Molecular Studies of the Pathogenic Free-living Amoeba, *Acanthamoeba*

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Abstract

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Ubiquitous amoebae from the genus *Acanthamoeba* are associated with two main serious infections: The more common eye disease acanthamoeba keratitis (AK), which can result in blindness, and the rare and often fatal disease affecting the central nervous system, granulomatous amoebic encephalitis (GAE).

The traditional morphological taxonomic system for *Acanthamoeba* is based on cyst size and shape, and divides the amoebae into three groups (I, II and III). Since the discovery that cyst shape can be modified by culture conditions, the classic system has become largely redundant. A more robust system has been developed, based on the nucleotide sequence of the 18S rRNA (*Rns*) gene. It types *Acanthamoeba* into 15 T-groups, with most species including environmental and clinical clumped into three groups T3, T4 and T11, with little resolution between them. Although speciation does not help cure patients directly, it can provide valuable information regarding disease epidemiology and ultimately benefit patient prognosis.

Here a system to better resolve strains has been developed, using the mitochondrial cytochrome oxidase subunit 1 and 2 (*cox1/2*) gene sequence. When used in conjunction with the T-group system, resolution between strains including those with a T3, T4 or T11 genotype is obtained. Additionally the combined approach identified a mixed infection in a patient suffering with AK, and the occurrence of *Acanthamoeba* strains with multiple alleles of 18S and *cox1/2* genes.

The combined use of both genotyping systems was used to investigate an unprecedented outbreak of GAE within a Swedish hospital. Results confirmed *Acanthamoeba* had infected several immunocompromised paediatrics from a single ICU, and the source was from within the unit's water system. *In vitro* assays were used to test the strains pathogenic abilities and sensitivities to antimicrobial compounds, identifying if they are more virulent than typical strains of *Acanthamoeba*.

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Abbreviations

AK	Acanthamoeba keratitis
APS	Ammonium persulfate
ARB	Amoeba-resistant bacteria
ATCC	American Type Culture Collection
CCAP	Culture Collection of Algae and Protozoa
CFU	Colony forming units
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computerised tomography
DH5α	*Escherichia coli* (ATTC 53868)
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotides
dPBS	Dulbecco's Phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FBS	Foetal bovine serum
FLA	Free-living amoeba
GMS	Gomori methamine silver
HCl	Hydrochloric acid
IIF	Indirect immunofluorescence
IPTG	Isopropyl-β-D-thiogalactoside
KH_2PO_4	Potassium dihydrogen orthophosphate
LB	Luria-Bertani

LSHTM	London School of Hygiene & Tropical Medicine
LSU	Large subunit
$MgCl_2$	Magnesium chloride
MP	Maximum parsimony
MRI	Magnetic resonance imagery
MRSA	Methicillin-resistant *Staphylococcus aureus*
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NHS	Normal human serum
nH_2O	Nanopure water
NJ	Neighbour-joining
$(NH_4)_2SO_4$	Ammonium sulphate
NNA	Non-nutrient agar
PAGE	Polyacrylamide gel
PAM	Primary amoebic meningoencephalitis
PAS	Periodic acid-Schiff
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PNACL	Protein Nucleic Acid Chemistry Laboratory
RE	Restriction enzymes
Rnl	The large subunit rRNA gene
Rns	The gene encoding for 18S
SDS	Sodium dodecyl sulphate
SOB	Super optimal broth
SOC	SOB with catabolite repression (added glucose)

SSU	Small subunit
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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Arch U.K. Biocides	Arch U.K. Biocides Ltd, Wheldon Road, Castleford, West Yorkshire, WF10 2JT, U.K.
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1 INTRODUCTION

1.1 Free-living amoebae

Amoebae are both ecologically and medically important, and are named from the Greek word for change (amoibe), referring to their continual changing shape as they move and feed.

This ecologically important and diverse group are ubiquitous throughout the environment. Within soil ecosystems naked amoebae consume large numbers of bacteria, and are thought to have a key role (Rodriguez-Zaragoza, 1994) comparable to that of flagellates within aquatic systems (Ekelund and Ronn, 1994). Several amoebae species are significant within marine ecosystems as both consumers and producers, and some are known to harbour symbiotic algae (Gast *et al*., 2009). There are also free-living amoebae (FLA) that have the potential to live a parasitic lifestyle and are called amphizoic in recognition of their endozoic existence (Page, 1988). Studies of these parasitic amoebae began in the late 19th century with the enteric pathogen *Entamoeba histolytica* (Lesh, 1975).

Amoebae are a group of unicellular eukaryotic microorganisms that include *Chaos carolinense* the giant amoebae, the prototype *Amoebe proteus*, and parasites such as *E. histolytica*, *Naegleria fowleri* and *Acanthamoeba* spp. Amoebae are found throughout the environment from pole to pole with habitats including soil, sand, seawater and freshwater.

Historically its taxonomy has been unsettled and variable, and this continues today despite new data from a series of recent genomic sequencing studies (De Jonckheere, 2004, Gast *et al.*, 1996, Horn *et al.*, 1999, Nassonova *et al.*, 2010, Schroeder-Diedrich *et al.*, 1998, Smirnov *et al.*, 2007, Stothard *et al.*, 1998, Weekers *et al.*, 1994). Under the original taxonomic system, the phylum protozoa,

was divided into four groups with amoebae in sarcodina. However a second system has been proposed classifying eukaryotes into six supergroups, with amoebae in Amoebozoa (Adl *et al*., 2005). All taxonomic classification difficulties are further compounded because amoebae are a polyphyletic group, having arisen from different branches of the protozoal evolutionary tree (Schuster and Visvesvara, 2004).

Amoebozoa (Lühe, 1913, emend (Cavalier-Smith, 1998)) although morphological diverse has several distinguishing features. Pseudopodia (Greek: false feet) are extended from the cell surface to achieve the distinctive amoeboid locomotion: they are non-eruptive and morphologically variable. Groups commonly have subpseudopodia. They can be naked and surrounded by only a plasma membrane, or testate with a partial single-chambered protective layer called a test. They are usually uninucleate, rarely binucleate but occasionally multinucleate. Most groups are at least dimorphic and able form protective cysts when environmental conditions become adverse.

Pseudopodia are used for both locomotion and feeding: As a chemotaxis response they detect concentration gradients of nutrients and other substances in their surrounding environment. The pseudopodia extend and retract with cytoplasmic streaming (Allen, 1961, Taylor, 1977) which results in the flow of organelles through the cytoplasm within the cell. Only in the trophic form, can the amoebae move and feed on surrounding nutrients and microorganisms by phagocytosis. Pseudopodia surround the food and engulf it, before digestion occurs within a phagolysosome. The cyst phase is a dormant resting stage, maintained until the surrounding environment becomes favourable again.

Several amoebae spp. have been recognised as models for studying cell mechanisms, one such species is *A. castellanii*, (ATCC 30010, Neff strain), which has been used extensively. However a variety of different species have been used to investigate and research areas including, means of locomotion (Allen *et al.*, 1965, Klopocka *et al.*, 2009, Pollard, 1981, Sinard *et al.*, 1989), phagocytosis (Obaray and Coakley, 2001), chemotaxis response (Levchenko and Iglesias, 2002), intracellular communication (Mogoa *et al.*, 2010, Prusch and Roscoe, 1993) and cell differentiation (Eichinger *et al.*, 1999). The ability to encyst within a protective outer layer when conditions become adverse provides protection from desiccation, starvation, temperature extremes and many chemical disinfectants. Some groups specifically *Naegleria*, can also have a flagellate stage.

In some circumstances, amoebae may act as environmental reservoirs, and be exploited to take up microorganisms, which survive phagocytosis and multiply within the amoebae (Axelsson-Olsson *et al.*, 2005). Associations such as these may represent how bacteria adapted and evolved to survive within eukaryotic cells (Molmeret *et al.*, 2005). Amoebae have even been described as the Trojan horse of some human diseases (Alsam *et al.*, 2006, Barker and Brown, 1994, Greub and Raoult, 2004): With some of these microorganisms becoming more virulent whilst within their amoebae host (Cirillo *et al.*, 1997).

Acanthamoeba can act as a host to *Staphylococcus aureus* (MRSA) (Huws *et al.*, 2005), *Legionella* spp. (Rowbotham, 1998), *Clamydia*-related symbiont (Horn and Wagner, 2004), *Cryptococccus neoforans* (Steenbergen *et al.*, 2001), *L. monocytogenes* (Ly and Muller, 1990), *Mycobacterium* spp. (Adekambi *et al.*, 2006), *Compylobacter jejuni* (Axelsson-Olsson *et al.*, 2005), and *Escherichia coli*

serotype K-1 (Alsam *et al*., 2006) and serotype O157 (Barker *et al*., 1999), and may act as a vector to transmit these pathogens to susceptible hosts, providing a route of entry into the human body. The exact mechanisms of bacterial-amoebae interaction are not understood. As amoebae feed on bacteria, how some pathogenic bacteria survive within the amoebae, while other non-pathogens are killed, is unclear (Alsam *et al.*, 2006).

The term endosymbiotic is commonly used to describe microorganisms within amoebae and their relationship to each other. However studies have shown microorganisms do not represent true endosymbionts of amoeba, since they can be both endosymbiotic or lytic, depending on environmental conditions, so the term 'amoeba-resistant bacteria (ARB)' has been coined as an alternative (Greub *et al*., 2004).

Amoebae have increasingly been recognised as important pathogens, with the cases of amebiasis increasing in frequency (Martinez, 1997). Infection occurs largely in immunocompromised rather than otherwise healthy individuals. Medically important amoebae that bridge the gap between parasitic and free-living include *Acanthamoeba* spp., *Balamuthia mandrillaris*, *N. fowleri*, and most recently *Sappina pedata* (Gelman *et al*., 2001, Qvarnstrom *et al*., 2009). Disease pathophysiology helps to distinguish the infecting agent.

Acanthamoeba and *Balamuthia* are opportunistic pathogens, which can cause devastating infections in immunocompromised individuals (either granulomatous amoebic encephalitis (GAE) or cutaneous infections**)**. *Acanthamoeba* can also cause a non-opportunistic ocular infection known as amoebic (or acanthamoeba) keratitis (AK). *Naegleria fowleri* causes a fatal non-opportunistic meningoencephalitis known as primary amoebic meningoencephalitis

(PAM). Since the discovery of encephalitis caused by *S. pedata* in an otherwise healthy young man is so recent, this suggests that there are probably other amoebae capable of causing fatal infections in humans (Qvarnstrom *et al*., 2009).

1.2 Acanthamoeba

Acanthamoeba was first observed as a contaminant of trypsinised monkey-kidney cells in tissue cultures in 1957 (Jahnes *et al*., 1957), but it was not until the following year that the pathogenic potential of these amoebae was uncovered during the development of a polio vaccine (Culbertson *et al*., 1958). Culbertson and colleagues used contaminated monkey-kidney cell culture fluids to inoculate immunosuppressed monkeys and mice, resulting in fatal encephalitis or encephalomyelitis of the hosts within days. Initially the contaminant was thought to be an unknown virus. However, upon histological examinations of the monkeys and mice, and microscopy of the culture fluids the contaminant was identified as *Acanthamoeba*. The first reported human infection by *Acanthamoeba* was described in 1972 (Jager and Stamm, 1972). It was not until almost a decade later that *Acanthamoeba* was characterised as an opportunistic organism, which infects debilitated or chronically ill patients (Martinez, 1980).

These parasitic FLA are not as well adapted to parasitism as classic parasitic protozoa such as *Plasmodium* spp. and *Leishmania* spp. Not only do the amoebae invariably kill their host, but also they have not yet evolved to survive within them for long enough to ensure transmission to a new host. Consequently host-to-host transmission of these amoebic diseases has not yet occurred.

Acanthamoeba are incredibly versatile and resilient, as is demonstrated by their ubiquitous distribution throughout nature and man-made habitats. Within soil and water samples they are documented as being the most common amoebae if not most common protozoan (Page, 1988), and they are found almost everywhere there is water. Their tolerance of marked extremes in temperature, osmolarity, oxygen availability, water potential and pH allows them to survive in such a wide variety of locations. As does their ability to encyst when conditions become especially adverse and survival is threatened.

Such universal distribution means they are not dependent on a host for transmission and spread. Surprisingly, despite the potential opportunities, infection of humans by *Acanthamoeba* is rare, and largely limited to immunocompromised hosts. *Acanthamoeba* are responsible for several forms of disease. They can infect eyes and cause Acanthamoeba Keratitis (AK) (often occurring in healthy individuals), as well as cutaneous infections, and disseminated infections of the lungs, the central nervous system (CNS), and the brain, known as Granulomatous Amoebic Encephalitis (GAE).

So far today there are approximately 24 named species of *Acanthamoeba*, but not all appear to be pathogenic (Visvesvara *et al*., 2007b). The first to be isolated was *A. polyphaga* from dust, it was assigned the name *Amoeba polyhagus* (Puschkarew, 1913), but was later re-described, and named *A. polyphaga* (Page, 1967). *A. castellanii* was discovered as a contaminant of a yeast culture of *Cryptococccus pararoseus* (Castellani, 1930), which was later named *Hartmannella castellanii* (Douglas, 1930). In 1931, the genus *Acanthamoeba* was

established (Volkonsky, 1931) and ultimately distinguished from *Hartmannella*. Only then was *H. castellanii* reclassified as *A. castellanii* (Volkonsky, 1931).

The genus continued to be the subject of controversy, and was revised, discarded, and finally redefined: But this time the earlier defining characteristics of pointed spindles at mitosis, general form, locomotion and the appearance of cysts, were replaced by more definitive features of cyst structure and presence of acanthapodia (also known as fine finger-like pseudopodia) (Page, 1967).

As taxonomic techniques have become more refined there is now less movement of species within the grouping system, which has stopped the historical practice of using the genus names *Acanthamoeba* and *Hartmannella* interchangeably. *Hartmannella* is a distinctly different amoeba (Page, 1967), which has never been reliably associated with human pathologies (Cleland *et al.*, 1982, Culbertson, 1971, Jager and Stamm, 1972).

1.3 Acanthamoeba ecology

Acanthamoeba are dimorphic, and exist as either a cyst or as a trophozoite. The trophozoites are the larger active feeding form, and ranges in size from 15 µm to 45 µm. Distinguishing features of trophic amoebae are fine finger-like pseudopodia, a nucleus containing a distinctive centrally located nucleolus, and a contractile vacuole found in the cytoplasm integral to maintaining osmotic equilibrium. The cytoplasm is granular and contains many mitochondria, lysosomes, ribosomes, and contractile vacuoles. Cysts are smaller than trophozoites and range from 10 µm to 25 µm. They are non-motile, resistant forms protected by an obvious inner and outer wall, and are formed in response to a hostile environment. Amoebae can exist as a cyst for many years if necessary until more favourable conditions

return. To continue trophic growth, dormant amoebae emerge through a pore or ostiole, located on the cell wall at the junction of the ectocyst and endocyst (Martinez, 1997), and covered by a protective flap or operculum.

Acanthamoeba are extremely abundant throughout nature and found everywhere associated with water, lakes, ponds, oceans, in soil, and even dust within the air. They have also been found in many man-made environments, including baths, heating/ventilation/air conditioning systems, cold water storage tanks, swimming pools, cooling towers of electric and nuclear power plants, humidifiers, Jacuzzi tubs, dialysis machines, hospital hydrotherapy pools, dental irrigation units, bottled water, contact lens solutions, bacterial, fungal and mammalian cell cultures, and medical equipment (Martinez, 1997, Schuster and Visvesvara, 2004, Visvesvara *et al.*, 2007b). They have even been isolated from ear discharge, skin lesions, corneal biopsies, cerebrospinal fluid (CSF), pulmonary secretions, mandibular autografts, brain necropsies, and nasophayngeal mucosa.

Acanthamoeba can inhabit such a wide variety of locations because they are tolerant of marked extremes in temperature, osmolarity, oxygen availability, water potential and pH. Additionally they have the ability to encyst when conditions become especially adverse and survival is threatened. Studies have shown cysts can survive freeze thaw refreeze cryotherapeutic methods reaching temperatures of between -50 to -130°C (Meisler *et al*., 1986) although results from a later study suggest that cyst survival is inversely linked to rate of freezing (Matoba *et al*., 1989). *Acanthamoeba* cysts can also endure periods of up to 8 months at low temperatures of –10, 4, 10 and 15°C (Biddick *et al*., 1984), pH 2.0, moist heat of

60°C for 60 min (Kilvington, 1991), gamma irradiation (250 K rads) and ultraviolet radiation (800 mJ/cm^2) (Aksozek *et al*., 2002).

Acanthamoeba have also been shown to remain viable over extended periods of time. Cysts stored in a state of desiccation for 20 years have hatched and resumed trophic growth (Sriram *et al*., 2008).

However some success has been shown in inactivating *Acanthamoeba* cysts using solar disinfection (a simulated global solar irradiance of 850 WM^{-2}) providing the water containing the organisms reaches a temperature of between 50-55°C for 6 or 4 hours respectively (Heaselgrave *et al*., 2006).

Several environmental factors govern the distribution of *Acanthamoeba*, namely organic matter. Bacteria must be readily available for the amoebae to feed upon and thrive. Consequently *Acanthamoeba* flourish within biofilm, where food is in continuous supply. Temperature is also significant, and must be optimal for strains to thrive; however most can withstand a broad range. Some strains, particularly clinical isolates, have enhanced growth at warmer temperatures (>37°C). Those isolated from corneal infections generally have an optimal growth temperature of ~30°C (Schuster and Visvesvara, 1998). Pathogenic strains of *Acanthamoeba* able to survive within mammals must be able to actively grow at temperatures of at least 37°C. Thermotolerant strains have been isolated from many biotopes. Including soil samples collected from the island of Tenerife, Canary Islands, Spain, which showed 90.6% (39 of the 43 isolates) of those collected, exhibited thermotolerance at 37°C (Lorenzo-Morales *et al*., 2005). While samples collected from soil in Talbot County, Maryland, USA showed 81.9% (17 of the 21 isolates) displayed thermotolerance between 37-39°C (Sawyer, 1989).

Thermotolerance is not compulsory for species that infect the cornea, as corneal temperature is around 5°C lower than the body between 32-35°C, consequently most species of *Acanthamoeba* have the potential to become opportunistic and colonise the corneal surface.

1.4 *Acanthamoeba* cultivation

As a general rule *Acanthamoeba* isolates adapt readily to axenic growth, and as a consequence are an attractive model to use for morphological, biochemical, nutritional and molecular studies: Often with the type strain *A. castellanii* Neff (ATCC 30010) as the most popular choice of species.

Axenic growth for *Acanthamoeba* is established through a series of steps based on the 'walk out' method (Neff, 1958). In this method amoebae are isolated *in vitro* on non-nutrient agar (NNA) plates covered with a monolayer or streak of gram-negative bacterial food such as *E. coli* or *Enterobacter aerogenes*. The bacteria do not have a food source present and so cannot multiply. Any amoebae present will crawl as they feed on the bacteria, producing plaque-like clearings in the bacterial lawn during early growth stage. Once the food source/bacteria has been exhausted the *Acanthamoeba* will encyst.

Providing the plates are sealed so they cannot dry out and are stored at 4°C, the cysts will remain viable for long periods of time. Additionally fresh amoebae cultures can be maintained within the laboratory indefinitely, by transplanting a small piece of agar containing trophozoites or cysts on to a fresh bacteria-seeded NNA plate, and placing it isolate-side down. However it must be noted that this method does not guarantee to establish monocultures of all isolates in a mixed

sample, as the slower growing amoebae are likely to be outrun by any faster dividing amoebae.

To establish axenic growth, amoebae are dislodged from the plate surface using a weak saline solution and are washed by centrifugation removing extraneous bacteria, before being placed into sterile flasks containing liquid culture medium and antibiotics. The most effective combination of antibiotics used to inhibit residual bacteria within this enriched culture medium is penicillin and streptomycin, or gentamycin. This method works for most isolates but some, in particular clinical specimens, may require additional nutrients such as foetal calf serum and further vitamins (Schuster, 2002).

There are several considerations that should be made when designing experiments using a strain of *Acanthamoeba* that has been cultured for many generations. As several studies have demonstrated that traits of strains can change over long-term culture. It has been shown that strains can develop a reduced tolerance to temperature, after long-term axenic culture (Pumidonming *et al*., 2010). While some exhibit a reduced resistance to therapeutic agents, shown by the comparison of two cultures of *A. polyphaga* Ros (one having been in continuous culture since 1991, and the other cryopreserved since 1991), both tested against a multipurpose contact lens solution (containing polyhexamethylene biguanide (PHMB) 1mg/ml) (Hughes *et al*., 2003).

There may be many other traits that could also have been lost when cultured for a substantial period of time in an artificial environment. In the case of assessing the efficacies of contact lens disinfectant solutions against bacteria and fungi a limit has been set, ensuring strains are passaged no more than five times from the original

culture, perhaps a similar limit should be taken into consideration when experimenting with *Acanthamoeba*.

1.5 Acanthamoeba epidemiology

There are surprisingly few cases of *Acanthamoeba* infections considering the ubiquitous nature of the amoebae and the opportunities for contact. However, unsurprisingly it has been shown that more than 80% of the normal population possesses anti-*Acanthamoeba* antibodies (Chappell *et al.*, 2001, Cursons *et al.*, 1980): Allowing most typically healthy individuals the ability to combat *Acanthamoeba* and not develop an infection when exposed to the amoebae. For an infection to become established, specific predisposing factors must occur.

Breaks in the skin are the most likely mode of infection into the body. However *Acanthamoeba* have been isolated from the nasal mucosa of healthy individuals (Cerva *et al*., 1973), probably carried there as cysts on air currents or the wind (Rodriguez-Zaragoza, 1994). So *Acanthamoeba* may be able to invade through the upper respiratory tract, again given the right circumstances (Schuster and Visvesvara, 2004). However the disease incubation period is unknown and weeks to months may pass following an infection before the symptoms become apparent. As a consequence of this time delay and the wide distribution of the amoebae within the environment the precise portal of infection is masked. Once inside the body, amoebae can spread through the blood to organs and the CNS.

Acanthamoeba infections within the body can result in a host of diseases including GAE, nasopharyngeal, cutaneous, and disseminated infections, in addition to AK affecting the eye. GAE is an opportunistic disease, affecting immunocompromised individuals and therefore has no pattern of seasonality and

can occur at anytime of the year. However the incidence of GAE has increased with the HIV/AIDS epidemic, because as a consequence more people are now immunosuppressed and are therefore susceptible.

Acanthamebiasis primarily occurs in debilitated often chronically ill patients who are immunocompromised or immunosuppressed often with HIV/AIDS, or who have undergone organ transplants.

