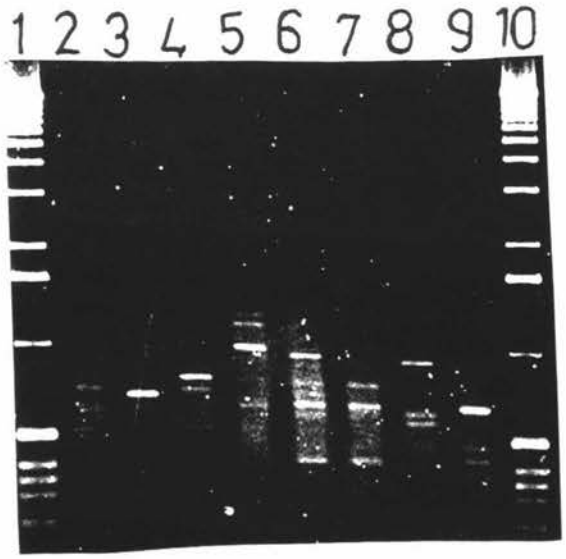
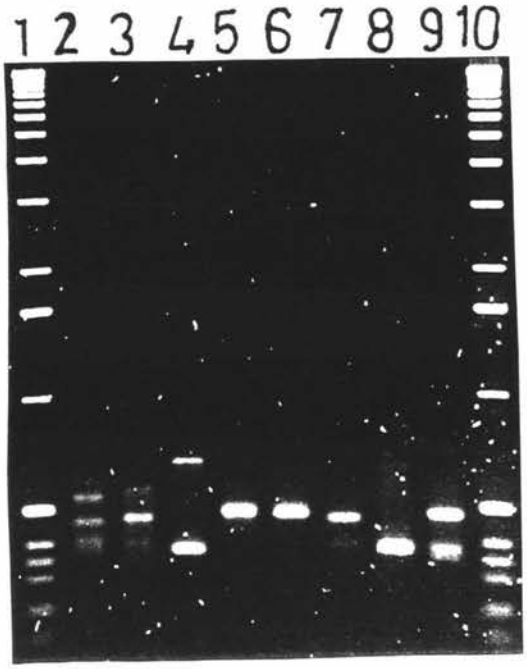

1 2 3 4 5 6 7 8 9 10

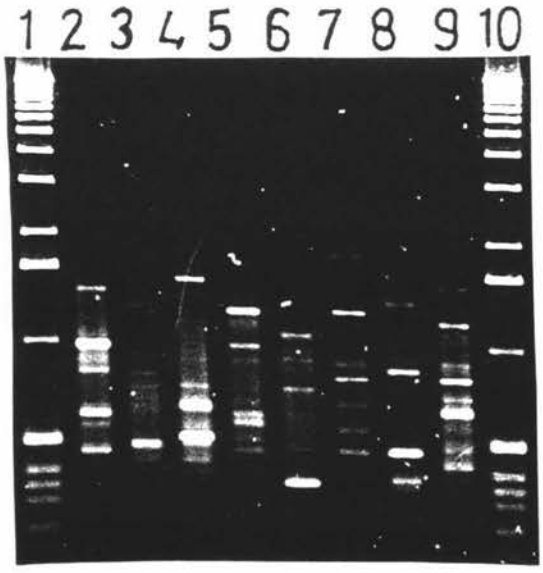
FIGURE 3.2.11: Examination of the heterogeneity of 8 isolates of Acanthamoeba by RAPD analysis with the primer P2.

The samples (left to right) are: Lane 1, 1kb ladder; Lane 2, Isolate 9; Lane 3, Isolate 19; Lane 4, Isolate 23; Lane 5, Isolate 24; Lane 6, Isolate 25; Lane 7, Isolate 63*; Lane 8, Isolate AU; Lane 9, Isolate HM2; Lane 10, 1kb ladder.

FIGURE 3.2.12: Examination of the heterogeneity of 8 isolates of Acanthamoeba by RAPD analysis with the primer P2.

The samples (left to right) are: Lane 1, 1kb ladder; Lane 2, Isolate 10; Lane 3, Isolate 15; Lane 4, Isolate 17; Lane 5, Isolate 10*; Lane 6, Isolate 27*; Lane 7, Isolate 75*; Lane 8, Isolate 90*; Lane 9, Isolate A1; Lane 10, 1kb ladder.


1 2 3 4 5 6 7 8 9 10


1 2 3 4 5 6 7 8 9 10

3.3 CONCLUSIONS:

Examination of 16 isolates with primers APO1, APO2, AM1, AM2, P1 and P2, showed each of the banding patterns, resulting from AP-PCR analysis, were unique to the isolate tested. Further, there were few bands which occurred in more than one isolate, with insufficient similarities to form groupings of isolates.
It was therefore impossible to correlate AP-PCR results with the drug sensitivity results of Chapter Four.

The results of this AP-PCR analysis may contribute to the questions regarding the taxonomy of the *Acanthamoeba* genus.
This genus is well-established, however, as discussed earlier, the subgeneric classification is still unclear, and subject to continuing debate.
Speciation by morphology has been complicated by variation in form, even within a clone (Badenoch *et al*, 1995), and more recent subcellular approaches have failed to resolve such confusion.
The majority of the isolates examined in this chapter fall into Pussard-Pons Group II and the results do not suggest any groups which may indicate species. This begs the question of whether species, as a concept, is possible within the high genetic heterogeneity of the *Acanthamoeba* genus.

CHAPTER FOUR: *In vitro* drug sensitivity tests against *Acanthamoeba* species

4.0 INTRODUCTION

One of the most distressing aspects for people suffering with acanthamoebic keratitis is knowing that there is no 100% cure, short of removing part or all of the infected eyeball. Recent history has shown that no one chemotherapeutic agent is effective in all cases of acanthamoebic keratitis. (Asbell, 1993, John, 1993, Bacon *et al*, 1993, Horne *et al*, 1994)
It is likely that different species and even strains of *Acanthamoeba* have different drug sensitivities.

Two chemotherapeutic agents were selected for a preliminary study into drug sensitivities in *Acanthamoeba* species and strains.
The first of these was Brolene, an over-the-counter eye drop for the treatment of minor eye infections. This preparation contains 0.1% propamidine isethionate, which inhibits DNA synthesis, and has been shown to have some efficacy against *Acanthamoeba* spp.
The second was Baquacil (20% polyhexamethylene biguanide (PHMB)), a pool disinfectant acting as an inhibitor of membrane function and which, in very diluted amounts, has been used experimentally in the treatment of acanthamoebic keratitis with promising results.

4.1 MATERIALS AND METHODS

i. PYG media for axenic culture
See 2.1.1

ii. PAS agar for monoxenic culture
See 2.1.1

iii. Brolene (0.1% w/v propamidine isethionate)

iv. Baquacil (20% w/w polyhexamethylene biguanide HCl)

v. *Acanthamoeba* isolates
See Appendix 1

Initially it was necessary to find a method for quantitatively evaluating the results of the in vitro drug testing.
A brief summary of each of these methods, accompanied by an evaluation, follows.

Evaluation by Inverted Microscope (Hay *et al*, 1994)

Following chemotherapeutic exposure, isolates were washed, incubated in PYG media and examined under an inverted microscope. In this method sensitivity is

assessed by complete lysis or degeneration of trophozoites and non-viability of cysts.

This method was deemed unsuitable for the purpose of this thesis because it did not give a quantitative result, and relies on a purely subjective analysis by the operator.

Evaluation by MTT

The pale yellow redox indicator 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is reduced to a dark blue end-product, MTT-formazan, by the mitochondrial dehydrogenases of living cells (Hodgson *et al*, 1994). This reduction can be measured spectophotometrically at a wavelength of 570nm.

PYG media was spiked with *Acanthamoeba* cultures of varying concentrations and the preparations incubated with MTT.

After spectrophotometric analysis this method was rejected because results from several spiked samples showed much less activity than a sterile media control. A trendline could be established but, as a baseline could not be determined, results were not able to be quantified.

Evaluation of Haemocytometer and Phase Contrast Microscopy

Under phase contrast, non-viable *Acanthamoeba* trophozoites appear golden yellow, and have no discernible internal structures or acanthapodia. Viable cysts appear pale yellow, the double wall is intact and contents look uniform in appearance. In this method one full haemocytometer grid was examined and the percentage of viable organisms determined.

This method showed promise. Phase contrast seemed to show clear differences between viable and non-viable organisms, allowing quantitative assessment of drug sensitivity tests.

There were two concerns with this method.

1. That phase contrast evaluation may not be accurate, particularly when identifying viable cysts.

2. That the quantitative results gained may not be statistically accurate.

Two further methods were used to address these concerns.

a) Cultures assessed by phase contrast were washed and used to inoculate PYG and PAS media. Cultures were incubated at 30°C and growth examined at 24, 48, and 72 hours. The amount of growth in these results correlated well with the numbers of viable organisms detected by phase contrast.

b) As only one full haemocytometer grid was counted for each culture of the experiment, there was some concern that this was not statistically significant. Thus, a recognised statistical method was used as a comparison. In this method 1000 organisms were examined under phase contrast, from each experimental culture, and the percentage of viable organisms assessed.

These results were compared with those from the haemocytometer counts. The two sets of results compared very well, with only 1-2% variation between them.

As the results from the above two methods alleviate any concerns with the phase contrast/haemocytometer method, this is the method used in all following experiments.

Thirty *Acanthamoeba* isolates were grown in PYG media without added antibiotics for approximately 72 hours at 30°C. The cultures were examined under phase contrast microscopy and the numbers of viable organisms, both trophozoites and cysts, assessed. Cells were pelleted by centrifugation at 4000g for five minutes and the pellet resuspended in PYG media to give approximately 2 x 10^4 viable organisms, per 100µl. A mixture of trophozoites and cysts was used to imitate the clinical infection.

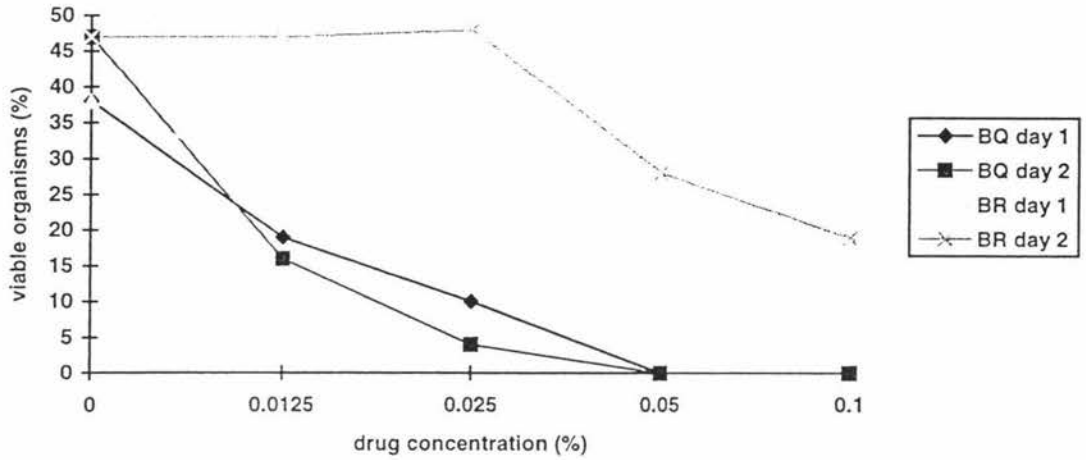
Drug screening was performed using sterile 96-well microtitre plates. A working solution of Baquacil was prepared by dilution from 20% PHMB to 0.1% PHMB with sterile distilled water. Serial dilutions of 0.1% PHMB (Baquacil) and 0.1% propamidine isethionate (Brolene) were made (0.1% - 0.0125%) in distilled water to give a volume of 100µl in each well. To each of the wells 100µl cell suspension was added. Controls were included in all experiments with distilled water replacing the drug solution. The plates, and contents were placed on a shaker and incubated at 30°C for 48 hours. At 24 and 48 hours aliquots were removed and the percentage of viable organisms determined, using a haemocytometer in phase contrast. All experiments were performed in duplicate.

4.2 RESULTS

The results of in vitro drug sensitivity tests against 30 *Acanthamoeba* isolates are shown in figures 4.2.1 - 4.2.30.

FIGURE 4.2.1: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 63* following incubation for 24 hours (day 1) and 48 hours (day 2).

