

Detection and Differentiation of Pathogenic *Acanthamoeba*.

Thesis presented for the degree of

Doctor of Philosophy

By

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Dedicated to my Father

“Hafeez Ahmed Khan”

and my Mother

“Khalida Khanum”

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LIST OF ABBREVIATIONS

| | |
|-------------------------------|---|
| AIDS | Acquired immunodeficiency syndrome |
| Amp | Ampicillin |
| AO | Aldehyde oxidase |
| ADH | Aldehyde dehydrogenase |
| ATCC | American Type Culture Collection |
| BSA | Bovine serum albumin |
| BIS-TRIS PROPANE | 1,3-bis[tris(Hydroxymethyl)methylamino]propane) |
| CAGE | Cellulose acetate gel electrophoresis |
| CFW | Calcofluor white |
| CCDC | Charged couple device camera |
| CCAP | Culture Collection of Algae and Protozoa |
| DTT | Dithiothreitol |
| ELISA | Enzyme linked immunosorbent assay |
| EDTA | Ethylene diamine tetra-acetic acid |
| EST | Esterases |
| ϵ -ACA | ϵ -amino-caproic acid |
| F6P | Fructose-6-phosphate |
| FACS | Fluorescent activated cell sorter |
| G-1-P+G-1, 6 diP | Glucose-1-phosphate + glucose-1, 6-diphosphate |
| G6PDH | Glucose-6-phosphate dehydrogenase |
| H ₂ O ₂ | Hydrogen peroxide |
| H-E | Hematoxylin-eosin |

| | |
|------|---|
| IDH | Isocitrate dehydrogenase |
| IFA | Indirect fluorescent antibody |
| ITS | Internal transcribed spacer |
| kan | Kanamycin |
| MES | N-[2-Morpholino]ethanesulfonic acid |
| MTT | Dimethyl thiazole..diphenyltetrazolium bromide thiazole blue |
| MDH | Malate dehydrogenase |
| NADP | Nicotinamide dinucleotide phosphate |
| NAD | Nicotinamide dinucleotide |
| PHMB | Polyhexamethylene biguanide |
| PBS | Phosphate buffer saline |
| PAS | Page's amoeba saline |
| PCR | Polymerase chain reaction |
| PHLS | Public health laboratory services |
| PGI | Phosphoglucose isomerase |
| PEPD | Prolidase |
| PMS | Phenazine methosulphate |
| pfu | Plaque forming unit |
| PEG | Polyethylene glycol |
| RFLP | Restriction fragment length polymorphism |
| SOD | Superoxide dismutase |
| XDH | Xanthine dehydrogenase |

SUMMARY

Acanthamoeba are free-living and opportunistic protozoa that are ubiquitous in nature. Human infection due to *Acanthamoeba* can involve the brain, skin, lung and eyes. However, eye infection, *Acanthamoeba* keratitis, is the most common infection. *Acanthamoeba* keratitis is a sight threatening corneal disease producing a progressive, blinding infection of the corneal surface.

Several species of *Acanthamoeba* have been identified as human pathogens, however, current laboratory techniques for identification and speciation of *Acanthamoeba* are time consuming and laborious.

The purpose of this study was to develop and improve current methods for the detection and differentiation of *Acanthamoeba* spp. Initially a number of isolates and species were obtained and their pathogenicity determined using animal model studies and cytopathic effect assays. Based on these data *Acanthamoeba* were classified in pathogenic and non-pathogenic groups. Using this information a variety of techniques were developed to differentiate these groups. Simple plating assays were used to differentiate pathogenic *Acanthamoeba*. We successfully differentiated pathogenic and non-pathogenic *Acanthamoeba* on the basis of physical characteristics such as growth at 37°C and ability to grow in the presence of 1 M mannitol. Morphological characteristics were observed using scanning electron microscopy. Although these were useful, not all *Acanthamoeba* tested could be differentiated.

Due to the increased analytical sensitivity and and specificity, PCR based assays were used to identify and speciate *Acanthamoeba* spp. A new DNA extraction method was developed which can be directly used with the given specimen without the prior need

of cultivation of parasites. This method together with *Acanthamoeba* specific primers makes an ideal assay for the detection of *Acanthamoeba*. Using this assay we were able to identify *Acanthamoeba* upto 5 cells. Ribosomal DNA sequencing was performed to aid in differentiation of pathogenic and non-pathogenic *Acanthamoeba*. *Acanthamoeba* isoates from different sources (environmental and eye infections) were clustered into distinct genotypes according to their rDNA sequence. All tested pathogenic *Acanthamoeba* fell into one group indicating that virulence may be associated with specific cluster of cladistic groups of *Acanthamoeba*.

Owing to simplicity and popularity of immunoassays as diagnostic methods we isolated antibody fragments from a phage display antibody library which can be potentially used for the clinical diagnosis of *Acanthamoeba* spp. These antibody fragments were tested against a range of different organisms and cell types for their specificities.

In a recent study by Cao et al. (1998), a correlation between proteases and rabbit primary corneal epithelial cell damage has been shown but the study is limited as only one isolate of *Acanthamoeba* was used. In our study we have shown clear differentiation between pathogenic and non-pathogenic species of *Acanthamoeba* on the basis of extracellular protease activity. In addition we have also observed a contact-dependent protease when whole pathogenic *Acanthamoeba* were added to primary corneal epithelial cell monolayers. These data will help in furthering our understanding of the basic mechanisms of *Acanthamoeba* pathogenicity and thus to prevent the development of infection.

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CHAPTER 1

Introduction

Acanthamoeba are known as opportunistic or pathogens of man, and many species have been isolated from a diverse number of environmental sources such as dust, soil, sand, rivers, streams, ponds and tap water. They have also been isolated from bottled drinking water, swimming pools, physiotherapy pools, sea water, industrial cooling water, air conditioners, sewage, compost and dialysis units as well as a variety of animal species including fish tissues, South Korea (Moura *et al.* 1992), human brain, (Ofori-Kwakye *et al.* 1986; Wiley *et al.* 1987) and yeast culture (Page 1988). *Acanthamoeba* can survive adverse conditions by forming resistant cysts. These cysts are impervious to inorganic chlorine up to 50 ppm, trophozoites (vegetative cells) are sensitive to chlorine at 2 ppm but even this concentration is well in excess of that achieved in public water supplies (<1 ppm, Seal *et al.* 1994; Badenoch *et al.* 1994; John 1993; Ma *et al.* 1990; Auran *et al.* 1987; Jones 1986).

Some species can cause a fatal chronic granulomatous meningoencephalitis involving the thalamus and brain stem (Di Gregorio *et al.* 1991; Friedland *et al.* 1992; Gonzalez *et al.* 1986; Gordon *et al.* 1992). The eye, skin and subcutaneous adipose tissue, lung, lymph nodes, adrenals, pancreas, thyroid, breast and ethmoid sinus may also be infected by the amoebic trophozoites. *Acanthamoeba* infections are more frequent in AIDS patients however the most common infection caused by this organism is a keratitis associated with contact lens use (Badenoch *et al.* 1994; Ma *et al.* 1990; John 1993; Auran *et al.* 1987).

Acanthamoeba keratitis infection is now recognised in Europe, Asia, Australia, U.S.A and parts of South Africa and has increased during the last 2 decades as shown in Figure 1 and 2 (Wilhelmus 1991).

Fig. 1. Cumulative number of reported cases of *Acanthamoeba* keratitis in the United States (Wilhelmus 1991).

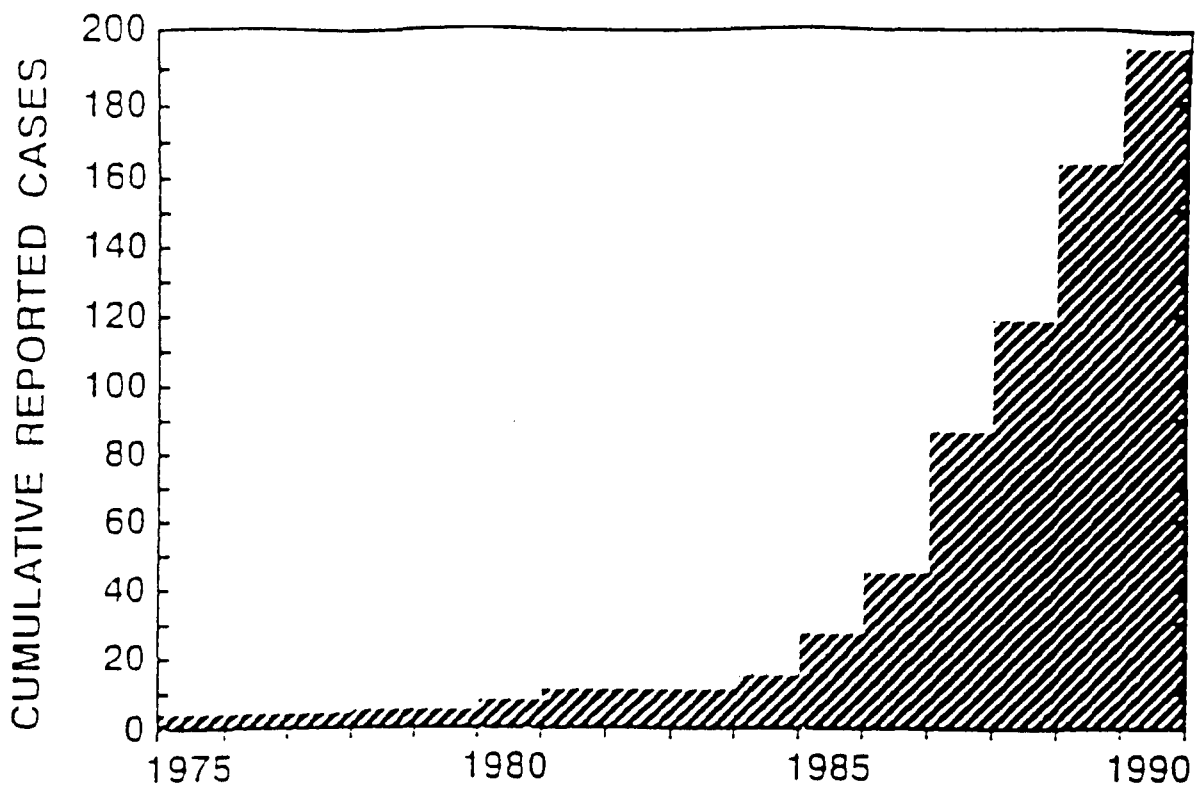
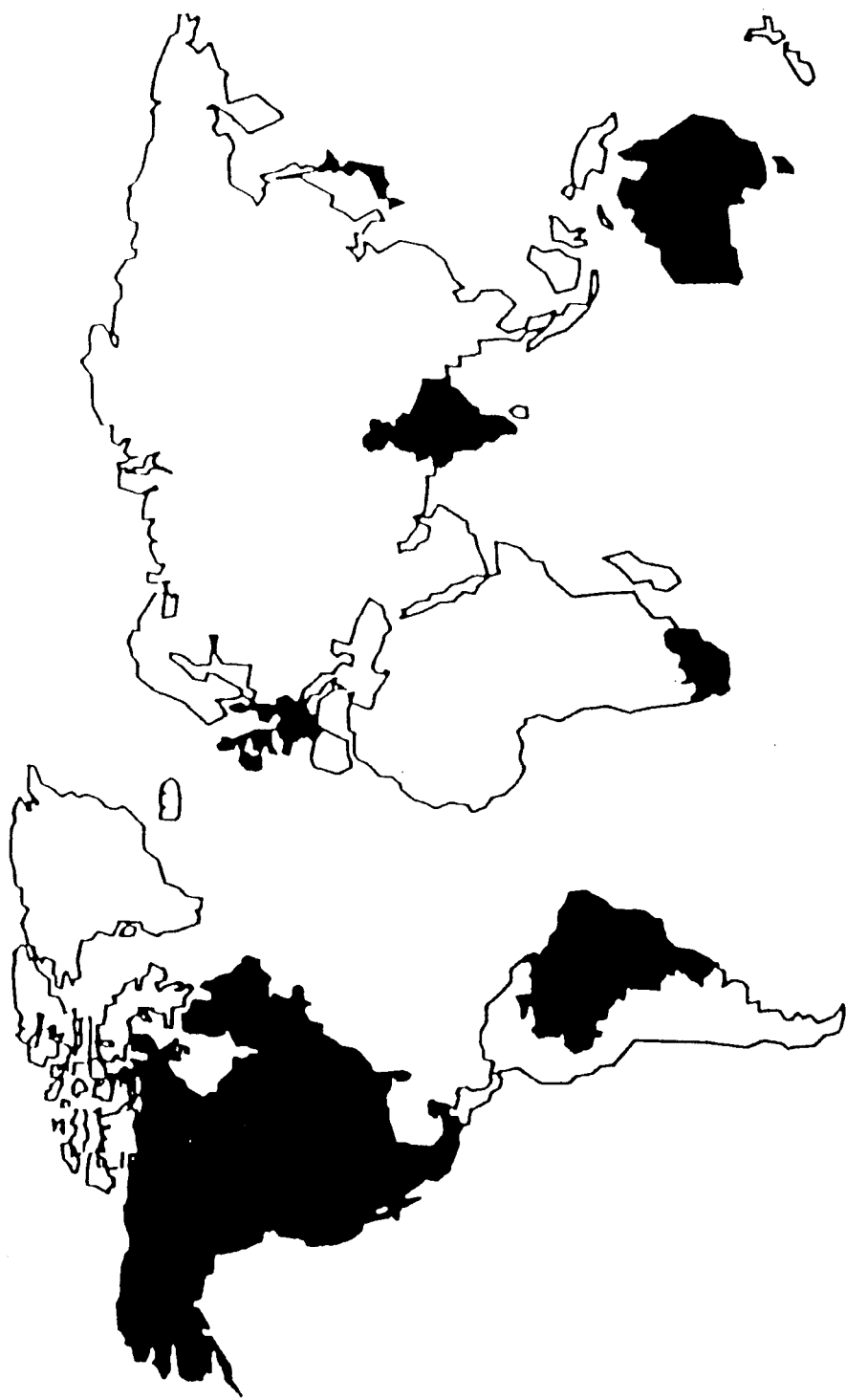
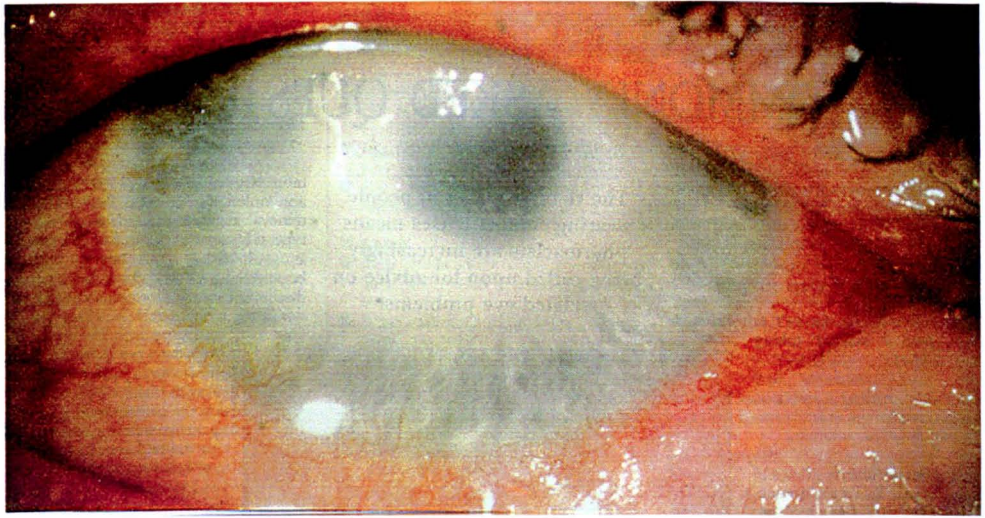


Fig. 2. Countries reporting cases of *Acanthamoeba* keratitis (Wilhelmus 1991).



According to a recent survey by Stehr-Green *et al.* (1989), it was estimated that there are approximately 24 million contact lens wearers in the USA and this number is expected to increase to over 40 million by the next decade. In a study by Mathers *et al.* (1996), a sharp increase from 2 cases per year to 30 cases per year has been observed at the University of Iowa Hospital, USA. With increasing contact lens use there must be continued awareness of the public health risks associated with their use (Stehr-Green *et al.* 1989). Corneal infections due to *Acanthamoeba* are a potentially serious problem for contact lens wearers. *Acanthamoeba* keratitis usually develops over a period of weeks to months and is characterised by severe ocular pain, inflammation and affected vision. Infection is initiated by direct contact of the amoebae with cornea, which may be introduced through minor corneal trauma and by exposure of the contact lens to contaminated water (home made saline solutions). When a contact lens is placed in non-sterile saline for cleaning, any microorganisms present may grow and adhere on the protein deposited on the lens during use. Once a microbial population is established on the lens, amoeba can attach and grow. When the lens is placed over the cornea, amoeba can become established as part of conjunctive flora and may invade the corneal stroma through the epithelium leading to infection. Clinically, the cornea becomes infiltrated and cloudy, and the patient experiences severe pain, iritis, glaucoma and cataract formation (Fig. 3). If the infection is not managed properly loss of vision can occur (John 1993; Ma *et al.* 1990; Jones *et al.* 1975; Badenoch *et al.* 1994; Auran *et al.* 1987).

Fig. 3. An *Acanthamoeba* infected eye (John 1996).



In a survey by Grey *et al.* (1995), 101 contact lens cases from individuals with no clinical infection were assayed for microbial contamination. Eighty one percent were contaminated with microorganisms. Bacteria were cultured from 77% of them, 24% grew fungi, and 20% protozoa. Of this 20%, 8% contained *Acanthamoeba* spp. Cohen *et al.* (1996), studied the causes of infectious keratitis (Table 1), this data supports the results by Grey *et al.* (1995). From these data it can be seen that the decrease in total number of eye infections was not paralleled by a decrease in *Acanthamoeba* keratitis infections.

1.1 - Life Cycle of *Acanthamoeba*

Acanthamoeba spp. has a two stage life cycle consisting of trophozoite and cyst forms (Fig. 4 & 5). Both trophozoites and cysts contain a single prominent nucleus. Slender acanthopodia project from the surface of trophozoites allowing for slow motility. Trophozoites encyst under adverse environmental conditions (lack of nutrients, high or low temperatures) and are resistant to desiccation. Cyst size varies from 13 to 30 μm and trophozoite size varies from 17-56 μm depending upon species. Cysts contain an outer wall (ectocyst) that is often wrinkled or rippled and an inner wall (endocyst) that may be polygonal, triangular, round or oval in shape. Cysts will excyst to yield trophozoites when environmental conditions are favourable (nutrients, oxygen and temperature between 25-30°C), (John 1993; Ma *et al.* 1990; Auran *et al.* 1987).

Table 1. Causes of infectious keratitis in Wills Eye Hospital, USA from 1988-1995 (Cohen *et al.* 1996). Results are represented in numbers.

| Organism | 1988-89 | 1990-91 | 1992 | 1993 | 1994 | 1995 | Total |
|-----------------------|---------|---------|------|------|------|------|-------|
| <i>Pseudomonas</i> | 20 | 17 | 6 | 6 | 2 | 2 | 53 |
| <i>Staphylococcus</i> | 9 | 11 | 2 | 10 | 3 | 1 | 36 |
| <i>Acanthamoeba</i> | 2 | 5 | 0 | 4 | 2 | 3 | 16 |
| Other organisms | 13 | 11 | 2 | 6 | 3 | 3 | 38 |
| No growth | 7 | 27 | 2 | 5 | 3 | 1 | 45 |
| No culture | 0 | 0 | 9 | 10 | 5 | 6 | 30 |
| Total | 51 | 71 | 21 | 41 | 18 | 16 | 218 |

Fig. 4. Two stage life cycle of *Acanthamoeba*: trophozoite and cyst (Martinez 1985).

VEGETATIVE FORM OF
A TROPHOZOITE

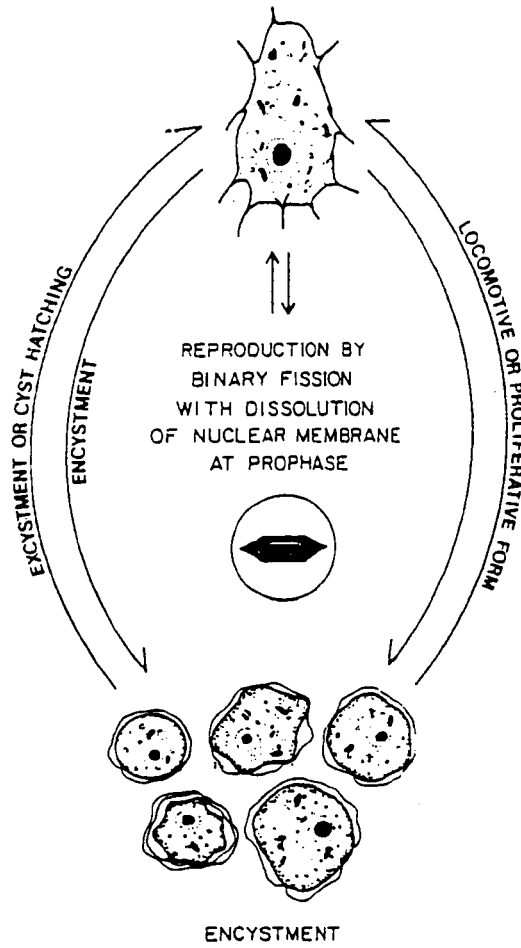
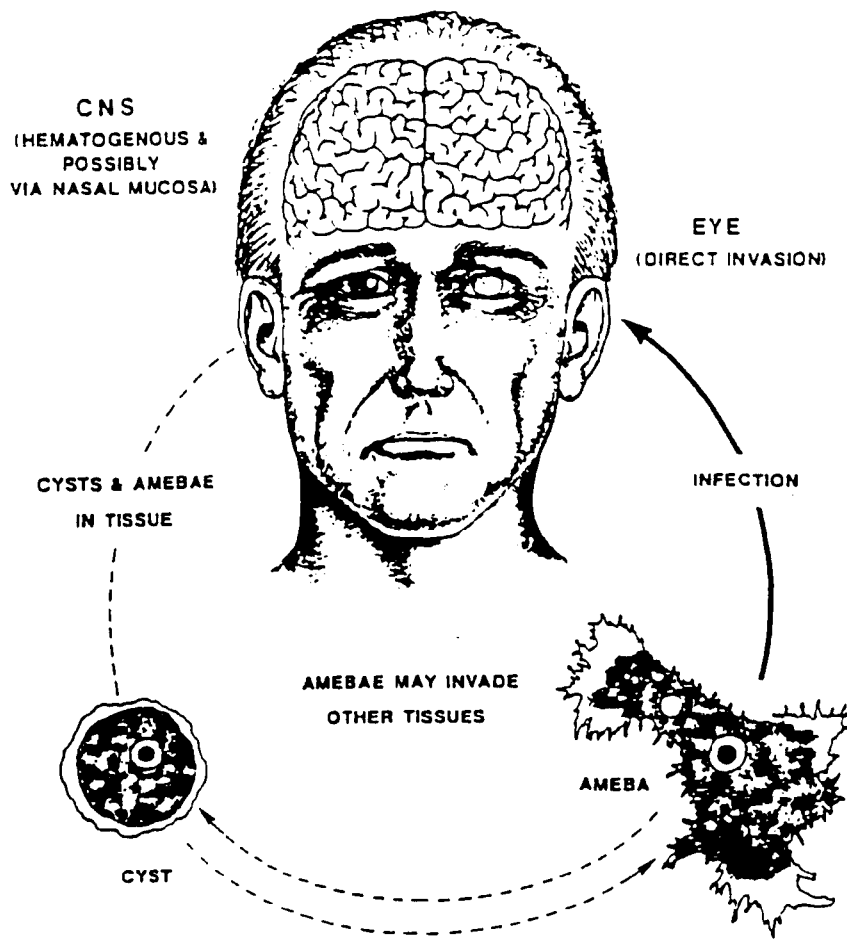


Fig. 5. Life cycle of *Acanthamoeba* sp. and human infection (Wilhelmus 1991).



1.2 - Taxonomy

In 1930, Castellani discovered an amoeba in a culture of the fungus *Cryptococcus pararoseus*. Castellani placed this amoeba in the genus *Hartmannella* and named it *Hartmannella castellanii*, 1930. Later, Volkonsky (1931) subdivided the *Hartmannella* genus into three genera.

1- *Hartmannella*: Amoebae characterised by round, smooth walled cysts.

2- *Glaeseria*: Amoebae characterised by nuclear division in the cysts.

3- *Acanthamoeba*: Amoeba characterised by the appearance of pointed spindles at mitosis and double walled cysts and an irregular outer layer were placed in this genus.

Singh (1952) and Singh and Das (1970) stated that the classification of amoeba by morphology, locomotion and appearance of cysts was of no phylogenetic value and that these characteristics were not diagnostic. They concluded that the shape of the mitotic spindle was inadequate as a generic character and discarded the genus *Acanthamoeba*.

In 1966, Pussard stated that he agreed with Singh (1952) that spindle shape was an unsatisfactory feature for species differentiation but considered the distinctive morphology of the cyst to be decisive character at the generic level and recognized the genus *Acanthamoeba*.

After studying several strains of *Hartmannella* and *Acanthamoeba*, Page (1967) also concluded that the shape of the spindle was a doubtful criterion for species differentiation. He considered the presence of acanthopodia and the structure of the cyst to be sufficiently distinctive and concluded that the generic designations of *Hartmannella* and *Acanthamoeba* were justified because of trophic and cyst characters. He also stated

that the genus *Hartmannella* had nothing in common with *Acanthamoeba* except for a general mitotic pattern, which is a property shared with many other amoeba.

In 1975, Sawyer and Griffin created the family Acanthamoebidae, and Page designated the suborder Acanthopodina under the order Amoebida. However in 1988, Page separated *Acanthamoeba* and *Hartmannella* at the ordinal level by creating a new order, Acanthopodida that includes the family Acanthamoebidae. Page (1988) placed *Hartmannella* in the family Hartmannellidae. The current position of *Acanthamoeba* in relation to *Hartmannella*, *Naegleria* and other free living amoebae is shown in the taxonomic scheme of the Society of Protozoologist is shown in Table 2 (Visvesvara 1991).

In 1977, Pussard and Pons used morphological features of the cysts to distinguish among the different species of *Acanthamoeba* and divided the genus (18 species) into three groups. Later De Jonckheere (1987) and Page (1988) accepted the classification of Pussard and Pons.

Group 1:

It consists of four species including *A. astronyxis*, *A. comandoni*, *A. echinulata* and *A. tubiashi*, all characterised by large trophozoites, ectocyst and endocyst are widely separated.

- 1) Less than 6 arms, Average diameter of cysts is $\geq 18 \mu\text{m}$, *A. astronyxis*.
- 2) 6-10 arms, Average diameter of cysts is $\geq 25.6 \mu\text{m}$, *A. comandoni*.
- 3) 12-14 arms, Average diameter of cysts are $\geq 25 \mu\text{m}$, *A. echinulata*.

Table 2. Position of *Acanthamoeba* in the taxonomic scheme (Visvesvara *et al.* 1991).

| | | | |
|------------|--------------------|---------------------|--|
| Kingdom | | Protista | |
| Subkingdom | | Protozoa | |
| Phylum | | Sarcomastigophora | |
| Subphylum | | Sarcodina | |
| Superclass | | Rhizopodea | |
| Class | | Lobosea | |
| Subclass | | Gymnamoebia | |
| Order | | Amoebida | |
| Suborder | Tubulina | Acanthopodina | |
| Family | Hartmannellidae | Acanthamoebidae | Vahlkampfiidae |
| Genus | <i>Hartmanella</i> | <i>Acanthamoeba</i> | <i>Naegleria</i> <i>Vahlkampfia</i> |

4) Average diameter of cysts is $\geq 22.6 \mu\text{m}$, *A. tubiashi*.

Group 2:

This is the largest group and includes 11 species, which are the most widespread and commonly isolated *Acanthamoeba*. Ectocyst and endocyst are either close together or widely separated. Ectocyst may be thick, thin and endocyst may be polygonal, triangular or round. Now includes *A. mauritaniensis*, *A. castellanii*, *A. polyphaga*, *A. quina*, *A. divionensis*, *A. triangularis*, *A. lugdunensis*, *A. griffini*, *A. rhysodes*, *A. paradivionensis* and *A. hatchetti*. The mean diameter of the cysts is $< 18 \mu\text{m}$ and usually do not have well-developed arms.

Group 3:

Five species are included in group 3: *A. palestinensis*, *A. culbertsoni*, *A. royreba*, *A. lenticulata*, *A. pustulosa*. The mean diameter of the cysts is usually $< 18 \mu\text{m}$.

Ectocyst in this group is thin and endocyst may have 3-5 gentle corners

1i) Endocyst round but not regular, *A. palestinensis*.

1ii) Endocyst regularly round, *A. culbertsoni*.

These characters are shown in the figures 6 and 7.

A. tubiashi in group 1 and *A. hatchetti* in group 2 were added later by Visvesvara.

Fig. 6. Differentiating cyst characters of various *Acanthamoeba* spp.

1a. *A. echinulata*; 1b. *A. astronyxis*; 2a. *A. castellanii*; 2b. *A. mauritaniensis*; 2c. *A. polyphaga*; 2d. *A. lugdunensis*; 2e. *A. quina*; 2f. *A. rhysodes*; 2g. *A. divionensis*; 2h. *A. paradiunionensis*; 2i. *A. griffini*; 2j. *A. triangularis*; 3a. *A. palestinensis*; 3b. *A. culbertsoni*; 3c. *A. lenticulata*; 3d. *A. royreba* (Pussard 1977).

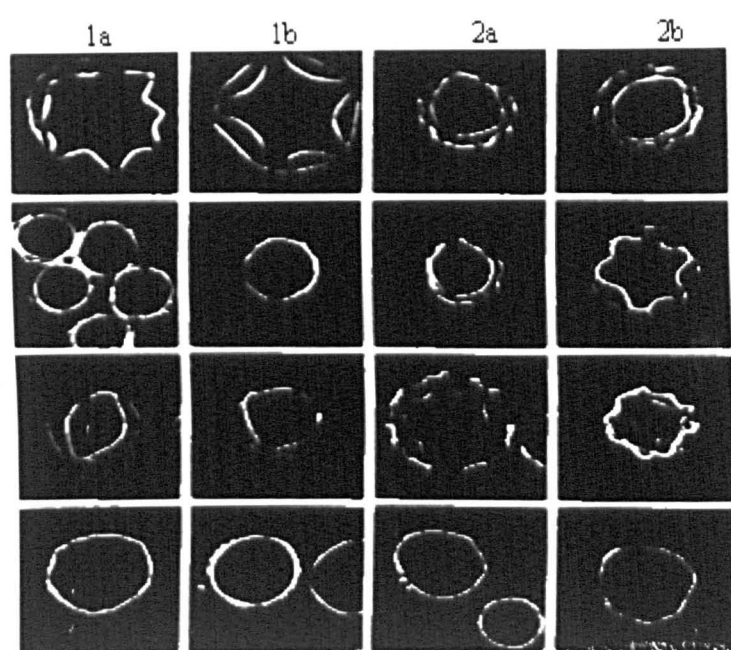
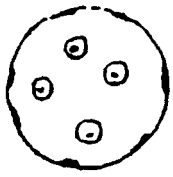


Fig. 7. Cysts characters of different species of *Acanthamoeba* spp.

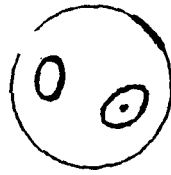
1a. *A. comandoni*; 2a. *A. rhysodes*; 2b. *A. griffini*; 2c. *A. divionensis*; 2d. *A. castellanii*; 2e. *A. triangularis*; 2f. *A. quina*; 2g. *A. polyphaga*; *A. lugdunensis*; 3a. *A. palestinensis*; 3b. *A. culbertsoni*; 3c. *A. lenticulata*; 3d. *A. royreba* (Pussard 1977).



1a



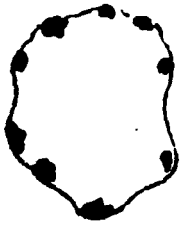
2a



2b



2c



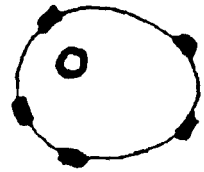
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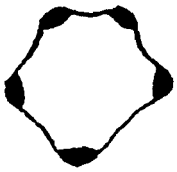
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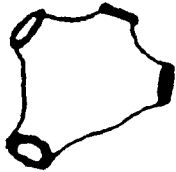
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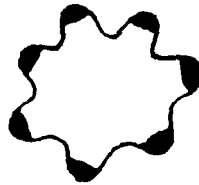
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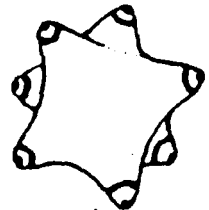
3a



3b



3c



3d

From above it is obvious that identification of the various species of *Acanthamoeba* spp. by morphological features alone is not possible. More studies are needed such as optimal growth, antigenic relationships, isoenzyme electrophoresis, banding patterns of electrophoretically separated proteins and restriction fragment length polymorphism of the mitochondrial or genomic DNA may have to be used for the identification of species of the genus *Acanthamoeba* particularly those in group 2 and 3 (De Jonckheere 1987; Costas 1984; Daggett 1982; Bogler 1983). Also Sawyer first discovered that ionic strength of the growth medium could alter the shape of cyst walls (Sawyer 1971), thus substantially reducing the reliability of cyst morphology as a taxonomic characteristic. Differentiation of some pathogenic species including *A. castellanii*, *A. polyphaga* and *A. rhysodes* is very uncertain (Warhurst 1985).

1.3 - Pathogenic *Acanthamoeba*

It is important to differentiate between pathogenic and non-pathogenic *Acanthamoeba* species as *Acanthamoeba* may be present but may not be the cause of infection. At present morphologic taxonomic criteria are generally unable to distinguish pathogenic from non-pathogenic strains (John 1993). There are a total of 19 species of *Acanthamoeba* among which 7 are known to be pathogenic to humans (Pussard 1977). The following species were thought to be pathogenic to humans.

1- *A. astronyxis*

2- *A. castellanii*

3- *A. culbertsoni*

4- *A. hatchetti*

5- *A. palestinensis*

6- *A. polyphaga*

7- *A. rhysodes*

The following species are thought to be non-pathogenic to humans.

8- *A. comandoni*

9- *A. echinulata*

10- *A. mauritaniensis*

11- *A. lugdunensis*

12- *A. quina*

13- *A. divionensis*

14- *A. paradivionensis*

15- *A. griffini*

16- *A. triangularis*

17- *A. lenticulata*

18- *A. pustulosa*

19- *A. royreba*

Later (1980), DeJonckheere tested 36 strains belonging to 19 different species of *Acanthamoeba* for cytopathic effects in Vero cell cultures and virulence in mice.

According to his study, he identified the following species or strains as are pathogenic: *A. castellanii*, *A. culbertsoni*, *A. hatchetti*, *A. palestinensis*, *A. polyphaga*, *A. rhysodes*, *A. lugdunensis*, *A. quina*, *A. lenticulata*, *A. royreba*, *A. divionensis* and *A. comandoni*. The following species / strains did not show virulence or cytopathic effects. *A. astronyxis*, *A.*

echinulata, *A. mauritaniensis*, *A. paratuberculosis*, *A. griffini*, *A. triangularis* and *A. pustulosa*. A study by Riany (PhD thesis), suggests that 3 strains of *A. quina* are virulent to mice intranasally. The same 3 strains in study by De Jonckheere (1980), showed that only one strain is virulent to mice intracerebrally. The study by Riany shows differences in enzymatic activities, isoelectric focusing patterns of proteins and immunoelectrophoresis between these 3 strains. Thus, the species identity of these 3 strains is questioned.

In conclusion, members of the genus *Acanthamoeba* are being isolated frequently from clinical specimens and are known to cause painful eye infections and fatal diseases of the CNS in humans (De Jonckheere 1987), it is important that the organism be correctly identified.

1.4 - Current methods of detection and differentiation

A number of methods including microscopy, staining, isoenzyme analysis and polymerase chain reaction have been used in the detection and differentiation of *Acanthamoeba* spp. Each method will be discussed individually in the following chapters.

From the information above it can be seen that *Acanthamoeba* keratitis is of importance due to the continuing increase in the number of infections. Methods of detection and differentiation are inadequate as they rely on culturing and microscopy, which could take several weeks. Thus, there is need to develop a rapid detection method for *Acanthamoeba* spp.

1.5 - Aims

The aims of this project were to design robust methods for the detection and differentiation of *Acanthamoeba* and to compare them with the current methods. The techniques that have employed include culturing, microscopy, isoenzyme and PCR based methods. In addition, we have also used a phage antibody display library for the isolation of *Acanthamoeba* specific antibodies. We have also studied molarity and temperature tolerance studies, rDNA sequencing and extracellular protease activity for the differentiation of pathogenic and non-pathogenic *Acanthamoeba* and to understand the basic mechanisms which are involved in the pathogenicity of *Acanthamoeba*.

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Chapter 2

Pathogenicity of *Acanthamoeba* species / isolates

2.1 - SUMMARY

Growth assays, cytopathic effect assays and animal model studies were used to differentiate pathogenic and non-pathogenic species / isolates of *Acanthamoeba*. In growth assays, *Acanthamoeba* were inoculated on non-nutrient agar plates at different pH (3 - 9.5), temperatures (30-37°C) and different concentrations of mannitol were added to non-nutrient agar plates to differentiate *Acanthamoeba* on the basis of osmotolerance. In cytopathic effect assays, primary corneal epithelial cell cultures were grown in 4-well plates which were infected with whole pathogenic and non-pathogenic *Acanthamoeba*

In animal model studies, pathogenic and non-pathogenic *Acanthamoeba* were incubated with artificial contact lenses (3 mm dialysis tubing) for 24 h at 35°C and placed over abraded mice corneas. On day 7 contact lenses were removed and the presence or absence of infection recorded. In growth assays, osmolality and temperatures showed differentiation between pathogenic and non-pathogenic *Acanthamoeba*. Pathogenic *Acanthamoeba* grew at 37°C as well as on 1 M mannitol plates. Non-pathogenic *Acanthamoeba* did not show any growth at 37°C or on 1 M mannitol plates. CPE assays showed clear differentiation as only pathogenic species / isolates of *Acanthamoeba* produced corneal epithelial cell damage. Furthermore animal model studies confirmed CPE results in differentiation of pathogenic and non-pathogenic species / isolates of *Acanthamoeba*.