However some cases have been described in individuals who seem not to be immunocompromised (Singhal *et al*., 2001). Acanthamebiasis has also been reported in horses (Kinde *et al*., 2007), dogs (Dubey *et al*., 2005, Pearce *et al*., 1985), a toucan (from which the etiological agent was isolated, cultured, grown at 44°C and therefore shown to be thermotolerant, and genotyped to the T4 group (Visvesvara *et al*., 2007a), turkeys, sheep, a kangaroo, reptiles, amphibians, fish (Dykova and Lom, 2004), invertebrates, pigs, rabbits, pigeons and cattle (Cerva, 1981, Cirillo *et al.*, 1997, Kadlec, 1978, Schuster and Visvesvara, 2004).

Models for encephalitis using the mouse as host have provided information on virulence, epidemiology and the course of the disease (Mazur *et al*., 1995). Not all species, strains and isolates of *Acanthamoeba* are pathogenic, and those that are, vary in their virulence. *Acanthamoeba* virulence is measured by the period of time from inoculation to the onset of symptoms leading to death, and the number of animals that have died as a consequence, as well as the dose of inoculants required to cause encephalitis (Schuster and Visvesvara, 2004). Virulence is also indicated by the ability of *Acanthamoeba* to cause cytopathology in tissue cultures (Cursons *et al*., 1980, De Jonckheere, 1980, Niszl *et al*., 1998).

Some strains maintained in axenic conditions for prolonged periods of time, have been shown to lose their virulence (Mazur *et al*., 1995) (although this is not

always the case (Niszl *et al*., 1998)), their encystment capacity, and change their susceptibility to drug treatments (Hughes *et al*., 2003). However most attenuated characteristics including virulence can be restored if passaged in series through human Hep-2 cell monolayer (Kohsler *et al*., 2009) or *in vivo* via intranasal inoculation into mice and ultimately the brain (Xuan *et al*., 2009).

Acanthamoeba is the etiological agent for AK. The disease can occur in otherwise healthy individuals who almost always are contact lens wearers. Observations made studying rat and mouse models have shown mode of infection usually occurs following a trauma to the eye and then wearing contact lenses (Ren and Wu, 2010). With poor contact lens (and lens case) hygiene being a major contributing factor to acquiring the disease. Infection does not automatically occur in both eyes. However, there has been one case of fatal GAE with associated uveitis and pharyngitis recorded in the literature, but never a case of GAE having developed from AK (Jones *et al.*, 1975, Visvesvara, 2010).

The precise incidence of AK is unknown, as the disease is not notifiable, however it has undoubtedly been on the increase as more people wear contact lenses today than previously. There was a recent outbreak of AK detected in the USA in 2006. Initially the cases were identified within the Chicago area by Illinois Department of Public Health, and brought to the attention of the Centers for Disease Control and Prevention (CDC). Who performed several surveys, one of which was a retrospective survey of ophthalmology centres across the USA. Their results showed an increase in the number of culture-confirmed cases during 2004-2006 compared with 1999-2003, in ten centres over nine states. Following a national outbreak investigation, analysis indicated the odds of ever having used the

multipurpose contact lens solution, Complete MoisturePlus (AMOCMP) manufactured by Advanced Medical Optics (now known as Abbotts Medical Optics) were 20 times greater for AK patients than for controls. Following communication with the food and drug administration (FDA, AMO voluntarily recalled AMOCMP worldwide (Verani *et al*., 2009).

1.6 Granulomatous amoebic encephalitis (GAE)

Acanthamoeba are one of the causative agents of granulomatous amoebic encephalitis also known as GAE. It is a rare and often fatal infection, found most commonly in immune compromised or severely debilitated individuals. GAE was first observed in 1972 (Jager and Stamm, 1972) with less than 200 cases caused by *Acanthamoeba* documented within the literature (Schuster and Visvesvara, 2004). This however is likely to be a false representation, as these infections are difficult to diagnose and distinguish from bacterial and other microbial infections even in the first world.

Headaches, slight fever, behavioural abnormalities, personality changes, stiff neck, nausea, hemiparesis, seizures, cranial nerve palsies, and typical signs of localised encephalopathy are all symptoms of the chronic progressive disease (da Rocha-Azevedo *et al.*, 2009, Marciano-Cabral, 2003, Schuster and Visvesvara, 2004, Walochnik *et al.*, 2008). As the clinical signs are not specific, the disease is often misdiagnosed. Differential diagnosis includes bacterial meningitis, viral encephalitis, neurocyticerosis, and brain tumors (da Rocha-Azevedo *et al.*, 2009, Matson *et al.*, 1988, Ofori-Kwakye *et al.*, 1986).

Diagnosis if made, is almost always post-mortem following brain tissue biopsies, and indirect immunofluorescence (IIF) staining of tissue sections (Bloch and Schuster, 2005, Schuster *et al.*, 2006b, Schuster and Visvesvara, 2004).

Medical image scanning to view the brain, such as computerised tomography (CT) and magnetic resonance imaging (MRI) can be used to visualise the lesions caused by *Acanthamoeba*, but these lesions are not specific enough to the disease to base a diagnosis on (McKellar *et al*., 2006).

A diagnosis of GAE has been made pre-mortem, and was achieved as a result of positive *Acanthamoeba*-specific PCR of several biopsy tissue and fluid specimens, including cerebrospinal fluid (CSF), bronchoalveolar lavage specimens (BAL), skin, lung, and brain tissue (from the main lesion): All of which had been *Acanthamoeba* culture negative when tested (Walochnik *et al*., 2008).

PCR is a proven invaluable diagnostic tool, used to detect *Acanthamoeba* infections. A reliable primer pair designed and developed from the complete DNA gene sequence of the 18S ribosomal gene (18S rDNA: *Rns*), known as JDP1 and JDP2, have the ability to detect all known *Acanthamoeba* subgroups successfully, including those from the environment, AK and GAE patients (Stothard *et al*., 1998). These primers within a PCR assay produce a specific amplimer of around 500 bp, and from this sequence variation has allowed the development of a highly sensitive T-group typing system. The system has even been used to determine an epidemiological association between a keratitis-causing strain of *Acanthamoeba*, the patient, their contact lens storage case and their domestic water supply (Ledee *et al*., 1996). On going studies have identified T-groups 1-15, with the majority of GAE and AK causing amoebae in the T4 subgroup (Gast, 2001, Hewett, 2003, Horn *et al.*, 1999, Schroeder *et al.*, 2001, Stothard *et al.*, 1998).

T-group typing is not the only molecular diagnostic technique for detecting *Acanthamoeba*. Comparison of sequence variation of the mitochondrial 16S rRNA genes (Ledee *et al*., 2003), Restriction fragment length polymorphisms (RFLP) nuclear and mitochondrial rRNA (Kilvington *et al*., 1991, Kilvington *et al*., 2004), and fluorescent probes to hybridise with *Acanthamoeba* DNA (Stothard *et al*., 1999). Real time PCR assays have also been highly successful at determining *Acanthamoeba* infections; they are rapid and particularly sensitive, with the ability to be effective even with a low concentration of template DNA (less than 10 cells) (Qvarnstrom *et al.*, 2006, Riviere *et al.*, 2006).

In addition to the molecular diagnostic techniques available to identify *Acanthamoeba* infections, there are also many molecular methods. Although rare, amoebae can be identified in wet preparations of CSF samples or those that have been giemsa or H & E stained (Marciano-Cabral, 2003, Martinez, 1997, Seijo Martinez *et al.*, 2000, Sharma *et al.*, 1993). Identification of amoebae in both trophozoite and cyst stages can also be made from biopsied patient samples, which have been formalin-fixed or paraffin-embedded (da Rocha-Azevedo *et al*., 2009), using techniques including immunohistochemistry and fluorescent microscopy. Rabbit generated, anti-*Acanthamoeba* antibodies are incubated with patient samples, followed by a secondary incubation with anti-rabbit IgG associated with a fluorescent marker such as FITC, and observed under fluorescent microscopy (Culbertson and Harper, 1984). More recently species-specific monoclonal antibodies for *A. castellanii, A. polyphaga, A. lenticulata, and A. culbertsoni* have been developed (Guarner *et al*., 2007).

Typically GAE infections occur in the CNS tissue, but may involve the lungs (Khan, 2006). The incubation period is unknown, and weeks to months may elapse before the onset of the disease becomes apparent. This time delay obscures the precise route of entry of the amoebae into the body, but the most likely portal is the skin, olfactory neuroepithelium (Visvesvara *et al*., 2007b, Walochnik *et al*., 2008) and in specific circumstances orally (Thamprasert *et al*., 1993). Once inside the body, the amoebae most likely gain access into the CNS and lungs by hematogenous dissemination, or by passing directly through the neuroepithelium (Marciano-Cabral, 2003).

Acanthamoeba can also cause lesions in the skin, but these are more often reported in association with HIV positive patients (da Rocha-Azevedo *et al.*, 2009, Torno *et al.*, 2000). Cutaneous acanthamebiasis presents with multiple hard erythematous nodules, papules, or ulcers, across the surface of the body (May *et al*., 1992). If they occur simultaneously with CNS symptoms their presence is often indicative of an infection by *Acanthamoeba* (da Rocha-Azevedo *et al*., 2009, Khan, 2006).

Pathogenesis of GAE is extremely complex and not yet fully understood, and as a result, prognosis is extremely poor. As the majority of people who contact GAE are often immune compromised, debilitated and/or chronically ill, these predisposing factors are further compounded by the difficulty associated with diagnosis. Treatment of *Acanthamoeba* infections is further hampered by the amoebae ability to encyst when environmental conditions are detrimental (such as in the presence of treatment drugs) and remain so until conditions become more favourable.

However several patients have been diagnosed early enough and have subsequently been successful treated (Lackner *et al.*, 2010, Seijo Martinez *et al.*, 2000, Walia *et al.*, 2007, Walochnik *et al.*, 2008). Each of these patients has been treated with a different antimicrobial, as often one drug treatment used successfully in one patient is ineffective in another. Several factors are influential to the outcome of the disease, these include: how early drug treatments are initiated; host immune status; infective dose of the amoebae; antimicrobial sensitivity of the strain and its virulence (Schuster and Visvesvara, 2004).

Drug treatments used clinically include amphotericin B, azithromycin, fluconazole, 5-fluorocytosine (flucytosine), pentamidine isethionate, meropenem, linezolid, moxifloxacin, miltefosine, amikacin, voriconazole and sulfadiazine treated (Lackner *et al.*, 2010, Schuster and Visvesvara, 2004, Seijo Martinez *et al.*, 2000, Walia *et al.*, 2007, Walochnik *et al.*, 2008).

1.7 Acanthamoeba keratitis (AK)

Acanthamoeba keratitis (AK) is the sight threatening, acute, progressive and extremely painful ulceration of the cornea caused by species of *Acanthamoeba*. The infection almost always occurs in immune competent individuals who are contact lens wearers, or who have minor corneal abrasions.

AK was first recognised in 1973 in a rancher from South Texas, USA, who had a history of trauma in one eye (Jones *et al.*, 1975). Since then, year on year the numbers of cases reported within the literature have continued to rise, however the infection is not classified as a notifiable disease. Contact lenses are a risk factor associated with AK, primarily in the users of soft contact lenses (Stehr-Green *et al.*, 1989). Incidence levels for the USA are estimated at an annual incidence of 1-2

cases per million contact lens wearer (Schaumberg *et al*., 1998). Mean while the estimates for the U.K. are higher at 1 in 30,000 contact lens users (Seal, 2003). In one decade between 1990 and 2000, 180 cases from the U.K. were detailed in the literature (Radford *et al*., 1995, Radford *et al*., 1998, Radford *et al*., 2002) with most patients referred for treatment to Moorfields Eye Hospital, London, U.K.. Several factors are likely to be linked to the observed increase in AK cases, namely the better understanding of the infection we have now, which has led to an increase in the number of correct diagnoses made, and the years on year increase in number of people wearing contact lens.

Typically AK occurs in one eye only, but bilateral AK has been described, often developing as a complication of the initial infection (Lee and Gotay, 2010, Wilhelmus *et al.*, 2008). Symptoms of AK are not specific initially, and include severe eye pain, eye redness, photophobia, blurred vision, and a sensation of something in the eye, along with excessive tearing. Clinical symptoms develop to include corneal inflammation leading to the formation of a ring-like stromal infiltrate, corneal oedema, and erosion of the corneal epithelial cells.

AK suffers experience disproportionate eye pain, which thought to be linked to radial keratoneuritis and the trophozoites found along the corneal nerves, leading to thickening and distortion of these nerves (Yoo *et al*., 2004). Later stages of the infection can result in epithelial denudation, stromal necrosis (da Rocha-Azevedo *et al*., 2009), nerve oedema and retinal detachment, and if miss-diagnosed or a delay in treatment occurs, the infection will almost certainly lead to blindness as the necrotic region spreads inwards (Niederkorn *et al*., 1999).

A waxing and waning of the clinical course is usually a good diagnostic indicator of AK. This trend is caused because *Acanthamoeba* are often partly susceptible to non-protozoan treatments. An additional potential complication of diagnosis and therefore management is a secondary infection due to bacteria. Differential diagnosis of AK should be considered, as keratitis caused by *Herpes simplex* has dendriform-appearing lesions on the cornea (Martinez 1997), while *Pseudomonas aeruginosa*, has a similar type of infiltration (Clarke and Niederkorn, 2006).

AK can be diagnosed using the same techniques as for GAE, however cyst and trophozoite stages can also be detected and identified in ocular samples, either from biopsies or less invasive corneal scrapes, however considerable expertise is a necessity. The samples are smeared onto glass slides and visualised with bright field microscopy. Or wet mount preparations can be made using 10% KOH (Bharathi *et al*., 2006). Wet mounts stained with Giemsa or with H & E can be used to readily detect cysts, while trophozoites are harder to distinguish, as they resemble inflammatory cells (Bharathi *et al*., 2006, da Rocha-Azevedo *et al*., 2009). Again, as with GAE, IIF, monoclonal antibodies and fluorescent microscopy can be used to detect *Acanthamoeba* in corneal samples, contact lenses and lens cases (Inoue *et al.*, 1999, Leher *et al.*, 1999, Mietz and Font, 1997). There are several fluorescent stains effective against *Acanthamoeba* including calcofluor white, fungiflora, Acridine orange, Periodic acid-Schiff (PAS), and Gomori methamine silver (GMS) (Hahn *et al*., 1998, Inoue *et al*., 1999, Vemuganti *et al*., 2000, Wilhelmus *et al*., 2008). Confocal microscopy has also been shown to be useful in detecting AK

(Vaddavalli *et al*., 2011), but specialised equipment and experienced observers are required.

Molecular techniques highlighted for diagnosing GAE infections as effective with AK. Such techniques have made it possible to trace the source of AK infections back to taps in the patient's home (Booton *et al.*, 2002, Kilvington *et al.*, 2004, Ledee *et al*., 1996).

Acanthamoeba are able to infect a cornea and establish an infection, when specific circumstances arise. If there is corneal trauma in association with contaminated water, or the wearing of contact lenses maintained with a poor hygiene routine. AK infections principally occur in individuals, who wear soft contact lenses, although infections have been documented in some wearing hard contact lenses as well (Moore *et al*., 1987, Srinivasan *et al*., 1993). *Acanthamoeba* cyst more readily attach to soft contact lenses which have a higher water content than rigid hard lenses (Sharma *et al*., 1995).

Epidemic factors also linked to AK are swimming in contact lenses (Radford *et al*., 2002), a failure to maintain proper lens care i.e. using unsterile contact lens solutions, inadequate disinfection of the lenses, not soaking the lenses for the full amount of their recommended period, storing the lenses in a dirty case and/or washing the lenses in homemade saline solutions (Martinez and Visvesvara, 1997, Radford *et al.*, 2002).

As a result of wearing contact lenses (hard or soft)**,** minor abrasion of the corneal epithelium can occur, allowing the infection to take hold (Visvesvara *et al*., 2007b). Once *Acanthamoeba* trophozoites have adhered to the surface of the corneal epithelial cells, mediated by mannose-bonding protein (MBP) expressed on the

surface of the amoeba, damage is inflicted by phagocytosis of the host cells (Clarke and Niederkorn, 2006). *In vitro* studies with *Acanthamoeba* and rat B103 neuroblastoma cells, viewed by scanning and transmission electron microscopy, have shown phagocytosis. Following contact between the two cells types, membrane blebbing of the nerve cells occurred, leading to either lysis of the nerve cell, or ingestion of the nerve cell via amebastome-like (food cups) and channelling the ingested cell into intracytoplasmic food vacuoles (Pettit *et al*., 1996).

To ensure a good prognosis, early diagnosis and appropriate treatment of AK is vital. The first successful treatment of AK was reported in 1985, using an antimicrobial agent belonging to the aromatic diamidine group of compounds, known as propamidine isethionate and available as Brolene 0.1% w/v (Sanofi-Aventis, Guilford, U.K.). This used in combination with Neosporin® (Johnson & Johnson, New Brunswick, USA), and was Europe's first-line medical treatment until the mid 1990's. When resistance to propamidine isethionate was detected in some strains of *Acanthamoeba* and studies showed it to be only weakly cysticidal (Perrine *et al*., 1995). The search has continued for other superior homologues and alternative drug treatments such as the bis-biguanides, alexidine and chlorhexidine. Alexidine has a cytopathic affect on *Acanthamoeba*, not unlike chlorhexidine, with 10 mg/mL being affective against trophozoites and 100 mg/mL against cysts. *In vivo* experiments carried out using Chinese hamster corneas showed alexidine to be less toxic to corneal epithelial cells than chlorhexidine at 100 mg/mL (Alizadeh *et al*., 2009). More recently i*n vitro* studies have shown *Acanthamoeba* to be susceptible to PHMB (Hughes *et a*l 2003), showing consistent cysticidal activity.

Now the recommended treatment regimes for AK are chlorhexidine with propamidine, or its polymeric equivalent PHMB originally with propamidine but now combined with hexamidine isethionate (Hexomedine, Sanofi-Aventis) (Seal, 2003). The biguanides are a class of cationic disinfectants, and the topical use of them is recommended as the only effective therapy for resistant encysted forms of *Acanthamoeba in vitro*, and likely *in vivo* (Dart *et al* 2009). If the treatment is administered promptly enough a response to AK is expected within one week, with total eradication of the active infection to occur within eight weeks. A stark contrast to the four months of treatment needed if using Brolene and neomycin (Seal, 2003).

If the infection has not responded to topical biguanide, the next treatment to attempt is the use of steroids, however this is controversial (Dart *et al*., 2009). As a last resort in extreme cases that have shown no signs of improvement therapeutic keratoplasty is then recommend (Dart *et al* 2009).

1.8 Taxonomy and Classification

So far today there are approximately 24 named species of *Acanthamoeba* based on morphological characteristics (Schuster and Visvesvara, 2004, Visvesvara *et al*., 2007b). Several of which do have pathogenic potential, and include *A. polyphaga*, *A. rhysodes*, *A. quina*, *A. griffini*, *A. lugdunensis*, *A. castellanii*, *A. culbertsoni*, *A. healyi* and *A. hatchetti* (Ledee *et al*., 1996, Schaumberg *et al*., 1998, Schuster and Visvesvara, 2004, Yu *et al*., 2004).

The genus *Acanthamoeba*, is divided into three morphological categories (I, II, and III) based on distinguishing features of the cyst, including diameter, and shape of both endocyst and ectocyst walls (Pussard and Pons, 1977). Today the most comprehensive dichotomous key for morphological taxonomy is based upon

the three group system, and is nearly 20 years old (Page, 1988). *Acanthamoeba* cysts are double walled, with an inner layer or endocyst composed of cellulose, and an outer made up of lipids and proteins known as the ectocyst (Blanton and Villemez, 1978). Group I amoebae have large cysts with a diameter of 18 µm or more, with stellate endocysts and smooth or wrinkled ectocysts (Table 1). Amoebae in group-II are the most common. This group is also the largest and includes the majority of the potentially pathogenic species (Table 1). Their cyst diameter is 18 µm or less, with polyhedric, globular, ovoid, or stellate endocysts and wavy ectocysts. Group III amoebae have cysts of less than 19 µm with ovoid or globular endocysts and wavy or smooth ectocysts (Table 1). The reliability of using cyst morphology as a taxonomic characteristic came into question once it was observed that ionic concentration of the growth media can alter cyst morphology (Sawyer, 1971).

Immunological, biochemical and physiological methods have been used in the past to attempt to type and identify *Acanthamoeba* species. Techniques used include western blotting and immunofluorescence, however they have been inconclusive at distinguishing between *Acanthamoeba* species.

More recently a system has been proposed to classify *Acanthamoeba* based on 18S rDNA (*Rns*) gene phylogeny (Gast *et al*., 1996, Schroeder-Diedrich *et al*., 1998). 18S ribosomal RNA is the structural RNA for the small component of the eukaryotic cytoplasmic ribosome: Which has a slow evolutionary rate making it an ideal candidate to study and base reconstruction of ancestral divergences on. *Acanthamoeba* are grouped based on sequence variation into evolutionary clades.

So far 15 clades have been recognised, each containing species or complexes of closely related species: T1-T12 (Gast *et al.*, 1996, Schroeder-Diedrich *et al.*, 1998), T13 (Horn *et al*., 1999), T14 (Gast, 2001), and T15 (Hewett, 2003). The group T4 contains the majority of species with pathogenic potential (Stothard *et al*., 1998). Table 1, shows examples of morphologically designated *Acanthamoeba*, and how they are grouped according to T-group genotyping.

Table 1. Morphologically designated *Acanthamoeba species* assigned to T-groups based on phylogenetic analysis of 18s sequence variations.