FIGURE 4.2.2: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 25 following incubation for 24 hours (day 1) and 48 hours (day 2).


viable organisms (%)
50
45
40
35
30
25
20
15
10
5
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

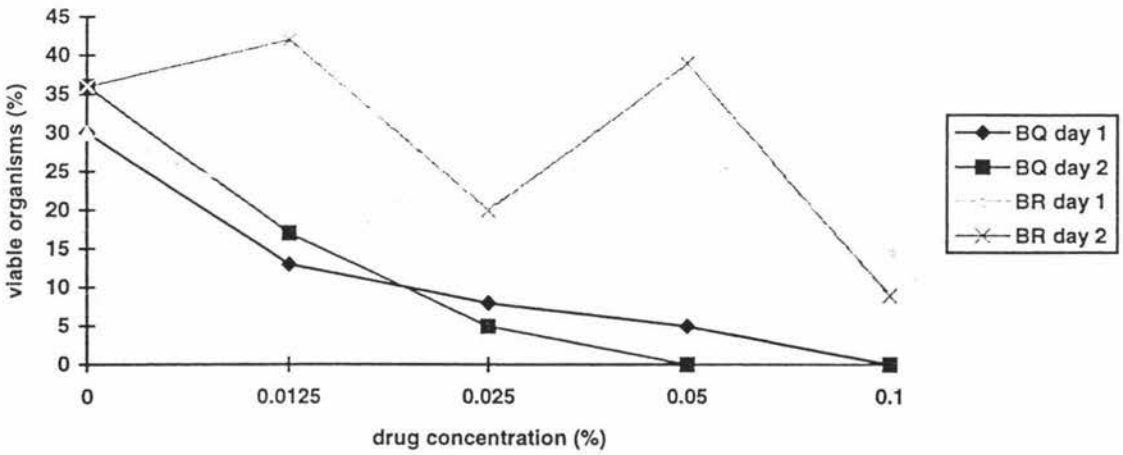
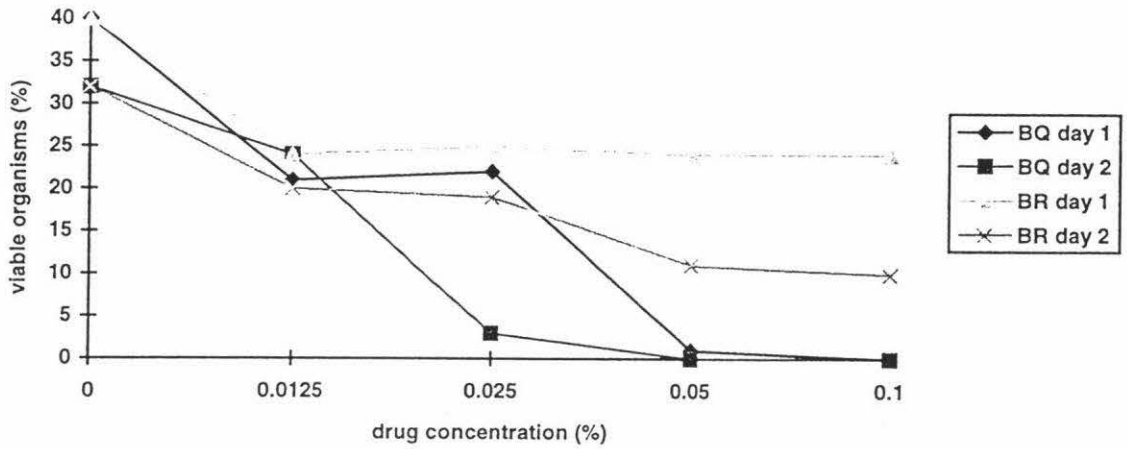

viable organisms (%)
45
40
35
30
25
20
15
10
5
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

FIGURE 4.2.3: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 24 following incubation for 24 hours (day 1) and 48 hours (day 2).

FIGURE 4.2.4: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate AU following incubation for 24 hours (day 1) and 48 hours (day 2).


viable organisms (%)
40
35
30
25
20
15
10
5
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

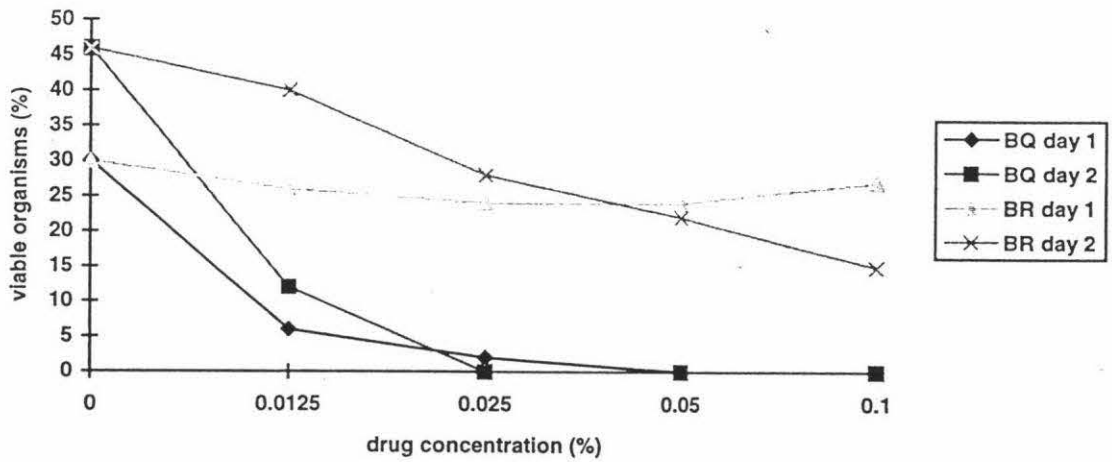
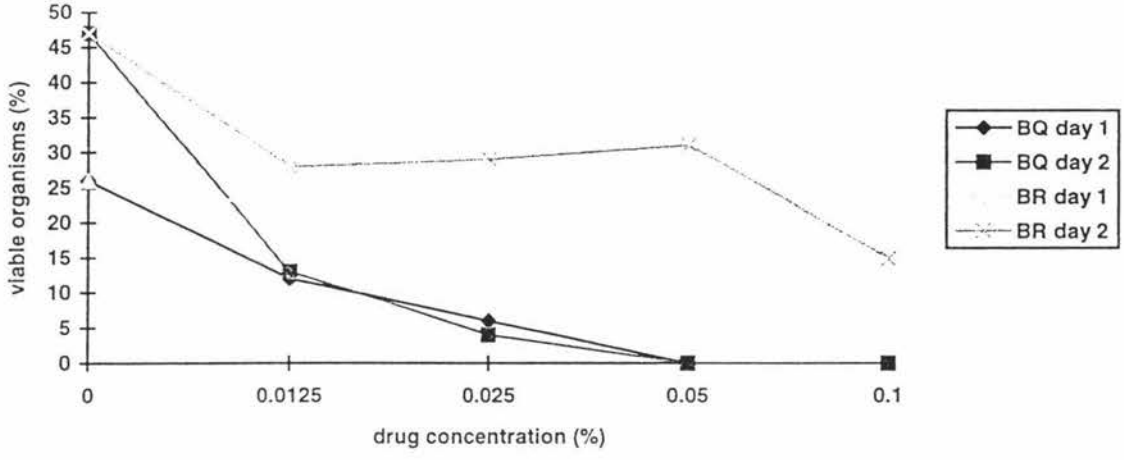

viable organisms (%)
50
45
40
35
30
25
20
15
10
5
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

FIGURE 4.2.5: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 9 following incubation for 24 hours (day 1) and 48 hours (day 2).

FIGURE 4.2.6: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 23 following incubation for 24 hours (day 1) and 48 hours (day 2).


viable organisms (%)
50
45
40
35
30
25
20
15
10
5
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

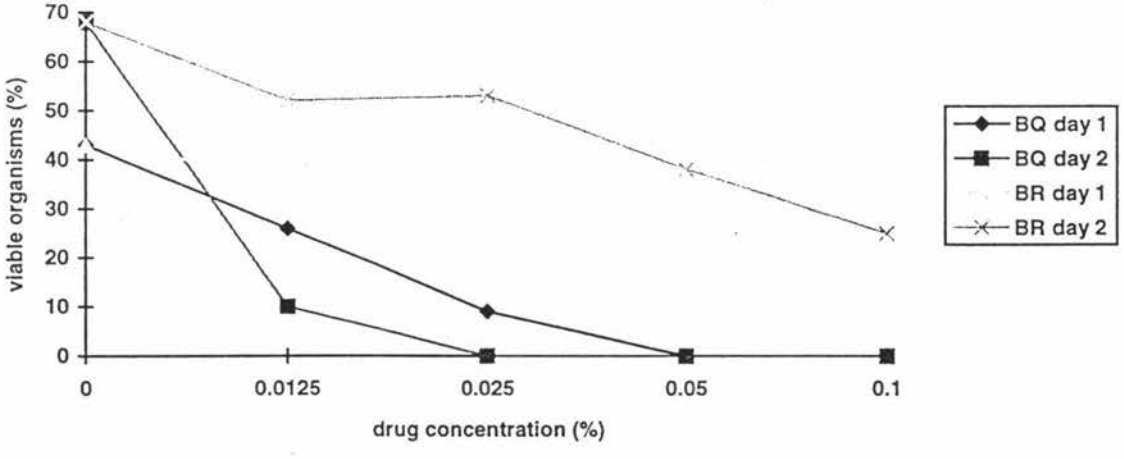
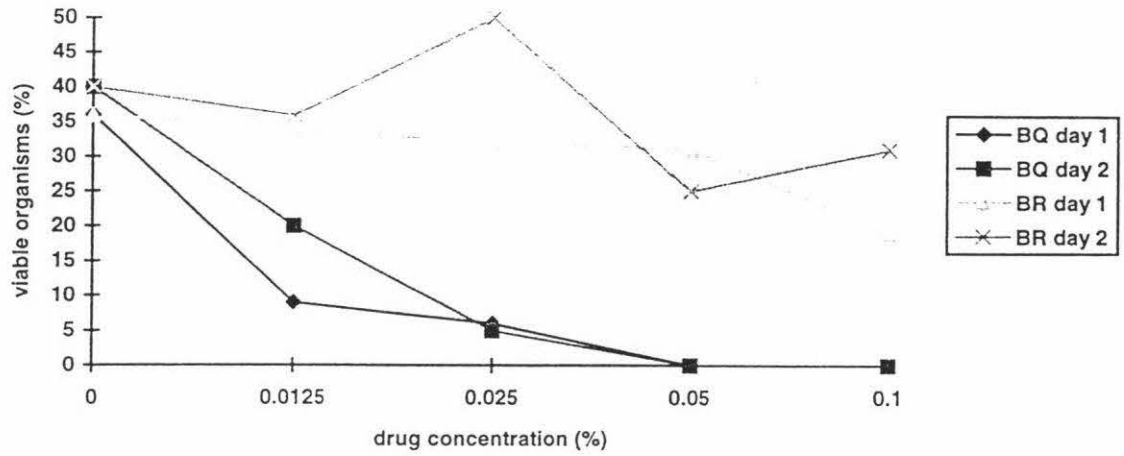

viable organisms (%)
70
60
50
40
30
20
10
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

FIGURE 4.2.7: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate HM2 following incubation for 24 hours (day 1) and 48 hours (day 2).

FIGURE 4.2.8: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 19 following incubation for 24 hours (day 1) and 48 hours (day 2).


viable organisms (%)
50
45
40
35
30
25
20
15
10
5
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

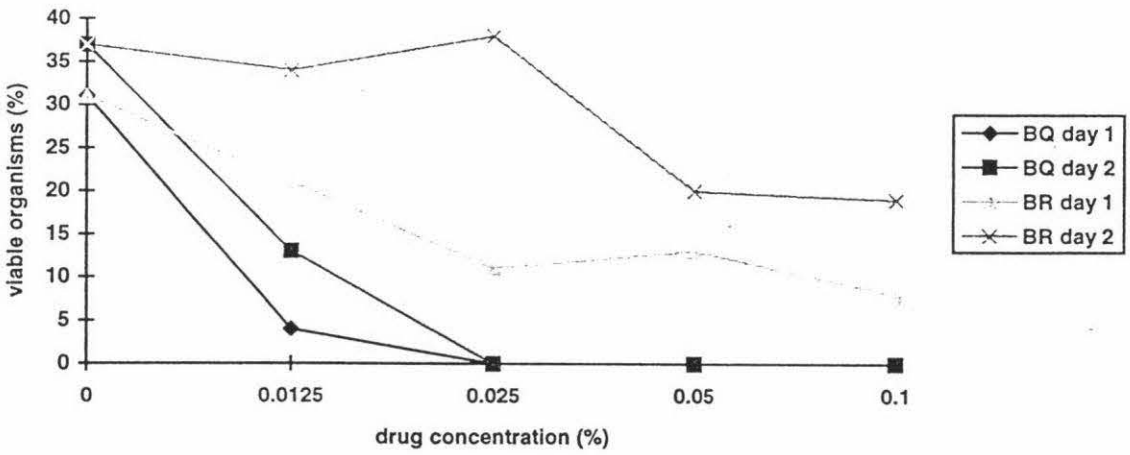
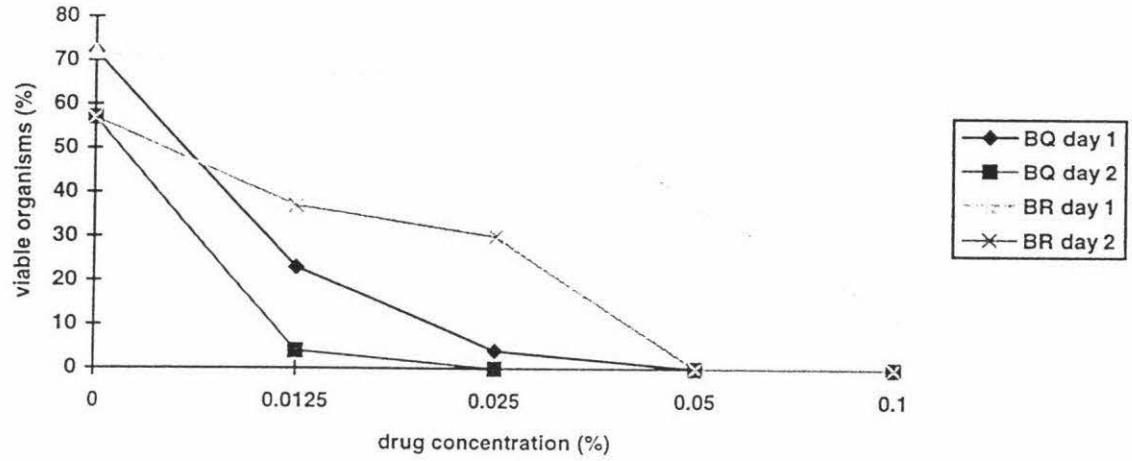

viable organisms (%)
40
35
30
25
20
15
10
5
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

FIGURE 4.2.9: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 10 following incubation for 24 hours (day 1) and 48 hours (day 2).