2.2 - INTRODUCTION

Acanthamoeba keratitis is characterised by intense pain and a slowly worsening clinical course, if not diagnosed early and treated aggressively, the infection may spread not only to deep stroma but also to other ocular tissues (Yang *et al.* 1997). At present, diagnosis of the disease is not straightforward and treatment is problematic, consisting of hourly topical application of a combination of drugs for an extended period of time (Yang *et al.* 1997).

In view of the devastating nature of the disease and the problems associated with the therapy, our goals are to find a means to rapidly identify *Acanthamoeba* and to differentiate pathogenic and non-pathogenic species / isolates of *Acanthamoeba*. In an attempt to develop an assay for differentiation of pathogenic and non-pathogenic *Acanthamoeba* it is important to induce *Acanthamoeba* keratitis in an animal model with pathogenic *Acanthamoeba* only. In recent years studies with animal models have made important contributions to our understanding of the role of the mucosal immune system in the pathogenesis of *Acanthamoeba* keratitis (Alizadeh *et al.* 1985; Van Klink *et al.* 1997). In their study whole *Acanthamoeba* lysates given orally protected against *Acanthamoeba* keratitis in a pig model. Other animal model studies (pigs and hamsters) have been used to develop contact lens induced *Acanthamoeba* keratitis (He *et al.* 1992; Alizadeh *et al.* 1995; Van Klink *et al.* 1997). Since these animals are expensive and difficult to handle we have described the contact lens induced *Acanthamoeba* keratitis in mice. Animal model system using mice has advantage over other animal models as mice are cheaper, easy to handle and most laboratories are familiar in their use.

In another study by Cao *et al.* (1998), cytopathic effects were shown on rabbit primary corneal epithelial cell cultures but this study was limited as only one *Acanthamoeba* isolate was used. In our study we have shown the potential use of this method in differentiation of pathogenic and non-pathogenic *Acanthamoeba*.

Although these methods are very reliable and confirmatory for the differentiation of pathogenic *Acanthamoeba*, they require a large number of parasites, are time consuming, expensive and laborious. In other studies growth assays (high temperatures) have been used in order to differentiate pathogenic *Acanthamoeba* (De Jonckheere 1980) but the results did not correlate with the cytopathic effect assays. Thus there is a need to develop simple, cheaper and easy to perform assay to differentiate between pathogenic and non-pathogenic species / isolates of *Acanthamoeba*.

2.3 - MATERIALS AND METHODS

2.3.1 - Culture of amoeba

Non-nutrient agar plates were prepared by adding 10 g of Oxoid no. 1 agar (non-nutrient agar) 1 litre of amoeba saline (PAS) [OXOID Ltd., Basingtoke, Hampshire, England] and the pH was adjusted to 6.9 with KOH before autoclaving at 121°C/15min. These plates were then seeded with *K. aerogenes*. This was performed by adding 5-10 ml of a 12-16 h grown *K. aerogenes* culture to plates. These plates were left for 2 min. and excess culture was poured onto the next plate. Plates were left to dry in a laminar flow hood. This method was used as glass spreaders scratch the surface of Agar thus making observations of *Acanthamoeba* difficult.

Acanthamoeba were also grown axenically in 250 ml conical flasks. These contained 100 ml PYG medium {proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v) and glucose 1.5% (w/v)}. This medium was autoclaved at 121°C for 15 min. Flasks were incubated at 30°C without shaking, cultures reached mid log phase after 7 days. After this period cells were either harvested or passaged into fresh medium (PYG). In total, 8 *Acanthamoeba* were used in this part of study, among them 2 were isolated from human infected eye and other 6 were obtained from either CCAP or ATCC as described in Table 1.

2.3.2 - Growth assays for differentiation of *Acanthamoeba*

pH:

Growth of pathogenic and non-pathogenic *Acanthamoeba* over a range of pH between 3-9.5 was tested to look at the effects on the growth of pathogenic and non-pathogenic species of *Acanthamoeba* on non-nutrient agar plates. Following buffers were used to adjust pH of the plates: Citric acid – Tris (pH 3 – 5.5), MES (pH 5.5 – 6.7), and BIS-TRIS PROPANE (pH 6.3 – 9.5). Growth of organisms was determined by measuring the diameter of clearing zones at 24, 48 and 72 h. All plating assays were performed in triplicate with each *Acanthamoeba* on individual plates.

Temperature:

Non-nutrient agar plates seeded with *K. aerogenes* were inoculated with *Acanthamoeba* and incubated at 10°C, 15°C, 20°C, 25°C, 30°C, 37°C and 42°C.

Table 1. *Acanthamoeba* used in this Chapter.

| No | Species | Strain | Source |
|----|-----------------------------------|--------------|----------------------------------|
| 1 | <i>A. astronyxis</i> | CCAP 1534/1 | Freshwater USA 1944 |
| 2 | <i>A. royreba</i> | CCAP 1501/7 | BeWo tissue culture, USA 1977 |
| 3 | <i>A. palestinensis</i> | CCAP 1547/1 | Soil, Israel 1933 |
| 4 | <i>A. polyphaga</i> | CCAP 1501/3C | Freshwater USA 1967 |
| 5 | <i>A. castellanii</i> | ATCC 30234 | Freshwater USA |
| 6 | <i>Acanthamoeba</i> sp. (Ros) | | U.K. keratitis |
| 7 | <i>Acanthamoeba</i> sp. (Nich) | | U.K. keratitis |
| 8 | <i>A. polyphaga</i> | ATCC 30871 | Freshwater, USA |

Osmotolerance:

Non-nutrient agar plates (in distilled H₂O) supplemented with 0.1, 0.3, 0.5, 1.0 and 1.5 mannitol with and without 1 mM betaine were prepared and seeded with *K. aerogenes* as described previously. *Acanthamoeba* (100 cells) were inoculated onto these plates and incubated at 30°C.

2.3.3 - Cytopathic effect assays (CPE)

Primary rabbit corneal epithelial cell culture:

Primary cell cultures of rabbit corneal epithelium were prepared according to published protocols (Jumblatt *et al.* 1983; Panjwani *et al.* 1990) using rabbit eyes from Pel-Freez Biologicals (Rogers, Ark.). Briefly, corneas were excised using a scalpel blade and placed in 100 mm petri dish containing Hank's Balanced Salt Solution (HBSS, Gibco BRL, Life Tech.). Corneas were disinfected with neosporin (Bausch & Lomb®), penicillin-streptomycin and fungizone (Gibco BRL, Life Tech.) for 10 min. Using forceps epithelium stroma was stripped from endothelium stroma and epi-stroma and was placed in a 10 cm dish containing HBSS. A solution of 2 mg/ml Dispase (Boehringer-Mannheim) was prepared in HBSS and 10-15 ml was used for 20 corneas in a petri dish. Corneas were incubated at 37°C in 5% CO₂ for 1 h. These were then washed with SHEM {Foetal bovine serum (15% w/v), gentamycin (40 µg/ml), insulin (5 µg/ml), cholera toxin (0.1 µg/ml), vitamin F12 (40%), DMEM (40%), DMSO (0.5%), EGF growth factor 10 ng / ml}}, (Gibco BRL, Life Tech.). Under the stereo microscope epithelial layer were gently scraped with a forceps and epithelial sheets were put into a 4-well plate containing SHEM medium. Stroma and other debris were removed. Epithelium from 1-2 cornea was put in

each well of 4-well plate with 0.2 ml of media and distributed evenly to ensure proper growth. Cells were incubated at 37°C in 5% CO₂ incubator. The next day media was aspirated off and 2 ml fresh medium was added. Fresh medium was added every 3 days. Cultures became confluent within 10-14 days.

CPE assays:

Pathogenicity of whole *Acanthamoeba* cells was assayed by observing disruption of epithelial cell monolayers essentially as described by Cao *et al.* (1998). Briefly, primary corneal epithelial cells were grown to a monolayer in 4 well plates. Pathogenic and non-pathogenic *Acanthamoeba* were added to these monolayers and plates were incubated at 37°C in a 5% CO₂ incubator for 12-24h. Monolayers were either visualized by eosin staining or cytotoxicity determined using Lactate dehydrogenase assay (Cytotoxicity detection kit, Boehringer Mannheim). For this assay hydrogen peroxide (1% v/v) was used to give 100% cell death. A control with epithelial cells only was used to give zero value. The effects of ACM on epithelial cells were also assayed using cytopathic and LDH assays.

2.3.4 - Animal model studies

Preparation of model contact lens:

Three mm discs of sterilised dialysis tubing (model lens), (Spectrum Medical Industries, Los Angeles, CA., USA) were prepared using a trephine and incubated with *Acanthamoeba* in 96 well plate (200 µl / disc, 3 x 10⁶ parasites / ml of PYG). One disc per well was used. Plates were incubated at 35°C for 24 h.

Surgery and clinical observations:

Animals were first anesthetized with Metofane (Mallinckrodt Veterinary Inc., Mundelein IL) inhalation and then by intraperitoneal injection of sodium pentobarbital (80 mg / kg, Abbot Laboratories, North Chicago, IL., USA). Only one eye in each animal was used and cornea were abraded by a cotton applicator. This step is essential as it is well-established that injury is a prerequisite for the development of infection (He *et al.* 1992; Aswad *et al.* 1989; Van Klink *et al.* 1997). Our preliminary studies (data not shown) have shown that the infection does not develop unless the corneas are abraded. The parasite-laden model contact lens was placed on the surface of the abraded cornea. Eyelids were closed by sutures (6/0 ethilon sutures, Ethicon, Inc., Somerville, NJ, USA). Control animals were processed the same way except that the membrane discs were incubated in the media alone without the amoeba. Post procedure 0.1-0.5 mg/kg/mouse of buprenorphine hydrochloride (Buprenex®, Reckitt and Colman Products Ltd., England) was given to each animal every 12 h to minimize pain. Animals were observed for 7 days. Sutures and parasite-laden contact lenses were removed on day 7 post infection, and the eyes were examined for epithelial defects, stromal edema and opacity under an operating microscope immediately after opening the sutures. Experimental eyes were compared with the normal eyes. Also Richardson's stain was used to look at the epithelial cell defects.

Isolation of viable parasites:

The superficial corneal epithelium was gently swabbed with a sterile cotton applicator and the specimen was cultured for the isolation of viable parasites. Specimens

Isolation of viable parasites:

The superficial corneal epithelium was gently swabbed with a sterile cotton applicator and the specimen was cultured for the isolation of viable parasites. Specimens were inoculated on to a lawn of *K. aerogenes* on non-nutrient agar plates. On some occasions, epithelial specimens were smeared directly on to *K. aerogenes* seeded plates. Plates were incubated at 30°C and observed daily for amoeba growth.

2.4 - RESULTS

2.4.1 - Growth assays

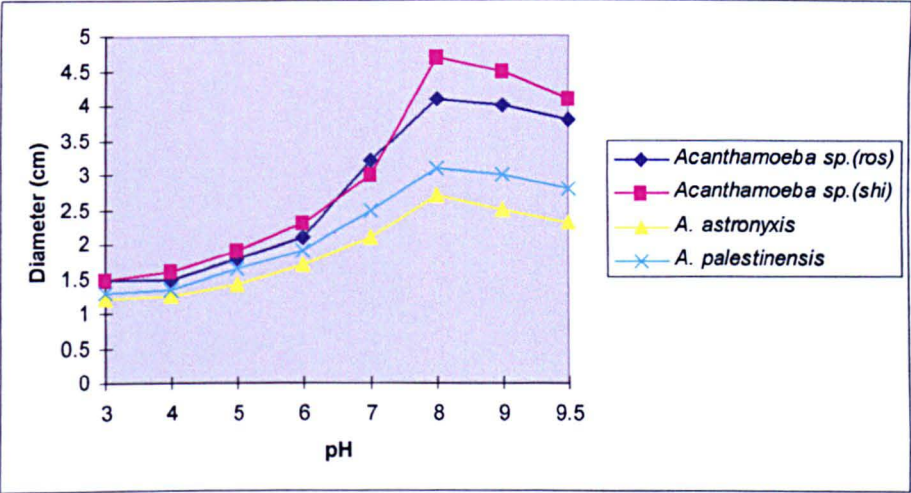
pH and Temperature:

All *Acanthamoeba* spp. grew over the full pH range 3-9.5 (Fig. 1a) with the optimal growth between pH 6.5-8.2. All *Acanthamoeba* spp. grew at temperatures below 35°C but when higher temperatures were tested (37-42°C) only pathogenic species grew except *A. griffini* and *A. royreba* which showed slower growth (Fig. 1b).

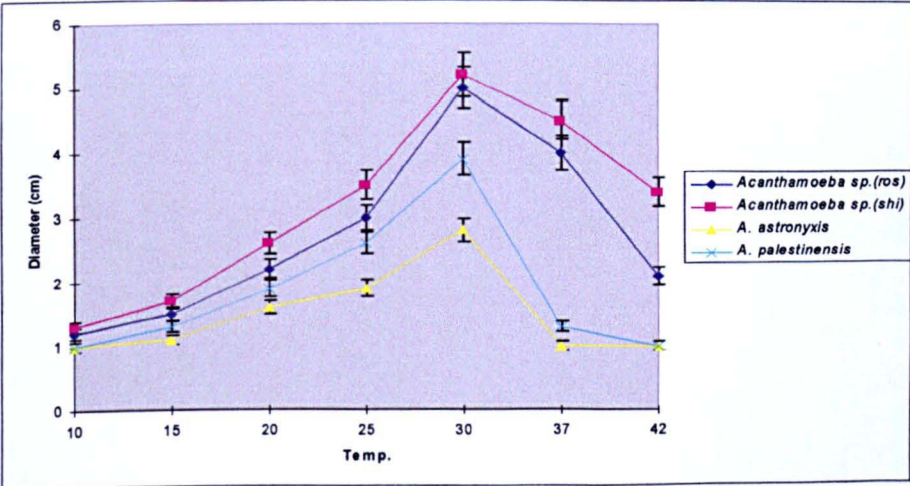
Osmolality:

Inoculation of *Acanthamoeba* (100 cells) onto plates with different concentrations of mannitol showed that non-pathogens are inhibited at conc. >0.5 – 1 M. (Fig. 1c). For differentiation purposes 0.5-1.0 M mannitol plates gave clear results as within 24 hours clearing zones can be seen in pathogenic but not in non-pathogenic species of *Acanthamoeba* (Fig. 2). Adding betaine to the plates reversed the effects of mannitol on the growth of *Acanthamoeba* (data not shown).

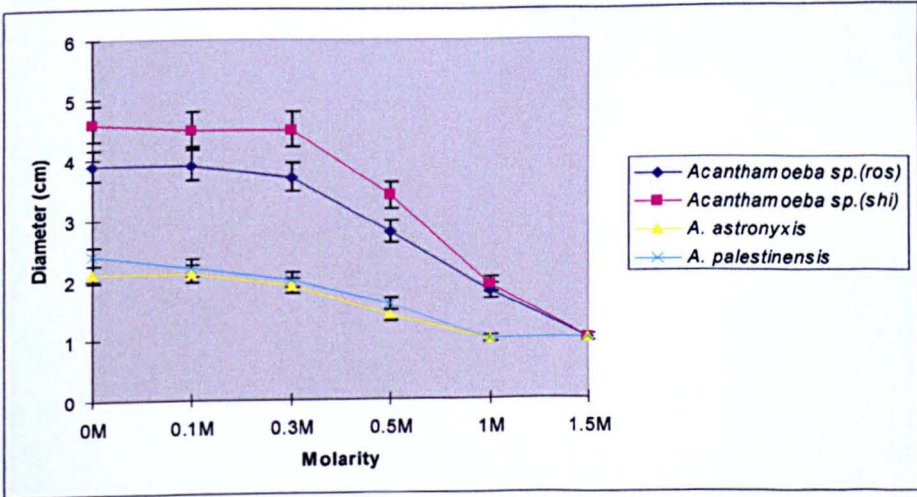
Fig. 1. Graphs showing the various physical parameters on the growth of *Acanthamoeba* after 72 h. Initial inoculation size was 1 cm. a) showing growth of pathogenic and non-pathogenic species on non-nutrient agar plates using different pH, b) using different incubation temperatures and c) different molar concentrations (using mannitol) plates. Each assay was performed in triplicates (n = 3), error bars represents \pm SD.



a)

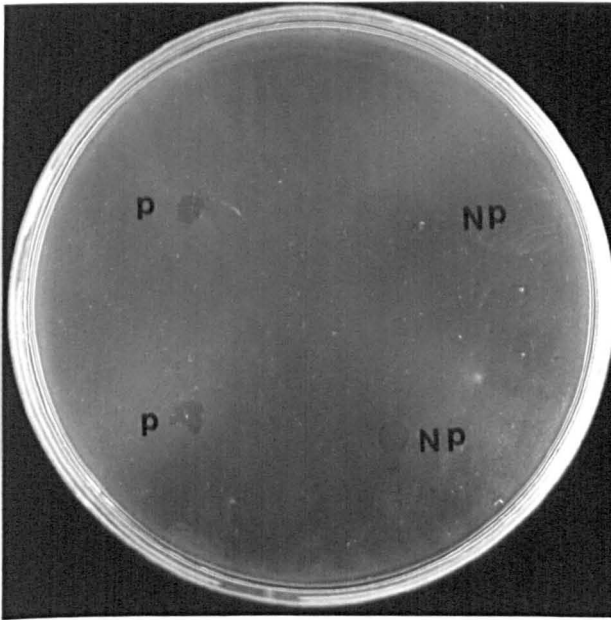


b)

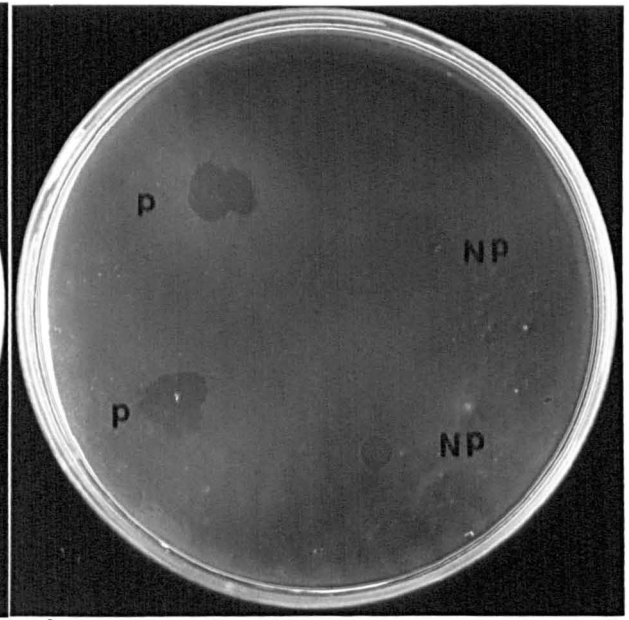


c)

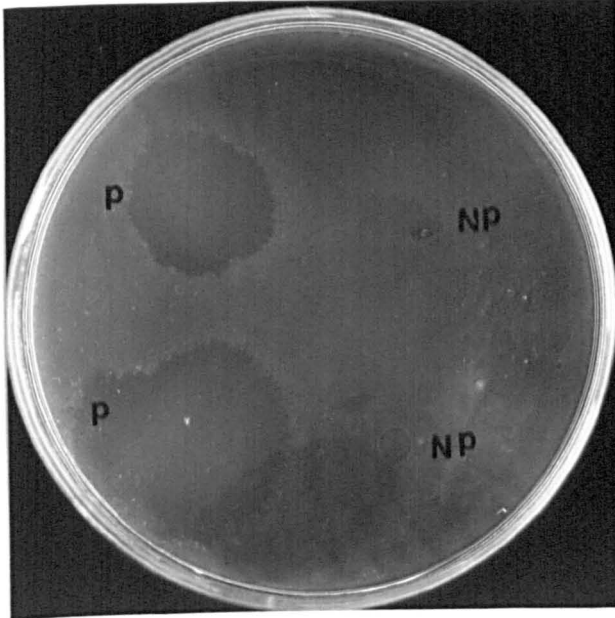
Fig. 2. Showing the comparison of growth between pathogenic and non-pathogenic species of *Acanthamoeba* on 1M mannitol non-nutrient agar plates at 30°C at a) 0, b) 24, c) 48 and d) 72 h. P represents pathogenic species of *Acanthamoeba*. See Table 2 for strain designation.



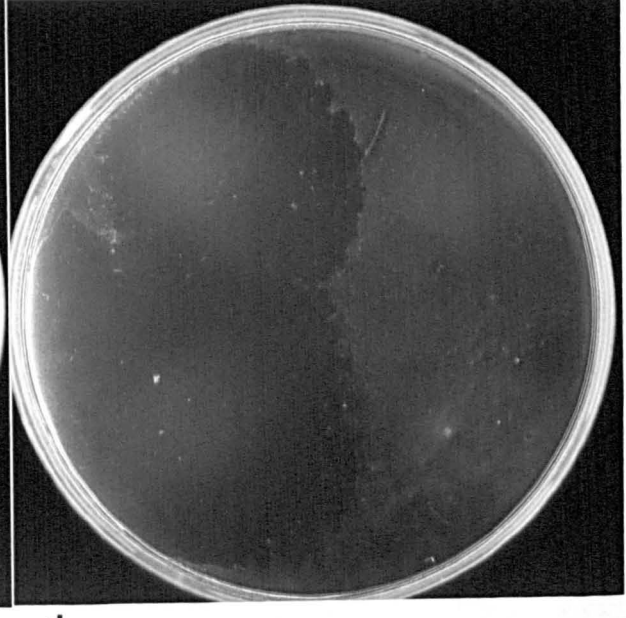
a



b



c



d

Better differentiation between pathogenic and non-pathogenic *Acanthamoeba* after 72 h can be observed in plates without mannitol as compared to 1 M mannitol plates (Fig. 1c). But the fact that viable trophozoites from pathogenic and non-pathogenic *Acanthamoeba* were observed on plates without mannitol made it difficult to differentiate the organisms. Non-pathogenic strains showed no trophozoites (no growth) on plates with 1 M mannitol as all inoculated *Acanthamoeba* encysted within 24 h.

2.4.2 - CPE assays

Staining the cell monolayers revealed that only pathogenic species of *Acanthamoeba* disrupted epithelial cell monolayers after 12h. (Fig. 3). The results for the LDH assays showed that pathogenic species of *Acanthamoeba* produced significant cytotoxicity (>94%) in epithelial cells after incubation for 24h (Table 2). No epithelial cell cytotoxicity was detected in incubations with non-pathogenic species (Table 2).

2.4.3 - Animal model studies

All mice exposed to pathogenic *Acanthamoeba*-laden contact lens developed keratitis closely resembling *Acanthamoeba* keratitis in humans (Fig. 4 & Table 3). Acute signs of infection became apparent between 6 and 7 days of post infection. Eyelids were swollen and corneal stroma became moderately opaque (Fig. 4). Viable parasites were isolated from both corneal smears and corneal scrapings. In staining with Richardson's stain, epithelial cell damage was observed (Fig. 5).

Fig. 3. The effects of whole *Acanthamoeba* cells (pathogens and non-pathogens) on primary corneal epithelial cells. All tested pathogenic strains showing clearing zones. P represents pathogenic and NP represents non-pathogenic species / isolates.

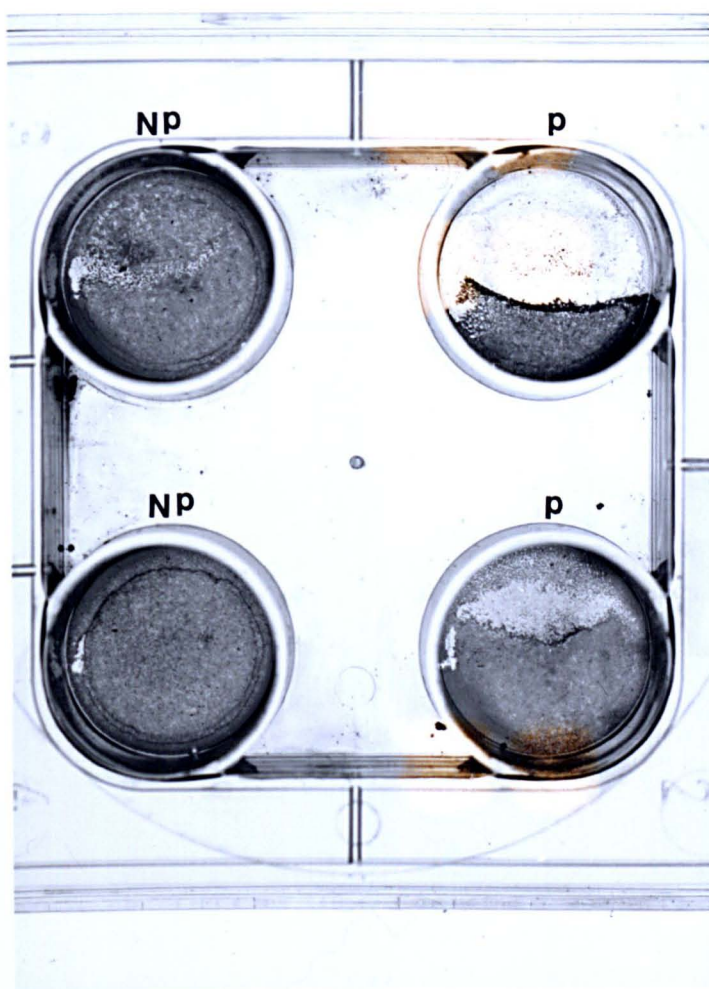


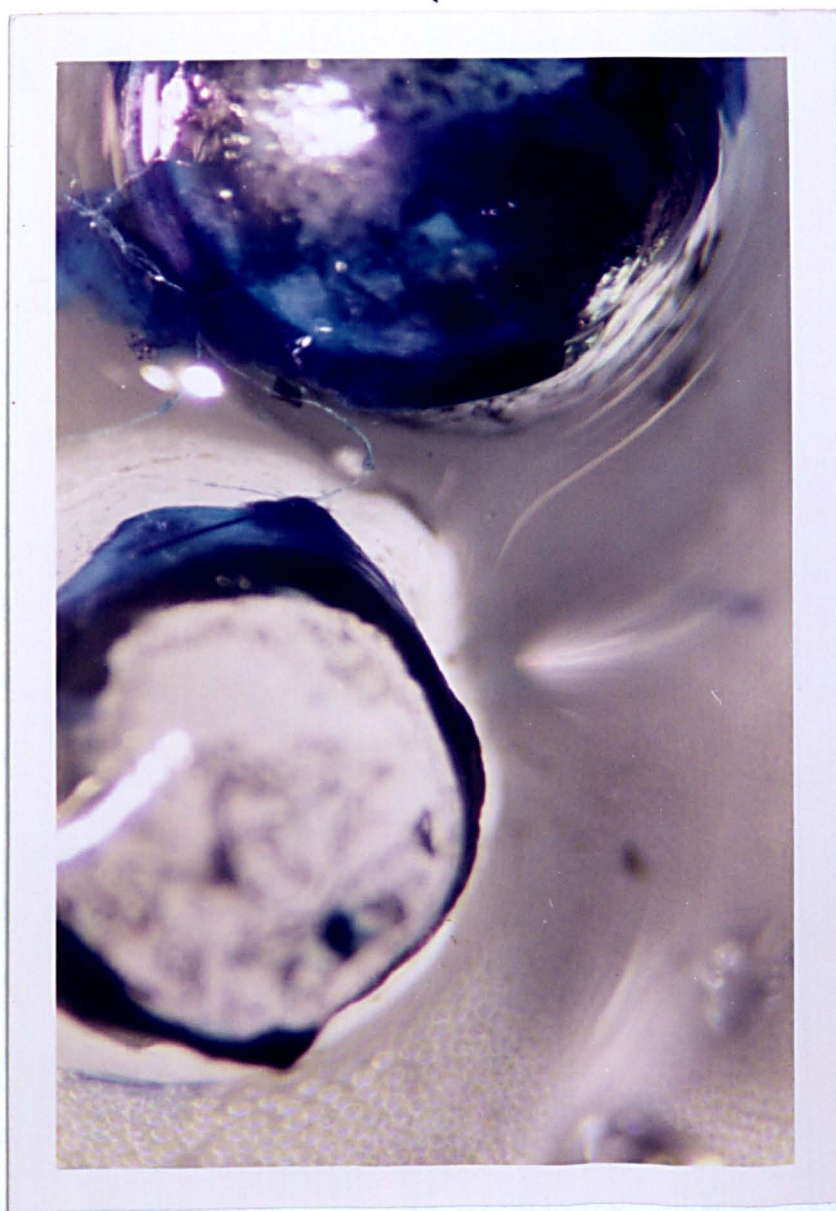
Table 2. Cytotoxicity determined using LDH assays. Pathogenic species (P) and non-pathogenic (NP). Each assay was performed in triplicate (n=3), \pm represents SD.

| <i>Acanthamoeba</i> sp. | % Cytotoxicity |
|---|----------------|
| <i>Acanthamoeba</i> sp. (Sp1, Ros) {P} | 99% \pm 0.42 |
| <i>Acanthamoeba</i> sp. (Sp2, Nich) {P} | 95% \pm 0.42 |
| <i>A. palestinensis</i> {NP} | 0% |
| <i>A. astronyxis</i> {NP} | 0% |

Fig. 4. Clinical appearance of acute *Acanthamoeba* infection in the mice (day 7). Diffuse infiltration and opacity can be observed. Infected eye is shown by arrow.



Fig. 5. Representative clinical appearance of epithelial cell damage using Richardson's stain. Infected eye is shown by arrow.



2.5 - DISCUSSION

A classification of *Acanthamoeba* based on morphological characteristics is still in use (Visvesara 1991), however, attempts to correlate pathogenicity to species using this scheme has proven difficult and thus presents a problem for clinical diagnosis.

In serological studies by Silvany *et al.* (1990) and Cerva *et al.* (1989) it was reported that 52% and 100% of the normal humans tested possessed antibody titers to *A. culbertsoni* and *A. castellanii* respectively. In our animal model studies infection was successfully induced with *A. castellanii* (Table 3) suggesting a need to identify pathogenic strains of *Acanthamoeba*.

We have successfully used simple growth assays in comparison with cytopathic effect assays and animal model studies to identify the pathogenic species / isolates of *Acanthamoeba* (Table 3). These plating assays were found very easy to perform with no required skill and can be potentially used for clinical diagnosis of pathogenic *Acanthamoeba*. We were not able to find any differences in pathogenic and non-pathogenic *Acanthamoeba* on different pH profile. Temperature produced a useful differentiation between pathogenic and non-pathogenic *Acanthamoeba* except *A. royreba* and *A. griffini*, which showed slower growth. In addition one of the non-pathogenic isolate, *A. palestinensis* showed growth at 37°C after 72 h (data not shown). However, differentiation of pathogenic and non-pathogenic species of *Acanthamoeba* can be easily performed on plates with 0.5-1.0 M mannitol plates, as, only pathogenic species / isolates of *Acanthamoeba* showed to be osmotolerant compared with non-pathogens.

Table 3. Showing differences between pathogenic and non-pathogenic *Acanthamoeba*.

| <i>Acanthamoeba</i> sp. | Source | Growth at 37°C | Osmotolerance growth assays (1M mannitol) | CPE results for epithelial cell damage | Mice <i>in vivo</i> eye infection |
|-------------------------------------|---------------------|----------------|---|--|-----------------------------------|
| <i>A. castellanii</i> ATCC 30234 | Fresh Water | + | + | + | + |
| <i>Acanthamoeba</i> sp. (Nich) | Eye infection | + | + | + | + |
| <i>Acanthamoeba</i> sp. (Ros) | Eye infection | + | + | + | + |
| <i>A. polyphaga</i> ATCC 30871 | Freshwater | + | + | + | + |
| <i>A. royreba</i> CCAP 1501/7 | BeWo tissue culture | SG | SG | PD | NI |
| <i>A. polyphaga</i> CCAP 1501/3c | Freshwater | NG | NG | ND | NI |
| <i>A. palestinensis</i> CCAP 1547/1 | Soil | NG | NG | ND | NI |
| <i>A. astronyxis</i> CCAP 1534/1 | Freshwater | NG | NG | ND | NI |

Where SG is slow growth, NG is no growth, NA is no amplification, PD is partial degradation, ND is no damage and NI is no infection.

Results from mannitol plating assays corroborated cytopathic effect assays results and animal model studies in differentiation of pathogenic *Acanthamoeba*. Due to the simplicity, this method can be potentially used for differentiation for clinical purposes.

Mechanisms of *Acanthamoeba* induced cytopathic effects are unknown but here we present the data suggesting that pathogenic *Acanthamoeba* can be easily differentiated on the basis of cytopathic effect assays. In our studies we have shown that *Acanthamoeba* keratitis can be induced in mice. The chronic nature of the infection and the appearance of corneal opacity, infiltration and epithelial cell damage are strikingly similar to those present in human *Acanthamoeba* keratitis. The strong correlation between the clinical and pathologic features of contact lens-induced *Acanthamoeba* keratitis in the mice eye, make this animal model a valuable tool for further investigations of the immunology, cell biology and therapy for *Acanthamoeba* keratitis. These results are supported by *in vitro* studies that demonstrated the cytopathic effects of pathogenic *Acanthamoeba* on primary corneal epithelial cell cultures (Table 3). These animal model studies should serve as a valuable tool to permit investigations designed to identify the factors that promote or prevent the development of *Acanthamoeba* keratitis.

2.6 - REFERENCES

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Chapter 3

Morphological and Isoenzyme Characterisation of *Acanthamoeba* spp.

3.1 - SUMMARY

Microscopical and isoenzyme analyses were used for the identification and differentiation of *Acanthamoeba* spp. For microscopical identification, samples (Environmental and clinical) were inoculated on non-nutrient agar plates overlaid with *Klebsiella aerogenes* or *Escherichia coli*. Plates were incubated at 30°C (optimal growth temperature for all species / strains of *Acanthamoeba*). Plates were observed for trophozoites and cysts every day. For axenic cultivation, single cells were isolated using an inverted microscope and grown on a non-nutrient agar plate overlaid with UV killed *K. aerogenes*, then incubated at 30°C. After *Acanthamoeba* had grown (24-48 h), stamp sized agar piece (10 mm²) containing *Acanthamoeba* was transferred to PYG medium. *Acanthamoeba* were successfully isolated using this method.

Scanning electron microscopy (SEM) was used to examine morphological differences among various *Acanthamoeba* spp. Isoenzyme analyses were also performed on axenically grown *Acanthamoeba* and a number of enzymes were assayed. Scanning electron microscopy revealed that all tested pathogenic *Acanthamoeba* strains have large number of acanthopodia on their surface in comparison to the non-pathogenic strains. Isoenzyme analysis showed differentiation among different species of *Acanthamoeba*. Interestingly, differentiation was found within 2 strains of the same species, *A. polyphaga*.

3.2 - INTRODUCTION

Microscopy is a powerful tool for identification of most protozoans. Since microscopic identification is based on morphological characteristics, skill and use of robust keys for identification are required. Examiners must have familiarity with the morphological characteristics of *Acanthamoeba* species otherwise diagnosis may require histological examination of material obtained by corneal biopsy or keratoplasty (Epstein *et al.* 1986).

A number of microscopic methods for *Acanthamoeba* identification have been used as described below:

3.2.1 - Confocal and light microscopy

Confocal microscopy is a new technique that has potential as a non-invasive diagnostic imaging method. It was used by Winchester *et al.* (1995) to investigate the cornea in *in vivo* animal studies. Confocal microscope has advantages over conventional optical microscopes in that it can image layers within the substance of a specimen of substantial thickness. Therefore, it is effective in imaging the cornea. In preparations for microscopic observations a drop of topical anesthetic is placed on the patients cornea and 2% hydroxymethylcellulose is placed on the end of the objective lens. Imaging time used is less than 5 min (Winchester *et al.* 1995). However resolution of *in vivo* confocal microscopy of the cornea is not equal to routine light microscopy of fixed histologic specimens. Thus, visually identification of *Acanthamoeba* is not reliable. Also small eye movements interfere with confocal microscopy imaging. The eye must remain still and for this reason, it is not a diagnostic option for children or patients with nystagmus (restless).

The identification of amoeba cysts on the surface of the patient contact lens by light microscopy can provide rapid diagnosis of *Acanthamoeba* keratitis. This is a simple, fast and non-invasive technique. In an experiment by Karla *et al.* (1989), a small fragment of the contact lens was excised with sterile scissors and forceps and placed on a sterile agar plate. Both anterior and posterior surfaces of contact lens fragment were examined with a standard light microscope using magnification of x200 and x400 and many cysts were observed. Direct examination of the contact lens with light microscopy can help to confirm the clinical diagnosis of *Acanthamoeba* keratitis. However, accurate clinical diagnosis of *Acanthamoeba* keratitis requires a high degree of suspicion because *Acanthamoeba* keratitis is often misdiagnosed as *herpes simplex* keratitis (Aversa *et al.* 1995).

3.2.2 - Filter culture technique for non-invasive diagnosis

In this method (Gradus *et al.* 1989), corneal scrapings or tissues were obtained by surgical procedure and were transported in a low osmolar transport solution. Pieces of tissues were then smeared in the centre of a non-nutrient agar plate seeded with *E. coli*. Plates were incubated at 35°C and were examined every 24-48 h. Four of the 7 cases studied gave a positive result within 72 hours, the other 3 were positive in 4-5 days.

Wilhelmus *et al.* (1986) used Calcofluor white (CFW); a fluorescent dye with an affinity for the polysaccharide polymers found in amoeba cysts. They compared calcofluor white with gram and giemsa stain. Their results are shown in Table 1.

Table 1. Comparison of Calcofluor White with other laboratory stains in the diagnosis of *Acanthamoeba sp.* Wilhelmus *et al.* (1986).

| Case | CFW | Gram or Giemsa | detection in culture | Organisms |
|------|-----|-------------------|-------------------------|-----------------------|
| 1 | + | + | 13 days | <i>A. castellanii</i> |
| 2 | + | - | 3 days | <i>A. polyphaga</i> |
| 3 | + | - | 28 days | <i>A. rhysodes</i> |
| 4 | + | - | No growth | No growth |
| 5 | + | - | 1 day | <i>A. castellanii</i> |

All the techniques described above rely on microscopy and are thus open to human error. Misdiagnosis of *Acanthamoeba* keratitis can lead to significant delay in proper diagnosis and treatment.

3.2.3 - Species differentiation using isoenzymes

Isoenzyme typing is a method for identification to the species level of protozoans. Isoenzyme analysis has been useful in *Acanthamoeba*, *Naegleria*, *Giardia*, *Vahlkampfia* and *Entamoeba* (Moss *et al.* 1992). Isoenzymes can be run either on cellulose acetate or starch gels. Cellulose acetate gels have the advantage over starch gels that they can be run faster thus avoiding the need to keep them cold. With some isoenzymes, cellulose acetate gels do not give such good banding patterns and thus reduce differentiation. Starch gels in comparison take longer to run, but are cheaper and give good differentiation.