T group	**Species**	**Strain ID**	**Reference**	**Morphological group**
T1	*A. castellanii*	ATCC 50494	(Dudley *et al*., 2005)	II
T2	*A. palestinensis*	ATCC 30870	(Gast *et al*., 1996)	III
T3	*A. griffinii*	ATCC 30731	(Gast *et al*., 1996)	II
T4	*A. rhysodes*	ATCC 50368	(Gast *et al*., 1996)	II
	A. polyphaga	ATCC 30971	(Stothard *et al*., 1998)	II
	A. castellanii	ATCC 50374	(Gast *et al*., 1996)	II
	A. lugdenensis	L3a	(Kong, 2009)	II

T5	*A. lenticulata*	ATCC 30841	(Stothard *et al*., 1998)	III
T6	*A. palestinensis*	ATCC 50708	(Stothard *et al*., 1998)	III
T7	*A. astonyxis*	ATCC 30137	(Stothard *et al*., 1998)	I
T8	*A. tubiashi*	ATCC 30867	(Stothard *et al*., 1998)	III
T9	*A. comandoni*	ATCC 30135	(Stothard *et al*., 1998)	I
T10	*A. culbertsoni*	ATCC 30171	(Stothard *et al*., 1998)	III
T11	*A. hatchetti*	Sawyer:NMFS	(Stothard *et al*., 1998)	III
	A. stevensoni	RB:F1	(Kong, 2009)	II
T12	*A. healyi*	CDC1283:V013	(Stothard *et al*., 1998)	III
T13	*Acanthamoeba* sp. *	-	(Horn *et al*., 1999)	-
T14	*Acanthamoeba* sp. *	-	(Horn *et al*., 1999)	-
T15	*A. jacobsi*	ATCC 30732	(Hewett, 2003)	III

*: Without morphological designation; -: Not assigned.

Still despite the recent advances in identification and typing of *Acanthamoeba* and all the information available in the literature there are still many uncertainties, confusions and complications to unravel. Which is unsurprising, considering the potential margin of error through misidentification of isolates, possible mislabelling of culture tubes, and/or cross-contamination of cultures. To highlight just one example, a group have analysed mitochondrial DNA restriction fragment length polymorphisms (mt RFLP), as well as sequences of both the nuclear 18S rDNA and mitochondrial 16S rDNA, of four morphological group II *Acanthamoeba*. From their results they have proposed that *A. divionensis*, *A. paradivionensis* and *A. mauritaniensis* all be regarded as synonyms for *A. rhysodes* (Liu *et al*., 2005).

1.9 Molecular biology of Acanthamoeba

There has been a lack of interest in studying the molecular biology of *Acanthamoeba*, but with the advent of recombinant DNA techniques, genomes of the organisms have now become more assessable for studies. The recent outbreaks of AK, and the realisation that *Acanthamoeba* can harbour pathogenic bacteria, and therefore serve as a vector in human infections, have all boosted the current interest in *Acanthamoeba*.

Acanthamoeba replicate by mitosis (Jantzen *et al*., 1990), with the nuclear membrane disappearing during division (Ma *et al*., 1990). As yet there has been no evidence of sexual reproduction occurring in *Acanthamoeba* (Yin and Henney Jnr, 1997). Evidence suggests that nuclear chromosomes are numerous and small with some ranging from 200 Kb to larger than 2 Mb (Byers, 1986, Byers *et al*., 1990, Rimm *et al*., 1988). *Acanthamoeba* trophozoites normally possess one nucleus,

which is approximately 16.6% of the size of the trophozoite (Khan, 2006). The nucleus does not contain any membrane bound sub components, but does contain a large centrally located nucleolus, which is also a prominent morphological characteristic.

The genome of *A. castellanii* Neff has been estimated at 33 Mb, and made up of approximately 60% GC (Byers, 1986). With a DNA content most commonly estimated at 1.28 pg/amoeba (Adam *et al*., 1969). Nuclear DNA content varies throughout culture growth, and decreases by approximately 50% (in unagitated cells) during the transition from log to post-log phase, and is most likely linked with preparations of the cell to encyst (Byers *et al*., 1969).

Mitochondrial DNA content has also been shown to vary throughout culture, with measurements of log phase *A. castellanii* Neff containing an average of 0.15 pg/amoeba, and reducing to 0.01-0.02 pg/amoeba during encystment (Byers, 1986).

In recent years the mitochondrial genome of *Acanthamoeba* has been fully sequenced, as part of collaboration to explore mitochondrial genome organisation and evolution within protists. *Acanthamoeba* mitochondrial genome is a circular molecule consisting of 41.6 Kb, with an AT content of 70.6%. Which contains the genes that code for both large and small subunit rDNA, 16 tRNA, 8 open reading frames (ORF) of undetermined function and 33 proteins. All of these are found in the same transcriptional orientation and make up 93.2% of the total sequence (Burger *et al*., 1995). A peculiar feature of the mitochondrial genome of *A. castellanii* is the presence of a single continuous ORF (*cox1/2*) encoding subunits 1 and 2 of cytochrome oxidase (COX1 and COX2) (Burger *et al*., 1995).

Phylogenetic trees based on sequences of nuclear rDNA, place *A. castellanii* on a branch with, or near (as an out group to) green algae and land plants. But as

small subunit SSU rDNA databases have expanded, *Acanthamoeba* has moved away from the algal and plant clade, to a sister branch of animals and fungi (Wainright *et al*., 1993) or outside of the multicellular lineages altogether (Cavalier-Smith, 1993). However one group has discovered evidence of horizontal gene transfer between algal chloroplasts and *Acanthamoeba* mitochondria (Lonergan and Gray 1994). By sequencing from the mitochondria of *A. castellanii*, a 7,778 bp region containing single-copy large subunit (LSU) and small (SSU) rRNA genes, they identified three group I introns within the LSU rRNA gene (*rnl*). The introns are placed within highly conserved regions and each possesses a freestanding open reading frame (ORF). One of the introns was found to be identical to that of the single group I intron in the chloroplast *rnl* of green algae *Chlamydomonas reinhardtii*, and structurally homologous within the core region and the ORF they encode. Suggesting intron movement has occurred between mitochondria and chloroplasts, either intracellularly in a photosynthetic, remote common ancestor of *A. castellanii* and *C. reinhardtii* or, more recently as a result of an intercellular exchange of genetic material (Lonergan and Gray 1994).

With the continued search for new data, this theory has developed as a result of the complete sequencing of the *Acanthamoeba* mitochondrial genome. The overall size of *A. castellanii*'s mt DNA, its gene content and organisation most closely resembles that of the chlorophycean algae *Prototheca wickerhamii*, than *C. reinhardtii*. Comparison of the mitochondria from all three organisms shows *A. castellanii* and *P. wickerhamii* to have almost identical respiratory and ribosomal protein genes, while *C. reinhardtii* does not encode any ribosomal proteins and lacks several standard respiratory genes. The authors argue that the results can be interpreted in two possible scenarios, either *C. reinhardtii* does not share a common

ancestry with land plants and *A. castellanii*, or more likely, that they do all share a common ancestor, and *C. reinhardtii* has diverged radically and lost many of its former gene content (Burger *et al*., 1995).

Ribosomes and their associated sequences have been extensively studied throughout biology as a means to better understand evolutionary origins. As a consequence the ribosomal biology of *Acanthamoeba* is one of the better-understood areas of its molecular biology.

One of the most studied and frequently used genes throughout eukaryotic biology is a component of the small ribosomal subunit 40S, known as the small subunit (SSU) 18S rRNA. SSU 18S is the structural RNA for the small component of the cytoplasmic ribosomes, and is therefore integral to protein synthesis in all living cells. Molecular analysis using 18S data to understand evolutionary divergences has become extremely popular. Several factors make SSU 18S such a prime target for molecular analysis, both its slow evolutionary rate and repetition throughout the genome provide ample template for PCR. Additionally, the gene is usually flanked with highly conserved regions, therefore making it relatively easy to locate at the outset of the studies, and so readily accessible.

Amoebae rDNA coding sequences are arranged as is typical for eukaryotic ribosomal gene repeat units; they contain one set of 5' 18S, 5.8S and 28S 3' genes. Between these genes and neighbouring sets are spacer regions or internally transcribed spacers (ITS). *Acanthamoeba* rRNA repeats unit is 12 Kb, containing approximately 600 copies (Byers *et al*., 1990), while the coding sequence for the 18S rRNA gene is 2,303 bp long.

Acanthamoeba nuclear SSU 18S rRNA genes (18S rDNA; *Rns*) have been fairly extensively studied and are at present receiving much attention, in the hope of understanding why some strains appear pathogenic and others do not. Group I introns within the rRNA sequence have been found in *A. griffinii* and *A. lenticulata* (Gast *et al*., 1994, Schroeder-Diedrich *et al*., 1998), increasing the size of their nuclear rRNA genes to approximately 2,800 bp, compared with 2,300 bp. Sequence analysis of the *Rns* has allowed the development of a classification scheme, allowing *Acanthamoeba* to be typed into one of a possible 15 *Rns* genotypes known as T-groups (Booton *et al.*, 2005, Gast *et al.*, 1994, Hewett, 2003, Stothard *et al.*, 1998).

Many amino acid sequences have been studied and published, these include complete and/or partial, mRNA, RNA and genomic sequences. The literature and GenBank are dominated by 18S sequences from many *Acanthamoeba* sp., but other sequences available include: actin I (Nellen and Gallwitz, 1982); myosin heavy chains I (Brzeska *et al*., 1999); 26S (Lai and Henney, 1993); 5S (Zwick *et al*., 1991); lactate dehydrogenase-like (Watkins and Gray, 2006); mannose-binding protein (Garate *et al*., 2004); polyubiquitin (Hu and Henney, 1997) and profilin I and II (Pollard and Rimm, 1991).

Although slow to start, the field of *Acanthamoeba* molecular biology has moved forwards rapidly. Evidence has recently been presented to include a new genotype of 18S, T16 (Corsaro and Venditti, 2010). While data also suggests analysis of microsatellites found within the ITS located between 18S and 5,8S, could be a potential candidate to further distinguish within the clades, especially T4 (Kohsler *et al*., 2006).

1.10 Aims

The aims of this thesis will be to study the molecular biology of *Acanthamoeba* with the aim of developing a genotyping system to better resolve the *Acanthamoeba* genus. An improved genotyping system could prove invaluable for epidemiological fingerprinting and species resolution in the currently muddled group. The new system will be put to the test in a case study of an outbreak of GAE within a Swedish hospital. The following chapters will first introduce the subject being investigated, and then outline the aims, methods, and any results found, before a discussion is presented.

2 DNA TYPING OF *ACANTHAMOEBA* SP.

2.1 Introduction

The classic morphological typing system divides the *Acanthamoeba* genus into three groups based on morphological characteristics of the cysts including size and shape (Pussard and Pons, 1977): Group I species, have relatively large cysts with a diameter of at least 18 µm, with distinctly stellate endocysts and smooth or slightly wrinkled ectocysts. Group II *Acanthamoeba* have a diameter of 18 µm or less, with polyhedric, globular, ovoid, or stellate endocyst and wavy ectocyst. While those classified to group III, have cyst diameters of less than 19 µm with ovoid or globular endocysts, and wavy or smooth ectocysts.

The reliability of using cyst morphology as a taxonomic characteristic came into question with the observation that variations in the ionic concentration of the growth media can alter cyst morphology (Sawyer, 1971, Stratford and Griffiths, 1978). As a consequence the morphological classification scheme has been challenged by the use of isoenzyme electrophoretic patterns to study intragenetic relationships: Results have been mixed, with some good correlations between isoenzyme patterns and morphological groups (Moura *et al*., 1992), and those that contradicted their morphological group designations' (Daggett *et al.*, 1985, De Jonckheere, 1983).

Isolation, cultivation and subsequent microscopy were once the main method of *Acanthamoeba* identification. However due to the ambiguities of the morphological system, these techniques have now been superseded: Although they remain a vital component, as both a useful method of diagnosis to genus level, and to cultivate sufficient numbers of cells for downstream assays. Attempts have been made to develop molecular diagnostic techniques for studying *Acanthamoeba* and

its associated diseases: These include techniques such as PCR amplification of nuclear SSU 18S (Gast *et al*., 1996, Gunderson and Sogin, 1986), mitochondrial cytochrome oxidase subunit 1 and 2 (*cox1/2*) gene (Kilvington *et al*., 2004), mitochondrial 16S rRNA genes (Chung *et al*., 1998), as well as PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of nuclear and/or mitochondrial rDNA (Yu *et al*., 1999) and fluorescent probes to hybridise with *Acanthamoeba* DNA (Stothard *et al*., 1999). Ultimately molecular developments have resulted in a phylogenetic typing system, which is both genus and subgenus-specific, allowing *Acanthamoeba* to be classified based on 18S sequence genotype: This system can assign isolates representing all three morphological groups to one of a possible 15 T-groups (Booton *et al.*, 2005, Gast *et al.*, 1994, Hewett, 2003, Stothard *et al.*, 1998), based on sequence similarities of SSU 18S rDNA. The T-group system has proven to be a sensitive tool, with the ability to detect very small numbers of trophic amoebae in samples (Schroeder *et al*., 2001). Eukaryotes have many copies of rRNA genes found repeatedly throughout the genome, providing an excess of template available for PCR.

Although the 18S sequence typing system for *Acanthamoeba* is highly sensitive and useful, it is not without its own specific complications. Such as multiple alleles, where some isolates have been found to have three alleles of 18S (Ledee *et al*., 1998). A second complication is the presence of Group 1 introns in some species, such as *A. griffinii* (S-7; ATCC 30731) and *A. lenticulata* (PD2S; ATCC 30841) (Gast, 2001, Gast *et al*., 1994), which distorts interstrain relationships. The 18S T-group genotyping system is also not particularly robust and clumps together both disease causing strains, into single clades with non-pathogenic environmental strains (Booton *et al*., 2005). Of the 15 recognised

groups, T4 is by far the largest with little resolution provided between the isolates. The development of a second system has been attempted to better resolve T4 using mt 16S rRNA sequences, but was unsuccessful (Ledee *et al*., 2003).

For a second system to be developed successfully, a gene suitable for phylogenetic comparisons needs a high level of diversity. A key enzyme in aerobic metabolism is mitochondrial cytochrome oxidase: Which is a component of the respiratory chain, and involved with transfer of electrons from cytochrome c to oxygen. Unusually within *Acanthamoeba* this gene named *cox1/2*, encodes for both subunits of cytochrome oxidase and specified by a single continuous ORF (Burger *et al*., 1995). Cytochrome oxidase genes have already been used for phylogenetic typing systems in a variety of different organisms, including crayfish (Yue *et al*., 2008), *Echinococcus granulosus* (Villalobos *et al*., 2007) and *Trypanosoma cruzi* (Burgos *et al*., 2008).

The mitochondrial gene *cox1/2* is a suitable contender as an alternative target for sequence analysis typing, to be used as a target for rapid and sensitive diagnosis of AK. Mitochondrial DNA has a large copy number in *Acanthamoeba*, approximately 3,300 for a log phase amoebae of the Neff strain (Byers, 1986), and analysis has shown it to have a large degree of nucleotide variation. Phylogenetic analysis using c*ox1/2* has already been used to differentiate eight patient isolates, as well as for six of them, matching their sequence homology with their respective tap water isolates (Kilvington *et al*., 2004).

Recent studies have shown multiple housekeeping genes can be concatenated to form a single super-gene alignment for building more robust phylogenetic trees, which provide better discrimination power (Devulder *et al*., 2005, Gadagkar *et al*., 2005, Kurtzman and Robnett, 2007). The use of the

concatenated gene approach within *Acanthamoeba*, using the well documented SSU 18S rDNA gene from the T-group system, in combination with another housekeeping gene, such as mt *cox1/2* could yield more accurate trees with greater powers of discrimination and ultimately provide the potential to phylogenetically classify the subgenus and improve the diagnosis of AK.

2.1.1 Aims

To investigate the phylogeny and relatedness of multiple *Acanthamoeba* species including both environmental and disease causing strains, by amplifying and analysing two distinct genes, the housekeeping gene mt cytochrome oxidase subunit 1 and 2 (*cox1/2*) gene, and the well documented SSU 18S rDNA (*Rns*) gene, making comparisons between their genotypes and morphological classifications. Attempts will then be made to use the concatenated gene approach, combining the 18S gene, with the *cox1/2* gene, to yield more accurate trees with greater powers of discrimination. With the ultimate aim of providing a tool to improve the phylogenetic techniques to classify the subgenus and improve the diagnosis of AK, ideally differentiating between pathogenic and non-pathogenic strains.

2.2 Materials and Methods

2.2.1 Chemicals

Chemicals were obtained from Sigma-Aldrich (Gillingham, U.K.) unless otherwise stated. They were all sterilised by either autoclaving or by passage through a 0.2 µm- Acrodisc® syringe filter (Pall Life Sciences, Portsmouth, U.K.) prior to use.

2.2.2 Organisms

All strains of *Acanthamoeba* used in this study are held within the culture collection of Dr Simon Kilvington, Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, U.K (Table 2). Unique AK clinical isolates were obtained from patients receiving treatment at Moorfields Eye Hospital, London, U.K. Corneal scrapes were collected and kindly donated by Mr John Dart of Moorfields Eye Hospital, London, U.K: Corneal scrapes were placed, stored and transported on agar slopes, ready for recovery and culturing techniques to be carried out at the University of Leicester under the guidance of Dr Simon Kilvington.

Table 2. Microorganisms used for these studies.

Species	**CCAP/ ATCC**	**Strain name**	**T group**	**Morph. group**	**Source**
A. astronyxis [a]	30137	Ray and Hayes	T7	I	Soil, California, USA

A. castellanii [a]	1501/1a **50373**	Neff/ OS4-7B	T4	II	Soil, California, USA
A. comandoni [a]	1501/5	-	-	-	Soil, France
A. culbertsoni [a]	30171	Lilly A-1 [AC-001]	T10	III	Primary monkey kidney tissue culture, India
A. griffinii [a]	1501/4	-	T3	II	Marine beach, Connecticut, USA
A. hatchetti [a]	30730	Bh2	T11	II	Sediment, Baltimore Harbour, Maryland, USA
A. healyi [a]	-	CDC1283 VO13	T12	III	GAE, brain, Barbados, BWI
A. lenticulata [a]	30841	PD2S	T5	III	Swimming pool, France
A. palestinensis [a]	30870 1547/1	AC-014	T2	III	Soil, Israel
A. palestinensis [a]	50708 1501/3c	OX-1/2802	T6	III	Swimming pool, France
A. polyphaga [a]	1501/3g	-	-	-	AK, USA
A. palestinensis [a]	1547/1	-	-	-	-

A. polyphaga [a]	30873 1501/3d	Nagington	T4	II	AK, UK
A. polyphaga [a]	-	Ros	T4	II	AK, UK
Acanthamoeba sp. [a]	-	Environmental 1	-	-	Cold water storage tank, UK
Acanthamoeba sp. [c]	-	A Keratitis (AK) 1 to 19	-	-	AK, UK
Acanthamoeba sp. [b]	-	AK95/1153	-	-	AK, UK
A. tubiashi [a]	30876	-	T8	I	Freshwater, Maryland, USA

[a] Dr S. Kilvington, University of Leicester; [b] Prof. D. Warhurst, London School of Hygiene and Tropical Medicine (LSHTM); [c] Mr J. Dart, Moorfields Eye Hospital; -: no information available.

2.2.3 Monoxenic culture of *Acanthamoeba*

Acanthamoeba trophozoites were cultured by monoxenic growth on non-nutrient agar plates seeded with a lawn of *E. coli* (NNA-*E. coli*) (Page, 1988). The NNA media was comprised of 1.5% plain agar (Agar No. 1, Lab M™, Bury U.K.) and 1 tablet of ¼ strength Ringer's solution per 500 ml of deionised water. The agar mix was autoclaved at 121°C for 15 minutes, and allowed to cool to 50°C before being poured into plates, and left to dry overnight at 37°C.

Once dried, the agar surface is seeded with 2-3 drops of a dense suspension of bacteria *E. coli* strain JM101 (ATCC 33876) (see 2.2.3.1). The bacteria are spread evenly over the surface with a sterile bacteria spreader, and the plates left to

dry at room temperature. Seeded plates remain viable for two weeks, if stored at 4°C.

For routine maintenance of *Acanthamoeba*, amoebae seeded plates were cultured in air at 32°C in sealed polythene bags. Cultures were refreshed weekly by excising a one cm^2 region of agar from the leading edge of the plaque, containing numerous trophozoites, and placing it face down onto the centre of a fresh NNA-*E. coli* plate for incubation as described above. To isolate and clone single cysts from a mixed culture, individual cysts were transferred by micro capillary manipulation on to a fresh NNA-*E. coli*, and again incubated as above.

2.2.3.1 Preparation of *E. coli* food source stock

To make the stock suspension, *E. coli* was streaked on to a Luria-Bertani (LB) agar plate using a sterile disposable loop (Fisher Scientific UK). The agar plates made comprises of 7.5 g plain agar (Lab M™), and 12.5 g of Difco LB powder (BD Biosciences, Oxford, U.K.) per 500 ml of distilled water, and autoclaved at 121°C for 15 minutes, and allowed to cool to 50°C before being poured into plates, and left to dry overnight at 37°C (plates are viable for 2 weeks when stored at 4°C). Once inoculated, the LB plates were incubated overnight at 37°C allowing colonies to form.

Distinct single colonies were picked with a sterile disposable loop (Fisher Scientific U.K.), and transferred to a 175 cm2 tissue culture flask (Nunc- Fisher Scientific U.K.) with 100 ml of LB broth (comprising of 12.5 g of Difco LB powder (BD Biosciences) in 500 ml of distilled water, and autoclaved at 121°C for 15 minutes), and propagated overnight at 37°C in a shaking incubator. The suspension

of *E. coli* was harvested under aseptic conditions and transferred to two 50 ml polypropylene tubes. The bacteria were pelleted by centrifugation at 2,000 x *g* for 30 minutes, and the supernatant discarded. Next, all culture media was removed preventing additional multiplication of the bacteria, by resuspending and thereby washing the pellet in ¼ strength Ringer's solution. This wash step was carried out a total of three times, with the final pellet being resuspended in 10 ml of ¼ strength Ringer's solution.

The stock suspension of bacteria as a food source can be stored at 4°C for up to two weeks.

2.2.4 Axenic culture of *Acanthamoeba*

Where possible, strains were adapted to axenic growth in a semi-defined media (Hughes and Kilvington, 2001), comprising of 20 g Biosate peptone (BD), 5 g Glucose, 0.3 g Potassium dihydrogen orthophosphate (KH_2PO_4), 10 mg Vitamin B_{12}, 15 mg L-Methionine per 900 ml of double distilled water, and if necessary pH was adjusted to 6.5-6.6 with 1 M sodium hydroxide (NaOH). The culture media was divided in volumes of 225 ml and autoclaved at 121˚C for 15 minutes. Prior to use, penicillin/streptomycin solution (to a final concentration of 100 U/ml and 0.1 mg/ml, respectively) was added to all the media aliquots. These were then made up to 250 ml with either sterile distilled water, or 10% heat inactivated foetal calf serum (Invitrogen Ltd, Paisley, U.K.) depending on the strain of *Acanthamoeba* being cultured. The complete media can be stored at 4˚C, and used within one month.