FIGURE 4.2.10: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 15 following incubation for 24 hours (day 1) and 48 hours (day 2).


viable organisms (%)
80
70
60
50
40
30
20
10
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

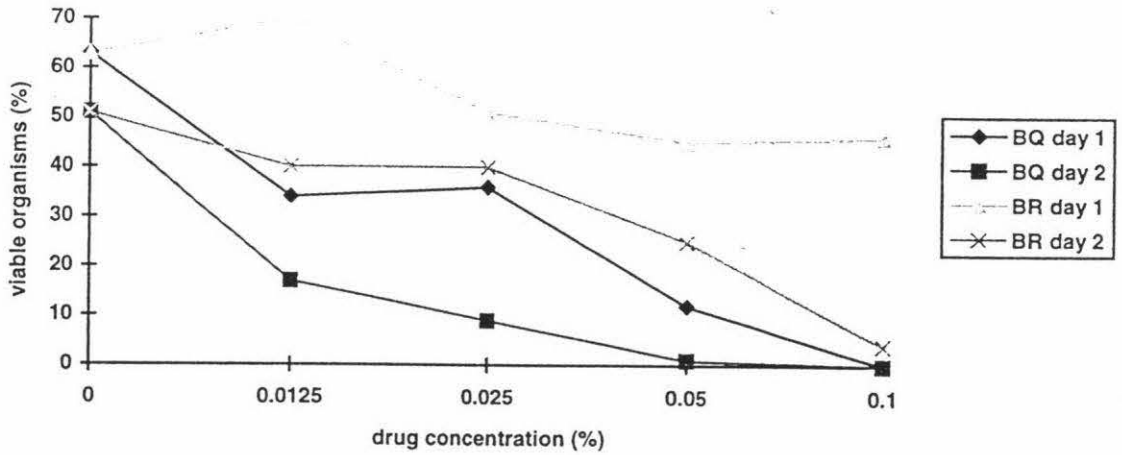
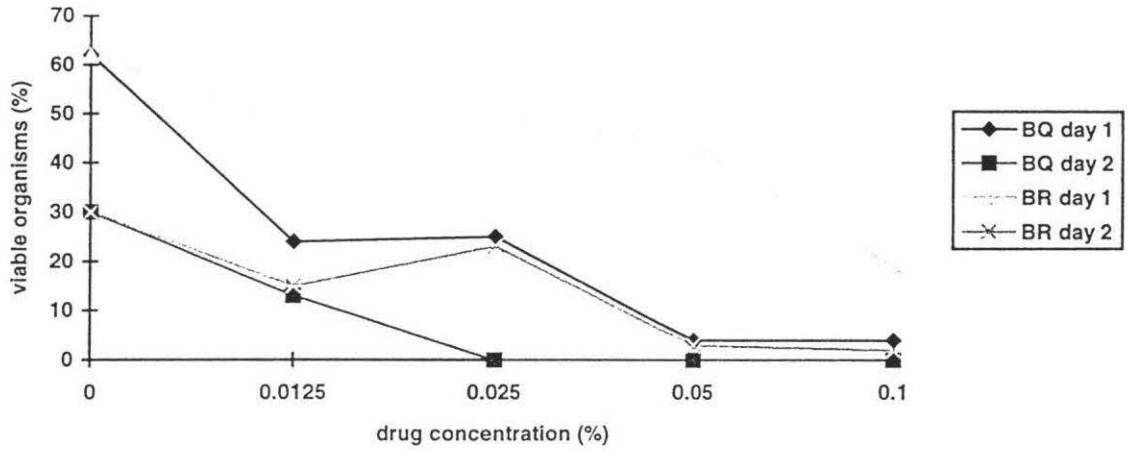

viable organisms (%)
70
60
50
40
30
20
10
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

FIGURE 4.2.11: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 17 following incubation for 24 hours (day 1) and 48 hours (day 2).

FIGURE 4.2.12: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 10* following incubation for 24 hours (day 1) and 48 hours (day 2).


70
60
50
40
30
20
10
0
viable organisms (%)
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

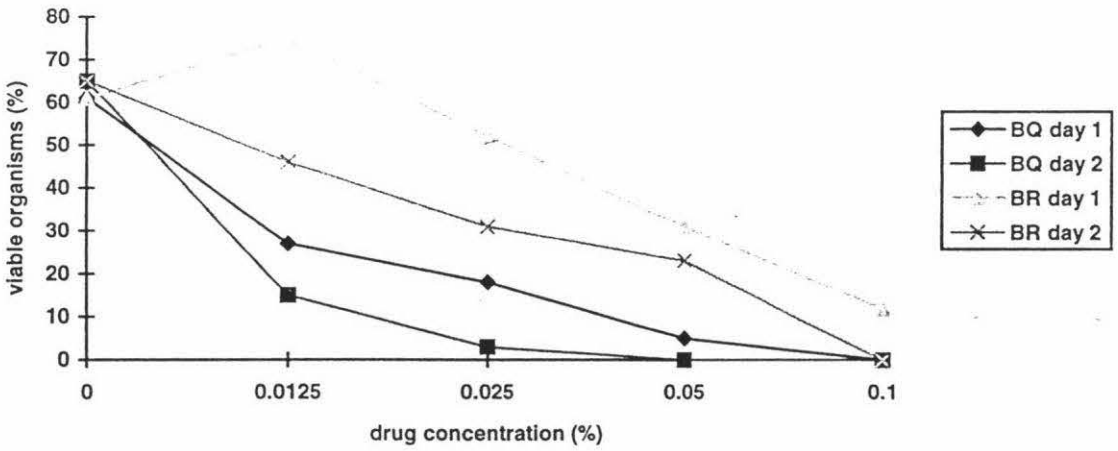
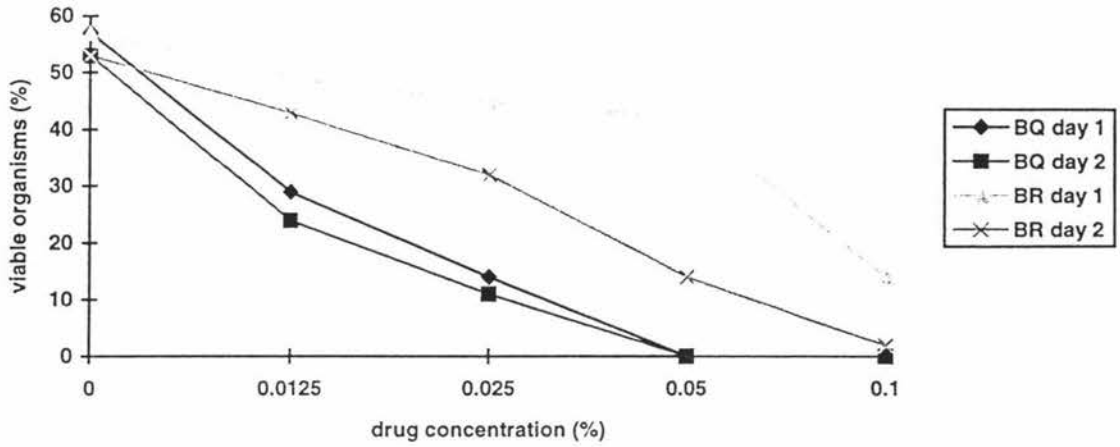

80
70
60
50
40
30
20
10
0
viable organisms (%)
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

FIGURE 4.2.13: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 27* following incubation for 24 hours (day 1) and 48 hours (day 2).

FIGURE 4.2.14: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 75* following incubation for 24 hours (day 1) and 48 hours (day 2).


viable organisms (%)
60
50
40
30
20
10
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

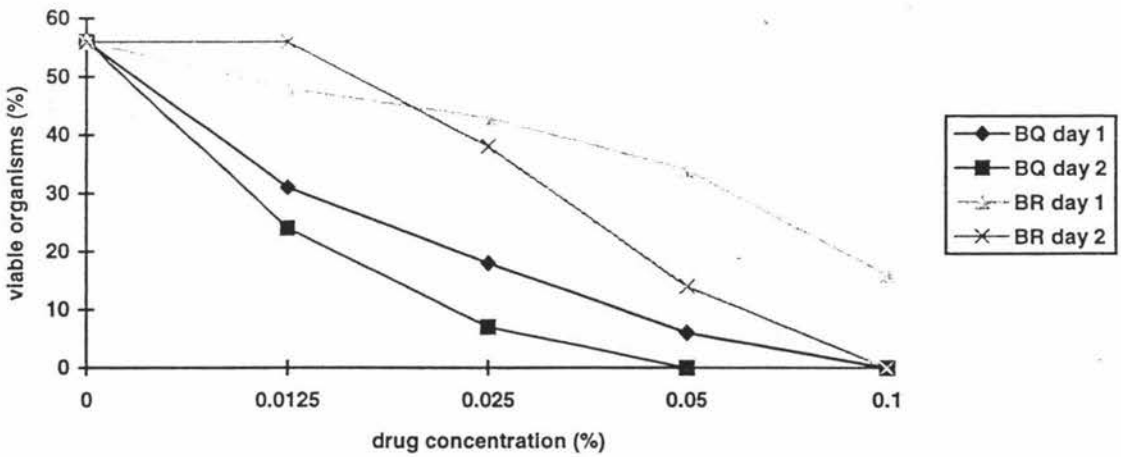
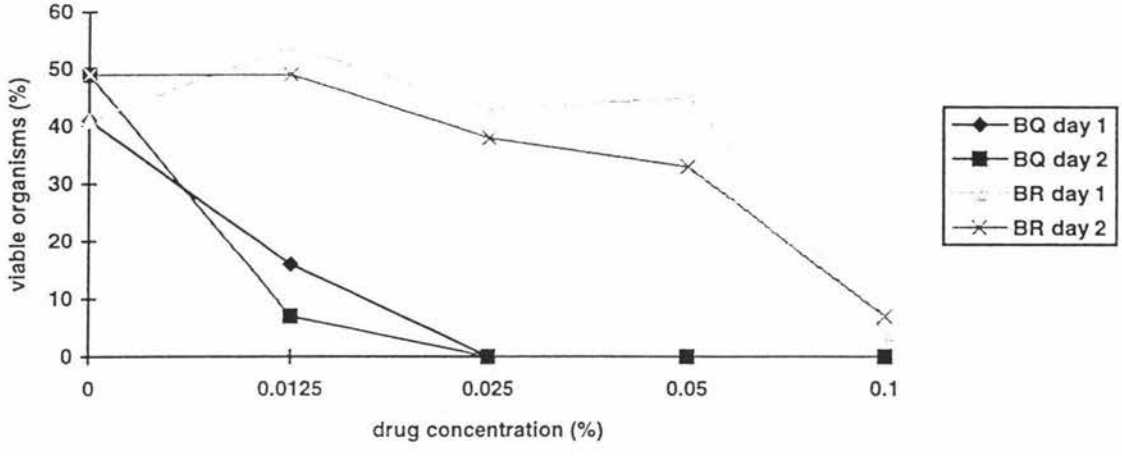

viable organisms (%)
60
50
40
30
20
10
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

FIGURE 4.2.15: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 90* following incubation for 24 hours (day 1) and 48 hours (day 2).

FIGURE 4.2.16: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate A1 following incubation for 24 hours (day 1) and 48 hours (day 2).