3.3 - MATERIALS AND METHODS

All *Acanthamoeba* species used in this study were obtained from either CCAP (Culture Collection of Algae and Protozoa), ATCC (American Type Culture Collection) or from Dr. S. Kilvington (Leicester Public Health Laboratories Services) as shown in Table 2.

3.3.1 - Plating Assays

Acanthamoeba spp. were isolated from different environments including soil, tap water, canal, river, sea, wash basins and distilled wash bottles using bacterial seeded non-nutrient agar plates.

Table 2. *Acanthamoeba* tested in this Chapter.

| No | Species | Strain | Source | Pathogenicity tested by CPE assays (Chapter 2) |
|----|--------------------------------|--------------|-------------------------------|--|
| 1 | <i>A. astronyxis</i> | CCAP 1534/1 | Freshwater USA 1944 | Non-pathogen |
| 2 | <i>A. royreba</i> | CCAP 1501/7 | BeWo tissue culture, USA 1977 | Non-pathogen |
| 3 | <i>A. palestinensis</i> | CCAP 1547/1 | Soil, Israel 1933 | Non-pathogen |
| 4 | <i>A. polyphaga</i> | CCAP 1501/3C | Freshwater USA 1967 | Non-pathogen |
| 5 | <i>A. castellanii</i> | ATCC 30234 | Freshwater USA | Pathogen |
| 6 | <i>Acanthamoeba</i> sp. (Ros) | | U.K. keratitis | Pathogen |
| 7 | <i>Acanthamoeba</i> sp. (Nich) | | U.K. keratitis | Pathogen |
| 8 | <i>A. polyphaga</i> | ATCC 30871 | Freshwater, USA | Pathogen |
| 9 | <i>A. griffini</i> | CCAP 1501/4 | Marine, USA 1962 | Unknown |

Non-nutrient agar plates seeded with *K. aerogenes* were prepared as described in Chapter 2. In addition, plates were seeded with *E. coli* also to compare the differences in amoeba growth.

Soil, sand and wash basin environmental isolates were suspended in PAS and centrifuged at 800 x g for 10 min. and 50-100 µl of supernatant were inoculated on the plates. River, canal and sea samples were directly inoculated on to plates. Wash bottle water and tap water (approx. 500 ml) were filtered through a 0.45 µm filter, and these filters were placed directly on to the plates. Plates were examined daily for protozoan growth.

Axenic culturing:

Plate cultures were axenised by removing single cells (using an inverted microscope) and transferred into fresh non-nutrient agar plates seeded with UV (10 min with 254 nm wavelength using Stratalinker™) killed *K. aerogenes*. After 24-48 h a stamp sized agar piece (10 mm²) was removed and incubated in 10 ml of PYG medium in universal bottles. Samples were incubated at 30°C and examined daily for growth of *Acanthamoeba*. For SEM and isoenzyme analyses, *Acanthamoeba* strains were grown in 250 ml conical flasks containing 100 ml of PYG medium at 30°C without shaking as described in Chapter 2.

3.3.2 - Microscopy

Fluorescence Microscopy:

Calcofluor white (CFW) was used to stain *Acanthamoeba* (Wilhelmus *et al.* 1986). Briefly, a suspension of parasites were placed on to slides, air dried and then fixed with methanol for 3-5 min. Several drops of 0.1% (w/v) CFW solution and 0.1% (w/v) Evans blue dissolved in distilled water were added. After 5 min., excess stain was removed and coverslips were applied. Slides were examined by fluorescence microscopy.

3.3.3 - Scanning Electron Microscopy (SEM)

Cells were prepared for SEM using the following protocol: Coverslips were cleaned by soaking in 100% ethanol, wiped with lint free paper and air dried for at least 1 h. A drop of poly-L-lysine (Sigma- 0.1% w/v in dH₂O) was placed on each coverslip, dried overnight, and then rinsed in distilled water. *Acanthamoeba* in PAS were placed on the coverslip, left for 5-10 min. to allow adherence to the coverslips, the coverslips with cells were washed with 2.5% glutaraldehyde at 4°C, and left overnight. The following day, coverslips were washed again with ice cold PAS and left overnight. Samples were finally washed with distilled water at room temperature and left for 30 min. Samples were then dehydrated in a series of acetone and water mixtures from 30% acetone in water to 100% acetone. Samples were finally heated at 36°C, mounted, and observed under SEM.

3.3.4 - Species differentiation using isoenzymes

Enzyme extraction:

Mid log phase *Acanthamoeba* cells (100 ml) were harvested by centrifugation at 800 x g for 10 min. Pelleted cells were resuspended in 40 ml of ice cold PAS and centrifuged again at 800 x g for 10 min. This process was repeated twice. The washed cell pellet was then resuspended in an equal volume of enzyme stabilizer (Stock enzyme stabilizer solution 1000 x conc. [200 mM EDTA, pH 7.0 and 200 mM DTT, once dissolved, 200 mM of ϵ -ACA is added]. The suspension (pelleted cells and enzyme stabilizer) was freeze-thawed 3 x in liquid N₂, transferred to microcentrifuge tubes then centrifuged at 100 x g for 30 min. at 4°C. The supernatant containing enzymes was then beaded. To prepare beads, 15 μ l of enzyme suspension was dropped in a pyrex[®] beaker containing liquid N₂, after freezing, beads were placed in cryotubes for storage.

Cellulose Acetate Gel Electrophoresis (CAGE):

For CAGE, 76 mm x 76 mm Titan[®] III cellulose acetate plates (Helena laboratories) were presoaked in the running buffer overnight prior to use. When used for electrophoresis, plates were placed above a wick and both reservoirs were filled with Tris glycine buffer (TG buffer, pH 8.0 appendix 1). Using an applicator, extracts were applied one or more times to the same position on the plate (acetate side) depending on the enzyme (for high activity enzyme i.e., PGI one application was sufficient and for low activity enzyme, i.e., TPI, 3 or 4 applications were required). The plates were placed acetate side down on the wicks in the electrophoresis tank. The loading zone was positioned at the cathode end of the tank for all enzymes. Enzyme activity was assayed

for Aldehyde oxidase (AO), Xanthine dehydrogenase (XDH), Isocitrate dehydrogenase (IDH), Phosphoglucose isomerase (PGI), Aldehyde dehydrogenase (ADH), Esterases (ES), Glucose-6-phosphate dehydrogenase (G6PD) and Malate dehydrogenase (MDH). Electrophoresis was performed at room temperature and gels were run at 200 volts for 25 min. Gel staining stocks of the chemicals were made and kept at 4°C for up to 1 month. Detailed stain recipes are listed in the appendix 6. Gels were viewed and photographed under either UV or white light.

Starch Gel Electrophoresis (SGE):

For starch gel electrophoresis, 140 mm x 80 mm glass plates were used. The starch gel was prepared by adding 2.8 g of hydrolysed potato starch (Sigma laboratories) in 40 ml of the appropriate buffer (Table 3) and heated to boiling, degassed and quickly poured on to the plate. The plate was placed at 4°C approx. 20-30 min. Using a template, slots were made, one third down the gel from the cathode. Sterile sewing threads (3 mm in diameter) were dipped in the extract and transferred to slots using fine forceps. The forceps were washed in appropriate buffer after loading each sample. The plate was transferred to the electrophoresis tank (cooling block) and wicks were placed over the starch gel making sure that one end of each wick was in the buffer. Electrophoresis was carried out as appropriate for each enzyme (Table 3).

Starch gels were used to look at the activity of Malate dehydrogenase (MDH), Glucosephospho-isomerase (GPI), Phosphoglucumutase (PGM), Prolidase (PEPD), Esterases (ES) and 6-phosphogluco-dehydrogenase (6PGD) enzymes. Stains solutions were prepared in 1 % agar (see appendix 7) and poured over the

Table 3. Showing conditions used for starch gel electrophoresis.

| Enzyme | Tank Buffer | Gel Buffer (dilution-H ₂ O) | Time | Volts |
|----------------------------------|--|--|-------|-------|
| 6-Phosphogluconate dehydrogenase | 0.1M TMEB pH7.4 | 1/5 | 2.5 h | 300V |
| Esterases | 0.1M TMEB pH7.4 | 1/5 | 2.5 h | 300V |
| Glucose phosphoisomerase | 0.1M TMEB pH7.4 | 1/5 | 2.5 h | 300V |
| Phosphoglucomutase | 0.1M TBEM pH9.0 | 1/5 | 2.5 h | 250V |
| Malate dehydrogenase | 0.1M STEC pH9.5 | 2/3 | 4.0 h | 120V |
| Prolidase | 0.15M tris/0.1MNaH ₂ PO ₄ .2H ₂ O pH8.2 | 1/10 | 2.0 h | 300V |

Stains solutions were prepared in 1% agar (see appendix 7) and poured over the starch gel, left to set for 2-3 min. and then transferred to a 37°C incubator to develop. The gels were viewed and photographed under either UV or white light.

3.4 - RESULTS

3.4.1 - Plating assays

Acanthamoeba were isolated (characteristic acanthopodia) from samples on non-nutrient agar plates overlaid with *K. aerogenes* (Fig. 1), while plates overlaid with *E. coli* did not support the growth of *Acanthamoeba*.

Environmental isolates yielded a wide distribution of *Acanthamoeba*.

Acanthamoeba were isolated from all environments (Table 4) except tap water. Among 5 samples from tap water and wash basins, 2 were positive for *Acanthamoeba* sp. (Table 4). Interestingly *Acanthamoeba* were also isolated from a distilled H₂O-wash bottle in my lab.

3.4.2 - CFW staining

Staining of *Acanthamoeba* using CFW stain showed significant fluorescence both in trophozoites and cysts of all *Acanthamoeba* species / strains tested (Fig. 2 a, b). The specificity of this method was assessed by staining other organisms such as bacteria, fungi, and other free-living amoeba. All cells tested showed significant fluorescence with CFW (Table 5).

Fig. 1. *Acanthamoeba* trophozoite (characteristic acanthopodia shown by →) and cyst (shown by •→) on a non-nutrient agar plate seeded with *K. aerogenes* (x400).

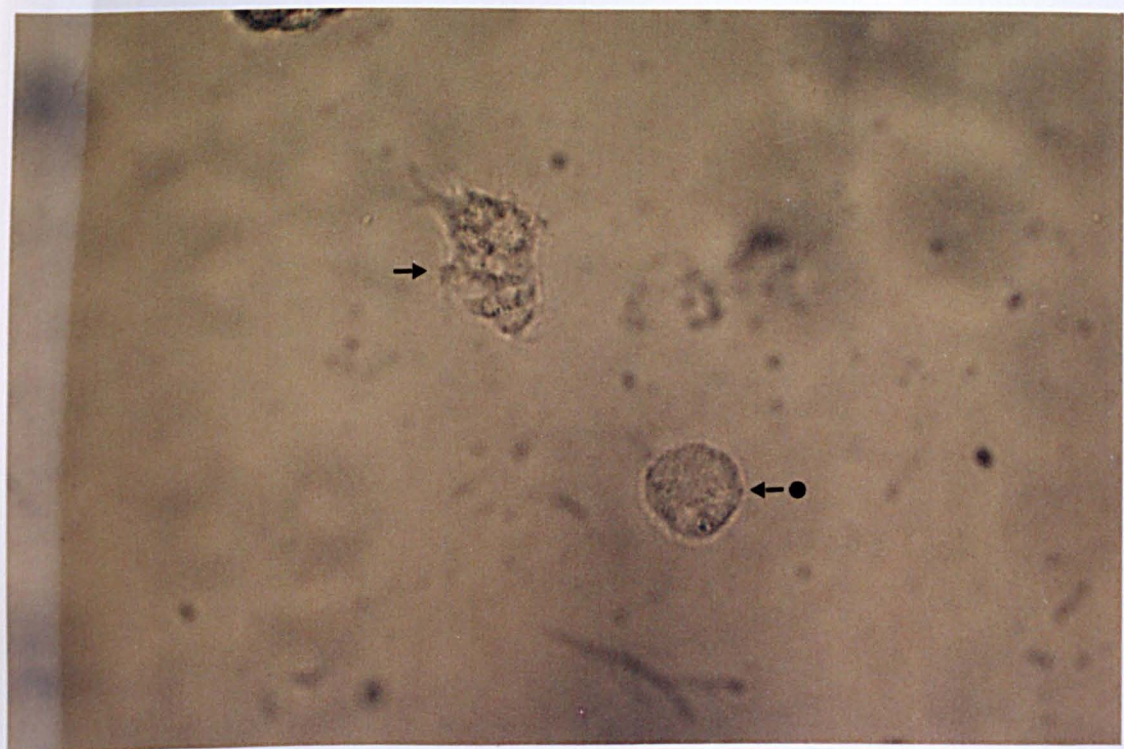
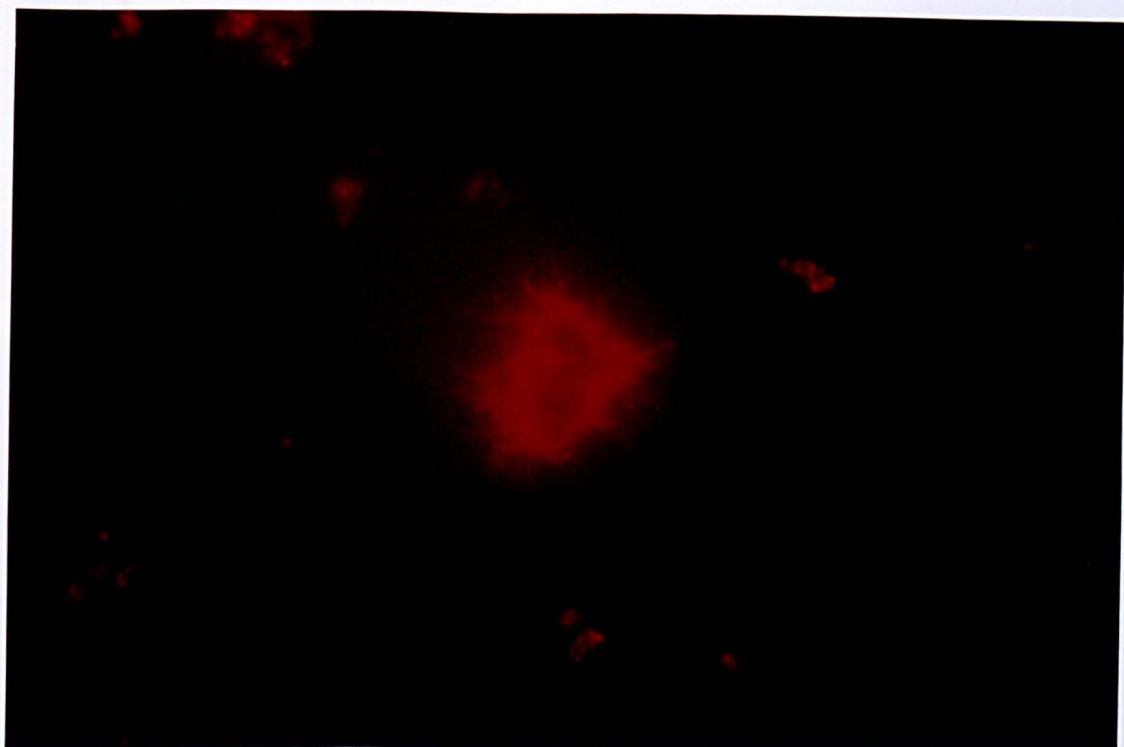


Table 4. Environmentally isolated *Acanthamoeba*.

| Source | Organism |
|---|-----------------------------|
| Leven Canal, Beverley, UK | <i>Acanthamoeba</i> sp. 2/2 |
| River Hull, Hull, UK | 2/2 |
| Humber Estuary, Hull, UK | 2/2 |
| Soil, Car Park, Univ. Hull, Hull, UK | 2/2 |
| Soil, Pearson Park, Hull, UK | 3/3 |
| Soil, Near Gym, Univ. Hull, Hull, UK | 2/2 |
| Soil, Near Admin. Building, Univ. Hull, Hull, UK | 2/2 |
| Soil, Cottingham Road, Hull, UK | 2/2 |
| Distilled H ₂ O wash bottle, Lab 303, Dept. of Biological Sciences, Univ. Hull, Hull, UK | 1/1 |
| Wash basin waste (2 samples from different basins) | 2/2 |
| Tap water (3 samples from different sources) | 0/3 |

Fig. 2. Calcofluor white staining of *Acanthamoeba* a) trophozoite and b) cyst (x400).



a



b

Table 5. Showing the CFW fluorescence with other amoeba and bacterial cells.

| Organism | Fluorescence with CFW |
|-----------------------------------|--------------------------|
| <i>Acanthamoeba palestinensis</i> | + |
| <i>A. castellanii</i> | + |
| <i>A. polyphaga</i> | + |
| <i>A. astronyxis</i> | + |
| <i>A. griffinii</i> | + |
| <i>Acanthamoeba</i> sp. (ros) | + |
| <i>Acanthamoeba</i> sp. (nich) | + |

| | |
|---------------------------------|---|
| <i>Hartmannella</i> sp.* | + |
| <i>Candida albicans</i> * | + |
| <i>Escherichia coli</i> * | + |
| <i>Nisseria</i> sp.* | + |
| <i>Pseudomonas aeruginosa</i> * | + |
| <i>Klebsiella aerogenes</i> * | + |

*These organisms are common causes of eye infection.

3.4.3 – S. E. M

Scanning electron microscopy showed the presence of characteristic acanthopodia (Fig. 3). The tested non-pathogenic strains of *Acanthamoeba* have <20 acanthopodia while the pathogenic of *Acanthamoeba* have >100 acanthopodia per cell (Fig. 4 & 3 respectively). In one non-pathogenic *Acanthamoeba*, (*A. royreba*) >100 acanthopodia per cell were observed (data not shown).

3.4.4 - Species differentiation using isoenzymes

Acanthamoeba species / strains can be differentiated using isoenzyme analysis on cellulose acetate gels. Xanthine dehydrogenase shows a clear distinction between *A. astronyxis* and other *Acanthamoeba* tested. *Acanthamoeba castellanii* and *Acanthamoeba* sp. (isolate, ros) could not be differentiated using this enzyme (Fig. 5). The banding pattern for AO in *A. astronyxis* was again quite different from all other *Acanthamoeba* species tested (Fig. 6). Isoenzyme analysis on starch gel was also useful. The MDH pattern was able to differentiate among the *Acanthamoeba* species tested except that a similar enzyme pattern appeared for *A. castellanii* and *Acanthamoeba* sp. (ros), (Fig. 7). Malate dehydrogenase gave different banding patterns for *A. polyphaga* ATCC 30871 and *A. polyphaga* CCAP 1501/3C. Aminoacyl-proline peptidase (prolidase, EC 3.4.13.9) gave no differentiation between *A. polyphaga* ATCC 30871 and *A. polyphaga* CCAP 1501/3C but all other tested species of *Acanthamoeba* could be differentiated (Fig. 8).

Fig. 3. Scanning electron micrograph of *A. castellanii* (pathogenic).

L= SE1

EHT= 15.0 kV

WD= 8

mm

5.00µm

PHOTO= 4

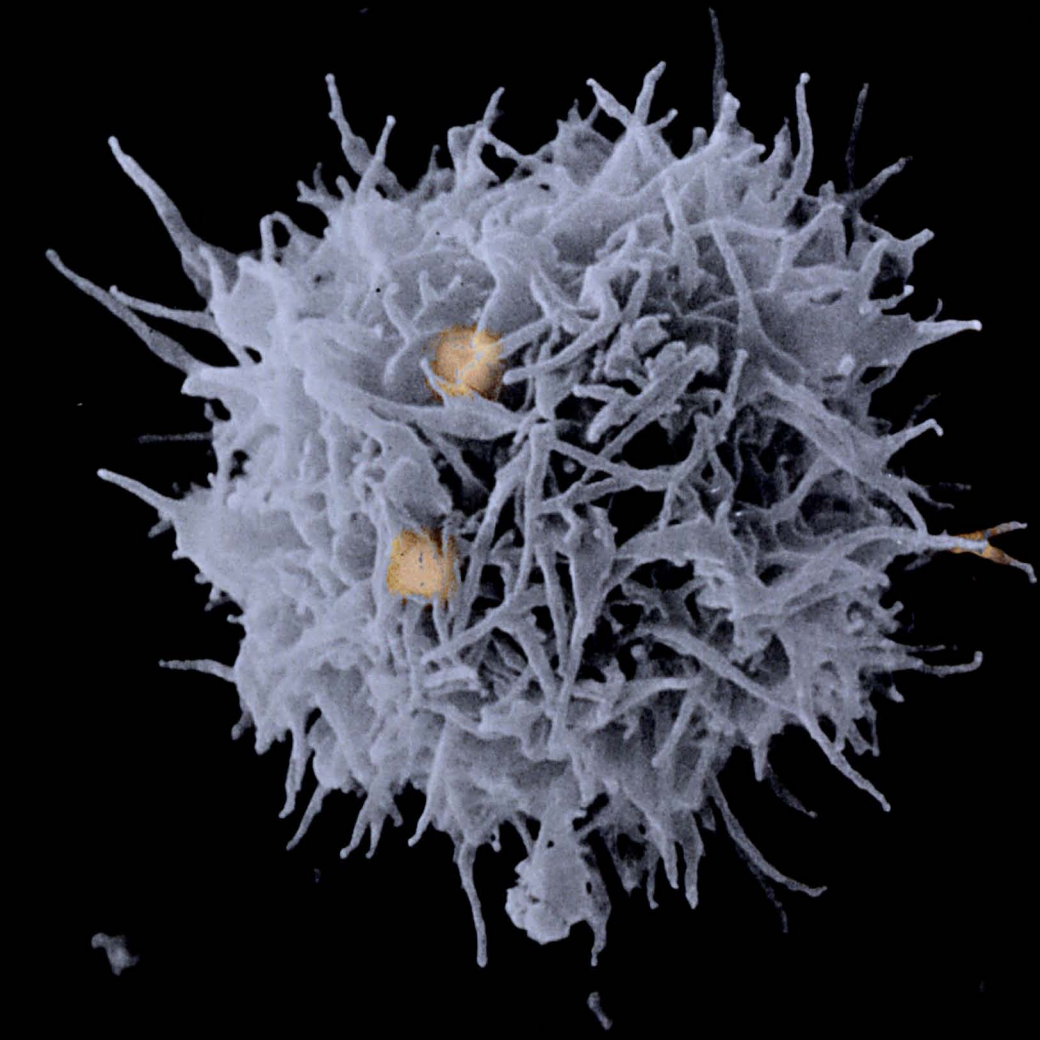


Fig. 4. Scanning electron micrograph of *A. griffini* (non-pathogenic).

PHOTO= 11

L= SE1 EHT= 15.0 KV WD= 5 mm
5.00µm

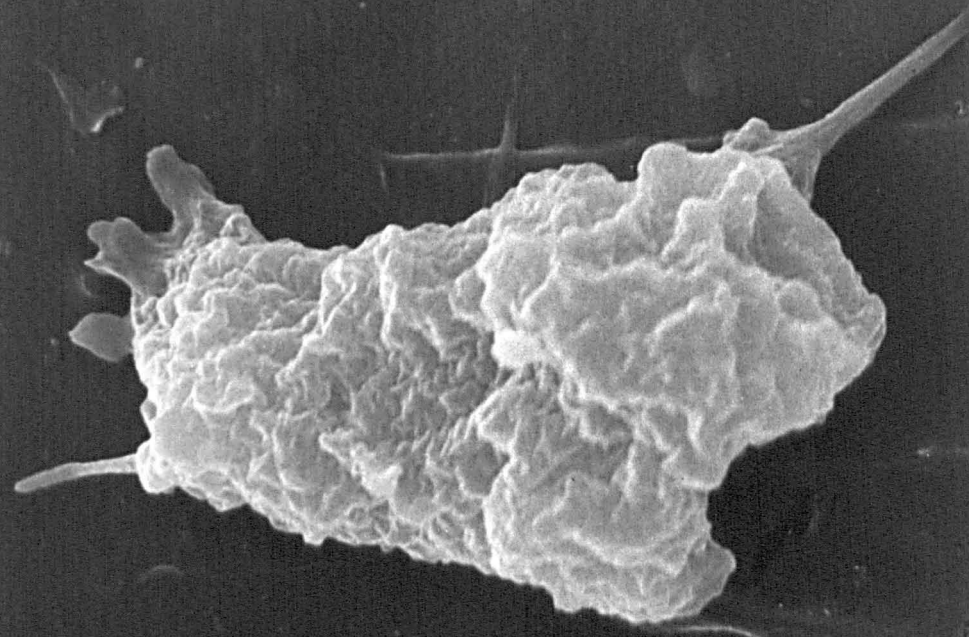


Fig. 5. Xanthine dehydrogenase (XDH) showing isoenzyme banding patterns of *Acanthamoeba* sp. using CAGE. Lane 1, *A. astronyxis*; lane 2, *A. griffini*; lane 3, *A. palestinensis*; lane 4, *A. polyphaga* CCAP 1501/3C; lane 5, *A. castellanii*; lane 6, *Acanthamoeba* sp. (ros); lane 7, *Acanthamoeba* sp. (nich); lane 8, *Acanthamoeba* sp. (shi) and lane 9 is *A. polyphaga* ATCC 30871.

1 2 3 4 5 6 7 8

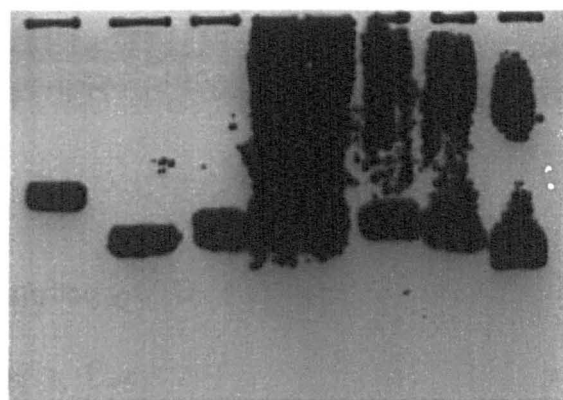


Fig. 6. Aldehyde oxidase (AO) showing isoenzyme banding patterns of *Acanthamoeba* sp. using CAGE. Lane 1, *A. astronyxis*; lane 2, *A. griffini*; lane 3, *A. palestinensis*; lane 4, *A. polyphaga* CCAP 1501/3C; lane 5, *A. castellanii*; lane 6, *Acanthamoeba* sp. (ros); lane 7, *Acanthamoeba* sp. (nich); lane 8, *Acanthamoeba* sp. (shi) and lane 9 is *A. polyphaga* ATCC 30871.

1 2 3 4 5 6 7 8 9

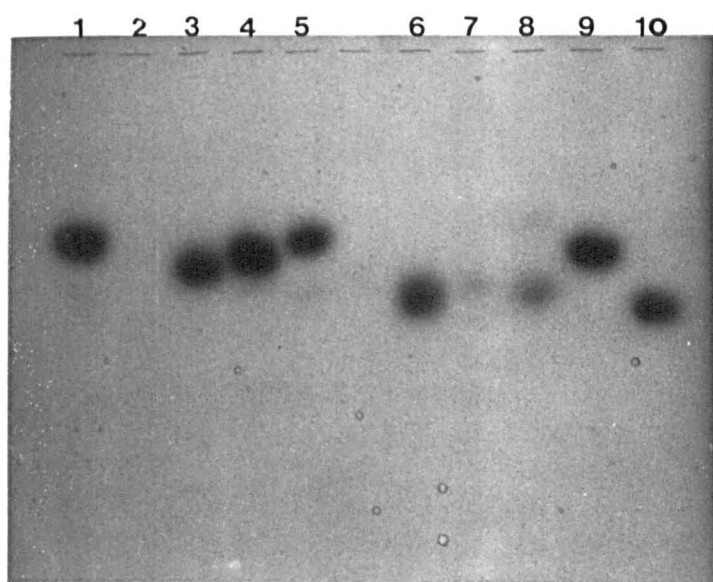


Fig. 7. Malate dehydrogenase (MDH) showing isoenzyme banding patterns of *Acanthamoeba* sp. using SGE. Lane 1, *A. astronyxis*; lane 2, *A. griffini*; lane 3, *A. palestinensis*; lane 4, *A. polyphaga* CCAP 1501/3C; lane 5, *A. castellanii*; lane 6, *Acanthamoeba* sp. (ros); lane 7, *Acanthamoeba* sp. (nich); lane 8, *Acanthamoeba* sp. (shi) and lane 9 is *A. polyphaga* ATCC 30871.

1 2 3 4 5 6 7 8 9



Fig. 8. Prolidase (PEPD) showing isoenzyme banding patterns of *Acanthamoeba* sp. using SGE. Lane 1, *Acanthamoeba* sp. (keh); lane 2, *A. astronyxis*; lane 3, *A. griffini*; lane 4, *A. palestinensis*; lane 5, *A. polyphaga* CCAP 1501/3C; lane 6, *A. castellanii*; lane 7, *Acanthamoeba* sp. (ros); lane 8, *Acanthamoeba* sp. (nich); lane 9, *Acanthamoeba* sp. (shi) and lane 10 is *A. polyphaga* ATCC 30871.



3.5 - DISCUSSION

We were able to successfully isolate *Acanthamoeba* on non-nutrient agar plates from a variety of sources (Table 4). Also since non-nutrient agar plates are used bacterial or fungal growth is minimized. Problems with this method are that only viable / culturable cells can be identified.

While PCR based assays are more sensitive, specific and can detect non-culturable organisms, however, these assays are more expensive and are not very common in developing countries. Plating assay can be potentially used in clinical applications as they are cheaper and simple.

Environmental isolates showed the wide distribution of *Acanthamoeba* spp. These data were obtained from a very small number of samples, however, these data suggest that there is a need for further epidemiological studies of *Acanthamoeba* differentiation to give a rationale for people at risk.

Scanning electron microscopy clearly shows the morphological characters of *Acanthamoeba* such as acanthopodia. Scanning electron microscopy may thus help in the differentiation of pathogenic and non-pathogenic *Acanthamoeba* however, this technique requires a large number of cells, expensive, labour intensive and thus limited in its clinical use. Also, this technique can not be used in *Acanthamoeba* species differentiation.

Species differentiation using isoenzyme analysis can provide information about the tested *Acanthamoeba* to some extent. Cellulose acetate gel electrophoresis showed the best differentiation using XDH and AO enzymes. The fact that *A. castellanii* and *Acanthamoeba* sp. (ros) could not be differentiated in all tested enzymes suggest that

Acanthamoeba sp. (ros) may well be *A. castellanii*. Starch gel electrophoresis was also useful in differentiating *Acanthamoeba* species / strains. *A. castellanii* and *Acanthamoeba* sp. (ros) showed the same banding pattern thus correlating the data observed in CAGE assays. Interestingly, two *A. polyphaga* strains (*A. polyphaga* ATCC 30871 & *A. polyphaga* CCAP 1501/3c) also showed different bands for the MDH enzyme suggesting heterogeneity within the same species. Again the drawback to this technique is that it requires a large number of parasites which indirectly requires isolation of *Acanthamoeba* and axenic culturing which could take several weeks.

Also, in a study by De Jonckheere (1983), 30 axenically grown strains belonging to 15 different species of *Acanthamoeba* were investigated for isoenzyme patterns. In our results different banding patterns were found within the same species. This data suggest isoenzyme patterns must not be used as the sole criterion to establish a new species nor should a morphological analysis be used alone. In another study by De Jonckheere (1987) *A. pustulosa* and *A. palestinensis* were found to have identical isoenzyme patterns although previously Costas *et al.* (1984), reported distinct differences in the acid phosphatase and esterase isoenzyme profiles of the two species. Thus a variety of different criteria should be investigated to describe new species such as isoenzyme electrophoresis, RFLP's, rDNA sequencing and banding patterns of electrophoretically separated proteins.

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Chapter 4

Molecular Approaches for Detection and Speciation of *Acanthamoeba* spp.

4.1 - SUMMARY

PCR based assays using primers designed by Vodkin *et al.* (1992) were developed as a method for the rapid and specific detection of *Acanthamoeba* at the genus level, ACARNA 1383-1655. These amplify ribosomal DNA and give a product of 272 bp. Another primer pair was designed to amplify a 284 bp product from 18S rDNA of *Acanthamoeba griffini*. An additional primer set (NA1L-R) was used to amplify a 910-1170 bp *Acanthamoeba*-specific rDNA product. Restriction enzyme analyses were performed on this product to differentiate *Acanthamoeba* spp. using a variety of restriction enzymes. A simple and rapid DNA extraction method using insta-gene matrix (chelex) was developed for use directly with the given specimen without the need for prior cultivation of *Acanthamoeba* parasites. Since there is no DNA precipitation or washes involved, thus loss of DNA is minimized. Ribosomal DNA sequencing was performed using fluorescently labelled NA1 L-R primers on the ALFexpress™ (Pharmacia Biotech.) automated sequencer .

Genus-specific *Acanthamoeba* PCR amplification was observed using both ACARNA 1383-1655 as well as NA1 L-R primers. Using this new DNA extraction method, it was possible to amplify *Acanthamoeba* DNA from as few as five cells but only with ACARNA primers. Restriction enzyme analyses showed variation among all *Acanthamoeba* spp. tested. Ribosomal DNA sequences were generated for 14 different *Acanthamoeba* species / strains. After comparison with the available sequences in Genbank our data showed some correlation although results were polymorphic among the strains assigned to the same species. A consensus parsimony tree was generated which showed 2 subclusters with all the pathogenic strains grouping together. Also 2 sequence

types of morphological group 1 (*A. astronyxis* & *A. comandoni*) showed the earliest divergence in the genus *Acanthamoeba* (based on comparison of the most conserved region of the alignment to *Hartmannella*, a close relative to *Acanthamoeba*). This agrees with the data from Stothard *et al.* (1998).

4.2 - INTRODUCTION

4.2.1 - Polymerase chain reaction (PCR)

The use of PCR as a diagnostic tool has increased during the last 10 years because it is a sensitive method and amplification can be achieved from a single cell (Abbaszadegan *et al.* 1991). This method is increasingly being used to identify parasitic organisms however the scarcity of detailed molecular analysis of many parasites limits its use. Usually PCR can be performed within 2-3 hours, and samples can be directly assayed without the prior need of cultivation making it a rapid assay. To devise a PCR based assay, a target sequence is required. If a genus specific assay is developed, the target sequence should be highly conserved (such as ribosomal DNA sequence) to allow primers to be designed from non-polymorphic regions. Following the identification of a conserved sequence, the next step is to design primers from that sequence and to optimize the conditions for PCR (conc. of magnesium chloride, nucleotide triphosphates and primer concentration). Finally, it is important to select the proper temperature for annealing and the duration of time for each step.

PCR-based assays have been successfully employed for a number of protozoans including *Plasmodium* (Waters *et al.* 1989; Mathiopoulos *et al.* 1993), *Leishmania* (Uliana *et al.* 1991), *Toxoplasma* (Cazenave *et al.* 1991; Guay *et al.* 1993; MacPherson *et*

al. 1993; Verhofstede *et al.* 1993), *Entamoeba histolytica* (Garfink *et al.* 1989; Bracha *et al.* 1990), *Naegleria* (Sparagano *et al.* 1993), *Cryptosporidium parvum* (Laxer *et al.* 1991; Laxer *et al.* 1992) and *Giardia* (Mahbubani *et al.* 1991; Mahbubani *et al.* 1992). PCR has also been used for the detection of *Acanthamoeba* (Vodkin *et al.* 1992) but needed to be further refined to make it relevant for clinical purposes.

4.2.2 - Restriction enzyme analysis and DNA sequencing

Analysis of parasite species and strains can be made (among other techniques) by examination of the patterns of digestion of their DNA by specific restriction endonucleases or by DNA sequencing. The restriction banding patterns depends on the nucleotide sequence recognized by restriction enzymes. Genetic polymorphism resulting even from a single base pair change can yield different electrophoretic patterns if this change creates or eliminates a restriction enzyme site. In DNA sequencing a change from a single base pair may be detected even in the absence of any phenotype change or restriction site involvement. Both analyses therefore offer a powerful and direct probe into the parasite genotype, quite distinct from other methods (Morphological study, isoenzyme analysis and protein pattern analysis). However, DNA sequencing is more sensitive as more genetic characters are surveyed and thus serves as a sensitive marker of genetic change and is therefore more useful in identification of species and strains of the parasites. Although species identification by cyst morphology has been used in the genus *Acanthamoeba* (Page, 1967; Pussard *et al.* 1977; Singh *et al.* 1979), high morphological variability within one species (Page, 1988) limited the utility of morphology alone as a taxonomic tool (Visvesvara, 1991). Kong *et al.* (1995); Chung *et al.* (1996), used isoelectric focusing and

mitochondrial DNA-RFLP for the species differentiation of *Acanthamoeba*, but the results appeared highly polymorphic among strains assigned to the same species. Similar results were obtained by Bogler *et al.* (1983); Yagita *et al.* (1990). The high intraspecific heterogeneity, and the fact that both methods require considerable number of trophozoites are also shortcomings. Therefore, comparisons of highly conserved sequences which have a central function and therefore change only slowly during evolution can reveal phylogenetic relationships between organisms (Sogin *et al.* 1989). The small subunit ribosomal RNAs (ssu rRNAs) are recognized as being particularly well suited for estimating phylogenetic relationships between even the most divergent taxa (Woese 1987). However the generation of sequence data is labour intensive and expensive. In order to analyse numerous strains, less expensive and easier methods need to be developed. In this study new refined and reproducible methods have been applied to aid our understanding in some of the unresolved strains of *Acanthamoeba*.

4.3 - MATERIALS AND METHODS

4.3.1 - PCR

DNA extraction:

Total DNA was extracted from *Acanthamoeba* using the following protocol: mid log phase *Acanthamoeba* were centrifuged at 800 x g for 5 min, washed in PAS three times, and the pellet was resuspended in 1 ml of PAS. To lyse the cells, the pellet was resuspended in 2 K buffer (100 mM KCl, 40 mM Tris, 5 mM MgCl₂, 1% (w/v) Tween and 200 µg/ml Proteinase K) and incubated at 55°C for 1 h followed by incubation

at 99.9°C for 10 min to denature proteinase K. Lysed cells were centrifuged again and the supernatant was used as DNA template. Another DNA extraction method was developed to directly use with clinical specimen as follows:

Various number of *Acanthamoeba* cells including 5, 10, and 50 were diluted in 0.5 ml microcentrifuge tube in different volumes. Insta gene matrix (chelex, chelating agent, Bio-Rad Labs.) was vortexed vigourously and 30 µl of chelex was added to each tube. It is important that chelex is vortexed each time as beads settle down quickly. Tubes were incubated at 56°C for 20 min and then at 99.9°C for 10 min in a solid block thermocycler (Genetic Research Instrumentation Ltd.). Tubes were pulse spun in a microcentrifuge and supernatant was removed carefully. The supernatant (5-10 µl) was used as DNA template.