Before axenic culture could be obtained superfluous bacteria surrounding the *Acanthamoeba* was removed by treating cysts with 2% (v/v) hydrochloric acid

(HCl) for 24 hours (Kilvington and White, 1994). All traces of HCl were then removed by washing the cysts in sterile deionised water and pelleting with centrifugation at 1,000 x *g* for 3 minutes, this wash step is repeated three times. Washed cysts are inoculated into flat-sided Nunclon™ Surface tissue culture tubes (Fisher Scientific UK) containing 3 ml of culture media, and incubated in air at 32°C. Under these conditions, excystation should occur and any emergent trophozoites can adapt and replicate in the media. Axenic strains were successfully maintained in 25 cm^2 (small) tissue culture flasks (Nunc- Fisher Scientific UK).

Cultures were maintained on a weekly basis by harvesting the amoebae under axenic conditions and leaving a seeding stock replenished with fresh media, this ensured cells did not become over confluent and were typically maintained at mid log phase when metabolism is greatest. When required, cell density was assessed microscopically using a modified Fuchs Rosenthal haemocytometer (SLS, Nottingham, U.K.) and adjusted accordingly. When a higher density of cells was required, a seeding stock was inoculated into an appropriately sized tissue culture flask (Nunc- Fisher Scientific UK) under axenic conditions and incubated at as before.

2.2.5 Cryopreservation of *Acanthamoeba*

As a standard procedure *Acanthamoeba* isolates were cryopreserved to maintain integrity and avoid potential subculturing cross contamination.

Axenic trophozoites were grown to late log phase (approximately 1 x 10^6), and then pelleted by centrifugation (500 x *g*, for 5 minutes). The pellet was resuspended in (heat- inactivated) foetal bovine serum (FBS) (Gibco®, Invitrogen, Paisley, U.K.) supplemented with 5% DMSO and 0.5 ml volumes were transferred to cryogenic vials

(Nalgene- Fisher Scientific UK). The vials were immediately placed in a cryopreservation unit- 5100 Cryo 1°C freezing container "Mr. Frosty", (Nalgene-Fisher Scientific UK). The commercially available unit is designed specifically for freezing organisms in cryogenic vials, and contains a special compartment to house propan-2-ol ensuring it surrounds the vials. The cryopreservation unit is placed on the bottom shelf of an -80°C freezer (Sanyo Gallenkamp, Loughborough, U.K.) for a minimum of four hours. Once in the freezer the container and propan-2-ol controls the cooling rate of the cells to 1°C /minute when they are in the presence of a cryoprotective agent commonly dimethyl sulfoxide (DMSO), therefore preventing intracellular ice crystal formation and cellular damage.

The vials containing strains were then catalogued and plunged into liquid nitrogen at -196°C, for long-term storage.

Recovery of a cryopreserved strain was achieved by rapidly thawing the relevant vial in a 37°C water bath. Then inoculating the cells into fresh culture media warmed to 32°C; although not essential, the media was usually supplemented with 10% FBS. To reduce any toxicity caused by the DMSO the media was replaced after six hours. After 24 hours incubation, the recovered trophozoites should have adhered to the wall of the flat-sided Nunclon™ Surface tissue culture tube (Fisher Scientific UK); again the culture media was replaced with a fresh supply. Confluent trophozoite growth was usually observed after a further 48 hours incubation 32°C.

2.2.5.1 Cryopreservation of bacteria

Bacteria were cryopreserved, by suspending the cells in LB broth containing 5% DMSO and rapidly freezing catalogued vials directly at -80°C (Sanyo) for long-term storage. Cells were recovered by rapid thawing at 37°C, and inoculated on to appropriate agar media or broth for incubation at 37°C. Again media was replaced after six hours to reduce any toxicity caused by the DMSO.

2.2.6 *Acanthamoeba* DNA isolation

Axenic *Acanthamoeba* cultures were harvested for high quality genomic DNA extraction by the UNSET method (Gast *et al*., 1994). If attempts to axenise strains had failed, the cells were continued to be maintained and propagated by the slower method of monoxenic culturing (section 2.2.3), *Acanthamoeba* cells and subsequently DNA was collected directly from the agar surface, with resulting lower concentrations of recovered DNA (see section 2.2.6.1).

Cells were harvested by centrifugation at 1,000 x *g* for 5 minutes, and the supernatant gently decanted. Cells were resuspended in 10 ml of ice cold Dulbecco's phosphate buffered saline (dPBS) (one tablet (Oxoid, Basingstoke, U.K.) dissolved in 100 ml, autoclaved at 121˚C for 15 minutes), and transferred to a 14 ml polypropylene centrifuge tube. Once washed, the cells were pelleted by centrifugation at 1,000 x *g* for 5 minutes, and the supernatant removed. 3 ml of UNSET buffer lysis was added (8M urea, 0.15M NaCl, 2% sarkosyl, 1mM EDTA, 0.1M Tris, per 1,000 ml of nH_2O, adjusted to pH 8.0 if necessary) and the solution gently inverted for approximately 10 seconds until lysate clears. Immediately DNA was extracted with the addition of 3 ml of phenol-chloroform (1:1), and gently

rocked from side to side ensuring the phases are completely mixed. The phases were separated by centrifugation at 2,000 x *g* for 2 minutes and the lower phase removed, using a fine tip pipette. These steps were repeated with a further 3 ml of phenol-chloroform (1:1), and followed by 3 ml of chloroform: isoamyl alcohol (24:1). With these steps culminating in a final spin of 2,000 x *g* for 15 minutes. The upper aqueous phase then transferred to a fresh polypropylene tube, and 0.8 volumes of ice-cold isopropanol added. The solution was gently mixed before being held at room temperature for 30 minutes or overnight at 4˚C. All precipitated DNA was pelleted by centrifugation at 2,000 x *g* for 15 minutes. The resulting pellet was washed with 1 ml of 70% ethanol, the pellet dislodged and recentrifuged at 2,000 x *g* for 5 minutes. All supernatant removed with a fine bore pipette. This ethanol wash step was repeated twice more. After the final removal of the supernatant, all remaining alcohol was evaporated in an air incubator at 32˚C (approximately 20 minutes) with the cap loosened. Ultimately the pellet was left to dissolve at 4˚C in 200-400 mL of $TE_{0.1}$ buffer (10mM Tris HCl, 0.1mM EDTA, autoclaved at 121˚C, and stored at 4˚C), containing RNase A (5 µl/ml) to remove any RNA.

The presence of the DNA was determined by running an aliquot on an agarose TAE gel (see section 2.2.7): With DNA quantity obtained using GeneSnap (version 7.08) (Syngene, Cambridge, U.K.) with the G: BOX XT gel documentation system (Syngene).

2.2.6.1 DNA extraction from *Acanthamoeba* in monoxenic culture

To harvest *Acanthamoeba* from the surface of the NNA plates, cells must be washed off the agar with 5 ml of ice cold dPBS, squirted repeatedly across the

surface until the amoebae cells are dislodged. The solution is then transferred to a 14 ml polypropylene tube, and centrifuged at 1,000 x *g* for five minutes. The supernatant was gently decanted, and the remaining amoebae washed within the tube with 10 ml of ice-cold dPBS and centrifuged again. This wash stage was repeated three times, to remove any remaining *E. coli*, before DNA extraction could commence (section 2.2.6).

2.2.7 Agarose gel electrophoresis

PCR product/DNA samples were separated in 1.2% 1X-Tris-acetate (TAE)-agarose gels (TAE 10X stock solution: 48.4 g Trizma base, 11.4 ml glacial acetic acid, 20 ml EDTA (0.5 M, pH 8.0) per 1,000 ml nH_2O) at 2 v/cm (approximately one hour) containing ethidium bromide (EtBr) 0.5 μg/ml, and visualised with a G: BOX XT gel documentation system (Syngene).

If required, a specific band can be isolated from a reaction containing multiple bands. Specific sized DNA fragments can be separated directly from a lower percentage agarose gel. Certain parameters must be followed to prevent degradation and cutting of the DNA caused by prolonged exposure to UV light. The DNA sample is separated in a 0.8% 1X-TAE agarose gel, at 2 v/cm (approximately one hour) containing EtBr (0.5 μg/ml). Prior to UV exposure the gel is placed on a glass plate, and the DNA fragment of interest is excised quickly with a sterile scalpel blade, and transferred into a 1.5 ml eppendorf tube.

The quantity of DNA was obtained using GeneSnap (version 7.08) (Syngene) with the G: BOX XT gel documentation system (Syngene).

2.2.8 Primer design

To obtain gene sequences from such a broad range of *Acanthamoeba* isolates several primer pairs were used. The JDP and 18s primers were obtained directly from the literature, although modification was made to 18sR (SSU1) to improve its specificity (Table 3), and the cytochrome oxidase (cox) primers were designed specifically for this study (Table 3). Primers were identified using GeneFisher software (Giegerich, 1996), and constructed by Eurofins MWG Operon (Eurofins MWG Operon, London, U.K.). Following primer optimisation reactions, basic PCR was carried out with the relevant primer specific parameters shown in (Table 3).

2.2.9 PCR amplification of DNA

PCR amplification of DNA was performed in either a 96-well GeneAmp® PCR System 9700 (Applied Biosystems, Warrington, U.K.) or a 24-well Perkin-Elmer GeneAmp® PCR system 2400 (Applied Biosystems). Using either a standard pre-made PCR reaction mixture (2X Reddymix™ PCR master mix; 1.5 mM $MgCl_{2;}$ ABGene, Surrey, U.K.), or one prepared using individual components (ABGene). All oligonucleotide primers were purchased from Eurofins MWG Operon.

To complete the pre-prepared PCR mix (2X Reddymix™; ABGene), approximately 100 ng of genomic DNA template was added, as well as 1 μM of both forward and reverse primers, and if necessary the $MgCl_2$ concentration was upwards adjusted from the initial concentration of 1.5 μM. Finally the mixture was made up to a total volume of 50 μl PCR mix with nH_2O.

The PCR mixture prepared with individual components consisted of approximately 100 ng of genomic DNA with 1X reaction buffer, magnesium

chloride ($MgCl_2$) (1.5 mM - 2.5 mM), 400 µM of dNTP, 1 µM of each forward and reverse primer, 1X sucrose dye (10X: 20% sucrose, 2 mM cresol red), and 1.5 U/µl of Red Hot DNA Polymerase (ABGene, Surrey, U.K.). The mixture was then increased to a total volume of 50 µl with nH_2O.

Thermal cycling conditions varied according to the oligonucleotides used within the reaction, but a typical cycle comprised of an initial DNA denaturing stage at 95˚C for 5 minutes, followed by 35 cycles of 95˚C for 30 seconds, 50˚C - 62˚C for 30 seconds (primer annealing depending on primer Tm), followed by 72˚C for amplification of product. The extension time was dependent on the size of the product being produced (30 seconds per 500 bp (Sambrook *et al*., 1989)). Followed by a final extension step for 10 minutes at 72˚C, however if the product was to be cloned in a TA vector this duration of this step was increased to 30 minutes. Finally the reaction was held at 14˚C, before a small quantity of the product was analysed by agarose gel electrophoresis (section 2.2.7).

If PCR products of the expected size were obtained the appropriate bands were purified by one of the techniques in section 2.2.10.

2.2.10 Purification of PCR products

As a general rule, the PEG method (2.2.10.1) was used for screening to confirm correct band size. While for purifying DNA ready for insertion into plasmids, the use of Microcon® centrifugal filters (device YM-50) (Millipore) were favoured as they produced the best sequencing results and therefore the most pure DNA of the three methods (2.2.10.1.i). If multiple DNA bands were produced by PCR method 2.2.10.2 was used to isolate the DNA of the correct size.

2.2.10.1 PEG purification of single bands

Production of a single band allows target DNA to be precipitated directly with a non-ionic water-soluble uncharged polymer such as polyethylene glycol (PEG) (Cole, 1991), which can then be dissolved in nH_2O. Or centrifuged in a single-spin Microcon® centrifugal filter device (YM-50) (Millipore, Watford, U.K.) that also results in sequencing or cloning grade DNA.

For the precipitation of DNA using PEG, 50 µl of 20% PEG (2.5 M NaCl) (10 g PEG 8,000, 7.3 g NaCl, up to 50 ml with nH_2O) was added to a 1.5 ml eppendorf, with 45 µl of PCR mix, and mixed by repeated pipetting, before being incubated for 15 minutes at 37°C. The mixture was then centrifuged at 12,000 x *g* for 15 minutes, and the supernatant carefully removed. The DNA pellet was washed with 0.5 ml of cold (4°C) 70% ethanol (EtOH), and again centrifuged at 12,000 x *g* for 5 minutes. The EtOH was again carefully removed, with any remaining alcohol allowed to evaporate during incubation at 32°C. Once completely dried the purified PCR products were dissolved in 20 µl of nH_2O (by pipetting several times and warming at 37°C the process can be assisted). Recovery can be confirmed by running 2 µl on an agarose gel (section 2.2.7).

2.2.10.1.i Micron®–PCR purification of single bands

The alternative method using Microcon®–PCR filter purification units (Millipore), and the supplied instructions, results an average 95% recovery rate. The system relies on an ultracel YM membrane and centrifugal force. The Microcon sample reservoir was inserted into the vial, and the PCR solution carefully pipetted into the reservoir (avoiding contact with the membrane), and the lid is sealed. The assemblage was then centrifuged at 14,000 x *g* for 12 minutes, at 25°C. Finally the

sample reservoir was removed from the original vial, and transferred inverted, to a new 1.5 ml eppendorf, and again centrifuged, but this time at 1,000 x *g* for 3 minutes, collecting the now purified PCR product into the base of the new eppendorf.

2.2.10.2 Target DNA purification from a multiple band PCR

If PCR amplification resulted in multiple bands, purification by an alternative method is necessary. Products from the reaction mix were separated in a 0.8% agarose gel, and the target band excised with a sterile scalpel. The gel segment containing the band was heated to 60°C with 3X 7 M guanidine acetate (67 g Guanidine hydrochloride, 20 ml Potassium acetate 3 M, pH 4.8 (29.4 g Potassium acetate, 11.5 ml Glacial acetic acid, to 100 ml with nH_2O, autoclaved at 121°C for 15 minutes, and stored at 4°C), total volume was increased to 90 ml and the pH adjusted to 5.5 with NaOH (10 M) if necessary), whilst warming and stirring. Volume is then increased to 100 ml and the solution passed through a 0.45 µM filter membrane), until the agarose gel dissolves. The chaotropic solute guanidine acetate increases the entropy of the system and disrupts the hydrogen bonds of DNA, forming a hydrophobic environment. Driven by this dehydration, when salt concentrations are high, the positively charged salt ions form a salt bridge between the negatively charged nucleic acids and the negatively charged silica. Proteins metabolites and other contaminants do not bind to the silica with the nucleic acids (Boom *et al.*, 1990, Nelson, 1992, Vogelstein and Gillespie, 1979). Accordingly 20 µl of silica in nH_2O (300 mg/ml) was added to the reaction (2 g silica suspended in 20 ml nH_2O, and allowed to settle for two hours. The milky supernatant removed again and the silica resuspended in a further 20 ml of nH_2O, these steps repeated twice more, before the remaining volume of silica was estimated, and the silica re-

suspended in 2 volumes of nH_2O). Ensuring it was fully in-suspension before doing so. The PCR reaction, guanidine acetate and silica mixture was vortexed and incubated at ambient temperature for 5 – 10 minutes with occasional further gentle mixing. The mixture was centrifuged at 10,000 x *g* for 10 seconds and the supernatant removed. The DNA bound silica was washed three times in 80% isopropanol, spinning at 10,000 x *g* for 10 seconds and removing all supernatant, between washes. Ensuring the eppendorf cap was opened, the DNA bound silica was incubated at 37°C for approximately 10 minutes ensuring the pellet was completely air-dried. The DNA was then dissolved in 20 µl of pre-warmed nH_2O, and incubated for 5 minutes at 60°C (with occasional mixing). Finally the silica was removed by centrifuging at 10,000 x *g* for 1 minute, and the supernatant containing the DNA was recovered in to a fresh eppendorf.

2.2.11 Ligation of DNA into cloning vectors

Ligation reactions were carried out in a 0.5 µl eppendorf. In a standard reaction containing 3.5 µl of amplified *cox1/2* or 18s PCR product, 5µl ligation buffer (2X), 25 ng linearised pGEM®-T Easy vector (Promega UK, Southampton, U.K.), 3 Weiss units of T4 DNA ligase (Promega UK), and nH_2O to a final volume of 10 µl were gently mixed by pipetting (so as not to form air bubbles). Ligation mixtures were incubated at ambient temperature for one hour, or 48 hours at 14°C.

A small aliquot of the modified plasmids were visualised and quantified by 1.5% agarose gel electrophoresis (detailed in section 2.2.7).

2.2.12 Production of ultra competent *E. coli* cells

Ultra competent *E. coli* cells were produced for transformation using a simplified method, based on the technique developed by Inoue (Inoue, 1990), and produces cells with an efficiency of $>10^8$ cfu/µg of plasmid DNA.

One ml of an overnight culture of *E. coli* DH5α (ATCC 53868) was added to a 2 L flask containing 250 ml of super optimal broth (SOB) (20 g Bacto™ tryptone (BD Biosciences), 5 g Bacto™ Yeast extract (BD Biosciences), 0.5 g NaCl, per 950 ml of nH_2O; Dissolved by shaking, then added 2.5 ml KCl (1M), adjusted to pH 7 with NaOH (5 M), and made up to 990 ml with nH_2O: Autoclaved at 121°C for 15 minutes, and finished by added 5 ml $MgCL_2$ (2 M), and $MgSO_4$ (2 M)).

Over a period of approximately two days, the cells were incubated at 18°C in a shake incubator (200-250 rpm), until an OD_{600} = 0.6 (optimal but 0.4 to 1 will work). Once the correct OD had been reached, the cells were placed on ice for 10 minutes, before being pelleted at 2,500 x *g* for 10 minutes at 4°C.

Next the cells were carefully resuspended without air bubbles in 80 ml of ice cold transformation buffer (TB) (3.36 g PIPES, 2.2 g $CaCl_2$, 18.64 g KCl, per 900 ml nH_2O: pH adjusted to 6.7 with KOH (5 M), whilst slowly stirring 10.88 g manganese chloride ($MnCl_2$) was added: Volume adjusted to 1 L with nH_2O: Filter sterilised and stored at 4°C). The cells were stored on ice for 10 minutes, before being pelleted again at 2,500 x *g* for 10 minutes at 4°C. Cells were carefully resuspended (air bubble free) in 20 ml ice cold TB: 7% DMSO was added and the solution placed on ice for 10 minutes. Aliquots of 0.5-1 ml quantities were transferred to cooled cryotubes and frozen in liquid N_2. The cells can be stored for up to a one-year at –70°C.

2.2.13 Heat shock transformation of ultra competent *E. coli*

The fragile ultra competent *E. coli* were thawed slowly on ice, for approximately 30 minutes. The solution was distributed in 200 µl aliquots, into cooled 1.5 ml eppendorfs and placed on ice. Added to one aliquot was 100 ng of circular pGEM®-T Easy plasmids now complete with inserts, while the control aliquot had an addition of 2 µl of $TE_{0.1}$ as a substitute for the lack of plasmid. The tubes were incubated on ice for 30 minutes, with the occasional gentle mix. The incubated solutions were exposed to a heat pulse without agitation at 42°C for 45 seconds, and transferred to an ice bath for 2 minutes. Next is the addition of 0.8 ml of SOC (SOB, with 20 mM filter sterilised 1 M glucose (super optimal broth with catabolite repression)), and the cells are left to recover and multiply in a shaking incubator (200-250 rpm) at 37°C.

Following incubation, identification of successful transformants is carried out on specific selective LB agar plates. A 200 µl aliquot of the transformation mix and control were plated on to LB agar indicator plates containing ampicillin (100 µg/ml), which have been surface supplemented with the blue/white screening indicator solutions, X-gal- 5-bromo-4-chloro-3-indolyl-β-D-galactoside in dimethylformamide (80µg/ml), and Isopropylthio-β-D-galactoside in nH_2O (IPTG) (0.5mM): These solutions must be added respectively, with each being left for a period of time to allow the liquid to evaporate. Once the surface is dry, the plates can be inoculated with transformed bacteria.

When antibiotic pressure is required, the agar is heated until molten but allowed to cool to 50°C before the addition of the required quantity of antibiotic. The bottle is then gently shaken to ensure even distribution throughout the solution.

In addition, the remaining solution from the test transformation mix was centrifuged, and the supernatant removed. The resultant pellet of cells was also plated on to an LB agar ampicillin indicator plate. All seeded plates were incubated overnight at 37°C.

Multiple second round selections on selective indicator LB agar were made of successful transformants to ensure single colonies are isolated. From this second plate distinct colonies were selected for cultivation in LB broth (section 2.2.3.1) with continued selective pressure of ampicillin at 100µg/ml, in a shake culture of 37°C, revolving at 180 rpm.

2.2.14 Restriction enzyme digestion

In a 0.5 ml eppendorf, the following components were mixed to produce a 2X restriction enzyme digestion, 2 µl of nH_2O, 1 µl of restriction enzyme EcoR 1 (Promega UK) and 2 µl of 10X enzyme buffer H (1X: 90mM Tris-HCl, 10mM $MgCl_2$, 50mM NaCl, pH 7.5) (Promega UK) (8-12 U/µl), (the enzyme must be kept on ice until returned to -20°C). Added to the reaction mixture was 15 µl of ligated target DNA (pGEM®-T Easy plasmids with insert). The mixture was incubated for 4-12 hours, at 37°C, and a small amount of the reaction was resolved by agarose gel electrophoresis (as in section 2.2.7).

Table 3. Primers (5' to 3') used for gene sequence typing of *Acanthamoeba* spp. SSU 18S rDNA and *cox1/2*, with corresponding PCR parameters.