60
50
40
30
20
10
0
viable organisms (%)
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

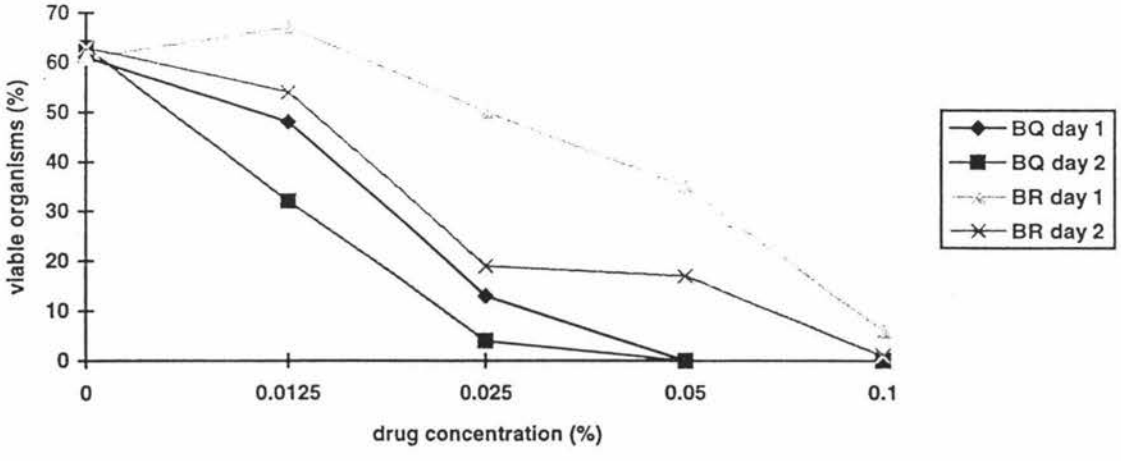
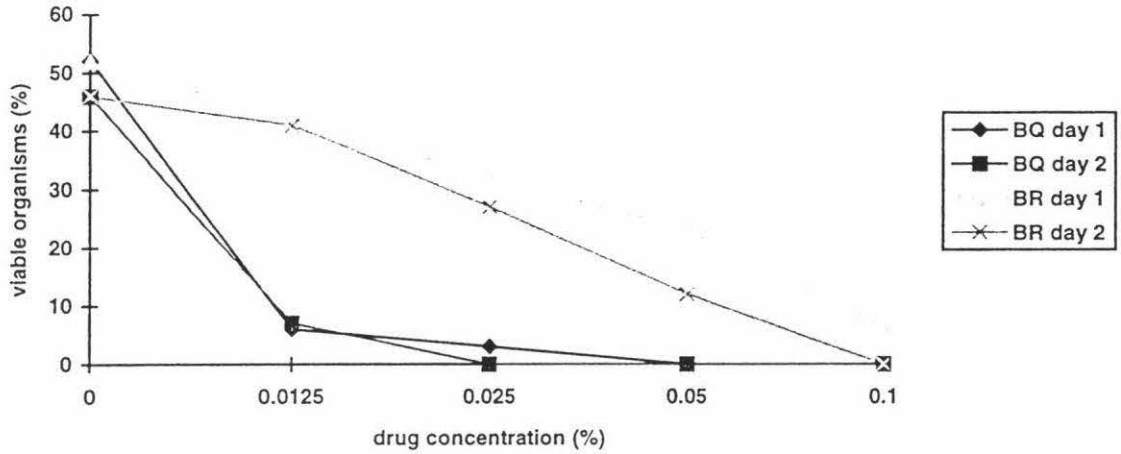

70
60
50
40
30
20
10
0
viable organisms (%)
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

FIGURE 4.2.17: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 3 following incubation for 24 hours (day 1) and 48 hours (day 2).

FIGURE 4.2.18: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 20 following incubation for 24 hours (day 1) and 48 hours (day 2).


60
50
40
30
20
10
0
viable organisms (%)
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

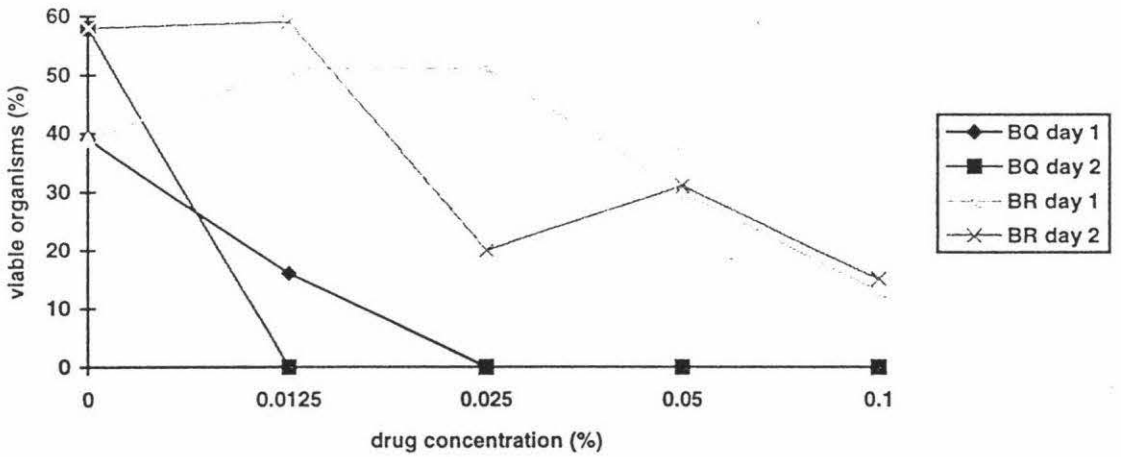
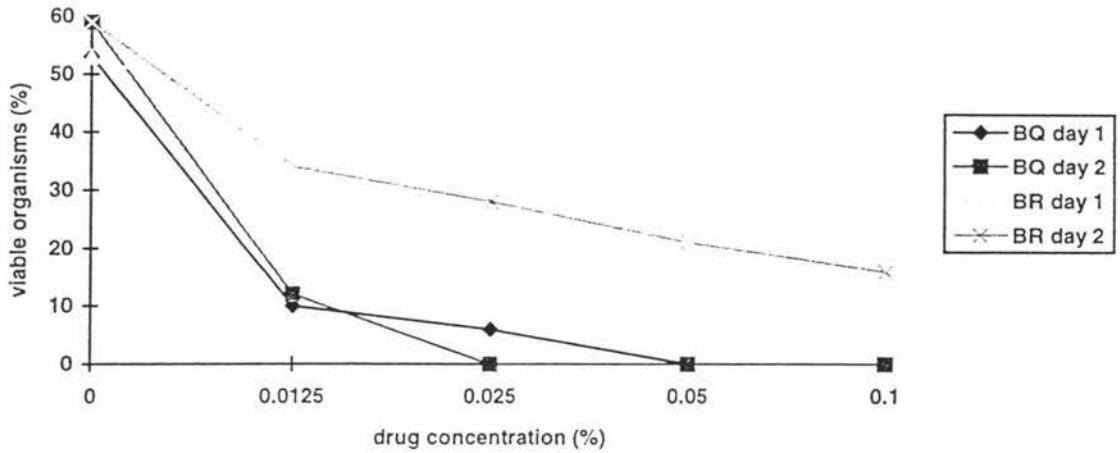

60
50
40
30
20
10
0
viable organisms (%)
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

FIGURE 4.2.19: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 18 following incubation for 24 hours (day 1) and 48 hours (day 2).

FIGURE 4.2.20: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 68* following incubation for 24 hours (day 1) and 48 hours (day 2).


viable organisms (%)
60
50
40
30
20
10
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

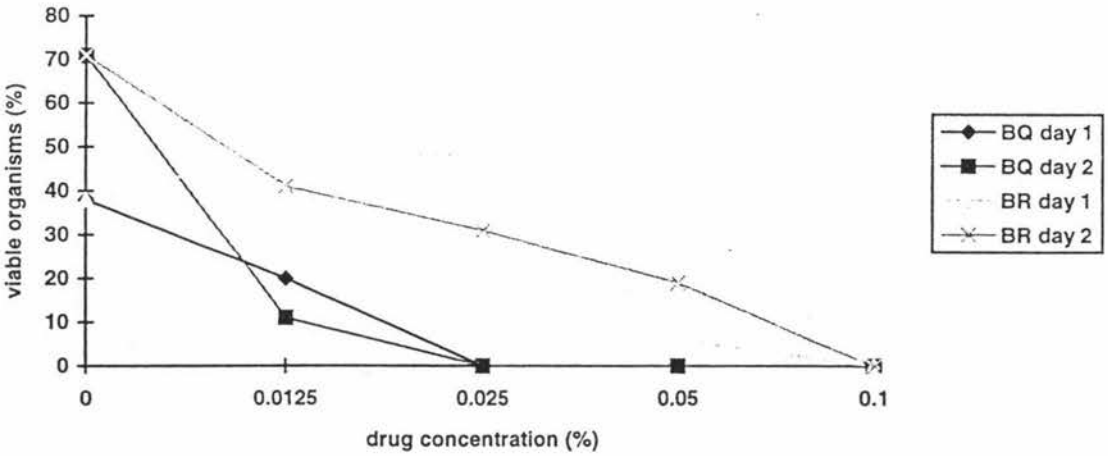
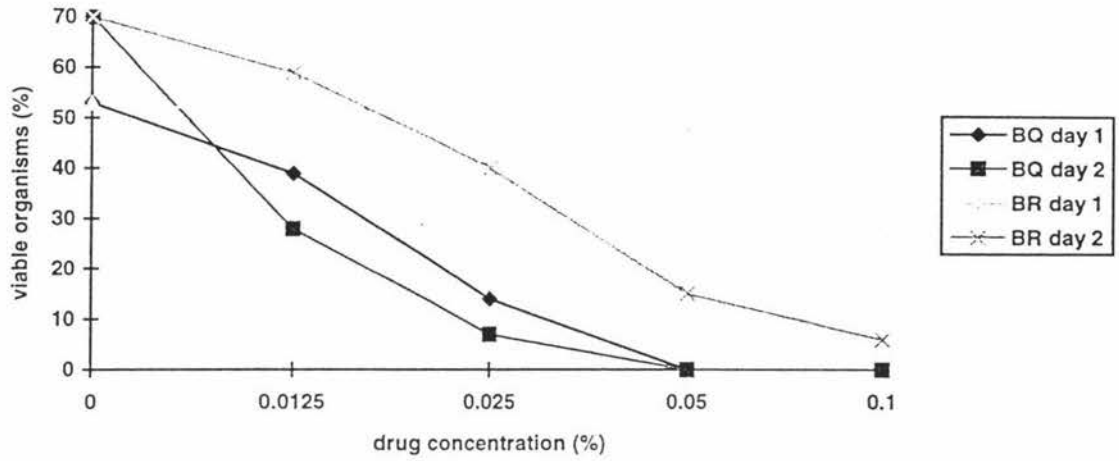

viable organisms (%)
80
70
60
50
40
30
20
10
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

FIGURE 4.2.21: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate HM1 following incubation for 24 hours (day 1) and 48 hours (day 2).

FIGURE 4.2.22: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 2 following incubation for 24 hours (day 1) and 48 hours (day 2).


viable organisms (%)
70
60
50
40
30
20
10
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

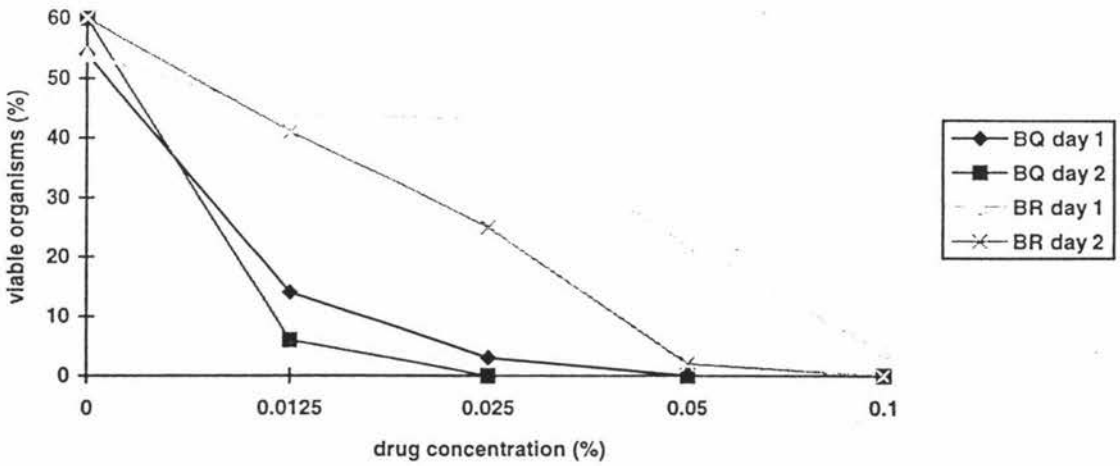
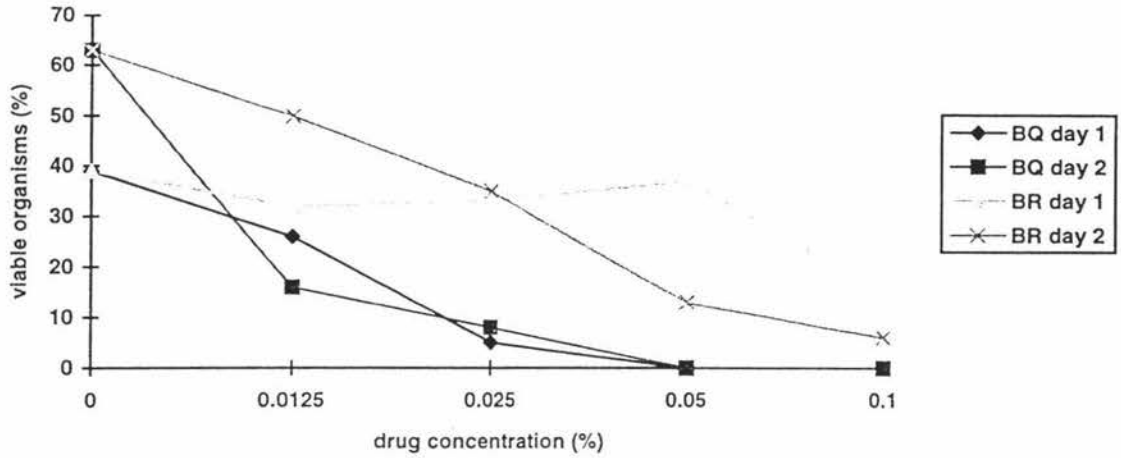

viable organisms (%)
60
50
40
30
20
10
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

FIGURE 4.2.23: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 4 following incubation for 24 hours (day 1) and 48 hours (day 2).