Primers:

For this study a variety of primers were used including a range of published sequences shown in Table 1. However we have also designed primers (Primer design, web site- <http://www.bmb.psu.edu/597a/students/bab10/links.html>) using rDNA sequences of *Acanthamoeba* spp. from GenBank.

Table 1. *Acanthamoeba* specific primers.

| Template | Sequence | Identifies | Expected product | Ref. |
|--|--|---------------------|------------------|------------------------------|
| 18S rDNA ACARNA. for 1383 ACARNA. rev 1655 | TCCCCTAGCAGCTTGTG GTTAAGGTCTCGTTCGTTA | Genus | 272 bp | Vodkin <i>et al.</i> 1992 |
| 18S rDNA NA1L NA1R | TTTGAATTCGCTCCAATAGCGTATATTAA TTTGAATTCAGAAAGAGCTATCAATCTGT | Genus | 910-1170 bp | Gunderson <i>et al.</i> 1986 |
| 18S rDNA Ac6/10. for Ac6/210. rev | GGCGAAGAACCTGCATCAGC CAAACCAACTCCCGAGCCA | Pathogenic | 195 bp | Daniel <i>et al.</i> 1997 |
| 18S rDNA AGL AGR | ATTTTCAGTTGGTTTTGCCG GTGAGCGACAACAAAGACGA | <i>A. griffinii</i> | 284 bp | Our project |

Method for PCR analysis:

PCR was performed in a 50µl volume containing 1.25 U Taq polymerase (Pharmacia Biotech), 0.1-1.0 ng DNA (measured using Gene Quant, Pharmacia biotech), 3mM MgCl₂ and 0.5µM primer. In all PCR assays, amplified DNA was electrophoresed on a 2% agarose gel in 1 x TAE buffer {For 50 x TAE, (2 M Tris, 0.05 M EDTA, 5.7% Glacial acetic acid)} adjusted to pH 8.0. Gels were stained using 0.5 µg / ml of ethidium bromide and visualized under UV illumination. PCR cycles used for the genus specific primer ACARNA 1383-1655 are as follows.

| | | | | |
|--------|----|------|-------|--|
| Step 1 | | 95°C | 2 min | 1 cycle |
| Step 2 | a) | 94°C | 1 min | No. of cycles used for step 2 is 40 |
| | b) | 47°C | 1 min | |
| | c) | 72°C | 1 min | |
| Step 3 | | 72°C | 5 min | 1 cycle |

Specificity and sensitivity:

The specificity of primers was determined using DNA from *Escherichia coli*, *Giardia*, *Daphnia*, *Saccharomyces* and human corneal epithelial cells (Obtained from Dr. Ian Adams, Dept of Biological Science, University of Hull, Hull, UK) using the same PCR cycles. The sensitivity of primers was also tested by extracting DNA from varying numbers of *Acanthamoeba* cells using newly developed DNA extraction method.

Primers designed to differentiate between pathogenic and non-pathogenic species of *Acanthamoeba* were also tested. According to Daniel *et al.* (1997), the expected 195

bp band should only amplify DNA from pathogenic *Acanthamoeba*. The same PCR conditions were used as described for ACARNA 1383-1655 but the annealing temperature was increased to 64°C from 47°C.

A. griffini specific primers (AGL-R) were designed using the 18S rDNA sequence of *A. griffini* from GenBank. Amplification was performed using the same PCR cycles as described above but with an annealing temperature of 50°C.

4.3.2 - Restriction enzyme analysis

For restriction enzyme analysis, DNA from all *Acanthamoeba* spp. was amplified using primers NA1L-R and the conditions described above but with 4 mM MgCl₂. PCR cycles used are as follows.

| | | | | |
|--------|----|------|--------|--|
| Step 1 | | 95°C | 2 min | 1 cycle |
| Step 2 | a) | 94°C | 1 min | Number of cycles used for this step is 30 |
| | b) | 55°C | 1 min | |
| | c) | 72°C | 2 min | |
| Step 3 | | 72°C | 10 min | 1 cycle |

Amplified DNA was extracted from agarose gels using glassmilk (NBL Gene Sciences), using following protocol: Briefly 100 µl of NaI solution (90.8 g NaI; 1.5 g Na₂SO₃ dissolved in 100 ml of distilled H₂O) were added to agarose piece containing amplified DNA in a 0.5 ml microcentrifuge tube and incubated at 55°C for 5 min. After vortexing 10 µl of glass milk was added to the DNA-NaI mixture and incubated on ice for 5 min. Glass milk was pelleted by pulse spin centrifugation in a microcentrifuge and the

supernatant discarded. The pellet was washed twice with New buffer (50 % ethanol, 0.1 M NaCl, 10 mM Tris HCl, (pH 7.5), 1 mM EDTA). Finally 10-20 µl of distilled H₂O was added to the pellet and incubated at 55°C for 2-3 min and pulse spun again for 30 sec in a microcentrifuge. Supernatant was carefully removed and used for restriction enzyme analysis. The following restriction enzymes were used: *AluI*, *CfoI*, *CspI*, *DdeI*, *EcoRI*, *HinfI*, *HaeIII*, *HhaI*, *HindIII*, *SmaI*, and *TaqI*. All enzymes were incubated with amplified DNA for 2h at 37°C. A wide range of environmentally isolated *Acanthamoeba* (15 in total, Table 2) were tested for restriction enzyme analyses. Known species of *Acanthamoeba* were used to compare the restriction banding patterns (Table 3).

4.3.3 - DNA sequencing and Phylogenetic analysis

DNA sequencing was performed using cyanin (Cy5) labelled NA1L-R primers (Pharmacia Biotech.), and cycle sequencing. Briefly, PCR products (double stranded) were mixed with a single primer, dNTPs, ddNTPs and a thermostable DNA polymerase (Amersham, Pharmacia Biotech.). Linear amplification was performed in a thermal cycler for 20 cycles. Each cycle consists of annealing, extension and thermal denaturation as in a normal PCR. Sequencing products were separated on polyacrylamide gel followed by detection with automated ALFexpress™ sequencer. Nucleotide sequences were aligned with the assistance of the program CLUSTAL (Higgins *et al.* 1998) and manually edited by ESEE.

Table 2. Environmental and clinical isolates of *Acanthamoeba* tested in this study.

+ represents presence of *Acanthamoeba*.

| Source | Organism |
|---|-----------------------------|
| Leven Canal, Beverley, UK | <i>Acanthamoeba</i> sp. (+) |
| River Hull, Hull, UK | + |
| Estuary, Hull, UK | + |
| Soil, Car Park, Univ. Hull, Hull, UK | + |
| Soil, Pearson Park, Hull, UK | + |
| Wash basin waste, Hull, UK | + |
| Soil, Near Gym, Univ. Hull, Hull, UK | + |
| Soil, Near Admin. Building, Univ. Hull, Hull, UK | + |
| Soil, Cottingham Road, Hull, UK | + |
| Distilled H ₂ O wash bottle, Lab 303, Dept. of Biological Sciences, Univ. Hull, Hull, UK | + |
| Tap water, Leicester, UK | + |
| Infected eye (Ros), Phublic Health Labs. (PHLS), Leicester, UK | + |
| Infected eye (Nich), PHLS, Leicester, UK | + |
| Infected eye (Esbc4), PHLS, Leicester, UK | + |
| Infected eye (Curton), PHLS, Leicester, UK | + |
| Infected eye (Shi), PHLS, Leicester, UK | + |

Table 3. Known species of *Acanthamoeba* used in this chapter.

| No | Species | Strain |
|----|-------------------------|--------------|
| 1 | <i>A. astronyxis</i> | CCAP 1534/1 |
| 2 | <i>A. royreba</i> | CCAP 1501/7 |
| 3 | <i>A. palestinensis</i> | CCAP 1547/1 |
| 4 | <i>A. polyphaga</i> | ATCC 30871 |
| 5 | <i>A. polyphaga</i> | CCAP 1501/3C |
| 6 | <i>A. griffini</i> | CCAP 1501/4 |
| 7 | <i>A. comandoni</i> | CCAP 1501/5 |
| 8 | <i>A. castellanii</i> | ATCC 30234 |

(EyeBall Sequence Editor, Cabot *et al.* 1989) and MacClade (Madisson *et al.* 1992). One master alignment was made and used for all further phylogenetic reconstructions. Regions of ambiguous alignment were removed from the master alignment. Once aligned, nucleotide sequences were compared. Phylogenetic analyses were performed using PAUP package (Ver. 4.0b, Swofford 1998). *Hartmannella*, the closest relative to *Acanthamoeba* was used as the outgroup. Alignment, editing and phylogenetic analyses were performed with the help of Dr. David Lunt (Dept of Biological Science, University of Hull, Hull, UK)

4.4 - RESULTS

4.4.1 - Polymerase chain reaction

The primers ACARNA 1383-1655 gave a single band of 272 bp length from all species / isolates of *Acanthamoeba* tested (Fig. 1). The DNA from up to 5 cells could be detected with ACARNA 1383-1655 primer pair using newly developed DNA extraction method (Fig. 2).

Primers pair (Ac6 10-210) was used to amplify an internal transcribed spacer region from pathogenic strains of *Acanthamoeba*, gave a discrete band of 195 bp length with pathogenic species / strains of *Acanthamoeba* (Fig. 3). AGL-R primers specifically amplified DNA from *A. griffini* but did not amplify any other *Acanthamoeba* species tested (Fig. 4).

Fig. 1. Amplification of 18S rDNA of *Acanthamoeba* sp. using primers ACARNA 1383-1655 for genus specific identification. Lane 1, Negative control; lane 2, *Acanthamoeba* sp. (Canal); lane 3, *Acanthamoeba* sp. (River Hull); lane 4, *A. palestinensis*; lane 5, *A. polyphaga* CCAP 1501/3C; lane 6, *A. polyphaga* ATCC 30871; lane 7, *A. castellanii*; lane 8, *A. comandoni*; lane 9, *A. royreba*; lane 10, *Acanthamoeba* sp. (Ros); lane 11, *Acanthamoeba* sp. (Shi); lane 12, *Acanthamoeba* sp. (Nich); lane 13, *Acanthamoeba* sp. (Keh); lane 14, *Acanthamoeba* sp. (Leic/tap); lane 15, *Acanthamoeba* sp. (Wash bottle); lane 16, *Acanthamoeba* sp. (Soil, Pearson Park); lane 17, *A. astronyxis*; lane 18, *A. griffini*; lane 19, *Acanthamoeba* sp. (Estuary) and lane 20 is 100 bp DNA ladder.

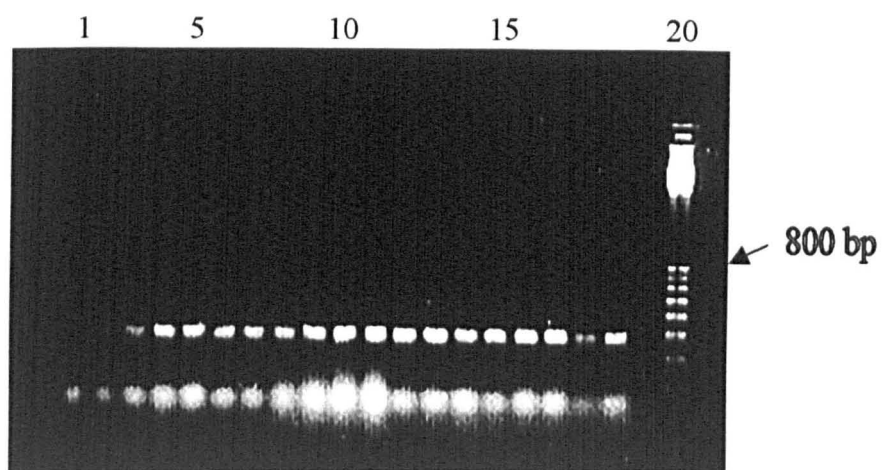


Fig. 2. Showing sensitivity of primers ACARNA 1383-1655. Lane 1, Negative control; lane 2, 50 cells; lane 3, 10 cells; lane 4, 5 cells; lane 5, 1000 cells and lane 12 is 100 bp ladder. Lane 6-11, DNA extraction using distilled H₂O.

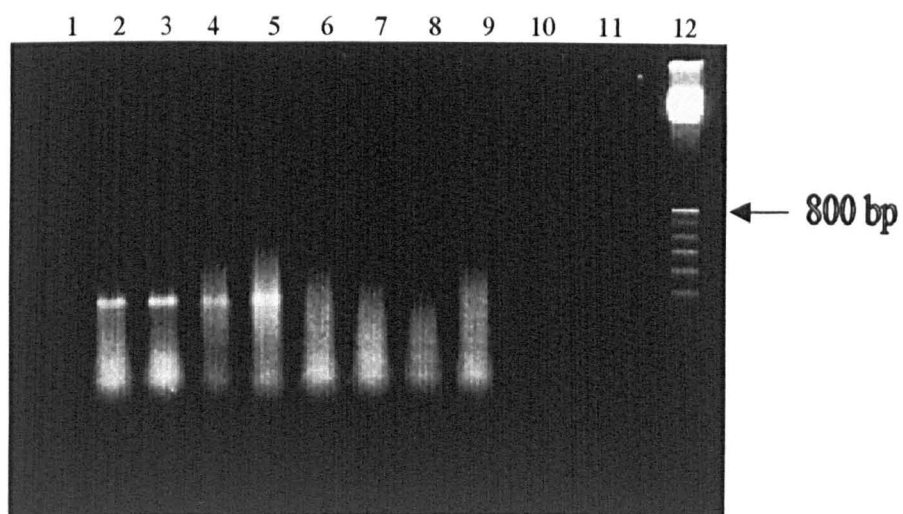


Fig. 3. Amplification of pathogenic species of *Acanthamoeba* using primers Ac6 10-210. Lane 1, 100 bp DNA ladder; lane 2, *A. polyphaga* ATCC 30871; lane 3, *A. castellanii*; lane 4, *Acanthamoeba* sp. (Shi); lane 5, *Acanthamoeba* sp. (Nich); lane 6, *A. palestinensis*; lane 7, *A. comandoni*; lane 8, *A. astronyxis*; lane 9, *A. polyphaga* CCAP 1501/3c; lane 10 is negative control

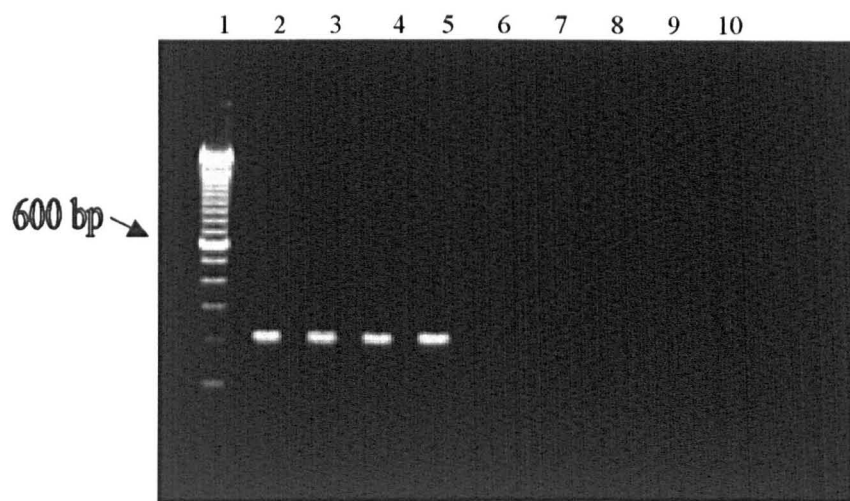
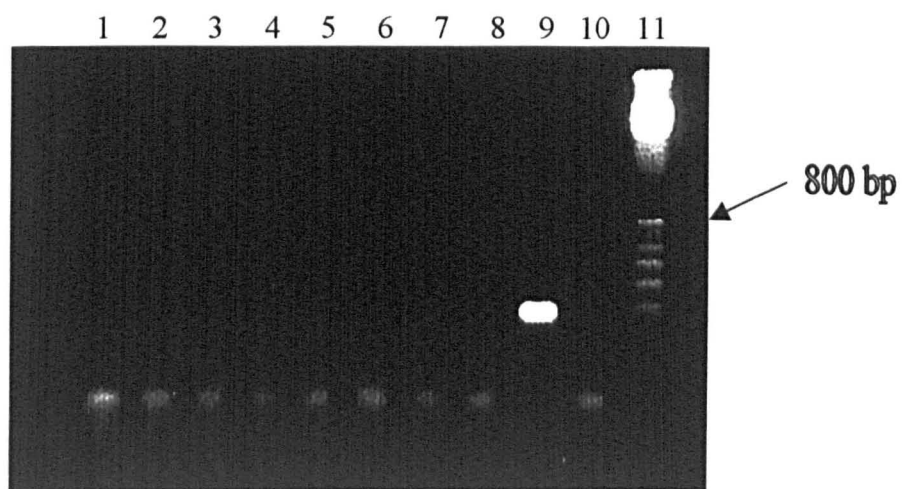


Fig. 4. Primers AGL-R designed for species specific *A. griffini* amplification. Lane 1, Negative control; lane 2, *A. astronyxis*; lane 3, *A. royreba*; lane 4, *A. palestinensis*; lane 5, *A. polyphaga* CCAP 1501/3C; lane 6, *A. polyphaga* ATCC 30871; lane 7, *A. castellanii*; lane 8, *A. comandoni*; lane 9, *A. griffini*; lane 10, *Acanthamoeba* sp. (Ros) and lane 11 is 100 bp DNA ladder.



4.4.2 - Restriction enzyme analysis:

The genus specific primer NA1L-R gave a SSU rDNA product of 910-1170 bp in length from all species / isolates of *Acanthamoeba* tested (Fig. 5).

Restriction digests of PCR amplified *Acanthamoeba* DNA from different species were distinct. Restriction enzyme, *Hae*III, produced a useful range of restriction patterns for all *Acanthamoeba* species tested (Fig. 6) and environmental and clinically isolated *Acanthamoeba* species / strains (Leicester & Hull, U.K.) were also differentiated on the basis of restriction banding patterns. *Acanthamoeba* sp. (Ros), gave similar banding pattern as *A. castellanii* in all enzymes tested including *Hae*III, *Cfo*I (Fig. 6) and *Hin*fl, *Alu*I (Fig. 7). *Acanthamoeba polyphaga* ATCC 30871 and *A. polyphaga* CCAP 1501/3c could be easily differentiated using *Cfo*I restriction enzyme (Fig. 6, lane 1,5) and *Alu*I (Fig. 7, lane 1,4). *Acanthamoeba* sp. (Canal), *Acanthamoeba* sp. (Soil, Pearson Park) and *Acanthamoeba* sp. (River) gave completely different patterns which did not match any of the known species tested (Fig. 8, lane 7, 10, 13). *Acanthamoeba* sp. (Soil, Car Park) gave similar patterns to *A. griffini* (Fig. 8, lane 12, 3). *Acanthamoeba* sp. (Wash basin, 2 samples) gave patterns similar to *A. palestinensis* (Fig. 8, lane 14, 15, 2). *Acanthamoeba* sp. (Esbc4) and *Acanthamoeba* sp. (Estuary) gave restriction patterns similar to *A. polyphaga* ATCC30871 (Fig. 8, lane 8, 11, 4). *Acanthamoeba* sp. (Curtan) gave similar patterns to *A. castellanii* (Fig. 8). Restriction digests using other enzymes gave information in differentiating unresolved *Acanthamoeba* (Fig. 9).

Fig. 5. Showing amplification of 18S rDNA of *Acanthamoeba* sp. using primers NA1L-R. Lane 1, 100 bp DNA ladder; lane 2, *A. royreba*; lane 3, *A. griffini*; lane 4, *A. palestinensis*; lane 5, *A. polyphaga* CCAP 1501/3C; lane 6, *A. polyphaga* ATCC 30871; lane 7, *A. castellanii*; lane 8, *Acanthamoeba* sp. (Nich); lane 9, *Acanthamoeba* sp. (Esbc4); lane 10, *Acanthamoeba* sp. (Ros); lane 11, *Acanthamoeba* sp. (Leic/tap); lane 12, *Acanthamoeba* sp. (Dargon); lane 13, *Acanthamoeba* sp. (Shi); lane 14, *Acanthamoeba* sp. (Estuary); lane 15, *Acanthamoeba* sp. (River) and lane 16 is negative control.

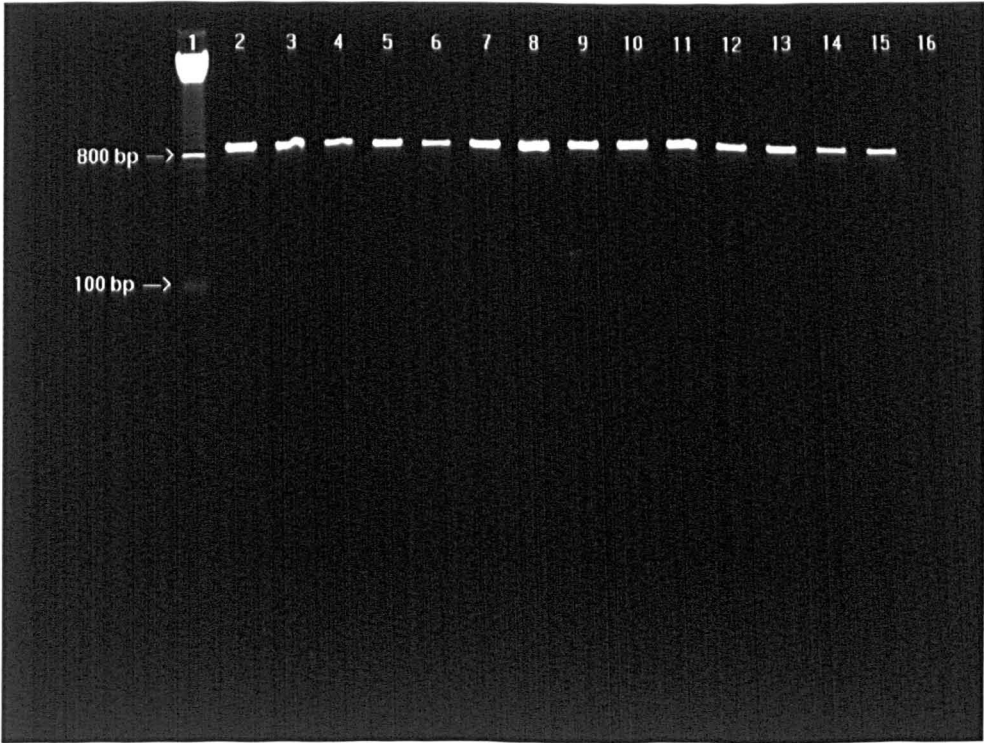


Fig. 6. Showing restriction enzyme analysis of *Acanthamoeba* species using enzymes CfoI (lane 1-7) and HaeIII (lane 8-12). Lane 1, *A. polyphaga* ATCC 30871; lane 2, *Acanthamoeba* sp. (Nich); lane 3, *Acanthamoeba* sp. (Ros); lane 4, *A. castellanii*; lane 5, *A. polyphaga* CCAP 1501/3c; lane 6, *A. palestinensis*; lane 7, *A. griffini*; lane 8, *Acanthamoeba* sp. (Ros); lane 9, *A. castellanii*; lane 10, *A. polyphaga* CCAP 1501/3c; lane 11, *A. palestinensis*; lane 12, *A. griffini* and lane 13 is 100 bp DNA ladder.

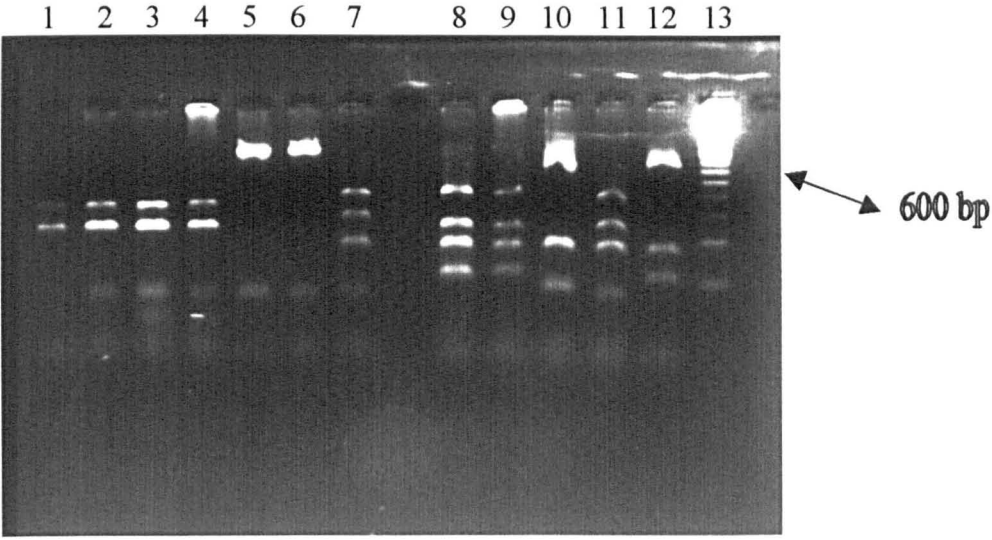


Fig. 7. Showing restriction enzyme analysis using AluI (lane 1-6) and HinfI (7-13). Lane 1, *A. polyphaga* ATCC 30871; lane 2, *Acanthamoeba* sp. (Ros); lane 3, *A. castellanii*; lane 4, *A. polyphaga* CCAP 1501/3c; lane 5, *A. palestinensis*; lane 6, *A. griffini*; lane 7, *A. polyphaga* ATCC 30871; lane 8, *Acanthamoeba* sp. (Nich); lane 9, *Acanthamoeba* sp. (Ros); lane 10, *A. castellanii*; lane 11, *A. polyphaga* CCAP 1501/3c; lane 12, *A. palestinensis*; lane 13, *A. griffini* and lane 14 is 100 bp DNA ladder

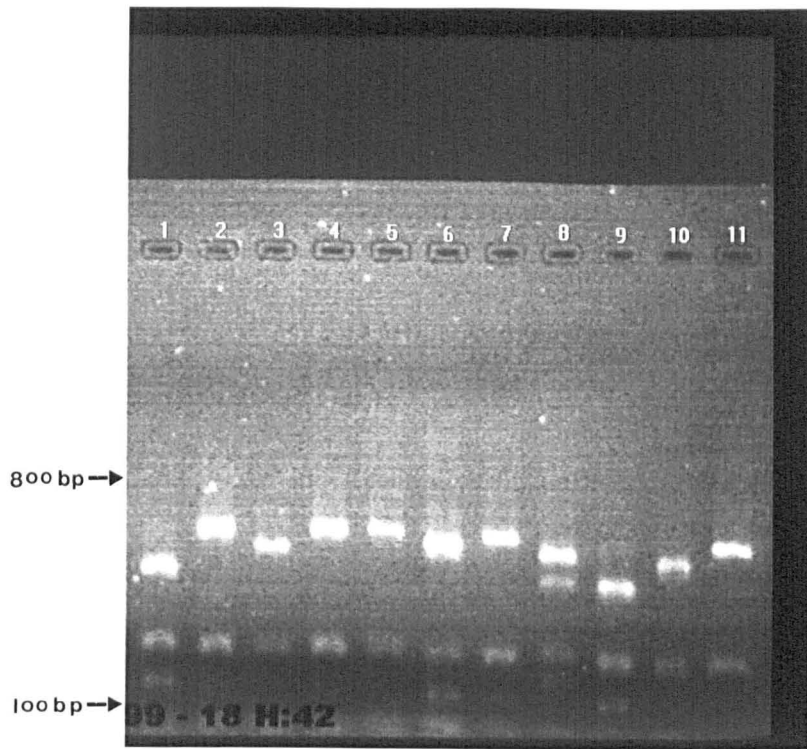


Fig. 8. Showing restriction enzyme analysis using HaeIII. Lane 1 is 100 bp DNA ladder; lane 2, *A. palestinensis*; lane 3, *A. griffini*; lane 4, *A. polyphaga* ATCC 30871; lane 5, *Acanthamoeba* sp. (Leicester/tap); lane 6, *Acanthamoeba* sp. (Curtan); lane 7, *Acanthamoeba* sp. (Canal); lane 8, *Acanthamoeba* sp. (Esbc4); lane 9, *A. polyphaga* CCAP 1501/3C; lane 10, *Acanthamoeba* sp. (River); lane 11, *Acanthamoeba* sp. (Estuary); lane 12, *Acanthamoeba* sp. (Soil, Car Park); lane 13, *Acanthamoeba* sp. (Soil, Pearson Park); lane 14, *Acanthamoeba* sp. (Wash basin); lane 15, *Acanthamoeba* sp. (Wash basin) and lane 16, *A. comandoni*.

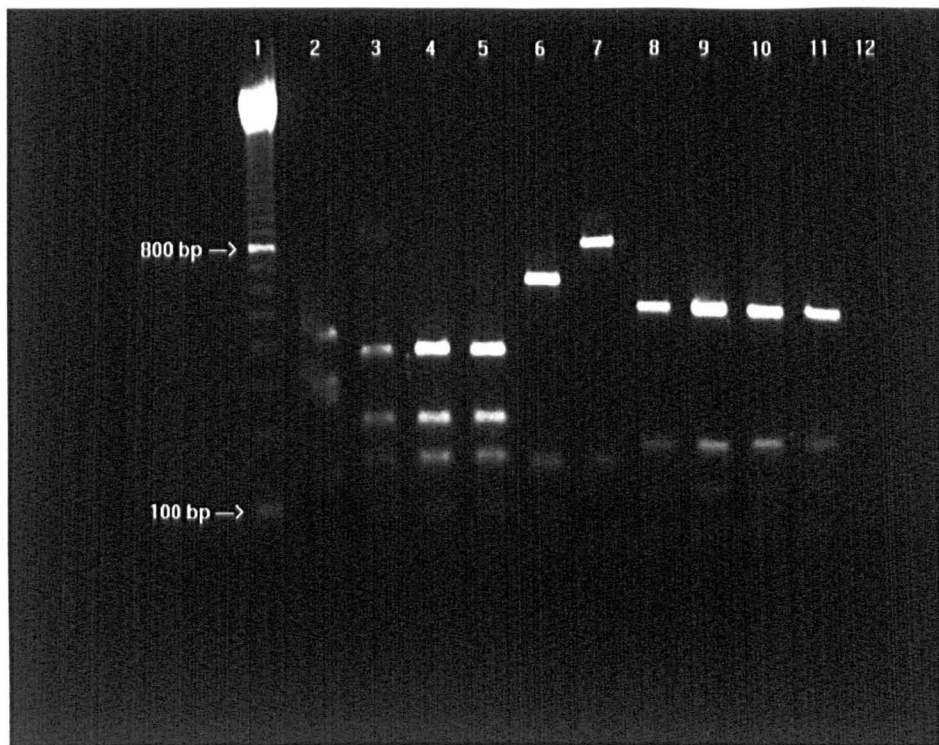


Fig. 9 a) Showing restriction enzyme analysis using HinfI. Lane 1, *A. palestinensis*; lane 2, *A. griffini*; lane 3, *A. polyphaga* ATCC 30871; lane 4, *Acanthamoeba* sp. (Leic/tap); lane 5, *Acanthamoeba* sp. (Curtan); lane 6, *Acanthamoeba* sp. (Canal); lane 7, *Acanthamoeba* sp. (Esbc4); lane 8, *A. polyphaga* CCAP 1501/3c; lane 9, *Acanthamoeba* sp. (River); lane 10, *Acanthamoeba* sp. (Estuary) and lane 11, *Acanthamoeba* sp. (Soil, Car Park).

b) Showing restriction enzyme analysis using HinfI. Lane 1, 100 bp DNA ladder; lane 2, *Acanthamoeba* sp. (Pearson Park); lane 3, *Acanthamoeba* sp. (Wash basin); lane 4, *Acanthamoeba* sp. (Shi); lane 5, *Acanthamoeba* sp. (Soil, Admin.); lane 6, *Acanthamoeba* sp. (Soil, Cottingham Rd.); lane 7, *Acanthamoeba* sp. (Thackray); lane 8, *Acanthamoeba* sp. (Wash bottle); lane 9, *A. comandoni*; lane 10, *Acanthamoeba* sp. (Dargon) and lane 11, *Acanthamoeba* sp. (Soil, gym) and lane 12 is negative control.



a



b

Acanthamoeba palestinensis and *A. polyphaga* ATCC 30871 (Fig. 8, lane 1, 3) were not easily differentiated using *Hae*III enzyme but gave different banding pattern using *Hinf*I (Fig. 9a, lane 1 & 3).

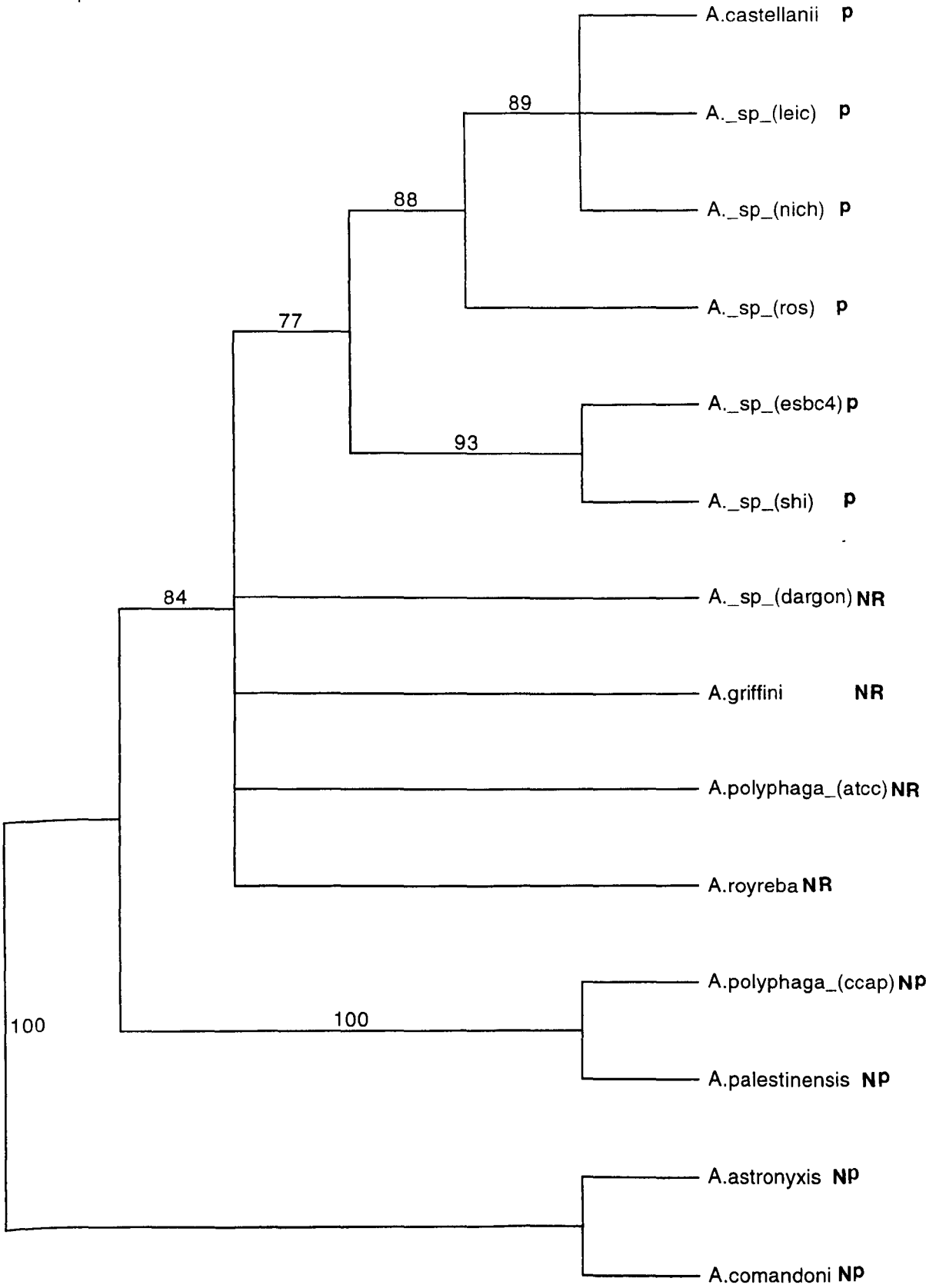
Acanthamoeba sp. (Dargon), *Acanthamoeba* sp. (Wash bottle) and *Acanthamoeba* sp. (Soil, gym) gave a similar banding pattern as *A. comandoni* using *Hinf*I (Fig. 9b). Two strains of *A. polyphaga*, ATCC 30871 and CCAP 1501/3c produced different banding patterns (Fig. 9a). Restriction enzyme analysis using *Hae*III showed that banding patterns of *A. comandoni* and *A. griffini* were very different from *A. polyphaga*, *A. palestinensis* and *A. castellanii*.

4.4.3 - DNA sequencing

Trees were generated by minimum evolution (Distance) and maximum parsimony criteria. The aligned sequence used to generate trees is shown in appendix 8. Tree topologies were almost identical irrespective of methods. In the parsimony trees nodes were checked using 100 bootstrap replicates and are marked on Fig. 10. Results suggest two subclusters of *Acanthamoeba* spp. tested. The phylogeny from all *Acanthamoeba* revealed that pathogenic and non-pathogenic taxa are the main subclusters and pathogenic taxa may well have diverged from non-pathogenic taxa as the tree was rooted by comparison of the most conserved region of the alignment to *Hartmannella* (a close relative to *Acanthamoeba*). Results were confirmed by mid-point rooting. However, *A. royreba*, *A. polyphaga* ATCC 30871, *A. griffini* and *Acanthamoeba* sp. (Dargon) were not fully resolved by the use of rDNA sequences.

Fig. 10. Showing bootstrap analysis of maximum parsimony tree. P represents pathogenic, NP represents non-pathogenic and NR (not resolved) represents avirulent strains. Numbers represents bootstrap values based on 100 replicates. Among avirulent strains *A. polyphaga* (ATCC 30871) and *Acanthamoeba* sp. (Dargon) showed pathogenic characters and *A. griffini* showed pathogenic characters in PCR and slow growth at 37°C and no growth on 1 M mannitol non-nutrient agar plates, <20 acanthopodia in SEM and low extracellular protease activity. *Acanthamoeba royreba* showed >100 acanthopodia, higher extracellular protease activity but no amplification in PCR using pathogenic primer pair, no growth at 37°C or on 1 M mannitol non-nutrient agar plates.