Primers	**Sequence**	**$MgCl_2$ conc. MM**	**Annealing temp. °C**	**Extension time sec.**	**Product size bp**	**Paper cited**
***Acanthamoeba* 18S:**						
JDP1F	GGCCCAGATCGTTTACCGTGAA	1.5	60	45	450	(Schroeder *et al*., 2001)
JDP2R	TCTCACAAGCTGCTAGGGGAGTCA					
AC-892c	GTCAGAGGTGAAATTCTTGG	Sequencing primer				
18sF	CTGGTTGATCCTGCCAG	1.5	48	90	1.5-2 kb	(Weekers *et al*., 1994)
	Originally called SSU1					
18sR	GATCCTTCTGCAGGTTCACCTAC					Modified for this study
	Modified from SSU1					
18s588	CGCGCAAATTACCCAATC	Sequencing primer				
***Acanthamoeba cox1/2*:**						
CoxA125	ATGATTGG**H**GCTCC**N**GA**Y**ATGG	2.5	60	45	748	Designed for

CoxA873	TG**R**CCTCCCCATAATGTAGC					this study *
Cox1/2F	GAATTAGCTGCTCCGGGTTC	2 – 2.5	46 or 52	90	1.2 kb	(Kilvington *et*
Cox1/2R	TCAGGATAATCGGGGATCCTTC					*al*., 2004)
CoxA-486F	GC**H**GGTGCTATTACTATGCTTT	1.5	56 (60	30	566	Designed for
CoxA-1057R	CC**W**GCAAA**R**AA**R**GCAAAAACDGC		touchdown)			this study *
SeqCoxA	GGTGCTATTACTATGC	Sequencing primer				

*Wobble nucleotides for degenerate primers were those according to Operon: **H**, A/C/T; **W**, A/T; **R**, A/G; **N**, A/T/G/C; **Y**, C/T.

2.2.15 Plasmid purification

Several distinct white colonies were chosen at random from the LB agar (ampicillin 100 μg/ml) indicator plates, seeded with pGEM®-T Easy (plus insert) plasmids and transformed *E. coli*. A small section of each colony was inoculated into 5 ml universal tubes containing LB broth (ampicillin 100 μg/ml), and propagated overnight in a shaking incubator at 37°C, revolving at 180 rpm.

Plasmids were then purified with the relevant method depending on their downstream application.

For quick screening to confirm insert presence and size, the silica method (section 2.2.15.i) was selected. If plasmids were required for sequencing, either the comprehensive method (section 2.2.15.ii), or the plasmid purification kit (Qiagen Ltd, Crawley, U.K.) (2.2.15.iii) was carried out. As a general rule the comprehensive method was trialled first, but if failed at the sequencing stage as a result of impurities, plasmids were then purified using the commercial kit on the second attempt.

Once screening of the RE digests confirmed which colony contained a plasmid with the correct size insert, it was selected and propagated in an LB broth shake culture, before being purified for sequencing (as in section 2.2.15.iii).

2.2.15.1 Silica method of plasmid purification for screening

From an overnight culture of transformed *E. coli* DH5α, 1.4 ml was transferred into a 1.5 ml eppendorf, and pelleted at 6,000 x *g* for 2 minutes. Bacterial cell membranes were broken down and chromosomal DNA precipitated by the addition of lysis buffer, and GTE. The supernatant was removed, and the

remaining pellet resuspended in 120 µl GTE (50 mM Glucose, 25 mM Tris pH 8, 10 mM EDTA pH 8). Followed by the addition of 240 µl of lysis buffer (200 mM NaOH, 1% SDS, (Stored for up to one week at 4°C)). The tube was inverted six times, and incubated on ice for 5 minutes. Then 360 µl KAc (adjusted to pH 5.5 with acetic acid) was added, and the tube inverted six times, followed by centrifugation at 10,000 x g for 10 minutes. The supernatant was transferred to a new eppendorf, leaving behind any bacterial cell membranes.

To the new tube 20 µl of fully resuspended silica suspension was added (2.2.10.2), and the solution vortexed. The now DNA bound silica was pelleted by centrifugation at 10,000 x *g* for 10 seconds and all the supernatant removed. The silica was resuspended in 500 µl of wash solution (50 mM NaCl, 10 mM Tris pH 7.5, 2.5 mM EDTA, 50% ethanol) by gently pipetting. Once washed, the silica was again pelleted at 10,000 x *g* for 10 seconds, and the supernatant removed. A second spin was carried out, and any residual wash buffer removed. The silica was left to fully dry in their tubes with the caps open, in an incubator at 37°C for approximately 10 minutes. Finally the DNA was eluted into nH_2O, by resuspending the silica in 50 µl nH_2O and incubating at 60°C for 5 minutes (with occasional mixing). To remove the silica from the DNA in suspension, the solution was centrifuged at 12,000 x *g* for 1 minute, and the supernatant transferred to a new eppendorf.

Purified plasmids were visualised and quantified in 1.5% agarose gel electrophoresis (detailed in 2.2.7).

2.2.15.2 Comprehensive plasmid purification for sequencing

From an overnight culture of transformed *E. coli* DH5α, 1.4 ml was transferred into a 1.5 ml eppendorf, and pelleted at 12,000 x *g* for 1 minute, and the supernatant removed. Bacterial cell membranes were broken down and chromosomal DNA, precipitated by the addition of lysis buffer, and GTE. The supernatant was removed, and the remaining pellet resuspended in 200 µl GTE (50 mM Glucose, 25 mM Tris pH 8, 10 mM EDTA pH 8). Followed by the addition of 300 µl of lysis buffer (200 mM NaOH, 1% SDS), (Stored for up to one week at 4°C)). The solutions were mixed by tube inversion, and incubated on ice for exactly 5 minutes. A further addition of 300 µl KAc (adjusted to pH 5.5 with acetic acid) was carried out, and again mixed by inversion, and followed with a 5 minute incubation on ice. The solutions were then centrifuged at 12,000 x *g* for 10 minutes, and the supernatant transferred to a new eppendorf (between 700-750 µl). Rnase A to a final concentration of 20 µg/ml was added, and the solution left to incubate at 37°C for 20 minutes.

To remove unwanted proteins and oligosaccharides two chloroform extractions were carried out. Here 400 µl of chloroform was added, and the solutions mixed thoroughly. Centrifuging at 12,000 x *g* for 1 minute separated the phases, and the upper aqueous layer removed to a clean eppendorf. Two chloroform extractions were completed, and then DNA precipitated by adding equal volumes of isopropanol, and incubating on ice for 10 minutes. All DNA was pelleted by centrifugation at 12,000 x *g* for 15 minutes at ambient temperature. The surface of the pellet was then washed with 500 µl of 70% ethanol. All traces of the alcohol was removed, following a spin at 12,000 x *g* for 2 minutes, and air drying at 37°C for approximately 30 minutes. The DNA pellet was dissolved in 32 µl of nH_2O.

Once dissolved, 8 µl of NaCl (4 M) and 40 µl of 13% PEG 8,000, was added and thoroughly mixed, then incubated on ice for at least 20 minutes. The DNA was again pelleted by spinning at 12,000 x *g* for 15 minutes at 4°C. The supernatant removed, and the now translucent pellet washed in 70% ethanol, this step was carried out twice with 12,000 x *g* centrifugation between each wash. Following the washing steps all traces of ethanol was removed, after centrifugation at 12,000 x *g* for 5 minutes, and the pellet left to air dry at 37°C. Ultimately the pellet was dissolved in 25 µl nH_2O.

Purified plasmids were visualised and quantified by 1.5% agarose gel electrophoresis (detailed in 2.2.7).

2.2.15.3 QIAGEN® plasmid maxi kit

The comprehensive method for purifying plasmids for sequencing was used initially but replaced by QIAGEN® plasmid maxi kits (QIAGEN Ltd), also based on alkaline-lysis, and followed by binding of the free DNA to anion-exchange resin, therefore recovering the plasmids, before dissolving them in nH_2O.

Purified plasmids were visualised and quantified by 1.5% agarose gel electrophoresis (detailed in 2.2.7).

2.2.16 Sequencing

The inserts were commercially sequenced using the standard technique of automated sequencing at either the Protein Nucleic Acid Chemistry Laboratory (PNACL; University of Leicester, U.K.) using fluorescently labelled M13 or T7 primers in a 3730 DNA analyser automated sequencer (Applied Biosystems,

Warrington, U.K.), or at Eurofins MWG Operon using their value read sequencing service.

2.2.17 Sequence analysis

Sequence data was viewed on a chromatogram using Chromas software version 2.23 (http://www.technelysium.com.au/chromas.html); primer sites were excluded from analysis to reduce any forced bias. Sequences were then identified via a basic local alignment search tool (BLAST) (Altschul *et al*., 1997), using nucleotide blast (blastn) to locate other highly similar sequences for comparison. Multiple sequences of the same target region from different strains and species were aligned together using ClustalW (Larkin M.A. *et al*., 2007), through the BioEdit interface (version 7.0.4.1.) (Hall, 1999). With BioEdit, sequences can be aligned (ClustalW) and extraneous data including primer-binding sites can be easily removed, which removes any bias caused by their inclusion in the alignment. The software ClustalW is a general purpose multiple sequence alignment program for DNA, which aligns the sequences allowing single nucleotide differences to be identified. Finally phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5.05 (Kumar *et al*., 2004), including the concatenation of the two genes: The multiple aligned sequences were subject to phylogenetic reconstruction to produce a gene tree using neighbour-joining (NJ) distance analysis or maximum parsimony (MP) with the kimura two-parameter correction, and bootstrap support for all phylogenetic trees determined from 1000 replications.

2.3 Results

2.3.1 18S genotyping of strains

Sequences of 18S rDNA and mt *cox1/2* were obtained from 28 unique drug resistant strains, isolated by corneal scrapings from 17 patients. These strains were added to a range of 12 known isolates, including type species, from all three morphological groups and 11 T-groups. Sequence variation across ~204 bp and ~564 bp respectively, from the group of 40 isolates was analysed.

Phylogenetic relationships among isolates were examined by maximum parsimony (MP) and neighbour joining (NJ) analysis, and comparisons were made of any group's formed, bootstrap values, and tree topography.

Bootstrap values have been included to help determine groups, as it is generally considered that values of greater than 70 are evidence to support the distinction of the clade. Phylogenetic trees produced by both NJ and MP algorithms, do vary from each other (NJ: <48 = 35%; 49-94 = 50%; >95 = 15%. MP: <48 = 86%; 49-94 = 11%; >95 = 3%), with NJ trees resulting in a greater number of higher bootstrap values. However the clusters of taxa remain largely the same, although their position within the tree vary depending on the analysis used.

The 18S NJ tree (Figure 1) shows multiple species are clumped together in genotype groups T3, T4 and T11, with the maximum pairwise distance value between the three clades at 0.055 (T11 verses T3), and a minimum value of 0.033 (T3 verses T4 (Table 4)). The 18S tree highlights the close relationship between T3, T4 and T11 clades. With the T11 group located between two subsections of T3, where isolates AK 1a, AK 4, AK 6 and AK 14 are above the T11 cluster, and AK 5, AK 5a, AK 11 and *A. griffinii* 1501/4 are below (Figure 1). Also located a separate

branch of its own is the T4 isolate *A. castellanii* 1501/1a, which when analysed here clusters above T11 and below the upper T3 subsection.

Table 4. Estimates of evolutionary divergence over 18S sequence pairs between T-group genotypes. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura *et al*., 2004). The analysis involved 40 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 336 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011) in press.

	T11	**T3**	**T4**	**T6**	**T2**	**T10**	**T9**	**T8**	**T5**
T3	0.055	-	-	-	-	-	-	-	-
T4	0.034	0.033	-	-	-	-	-	-	-
T6	0.104	0.081	0.084	-	-	-	-	-	-
T2	0.123	0.109	0.111	0.034	-	-	-	-	-
T10	0.101	0.118	0.093	0.14	0.161	-	-	-	-
T9	0.221	0.236	0.219	0.276	0.29	0.229	-	-	-
T8	0.258	0.211	0.229	0.263	0.274	0.25	0.118	-	-
T5	0.091	0.091	0.072	0.079	0.07	0.1	0.229	0.249	-
T7	0.256	0.199	0.226	0.238	0.271	0.272	0.109	0.07	0.248

When comparing average pairwise distances of all the T-groups, maximum values occur between T2 verses T8 (0.274), and minimum between T3 and T4 (0.033) (Table 4). Differences between the closely related sequence types; T3, T4

and T11 in this study, are less than the previously recorded value of at least 5% (which is an arbitrary value) (Stothard *et al*., 1998). Here, dissimilarity values of 3.3% (T4 verses T3), 5.5% (T3 verses T11) and 3.4% (T4 verses T11) were obtained. Sequences types T2 and T6 also showed high sequence similarity, with a dissimilarity score of 3.4%. Differences between genotypes were always greater than within sequence types.

Of the 28 clinical AK isolates, 11 strains collected from eight patients were analysed and assigned to the T4 clade (Figure 1). While seven AK isolates from six patients were assigned to the clade T3 with *A. griffinii* 1501/4. The final 11 isolates from six patients were included on three branches within T11 with *A. hatchetti* Bh2.

Distinct sequence types placed at the very bottom of the tree are all morphological group I species, which have previously been assigned to T7 (*A. astronyxis* 30137), T8 (*A. tubiashi* 30876), and T9 (*A. comandoni* 1501/5). Placed between the lower morphological group I species, are the closely linked T2 and T6 species (*A. palestinensis* 1501/3c and *A. palestinensis* 1547/1), along with T9 *A. lenticulata* PD2S on a separate branch.

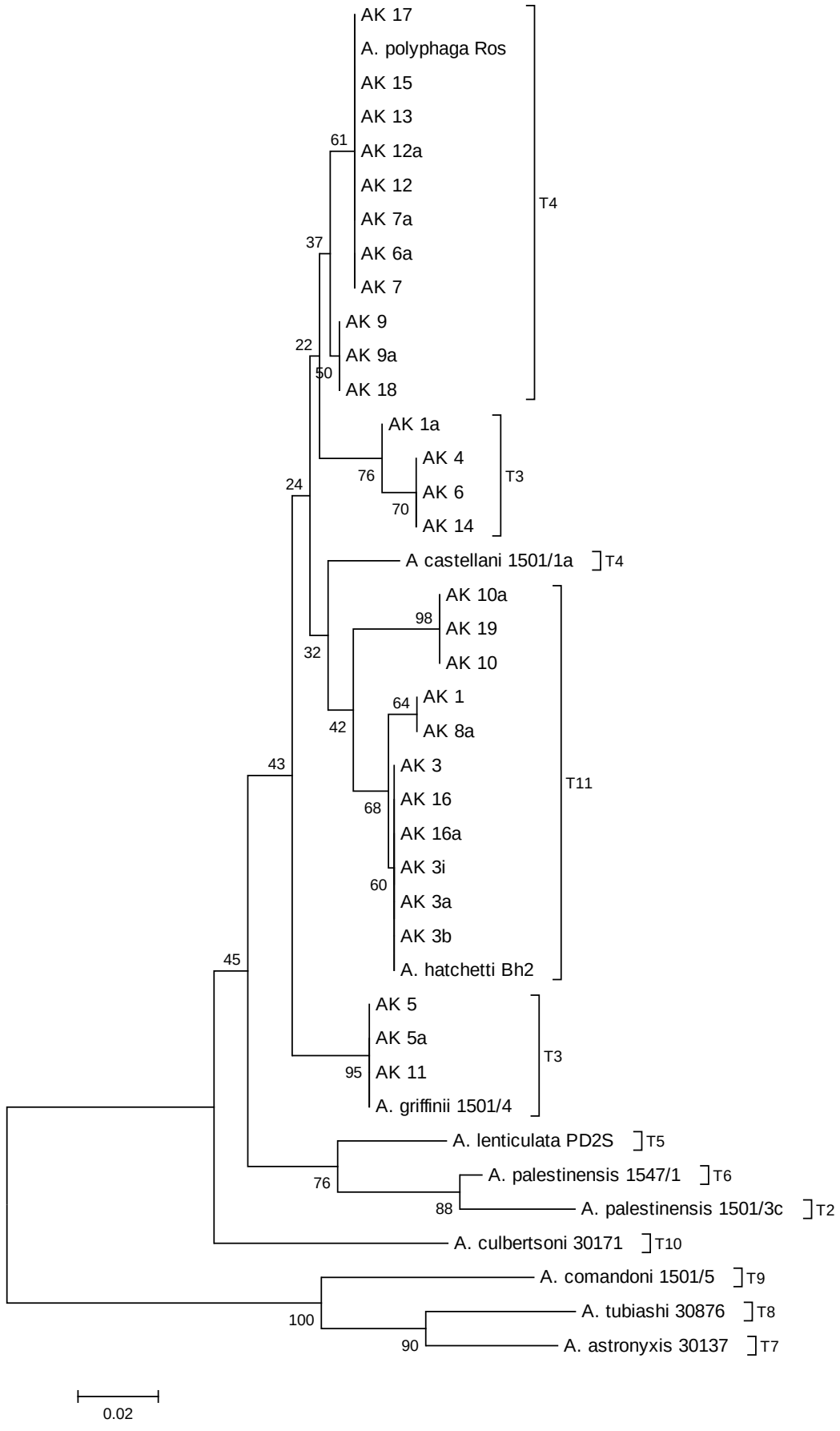
AK 17
A. polyphaga Ros
AK 15
AK 13
61
AK 12a
AK 12
AK 7a
37
AK 6a
AK 7
AK 9
22
AK 9a
50
AK 18
T4
AK 1a
AK 4
76
AK 6
70
AK 14
T3
24
A castellani 1501/1a
T4
AK 10a
98
AK 19
32
AK 10
64
AK 1
42
AK 8a
43
AK 3
AK 16
68
AK 16a
AK 3i
60
AK 3a
AK 3b
45
A. hatchetti Bh2
T11
AK 5
AK 5a
95
AK 11
A. griffinii 1501/4
T3
A. lenticulata PD2S
T5
76
A. palestinensis 1547/1
T6
88
A. palestinensis 1501/3c
T2
A. culbertsoni 30171
T10
A. comandoni 1501/5
T9
100
A. tubiashi 30876
T8
90
A. astronyxis 30137
T7
0.02

Figure 1. Neighbour joining distance tree based on partial 18S rDNA sequences of *Acanthamoeba* spp. The tree is unrooted and obtained by Kimura two-parameters correction for multiple substitutions using MEGA (5.05). The tree is based on reference bp from 1,175 to 1,379. The scale bar represents the corrected number of nucleotide substitutions per base using Kimura method. Designated T-groups are shown.

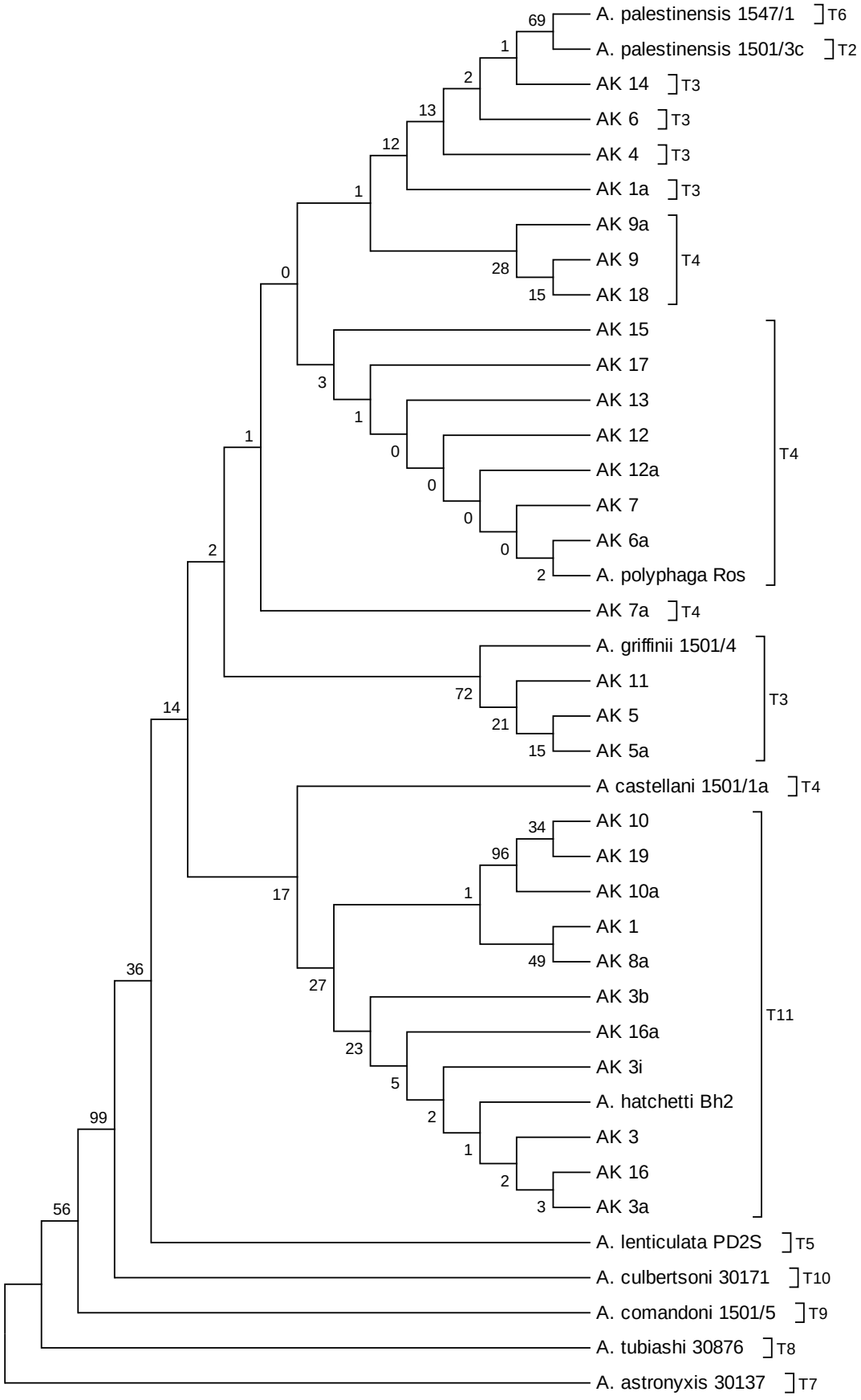
A. palestinensis 1547/1
T6
A. palestinensis 1501/3c
T2
AK 14
T3
AK 6
T3
AK 4
T3
AK 1a
T3
AK 9a
AK 9
AK 18
T4
AK 15
AK 17
AK 13
AK 12
AK 12a
AK 7
AK 6a
A. polyphaga Ros
T4
AK 7a
T4
A. griffinii 1501/4
AK 11
AK 5
AK 5a
T3
A castellani 1501/1a
T4
AK 10
AK 19
AK 10a
AK 1
AK 8a
AK 3b
AK 16a
AK 3i
A. hatchetti Bh2
AK 3
AK 16
AK 3a
T11
A. lenticulata PD2S
T5
A. culbertsoni 30171
T10
A. comandoni 1501/5
T9
A. tubiashi 30876
T8
A. astronyxis 30137
T7

Figure 2. Maximum parsimony distance tree based on partial 18S rDNA sequences of *Acanthamoeba* spp. The tree is unrooted and obtained by Kimura two-parameters correction for multiple substitutions using MEGA (5.05). The tree is based on reference bp from 1,175 to 1,379. Designated T-groups are shown.