FIGURE 4.2.24: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 1501 following incubation for 24 hours (day 1) and 48 hours (day 2).


70
60
50
40
30
20
10
0
viable organisms (%)
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

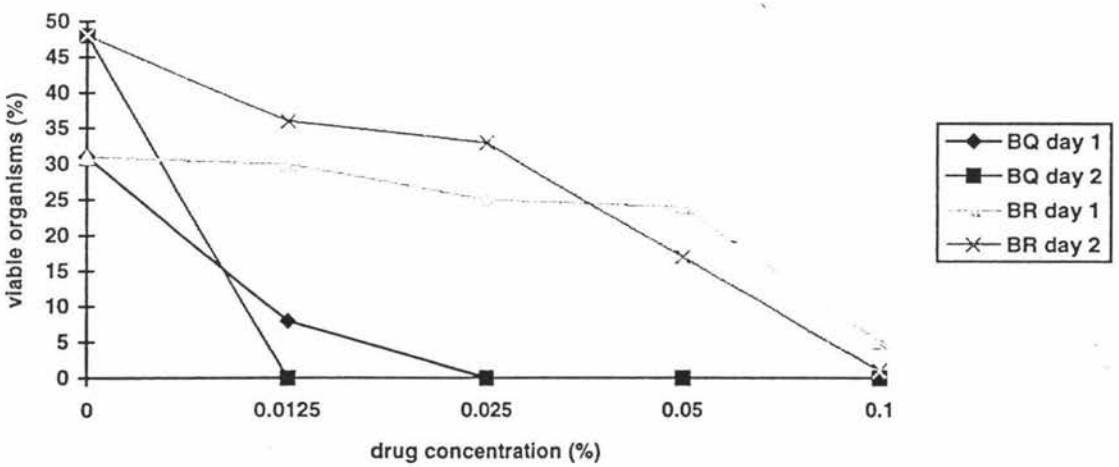
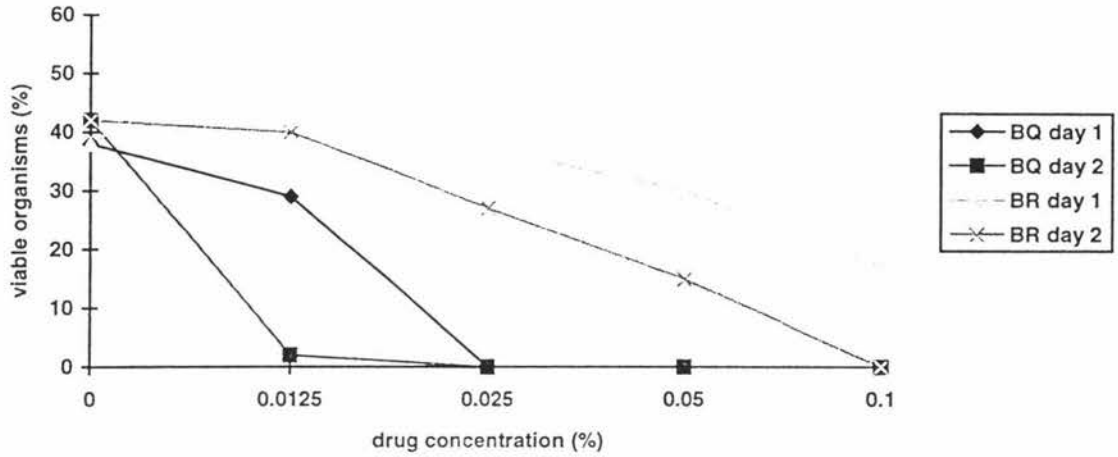

50
45
40
35
30
25
20
15
10
5
0
viable organisms (%)
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

FIGURE 4.2.25: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 24* following incubation for 24 hours (day 1) and 48 hours (day 2).

FIGURE 4.2.26: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 8 following incubation for 24 hours (day 1) and 48 hours (day 2).


viable organisms (%)
60
50
40
30
20
10
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

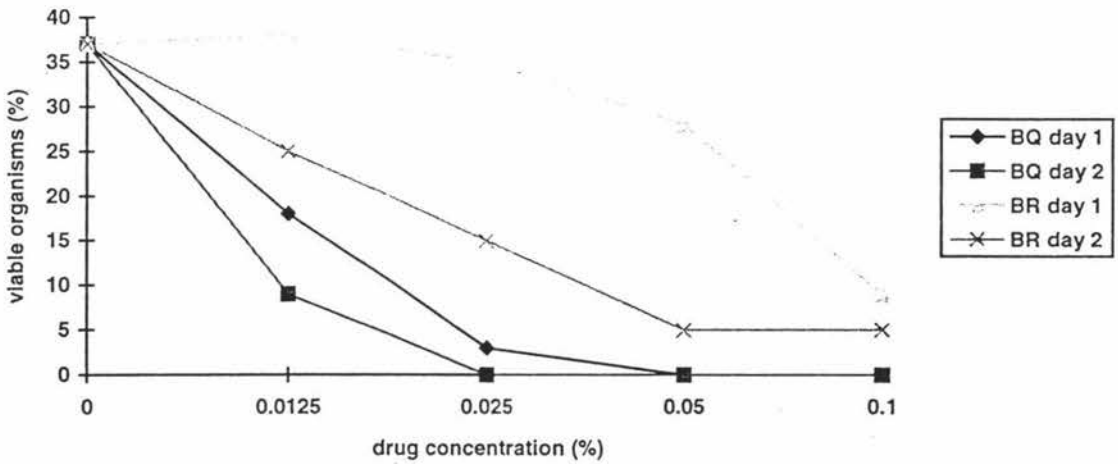
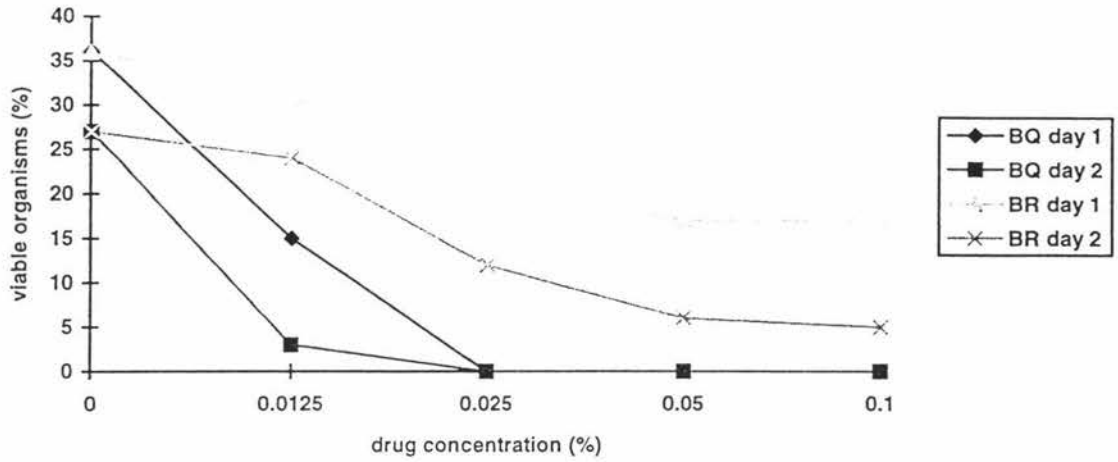

viable organisms (%)
40
35
30
25
20
15
10
5
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

FIGURE 4.2.27: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 26 following incubation for 24 hours (day 1) and 48 hours (day 2).

FIGURE 4.2.28: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 22 following incubation for 24 hours (day 1) and 48 hours (day 2).


viable organisms (%)
40
35
30
25
20
15
10
5
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

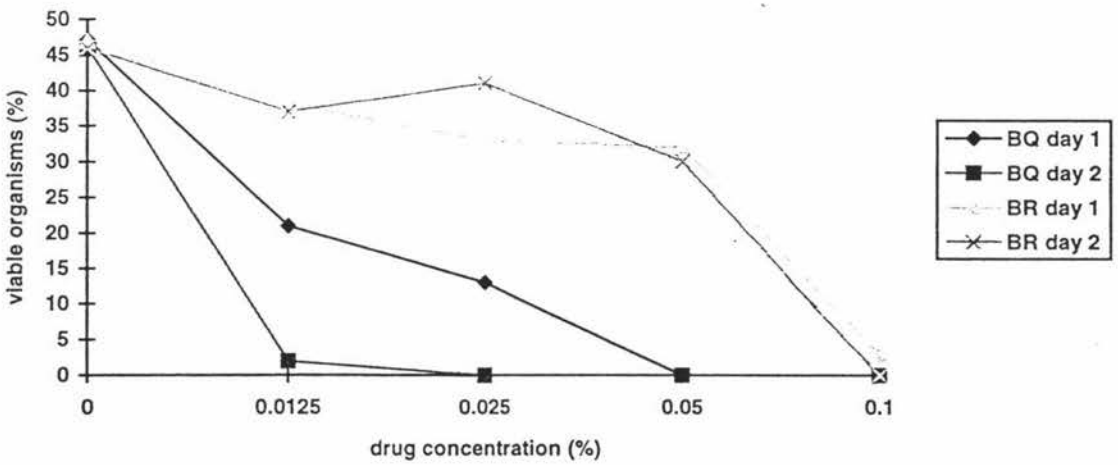
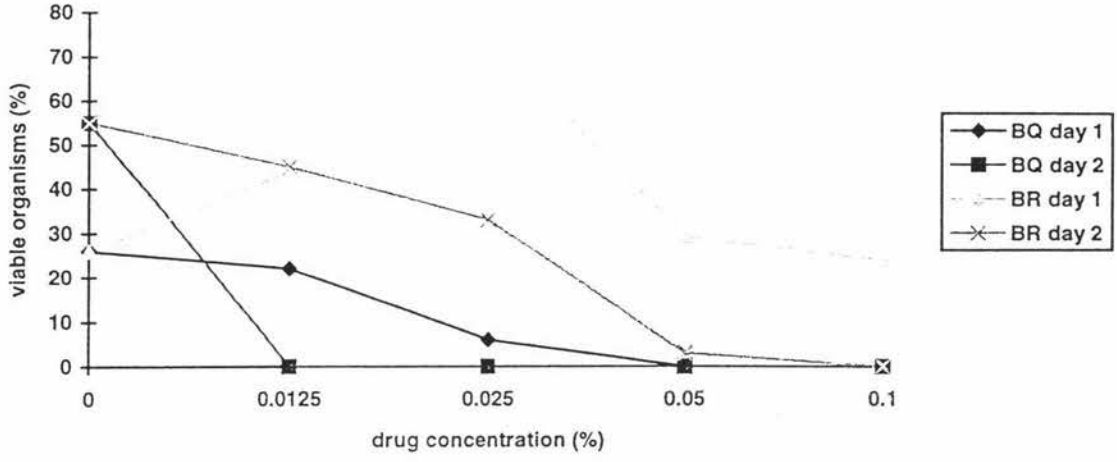
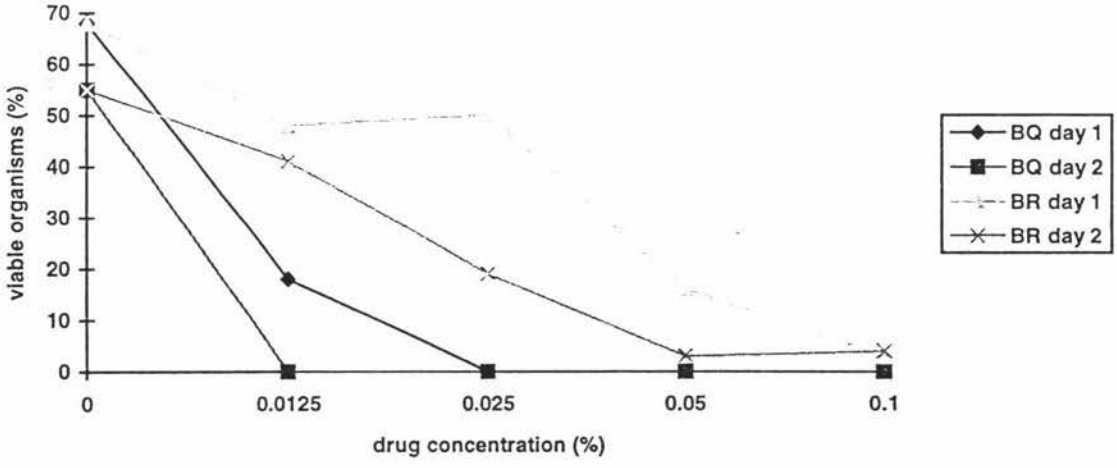

viable organisms (%)
50
45
40
35
30
25
20
15
10
5
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

FIGURE 4.2.29: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 16 following incubation for 24 hours (day 1) and 48 hours (day 2).