Bootstrap



Acanthamoeba polyphaga CCAP 1501/3c and *A. polyphaga* ATCC 30871 (2 strains of the same species) showed 11% sequence divergence (Table 4), thus correlating with the data obtained from restriction enzyme and isoenzyme analyses (See Chapter 3). Interestingly, *A. polyphaga* ATCC 30871 was placed in the pathogenic cluster and *A. polyphaga* CCAP 1501/3c in non-pathogenic cluster.

4.5 - DISCUSSION

Detection of *Acanthamoeba* at the generic level was achieved using primers ACARNA 1383-1655 from rDNA sequence of *Acanthamoeba castellanii*. A DNA extraction method has been successfully used which showed sensitivity up to 5 cells. This method can be directly performed with the given specimen. The whole DNA extraction method was performed in 30 min. at room temperature. Neither ice nor refrigeration is required. There is no precipitation step, eliminating problems with small amounts of DNA. No laborious hybridizations or the use of radionucleotides are required. Since ACARNA primers did not give any amplification with other amoebal, bacterial or fungal DNA, thus making this assay a simple, rapid, sensitive and specific method.

Pathogenic and non-pathogenic *Acanthamoeba* sp. can be differentiated using primers Ac6/10-210. This data is based on the analysis of a single locus in a limited number of *Acanthamoeba* species / strains which were tested for their cytopathic effects on corneal epithelial cells (Table 5). The major potential application of this primer pair is in the complex ecology of infections with pathogenic and non-pathogenic *Acanthamoeba*.

Table 4. Percent dissimilarities between and within sequence types.

| | | | | | | | | | | | | | | |
|----|------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| 1 | A.castellani | - | | | | | | | | | | | | |
| 2 | A.sp(leic) | 0.00410 | - | | | | | | | | | | | |
| 3 | A.sp(esbc4) | 0.01985 | 0.01555 | - | | | | | | | | | | |
| 4 | A.sp(shi) | 0.01591 | 0.01576 | 0.00829 | - | | | | | | | | | |
| 5 | A.sp(nich) | 0.00145 | 0.00131 | 0.01690 | 0.01445 | - | | | | | | | | |
| 6 | A.sp(ros) | 0.00419 | 0.00406 | 0.01419 | 0.01169 | 0.00273 | - | | | | | | | |
| 7 | A.polyphaga ccap | 0.10616 | 0.10497 | 0.10815 | 0.10699 | 0.10455 | 0.10344 | - | | | | | | |
| 8 | A.sp(dargon) | 0.05045 | 0.05053 | 0.05000 | 0.04867 | 0.04906 | 0.04747 | 0.10998 | - | | | | | |
| 9 | A.griffini | 0.04851 | 0.04861 | 0.04987 | 0.04873 | 0.04864 | 0.04559 | 0.10871 | 0.06297 | - | | | | |
| 10 | A.polyphaga atcc | 0.04315 | 0.04163 | 0.03843 | 0.03422 | 0.04017 | 0.03723 | 0.11616 | 0.05268 | 0.05004 | - | | | |
| 11 | A.palestinensis | 0.09531 | 0.09406 | 0.09694 | 0.09226 | 0.09379 | 0.09264 | 0.03652 | 0.09500 | 0.10161 | 0.10681 | - | | |
| 12 | A.royreba | 0.03574 | 0.03432 | 0.03837 | 0.03712 | 0.03287 | 0.03289 | 0.09793 | 0.04112 | 0.05661 | 0.04194 | 0.08487 | - | |
| 13 | A.astronyxis | 0.19535 | 0.19387 | 0.19857 | 0.20026 | 0.19239 | 0.19036 | 0.20017 | 0.19971 | 0.18970 | 0.19578 | 0.19502 | 0.18136 | - |
| 14 | A.comandoni | 0.20549 | 0.20406 | 0.20735 | 0.20945 | 0.20426 | 0.20011 | 0.20077 | 0.22843 | 0.20477 | 0.21369 | 0.20590 | 0.21148 | 0.09484 |

Table 5. Characteristics of *Acanthamoeba* used in DNA sequencing study.

| <i>Acanthamoeba</i> sp. | Growth at 37°C | Osmotolerance growth assays | PCR result using pathogenic specific primer pair | CPE results for epithelial cell damage | Mice <i>in vivo</i> eye infection |
|----------------------------------|----------------|-----------------------------|--|--|-----------------------------------|
| <i>A. castellanii</i> | + | + | + | + | + |
| Leic/tap | + | + | + | + | NP |
| Nich | + | + | + | + | + |
| Ros | + | + | + | + | + |
| Esbc4 | + | + | + | + | NP |
| Shi | + | + | + | + | NP |
| Dargon | + | + | + | + | NP |
| <i>A. griffini</i> | SG | SG | NA | PD | NP |
| <i>A. polyphaga</i> ATCC 30871 | + | + | + | + | + |
| <i>A. royreba</i> | SG | SG | NA | PD | NI |
| <i>A. polyphaga</i> CCAP 1501/3c | NG | NG | NA | ND | NI |
| <i>A. palestinensis</i> | NG | NG | NA | ND | NI |
| <i>A. astronyxis</i> | NG | NG | NA | ND | NI |
| <i>A. comandoni</i> | NG | NG | NA | ND | NP |

Where SG is slow growth, NG is no growth, NA is no amplification, PD is partial degradation of corneal epithelial monolayers, ND is no damage, NI is no infection and NP is not performed.

But in the application of this assay, a large number of specimens will need to be processed and the simplification of the assay using newly developed DNA extraction method as described above should make this more practicable. The alternative to this assay are antigen detection methods. At present, however, there are no available methods for distinguishing between pathogenic and non-pathogenic *Acanthamoeba*.

It is also shown that species specific primers can be designed to identify *Acanthamoeba* at the species level. In this study *A. griffini* specific primer pair (AG L-R) was designed from rDNA sequence which did not amplify DNA from any other tested species of *Acanthamoeba*. But this is an expensive and laborious method as *Acanthamoeba* has 20 species.

Restriction enzyme analysis is a rapid, reliable and specific method for species differentiation. It is very interesting that some isolates (CCAP 1501/3c & ATCC 30871) assigned to *A. polyphaga* (on the basis of cyst morphology) showed significant sequence divergence within the same species, therefore they could be recognised as a separate species. Also the above two isolates were observed using microscopic analysis to look at the cyst morphology which did not help in resolving the species differentiation. *Acanthamoeba castellanii* and *Acanthamoeba* sp. (Ros) showed the same banding patterns in all tested restriction enzymes. This supports our results observed in isoenzyme analysis. This study presents the applicability of PCR based restriction enzyme analysis as a tool for differentiation of *Acanthamoeba* spp. A few strains of *Acanthamoeba* gave the same restriction enzyme analysis for one enzyme but this could be resolved using more than one enzyme. However, we were able to identify *Acanthamoeba* species using this technique.

DNA sequencing gave interesting results and shows that rDNA sequence data may have potential in the species designation of unresolved *Acanthamoeba* sp. In this study *A. polyphaga* (Culture Collection of Algae Protozoa 1501/3c) and *A. polyphaga* (American Type Culture Collection 30871) were analysed for growth assays. *Acanthamoeba polyphaga* ATCC 30871 showed growth at temperatures of 37°C while *A. polyphaga* CCAP 1501/3c did not show any growth at this temperature (Table 4). In PCR, primers designed to amplify internal transcribed spacer (ITS) region from pathogenic *Acanthamoeba* gave a discrete product from *A. polyphaga* ATCC 30871 with no amplification from *A. polyphaga* CCAP 1501/3c (Table 4). In protease assays (See Chapter 6), *A. polyphaga* ATCC 30871 showed higher amounts of extracellular proteases as compared to *A. polyphaga* CCAP 1501/3c (Table 4). Interestingly when rDNA sequencing was performed to generate a parsimony tree, *A. polyphaga* CCAP 1501/3c was placed in the non-pathogenic subcluster and *A. polyphaga* ATCC 30871 in unresolved taxa (Fig. 10).

In a study by Stothard *et al.* (1998), *A. polyphaga* CCAP 1501/3c rDNA sequence resembled the rDNA sequence of group 3 isolates although previously Visvesvara (1991) identified this as *A. polyphaga* and placed in group 2 on the basis of cyst morphology. In order to look at the morphological characteristics of the cyst we examined the cyst of both *A. polyphaga* ATCC 30871 and *A. polyphaga* CCAP 1501/3c. Semi thin (0.5 µm) sections of cysts were made using a glass knife and stained with 1% toluidine blue (Sigma Labs.). Neither *A. polyphaga* CCAP 1501/3c (Fig. 11) nor *A. polyphaga* ATCC 30871 (Fig. 12) could be differentiated due to the limiting number of characteristics (number of arms) while rDNA sequencing is more reliable as there are more characteristics to

compare different strains. Thus our evidence supports that of Stothard *et al.* (1998) and suggest that *A. polyphaga* CCAP 1501/3c should be reclassified.

In other unresolved *Acanthamoeba*, *A. royreba* and *A. griffini* which could grow at 37°C if left for longer (Table 4) and did not gave any product with primer pair designed for pathogenic species of *Acanthamoeba*. These 2 strains showed extracellular protease activity intermediate between pathogenic and non-pathogenic taxa and when tested for cytopathic effect assays (CPE) some epithelial cell monolayer degradation could be observed (Table 4). In sequencing these 2 strains including with *Acanthamoeba* sp. (Dargon) and *A. polyphaga* ATCC 30871 remained unresolved (could not fit into either pathogenic or non-pathogenic group). From our study it can be concluded that pathogens may have evolved from non-pathogens as highly conserved sequence of the outgroup, *Hartmannella*, appeared to be closest to *A. astronyxis* and *A. comandoni* sequence indicating that they are most basal. This relationship was also confirmed by mid point rooting. The strains that show characters (sequence, CPE, growth assays) intermediate between pathogenic and non-pathogenic may well be avirulent. All tested virulent strains were subclustered into one group and the non-virulent into other group and the unresolved were placed between the two groups.

Fig. 11. Showing cyst morphology of *A. polyphaga* CCAP 1501/3c (x400).

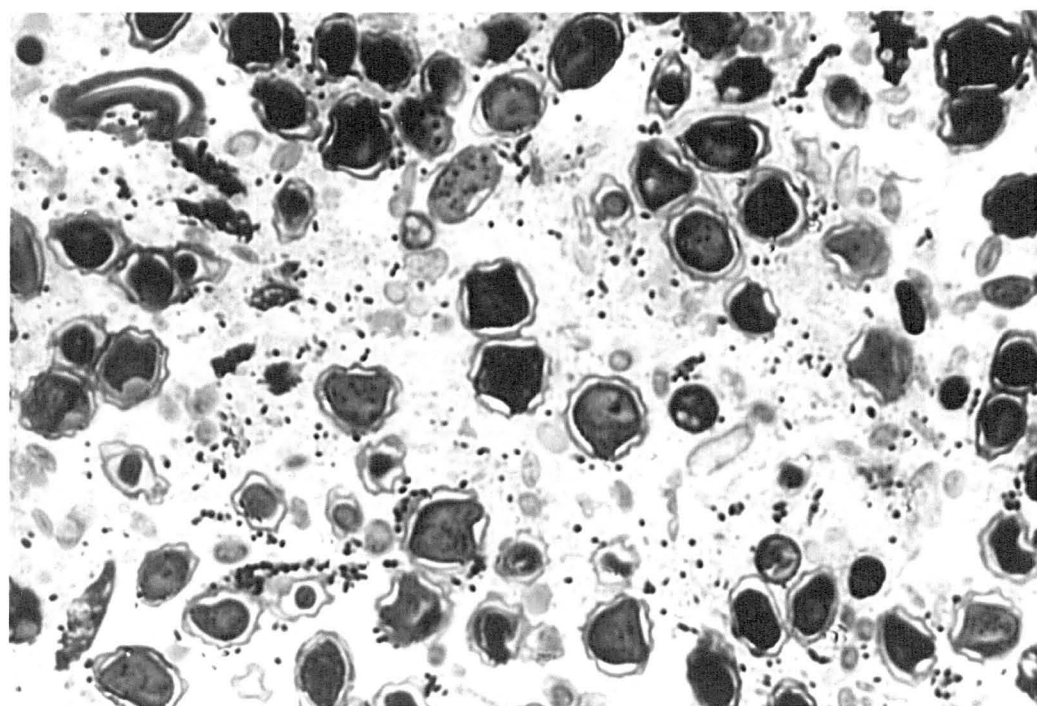
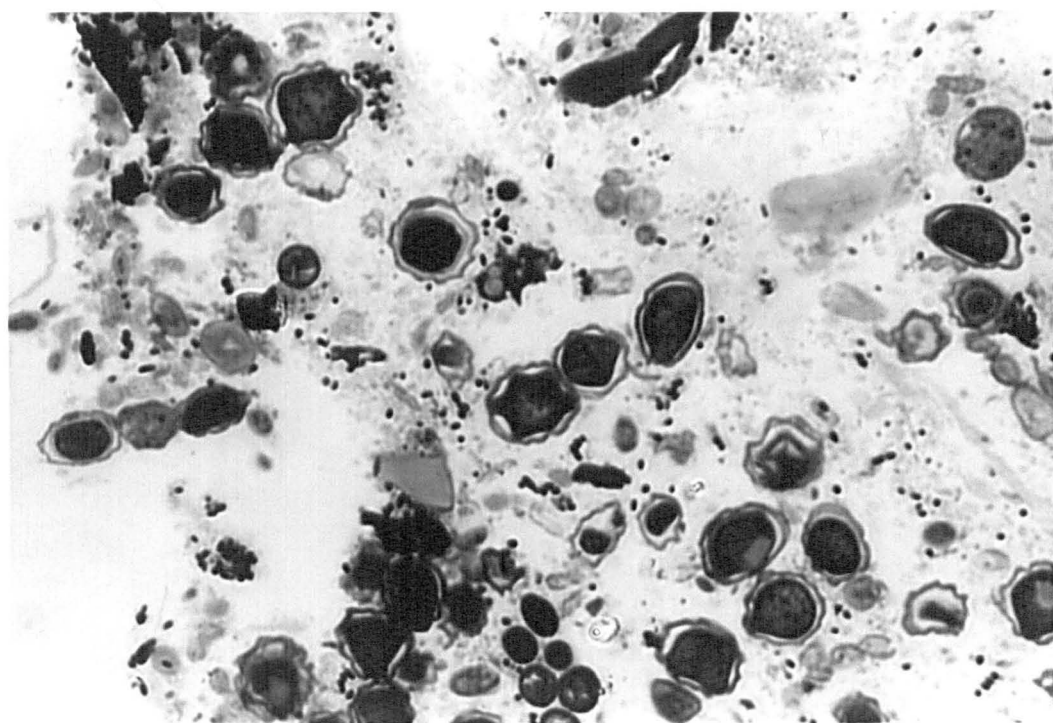


Fig. 12. Showing cyst morphology of *A. polyphaga* ATCC 30871 (x400).



As indicated above it is generally agreed that the identification of *Acanthamoeba* isolates at the species level is very problematical (Visvesvara 1991). Our studies indicate that there are inconsistencies between the classification of strains and the evolutionary lineages. Other methods including cyst morphology, isoenzyme analysis, RFLP's have been employed but we are unable to say which method is more promising for further resolving *Acanthamoeba* differentiation.

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CHAPTER 5

Use of Phage Display Antibodies for Clinical Diagnosis of *Acanthamoeba* spp.

5.1 - SUMMARY

Antibody fragments are generated by the random pairing of large diverse repertoires of variable heavy and light chain genes, derived by PCR from naive human lymphocytes, and cloned for expression of individual specificities on the surface of filamentous bacteriophage (Nissim *et al.* 1994, Phage library). This library contained a vast number of different antibody specificities varying from 10^7 to 10^{12} . In this study, we have used the library for the isolation of *Acanthamoeba* specific antibody fragments. The library was incubated with the whole fixed *Acanthamoeba* cells. Bound phages were separated by acid treatment and amplified in *E. coli*. Phages were incubated with *Hartmannella* to remove non-specific phages. Unbound phages were again incubated with *Acanthamoeba*, separated and amplified in *E. coli*. This process was repeated 3 times. Finally individual clones were grown and reacted with the whole *Acanthamoeba* cells using ELISA. Clones showing reactivity with *Acanthamoeba* were tested using flow cytometry and immunofluorescence. Cross reactivity was tested using a number of microorganisms and cell lines. Ten clones were isolated with the required specificity and sensitivity. Here we report the isolation of antibody fragments that can be used to detect *Acanthamoeba* specifically using immunofluorescence and flow cytometry thus providing the reagents for a specific and rapid detection assay.

5.2 - INTRODUCTION

Traditionally, approaches for identification of infectious diseases included culture techniques, serologic tests, and biochemical assays. Serodiagnosis was accomplished using precipitation and agglutination, but these assays are labor intensive, insensitive and non-specific (Goka *et al.* 1986; Nash *et al.* 1987a; Jokipii *et al.* 1988). Immunoassay methods became popular with the development of immunofluorescence assays. In these, either specific microbial antigen or microbial antigen specific antibodies are tested. Microbial antigens are easier to test as they are usually tested within the clinical specimen (Van Regenmortel 1992; Underwood 1988; Green *et al.* 1985; Stibbs *et al.* 1988). The lower limits of sensitivity for detecting antigen by immunofluorescence in most studies are approx. 100 pg – 1 ng. This level of sensitivity is sufficient to detect virtually all bacterial, viral or parasitic agents following laboratory culture but is not always sufficient to detect antigens directly in clinical specimens (Crafts *et al.* 1982; Nash *et al.* 1987; Villa *et al.* 1989). Although polyclonal antibodies have been successfully used as diagnostic reagents in infectious diseases, these tests have been difficult to standardize mainly due to broad specificity of the antiserum which can lead to undesirable cross reactions with other antigens (Underwood 1988; Van Regenmortel 1992).

On the other hand monoclonal antibodies (Kohler *et al.* 1975) have defined specificity and sensitivity when used in immunoassays. In immunoassays using monoclonal antibodies, microbial antigens can be directly tested within the clinical specimen. Monoclonal antibodies have been employed successfully for *C. trachomatis* (Caul *et al.* 1985; Mohanty *et al.* 1986), *H. influenzae* (Belmaaza *et al.* 1986; Groeneveld *et al.* 1987), *L. pneumophila* (Bibb *et al.* 1984; Brown *et al.* 1985), *N. meningitidis*

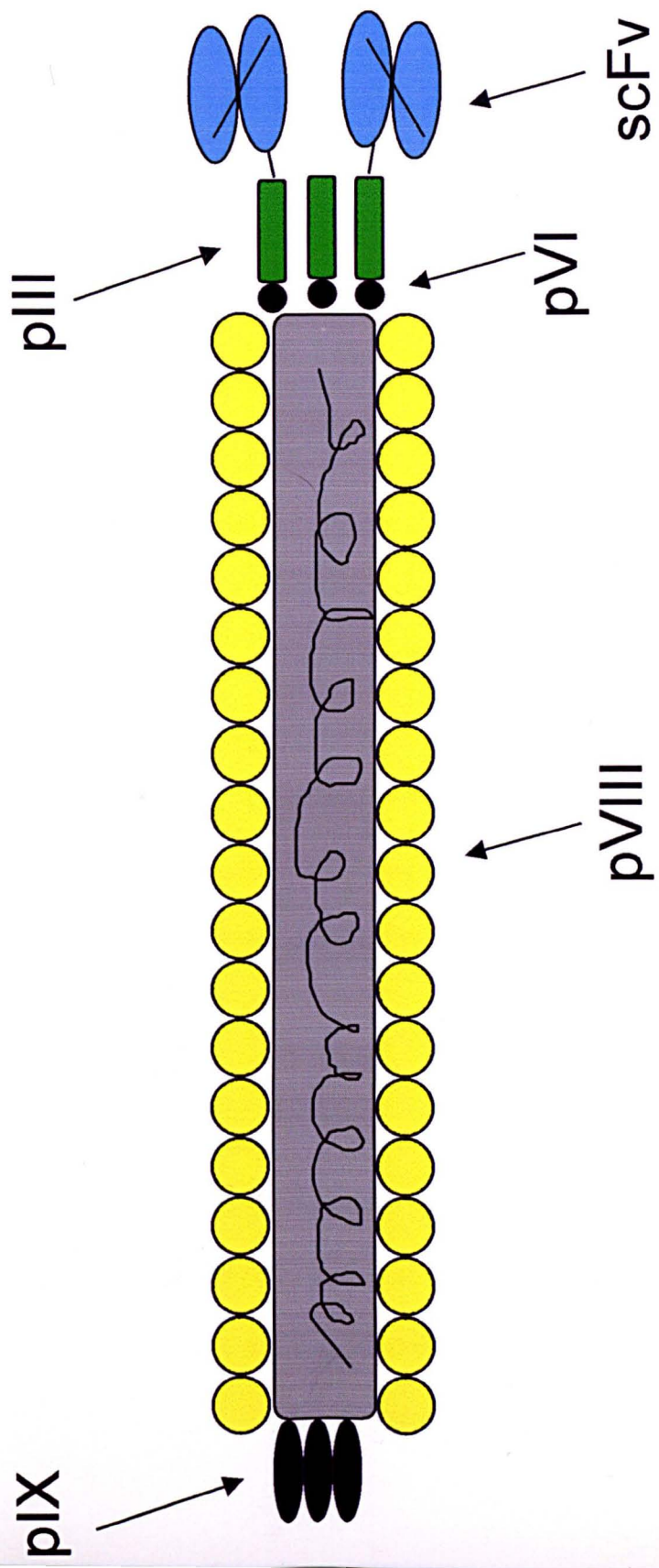
(Sugaswara *et al.* 1984), *Yersinia pestis* (Williams *et al.* 1984) and *E. histolytica* (Sengupta *et al.* 1993) *Giardia* (Engelkirk *et al.* 1990).

In general, monoclonal antibodies to microbial antigens have been produced by *in vivo* techniques following the fusion of myeloma cells with antibody secreting B-cells. The resultant continuous cell line (hybridoma) produces large quantities of homogeneous single epitope antibody. Antibodies from a given hybridoma are immunologically identical and react with one specific epitope on the antigen against which they are raised. A detailed review can be found in Harlow *et al.* (1988). Problems with this method are that antigens have to be immunogenic, animals are required, it could take several months before a useful antibody is isolated, and this is an expensive method. This has led to the development of phage antibody display libraries.

5.2.1 - Phage display technology

Phage display technology has been developed recently as an alternative to isolating specific antibodies (Winter *et al.* 1994). Antibody fragments are generated by the random pairing of large diverse repertoires of variable heavy and light chain genes, derived by PCR from naive human lymphocytes, and cloned for expression of individual specificities on the surface of filamentous bacteriophage (Fig. 1). A library contains antibody specificities varying from 10^7 to 10^{12} depending on how the library is constructed. This approach for generating antibodies has the major advantage that epitopes do not have to be immunogenic, i.e. antibody fragments can be isolated that recognise native cell surface structures. The first phage antibody display library was constructed by Smith (1985).

**Fig. 1. Showing a bacteriophage expressing single chain frangment Fab v-region (scFv).
PIII, pVI, pVIII and PIX are different surface proteins of bacteriophage.**



Phage antibody display technology has been successfully used for the isolation of monoclonal antibody fragments for carcino-embryonic antigen (CEA), the most widely used clinical tumour marker (Osbourn *et al.* 1996) and melanoma expressing antigens (Pereira *et al.* 1997). In this study, we report the potential use of phage antibody display technology as a diagnostic tool for isolating antibody fragments against microbial antigens.

5.3 - MATERIALS AND METHODS

5.3.1 - Preparation of parasites

Acanthamoeba were grown as described previously. Mid log phase cells were harvested by centrifugation at 800 x g for 10 min. Cells were resuspended in PAS and centrifuged at 800 x g for 10 min; this was repeated twice. Cells used for isolating antibody fragments, ELISA and FACS analysis were fixed in 50% (v/v) methanol:PAS. After fixation, cells were washed three times with PAS as described above. Organisms used for immunofluorescence microscopy were harvested by centrifugation and then placed onto slides prior to fixation. Organisms and cell types (Table 1) used as negative controls were obtained from Public Health Laboratories Services (PHLS), Hull, UK., except *Hartmannella* which was kindly provided by Dr. Simon Kilvington, PHLS, Leicester, UK.

5.3.2 - Preparation of bacteriophage

For bacteriophage preparation, the library stock or individual bacteriophage clones were added to a culture of *E. coli* (TG1) grown in 2 x TY {0.8%(w/v) NaCl, 1.0%(w/v) tryptone, 0.5%(w/v) yeast extract} supplemented with 100µg/ml ampicillin and 1% (w/v)

glucose. This culture was incubated at 37°C until the absorbance at 600 nm was between 0.4-0.5. VCS-M13 helper bacteriophage were then added to the culture and incubated for a further 30 min at 37°C without shaking. The culture was centrifuged at 1500 x g for 10 min., the pellet was resuspended in 2 x TY supplemented with 100 µg/ml ampicillin and 25 µg/ml kanamycin (to select for bacteriophage-containing clones) and incubated at 30°C overnight. The overnight culture was centrifuged at 10800 x g for 10 min. and the pellet resuspended in 1/5 volume of 20% (w/v) polyethylene glycol 6000 in 2.5M NaCl for 1h at 4°C. After incubation, pellet was washed 3 times with PAS and resuspended in PAS with 15% (v/v) glycerol and centrifuged at 1500 x g for 10 min. Finally, the supernatant containing the bacteriophage were filtered using a 0.45 µm prior to storage at -80°C. Bacteriophage titre was determined by serial dilution of infected *E. coli* (TG1) grown on TYE { 1.5%(w/v) Bacto-agar, 0.8% (w/v) NaCl, 1% (w/v) tryptone and 5% (w/v) yeast extract } supplemented with 25µg/ml kanamycin.

5.3.3 - Use of bacteriophage antibody display library

A human derived bacteriophage antibody library expressing single chain Fv fragments was obtained from G. Winter (Synthetic scFv library #1, Centre for Protein Engineering, MRC centre, Cambridge) (Nissim *et al.* 1994). To isolate *Acanthamoeba* specific antibody fragments the bacteriophage library (2×10^{11} bacteriophage) was added to a suspension of whole fixed cells (2×10^7) which had been blocked by incubation in MPAS (2% (w/v) dried milk powder, 1% (w/v) bovine serum albumin in PAS) at 37°C for 1 h prior to use. The mixture of bacteriophage and *Acanthamoeba* was incubated at 20°C

1 h prior to use. The mixture of bacteriophage and *Acanthamoeba* was incubated at 20°C with gentle shaking for 1 h then centrifuged at 400 x g for 5 min. The pellet was resuspended in 0.1% (w/v) BSA in PAS then centrifuged again; this process was repeated 10 times in total, to remove unbound bacteriophage. The pellet was resuspended in citric acid (76mM) and incubated at 20°C for 5 min with shaking to elute bound bacteriophage. The pH of the mixture was then adjusted to 7.0 by adding Tris-HCl (1M) pH 7.4. This procedure was termed a panning round (Fig. 2). The bacteriophage selected by this procedure were then amplified as described previously (Preparation of bacteriophage particles) except that bacteria infected with bacteriophage were spread onto TYE bio-assay dishes and incubated at 30°C overnight. The *E. coli* (TG1) containing bacteriophage were scraped from the plate and resuspended in 2 x TY containing 15% (w/v) glycerol and this suspension termed, library stock, was stored at -80°C.

To remove non-specifically binding bacteriophage a negative panning round against the amoeba *Hartmannella* sp. was performed as described for *Acanthamoeba* except that the supernatant containing unbound bacteriophage was retained (Fig. 2). Unbound bacteriophage were incubated with a fresh aliquot of *Hartmannella* and the unbound phage were retained. Three more incubations were performed using fresh aliquots of *Hartmannella*. The supernatant containing unbound phage obtained from the final incubation with *Hartmannella* was then incubated with 2×10^7 fixed and blocked *Acanthamoeba*. In summary, 4 panning rounds were performed in the following order: a positive pan against *Acanthamoeba*, a negative pan on *Hartmannella* followed immediately by a positive pan on *Acanthamoeba*, and finally 2 positive panning rounds against *Acanthamoeba* (Fig. 2).

The above procedure was also performed to isolate antibody fragments which could be used in differentiation of pathogenic *Acanthamoeba*. Four panning rounds were performed in the following order: a positive pan on pathogenic *Acanthamoeba* sp. (Ros), a negative pan on non-pathogenic *A. astronyxis*, and finally 2 positive panning rounds against pathogenic *Acanthamoeba* sp. (Ros).

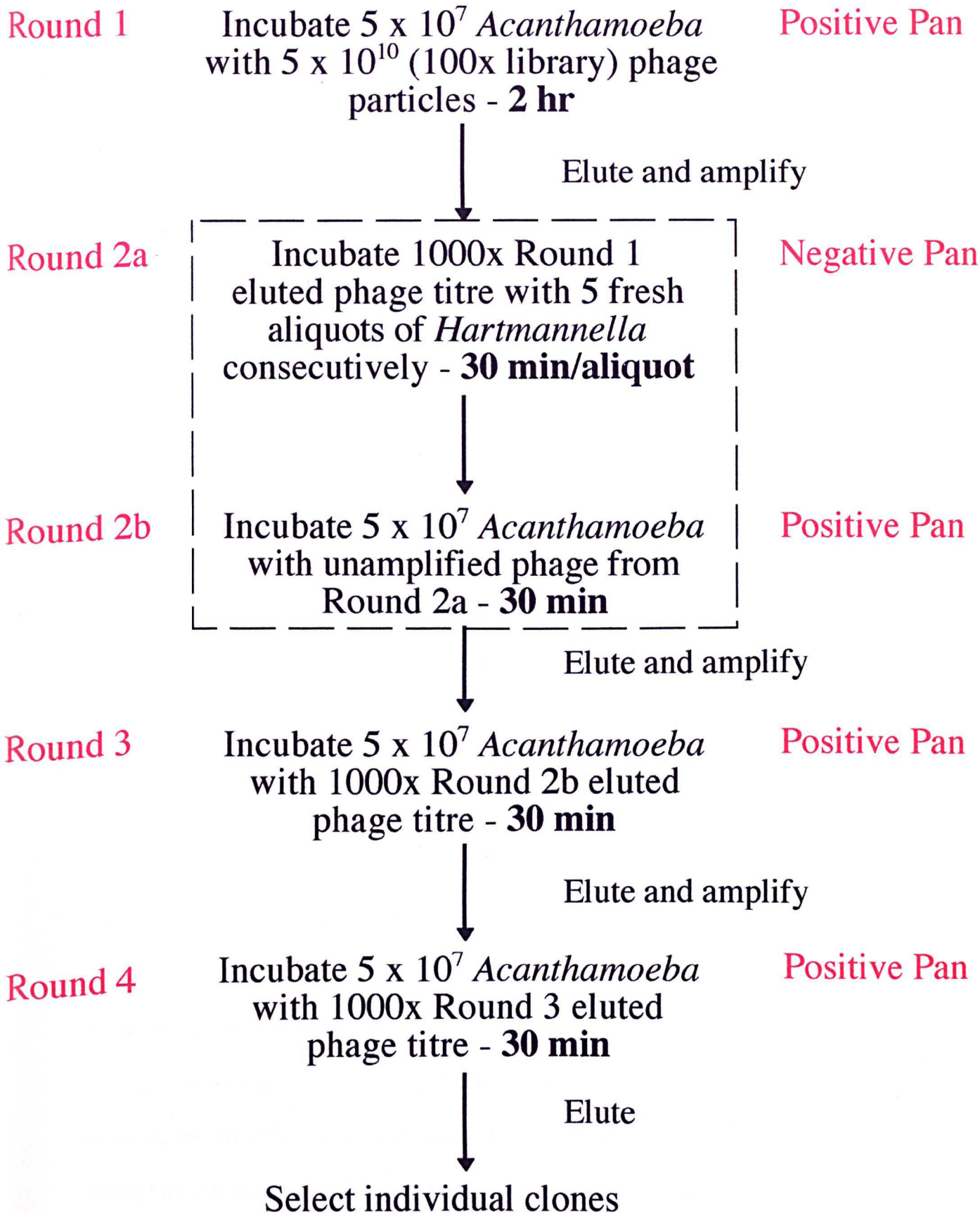
5.3.4 - PCR

PCR was performed to demonstrate the diversity of bacteriophage clones isolated after each panning round. The method used was essentially the same as described by Marks *et al.* (1991). PCR fragments were amplified directly from bacterial colonies using the previously described primers from V-regions, CDR-FOR (5' CAG GGT ACC TTG GCC CCA 3') and CDR-BACK (5' GTG TAT TAC TGT GCA AGA 3').

5.3.5 - ELISA

Single bacterial colonies were picked directly from the bio-assay dish and inoculated onto 2 x TY supplemented with 100 µg/ml ampicillin+1% (w/v) glucose and grown in 96 well round bottom plates overnight at 37°C. Glycerol stocks of the overnight incubations were made by adding 15% (w/v) glycerol and stored at -80°C until required.

Fig. 2. Showing panning protocol for the present study.



To rescue bacteriophage, 5 µl of the glycerol stock culture was transferred into a new 96 well plate containing 2 x TY and incubated at 37°C for 1h. VCS-M13 helper bacteriophage (1×10^9) were added to each well and incubated at 37°C for 30 min. without shaking and for 1h with shaking. Plate was then centrifuged at 400 x g for 10 min. and the supernatant aspirated. The pellet was resuspended in 2 x TY supplemented with 100 µg/ml ampicillin+50 µg/ml kanamycin and incubated overnight at 30°C with shaking (bacteriophage plate). Fixed and MPAS-blocked *Acanthamoeba* (2×10^5) were added to a conical well plate and centrifuged at 200 x g for 5 min. The supernatant was carefully removed and the wells washed with PAS. Bacteriophage plates grown overnight were centrifuged at 400 x g for 10 min and the supernatant was used as a source of the bacteriophage clones. To the plate containing immobilised *Acanthamoeba*, individual bacteriophage clones were added to each well and the plate incubated with shaking at 20°C for 1h then washed with PAS. Sheep Anti M13-HRP [Pharmacia Biotech.] (diluted 1:500 in MPAS) was added to each well and incubated with shaking at 20°C for 1 h. Wells were washed twice with PAS before ABTS substrate (Vector Laboratories) was added. The plate was then incubated at 20°C for 30 min. in the dark. The supernatant was transferred to a flat bottom plate and the absorbance (405 - 690nm) determined.

5.3.6 - Flow Cytometry

Acanthamoeba (1×10^6) were fixed and blocked as before, mixed with 1×10^{12} bacteriophage from the appropriate clone and incubated at 4°C for 1 h. Cells were washed three times with 0.25% (w/v) bovine serum albumin in PAS (PAA). The final pellet was resuspended in sheep anti-M13 antibody (10 µg/ml, Pharmacia) and incubated at

4°C for 1h. Cells were washed twice with PAA, resuspended in fluorescein isothiocyanate conjugated anti-sheep IgG (10µg/ml, Vector Laboratories) and incubated at 4°C for 1h. Cells were washed twice as above and finally resuspended in PAA and analysed by flow cytometry. Fluorescence intensity was measured on a FACSCalibur (Becton Dickinson) using an excitation wavelength of 488 nm.

5.3.7 - Indirect immunofluorescence

Samples for fluorescence microscopy were prepared as for ELISA except that after incubation with the fluorescein isothiocyanate conjugated anti-sheep IgG, the suspension was spotted onto glass slides and allowed to dry. Slides were then viewed under normal and epi-fluorescent illumination using a Nikon E800 epi-fluorescence microscope equipped with a Chroma Technology 83000 filter set coupled to a Photometrics Sensys cooled CCD.

5.4 - RESULTS

5.4.1 - ELISA

Over 300 clones were analysed by ELISA. The 10 clones with the highest level of binding to *A. palestinensis* (OD $A_{405}-A_{690} > 0.8$) were selected for further study. All these clones showed cross reactivity with all other *Acanthamoeba* species studied (OD $A_{405}-A_{690}$ between 0.8-1.1). The polymerase chain reaction (Fig. 3) shows the diversity of the selected clones. For isolation of pathogenic *Acanthamoeba* specific antibody

fragments 200 clones were analysed by ELISA and 1 clone showed higher level of binding to *Acanthamoeba* sp. (Ros) compared to *A. astronyxis*.

5.4.2 - Flow Cytometry

Flow cytometry showed a high level of antibody fragment binding to *Acanthamoeba* cells with no binding to a range of other cell types (Table 1). Mean channel fluorescence for clone HPPG6 with *A. palestinensis* was 200 compared to 48 for cells stained with an irrelevant bacteriophage (Fig. 4). Other tested clones (HPPG8, HPPG11, HPPG28, HPPG31) also showed high level of binding to *Acanthamoeba* sp. (Fig. 5). Clone HPPG55, isolated for pathogenic *Acanthamoeba* sp. (Ros) showed mean channel fluorescence of 80 as compared to 20 for *A. astronyxis* (Fig. 6).

5.4.3 - Indirect immunofluorescence

The selected antibody fragments were also assessed by indirect immunofluorescence microscopy and were found to give similar results to flow cytometry. Figure 7 shows *A. palestinensis* stained with HPPG6. It can be seen from the photomicrograph that this antibody fragment binds uniformly, with a high intensity to all cells of *A. palestinensis*. Same antibody fragment when reacted with *Hartmannella* showed no fluorescence (Fig. 8).

Fig. 3. Showing the diversity of the selected clones. Lane 1-11, different clones and lane 12 is 10 bp DNA ladder.



Table 1. Reactivity of clone HPPG6 as determined by fluorescent microscopy.

| Organism | Reactivity |
|-----------------------------------|------------|
| <i>Acanthamoeba palestinensis</i> | + |
| <i>A. castellanii</i> | + |
| <i>A. polyphaga</i> | + |
| <i>A. astronyxis</i> | + |
| <i>A. griffinii</i> | + |
| <i>Acanthamoeba</i> sp. (ros) | + |
| <i>Acanthamoeba</i> sp. (nich) | + |
| Environmental isolates (7) | + |

| | |
|---------------------------------|---|
| <i>Hartmannella</i> sp.* | - |
| <i>Candida albicans</i> * | - |
| <i>Escherichia coli</i> * | - |
| <i>Haemophilus influenzae</i> * | - |
| <i>Neisseria</i> sp.* | - |
| <i>Pseudomonas aeruginosa</i> * | - |
| <i>Klebsiella aerogenes</i> * | - |
| Monocytes [#] | - |
| Lymphocytes [#] | - |
| Neutrophils [#] | - |

*These organisms are a common causes of eye infection.