2.3.2 Typing strains with *cox1/2* gene

Sequences of mt *cox1/2* were obtained from 28 unique drug resistant strains, isolated by corneal scrapings, from 17 patients. These strains were added to a range of 12 known isolates, including type species, from all three morphological groups and 11 T-groups. Sequence variation across ~564 bp, from the group of 40 isolates was analysed.

Phylogenetic relationships among isolates were examined by maximum parsimony (MP) (Figure 3) and NJ analyses (Figure 4).

When examined by NJ analysis as with *cox1/2*, *A. tubiashi* 30876 (T8) was found to be distinct from the main group of *Acanthamoeba* sp., but interestingly even more distinct was the species *A. hatchetti* Bh2 (Figure 4). By removing these two distinct species from the comparison, the branches of the tree become more elongated (data not shown). Unfortunately, despite continued efforts, mt *cox1/2* sequences could not be obtained for *Hartmannella* or *Balamuthia* spp. in order that they be included as an outlying species or true evolutionary ancestor, and allow *A. tubiashi* and *A. hatchetti* to remain within the main section of the tree.

Figure 3, displays phylogenetic relationships when examined by maximum parsimony and includes bootstrap values to help determine clades, as it is generally considered that values of greater than 70 are evidence to support the distinction of the clade. Phylogenetic trees produced by both NJ and MP algorithms, do not vary

extensively from each other (NJ: <48 = 32%; 49-94 = 42%; >95 = 19%. MP: <48 = 50%; 49-94 = 29%; >95 = 18%), and the clusters of taxa remain the same, although their position within the tree may vary depending on the analysis used. The outlying distinct taxa change, from *A. tubiashi* 30876 (T8) and *A. hatchetti* Bh2 (T11) with NJ, to group I species *A. astronyxis* 1534/1 with MP analysis.

Using *cox1/2* tree analysis with bootstrap values, eight groups can be identified (Figure 3 and 4) (Group A-H). Across these sequence types, the pathogenic strains including the Moorfield AK strains, are resolved into smaller clades, than the general clumping together formed by 18S T-group analysis. T11 isolates are no longer clustered together, especially relevant to the repeat isolates from Patient 3 (AK 3 AK 3i, AK 3a, AK 3b, AK 16, and AK 16a), which were all located in a tight clade by 18S (Figure 1 and 2), but are now distributed throughout *cox1/2* groups A, C, E, G and H. Within this study *A. castellanii* 1501/1a, AK 10a, *A. palestinensis* 1547/1, *A. griffini* 1501/4, *A. tubiashi* 30876, *A. hatchetti* Bh2, *A. astronyxis* 1534/1, *A. comandoni* 1501/5 and *A. palestinensis* 1501/3c cannot be assigned to a clade with a relevant bootstrap value of over 70.

Strains with pathogenic potential are found throughout the tree, and AK and encephalitis causing strains are not distinct from those which are thought be non-pathogenic. Interestingly, *cox1/2* groups were not limited to repeat isolate sequences but often contain isolates collected from different patients/locations. The range of pairwise distance values between the *cox1/2* sequence groups varies from a minimum of 0.036 (group B verses C), to a maximum of 0.286 (group H verses F) (Table 5).

Distinct species at the base of the *cox1/2* trees differs depending on analysis. By NJ *A. hatchetti* Bh2 and *A. tubiashi* 30876 are the distinct strains, but by MP analysis the outlying species is A. *astronyxis* 1534/1. Sequence similarity between the *cox1/2* groups is always higher than 5%, and the differences between sequence types is always greater than within sequence types.

Table 5. Estimates of evolutionary divergence over *cox1/2* sequence pairs between groups (A-G). The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura *et al*., 2004). The analysis involved 40 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 336 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011) in press.

	G	**C**	**E**	**H**	**B**	**A**	**D**
C	0.229	-	-	-	-	-	-
E	0.23	0.101	-	-	-	-	-
H	0.23	0.228	0.224	-	-	-	-
B	0.189	0.036	0.09	0.193	-	-	-

A	0.219	0.039	0.092	0.233	0.023	-	-
D	0.242	0.095	0.105	0.247	0.07	0.085	-
F	0.265	0.201	0.222	0.286	0.172	0.207	0.237

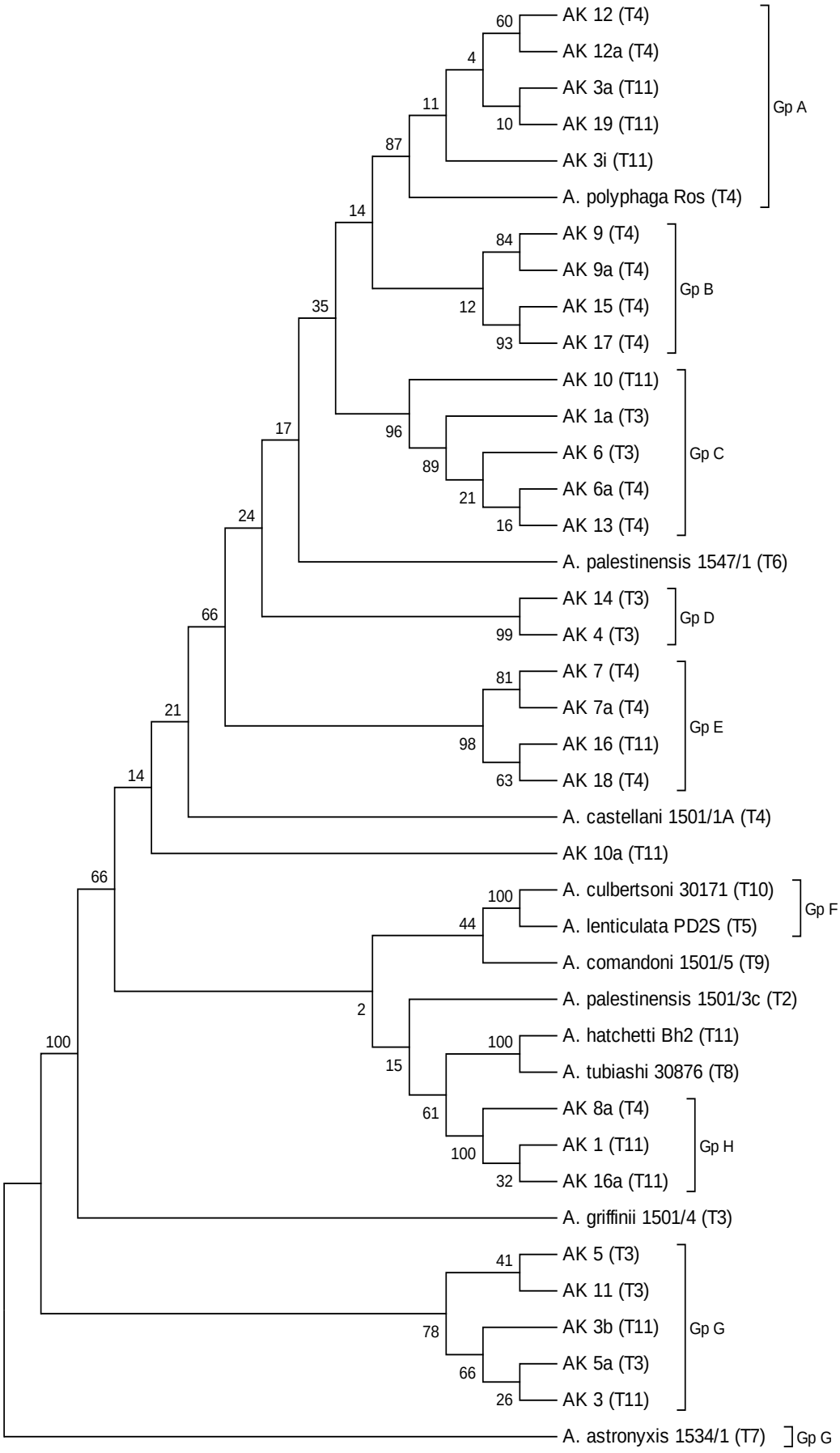
AK 12 (T4)
AK 12a (T4)
AK 3a (T11)
AK 19 (T11)
AK 3i (T11)
A. polyphaga Ros (T4)
Gp A
AK 9 (T4)
AK 9a (T4)
AK 15 (T4)
AK 17 (T4)
Gp B
AK 10 (T11)
AK 1a (T3)
AK 6 (T3)
AK 6a (T4)
AK 13 (T4)
Gp C
A. palestinensis 1547/1 (T6)
AK 14 (T3)
AK 4 (T3)
Gp D
AK 7 (T4)
AK 7a (T4)
AK 16 (T11)
AK 18 (T4)
Gp E
A. castellani 1501/1A (T4)
AK 10a (T11)
A. culbertsoni 30171 (T10)
A. lenticulata PD2S (T5)
Gp F
A. comandoni 1501/5 (T9)
A. palestinensis 1501/3c (T2)
A. hatchetti Bh2 (T11)
A. tubiashi 30876 (T8)
AK 8a (T4)
AK 1 (T11)
AK 16a (T11)
Gp H
A. griffinii 1501/4 (T3)
AK 5 (T3)
AK 11 (T3)
AK 3b (T11)
AK 5a (T3)
AK 3 (T11)
Gp G
A. astronyxis 1534/1 (T7)
Gp G

Figure 3. Maximum parsimony tree based on partial mt *cox1/2* sequences of *Acanthamoeba* spp, with *cox1/2* groups (A-H) and T-groups (T2-T11) included. The tree is unrooted and obtained by Kimura two-parameters correction for multiple substitutions using MEGA (5.05). The tree is based on reference bp 8,002 to 8,566. Bootstrap values have been included, based on 1,000 bootstrap values, and are placed at the nodes they apply to.

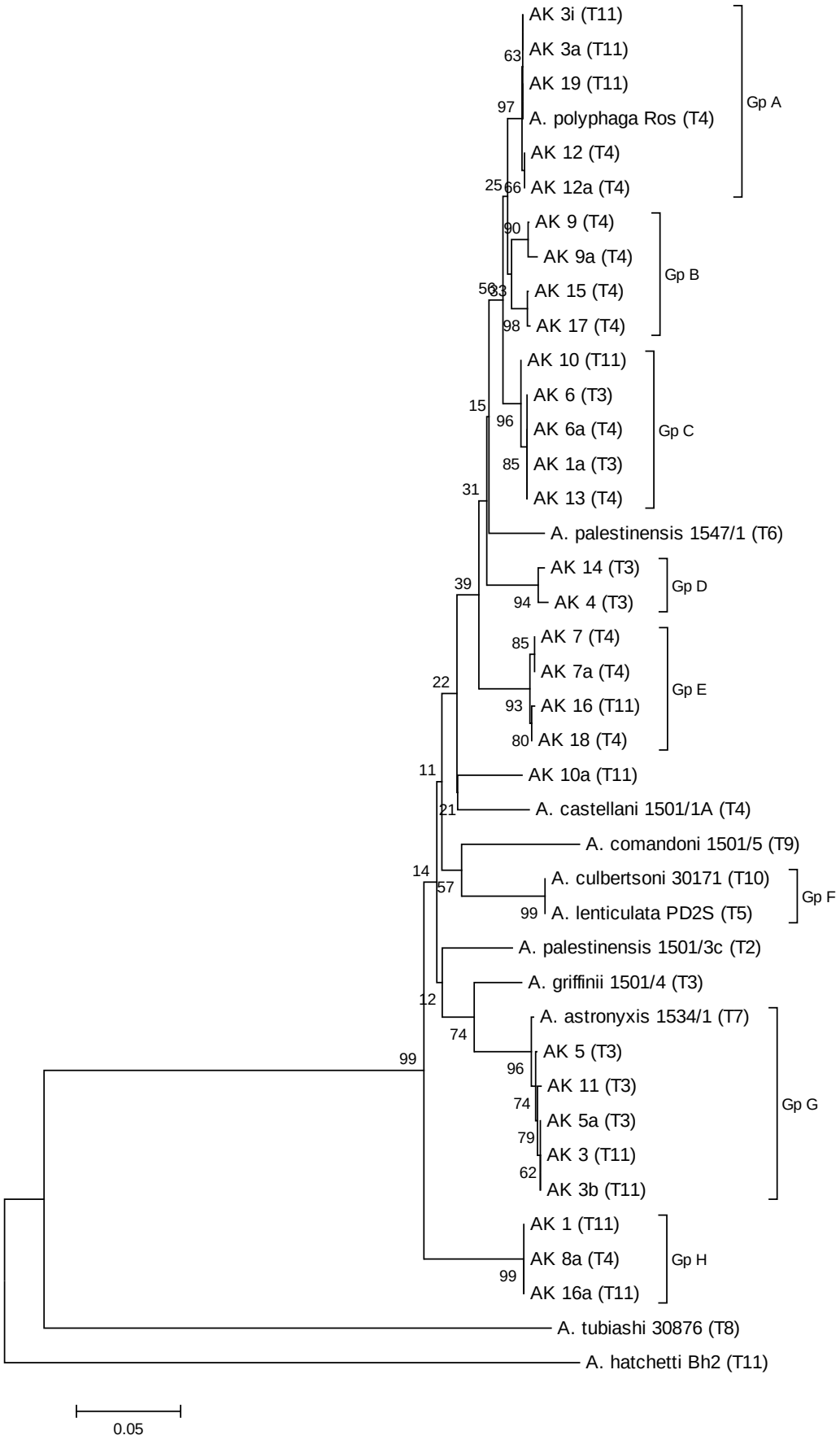
AK 3i (T11)
AK 3a (T11)
AK 19 (T11)
A. polyphaga Ros (T4)
AK 12 (T4)
AK 12a (T4)
Gp A
AK 9 (T4)
AK 9a (T4)
AK 15 (T4)
AK 17 (T4)
Gp B
AK 10 (T11)
AK 6 (T3)
AK 6a (T4)
AK 1a (T3)
AK 13 (T4)
Gp C
A. palestinensis 1547/1 (T6)
AK 14 (T3)
AK 4 (T3)
Gp D
AK 7 (T4)
AK 7a (T4)
AK 16 (T11)
AK 18 (T4)
Gp E
AK 10a (T11)
A. castellani 1501/1A (T4)
A. comandoni 1501/5 (T9)
A. culbertsoni 30171 (T10)
A. lenticulata PD2S (T5)
Gp F
A. palestinensis 1501/3c (T2)
A. griffinii 1501/4 (T3)
A. astronyxis 1534/1 (T7)
AK 5 (T3)
AK 11 (T3)
AK 5a (T3)
AK 3 (T11)
AK 3b (T11)
Gp G
AK 1 (T11)
AK 8a (T4)
AK 16a (T11)
Gp H
A. tubiashi 30876 (T8)
A. hatchetti Bh2 (T11)
0.05

Figure 4. Neighbour-joining distance tree based on partial mt *cox1/2* sequences of *Acanthamoeba* spp with *cox1/2* groups (A-H) and T-groups (T2-T11) included. The tree is unrooted and obtained by Kimura two-parameters correction for multiple substitutions using MEGA (5.05). The tree is based on reference bp 8,002 to 8,566. The scale bar represents the corrected number of nucleotide substitutions per base using Kimura method.

2.3.3 Introns, multiple alleles and mixed infections

Using repeat patient isolates evidence presented here suggests two patients contained species with multiple alleles.

Repeat samples include AK 1 and AK 1a from Patient one, AK 6 and AK 6a from Patient six, and AK 3, AK 3i, AK 3a, AK 3b, AK 16 and AK 16a from Patient 3. 18S NJ and MP tree analysis shows Patient one's isolate AK 1 in T11 clustering independently from the second isolate, AK 1a in T3. The T3 clade also contains an isolate from Patient six (AK 6a). While the other Patient six isolate, AK 6 clusters within T4 (Figure 1 and 2).

However by sequencing a second gene, *cox1/2*, Patient six's isolates AK 6 and AK 6a, are found to have sequence homology with a pairwise distance score of 0 (*cox1/2* group c by NJ and MP trees) (Figure 3 and 4), there by indicating multiple alleles of 18S but not *cox1/2*.

This was not so for Patient one's isolates (AK 1 and AK 1a), which showed sequence variation occurring in both *cox1/2* and 18S genes. The high dissimilarity sequence variation of *cox1/2* 10.2% (pairwise value of 0.102) and 18S (pairwise value 0.052) genes, suggests the presence of a mixed infection (Figure 1, 2, 3 and 4).

Isolates AK 3, AK 3i, AK 3a, AK 3b, AK 16 and AK 16a are all repeat strains collected from Patient three. When aligned by 18S they are 100% identical, and cluster tightly together on the same branch within T11, with *A. hatchetti* Bh2 (Figure 1 and 2). However by *cox1/2*, analysis of the data suggests that there are four copies of the gene within the strain. The isolates are spread out across the tree in five groups, A, C, E, G and H (Figure 3 and 4), with sequence dissimilarity values ranging from 4.6 to 10.2% (Table 6).

Within the region of the sequenced region of the *cox1/2* gene, analysis shows no indication of introns, while evidence for the presence of multiple alleles and mixed infections were found.

Table 6. Pairwise distance values between *cox1/2* sequences of repeat isolates from Patient three. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura *et al*., 2004). The analysis involved 40 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 336 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011) in press.

	AK 3	**AK 16**	**AK 16a**	**AK 3i**	**AK 3a**
AK 16	0.098	-	-	-	-
AK 16a	0.098	0.100	-	-	-
AK 3i	0.094	0.046	0.102	-	-
AK 3a	0.094	0.046	0.102	0.000	-
AK 3b	0.000	0.098	0.098	0.098	0.094

2.3.4 Concatenated sequence data

Trees were also analysed from 18S and cox1/*2* data, which had been concatenated together to determine which resulting tree from all three data sets provided the greatest resolution.

MP and NJ trees both contained 37 nodes supported by bootstrap values. The tree from NJ analysis (Figure 6) contains more bootstrap values of greater than 49 compared with MP trees (Figure 5) (NJ- <48 = 24%, 49-94 = 46%, >95 = 32%;

MP- <48 = 32%, 49-94 = 46%, >95 = 16%). Both contain similar clade formation, with differing distinct strains at the base of the trees, following trees derived from *cox1*/*2* data alone. The distinct species by NJ are *A. tubiashi* 30876, and *A. hatchetti*, which by MP form their own group between cox1/*2* groups E and H. Leaving *A. astronyxis* 1534/1 to be the distinct species in concatenated MP trees. Another distinction between MP and NJ trees with concatenated data is the split of group B, with AK 15 and AK 17 in the top half, and AK 9 and AK 9a in the second half (Figure 5).

Clade formation of concatenated trees most resembles those of *cox1*/*2*, rather than 18S. However shorter branch lengths are found on the concatenated tree than those of *cox1*/*2*. The concatenated tree provides more resolution between species, rather than the clumping found with 18S trees, supported by a greater number of nodes with significant bootstrap values of greater than >95.

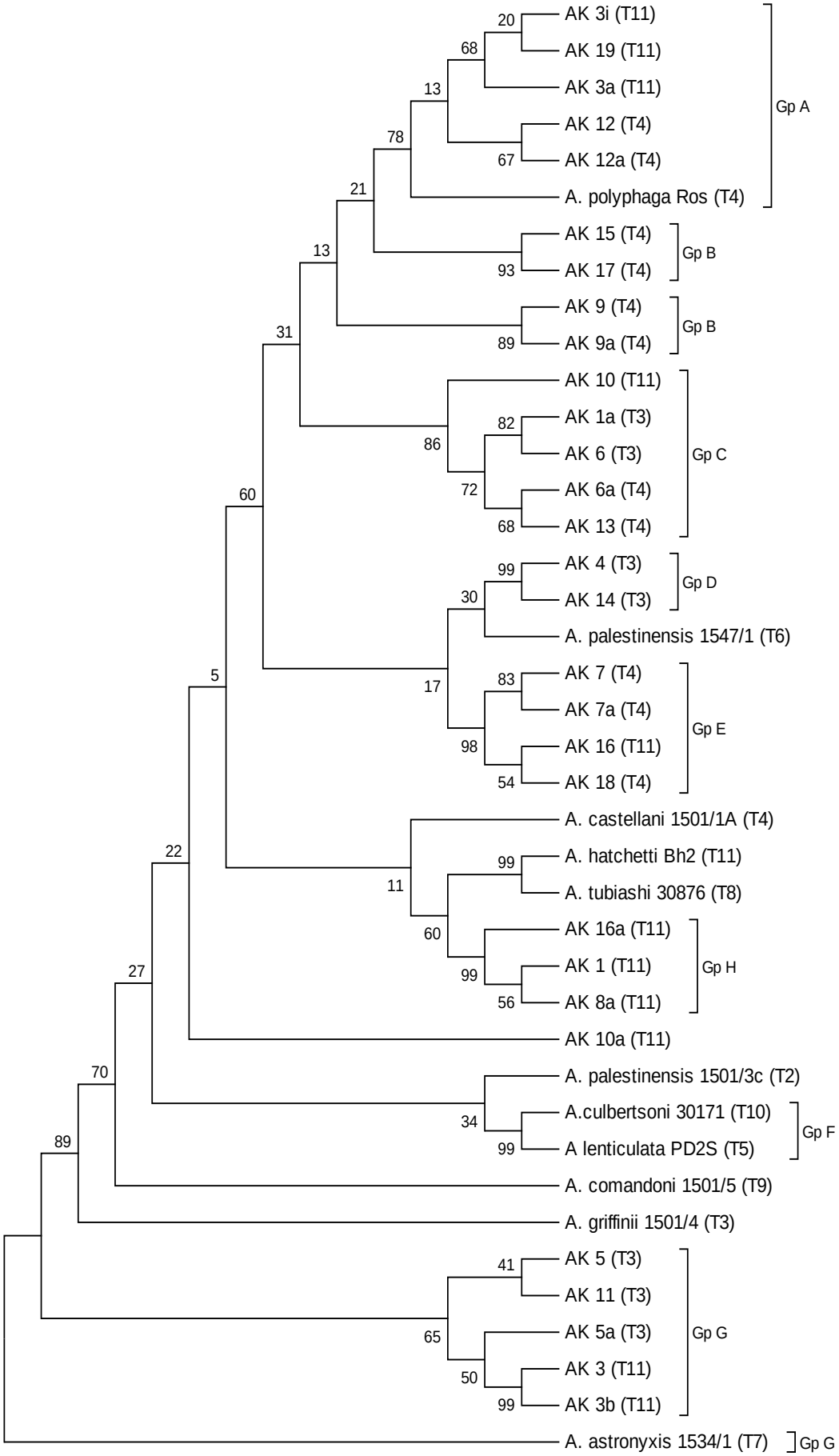
AK 3i (T11)
AK 19 (T11)
AK 3a (T11)
AK 12 (T4)
AK 12a (T4)
A. polyphaga Ros (T4)
Gp A
AK 15 (T4)
AK 17 (T4)
Gp B
AK 9 (T4)
AK 9a (T4)
Gp B
AK 10 (T11)
AK 1a (T3)
AK 6 (T3)
AK 6a (T4)
AK 13 (T4)
Gp C
AK 4 (T3)
AK 14 (T3)
Gp D
A. palestinensis 1547/1 (T6)
AK 7 (T4)
AK 7a (T4)
AK 16 (T11)
AK 18 (T4)
Gp E
A. castellani 1501/1A (T4)
A. hatchetti Bh2 (T11)
A. tubiashi 30876 (T8)
AK 16a (T11)
AK 1 (T11)
AK 8a (T11)
Gp H
AK 10a (T11)
A. palestinensis 1501/3c (T2)
A.culbertsoni 30171 (T10)
A lenticulata PD2S (T5)
Gp F
A. comandoni 1501/5 (T9)
A. griffinii 1501/4 (T3)
AK 5 (T3)
AK 11 (T3)
AK 5a (T3)
AK 3 (T11)
AK 3b (T11)
Gp G
A. astronyxis 1534/1 (T7)
Gp G

Figure 5. Maximum parsimony distance tree based on concatenated partial mt *cox1/2* with 18S sequences of *Acanthamoeba* spp with cox1/*2* groups (A-H) and T-groups (T2-T11) included. The tree is unrooted and obtained by Kimura two-parameters correction for multiple substitutions using MEGA (5.05). The tree is based on reference bp 8,002 to 8,566.