FIGURE 4.2.30: The Effect of Baquacil (BQ) and Brolene (BR) on Isolate 21 following incubation for 24 hours (day 1) and 48 hours (day 2).


viable organisms (%)
80
70
60
50
40
30
20
10
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2


viable organisms (%)
70
60
50
40
30
20
10
0
0
0.0125
0.025
0.05
0.1
drug concentration (%)
BQ day 1
BQ day 2
BR day 1
BR day 2

4.3 CONCLUSIONS

All isolates tested showed sensitivity to both Baquacil and Brolene. Brolene was consistently effective only at the maximum concentration tested (0.1%). Results at lower concentrations were erratic and, at times, seemed to show promotion of growth. Baquacil showed inhibition at all concentrations tested.
Although all isolates showed sensitivity to both agents tested, it is possible to determine differences in sensitivity between isolates.
The following table separates isolates into groups according to resistance to the two drugs tested.

Table 4.3.1

	GROUP 1 most sensitive	GROUP 2 moderately sensitive	GROUP 3 most resistant
BAQUACIL	**Isolates: 19, 90*, 20, 69*, 24*, 26, 1501, 16, 21**	**Isolates: 63, 9, 23, HM2, 10, 17, 27*, A1, 3, HM1, 2, 4, 8, 22, 18, AU**	**Isolates: 15, 10*, 75*, 24, 25**
BROLENE	**Isolates: 21, 16, 22, 24*, 1501, 2, 69*, 3, A1, 75*, 27*, 10*, 10**	**Isolates: 26, 8, 4, HM1, 90*, 17, 15**	**Isolates: 18, 19, HM2, 23, 9, AU, 24, 25, 63*, 20**

Parameters by which the above groups are defined.

Baquacil: Groups are defined by the lowest concentration where zero viability is reached and the time in which this activity occurs.

Group 1: ≤ 0.025 day 1 or 0.0125 day 2
Group 2: 0.05 day 1 or 0.025 day 2
Group 3: 0.1 day 1 or ≥ 0.05 day 2

Brolene: Groups are determined using only results at the maximum concentration tested (0.1% propamidine isethionate). Groupings were then made using the percentage reduction of viable organisms following 48 hours exposure to Brolene.

Group 1: ≥ 95% reduction in viable organisms
Group 2: 80% to 95% reduction in viable organisms
Group 3: < 80% reduction in viable organisms OR
>5% growth between days 1 and 2.

Within 48 hours 97% of all isolates tested reached zero viability at a concentration of 0.05% PHMB, and 100% of isolates tested reached zero viability at 0.1% PHMB within the same period of time. In experimental treatment of acanthamoebic keratitis with Baquacil a concentration of 0.02% PHMB is used. The results of this study would suggest that the concentration of PHMB be increased to at least 0.05% when used to treat acanthamoebic keratitis. Toxicity studies have shown that concentrations up to 20% of PHMB are tolerated by animal corneal epithelia (Seal *et al*, 1995).

In comparison to the results obtained with Baquacil, only 30% of all isolates tested reached zero viability within 48 hours of exposure to 0.1% propamidine isethionate (Brolene). However, 60% of isolates tested showed at least 80% reduction in viability within 48 hours of exposure to 0.1% propamidine isethionate.
It is likely that Brolene will be effective, used diligently at its maximum concentration, in many cases. However, it would be more satisfactory to perform further tests, on other chemotherapeutic agents, to find additional drugs with good activity against *Acanthamoeba* isolates. To minimise the likelihood of isolates developing resistance to currently effective treatments, combinations of drugs could be used.

A combination of Brolene and Baquacil was tested for a possible synergistic or additive effect against *Acanthamoeba* isolates. The results (not shown) indicated an additive effect may occur, but any increase in effectiveness over Baquacil alone was $\leq 2\%$ and therefore not statistically significant.

CHAPTER FIVE: General Discussion

The ultimate goal of this thesis was twofold. To develop a more rapid method of diagnosis of corneal infection by *Acanthamoeba* species and to determine whether identification of strains would correlate with drug sensitivities. Were these goals achieved the benefits would have immediate impact to both patient and clinician as cases of acanthamoebic keratitis could be identified sooner and the treatment regime customised to the infecting strain, thereby increasing the likelihood of cure, with minimum need for invasive, surgical procedures.
In attempting to achieve the goals the following areas were examined:

1. DNA extraction from *Acanthamoeba* species,
2. PCR analysis of *Acanthamoeba* species, and
3. Investigating resistance of *Acanthamoeba* species to two chemotherapeutic agents.

Prior to commencing this thesis there was no documented evidence of any one method which consistently and efficiently extracted DNA from *Acanthamoeba* species. This thesis compared ten DNA extraction methods with *Acanthamoeba* species for yield of DNA, purity of resulting DNA and reliability in PCR experiments. A summary of the results are shown in table 5.0.1.

Table 5.0.1: DNA Extraction Summary

Method	1	2	3	4	5	6	7	8	9	10
yield (mg/ml)	0.120	0.108	3.835	0.165	0.170	0.543	0.603	0.390	0.040	2.810
purity	3.0	1.6	1.14	3.2	1.34	2.03	1.59	1.55	0.45	2.01
PCR	no	yes	inc	no	no	inc	yes	yes	no	yes

KEY: no = no bands revealed in PCR analysis
inc= inconsistent PCR results
yes= consistent banding patterns with PCR analysis

Of the four methods which gave consistent PCR results it was decided to use the method giving the highest DNA yield, i.e. Method 10, throughout the rest of this thesis.

While this thesis has identified a DNA extraction method which gives a good yield of DNA and reliable results in PCR reactions, more work is required in this area. The true quantitative efficiency of DNA extraction from *Acanthamoeba* organisms has not been investigated.
In a diagnostic laboratory it is most desirable to obtain a result directly from a swab or biopsy sample. In these cases the numbers of *Acanthamoeba* cells is likely to be low. The methods tested in this thesis were examined using in excess of $1x10^4$ cells/ml.
The yield of DNA from these cells was low, in comparison to similar numbers of other organisms (Cursons, *pers comm.*). This may be due to low efficiency of extraction or to low amounts of DNA present in cells. Additionally, the spectophotometric analysis used in this thesis does not distinguish DNA from RNA. For practical purposes the DNA yield has been estimated as half the total nucleic acid yield.
Further research is required to determine the minimum number of *Acanthamoeba* cells required to provide sufficient quality and quantity of DNA for use in PCR reactions.

Should this number be far greater than that expected to be present in a clinical sample it will be necessary to develop a DNA extraction method with greater efficiency in extracting DNA from *Acanthamoeba* cells, or a more efficient PCR reaction using primers to different DNA regions of the Acanthamoebae.

Traditionally, the identification and classification of microbiolgical organisms has been carried out using characteristics such as morphology, nutritional requirements, antibiotic resistance, isoenzyme comparisons and phage sensitivity. More recently DNA based methods have been employed to augment this work. These methods include rRNA sequencing, strain-specific fluorescent oligonucleotide probes and the polymerase chain reaction. While fluorescent probes and PCR are very powerful methods, they both require sequence information from the relevant genes and must be custom built for each location within the genome of a species or strain.

In 1990 Welsh and McClelland published an alternative method which they called Arbitrarily Primed (AP) PCR. This technique can be applied to any species from which DNA can be obtained, and simply and rapidly generates a fingerprint of PCR products.
AP-PCR uses primers chosen without regard to the sequence of the genome to be fingerprinted, therefore requiring no prior knowledge of the molecular biology of the organisms being investigated.
The premise behind this method was that at a sufficiently low temperature primers will anneal to many sequences with a variety of mismatches. Some of these will be within a few hundred base pairs of each other and on opposite stands. Sequences between these primers will be PCR amplifiable. Amplification parameters are then manipulated to provide reproducible AP-PCR fingerprints.
Each primer used gives a different pattern of AP-PCR products, each with the potential of detecting polymorphisms between strains. Thus, the data produced allows the differentiation of even closely related strains of the same species.
If a common band is present for all isolates following AP-PCR analysis, this product can be sequenced and this sequence used to select a genus-specific primer pair. Likewise, should groups of isolates show banding patterns particular to that group it may be possible to generate additional specific primers for distinguishing species and/or strains.

As there is not a great deal of published *Acanthamoeba* DNA sequence, thus making selection of specific primers for PCR difficult, AP-PCR was chosen as a potential means for identifying and distinguishing *Acanthamoeba* isolates. AP-PCR is used for typing of isolates, rather than diagnostics, as amplification can occur with any DNA sample. In a clinical sample DNA from corneal cells, inflammatory cells and possibly co-contaminating bacteria, is likely to be present, in addition to that of any *Acanthamoeba* cells. Should the results of AP-PCR reveal groups useful for diagnosis or epidemiology it will be necessary to generate specific primer pairs for this purpose.

In many ways AP-PCR analysis mimics the kind of information provided by Restriction Fragment Length Polymorphisms (RFLP).
The RFLP method uses enzymes, restriction endonucleases, which cleave DNA at specific nucleotide sequences (restriction sites) that typically are 4-6 nucleotide pairs long. Digestion of DNA with these enzymes produces an assortment of DNA fragments of variable size (a restriction digest) which can be separated by agarose gel electrophoresis. Electrophoretic patterns obtained can then be compared and the

differences used in qualitative and quantitative measures of relatedness. (Byers *et al*, 1983)

RFLP has been used in several studies to examine the taxonomy of the *Acanthamoeba* genus. One such study was undertaken by Kilvington *et al* (1991) to differentiate species and strains of *Acanthamoeba* by RFLP analysis of mitochondrial DNA. The results demonstrated wide interstrain variation between morphologically defined species and similarities between strains of separate species. Of 33 isolates tested, 10 RFLP groups could be defined. Additionally, initial drug susceptibility testing suggested that RFLP analysis may correlate with innate resistances within strain groups. This method, however, did not differentiate strains isolated from one patient over a 7 month period which showed differing susceptibility to propamidine isethionate.
RFLP differentiates morphologically identical isolates but it is unclear whether it indicates intra or interspecies differences.
Several studies using RFLP to examine the taxonomy of the *Acanthamoeba* genus have not definitively determined species/strains or virulence groups. (Byers *et al*, 1983., Gautom *et al*, 1994., Yagita and Endo, 1990)
While both RFLP and AP-PCR provide similar data AP-PCR has some advantages. As with any PCR based system, AP-PCR requires only a small amount of DNA for each reaction. This reduces the number of DNA extractions required for a lengthy study. Banding patterns provided by RFLP analysis can be unwieldy due to large numbers of restriction sites, particularly when examining genomic DNA. Primers for AP-PCR identify larger nucleotide sequences and can be manipulated to provide optimum numbers of bands for analysis.

In this thesis six primers, Apo1, Apo2, Am1, Am2, P1 and P2, were used to amplify each of 16 *Acanthamoeba* isolates. The origins of these primers are as pairs of primers developed to identify the *Acanthamoeba* genus.

The present study examined the use of each of these primers with DNA from *Acanthamoeba* isolates and the system was optimised with respect to $MgCl_2$ concentration, DNA Taq polymerase concentration, dNTP concentration, primer concentration, buffer concentration, DNA concentration, cycle number, and annealing temperature. Following this optimisation, the DNA of 16 *Acanthamoeba* isolates was examined by AP-PCR analysis.

The results indicated that AP-PCR analysis can be applied to differentiate *Acanthamoeba* isolates, however these primers seemed to achieve that goal almost too well. Each of the isolates tested had a different banding pattern, with few bands shared between any of the isolates. It had been hoped that two or more groups would have been apparent which may have correlated with the differing drug sensitivities as determined in Chapter Four.

More recently Gast *et al* (1996) used RNA gene sequences in an attempt to provide a consistent classification system at the subgenus level of *Acanthamoeba*. This study is based on analysis of complete sequences of nuclear small ribosomal subunit RNA genes (*Rns*) of 18 strains from six species of *Acanthamoeba*. Within these 18 strains,

seventeen of the sequences determined differed from each other, leading the authors to conclude "no quantitative boundaries for species have been determined".

While taxonomic studies will continue, to date there has been no reliable method of classifying *Acanthamoeba* at any subgenus level. It is possible that the heterogeneity within this genus will continue to defy researchers wishing to catagorise these organisms.

Acanthamoeba isolates were tested for sensitivities to two chemotherapeutic agents. The first of these, Brolene, contains 0.1% propamidine isethionate. This preparation has been used in the treatment of acanthamoebic keratitis with mixed success. The second agent tested was Baquacil, 20% polyhexamethylene biquanide (PHMB), a pool disinfectant which, in a very diluted form, has been used experimentally for treatment of acanthamoebic keratitis with very promising results.
Serial dilutions of each drug was made and inoculated with a standardised concentration of *Acanthamobae*. All experiments included a control.