[#]These cell types may be found on eye swabs.

Fig. 4. Showing flow cytometry analysis of specificities of phage clone HPPG6 a) reactivity with *A. palestinensis* and b) with *Hartmannella* sp.

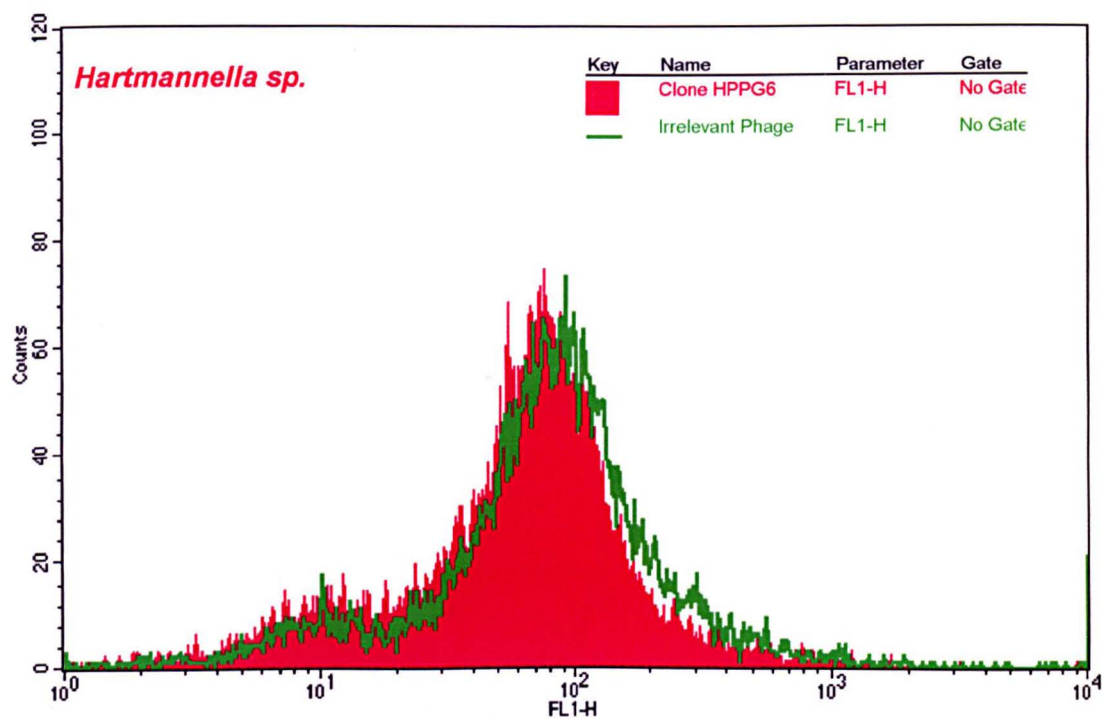
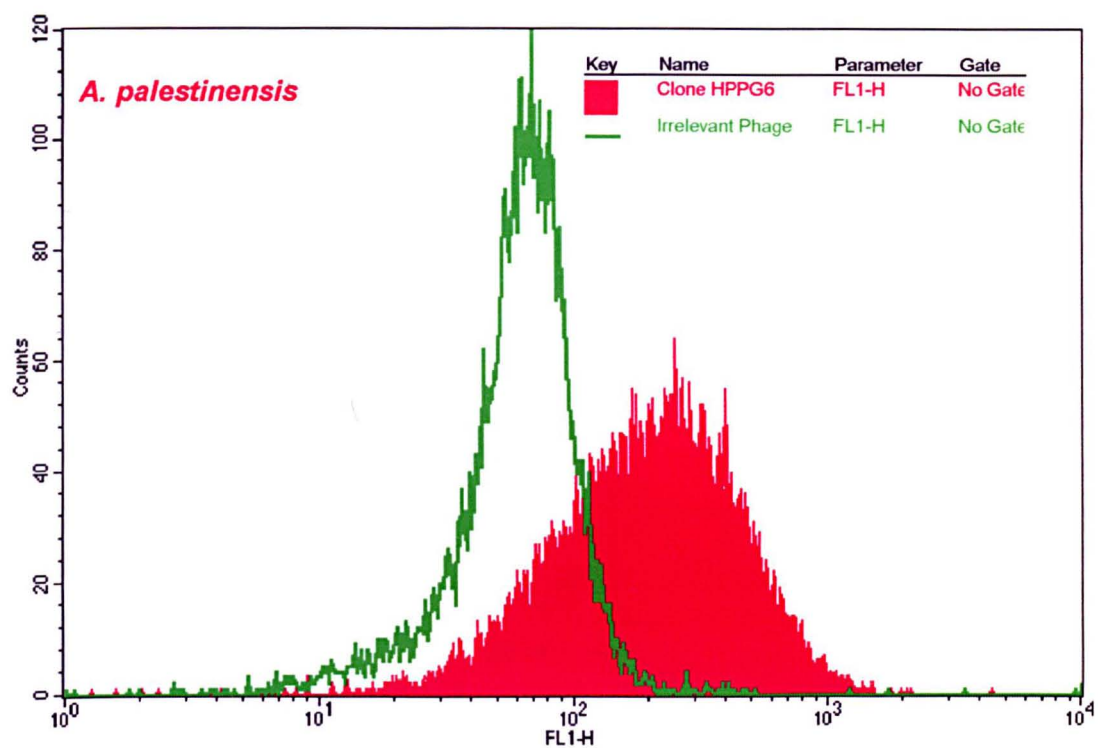


Fig. 5. Showing flow cytometry analysis of specificities of phage clones *with A. palestinensis*, a) Clone HPPG8; b) Clone HPPG11; c) Clone HPPG28 and d) HPPG31. Data 018 represents irrelevant phage.

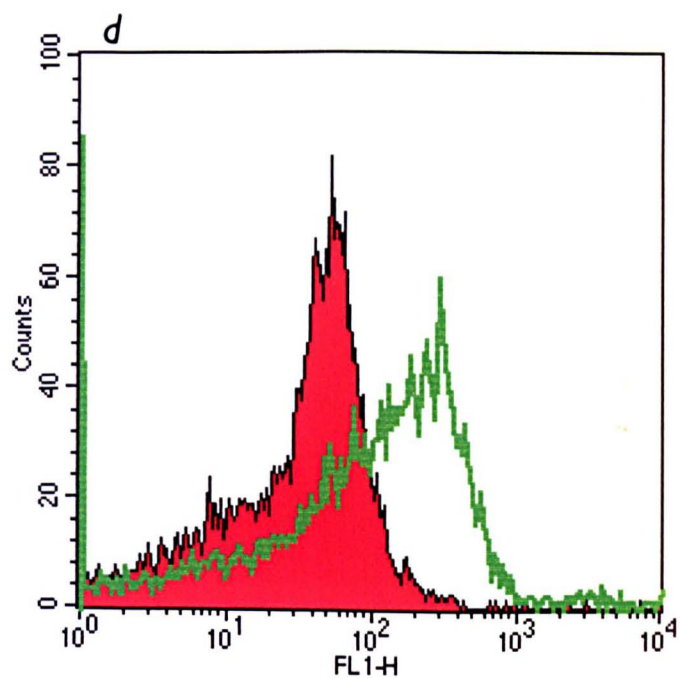
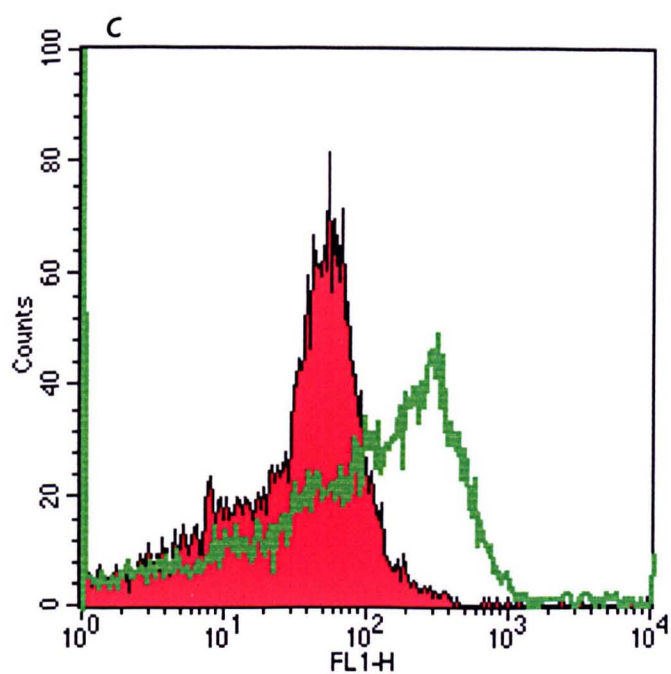
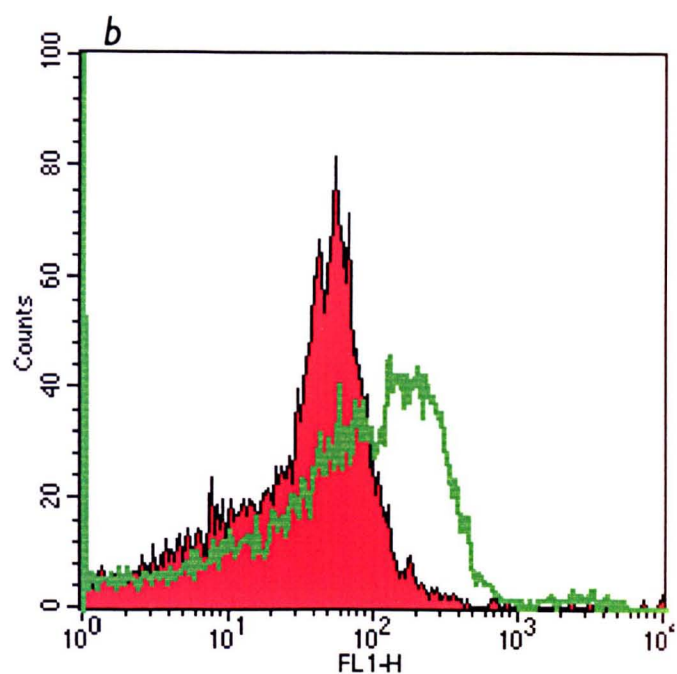
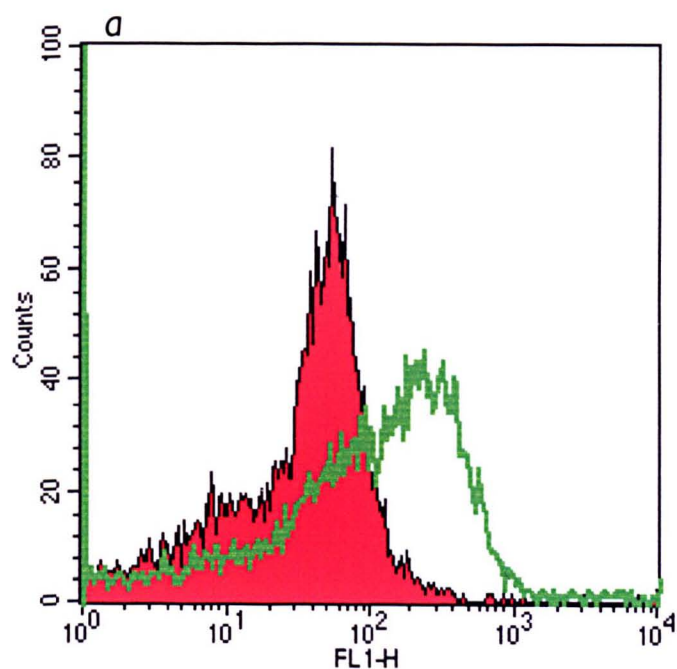


Fig. 6. Showing flow cytometry analysis of specificities of phage clone HPPG55 with a) *Acanthamoeba* sp. (Ros) and b) *A. astronyxis*.

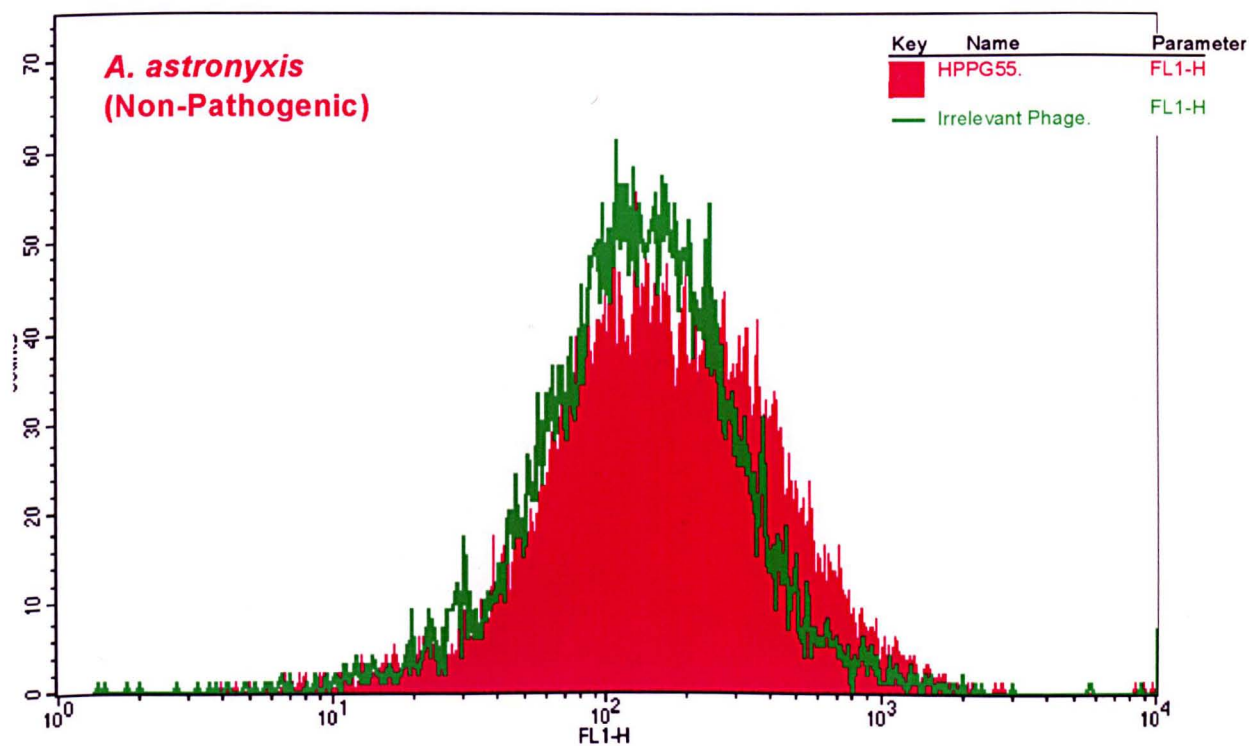
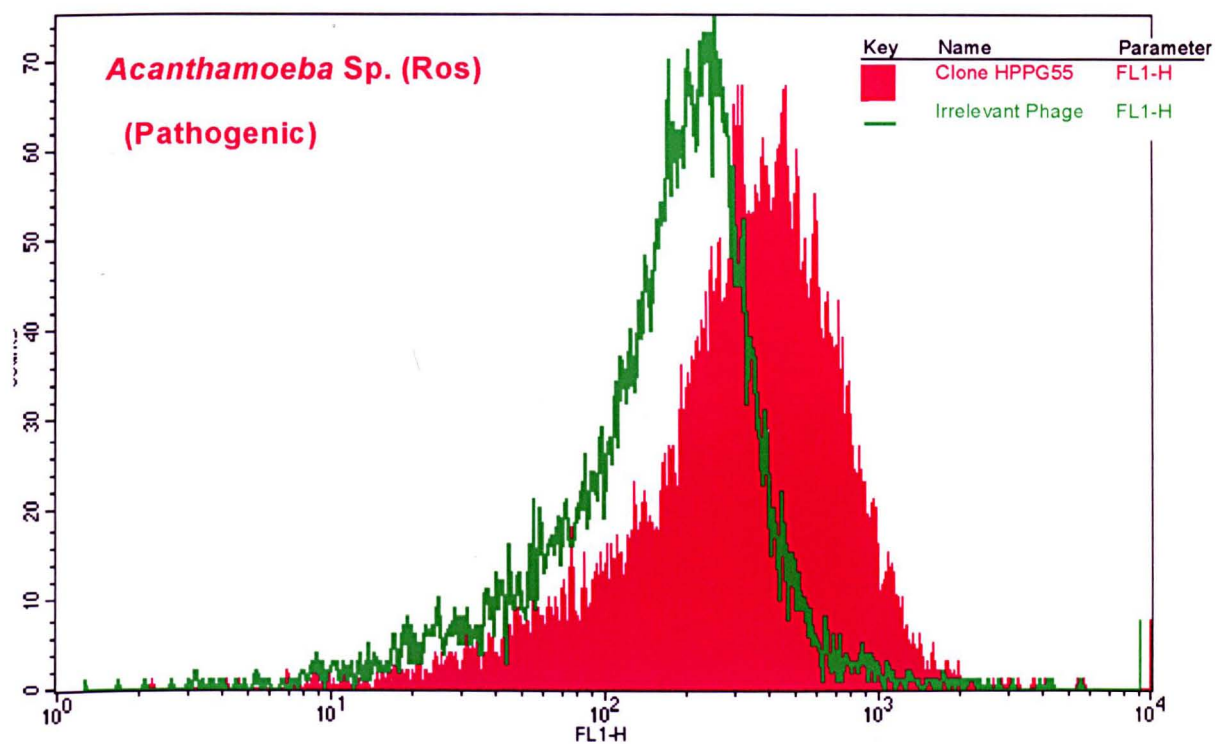
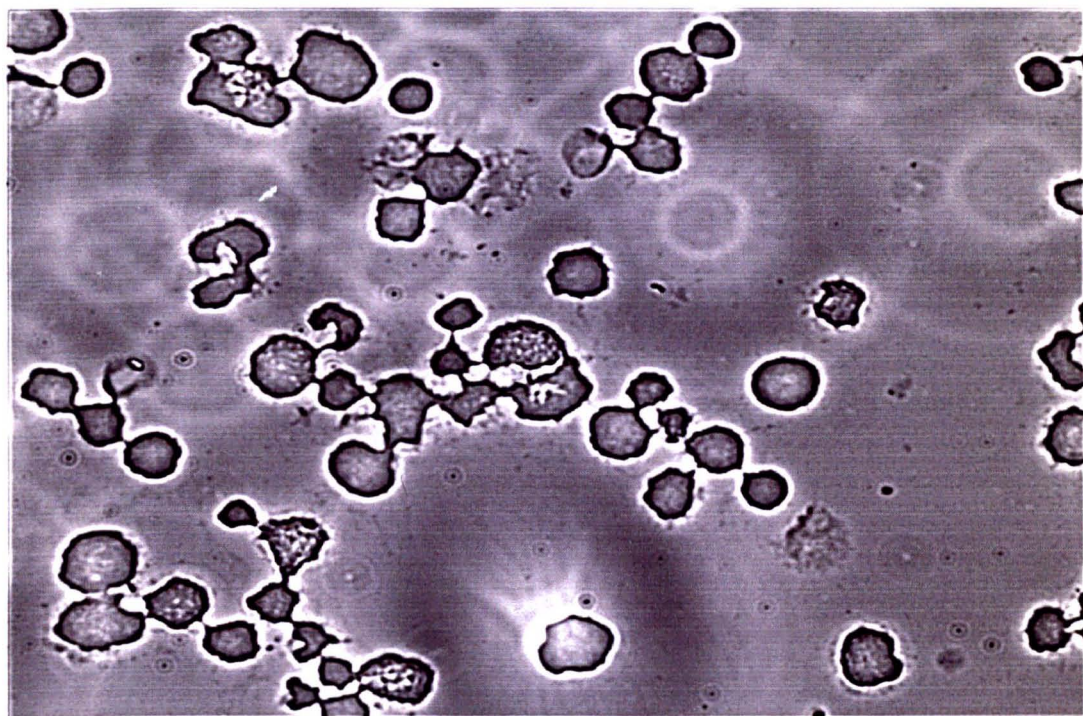
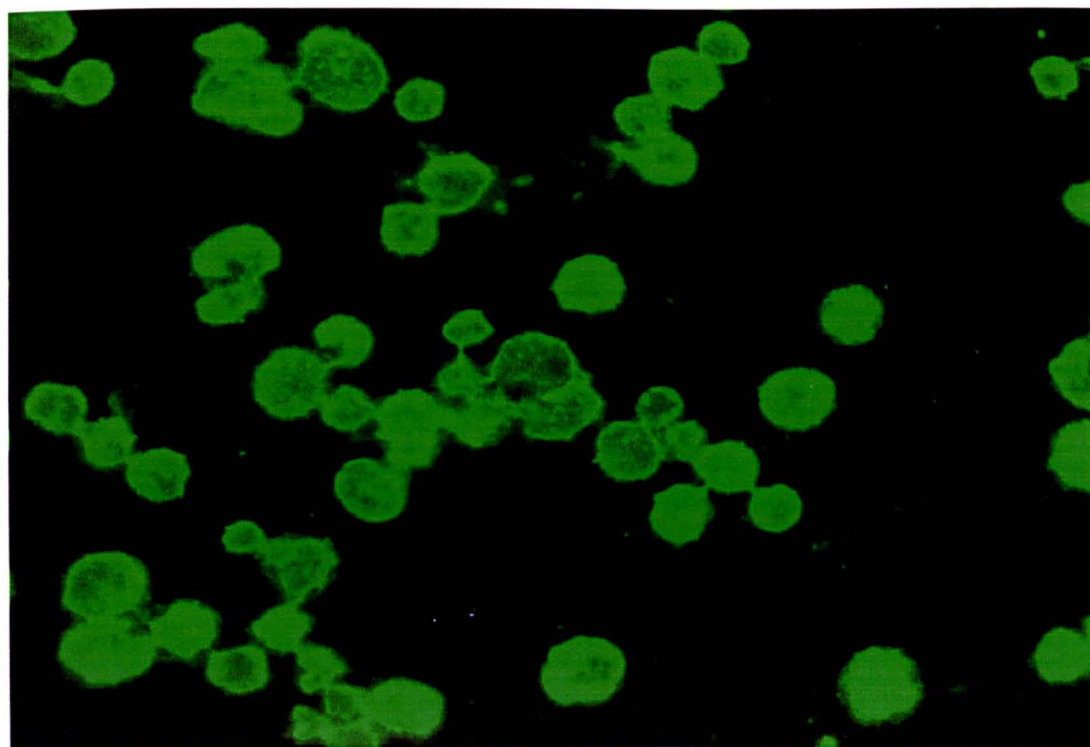


Fig. 7. Showing reactivity of phage clone HPPG6 against *A. palestinensis*. a) Under light microscope and b) Under fluorescent microscope.

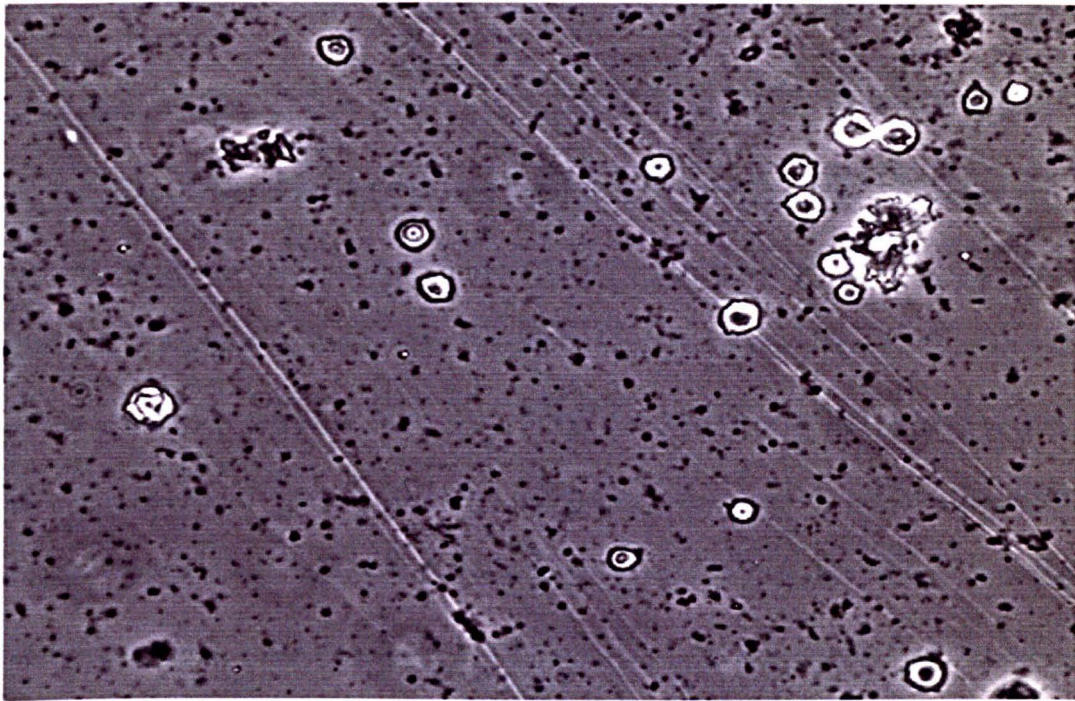


a)

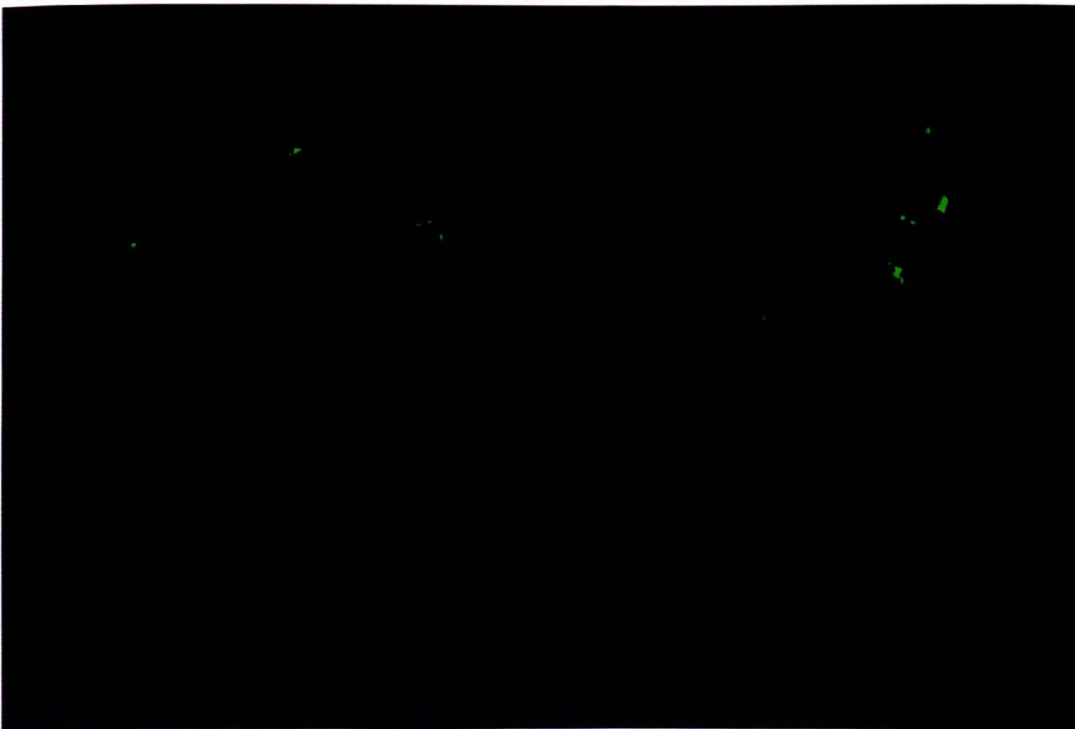


b)

Fig. 8. Showing reactivity of phage clone HPPG6 against *Hartmannella* sp. a) Under light microscope and b) Under fluorescent microscope.



a)



b)

5.5 - DISCUSSION

In this study we have shown that antibody fragments showing a high level of specificity for *Acanthamoeba sp.* can be isolated from a naive bacteriophage display library. This represents the first time that bacteriophage antibody display technology has been used in the development of an antibody for the identification of a microorganism. Previously, similar technology has been used successfully to isolate a melanoma-specific antibody, but this work required extensive screening of more than 1700 clones before a useful reagent was identified (Cai *et al.* 1995). It is significant that we have been able to isolate 10 clones with the desired specificities from an initial screen of only three hundred clones. This suggests that bacteriophage antibody display technology is ideally suited for the isolation of novel antibodies for use as research and diagnostic tools in clinical microbiology.

In our results we have also shown that these antibody fragments can be isolated to differentiate pathogenic and non-pathogenic *Acanthamoeba*. Although we did not observe the higher fluorescence as achieved with clones for genus specific identification, however, further studies are needed to isolate clones with high reactivity to pathogenic *Acanthamoeba* only. However, Our data suggest that antibody fragments can be isolated for genus and even species differentiation of *Acanthamoeba*.

Current methods for the rapid identification of *Acanthamoeba* involve staining preparations with Giemsa, calcofluor white, methylene blue or acridine orange. Culturing is also widely used in clinical laboratories for the reliable identification of *Acanthamoeba*.

However, accurate diagnosis and interpretation of results using these techniques requires a strong clinical suspicion of amoebic infection and highly trained personnel.

Other groups have utilised PCR as a rapid detection method for *Acanthamoeba* (Vodkin *et al.* 1992) but the specificity and sensitivity of this technique in a clinical environment has not been tested. Rabbit polyclonal antisera have also been used for the detection of *Acanthamoeba* keratitis and suspected *Acanthamoeba* meningoencephalitis. However, these reagents showed considerable cross reactivity with other cell types and once again the sensitivity of the assay has been questioned (Flores *et al.* 1990).

The need for a simple and rapid method for the specific detection of *Acanthamoeba* has become more urgent as *Acanthamoeba* keratitis becomes a more significant causative agent of eye keratitis, due to greater contact lens use and is increasingly associated with meningoencephalitis in immunocompromised individuals . We believe that the clones isolated in this study will form the basis of a rapid and unequivocal assay for the detection of *Acanthamoeba*. Thus bacteriophage antibody display libraries are potentially useful and powerful tools that allow the rapid generation of antibody reagents for use in diagnostic assays. Recently phage antibody display technology has been used in designing an antibody that recognises a given protein epitope (Kirkham *et al.* 1999).

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CHAPTER 6

Role of Proteases in Pathogenicity of *Acanthamoeba* spp.

6.1 - SUMMARY

Acanthamoeba keratitis is an eye threatening infection caused by pathogenic species of the genus *Acanthamoeba*. As not all *Acanthamoeba* spp. can cause keratitis, thus it is important to differentiate pathogenic species and isolates. Since extracellular proteases have been identified playing a role in ocular pathology associated with acanthamoebiasis (Mitro *et al.* 1994), we have used colorimetric, cytopathic and zymographic assays to differentiate extracellular protease activity in pathogenic and non-pathogenic *Acanthamoeba*.

Colorimetric and cytopathic assays showed clear differentiation among pathogenic and non-pathogenic *Acanthamoeba*. The zymographic assays showed various banding patterns for different strains of *Acanthamoeba*. In pathogenic *Acanthamoeba* all protease bands could be inhibited by PMSF suggesting serine type proteases while in non-pathogenic strains only partial inhibition was observed using PMSF. All pathogenic *Acanthamoeba* grown under normal environmental conditions with no epithelial cells showed one similar overexpressed protease band of approx. 107 kDa which was not observed in non-pathogenic *Acanthamoeba*. This protease is active from pH 5-9.5.

Cytopathic effect (CPE) and zymography assays were repeated using monolayers of primary corneal epithelial cells. Conditioned medium from pathogenic *Acanthamoeba* with primary corneal epithelial cells showed an inducible protease which was not observed in conditioned medium from non-pathogenic *Acanthamoeba* with primary corneal epithelial cells or from *Acanthamoeba* conditioned medium alone or primary corneal epithelial cells alone.

6.2 - INTRODUCTION

Proteinases are degradative enzymes which catalyse the total hydrolysis of proteins. Proteinases exhibit a variety of complex physiological functions. They play an important role in many pathological processes such as protein catabolism, blood coagulation, inflammation, tumor growth, release of hormones and transport of secretory proteins across membranes (Rao *et al.* 1998). In general, extracellular proteases catalyse the hydrolysis of large proteins into smaller molecules for subsequent absorption by the cell whereas intracellular proteases play a role in the regulation of cell function (Rao *et al.* 1998). The microbial extracellular proteases are primarily involved in keeping the cells alive by providing them with the necessary amino acid pool as nutrition (Mala *et al.* 1998). Advances in analytical techniques have demonstrated that proteases can also conduct highly specific and selective modifications of proteins such as activation of zymogenic forms of enzymes by limited proteolysis (Mala *et al.* 1998). Since proteases are physiological necessary for living organisms, they are found in a wide diversity of sources such as plants, animals and microorganisms. Proteases are subdivided into two major groups i.e., exopeptidases and endopeptidases depending on their site of action. Exopeptidases cleave the peptide bonds proximal to the amino or carboxy terminal of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate. Based on the functional group at the action site, proteases are classified into four groups i.e., serine, aspartic, cysteine and metallo proteases (Hartley 1960). In addition, there are a number of proteinases for which the catalytic mechanisms are unknown (Barrett *et al.* 1991). McKerrow *et al.* (1987) have shown that proteinases play an important role in tissue invasion, migration and host pathology of other amoeboid

parasite, *Entamoeba histolytica*. Proteinase activity has been reported in a number of other protozoa including *Giardia*, *Trypanosoma cruzi* and *Leishmania* (McKerrow *et al.* 1993; Hare *et al.* 1989; Bonaldo *et al.* 1991; Prina *et al.* 1990). The amount of proteolytic enzymes produced by *E. histolytica* has been correlated with the virulence showing the importance of proteinases (Gadasi *et al.* 1983; Munoz *et al.* 1984). The importance of proteinases in *Acanthamoeba* has also been shown by a other groups (Mitra *et al.* 1995; Mitro *et al.* 1994; Hadas *et al.* 1993; Cao *et al.* 1998). Excretory and secretory products from trophozoites of *Acanthamoeba* induced damage to collagen shields in an *in vitro* and an *in vivo* in rat cornea (He *et al.* 1990).

In this study we have shown that extracellular proteases of *Acanthamoeba* are useful markers for differentiating pathogenic from non-pathogenic strains.

6.3 - MATERIALS AND METHODS

6.3.1 - Culture of parasites and corneal epithelial cells

All chemicals were obtained from Sigma. All species and isolates of *Acanthamoeba* (Table 1) were grown axenically in PYG medium (See Chapter 2). In total, 9 *Acanthamoeba* were used, among them 2 were isolated from human infected eye and the remaining 7 were obtained from either CCAP or ATCC as described in Table 1.

Immortalized corneal epithelial cells were kindly provided by Mr. Walton (Dept. of Medicine, University of Hull, Hull, HU6 7RX, UK.) and grown in MEM without serum at 37°C in 5% CO₂. Primary cell cultures of rabbit corneal epithelium were prepared

according to published protocols (Jumblatt *et al.* 1983; Panjwani *et al.* 1990) using rabbit eyes from Pel-Freez Biologicals (Rogers, Ark. USA) as described in Chapter 2.

For the assays of extracellular protease activity, cytopathic effect and zymography *Acanthamoeba* (1×10^6) were incubated in MEM (Minimum Essential Medium) without serum at 37°C in a 5% CO₂ incubator for 24h. *Acanthamoeba* were removed from the medium by centrifugation (100 x g for 5 min) and the supernatant, *Acanthamoeba* conditioned medium (ACM), was used for the assays. UV killed *Acanthamoeba* were used to determine protease released from cells during the centrifugation process.

6.3.2 - Protease assays

Protease activity in conditioned medium was determined by a colorimetric method (Sarath *et al.* 1994) using azoalbumin or azocasein as substrates. Briefly, 200 µl of 1 mg / ml of substrate in PAS was incubated with 100 µl of ACM for 60 min. Reactions were stopped by adding 10% trichloro acetic acid (TCA). The mixture was shaken, then left for 15min. centrifuged (400 x g for 15 min) and finally, 1ml of supernatant was added to 1ml of NaOH (1 M). Absorbance of this solution was determined at 440 nm and then converted to units of protease activity as follows.

$$(\text{Absorbance}) / (\text{Extinction Coefficient}) (10^3) = \mu \text{ moles of dye}$$

and converted to units of enzyme using,

$$1 \text{ unit of enzyme} = 1 \mu \text{ mole substrate attacked per minute}$$

Table 1. *Acanthamoeba* tested in the present study.

| No | Species | Strain | Pathogenicity tested by CPE assays |
|----|--|--------------|------------------------------------|
| 1 | <i>A. astronyxis</i> (Sp7) | CCAP 1534/1 | Non-pathogen |
| 2 | <i>A. royreba</i> (Sp9) | CCAP 1501/7 | Non-pathogen |
| 3 | <i>A. palestinensis</i> (Sp8) | CCAP 1547/1 | Non-pathogen |
| 4 | <i>A. polyphaga</i> (Sp6) | CCAP 1501/3C | Non-pathogen |
| 5 | <i>A. castellanii</i> (Sp5) | ATCC 30234 | Pathogen |
| 6 | <i>Acanthamoeba</i> sp. (Sp1, Ros) | | Pathogen |
| 7 | <i>Acanthamoeba</i> sp. (Sp2, Nich) | | Pathogen |
| 8 | <i>A. polyphaga</i> (Sp4) | ATCC 30871 | Pathogen |
| 9 | <i>A. griffini</i> (Sp3) | CCAP 1501/4 | Unknown |

6.3.3 - Cytopathic effect assays (CPE)

Pathogenicity of whole *Acanthamoeba* cells was assayed by observing disruption of epithelial cell monolayers essentially as described by Cao *et al.* (1998). Briefly, primary and immortalized corneal epithelial cells were grown to a monolayer in 4 well plates. Pathogenic and non-pathogenic *Acanthamoeba* or ACM were added to these monolayers and plates were incubated at 37°C in a 5% CO₂ incubator for 12-24h. Monolayers were either visualized by eosin staining or cytotoxicity determined using LDH assay (Cytotoxicity detection kit, Boehringer Mannheim). For the assay hydrogen peroxide (1% v/v) was used to kill the cells and give a 100% control value. A control with epithelial cells only was used to give a zero value. The effects of ACM on epithelial cells were also assayed using the same methods.

In addition scanning electron microscopy was performed (as described in Chapter 3, section 3.3.3), on *A. castellanii* with rabbit primary corneal epithelial cell monolayers (after 1-2 h incubation at 37°C, 5% CO₂) in order to look at the adhesion and any morphological changes in *Acanthamoeba*.