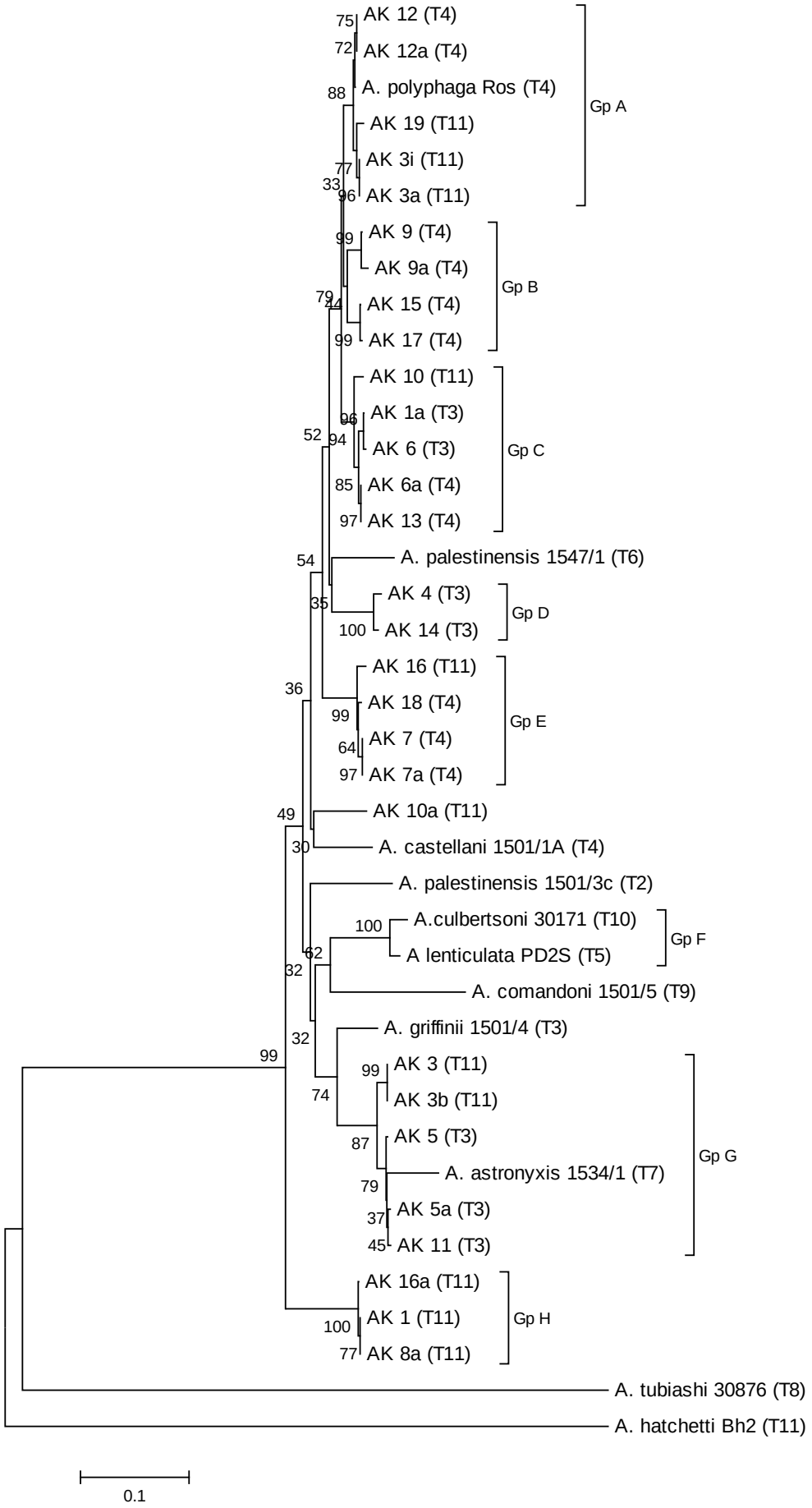
AK 12 (T4)
AK 12a (T4)
A. polyphaga Ros (T4)
AK 19 (T11)
AK 3i (T11)
AK 3a (T11)
Gp A
AK 9 (T4)
AK 9a (T4)
AK 15 (T4)
AK 17 (T4)
Gp B
AK 10 (T11)
AK 1a (T3)
AK 6 (T3)
AK 6a (T4)
AK 13 (T4)
Gp C
A. palestinensis 1547/1 (T6)
AK 4 (T3)
AK 14 (T3)
Gp D
AK 16 (T11)
AK 18 (T4)
AK 7 (T4)
AK 7a (T4)
Gp E
AK 10a (T11)
A. castellani 1501/1A (T4)
A. palestinensis 1501/3c (T2)
A.culbertsoni 30171 (T10)
A lenticulata PD2S (T5)
Gp F
A. comandoni 1501/5 (T9)
A. griffinii 1501/4 (T3)
AK 3 (T11)
AK 3b (T11)
AK 5 (T3)
A. astronyxis 1534/1 (T7)
AK 5a (T3)
AK 11 (T3)
Gp G
AK 16a (T11)
AK 1 (T11)
AK 8a (T11)
Gp H
A. tubiashi 30876 (T8)
A. hatchetti Bh2 (T11)
0.1

Figure 6. Neighbour-joining distance tree based on concatenated partial mt *cox1/2* with 18S sequences of *Acanthamoeba* spp with *cox1/2* groups (A-H) and T-groups (T2-T11) included. The tree is unrooted and obtained by Kimura two-parameters correction for multiple substitutions using MEGA (5.05). The tree is based on reference bp 8,002 to 8,566. The scale bar represents the corrected number of nucleotide substitutions per base using Kimura method.

2.4 Discussion

2.4.1 Genotyping *Acanthamoeba* sp. with 18S sequences

There are many examples of the use of 18S based molecular studies for epidemiological typing, to uncover relationships between patients with *Acanthamoeba* infections and their environments (Maghsood *et al.*, 2005, Schroeder *et al.*, 2001, Walochnik *et al.*, 2000). As all forms of life share features of structure and sequence within rRNA, analysis of it has been used to map phylogenetic history across organisms at all levels, including the discovery of a third kingdom the archaea, in addition to prokaryotes and eukaryotes (Woese *et al.*, 1990).

Within *Acanthamoeba*, rRNA genes show a relatively slow rate of change, and are a suitable candidate to base a genotyping system on (Gast *et al.*, 1996). From the similarities of the genotype clusters found by analysis of both nuclear (*Rns*) and mitochondrial (*rns*) small subunit rRNA genes, it can be assumed that phylogenetic clades based on 18S sequences represent evolutionary history (Ledee *et al.*, 2003). Thereby inferring all clades linked with AK (T2, T3, T4, T5, T6 and T11), and GAE (T1, T4, T10 and T12), each share evolutionary adaptations to have the potential to cause infections. In general T4 genotypes are responsible for most AK infections, such a marked phylogenetic localisation suggests either, innate pathogenic potential, a peculiarity of the gene sequence, or increased prevalence in the environment (Ledee *et al.*, 2003). Yet evidence has been presented to show the close relationship between an eye and a lung isolate when examined by 18S sequence, leading the authors to conclude that any pathogenic strain may be capable of infecting more than one type of tissue (Gast *et al.*, 1996).

By sequencing the 18S gene of *Acanthamoeba* it has been shown that most isolates, both environmental and clinical including those from all forms of

acanthamebiasis can be typed into the T4 genotype (Gast *et al.*, 1996, Schroeder *et al.*, 2001, Stothard *et al.*, 1999, Stothard *et al.*, 1998). In a single study examining 249 isolates, 179 of them belonged to T4. These isolates were obtained from both environmental and clinical sources from, AK patients and non-AK including CSF, brain, skin and lungs (Booton *et al*., 2005).

Isolates of the T4 genotype appear to be the predominant cause of AK and GAE, and the only genotypes yet to be linked to human disease are T7, T8, T9, T13, T14 and T15 (Khan, 2009). To find pathogenic strains classified to groups other than T4 is less common (Booton *et al.*, 2005, Gast *et al.*, 1996, Stothard *et al.*, 1999, Stothard *et al.*, 1998). However, exception has been shown in this study, with many of the isolates belonging to genotypes T3, T4 and T11, but as this study group contains largely AK causing strains it presents a strong bias towards pathogenic genotypes. From the clinical isolates collected from 17 patients, eight of the patients had isolates belonging to T4 (11 strains); six patients had isolates belonged to T3 (seven strains), and five patients were infected with T11 isolates (11 strains). Eleven of the possible 15 T-groups were included in this study, and of these, three belonged to non-pathogenic genotypes: As expected they clustered close to each other at the bottom of the tree, distinct from the main taxa, and formed clades T7, T8 and T9. Despite tree topology mainly following genotypes, with the exception of the split T3 group average sequence divergence between the genotypic clades T3, T4 and T11 based on pairwise sequence comparison are lower than the current arbitrary cut off value of 5% (T3-T4: 3.3%; T4-T11: 3.4%), (Gast *et al*., 1996). The close association between isolates from the three genotypes (T3/4/11) shown by pairwise sequence comparisons may explain why the in both MP and NJ trees T3 isolates are split into smaller clusters.

Although the 18S typing system has been used here to effectively classify a unique group of AK causing isolates, and consequently confirm the robustness of the current system. As expected, the system was not effective at resolving the species and provided little definition between them. Instead multiple isolates were clumped into a few large groups. A proposal has been put forward to rename *Acanthamoeba* strains by T-group rather than species name, saving confusion caused as a result of the historical classification system (Stothard *et al*., 1998). However given the clustering of many species into few clades by the T-group system, such renaming would most likely cause even more confusion to an already complicated taxonomic system. Especially given the close association between certain clades, where T2 and T6 have recently been referred to within the literature as T2-T6 clade (Corsaro and Venditti, 2010, Huang and Hsu, 2010, Niyyati *et al.*, 2009).

Even though partial 18S sequences were obtained from all study isolates, once subjected to phylogenetic analysis, distance trees with comparable topology to previous studies were produced. Amplification of a shorter sequence both increases the throughput of isolates, and reduces the potential for *taq* errors, without the loss of vital data for analysis.

With the recent increase in AK incidence, and the latest outbreak in the USA, more and more cases are being diagnosed. Ultimately this is leading to an increase in the development of resistance to drug treatments. Highlighting the need for an improved system with a greater power of differentiating than the current T-group genotyping system.

2.4.2 Genotyping based on *cox1/2* sequences

Although rRNA genes prove the most attractive and popular choice for intraspecific phylogeny and taxonomy studies, mitochondrial genes are also suitable for use as a resolving tool to better understand close phylogenetic relationships because they evolve approximately 10 fold faster than nuclear genes (Brown *et al*., 1982). The mitochondrial genome is most likely to be under different constraints, as it is located within a cell organelle rather than the nucleus (Ledee *et al*., 2003). The *Acanthamoeba* mitochondrial genome appears to be conserved and have evolved relatively slowly (Burger *et al*., 1995).

An earlier study has looked at sequence variation of cytochrome oxidase genes within *Acanthamoeba* species, revealing a large degree of nucleotide variation, enough to differentiate eight AK patient isolates, as well as matching six of them by sequence homology to their respective tap water isolates (Kilvington *et al*., 2004). Here the same mitochondrial gene *cox1/2* was trialled as a suitable contender for an alternative/additional target for genotyping *Acanthamoeba* species rather than the current 18S T-group system.

The high degree of nucleotide variation within *cox1/2* sequences of *Acanthamoeba* isolates made the search for suitable primers difficult. The primers were required to be *Acanthamoeba* specific, and suitable for use with as many strains as possible. A second objective was to design a pair of primers that produced a sequence product ideally <1,000 bp, but still include a region of high variability. The resulting primer pair CoxA-486F and CoxA-1057R is sensitive and highly effective, with the ability to work well with a range of unusual strains. Even amplifying the *cox1/2* gene of an atypical AK amoeba AK95/1153, where the 18S *Acanthamoeba* specific primers JDP1F and JDP2R failed, and only the universal

primers succeeded (18sF: 18sR). Sequence analysis of the strain AK95/1153 has provided surprising results with its cytochrome oxidase sequence matching *Acanthamoeba*, but its 18S sharing 96% homology with *F. aegytia*. Further research is needed to understand more of its genetics and evolution, but initial observations have revealed some binucleate cysts, further linking it to the *Flamella* genus. If confirmed as a *Flamella* sp., this would be first time the genus had been associated with a case of human keratitis.

Evidence has been presented here to indicate that an alternative system that has the ability to resolve *Acanthamoeba* isolates with pathogenic potential has been developed. Genotyping by *cox1/2* sequences does not group multiple pathogenic strains into a few clades, as does the T-group system. However, the majority of strains included in this study are AK causing isolates, the addition of environmental strains would show their effect on the branching patterns, and in turn highlight the robustness of the system.

2.4.3 Typing with concatenated gene sequences

Recent studies have shown multiple housekeeping genes can be concatenated to form one super-gene alignment for building more robust phylogenetic trees, which provide better discrimination power (Devulder *et al.*, 2005, Gadagkar *et al.*, 2005, Kurtzman and Robnett, 2007).

This concatenated approach within the *Acanthamoeba* genus using the well documented SSU 18S rDNA gene from the T group system, in combination with another housekeeping gene, the mitochondrial *cox1*/*2*, has resulted in trees with

greater powers of discrimination. Which ultimately improve the ability to phylogenetically classify the subgenus and improve the diagnosis of AK.

The phylogenetic trees based on concatenated data trees bears most resemblance to the trees based on *cox1*/*2* data, and increases the discrimination power of the phylogenetic analyses within *Acanthamoeba* spp., something that has not yet been achieved by the less discriminatory T-group system.

Results here do not contradict current opinions, now strongly biased towards using a multigene approach to increase the resolution power of identifying species (Devulder *et al*., 2005, Gadagkar *et al*., 2005, Kurtzman and Robnett, 2007). However, although the concatenated tree has more discriminatory capabilities than those with 18S data alone, little improvement is gained over using *cox1*/*2* data independently. The addition of even more genes in a concatenated dataset should be tested to ascertain if further discriminatory power would be obtained. Further analysis should also be considered using a larger data set than studied here.

2.4.4 Multiple alleles and mixed infections using 18S and *cox1/2*

Historically, evidence has suggested the presence of a single allele of the 18S gene occurring within *Acanthamoeba*, despite estimates suggesting that *A. castellanii* Neff has several hundred copies of the gene. More recent evidence has shown multiple alleles are actually present in at least seven isolates (Stothard *et al*., 1998). For the presence of multiple alleles of 18S to be due to errors by *taq* polymerase, the same allelic-specific mistakes would have to occur in two groups of isolates, which is unlikely. However multiple alleles can occur as a result of genetically different strains being present within the initial amoebae cultures.

The discovery of multiple alleles can be a benefit, and aid a better understanding of the course of the infection.

Using a repeat series of isolates collected from a single patient, a second study determined if drug resistance to propamidine had developed, or if the failure of the patient to respond to treatment was due to a mixed infection (Ledee *et al*., 1998). The presence of identical 18S multiple alleles in both drug-sensitive and drug-resistant isolates (pre and post treatment), led to the conclusion that resistance had developed throughout the cause of the treatment.

This study has shown the 18S gene sequence from two strains isolated from Patient 6 (AK 6 and AK 6a), cluster independently on two branches, each in a different clade, AK 6a in T4 and AK 6 in T3. However when analysed by *cox1/2* sequence, both strains show sequence homology, and are found together in *cox1*/*2* group c. Suggesting Patient 6 was infected with a strain of *Acanthamoeba* which has multiple alleles of it's 18S gene. Unlike here, the previous study which uncovered multiple alleles of 18S in seven isolates, found where they do occur, both alleles fall within the same 18S rDNA sequence type, which as a consequence is unlikely to have a major impact on genotyping (Stothard *et al*., 1998).

Analyses of 18S sequences from repeat isolates of Patient one (AK 1 and AK 1a) showed the presence of two sequence types (*cox1*/*2* c and h), with AK 1 located in T11, and AK 1a in T3. However, analysis of the *cox1/2* gene from both isolates shows a similar pattern, with two distinct sequence types. Evidence no longer points to multiple alleles in either genes, but instead a mixed infection within the one patient.

Evidence for the presence of multiple alleles second gene, *cox1/2*, was found in this study. Six isolates recovered from a single patient (three), collected as repeat

samples, demonstrated four sequence types (*cox1/2* a, e, g, and h), while they shared sequence homology when analysed by 18S. Multiple alleles have also been discovered in the *Acanthamoeba* lactic dehydrogenase gene (Ward and al-Abidin, 1988), but no others as yet have been identified. This is likely to change with the imminent release of the complete high-quality sequencing of *A. castellanii* Neff genome, which is currently in progress.

The use of two genes in relatively quick, and robust (in the case of 18S, and yet to be proven in *cox1/2*) PCR can rapidly confirm or rule out the possibility of mixed infections within a patient. Early detection of a mixed infection would benefit the patient by improving prognosis, and reducing the risk of drug resistance developing. In addition, a genotyping system capable of identifying multiple alleles allows drug resistance to be tracked throughout the course of the treatment.

2.4.5 Conclusion

Speciation of the amoebae involved in acanthamebiasis may have implications regarding epidemiology and the course of the infection, but is unlikely to have a huge effect on the treatment, while the discovery of a mixed infection in an individual may. The combined use of two systems to genotype can rapidly determine the occurrence of a mixed infection, where a single system would struggle.

Evidence here, supported previous studies (Stothard *et al*., 1998) identifying multiple alleles of 18S occurring within *Acanthamoeba*, and additionally recognised multiple alleles of the *cox1/2* gene present within the genus. Tracking multiples alleles of both genes and using them as epidemiological markers can track drug regime resistance.

The T-group system is proving an invaluable tool within epidemiological studies, and is continuing to evolve with the addition of new genotypes. However the T-group system does not effectively resolve the isolates with pathogenic potential, and clumps large numbers of strains within groups T3, T4 and T11. The search for an efficient, fast and robust system to differentiate between virulent and non-virulent strains has been an ongoing concern. This study has presented *cox1/2* as a suitable contender to 18S for use in a phylogenetic system in the epidemiological typing of *Acanthamoeba*, as it provides much more distinction between the strains than the T-group system. However, the combined use of both systems in this study led to the discovery of the mixed infection in Patient one. This had the potential to have been interpreted as multiple alleles had either system been used independently. Leading ultimately to the conclusion that the use of both systems to genotype *Acanthamoeba* is synergistic.

3 SWEDISH GAE STUDY

3.1 Introduction

Species of *Acanthamoeba* are one of the causative agents of granulomatous amoebic encephalitis also known as GAE. It is a rare disease, with less than 200 cases caused by *Acanthamoeba* documented within the literature (Schuster and Visvesvara, 2004). The skin and the olfactory neuroepithelium are the most likely route of infection (Walochnik *et al*., 2008). Once inside the body, the amoebae most likely gain access into the CSF by hematogenous, or by passing directly through the neuroepithelium (Marciano-Cabral, 2003). Typically the infections occur in the brain and/or the lungs of severely debilitated or immune suppressed individuals. The infection presents with hemorrhagic necrotising lesions, generally found in the cerebrum, cerebellum and brain stem, with symptoms including headaches, fever, behavioural abnormalities and hemiparesis (Marciano-Cabral, 2003, Martinez and Janitschke, 1985, Schuster and Visvesvara, 2004, Walochnik *et al.*, 2008).

Diagnosis is almost always made post-mortem following brain tissue biopsies, and indirect immunofluorescence (IIF) staining of tissue sections (Bloch and Schuster, 2005, Schuster and Visvesvara, 2004). However pre-mortem diagnosis of GAE has been reported within the literature. Achieved as a result of positive *Acanthamoeba*-specific PCR of several biopsy tissue and fluid specimens, including cerebrospinal fluid (CSF), bronchoalveolar lavage specimens (BAL), skin, lung, and brain tissue (from the main lesion). All of which had been *Acanthamoeba* culture negative when tested (Walochnik *et al*., 2008).

On a rare occasion, direct recovery of *Acanthamoeba* from CSF has been reported (Callicott, 1968, Sharma *et al*., 1993, Singhal *et al*., 2001), however amoebae are generally not found in the CSF.

PCR has proved to be an invaluable tool, used to diagnose *Acanthamoeba* infections. The highly sensitive T-group typing system has also been used to determine epidemiological association between a keratitis-causing strain of *Acanthamoeba*, the patient, their contact lens storage case and their domestic water supply (Ledee *et al*., 1996). An alternative system based on mt DNA profiles, comparing a region of the *cox1/2* gene, has also been used to link six patients and their home tap water isolates (Kilvington *et al*., 2004).

In an unprecedented case our laboratory received repeat clinical and water samples from three patients from a single ward within a Swedish hospital. The patients were immune compromised paediatrics with severe debilitating illnesses. Extraordinarily, they had all contracted fatal GAE whilst being treated on the same intensive care unit (ICU) within the hospital. The sample set included repeat CSF samples from all three patients and hospital water samples from four shower rooms, a pool, and the main in-water supply to the ward. Such an outbreak of *Acanthamoeba* GAE infections has never before been documented, as occurrences of cases previously have been sporadic involving individuals in isolated cases (Khan, 2006, Marciano-Cabral, 2003, Visvesvara *et al.*, 2007b).