The results showed a clear delineation of effectiveness between Brolene and Baquacil, with Baquacil consistently showing greater activity against both trophozoites and cysts of *Acanthamoeba* isolates.
Within 48 hours 97% of all isolates tested reached zero viability at a concentration of 0.05% PHMB, and 100% of isolates tested reached zero viability at 0.1% PHMB within the same period of time. In experimental treatment of acanthamoebic keratitis with Baquacil a concentration of 0.02% PHMB is used. The results of this study would suggest that the concentration of PHMB be increased to at least 0.05% when used to treat acanthamoebic keratitis. Toxicity studies have shown that concentrations up to 20% of PHMB are tolerated by animal corneal epithelia (Seal *et al*, 1995).

In comparison to the results obtained with Baquacil, only 30% of all isolates tested reached zero viability within 48 hours of exposure to 0.1% propamidine isethionate (Brolene). However, 60% of isolates tested showed at least 80% reduction in viability within 48 hours of exposure to 0.1% propamidine isethionate.
It was possible to group the isolates regarding sensitivities to each of the agents tested.
The results suggest that, ideally, Baquacil should be the drug of choice for treatment of acanthamoebic keratitis. However, Baquacil is not manufactured or licensed for therapeutic use, and thus caution must be exercised when administering as a medical treatment. Many clinicians may deem it prudent to reserve this chemical as a last resort treatment, preferring to try preparations such as Brolene as an initial attempt to resolve the infection.

It is likely that Brolene will be effective, used diligently at its maximum concentration, in many cases. However, it would be more satisfactory to perform further tests, on other chemotherapeutic agents, to find additional drugs effective against *Acanthamoeba* isolates. To minimise the likelihood of isolates developing resistance to currently effective treatments, combinations of drugs could be used.

Having identified a case of acanthamoebic keratitis the challenge is on to cure it. In this thesis two chemotherapeutic agents were tested. One of these, Baquacil, was very

effective at killing *Acanthamoeba* cells. It is unrealistic to think that one agent will be effective against all *Acanthamoeba* isolates, all of the time. Further research is necessary to identify other chemotherapeutic agents effective against *Acanthamoeba*. It would also be useful to investigate the abilities of *Acanthamoeba* isolates in developing resistances to these agents.
It is also desirable to have Baquacil manufacturing standards raised with the intention that it be licensed for medical use as a treatment for acanthamoebic keratitis.

The ultimate goal of this thesis was to form groups of isolates using PCR and drug sensitivities and to discover any correlation between these groups. The results of AP-PCR analysis however suggests a high genetic heterogeneity within the *Acanthamoeba* genus, thus preventing any correlation with drug sensitivity tests.
Areas of further research in this subject could include:

1. Further work with AP-PCR analysis, using different primers or a series of primers, to fully determine whether a common band or banding patterns can identify the genus and differentiate between species and strains of *Acanthamoeba* isolates.
2. Generation of genus, species, and possibly strain, or group, specific primers for *Acanthamoebae*.
3. Testing many more possible chemotherapeutic agents with *Acanthamoebae*.

APPENDIX 1:

Acanthamoeba Isolates

Isolate number	Isolate	Origin
1	Ma	USA
2	JAC/E6	Japan
3	JAC/E1	Japan
4	JAC/E8	Japan
5	Aikas	England
6	Barnard	England
7	Bar.L	France
8	JAC/E2	Japan
9	JAC/E4	Japan
10	JAC/E5	Japan
11	JAC/E9	Japan
12	Cook	England
13	1501/3d	England
14	Acker	England
15	W.Coln	England
16	Ell-Waidth	England
17	Jones	USA
18	289	USA
19	MDC	England
20	146	USA
21	Ac112	Australia
22	Ac119	Australia
23	TAC/E3	Thailand
24	Fernandez	USA
25	Haas	USA
26	IBI-7	Philippines
27	NZAU	New Zealand
28	NZCH	New Zealand
10*	unknown	unknown
24*	unknown	unknown
27*	unknown	unknown
63*	unknown	unknown
68*	unknown	unknown
75*	unknown	unknown
90*	unknown	unknown
A1	*A.culbertsoni*	USA
1501	*A.castellanii*	CCAP
HM1	NZHM1	New Zealand
HM2	NZHM2	New Zealand
HM3	NZHM3	New Zealand
AU	NZAU	New Zealand
CH	NZCH	New Zealand

APPENDIX 2:

Each molecular weight given below represents the molecular weight of a band in the 1kb ladder used throughout this thesis.

12216
11198
10180
9162
8144
7126
6108
5090
4072
3054
2036
1636
1018
517
506
396
344
298

REFERENCES:

Achar, S.B. (1991) Identification of Cyclic AMP-Dependent Protein Kinase Activity in *Acanthamoeba*. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S393, Mar-Apr.

Adam, K.M.G., Blewett, D.A., Flamm, W.G.(1969) The DNA of *Acanthamoeba* spp.; A Method for Extraction and its Characterization. Journal of Protozoology. **16(1)**: 6-11.

Akins, R.A., Gozs, S.M., Byers, T.J. (1985) Factors regulating the encystment enhancing activity (EEA) of *Acanthamoeba castellani*. Journal of General Microbiology **131**: 2609-17.

Anger, C., Ruff, D., Pomrenke, M., Kapadia, S. (1991) A Quantitative Bacterial Plaque Assay for the Enumeration of Viable *Acanthamoeba* Cells. [Extended Abstract]. Reviews of Infectious Diseases.**13Suppl. 5**: S396, Mar-Apr.

Anonymous. (1988) *Acanthamoeba* keratitis [Editorial]. Lancet 1988; **i**: 687-688.

Asbell, P.A. (1993) *Acanthamoeba* Keratitis: There and Back Again. The Mount Sinai Journal Of Medicine **60 (4)**: 279-282. September.

Bacon, A.S., Frazer, D.G., Dart, J.K.G., Matheson, M., Ficker, L.A.,Wright, P. (1993) A Review of 72 Consecutive Cases of *Acanthamoeba* Keratitis. 1984-1992. Eye **7 (6):**719-725.

Badenoch, P.R., Adams, M., Coster, D.J. (1995) Corneal Virulence, Cytopathic Effect on Human Keratocytes and Genetic Characterization of *Acanthamoeba*. International Journal for Parasitology **25 (2):** 229-239.

Badenoch, P.R., Johnson, A.M., Christy, P.E., Coster, D.J. (1991) A Model of *Acanthamoeba* Keratitis in the Rat. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S445, Mar-Apr.

Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wertheim-van Dillen, P.M.E., van der Noordaa, J. (1990) Rapid and Simple Method for Purification of Nucleic Acids. Journal of Clinical Microbiology **28(3):** 495-503.

Bottone, E.J., Madayag, R.M., Qureshi, M.N. (1992) *Acanthamoeba* Keratitis: Synergy between Amoebic and Bacterial Cocontaminants in Contact Lens Care Systems as a Prelude to Infection. Journal of Clinical Microbiology **30 (9):** 2447- 2450.

Brown, T.J., Cursons, R.T.M. (1987) Prophylaxis. *In* Amphizoic Amoebae Human Pathology. (Ed. E.G. Rondanelli). Piccin Nuova Libraria, S.p.A., Padua, Italy.

Buehler, P.O., Schein, O.D., Stamler, J.F., Verdier, D.D., Katz, J. (1992) The Increased Risk of Ulcerative Keratitis Among Disposable Soft Contact Lens Users. Archives of Ophthalmology **110 (11):** 1555-8.

Byers, T.J., Bogler, S.A., Burianek, L.L. (1983) Analysis of Mitochondrial DNA Variation as an Approach to Systematic Relationships in the Genus *Acanthamoeba*. Journal of Protozoology **30 (2):** 198-203.

Byers, T.J., Hugo, E.R., Stewart, V.J. (1990) Genes of *Acanthamoeba*: DNA, RNA and Protein sequences (A Review). Journal of Protozoology **37(4):** 17S-25S.

Byers, T.J., Kim, B.G., King, L.E., Hugo, E.R. (1991) Molecular Aspects of the Cell Cycle and Encystment of *Acanthamoeba*. Reviews of Infectious Diseases. **13Suppl. 5**: S373-84, Mar-Apr.

Cohen, E.J., Higgens, S.E., Arentsen, J.J., Eagle, R.C., Jr., Laibson, P.R. (1991) Clinical Course of *Acanthamoeba* Keratitis. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S426, Mar-Apr.

Côté, M.A., Irvine, J.A., Rao, N.A., Trousdale, M.D. (1991) Evaluation of the Rabbit as a Model of *Acanthamoeba* Keratitis. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S443-4, Mar-Apr.

Cursons, R.T.M., Brown, T.J. (1976) Identification and classification of the aetiological agents of primary amebic meningo-encephalitis. New Zealand Journal of Marine and Freshwater Research **10**: 245-262.

Cursons, R.T.M. (1978) Pathogenic Free-Living Amoebae - Occurrence in New Zealand Thermal Regions Together with Investigations into their Disinfection, Immunity and Virulence. Ph.D. Thesis, Massey University, Palmerston North, New Zealand.

Dawson, M.W. (1982) Disinfection and Environmental Studies on Pathogenic Free-Living Amoebae. MSc Thesis, Massey University, Palmerston North, New Zealand.

De Jonckheere, J.F. (1987a) Taxonomy. *In* Amphizoic Amoebae Human Pathology.(Ed. E.G. Rondanelli). Piccin Nuova Libraria, S.p.A., Padua, Italy.

De Jonckheere, J.F. (1987b) Epidemiology. *In* Amphizoic Amoebae Human Pathology. (Ed. E.G. Rondanelli). Piccin Nuova Libraria, S.p.A., Padua, Italy.

De Jonckheere, J.F. (1991) Ecology of *Acanthamoeba*. Reviews of Infectious Diseases. **13Suppl. 5**: S385-7, Mar-Apr.

de Lamballerie, X., Chapel, F., Vignoli, C., Zandotti, C. (1994) Improved Current Methods for Amplification of DNA from Routinely Processed Liver Tissue by PCR. Journal of Clinical Pathology **47**: 466-467.

de Lamballerie, X., Zandotti, C., Vignoli, C., Bollet, C., de Micco, P. (1992) A One-Step Microbial DNA Extraction Method using "Chelex 100" Suitable for Gene Amplification. Res. Microbiol. **143**: 785-790.

Donald, J.J. (1979) The Susceptibility of Pathogenic Free-Living Amoebae to Chemotherapeutic Agents. MSc Thesis, Massey University, Palmerston North, New Zealand.

Elmsly, C.J. (1980) The In Vitro and In Vivo Testing of Chemotherapeutic Agents Against Pathogenic Free-Living Amoebae. MSc Thesis, Massey University, Palmerston North, New Zealand.

Farri, T.A., Warhurst, D.C., Marshall, T.F.deC. (1983) The Use of Infectivity Titrations for Measurement of the Viability of *Entamoeba histolytica* After Cryopreservation. Transactions of the Royal Society of Tropical Medicine and Hygiene **17(2):** 259.

Feldman, S.T., Speaker, M., Cleveland, P. (1991) Effect of Magainins on *Acanthamoeba castellani*. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S439, Mar-Apr.

Ferrante, A., Abell, T.J. (1986) Conditioned Medium from Stimulated Mononuclear Leukocytes Augments Human Neutrophil-Mediated Killing of a Virulent *Acanthamoeba* sp. Infection and Immunity **51(2):** 607-17.

Ferrante, A. (1991) Immunity to *Acanthamoeba*. Reviews of Infectious Diseases. **13Suppl. 5**: S403-409, Mar-Apr.

Gast, R.J., Ledee, D.R., Fuerst, P.A., Byers, T.J. (1996) Subgenus Systematics of *Acanthamoeba*: Four Nuclear 18S rDNA Sequence Types. Journal of Eukaryote Microbiology. **43(6):** 498-504

Gautom, R.K., Lory, S., Seyedirashti, S., Bergeron, D.L., Fritsche, T.R. (1994) Mitochondrial DNA Fingerprinting of *Acanthamoeba* spp. Isolated from Clinical and Environmental Sources. Journal of Clinical Microbiology **32 (4):** 1070-73.

Harf, C. (1991) Efficacy of Contact Lens Disinfecting Solutions for Prevention of *Acanthamoeba* Keratitis. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S413, Mar-Apr.

Horne, D.D., Frizell, M.E., Ingham, L., Jans, R.G., Gubash, S.M., Anand, C.M., Athar, M.A. (1994) *Acanthamoeba* Keratitis: An Emerging Clinical Problem. Canadian Medical Association Journal **150 (6):** 923-25.

Ionas, G (1989) A Study of the Heterogeneity of *Mycoplasma ovipneumoniae* Isolates by Examination of Their Proteins and Deoxyribonucleic Acid. MSc Thesis, Massey University, Palmerston North, New Zealand.

Jadin, J.B. (1987) History. *In* Amphizoic Amoebae Human Pathology. (Ed. E.G. Rondanelli). Piccin Nuova Libraria, S.p.A., Padua, Italy.