6.3.4 - Zymography

Polyacrylamide gels containing gelatin (2 mg / ml) were used for zymography of ACM from pathogenic and non-pathogenic species as well as from *Acanthamoeba* incubated with primary and immortalized epithelial cells essentially as described previously (Cao *et al.* 1998). For the assays, 5 µl of ACM were diluted with electrophoresis sample buffer (Tris-HCl , pH 6.8, containing 2% DTT, 20% SDS, 15% glycerol, 0.5% Bromophenol blue) and then applied to the separating gels containing 10% acrylamide

containing gelatin. After electrophoresis (electrophoresis buffer, 0.025M Tris, 0.192M glycine, 0.1% SDS, pH 8.3) gels were soaked in 2.5% w/v Triton-X-100 solution for 60 min. to remove SDS and incubated in a developing buffer (50mM Tris-HCl, pH 7.5, containing 10mM CaCl₂ at 37°C overnight). Some gels were incubated with 1, 10-phenanthroline, a metallo-proteinase inhibitor. Gels were finally stained with Coomassie Brilliant Blue. Areas of digestion were visualized as nonstaining regions of the gel. In some experiments, samples were pre-treated with phenylmethylsulfonyl fluoride (PMSF, 1mM), an inhibitor of serine proteinases, for 30 min prior to electrophoresis.

6.4 - RESULTS

6.4.1 - Protease assays

A clear distinction was observed between protease activity in pathogenic and non-pathogenic species of *Acanthamoeba* (Fig. 1). All pathogenic species showed significantly higher protease activity than that of the non-pathogenic species (p value: 0.000197, t-statistic: 13.0826, calculated using unpaired parametric t-test with SlideWrite Plus Version 3 for Windows) except for *A. griffini* (Sp3), which exhibited intermediate activity.

6.4.2 - CPE assays

Staining the cell monolayers revealed that only pathogenic species of *Acanthamoeba* (Fig. 2a) and ACM disrupted epithelial cell monolayers after 12 h. (Fig. 2b). The results for the LDH assays showed that pathogenic species of *Acanthamoeba* produced significant cytotoxicity (>94%) in epithelial cells after incubation for 24h. No epithelial cell cytotoxicity was detected in incubations with non-pathogenic species or ACM (Table 2).

Figure 1. Pathogenic *Acanthamoeba* showing elevated levels of proteases using colorimetric assay. Sp1 is *Acanthamoeba* sp. (Ros); Sp2 is *Acanthamoeba* sp. (Nich); Sp4 is *A. polyphaga* ATCC 30871; Sp5 is *A. castellanii*; Sp3 is *A. griffini*, Sp6 is *A. polyphaga* CCAP 1501/3c and Sp7 is *A. astronyxis*. Each assays was performed in triplicates (n = 3), error bar represents \pm SD.

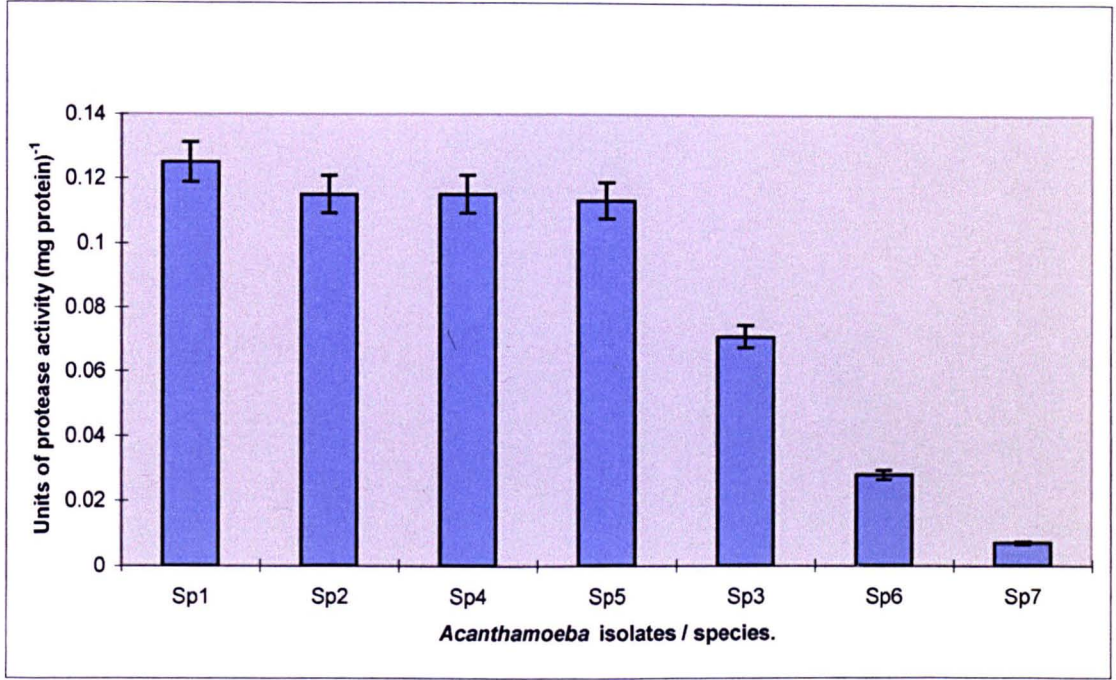
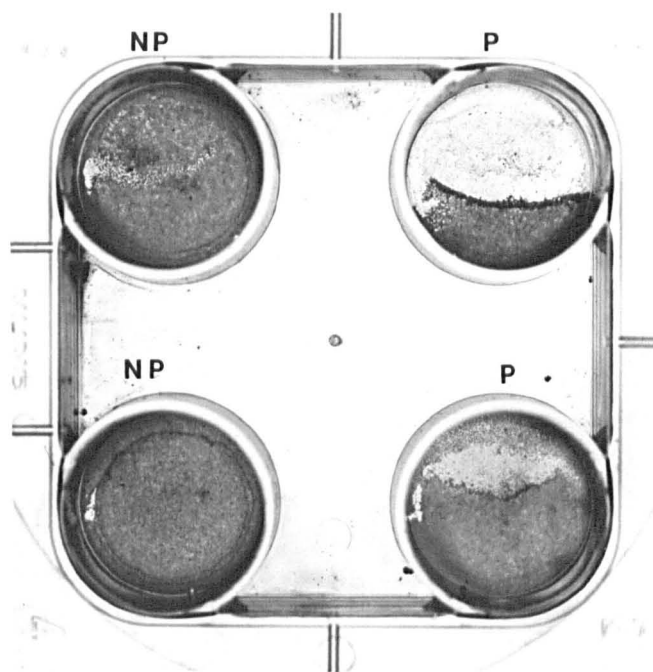
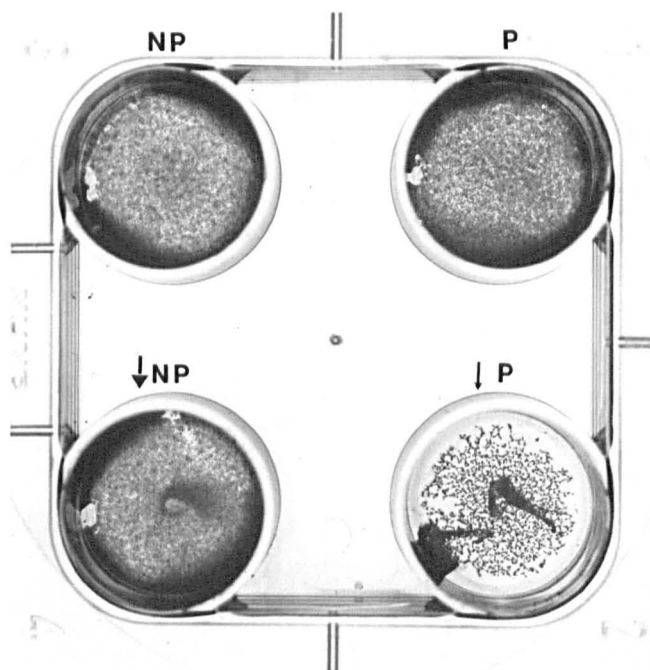


Figure 2a) The effects of whole *Acanthamoeba* cells (pathogens and non-pathogens) on immortalized corneal epithelial cells. All tested pathogenic strains showing clearing zones. P represents pathogenic and NP represents non-pathogenic species / isolates. 2b) The epithelial cell monolayer disruption using 10% & 30% ACM of *Acanthamoeba* sp. (Sp1 & Sp7), (well containing 30% ACM are shown by arrow).



a



b

Table 2. Cytotoxicity determined using LDH assays. Pathogenic species (P) and non-pathogenic (NP). Each assay was performed in triplicates (n=3), \pm represents SD.

| <i>Acanthamoeba</i> sp. | % Cytotoxicity |
|--|----------------|
| <i>Acanthamoeba</i> sp. (Sp1, Ros) {P} | 99% \pm 0.42 |
| 30% v/v of ACM from <i>Acanthamoeba</i> sp. (Sp1, Ros) [CO ₂] | 0% |
| 30% v/v of ACM from <i>Acanthamoeba</i> sp. (Sp1, Ros) [O ₂] | 0% |

Scanning electron microscopy results revealed the presence of amoebastomes (structures associated with the phagocytosis) and a firm binding to epithelial cell monolayer (Fig. 3).

6.4.3 - Zymography

Conditioned medium from all species of *Acanthamoeba* tested showed different protease banding patterns on gelatin gels (Fig. 4) and these were unchanged when cells were incubated with immortalized epithelial cells (Fig. 5, lane 1 & 2). However, conditioned medium from pathogenic species with immortalized epithelial cells, incubated in 5% or in atmospheric concentration of CO₂ (Fig. 5) showed a marked change. One protease becomes predominant when cells are incubated at atmospheric concentrations of CO₂. This protease has a molecular mass of 107 kDa (Fig. 6) and was inhibited by 1mM PMSF. The pH profile of its activity revealed that this protease is active between pH 5-9.5 (data not shown). Interestingly, this protease showed overexpression only in pathogenic species of *Acanthamoeba* (Fig. 6).

Conditioned medium from *Acanthamoeba* with primary epithelial cells was removed every 2 h and zymography was performed to look at any inducible protease activity. All pathogenic species show a discrete inducible protease of approx. 55 kDa (Fig. 7).

Fig. 3. Pathogenic *A. castellanii* adhering to a primary corneal epithelial cell prior to infection. The structures observed on the surface are amoebastomes. Photograph was taken using SEM.

L= SEI

EHT= 15.0 KV WD= 6 mm
5.00µm

PHOTO= 2

Ros

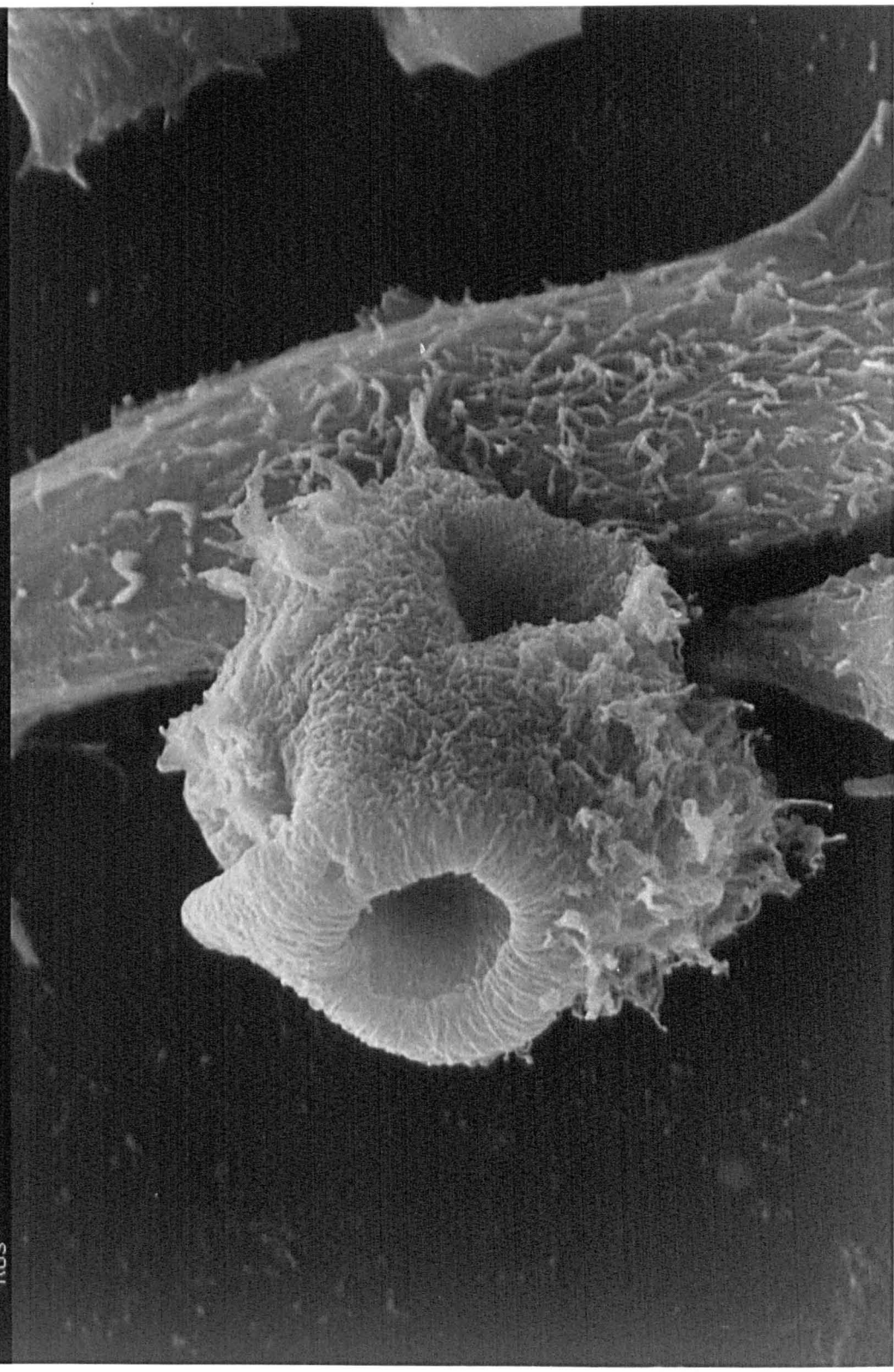


Fig. 4. Extracellular protease activity of different species of *Acanthamoeba* incubated in MEM alone. The gel was incubated at 37°C, pH 7.5 with 10 mM CaCl₂. Lane 1, *Acanthamoeba* sp. (Sp2, Nich); lane 2, *Acanthamoeba* sp. (Sp1, Ros); lane 3, *A. castellanii* (Sp5); lane 4, *A. polyphaga* ATCC 30871 (Sp4); lane 5, *A. griffini* (Sp3); lane 6, *A. polyphaga* CCAP 1501/3c (Sp6); lane 7, *A. astronyxis* (Sp7) and lane 8 is *A. palestinensis* (Sp8).

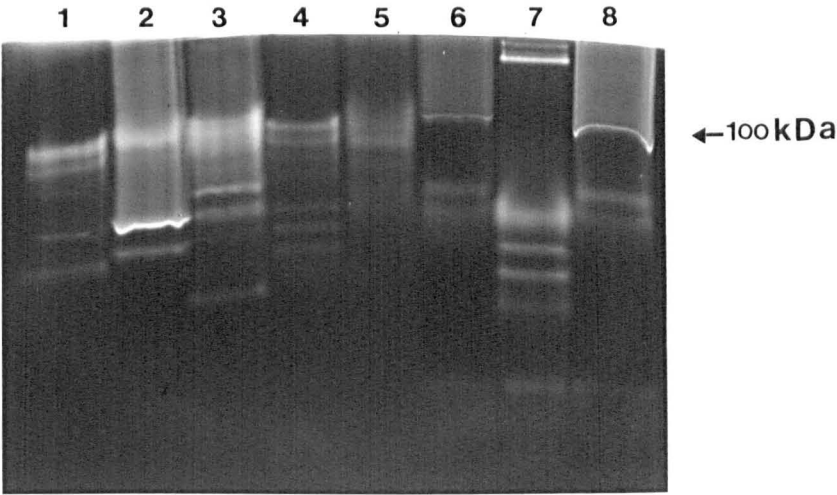


Fig. 5. Protease activity using different environmental conditions in pathogenic *Acanthamoeba* sp. (Sp2), with immortalized epithelial cells. Lane 1, ACM from *Acanthamoeba* (Sp2) incubated with epithelial cells; lane 2, *Acanthamoeba* (Sp2), incubated without epithelial cells at 37°C in 5% CO₂ incubator; lane 3, *Acanthamoeba* (Sp2) incubated without epithelial cells at 37°C in atmospheric concentration of CO₂; lane 4, ACM from Sp2 treated with 1mM PMSF showing complete inhibition; lane 5, ACM from *A. astronyxis* (Sp7) and lane 6 is ACM from *A. astronyxis* (Sp7) treated with 1mM PMSF showing partial inhibition.

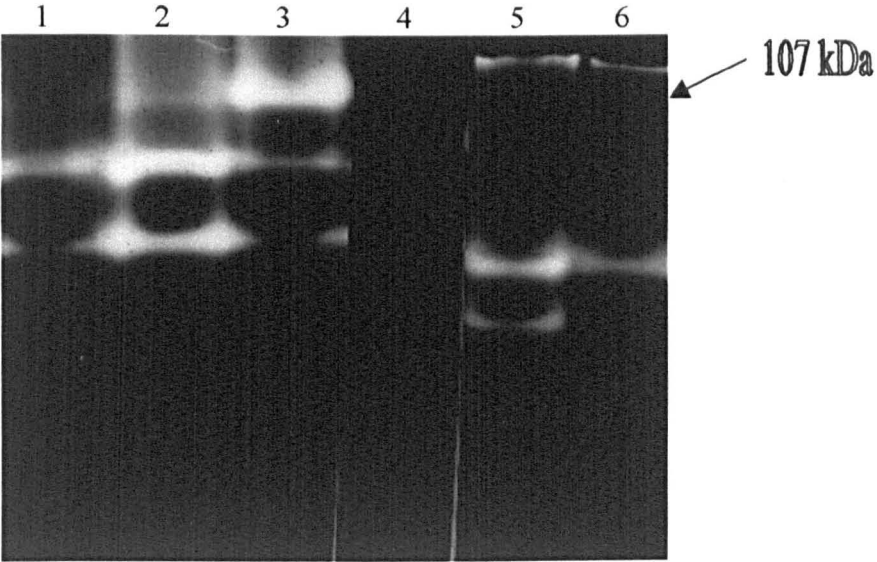
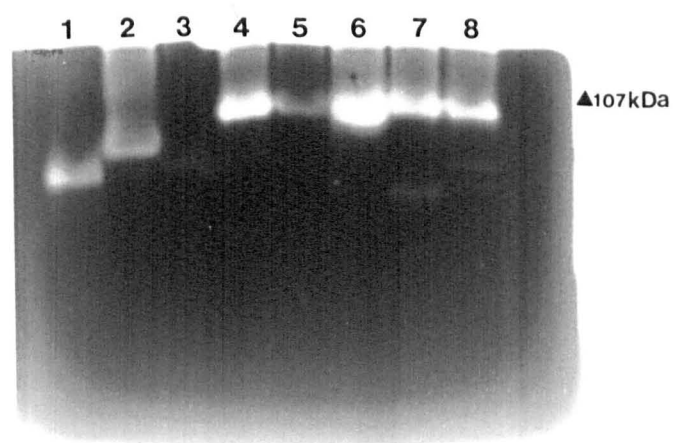


Fig. 6. Shows overexpression of a 107 kDa protease in pathogenic species of *Acanthamoeba* only. Lane 1, *A. astronyxis* (Sp7); lane 2, *A. palestinensis* (Sp8); lane 3, *A. polyphaga* CCAP 1501/3c (Sp6); lane 4, *Acanthamoeba* sp. (Sp1, Ros); lane 5, *A. griffini* (Sp3); lane 6, *A. castellanii* (Sp5); lane 7, *A. polyphaga* ATCC 30871 (Sp4) and lane 8 is *Acanthamoeba* sp. (Sp2, Nich).



Inhibitory studies showed that this protease can be inhibited by 1, 10-phenanthroline suggesting that it is a metallo-protease. This protease can be seen 6-8 h after the incubation of pathogenic *Acanthamoeba* with primary corneal epithelial cell cultures. Same samples ran after 6 days (kept at 4°C) did not show this protease. Interestingly, when samples are treated with PMSF prior running, this protease reappears or the activity gets better in the gel (Fig. 7). Non-pathogenic *Acanthamoeba* did not show any inducible protease (Fig. 8).

6.5 - DISCUSSION

In this study we have shown that pathogenic and non-pathogenic species of *Acanthamoeba* can be differentiated on the basis of extracellular protease activity using a simple colorimetric assay. Also cytotoxicity assays showed that pathogenic species produce significant levels of cytotoxicity. However, it is interesting that disaggregation of epithelial cells occurred when ACM, from pathogenic *Acanthamoeba* cells was added to corneal epithelial monolayers. This represents the first evidence that extracellular proteases in *Acanthamoeba* play some role in epithelial cell disaggregation.

All tested pathogenic *Acanthamoeba* in our study showed a variety of protease banding patterns in zymography but all these strains showed high protease activity in colorimetric assays highlighting the significance of total protease activity in differentiation of pathogenic and non-pathogenic *Acanthamoeba*.

Fig. 7. Zymogram showing an inducible protease in pathogenic species of *Acanthamoeba*. Lane 1, conditioned medium from primary corneal epithelial cells alone; lane 2, *Acanthamoeba* conditioned medium (ACM) from Sp2 (Nich) alone; lane 3 ACM from Sp2 treated with PMSF; lane 4, conditioned medium from Sp2 with epithelial cell monolayers; lane 5, conditioned medium from Sp2 with epithelial cells treated with PMSF; lane 6, ACM from Sp1 (Ros) alone; lane 7, ACM from Sp1 treated with PMSF; lane 8, conditioned medium from Sp1 with epithelial cells; lane 9, conditioned medium from Sp1 with epithelial cells treated with PMSF.

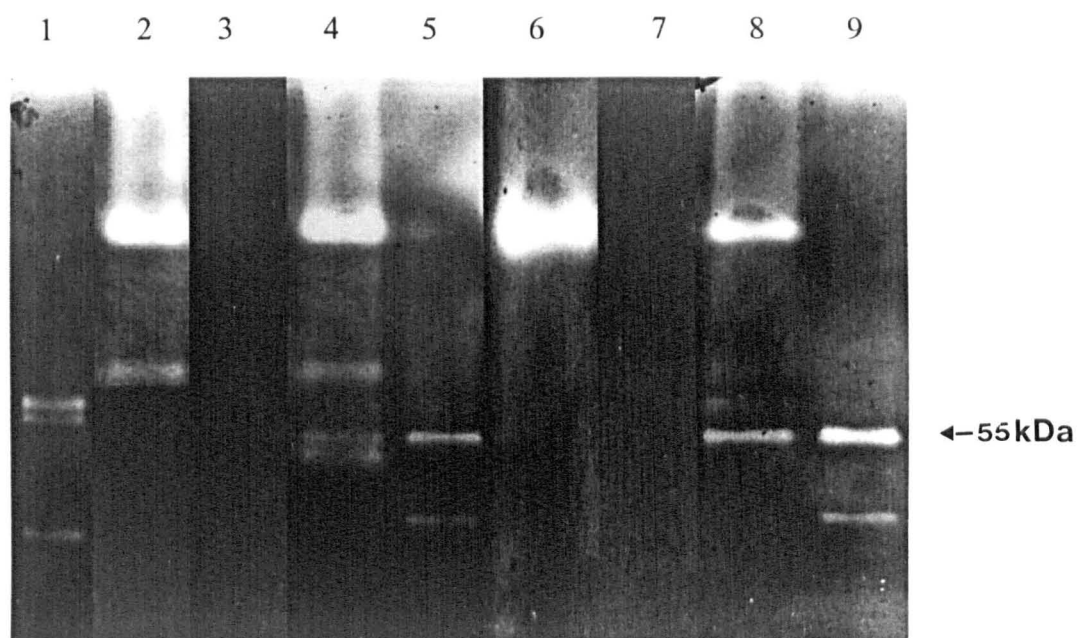
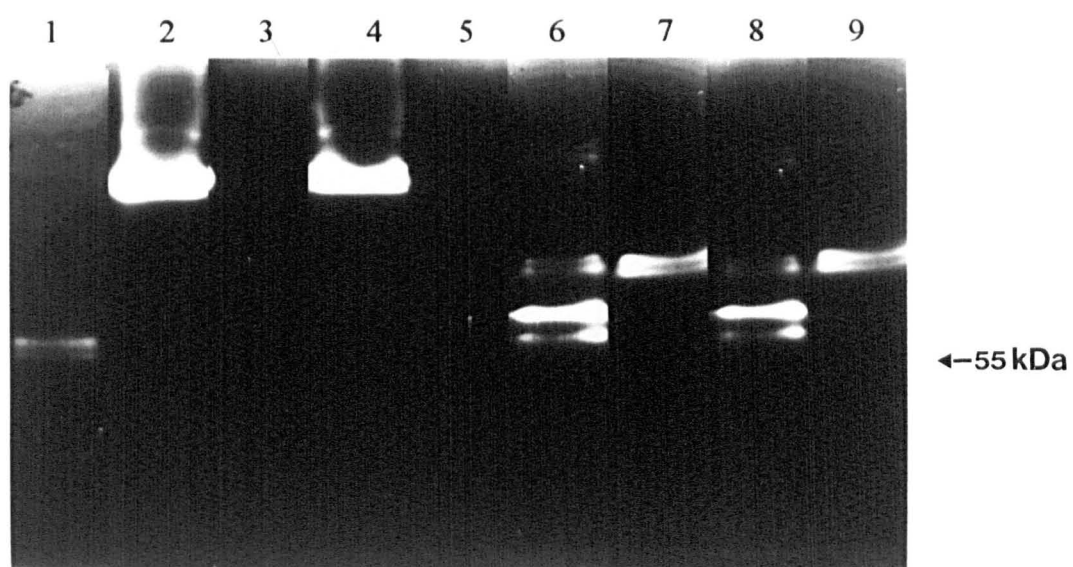


Fig. 8. Zymogram showing no inducible protease in non-pathogenic species of *Acanthamoeba*. Lane 1, conditioned medium from primary corneal epithelial cells alone; lane 2, *Acanthamoeba* conditioned medium (ACM) from Sp8 (*A. plaestinensis*) alone; lane 3 ACM from Sp8 treated with PMSF; lane 4, conditioned medium from Sp8 with epithelial cell monolayers; lane 5, conditioned medium from Sp8 with epithelial cells treated with PMSF; lane 6, ACM from Sp7 (*A. astronyxis*) alone; lane 7, ACM from Sp7 treated with PMSF; lane 8, conditioned medium from Sp7 with epithelial cells; lane 9, conditioned medium from Sp7 with epithelial cells treated with PMSF.



We have also shown that an inducible protease is observed, 6-8 h, after pathogenic *Acanthamoeba* introduced into primary corneal epithelial cell cultures thus confirming the results of Cao *et al.* (1998). In our results we have observed that all pathogenic *Acanthamoeba* tested secrete serine type proteases along with inducible metallo-protease. These serine type proteases may degrade the inducible metallo-protease as it can be recovered by treating the conditioned medium with PMSF prior running. Since the inducible metallo-protease was only observed in the first few hours of incubation with primary cultures, this suggest that the inducible protease may play some role in the initial stages of infection. In scanning electron microscopy amoebastomes were observed during the first 1-2 h of incubation of pathogenic *Acanthamoeba* with corneal epithelial cell monolayers. These structures are responsible for phagocytosis and thus may well be involved to some extent in the pathogenicity of *Acanthamoeba*. Phagocytosis has been shown in a number of protozoan parasites (Heat 1981; Rendon-Maldonado *et al.* 1998).

The mechanisms of *Acanthamoeba* induced cytopathic effects are not known, but here we present data suggesting that inducible metallo-protease may play a role in the initial stages of infection and then higher quantities of extracellular proteases in pathogenic strains compared with non-pathogenic may well be responsible for epithelial cell destruction. The proteinase activity in pathogenicity has already been reported for a number of protozoan parasites including *Entamoeba histolytica* (Reed *et al.* 1989, Keene *et al.* 1990), *Giardia lamblia* (Hare *et al.* 1989), *Leishmania amazonensis* (Prina *et al.* 1990) and *Trypanosoma cruzi* (Bonardo *et al.* 1991). Our data shows inducible protease as well as higher extracellular proteases in pathogenic species as compared to non-pathogens which could be used as a marker in differentiation of *Acanthamoeba* sp.

Moreover, overexpression of 107 kDa protease in pathogenic species / isolates of *Acanthamoeba* only could also be of diagnostic value in differentiation.

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CHAPTER 7

Discussion

Acanthamoeba spp. are ubiquitous free living protozoa found in a wide range of environments. They are resistant to disinfectants, temperature variation and desiccation and are responsible for two recognised diseases in humans, granulomatous amoebic encephalitis and keratitis (Walker 1996). Both infections are rare although latter is increasing following the association of between *Acanthamoeba* and contact lens (Walker 1996). *Acanthamoeba* keratitis, a serious corneal infection, was first recognized in 1973 (Jones *et al.* 1975). Usually *Acanthamoeba* keratitis is not diagnosed until pathologic examination of the excised corneal tissues. This is due to the lack of availability of rapid assays to identify and differentiate *Acanthamoeba* spp. In this study we have developed a number of robust methods that can be used for *Acanthamoeba* detection and differentiation. We have also performed some preliminary studies on proteases in order to understand the molecular basis of pathogenicity.

7.1 - Morphological and growth assay studies

Culturing is the most common method available for *Acanthamoeba* identification. Unlike usual techniques, culture for *Acanthamoeba* sp. uses bacteria as a source of nutrition. This is known as monoxenic culture. It has been reported as well as observed in our studies that the ability of *Acanthamoeba* sp. to grow is affected by the bacterial species chosen (Casemore *et al.* 1992). Higher temperatures have been used in order to differentiate various *Acanthamoeba* species (De Jonckheere 1980; Warhurst 1985). In our studies we observed successful differentiation of pathogenic and non-pathogenic *Acanthamoeba* on the basis of their ability to grow at high osmolarity (1 M) and temperatures (37°C), (See Chapter 2). Also we have found significant differences in the

morphology of S. E. M studies between pathogenic and non-pathogenic species of *Acanthamoeba* (See Chapetr 3).

7.2 - Isoenzymes

Previously, several groups have used analysis of isoenzyme electrophoretic patterns to test the morphological classification scheme. These studies discovered extensive diversity among isolates of *Acanthamoeba*. Moura *et al.* (1992), found good agreement between isoenzyme patterns and morphological groups but their study was limited and included only one isolate of *A. castellanii*. De Jonckheere (1983), Daggett *et al.* (1985) and Costas *et al.* (1986), studied larger groups of isolates (30, 71 and 37 strains respectively). Each study divided isolates of *Acanthamoeba* into several different groups that often were inconsistent with species and morphological groups designations. Weekers *et al.* (1997) used isoenzyme analysis to differentiate axenically and monoxenically grown *Acanthamoeba* and *Hartmannella* sp. They found differences in isoenzyme patterns when amoeba were cultured either on a bacterial substrate or in axenic cultures. Therefore, isoenzyme analysis for the identification of amoeba should be used with caution when different growth conditions are used to grow the amoeba. However for the purpose of strain identification or phylogenetic analysis axenically grown amoeba should be used. According to DeJonckheere (1987), *A. pustulosa* and *A. palestinensis* had identical isoenzyme patterns although previously Costas *et al.* (1984) recorded distinct differences in the acid phosphatase and esterase isoenzyme profiles of the two species thus there is a need for revision of the classification. In our assays, different isoenzyme patterns were observed for *A. polyphaga* ATCC 30871 and *A. polyphaga* CCAP 1501/3c

(See Chapter 3). Thus, isoenzyme patterns have highlighted ambiguities in the morphology based classification scheme but this approach itself does not address the phylogeny of *Acanthamoeba* due to its lack of reproducibility. Another drawback to this technique is the requirement of large number of axenically grown parasites which is both time consuming and laborious.

7.3 - Genotyping

In the past 20 years, genetic and molecular methods for characterizing pathogen strains have taken a major place in modern approaches in diagnostic and epidemiological studies of parasitic and other infectious diseases (Tibayrenc 1996). These techniques are more sensitive than the conventional morphological and isoenzyme methods. Studies on numerous protozoan parasites using these techniques have shown the inadequacies of assigning parasite species on the basis of morphology, host occurrence or site of infection. The most recent demonstration of this comes from studies on *Trichomonas*. *Trichomonas foetus* is a venereally transmitted pathogen of cattle, whereas the parasite *T. suis* occurs in the small intestine of pigs. However, species distinction has recently been thrown into question with convincing evidence from the molecular characterization of isolates from cattle and pigs, which has shown them to be genetically identical (Felleisen 1998).

Genetic characterization of pathogens allow their identification at the strain, subspecies or species levels and explore the impact of genetic diversity on relevant biomedical parameters such as virulence or resistance to drugs (Tibayrenc 1995; Tibayrenc 1996).

Gast *et al.* (1996) investigated 18S rRNA gene phylogeny using 18 isolates of *Acanthamoeba*. The amount of dissimilarity among the 18 isolates ranged from 0 – 11.9%. Fifteen of the isolates formed a tight phylogenetic cluster and the remaining three isolates, each had sequences that were very distinct from the major group. In a recent study Stothard *et al.* (1998), examined 18S rDNA sequences variation in a group of 53 strains that includes 18 isolates of Gast study plus 35 new isolates. Results showed 12 sequences types and have discovered that morphological group 1 isolates are so highly divergent that they probably could represent one or more unique genera. Our studies agrees with this data in *Acanthamoeba* phylogeny, however, we have also tested *Acanthamoeba* strains for their cytopathic effects on corneal epithelial cells as well as their pathogenic effects in animal model studies and found the virulent strains and non-virulent strains cluster separately with a group that are unresolved and represent avirulent strains (See Chapter 4). This sequence data suggest an early divergence of non-pathogenic species from the remaining taxa (based on the sequence analysis of *Hartmannella* as a out-group) after the diversification from a common ancestor (Free-living amoeba). Our results also show that the divergence of non-pathogenic taxa is followed by diversification of isolates that exhibit characteristics intermediate between pathogenic and non-pathogenic cluster and finally the divergence of a monophyletic cluster of pathogenic isolates. The large degree of genetic divergence between non-pathogenic (*A. astronyxis*, *A. comandoni*) and the rest of the genus suggest that they could be considered members of a different genera. Byers *et al.* (1990), have shown that the sequences of nuclear DNA, RNA and proteins, available for *Acanthamoeba* indicate that the genes of this amoeba are similar to those found in other organisms. These studies show that based on rRNA sequences,

Acanthamoeba genes are as closely related to those of higher organisms as to those of other protozoans. In a study by Gunderson et al. (1986) it was shown that SSU rRNA coding region of *A. castellanii* is 30% larger than those reported from other eukaryotes. Occasionally, it is shown that there is a correlation between the size of rRNA molecules and the phylogenetic position of eukaryotes possessing than with higher eukaryotes having the larger rRNA molecules (De La Cruz et al. 1985; Villalba et al. 1985). Thus there is a need for information about the functional organization of regulatory sequences for other genes. However, our data together with these studies will aid in diagnostic and differentiation of pathogenic *Acanthamoeba* as well as help in our understanding of the evolutionary history of pathogenic *Acanthamoeba*.

However, we have developed new method for DNA isolation which together with PCR may well be used for the clinical diagnosis of *Acanthamoeba* (See Chapter 4). This method will help in identifying amoeba in tissues. These techniques will and already have played a role in studies of the epidemiology, taxonomy and pathogenesis of parasite infections in humans. Enthusiasm for new molecular tools and methods of analysis should not be taken to mean for abandonment of old and proven techniques. Both morphological based assays and isoenzyme analysis (Chapter 3) have made major contributions and continue to be extremely cost-effective approaches for many applications.

7.4 - Phage display technology

Immunofluorescence methods provide a sensitive and specific means^a for the detection of protozoan parasites. It is thought that immunofluorescence antibody tests are more sensitive than direct microscopy, however there is little published critical data

analysis (Casemore 1992). Villa (1989) reported successful immunofluorescence antibody tests against *Giardia* using both polyclonal and monoclonal antibodies. Enzyme immunoassays have been used in the detection of *Giardia* antigen in faecal material employing the antigen capture format (Green *et al.* 1985; Stibbs *et al.* 1988). However, these antibodies have been produced from the serum of animals immunized with a particular antigen thus making it labor intensive, expensive and involve use of animals.

Filamentous phage have been shown to be an excellent approach for the production of antibodies mainly because epitopes often retain their native confirmation. This is perhaps one of the biggest advantages of phage display over bacterial expression vectors (Gram 1993). The potential of antibodies as diagnostic and therapeutic reagents has stimulated production of phage display libraries bearing antibody repertoires (O'Neil *et al.* 1995; Winter 1994). This approach mimic the *in vivo* production of antibodies and can eliminate the need for immunization and hybridoma technology (Clackson 1991). Phage display technology permits the selection of human antibody fragments from large libraries constructed from the B cells of individual encoding antibodies. The procedure is rapid and independent of the immunogenicity of the target antigen. Panning procedures on whole eukaryotic cells and intact tissues facilitates the isolation of antibodies against novel membrane molecules and epitopes, even when the target cells are rare or of loose phenotypic characteristics upon preparations. Engineering steps will further allow the antibody fragments to meet specific needs in biological effector and cellular interaction functions (De Kruif *et al.* 1996). These characteristics render this technology a valuable research tool and an approach to the production of human monoclonal antibodies for immunotherapy. Antibody based therapies have been shown against both extracellular and

intracellular pathogens. The monoclonal antibody-mediated therapy to *Cryptococcus neoformans* has shown in murine models of cryptococcosis (Mukherjee *et al.* 1994; Dromer *et al.* 1991; Mukherjee *et al.* 1995) and against *Mycobacterium tuberculosis* (Glatman-Freedman *et al.* 1998). Antibodies-mediated therapy have also been shown against intracellular pathogens or their products. Incubation of antibody with *Toxoplasma gondii* can inhibit intracellular replication of this organism after penetration in host cells (Mineo *et al.* 1994; Shirahata *et al.* 1976). Antibodies have also been shown to interfere with cellular entry mechanisms for several other intracellular pathogens. Antibodies raised against *Cryptosporidium parvum*, *Salmonella* spp. *Chlamydia* spp. Might protect by preventing into normal host cells (Michetti *et al.* 1994; Barenfanger *et al.* 1974). The ability to target cell and organ-specific receptors may enable the localization of parasite specific markers in infected cells and tissues for therapy (Pasqualini *et al.* 1996). The applications of phage display antibodies are limitless. Our data as well as other studies have shown the rapid isolation of antibodies using whole cells without the need of purification of antigens, further antigens do not have to be immunogenic making this technology more useful.