John, D.T. (1993) Opportunistically Pathogenic Free-living Amoebae. *In* Parasitic Protozoa, 2nd Edition, Volume 3. (Eds. J.P. Kreier and J.R. Baker). Academic Press, Inc., San Diego.

John, T., Desai, D. Sahm, D. (1991) Adherance of *Acanthamoeba castellani* Cysts and Trophozoites to Extended Wear Soft Contact Lenses. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S419-420, Mar-Apr.

John, T., Lin, J., Sahm, D., Rockey, J.H. (1991) Effects of Corticosteroids in Experimental *Acanthamoeba* Keratitis. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S440-42, Mar-Apr.

Johns, K.J., Head, W.S., Robinson, R.D., Williams, T.E., O'Day, D.M. (1991) Examination of the Contact Lens with Light Microscopy: An Aid in Diagnosis of Acanthamoeba Keratitis. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S425, Mar-Apr.

Kilvington, S., Beeching, J.R., White, D.G. (1991) Differentiation of *Acanthamoeba* Strains from Infected Corneas and the Environment by Using Restriction Endonuclease Digestion of Whole - Cell DNA. Journal of Clinical Microbiology **29(2):** 310-314.

Kilvington, S., Larkin, D.F.P. (1990) *Acanthamoeba* Adherence to Contact Lenses and Removal by Cleaning Agents. Eye **4**: 589-590.

Kilvington, S. (1991) Moist-Heat Disinfection of *Acanthamoeba* Cysts. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S418, Mar-Apr.

Kilvington, S., Anthony, Y., Davies, D.J.G., Meakin, B.J. (1991) Effect of Contact Lens Disinfectants Against *Acanthamoeba* Cysts. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S414-5, Mar-Apr.

Lai, S., Asgari, M., Henney, H.R., Jr. (1994) Non-radioactive DNA Probe and Polymerase Chain Reaction Procedures for the Specific Detection of *Acanthamoeba*. Molecular and Cellular Probes **8**: 81-89.

Larkin, D.F.P., Easty, D.L. (1991) The Relationship of *Acanthamoeba* to the External Eye Flora. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S394-5, Mar-Apr.

Larkin, D.F.P., Kilvington, S., Easty, D.L. (1990) Contamination of Contact lens storage cases by *Acanthamoeba* and Bacteria. British Journal Of Ophthalmology. **74**: 133-5.

Ma, P., Visvesvara, G.S., Martinez, A.J., Theodore, F.H., Daggett, P.M., Sawyer, T.K. (1990) *Naegleria* and *Acanthamoeba* Infections: Review. Reviews of Infectious Diseases **12(3):** 490-513.

Martinez, A.J., Guerra, A.E., Garcia-Tamayo, J., Céspedes, G., González-Alfonzo, J.E., Visvesvara, G.S. (1994) Granulomatous Amebic Encephalitis: A Review and Report of a Spontaneous Case from Venezuela. Acta Neuropathologica **87(4):** 430-4

Martinez, A.J., Scaglia, M. (1987) Experimental Infections (animal model). *In* Amphizoic Amoebae Human Pathology. (Ed. E.G. Rondanelli). Piccin Nuova Libraria, S.p.A., Padua, Italy.

Martinez, A.J. (1987a) Pathogenesis. *In* Amphizoic Amoebae Human Pathology (Ed. E.G. Rondanelli). Piccin Nuova Libraria, S.p.A., Padua, Italy.

Martinez, A.J. (1987b) Clinical Manifestaions of Free-Living Amebic Infections. *In* Amphizoic Amoebae Human Pathology (Ed. E.G. Rondanelli). Piccin Nuova Libraria, S.p.A., Padua, Italy.

Martinez, A.J. (1987c) Anatomic-Pathology of Free-Living Amebic Infections. *In* Amphizoic Amoebae Human Pathology (Ed. E.G. Rondanelli). Piccin Nuova Libraria, S.p.A., Padua, Italy.

Martinez, A.J. (1985) Free-Living Amebas - Natural History, Prevention, Diagnosis, Pathology and Treatment of Disease. CRC Press Inc., Boca Raton, Florida.

Martinez, A.J., (1991) Infection of the Central Nervous System Due to *Acanthamoeba*. Reviews of Infectious Diseases. **13Suppl. 5**: S399-402, Mar-Apr.

Matthews, T.D., Frazer, D.G., Minassian, D.C., Radford, C.F., Dart, J.K.G. (1992) Risks of Keratitis and Patterns of Use With Disposable Contact Lenses. Archives of Ophthalmology **110 (11):** 1559-62.

McLaughlin, G.L., Stimac, J.E., Luke, J.M., Kuhlenschmidt, M.S., Vernon, R.A., Morton, L.D., Visvesvara G.S., Whiteley, H.D. (1991) Development of *Acanthamoeba*-Cornea Coincubation Assays. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S397-8, Mar-Apr.

Meisler, D.M., Rutherford, I. (1991) *Acanthamoeba* and Disinfection of Soft Contact Lenses. Reviews of Infectious Diseases. **13Suppl. 5**: S410-12, Mar-Apr.

Mergeryan, H. (1991) The prevalence of *Acanthamoeba* in the Human Environment. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S390-91, Mar-Apr.

Moore, M.B. (1990) *Acanthamoeba* Keratitis and Contact Lens Wear: The Patient Is At Fault. Cornea **9(Suppl. 1):** S33-S35.

Moore, M.B., Ubelaker, J., Silvany, R., Martin, J., McCulley, J.P. (1991) Scanning Electron Microscopy of *Acanthamoeba castellani*: Adherence to Surfaces of New and Used Contact Lenses and to Human Corneal Button Epithelium. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S423, Mar-Apr.

Morton, L.D., McLaughlin, G.L., Whiteley, H.E. (1991) Adherence Characteristics of Three Strains of *Acanthamoeba*. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S424, Mar-Apr.

Nilsson, S.E.G., Montan, P.G. (1994) The Hospitalized Cases of Contact Lens Induced Keratitis in Sweden and Their Relation to Lens Type and Wear Schedule: Results of a Three Year Retrospective Study. Contact Lens Association of Ophthalmologists Journal **20 (2):** 97-101.

Osato, M.S., Robinson, N.M., Wilhelmus, K.R., Jones, D.B. (1991) In Vitro Evaluation of Antimicrobial Compounds for Cysticidal Activity Against *Acanthamoeba*. Reviews of Infectious Diseases. **13Suppl. 5**: S431-5, Mar-Apr.

Palmer, M.L., Hyndiuk, R.A. (1993) Contact Lens-related Infectious Keratitis. International Ophthalmology Clinics **33 (1):** 23-49.

Parrish, C.M., O'Day, D.M. (1991) *Acanthamoeba* Keratitis Following Penetrating Keratoplasty In a Patient Without Other Identifiable Risk Factors. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S430, Mar-Apr.

Perkovich, B.T., Meisler, D.M., McMahon, J.T., Rutherford, I. (1991) Adherance of *Acanthamoeba* to Soft Contact Lenses. [Extended Abstract].Reviews of Infectious Diseases. **13Suppl. 5**: S421-2, Mar-Apr.

Pernin, P., Omar, S., Hullo, A. (1991) Morphological and Isoenzymatic Study of an Ocular Strain of *Acanthamoeba*. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S428-9, Mar-Apr.

Perry, H. (1993) Polymerase chain reaction from an immunohaematology viewpoint. New Zealand Journal of Medical Laboratory Science **47(1):** 38-39.

Rabinovitch, T., Scott S., Weissman, H., Ostler, B., Sheppard, J.D., Teikari, J. (1991) *Acanthamoeba* Keratitis: Clinical Signs and Analysis of Outcome. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5:** S427, Mar-Apr.

Rimm, D.L., Pollard, T.D., Hieter, P. (1988) Resolution of *Acanthamoeba castellani* Chromosomes by Pulsed Field Gel Electrophoresis and Construction of the Initial Linkage Map. Chromosoma (Berl) **97**: 219-223.

Rivera, F., Lares, F., Ramirez, E., Bonilla, P., Rodriguez, S., Labastida, A., Ortiz, R., Hernandez, D. (1991) Pathogenic *Acanthamoeba* Isolated During an Atmospheric Survey in Mexico City. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S388-9, Mar-Apr.

Rodriguez-Zaragoza, S. (1994) Ecology of Free-Living Amoebae. Critical Reviews in Microbiology **20(3):** 225-241.

Rondanelli, E.G., Carosi, G., Lanzarini, P., Filice, G. (1987) Ultrastructure of *Acanthamoeba-Naegleria* Free-Living Amoebae. *In* Amphizoic Amoebae Human Pathology (Ed. E.G. Rondanelli). Piccin Nuova Libraria, S.p.A., Padua, Italy.

Rondanelli E.G., Scaglia M. (1987) Pathogenic amphizoic amoebae (*Naegleria fowleri - Acanthamoeba* spp.). Morphology and Cytology. *In* Amphizoic Amoebae Human Pathology (Ed. E.G. Rondanelli). Piccin Nuova Libraria, S.p.A., Padua, Italy.

Rutherford, I., Katanik, M.T. Meisler, D.M. (1991) Efficacy of a Chlorhexidine Tablet System for Disinfection of Soft Contact Lenses Against *Acanthamoeba* Species. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S416-7, Mar-Apr.

Scrimshaw, B.J., Fellowes, A.P. (1993) Application of the polymerase chain reaction to the diagnostic laboratory. New Zealand Journal of Medical Laboratory Science **47(1):** 14-16.

Shukla, O.P., Kishore, P., Gupta, S., Srivastava, D.K. (1991) Potential Metabolic Targets in Chemotherapy for *Acanthamoeba* Infections: Polyamine Metabolism. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5:** S438, Mar-Apr.

Singh, B.N., Sharma, A.K. (1983) Antigenic relationship among seven species of *Acanthamoeba* using immobilization and gel diffusion precipitin tests. Indian Journal of Parasitology **7(2):** 149-163.

Stapleton, F., Seal, D.V., Dart, J. (1991) Possible Environmental Sources of *Acanthamoeba* Species that cause Keratitis in Contact Lens Wearers. [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S392, Mar-Apr.

Thong, Y.H., Ferrante, A. (1987) Experimental Pharmacology. *In* Amphizoic Amoebae Human Pathology (Ed. E.G. Rondanelli). Piccin Nuova Libraria, S.p.A., Padua, Italy.

Tomlinson, G. (1991) Screening for Chemical Inhibitors of Growth Rate, Encystment, and Excystment in *Acanthamoeba castellani.* [Extended Abstract]. Reviews of Infectious Diseases. **13Suppl. 5**: S436-7, Mar-Apr.

Visvesvara, G.S., Stehr-Green, J.K. (1990) Epidemiology of Free-Living Ameba Infections. Journal of Protozoology **37(4):** 25S-33S.

Visvesvara, G.S. (1987) Laboratory diagnosis. *In* Amphizoic Amoebae Human Pathology (Ed. E.G. Rondanelli). Piccin Nuova Libraria, S.p.A., Padua, Italy.

Visvesvara, G.S. (1991) Classification of *Acanthamoeba*. Reviews of Infectious Diseases. **13Suppl. 5**: S369-72, Mar-Apr

Visvesvara, G.S., Schuster, F.L., Martinez, A.J. (1993) Balamuthia mandrillaris, N. G., N. Sp., agent of amebic meningoencephalitis in humans and other animals. Journal of Eukaryote Microbiology. **40(4):** 504-14

Vodkin, M.H., Howe, D.K., Visvesvara, G.S., McLaughlin, G.L. (1992) Identification of *Acanthamoeba* at the Generic and Specific Levels Using the Polymerase Chain Reaction. Journal of Protozoology **39(3):** 378-385.

Warhurst, D.C., Carman, J.A., Mann, P.G. (1980) Survival of *Naegleria fowleri* Cysts at 4°C for Eight Months with Retention of Virulence [correspondence]. Transactions of the Royal Society of Tropical Medicine and Hygiene **74(6):** 832.

Warhurst, D.C., Wright, S.G. (1979) Cryopreservation of *Giardia intestinalis* [correspondence]. Transactions of the Royal Society of Tropical Medicine and Hygiene **73(5):** 601.

Welsh, J., McClelland, M. (1990) Fingerprinting Genomes Using PCR With Arbitrary Primers. Nucleic Acids Research **18(24):** 7213-18.

Wilhelmus, K.R. (1991) Introduction: The Increasing Importance of *Acanthamoeba*. Reviews of Infectious Diseases. **13 Suppl. 5**: S367-8, Mar-Apr.

Wilhelmus, K.R., and Jones, D.B. (1991) Program Planning for Research on *Acanthamoeba*. Reviews of Infectious Diseases. **13Suppl. 5**: S446-450, Mar-Apr.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V. (1990) DNA Polymorphisms Amplified by Arbitrary Primers are Useful as Genetic Markers. Nucleic Acids Research **18 (22):** 6531-6535.

Yagita, K., Endo, T. (1990) Restriction enzyme analysis of mitochondrial DNA of *Acanthamoeba* strains in Japan. Journal of Protozoology **37 (6):** 570-575.