Clearly phage display libraries can have many uses in parasitology research. They offer new possibilities for the study of host-parasite interactions, identification of cell and organic specific markers and the discovery of disease specific epitopes, all of which may be exploited in the development of new drugs and vaccine and constitute a potentially useful option against existing and newly emergent and intracellular pathogens (Mazanec *et al.* 1992; Mineo *et al.* 1994).

7.5 - Proteases

Microbial proteases, including cysteine, serine and metalloproteases have been implicated in certain parasitic and fungal infections (Mc Kerrow *et al.* 1991; Rippon *et al.* 1968; Maeda *et al.* 1989). For example a serine protease is the major proteolytic agent released by invading larvae of the trematode *Schistosoma mansoni* (Mc Kerrow *et al.* 1991) and a human isolate of the fungus *Entomophthora coronata* produces elastase whereas a soil strain of the same fungus does not (Rippon *et al.* 1968). In another study by Kaminishi *et al.* (1994), it has been shown that proteinases from *Candida albicans* and *Pseudomonas aeruginosa* are capable of activating various clotting factors suggesting microbial proteinases might play an important role in co-aggulation and insufficient peripheral circulation. This in turn reflect the pathogenicity of microorganisms. *Candida albicans* and *Candida tropicalis* are the medically important opportunistic pathogens causing infections in immunocompromised patients. Their secretory proteolytic activity is considered to be a major virulence factor (Rao *et al.* 1998).

Despite recent advances in this field, the pathogenic mechanisms of *Acanthamoeba* have not been fully elucidated. Contact-dependent and contact independent mechanisms have been suggested. Support for the first one arose from the observations that only adhered parasites destroy primary corneal epithelial cell cultures (Cao *et al.* 1998), also in these studies a contact-dependent metalloprotease was observed. However, only one *Acanthamoeba* isolate was used. Our studies were based on the observations of Cao *et al.* (1998). In our findings we tested 4 pathogenic and 3 non-pathogenic strains of *Acanthamoeba* and observed the contact dependent metalloprotease in the pathogenic strains only (See Chapter 6).

We have also shown the contact independent mechanisms by showing hydrolytic enzymes found to be released to the medium by *Acanthamoeba* and its *in vitro* cytopathic effects on corneal epithelial cell damage (See Chapter 6). The links between pathogenicity and increased levels of extracellular proteases that we have observed in preliminary studies suggest that pathogenic *Acanthamoeba* utilizes proteases to facilitate invasion of the host.

In addition to contact-dependent and contact-independent mechanisms which are involved in pathogenicity we have also shown the phagocytotic ability of *Acanthamoeba* based on the observations that parasite apparently ingests corneal epithelial cells by the formation of the amoebastomes (See Chapter 6, Fig. 3). Phagocytosis has been shown in a number of protozoan parasites including *Trichomonas vaginalis* in which its been shown to ingest different mammalian cells (Heat 1981; Brasseur *et al.* 1982; Gonzalez-Robels *et al.* 1995; Rendon-Maldonado *et al.* 1998).

In conclusion we suggest that pathogenicity is a complex process that involve both contact dependent and contact independent pathways in order to kill host cells quickly to reduce the degree to which defence can be induced. However, basic molecular and cell biology studies are needed in order to understand these mechanisms and to potentially using them for the rationale development of protection against the infection.

In our studies we have been able to isolate *Acanthamoeba* from a variety of environments showing the wide distribution of these parasites. *Acanthamoeba* has posed a threat to increasing populations of contact lens wearers and AIDS patients. These observations together with the fact that *Acanthamoeba* sp. and *Naegleria fowleri* can harbor pathogenic microorganisms such as *Legionella* (Rowbotham 1980; Rowbotham 1980ii; De Jonckheere 1987; Fields 1989; Newsome 1985) and or mycobacteria (Jadin

1975) and that *Acanthamoeba* sp. have been used to isolate *Legionella* sp. in the absence of direct isolation from the environment indicate the public health importance of these organisms. In addition *Acanthamoeba*'s capacity to firmly bind to contact lens and large number of contact lens wearers would lead to a prediction of a higher incidence of infection in future (Stehr-Green *et al.* 1989).

Ultimately, the identification and differentiation of pathogenic *Acanthamoeba* can be achieved by any one or combination of the methods described above promising to accelerate the pace of our understanding of this important and interesting class of pathogens.

7.6 - FUTURE STUDIES

At present, diagnosis of the disease is not straightforward and treatment is problematic, consisting of hourly topical application of a combination of drugs for an extended period of time. In view of the devastating nature of the disease and the problems associated with the therapy, our future goals are to find a means to identify individuals who are at risk and provide them with rationally designed strategies to protect against the infection. It is known that the adhesion of amoeba to corneal surface is mediated by interactions between a mannose binding protein on the surface of amoeba membranes and mannose glycoproteins on the surface of corneal epithelium (Cao *et al.* 1998; Yang *et al.* 1997; Jaison *et al.* 1998). We would like to investigate the potential of the amoeba mannose receptor to serve as a suitable candidate for vaccine development to prevent the infection. At present there are no satisfactory *in vitro* methods to study the role of the

immune system in pathogenesis of infections. In recent years studies with animal models have made important contributions to our understanding of the role of the mucosal immune system in the pathogenesis of *Acanthamoeba* keratitis (He *et al.* 1992; Alizadeh *et al.* 1995; Van Klink *et al.* 1997). These studies (Alizadeh *et al.* 1995; Van Klink *et al.* 1997) have shown that animals (hamsters and pigs) immunised by oral administration but not intramuscular injections, of *Acanthamoeba* antigens (total parasite extracts) developed protective immunity against *Acanthamoeba* keratitis. The animal which were protected did not exhibit high titre IgG serum antibodies, but rather possessed parasite specific IgA in their tears, intestinal wash and stool samples. The parasite specific IgA inhibited the binding of amoeba to corneal epithelium. These findings suggest that oral immunization may offer a possible means of protection against the infection. Ideally a well defined antigen of the parasite should be used for immunization. Because the amoeba mannose receptor plays a central role in the pathogenesis of *Acanthamoeba* keratitis (Yang *et al.* 1997; Cao *et al.* 1998), it is likely to be an ideal candidate for vaccine development to prevent infection. In an attempt to identify individuals who are at risk of developing *Acanthamoeba* keratitis and find an ideal antigen for immunization of the population at risk, we propose to investigate the immune response to various domains of the amoeba mannose receptor in mice model and will determine whether oral immunization with specific domains of the amoeba mannose receptor leads to an elevated antibody level in the tears of mice and which specific domain of the mannose receptor provides protection against infection. This study will contribute to the basic understanding of the pathogenic mechanisms and cell biology of infections in general.

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Khan, N. A., Greenman, J., Topping, K.P., Hough, V.C., Temple, G.S., & Paget, T.A. (1999). Isolation of *Acanthamoeba* specific antibodies from bacterial phage display library. *Journal of Clinical Microbiology*, (Accepted for Publication).

Khan, N. A., Temple, G.S., Robotham, T., Kilvington, S. & Paget, T.A. (1999). Comparison of the polymerase chain reaction and simple plating based assays for detection and differentiation of pathogenic and non-pathogenic *Acanthamoeba* spp. and their applications in clinical use. *Journal of Clinical Microbiology* (Submitted).

Khan, N. A., Jarroll, E. L., Walton, D. S., Panjwani, N., & Paget, T. A. (1999). Proteases: a marker for differentiation of pathogenic and non-pathogenic *Acanthamoeba*. *Infection and Immunity* (Submitted).

ABSTRACTS

Khan, N.A., Jarroll, E.L., Panjwani, N. & Paget, T.A. (May 1999). Proteases: a marker for differentiation of pathogenic and non-pathogenic *Acanthamoeba*. *Invest. Ophthalmol. Vis. Sci.* **40(4)**:88296.

Khan, N. A., Cao, Z., Paget, T.A., & Panjwani, N. (Sep 1999). The role of proteinases in the pathogenicity of *Acanthamoeba* keratitis. Cornea Meeting, Boston, MA, USA.

Khan, N. A., Lunt, D., & Paget, T. A. (June 1999), Pathogenicity in *Acanthamoeba*: markers and evolution. Annual Meeting of the Scottish Section of Royal Society of Tropical Medicine and Hygiene, Glasgow, Scotland, UK.

Khan, N. A., Greenman, J., Topping, K.P., Hough, V.C. & Paget, T.A. (Sep 1998) Isolation of novel antibodies from a phage display library for the identification of *Acanthamoeba*. 7th Annual Molecular Parasitology Meeting, Woods Hole, MA., USA.

Paget, T.A., **Khan, N.A.,** Topping, K.P., Hough, V.C. & Greenman, J. (Sep 1998). A comparison of methods for the rapid identification of pathogenic and non-pathogenic strains of *Acanthamoeba*. 23rd Annual Public Health Laboratory Service Conference, Warwick, England.

Greenman, J., Topping, K.P., **Khan, N.A.,** Hough, V.C., Monson, J.R.T. & Paget, T.A. (June 1998). The isolation of novel antibodies from a phage display library and their application to tumour biology and parasitology. An International Symposium of Phage Display Technology; Antibodies, proteins and peptides.

TALKS

Khan, N.A., Jarroll, E.L., Panjwani, N. & Paget, T.A. (Feb 1999). Proteases: a marker for differentiation of pathogenic and non-pathogenic *Acanthamoeba*. Royal Society of Tropical Medicine and Hygiene Meeting, London, England.

Khan, N.A., Jarroll, E.L., Panjwani, N. & Paget, T.A. (Sep 1999). Differentiation of pathogenic and non-pathogenic species of *Acanthamoeba*. 24th Annual Public Health Laboratory Service Conference, Warwick, England.

Khan, N. A., Lunt, D., & Paget, T. A. (June 1999), Pathogenicity in *Acanthamoeba*: markers and evolution. Annual Meeting of the Scottish Section of Royal Society of Tropical Medicine and Hygiene, Glasgow, Scotland, UK.

Hough, V.C., Loveday, R., Topping, K.P., Greenman, J., **Khan, N.A.**, Monson, J.R.T. & Paget, T.A. (Mar 1999). Isolation of phage display antibodies for use in tumour biology. Society of Surgical Oncology annual meeting, FortLauderdale, FL, USA.

3.7 - APPENDIX

| | | |
|---------------------------------|--|----------|
| 1- TG | 10 x Stock (1 L) | |
| | Tris | 30g |
| | Glycine | 144g |
| | EDTA | 18.6g |
| 2- 0.1 M STEC | 5 x Stock (1 L) pH 9.5 | |
| | Tris | 121.15g |
| | Tri-sodium citrate | 70.0g |
| | EDTA | 18.6g TG |
| 3- 0.1 M TMEM | 5 x Stock (1 L) pH 7.4 | |
| | Tris | 60.55g |
| | Maleic acid | 58.05g |
| | Magnesium acetate | 10.7g |
| | EDTA | 18.6g |
| 4- 0.1 M TBEM | 5 x Stock (1 L) pH 9.0 | |
| | Tris | 151.35g |
| | Boric acid | 17g |
| | Magnesium acetate | 8g |
| | EDTA | 18.6g |
| 5- 0.1 M Tris / 0.1 M Phosphate | 10 x Stock (1 L) pH 8.2 | |
| | Tris | 90.85g |
| | NaH ₂ PO ₄ . 2H ₂ O | 78g |

6- Staining recipe for Cellulose acetate gels { * indicates optional and ** indicates light sensitive, (Recipe were obtained from Herbert, P. D. N., & Beaton, M. J. 1989.

Methodologies for allozyme analysis using cellulose acetate electrophoresis, A Practical Handbook)}.

a) Xanthine dehydrogenase (XDH)

1.0 ml Tris HCl, pH=8.0*

1.5 ml NAD (10 mg/ml)

20 drops of Hypoxanthine

5 drops of MTT (5 mg/ml)

5 drops of PMS** (2 mg/ml)

2 ml of agar

b) Aldehyde Oxidase (AO)

0.6 ml Tris HCl, pH=8.0*

1.5 ml NAD (10 mg/ml)

1 drop of Benzaldehyde

5 drops of MTT (5 mg/ml)

5 drops of PMS** (2 mg/ml)

2 ml of agar

c) Malate dehydrogenase (MDH)

1 ml of Tris HCl, pH=8.0*

1.5 ml of NAD (10 mg/ml)

13 drops of Malic substrate (180 ml H₂O, 20 ml Tris HCl, pH=9.0, 3.68g L-Malic acid, adjust to pH=8.0)

5 drops of MTT (5 mg/ml)

5 drops of PMS** (2 mg/ml)

2 ml of agar

d) Esterases (ES)

2 ml of 0.1 M Tris maleate, pH=5.3

200 µl α-naphthyl acetate solution (10 ml H₂O, 10 ml acetone, 0.1 g α-naphthyl acetate)

10 drops of saturated fast red TR salt**

2 ml of agar

e) Isocitrate dehydrogenase (IDH)

1 ml of Tris HCl, pH=7.0*

1.5 ml of NADP (10 mg/ml)

15 drops of DL-Isocitric acid

8 drops of MgCl₂

5 drops of MTT (5 mg/ml)

5 drops of PMS** (2 mg/ml)

2 ml of agar

f) Alcohol dehydrogenase (ADH)

0.6 ml of Tris HCl, pH=7.0*

1.5 ml of NAD (10 mg/ml)

5 drops of MTT (5 mg/ml)

5 drops of PMS**

15 µl Hexokinase**

5 μ l G6PD**

2 ml of agar

g) Glucose-6-phosphate dehydrogenase (G6PD)

0.6 ml Tris HCl, pH=8.0*

1.5 ml of NADP (10 mg/ml)

12 drops of D-Glucose-6-phosphate (10 mg/ml)

6 drops of MgCl_2

5 drops of MTT (5 mg/ml)

5 drops of PMS** (2 mg/ml)

2 ml of agar

h) Phosphoglucose isomerase (PGI)

1 ml of Tris HCl, pH=8.0*

1.5 ml of NAD (10 mg/ml)

5 drops of Fructose-6-phosphate (10 mg/ml)

5 drops of MTT (5 mg/ml)

5 drops of PMS**

2 ml of agar

7- Staining recipe for Starch gels.

a) Prolidase (PEPD)

16 ml of 0.3M Tris HCl, pH=7.0

0.4 ml of 0.5M MnCl_2

20 mg L-leucyl L-proline

2 mg peroxidase

2 mg Snake venom

1 ml of 3-amino, 9et carbazole

15 ml agar

b) Malate dehydrogenase (MDH)

8 ml of 0.3 M Tris HCl, pH=8.0

1.2 ml of NAD (10 mg/ml)

2 ml of 1 M L-Malate pH=7.0

3 ml of dH₂O

1 ml of MTT (5 mg/ml)

1 ml of PMS (2 mg/ml)**

15 ml of agar

c) 6-Phosphogluconate dehydrogenase (6PGD)

12 ml of 0.3 M Tris HCl, pH=8.0

2 ml of 0.1 M MgCl₂

0.6 ml of NADP (10 mg/ml)

1 ml of 6PG (10 mg/ml)

1 ml of MTT (5 mg/ml)

1 ml of PMS (2 mg/ml)**

d) Esterases (ES)

6 ml of 0.1 M PO₄, pH=7.4

1 mg in 200 µl of acetone 4-methylumbilliferyl butyrate

Filter paper view under UV

e) Glucose phosphoisomerase (GPI)

13.4 ml of 0.3 M Tris HCl, pH=8.0

0.4 ml 1 M MgCl_2

1 ml of NADP (10 mg/ml)

10 μl of G6PD (1000 U/ml)

1.6 ml of F6P (10 mg/ml)

1 ml of MTT (5 mg/ml)

1 ml of PMS (2 mg/ml)**

15 ml of agar

f) Phosphoglucomutase (PGM)

12 ml of 0.3 M Tris HCl, pH=8.0

2 ml of 0.1 M MgCl_2

0.6 ml of NADP (10 mg/ml)

10 μl of G6PD (1000 U/ml)

1.5 ml of G-1-P+G1,6diP (20 mg/ml)

1 ml of MTT (5 mg/ml)

1 ml of PMS (2 mg/ml)**

15 ml of agar

8-
A.castellanii GTCAAACCGGCGAC-TGCGT-TGGC--GTTGCGGGCTCGGTCCG-----TCGGTG--
A. sp (leic) GTCAAACCGGCGAC-TGCGT-TGGC--GTTGCGGGCTCGGTCCG-----TCGGTG--
A. sp (esbc4) GTCAAACCGGCGAC-TGCGT-TGGC--GTTGCGGGCTCGGTCCG-----TCGGTG--
A. sp (shi) GTCAAAC'TGGCGGC-TGCGT-TGGC--GTTGCGGGCTCGGTCCG-----TCGGTG--
A. sp (nich) GTCAAACCGGCGAC-TGCGT-TGGC--GTTGCGGGCTCGGTCCG-----TCGGTG--
A. sp (ros) GTCAAACCGGCGAC-TGCGT-TGGC--GTTGCGGGCTCGGTCCG-----TCGGTG--
A.polyphaga (ccap) GTCAAACCGATGAT-TGCG--TGGC-TGTTGCGGGCTCGGCCCCG-----TTGAT---
A. sp (dargon) GTCAAACCGGCGAC-TCG-T-TGGC--GTTGCGGGCTCGGTCCG-----TTGGTG--
A.griffini GTCAAACCGGTGAC-TGCG--TGGC--GTTGCGGGCTCGGTCCG-----TCGGTG--
A.polyphaga (atcc) GTCAAACCGGCGAC-TGC-T-TGGC--GTTGCGGGCTCGGTCCG-----TC-GTG--
A.palestinensis GTCAAAC'TGGTGAC-TGCG--TGGT--GTTGCGGGCTCGGTCCA-----T TAGT---
A.royreba GTCAAACCGGCGACTTGCGTATGGC--GTTGCGGGCTCGGTCCG-----TCGGTG--
A.astronyxis GTGTGTGGGGCGACCTGCACATGGCACGAGGAGGGCTCGGTCCGCTTACCTGCCGTTG--
A.comandoni GTGTGCAAGGTGACTTGACATGGCATGAGGAGGGCTTGGTCCGCTACTCTACCAATTGTG

A.castellanii C--CCCAC--AAAGG-----GCTACTGGCGT-----GTCAACCGGCCCCGCCC
A. sp (leic) C--CCCAC--AAAGG-----GCTACTGGCGT-----GTCAACCGGCCCCGCCC
A. sp (esbc4) C--CCCAC--AAAGG-----GCTATCGGCGT-----GTCAACCGGCCCCGCCC
A. sp (shi) C--CCCAC--AAAGG-----GCTATCGGCGT-----GTCAACCGGCCCCGCCC
A. sp (nich) C--CCCAC--AAAGG-----GCTACTGGCGT-----GTCAACCGGCCCCGCCC
A. sp (ros) C--CCCAC--AAAGG-----GCTACTGGCGT-----GTCAACCGGCCCCGCCC
A.polyphaga (ccap) CATCCCAC---AAGG-----GTGATTAGCGT-----GTTAACCGGCCCCGCCC
A. sp (dargon) C--CCCAC--AAA-----GC-ATCAGCGT-----GTCAACCGGCCCCGCCC
A.griffini C-TCCCAC'TAAAA-----GC-ATCGGCGT-----GTCAACCGGCCCCGTTT
A.polyphaga (atcc) C-TCCCAC--AAA-----GC-ATCGGCGT-----GTCAACCGGCCCCGCCC
A.palestinensis CATCCCAC---AAGG-----GTGATTAGCGT-----GTTAACCGGCCCCGCCC
A.royreba C--CCCAC--AAAGG-----TTATCGGCGT-----GTCAACCGGCCCCGCCC
A.astronyxis C--GCCACCGAAAGGTG-----GGTCGTGGCGTGTGGGGGCGTGCAC'TGGCCCCGCC
A.comandoni CATGCCACTGAAAGGTGCATTGTGTGCAGTTGGGGT-----GTGCACTGGCCCCGCT

A.castellanii GTCCCCTCCTTCTGGATTCCCGTTCCCT-----GCTATTGAGTTAGT---GGGGACGT
A. sp (leic) GTCCCCTCCTTCTGGATTCCCGTTCCCT-----GCTATTGAGTTAGT---GGGGACGT
A. sp (esbc4) GTCCCCTCCTTCTGGATTCCCGTTCCCT-----GCTATTGAGTTAGT---GGGGACGT
A. sp (shi) GTCCCCTCCTTCTGGATTCCCGTTCCCT-----GCTATTGAGTTAGT---GGGGACGT
A. sp (nich) GTCCCCTCCTTCTGGATTCCCGTTCCCT-----GCTATTGAGTTAGT---GGGGACGT
A. sp (ros) GTCCCCTCCTTCTGGATTCCCGTTCCCT-----GCTATTGAGTTAGT---GGGGACGT
A.polyphaga (ccap) GTCCCCTCCTTCTGGATTCCCGTTCCCT-----GCTATTGAGTTAGT---GGGGACGT
A. sp (dargon) GTCCCCTCCTTCTGGATTCCCGTTCCCT-----GCTATTGAGTTAGT---GGGGACGT
A.griffini GTCCCCTCCTTCTGGATTCCCGTTCCCT-----GCTATTGAGTTAGT---GGGGACGT
A.polyphaga (atcc) GTCCCCTCCTTCTGGATTCCCGTTCCCT-----GCTATTGAGTTAGT---GGGGACGT
A.palestinensis GTCCCCTCCTTCTGGATTCCCGTTCCCT-----GCTATTGAGTTAGT---GGGGACGT
A.royreba GTCCCCTCCTTCTGGATTCCCGTTCCCT-----GCTATTGAGTTAGT---GGGGACGT
A.astronyxis GCTCCCCTCCTTCTGGATTTCCTCAGCC--GGCTATTAAC'TTAGCTCTGGGGGCGC
A.comandoni GCTCCCCTCCTTCTGGATTTCCTCAGTATTGGCTATTAAC'TTAGCCCTGGGGGCGC

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| A.castellanii | CACAGGGGGCTCATCGTCGTGCGGCTCAAAAC-CGT--GCGGGCGGTGGGT----CCCT |
| A. sp (leic) | CACAGGGGGCTCATCGTCGTGCGGCTCAAAAC-CGT--G-GCGGGCGGTGGGT----CCCT |
| A. sp (esbc4) | CACAGGGGGCTCATCGTCGTGCTCAAAAC-CGT--G-GCGGGCGGTGGTT----CCCT |
| A. sp (shi) | CACAGGGGGCTCATCGTCGTGCTGC---AAAC-CGTC-G-GCGGGCGGTGGGT----CCCT |
| A. sp (nich) | CACAGGGGGCTCATCGTCGTGCGGCTCAAAAC-CGT--G-GCGGGCGGTGGGT----CCCT |
| A. sp (ros) | CACAGGGGGCTCATCGTCGTGCGG-TC-AAAC-C---G-GCGGGCGGTGGGT----CCCT |
| A.polyphaga (ccap) | CACAGGGAGCTGT--CATTGCCTTC-----AC-----GGGCCAGTGATA----AGTTCCCT |
| A. sp (dargon) | CACAGGGGGTTCATCGTCGTGTCAG-C-----AAT-----G-GCAGTGGTGG----GTCCCT |
| A.griffini | CACAGGGAGCTCATCGTCGTGTCAG-C-----AAT-----GACGGCGCGG-TGAGTTCCT |
| A.polyphaga (atcc) | CACAGGGGGCCCATCGTCGT-GTCCTGCAAAT-----GGGCACGGCG-G--TGAGTCCCT |
| A.palestinensis | CACAGGGAGCTGTATCATTGCCTTC-----AC-----GGGCCAGTGGT---ACAGTTTCT |
| A.royreba | CACAGGGGGTCCATCGTCG--AGCC-----AACGCGTCGGGCCAGTGGT---ACAGT---T |
| A.astronyxis | CATCGGGTGTGCTGTTGCCTTAGCC-----C--AGTGTGCATTTTGGCACTCAGGGCAT |
| A.comandoni | CATAGGGCAGTGTGTTGCCT-----AAAC--AGTGTGCATTTTCC---T-GGGGCAT |

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| A.castellanii | GGGGCCCAGATCGTTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATCCAATTTT |
| A. sp (leic) | GGGGCCCAGATCGTTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATCCAATTTT |
| A. sp (esbc4) | GGGGCCCAGATCGTTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATCCAATTTT |
| A. sp (shi) | GGGGCCCAGATCGTTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATCCAATTTT |
| A. sp (nich) | GGGGCCCAGATCGTTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATCCAATTTT |
| A. sp (ros) | GGGGCCCAGATCGTTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATCCAATTTT |
| A.polyphaga (ccap) | GGGGCCCAGATCGTTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATTCAATTTT |
| A. sp (dargon) | GGGGCCCAGATCGTTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATCCAATTTT |
| A.griffini | GGGGCCCAGATCGTTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATCCAATTTT |
| A.polyphaga (atcc) | GGGGCCCAGATCGTTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATCCAATTTT |
| A.palestinensis | GGGGCCCAGATCGTTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATACAATTTT |
| A.royreba | GGGGCCCAGATCGTTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATTCAATTTT |
| A.astronyxis | GGGGCCCAGATCGTTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATA---TTTT |
| A.comandoni | GGGGCCCAGATCGTTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATA---TTTT |

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| A.castellanii | ---CTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCC-TATTTT |
| A. sp (leic) | ---CTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCC-TATTTT |
| A. sp (esbc4) | ---CTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCC-TATTTT |
| A. sp (shi) | ---CTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCCTTATTTT |
| A. sp (nich) | ---CTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCC-TATTTT |
| A. sp (ros) | ---CTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCC-TATTTT |
| A.polyphaga (ccap) | ---CTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCC-TCTTTT |
| A. sp (dargon) | ---CTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCC-TATTTT |
| A.griffini | ---CTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCC-TATTTT |
| A.polyphaga (atcc) | ---CTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCC-TATTTT |
| A.palestinensis | ---CTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCC-TATTTT |
| A.royreba | ---CTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCC-TATTTT |
| A.astronyxis | ATACTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTTCATGTTTC |
| A.comandoni | ATACTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTTTATGTTTT |

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| A.castellanii | CAGTTGGTTTGAGGACTAAGGGTAATGAATTATTAGGGATAGTTGGGGGGCATTAAATATT |
| A. sp (leic) | CAGTTGGTTTAAGGACTAGGGGTAATGA-TTAATAGGGATAGTTGGGGGGCATTAAATATT |
| A. sp (esbc4) | CAGTTGGTTTAGGGACTA-GGGTAATGATTTAATAGGGATAGTTGGGGG-CATTAATATT |
| A. sp (shi) | CAGTTGGTTTGAGGACTA-GGGTAATGA-TTAATAGGGATAGTTGGGGG-CATTAATATT |
| A. sp (nich) | CAGTTGGTTTGAGGACTA-GGGTAATGA-TTAATAGGGATAGTTGGGGG-CATTAATATT |
| A. sp (ros) | CAGTTGGTTTGAGGACTA-GGGTAATGA-TTAATAGGGATAGTTGGGGG-CATTAATATT |
| A.polyphaga (ccap) | CAGTTGGTTAGAGGATCA-GGGTAATGA-TTAATAGGGATAGTTGGGGG-CATTAATATT |
| A. sp (dargon) | CAGTTGGTTTGAGGACCA-GGGTAATGA-TTAATAGGGATAGTTGGGGG-CATTAATATT |
| A.griffini | CAGTTGGTTTGAAGACCA-GGGTAATGA-TTAATAGGGATAGTTGGGGGGCATTAAATATT |
| A.polyphaga (atcc) | CAGTTGGTTTGAGGACTA-GGGTAATGA-TTAATAGGGATAGTTGGGGG-CATTAATATT |
| A.palestinensis | CAGTTGGTTAGAGGATCA-GGGTAATGA-TTAATAGGGATAGTTGGGGG-CATTAATATT |
| A.royreba | CAGTTGGTTTGAGGACTA-GGGTAATGA-TTAATAGGGATAGTTGGGGG-CATTAATATT |
| A.astronyxis | GCGTTGGTTGGAGGACCA-GGGTAATGA-TTAATAGGGATAGTTGGGGG-CATTAATATT |
| A.comandoni | GTGTTGGTTGGAGGACCA-GGGTAATGA-TTAATAGAGATAGTTGGGGG-CATTAATATT |
| | |
| A.castellanii | TAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGAATTAACCTCTGCGAAAGCATCTG |
| A. sp (leic) | TAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGA-TTAACCTCTGCGAAAGCATCTG |
| A. sp (esbc4) | TAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGA-TTAACCTCTGCGAAAGCATCTG |
| A. sp (shi) | TAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGA-TTAACCTCTGCGAAAGCATCTG |
| A. sp (nich) | TAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGAATTAACCTCTGCGAAAGCATCTG |
| A. sp (ros) | TAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGA-TTAACCTCTGCGAAAGCATCTG |
| A.polyphaga (ccap) | TAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGA-TTAACCTCTGCGAAAGCATCTG |
| A. sp (dargon) | TAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGA-TTAACCTCTGCGAAAGCATCTG |
| A.griffini | TAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGA-TTAACCTCTGCGAAAGCATCTG |
| A.polyphaga (atcc) | TAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGA-TTAACCTCTGCGAAAGCATCTG |
| A.palestinensis | TAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGA-TTAACCTCTGCGAAAGCATCTG |
| A.royreba | TAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGA-TTAACCTCTGCGAAAGCATCTG |
| A.astronyxis | TAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGA-TTAACCTCTGCGAAAGCATCTG |
| A.comandoni | TAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGA-TTAACCTCTGCGAAAGCATCTG |
| | |
| A.castellanii | CCAAGGATGTTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCG |
| A. sp (leic) | CCAAGGATGTTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCG |
| A. sp (esbc4) | CCAAGGATGTTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCG |
| A. sp (shi) | CCAAGGATGTTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCG |
| A. sp (nich) | CCAAGGATGTTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCG |
| A. sp (ros) | CCAAGGATGTTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCG |
| A.polyphaga (ccap) | CCAAGGATGTTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCG |
| A. sp (dargon) | CCAAGGATGTTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCG |
| A.griffini | CCAAGGATGTTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCG |
| A.polyphaga (atcc) | CCAAGGATGTTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCG |
| A.palestinensis | CCAAGGATGTTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCG |
| A.royreba | CCAAGGATGTTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCG |
| A.astronyxis | CCAAGGATGTTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATTAGATACCG |
| A.comandoni | CCAAGGATGTTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATTAGATACCG |

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| A. castellanii | TCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACAC |
| A. sp (leic) | TCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACAC |
| A. sp (esbc4) | TCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACAC |
| A. sp (shi) | TCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACAC |
| A. sp (nich) | TCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACAC |
| A. sp (ros) | TCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACAC |
| A. polyphaga (ccap) | TCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGTATACAAAACAC |
| A. sp (dargon) | TCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACAC |
| A. griffini | TCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACAC |
| A. polyphaga (atcc) | TCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACAC |
| A. palestinensis | TCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACAC |
| A. royreba | TCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACAC |
| A. astronyxis | TCGTAGTCTTAACCATAAACCATGCCGACCAGCGATTGGGAGACGTTTCACACGGAGGTG |
| A. comandoni | TCGTAGTCTTAACCATAAACCATGCCGACCAGCGATTGGGAGACGTTTCACACGGGGGTG |

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| A. castellanii | -CACCATCGGCG---CGGTCGTCCTTGG--CGTCGGTCCTTCA---CGGGGCCGGCGCGA |
| A. sp (leic) | -CACCATCGGCG---CGGTCGTCCTTGG--CGTCGGTCCTTCA---CGGGGCCGGCGCGA |
| A. sp (esbc4) | -CACCATCGGTG---CGGTCGTCCTTGG--CGTCGGTCCTTTC-----GGGGCCGGCGCGG |
| A. sp (shi) | -CACCATCGGTG---CGGTCGTCCTTGG--CGTCGGTCCTTTC-----GGGGCCGGCGCGG |
| A. sp (nich) | -CACCATCGGCG--CCGGTCGTCCTTGG--CGTCGGTCCTTCA---CGGGGCCGGCGCGA |
| A. sp (ros) | -CACCATCGGCG---CGGTCGTCCTTGG--CGTCGGTCCTTCA---CGGGGCCGGCGCGA |
| A. polyphaga (ccap) | -CACCAT-----TACATAATTCAGTATA-TGGTCAGC---CAA-----TGGCC-GT-GTA |
| A. sp (dargon) | -CACCATCGGTG---CGGTCGTCCTTGGCACATCATTGGCGCAA--GCCAGTGGTGGCGGA |
| A. griffini | -CACCATGCGCATTGCGGGCGTCTTTGG--TGTCGCTCA--CAA---GGCGGCACC---- |
| A. polyphaga (atcc) | -CACCATCGGCG---CGGTCGTCCTTGG--CGTGTCTCGGCTT---CACGGCCGGGGCGC |
| A. palestinensis | -CACCA-C-----TACATAATTCAGTACATCGGCCAG---TCAAA---TGGTT-GT-GTA |
| A. royreba | -CACCATCGGCG---CGGTCGTCCTTGG--CGTTCGTG-TTCA---CGCACGAG-CGCGA |
| A. astronyxis | G--CC-TCGGG----GGGCA-TTTTTGC-TGTTCCGTGCGCAAGCGGCGGG--CGCAAT |
| A. comandoni | GCCCCATGGGG---AGAGTGCGTGGGGG----TGGCCGCTTCATGCGGTGGCCTCCTCGC |

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| A. castellanii | G----GGCGGCTTAGCCCG-GTGGCACCGGTGAATGACTCCCCCTAGCAGCTTGTGAGAAA |
| A. sp (leic) | G----GGCGGCTTAGCCCG-GTGGCACCGGTGAATGACTCCCCCTAGCAGCTTGTGAGAAA |
| A. sp (esbc4) | G----GACGGCTTAGCCCG-GTGGCACCGGTGAATGACTCCCCCTAGCAGCTTGTGAGAAA |
| A. sp (shi) | G----GGCGGCTTAGCCCG-GTGGCACCGGTGAATGACTCCCCCTAGCAGCTTGTGAGAAA |
| A. sp (nich) | G----GGCGGCTTAGCCCG-GTGGCACCGGTGAATGACTCCCCCTAGCAGCTTGTGAGAAA |
| A. sp (ros) | G----GGCGGCTTAGCCCG-GTGGCACCGGTGAATGACTCCCCCTAGCAGCTTGTGAGAAA |
| A. polyphaga (ccap) | TTGTTTGTAT-----AAAGAT-CCACCGGT-AATGACTCCCCCAGCAGCTTGTGAGAAA |
| A. sp (dargon) | ----GGGCGATTAGCCTG-ATGGCATCGGTGAATGACTCCCCCTAGCAGCTTGTGAGAAA |
| A. griffini | ---GGGGCGGCTTAGCTCGCATGGCACCGGTGAATGACTCCCCCTAGCAGCTTGTGAGAAA |
| A. polyphaga (atcc) | GCGAGGGCGGTTTAGCCCG-GTGGCACCGGTGAATGACTCCCCCTAGCAGCTTGTGAGAAA |
| A. palestinensis | TTGTTTGTGT-----ATGGCACCGGTGAATGACTCCCCCTAGCAGCTTGTGAGAAA |
| A. royreba | ----GGGCGGTTTAGCCCG-ATGGCACCGGTGAATGACTCCCCCTAGCAGCTTGTGAGAAA |
| A. astronyxis | GTGTCCCTTT---GGGGGTGCTTCCACTAAAGGATGACTCCCCCAGCAGCTTGTGAGAAA |
| A. comandoni | CTTTCCCTTT-GGGGGGTGCTTCCACTAAAGGATGACTCCCCCAGCAGCTTGTGAGAAA |

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| A.castellanii | TCAT-AAGTCCTTT-GGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTT-AAAGGAAT |
| A. sp (leic) | TCAT-AAGTCCTTT-GGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTT-AAAGGAAT |
| A. sp (esbc4) | TCAT-AAGTCCTTT-GGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTT--AAGGAAT |
| A. sp (shi) | TCAT-AAGTCCTTT--GGTTCCGGGG--AGTATGGTCGCAAGGCTGAAACTT--AAGGAAT |
| A. sp (nich) | TCAT-AAGTCCTTT-GGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTT-AAAGGAAT |
| A. sp (ros) | TCAT-AAGTCCTTT-GGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTT-AAAGGAAT |
| A.polyphaga (ccap) | TCAT-AAGTCCTTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTG-AACTTTAAAGGAAT |
| A. sp (dargon) | TCAT-AAGTCCTTT--GGTTCCGGGGG-AGTATGGTCGCAAG-CTGAAACTT-AAAGGAAT |
| A.griffini | TCAT-AAGTCCTTT-GGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTT-AAAGGAAT |
| A.polyphaga (atcc) | TCAT-AAGTCCTTT-GGGTTCCGGGG--AGTATGGTCGCAAG-CTGAA-CT---AAGGAAT |
| A.palestinensis | TCAT-AAGTCCTTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTCTAAAGGAAT |
| A.royreba | TCAT-AAGTCCTTT-GGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTT-AAAGGAAT |
| A.astronyxis | TCATCAAGTCCTTT-GGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTT-AAAGGAAT |
| A.comandoni | TCATCAAGTCCTTT-GGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTT-AAAGGAAT |
| | |
| A.castellanii | TGACGGAAGGGCACCACCA |
| A. sp (leic) | TGACGGAAGGGCACCACCA |
| A. sp (esbc4) | TGACG-AA-GGCACCACCA |
| A. sp (shi) | TGACG-AAGGGCACCACCA |
| A. sp (nich) | TGACGGAA-GGCACCACCA |
| A. sp (ros) | TGACG-AA-GGCACCACCA |
| A.polyphaga (ccap) | TGACGGAAGGGCGCCACCA |
| A. sp (dargon) | TGACGGAAGGGCACCACCA |
| A.griffini | TGACGGAAGGGCACCACCA |
| A.polyphaga (atcc) | TGACG-AA-GGCACCACCA |
| A.palestinensis | TGACGGAAGGGCACCACCA |
| A.royreba | TGACGGAAGGGCACCACCA |
| A.astronyxis | TGACGGAAGGGCACCACCA |
| A.comandoni | TGACGGAAGGGCACCACCA |