Research has shown it is possible to build a profile of pathogenicity using a range of *in vitro* assays, by assessing physiological characteristics of the amoebae such as ability: to tolerate higher temperatures and osmolarity; to produce extracellular proteases (Khan *et al*., 2000, Sissons *et al*., 2006); to cause cytotoxic effects on epithelial cells (Khan *et al.*, 2002, Walochnik *et al.*, 2000); to withstand human complement (Ferrante and Rowan-Kelly, 1983, Pumidonming *et al*., 2011, Toney and Marciano-Cabral, 1998); and show increased sensitivity particularly of

cysts to antimicrobials (Alizadeh *et al.*, 2009, Elder *et al.*, 1994, Lim *et al.*, 2000, Perrine *et al.*, 1995, Schuster and Visvesvara, 1998).

3.1.1 Aims

To build pathogenicity and molecular profiles of the infecting GAE *Acanthamoeba* strains isolated from the Swedish hospital. Strains will be identified and typed by morphology and genotype techniques, using 18S sequence analysis to determine T-group and mitochondrial *cox1*/*2* sequence analysis. The pathogenic potential of the isolates will be analysed by means of an array of *in vitro* tests, including trophozoite and cyst sensitivities to a range of antimicrobials. Analysis of the Swedish hospital samples will determine if the patients are infected with the same strain of *Acanthamoeba*, and if that strain is present within the hospital water system, thereby identifying the source of the infection.

3.2 Materials and Methods

3.2.1 Establishing amoebae cultures

To establish the presence of FLA within the samples, aliquots were initially cultured by monoxenic methods (section 2.2.3). All seeded NNA plates were individually sealed with parafilm, and groups of plates incubated together in plastic bags, at 30°C. Plates were observed at 24-hour intervals to check for growth. Attempts were made to establish all recovered FLA in axenic culture (section 2.2.4), occurring over a period of weeks to months.

Amoebae were identified using phase contrast microscopy (inverted) (Olympus CKX41), with a camera (Olympus, CAMEDIA C5050), following the dichotomous key for morphological taxonomy (Page, 1988).

Once maintained in axenic culture, strains were cryopreserved for future work (2.2.5).

All samples were collected from a single ward of Swedish hospital, and kindly donated by Dr Elisabet Holst, Department of Laboratory Medicine, Lund University, Lund, Sweden. Patient and hospital strains with as much corresponding information regarding sample origin as we have access to, is shown in Table 7. Unfortunately access has not been granted to additional information regarding the collection of samples, or clinical information.

In addition to Swedish hospital isolates reference strains were included in the analysis; AK associated *A. polyphaga* Ros, soil originating *A. castellanii* (Neff, 50370); GAE associated *A. culbertsoni* (30171) and *A. healyi*; and a strain not associated with disease *A. polyphaga* (30871).

3.2.2 Amoebae DNA extraction

Axenic *Acanthamoeba* cultures were harvested for genomic DNA extraction by the UNSET method (detailed in section 2.2.6). If attempts to axenise strains had failed, the cells were then maintained and propagated by the alternative slower method of monoxenic culturing (section 2.2.3). *Acanthamoeba* DNA was collected directly from the agar surface but at lower concentrations than if obtained from axenic culture. The cells were harvested by repeated washing of the NNA surface with 8 ml of ice cold dPBS and a pipette. Recovered cells were resuspended in 1 ml of ice cold dPBS, and subjected to DNA extraction by the UNSET method: Carried as described in section 2.2.6.1 but with the following modifications: 0.6 ml UNSET buffer; 0.6 ml phenol-chloroform (1:1); 0.6 ml chloroform:isoamyl alcohol (24:1); and resuspended in 20 µl of $TE_{0.1}$ buffer containing RNase A (5 µl/ml).

3.2.2.i Direct DNA extraction from CSF

DNA was extracted directly from CSF samples. Using a technique modified from the guanidine acetate method to purify target DNA following PCR (section 2.2.10.2).

Added to 25 µl of CSF, were 500 µl of 7 M guanidine acetate and 20 µl of silica in nH_2O (section 2.2.10.2); Guanidine acetate creates a hydrophobic environment, encouraging the nucleic acids to bind to the silica. The solution was then heated for five minutes at 55°C, mixed briefly by vortex, and incubated on ice for 10 minutes. The mixture was centrifuged at 10,000 x *g* for 10 seconds and the supernatant removed. The DNA bound silica was washed three times in 80% isopropanol (volumes of 500 µl, 180 µl and 180 µl, respectively), spinning at 10,000 x *g* for 10 seconds and all supernatant removed between washes. Ensuring

the eppendorf cap was opened, the DNA bound silica was incubated at ambient room temperature in a fume hood, for approximately 10 minutes, to allow the silica pellet to completely air-dry. Finally the DNA was dissolved in 20 µl of pre-warmed nH_2O, and incubated for 5 minutes at 55°C (with occasional mixing). The silica was removed by centrifuging at 10,000 x *g* for 1 minute, and the supernatant containing the DNA was recovered in to a fresh eppendorf.

3.2.3 DNA manipulation

Basic manipulation of DNA was carried out with PCR techniques as described in section 2.2.9, and purification of PCR products as in 2.2.10. Clone libraries within pGEM®-T Easy cloning vectors were established as described in 2.2.11, production of ultra competent *E. coli* as in 2.2.12, transformation as in 2.2.13, and purification of propagated plasmids as in 2.2.15. Plasmids were sequenced commercially as detailed in section 2.2.16. Sequenced results were analysed and manipulated as described in section 2.2.17. Isolates and clone libraries were preserved for future use by cryopreservation techniques described in section 2.2.5.

3.2.4 Temperature tolerance assay

The method of Khan and colleagues was used to assess the ability to tolerate temperature (Khan *et al.*, 2001). This method was carried out by inoculating axenic *Acanthamoeba* trophozoites (2.2.4) on to *E. coli* seeded NNA plates (as described in 2.2.3) and incubating them at 32°C and 37°C for up to 96 hours. Viability and growth of the amoebae was determined by microscopy, and identification of the leading edge of trophozoite migration across the plate surface.

3.2.5 Osmotolerance assay

The osmotolerance of the amoebae was assayed using a method of Khan and colleagues to determine pathogenic potential (Khan *et al.*, 2001). The method was carried out as for temperature tolerance, with the exception of replacing normal NNA plates (2.2.4) with NNA supplemented with 1 M mannitol (0.25 osmolar). Plates were incubated at 28°C and 37°C for up to 96 hours. Growth of the amoebae was determined by identification of the leading edge of trophozoite migration by phase contrast microscopy. Mannitol supplemented plates, inoculated with *A. culbertsoni* (30171) served as controls.

3.2.6 Protease secretion

A modified method of Maciver was used for extracellular protease secretion zymography (Edinburgh). This involved sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels containing bovine gelatine (0.1%) (Heussen and Dowdle, 1980), used to determine the presence of proteases in the *Acanthamoeba* culture medium of the strains. Gels were cast containing 2.5 ml 1.5M Tris pH8.8, 4.31 ml dH_2O, 100 µl 10% SDS, 44 µl of 22.5% bovine gelatine, 3 ml 30% acrylamide mix 50 µl 10% APS and 20 µl TEMED (modifications from (Heussen and Dowdle, 1980, Khan *et al.*, 2000)). *Acanthamoeba* culture medium (ACM) from pathogenic and non-pathogenic species (5 µl) was mixed with electrophoresis sample loading buffer (1:1) (2 ml glycerol, 0.5 g SDS, 8 ml 0.5M Tris, 0.2 ml 0.5% bromephnol blue), and then applied to the gels. Gels were run at 200 V (Mini-PROTEAN®, Tetra Cell, BIO-RAD), in 1X electrode (running) buffer pH 8.3 (10X: 3.03 g Tris base, 14.1 g glycine, 1 g SDS per 100 ml of dH_2O). After electrophoresis gels were incubated in solution (2.5% Triton X–100, 50mM Tris-

HCl pH 7.0) for 60 minutes, and then overnight in developing buffer (50mM Tris-HCl pH 7.0, 2mM $CaCl_2$) at room temperature. Gels were then stained with rocking for 60 minutes (0.1% Coomassie blue R-250, 40% methanol, 10% acetic acid), before being rinsed in tap water, and incubated with rocking in destain solution (10% acetic acid, 5% methanol) for 90 minutes.

Areas of gelatine digestion were visualised as non-staining regions of the gel. In experimental controls fresh culture media was used, which had not been in contact with *Acanthamoeba* isolates.

3.2.7 Complement fixing potential

The method of Pumidonming and colleagues was used to assess the complement fixing potential of the isolates (Pumidonming *et al.*, 2011). This method involved harvesting axenic trophozoites by centrifugation at 500 x *g* for 1 minute. Cells then were washed twice with ¼ strength Ringer's solution, quantified using a modified Fuchs Rosenthal haemocytometer, and adjusted to concentrations of 1 x 10^4 per ml with ¼ strength Ringer's solution.

By pooling the sera of four randomly selected healthy-individuals, normal human serum (NHS) was produced; Whole blood was collected, and allowed to clot, undisturbed at ambient temperature. All clots were removed by centrifugation at 1,500 x *g* for 10 minutes at 8˚C, and the sera removed using a 3ml Pasteur pipette. All sera were stored at –20˚C, in 0.5ml aliquots, to avoid freeze-thaw cycles (Invitrogen, 2007).

In a round-bottomed microtitre plate (VWR, Lutterworth, U.K.) 100 µl of trophozoite suspension was added to 100 µl of NHS. Heat inactivated NHS (56˚C

for 30 minutes) served as a control (Toney and Marciano-Cabral, 1998). Assay plates were maintained 28˚C for 1 hour, and the morphology of the trophozoites following incubation at 0, 5, 10, 20 and 60 minute time points, was examined using phase contrast microscopy (inverted) (equipped with a Canon EOS 60D camera).

Living trophozoites appeared bright and moving, while killed trophozoites had destroyed cell membranes. Effect of compliment on the amoebae was also determined by the formation of a pellet in the bottom of the well. Pellets were only formed in wells where lysis of the amoebae had occurred, and where healthy trophozoites remained.

3.2.8 Cytopathogenic potential

A modified method of Khan and colleagues was used to determine the cytopathic potential of the *Acanthamoeba* isolates (Khan *et al*., 2000). The method involved observing the degradation of epthelioid HeLa cell monolayer's by *Acanthamoeba* trophozoites. Where by HeLa cells were grown in 25 cm^2 tissue culture flasks, in 5 ml Dulbecco's Modified Eagle Medium (DMEM) (with L-glutamine) supplemented with 10% FBS (heat inactivated) and incubated in CO_2 (5%) at 37˚C, until 80% confluent. Adhered cells were washed in 10 ml dPBS, to remove all traces of growth media, and detached from the flask surface by the addition of 2.5 ml of 1X trypsin-EDTA solution and incubation at 32˚C for 5 minutes. Detached cells in suspension were added to 32.5 ml of pre-warmed (37˚C) DMEM, and 2 ml added to all wells of a 12 well microtitre plate (VWR). Again incubated in 5% CO_2 at 37˚C, until 80-100% confluent.

Acanthamoeba trophozoites were grown as described in 2.2.4, in 5 ml of semi-defined culture media in 25 cm^2 tissue culture flasks, to a confluency of > 80%. Cells were harvested, by centrifugation (500 x *g* for 1 minute) and washed in ¼ strength Ringer's solution (three times), before being quantified.

1000 trophozoites in 30 µl were added to each well of the microtitre plate containing a confluent monolayer of HeLa cell, and incubated in 5% CO_2 at 37˚C. Cytopathogenic effect by the *Acanthamoeba* was identified as areas of cleared HeLa cells, and observed by microscopy up to 96 hours post-inoculation. Controls with epitheloid cells incubated alone but with 30 µl of either ¼ strength Ringer's solution or culture medium (without *Acanthamoeba*) were maintained at the same conditions for comparisons to be made against.

3.2.9 Antimicrobial sensitivity assays

3.2.9.1 Trophozoites

The methods of Kilvington and colleagues were used for the trophozoite antimicrobial sensitivity assays (Elder *et al.*, 1994, Hughes and Kilvington, 2001). This involves trophozoites grown as described in 2.2.4, in 50 ml of culture media in 175 cm^2 tissue culture flasks with filter caps, to a confluency of > 80%. Cells were harvested and washed three times in dPBS-Tween (5% w/v Tween® 80), centrifuged at 500 x *g* for 5 minutes between each wash. Finally quantified using a modified Fuchs Rosenthal haemocytometer, and adjusted to concentrations of 2 x 10^4 per ml in culture medium.

To test trophozoite sensitivity against antimicrobials, stock solutions of the compounds, Voriconazole (a gift from Kemprotec Ltd, Maltby, U.K.), Hexamidine

Diisethionate (Désomedire 0.1%- Bausch & Lomb, Kingston upon Thames, U.K.), Polyhexamethylene biguanide (PHMB) (Cosmosil CQ- Arch U.K. Biocides Ltd, Castleford, U.K.), Natamycin (Pimaricin- Molekula Ltd, Audenshaw, U.K.) and Miltefosine were prepared at 1000 µg/ml in nH_2O (with the exception of Miltefosine diluted in 5% ethanol), and filter sterilised (0.2 µm). Antimicrobial compound dilutions were obtained by two-fold serial dilution of 100 µl with 100 µl of ¼ strength Ringer's solution across a flat-bottomed microtitre plate. Calibrated trophozoites (100 µl) were added to each well, and the plates covered and incubated in air, at 32˚C, for 48 hours. Compounds were assayed in triplicate, with controls containing ¼ strength Ringer's solution only without antimicrobial compounds.

Following incubation, trophozoites were examined by inverted microscopy, and compared to the controls. Comparison to the test wells allows the degree of trophozoite growth or destruction to be assessed. The Minimum Trophozoite Inhibitory Concentration (MTIC) and the Minimum Trophozoite Amoebacidal Concentration (MTAC) of each test compound against each *Acanthamoeba* strain was determined. MTIC is defined as 50% inhibition of trophozoite replication compared to controls, and MTAC, where all trophozoites are rounded, non-motile and floating or lysed (Elder *et al*., 1994, Hughes and Kilvington, 2001).

3.2.9.2 Cysts

The methods of Kilvington and colleagues were also used for the cyst antimicrobial sensitivity assays (Elder *et al.*, 1994, Hughes and Kilvington, 2001). This involves *Acanthamoeba* trophozoites grown as described in 2.2.4, in 50 ml of semi-defined culture media in 175 cm^2 tissue culture flasks with filter caps, to a confluency of > 80%. From late log-phase cultures, cysts were prepared in Neff's

constant pH encystment medium (Hughes and Kilvington, 2001, Neff *et al.*, 1964). Harvested trophozoites were washed in Neff's encystment medium, and centrifuged at 1000 x *g* for 5 minutes, three times. From the pellet approximately 10^7 trophozoites were added to 100 ml of Neff's medium in a 175 cm^2 tissue culture flasks with vent/close caps, and left to encyst in a shake incubator revolving at 100 rpm, at 32˚C, for seven days (Hughes and Kilvington, 2001). Following microscopic evaluation to confirm >90% of cysts were mature; cells were detached from the flask surface by gentle rubbing of the flask wall with a modified cyst remover. Made by inserting a sterile polyester tipped applicator (Pur-Wraps) (Puritan Medical products, Maine, USA) into the upper end (mouthpiece) of sterile 1 ml x 0.01 Sterilin micropipette (Barloworld Scientific Ltd, Stone, U.K.), before gently heating the micropipette in a Bunsen flame to cause the polystyrene pipette to soften, and allow the upper end to be manipulated with sterile forceps to have a 90˚ bend. Harvested cells were pelleted by centrifugation at 1000 x *g* for 5 minutes. The cyst pellet was then washed three times in ¼ strength Ringer's solution (2.2.3), and centrifuged at 1000 x *g* for 5 minutes. Finally cells were stored in ¼ strength Ringer's solution, and stored at 4˚C for use within 14 days.

Cysts were quantified using a modified Fuchs Rosenthal haemocytometer, and adjusted to concentrations of 2 x 10^4 per ml in ¼ strength Ringer's solution.

Antimicrobial cyst assays rely on the adherence of cysts to the bottom of the polycarbonate wells of the microtitre plates, even after exposure to antimicrobial compound and removal by washing. Cyst assays were carried out as for trophozoites, until the end of the 48 hour incubation.

After which adhered cysts were washed three times in ¼ strength Ringer's solution with 15 minute soaking between rinses at room temperature. Wells were then filled with 100 µl of ¼ strength Ringer's solution containing *E. coli* JM101 (ATCC 33876) at an $O.D._{540}$ of 0.1-0.2, and incubated at 32˚C for 7 days.

Plates were examined for the presence of trophozoites by inverted microscopy, and the Minimum Cystical Concentration (MCC) was recorded as the lowest concentration of antimicrobial compound that resulted in no excystment and trophozoite replication (Elder *et al*., 1994).

3.3 Results

3.3.1 Culture and morphological analysis

Diagnosis in suspected cases of GAE is isolation, cultivation and subsequent microscopy of *Acanthamoeba* directly from the patient, followed by the sensitive technique of 18S rDNA sequence analysis.

3.3.1.1 Patient one

Initial samples collected from the patient, tested culture positive for *Hartmannella* sp. (Table 7). *Hartmannella* has yet to be found as the causative agent of disease in humans, and so given the highly irregular occurrence of culturing this species from a human sample, additional specimens from the patient were requested. Of the subsequent samples, four were *Acanthamoeba* culture positive (Table 7). The *Hartmannella* isolated from Patient one, could only be maintained on seeded-NNA plates.

3.3.1.2 Patient two

The samples included five CSF specimens, collected over a period of five months, all tested culture positive for *Acanthamoeba* sp. (Table 7). Axenic culture was achieved but compared to the hospital water supply samples, growth remained consistently slower.

3.3.1.3 Patient three

Of the two samples collected from Patient three, both proved culture positive for *Acanthamoeba* sp. (Table 7). The earlier of the two specimens collected was a sample of CSF, followed by a second sample taken from a head wound, two months later. Again axenic culture was established but Patient three isolates remained consistently slower when compared to the growth rate achieved by hospital water supply samples.

3.3.1.4 Hospital water samples

Samples were collected from several areas within the ICU that the patients had access to, and were associated with water, including a swimming pool, and several shower rooms. The ICU pool and two shower rooms all tested culture positive for *Acanthamoeba*. Axenic culture was established for all isolated strains.

Culture results obtained from this study were relayed back to the hospital, which responded immediately to super chlorinate the water supply within the hospital, in an attempt to rid the system of the FLA. Following this treatment, subsequent samples were collected from the hospital, and subjected to prolonged repeated analyses, despite continued attempts the sample proved to be culture negative of FLA.

Repeat testing approximately one year later, identified the presence of *Acanthamoeba* (BL2997) (Table 7).

Table 7. FLA isolated from clinical and hospital samples collected in a Swedish hospital, with morphological identification of genus groups.

Source	Strain	Origin	Culture	Identification
Patient 1 [a]	GAE-1	CSF	+	*Hartmannella* sp.
	GAE- 1a	?	-	Na
	GAE- 1b	CSF	-	Na
	GAE- 1c	CSF	-	Na
	GAE- 1d	CSF	-	Na
	GAE- 1e	CSF	-	Na
	GAE- 1f	?	+	*Acanthamoeba* sp.
	GAE- 1g	?	+	*Acanthamoeba* sp.
	GAE- 1h	?	+	*Acanthamoeba* sp.
	GAE- 1i	?	+	*Acanthamoeba* sp.
Patient 2 [a]	GAE- 2	CSF	+	*Acanthamoeba* sp.
	GAE- 2a	CSF	+	*Acanthamoeba* sp.
	GAE- 2b	CSF	+	*Acanthamoeba* sp.
	GAE- 2c	CSF	+	*Acanthamoeba* sp.
	GAE- 2d	CSF	+	*Acanthamoeba* sp.
Patient 3 [a]	GAE- 3	CSF	+	*Acanthamoeba* sp.
	GAE- 3a	Head wound	+	*Acanthamoeba* sp.
ICU Pool [a]	Pool	Pool	+	*Acanthamoeba* sp.
Shower rooms [a]	SR- 3	Number 3	+	*Acanthamoeba* sp.
	SR- 15	Number 15	+	*Acanthamoeba* sp.
Post-super chlorination [a]	BL2997	ICU in-water supply	+	*Acanthamoeba* sp.

Na: Not applicable; +: Culture positive; -: Culture negative; ?: Source unknown; [a]: Donated by Dr E. Holst, Lund University, Lund, Sweden.

3.3.2 Molecular analysis

Despite being culture negative, *Acanthamoeba* DNA was isolated from a Patient one's CSF sample (GAE- 1b). The DNA was directly extracted using a method with guanidine acetate and silica.

Comparisons made between the PCR findings and those of the culture methods (Table 8), showed 100% obtainment of 18S and *cox1/2* sequence fragments from all culture positive samples. While from the six culture negative specimens, one PCR positive result was obtained.

3.3.2.1 18S phylogenetic analysis

A region of 18S rDNA was amplified, cloned and sequenced from all FLA isolated from the three patients, and the hospital, this included all *Acanthamoeba* strains and the single *Hartmannella* sp. from Patient one. Two 18S primer sets and corresponding sequencing primers, were used in this study, to reproduce sufficient sequence length (approximately 204 bp; 1,175-1,379 bp) to determine any variation for T-group differentiation (Schroeder *et al*., 2001). All *Acanthamoeba* 18S rDNA PCR's were carried out using the primer pair JDP1F and JDP2R (Schroeder *et al*., 2001), producing partial (450 bp) 18S rDNA products. For the *Hartmannella* sp., a larger (631 bp) 18S product was obtained with the use of universal primer pair 18S F & 18S R (Weekers *et al*., 1994).

Individual BLAST analysis was carried out on all sequences (Altschul *et al*., 1997), with nucleotide blast parameters optimised for highly similar sequences

(megablast): Results show the partial 631 bp sequence from Patient one's *Hartmannella* sp. matched by 99% to *H. vermiformis* (GenBank AY680840 (Kudryavtsev *et al*., 2005)) .

BLAST analysis of *Acanthamoeba* sp. isolated from Patient one, Patient two and Patient three, all matched 100% to *Acanthamoeba* sp. ATCC 50496 (GenBank ASU07408 (Gast *et al*., 1996)). The only *Acanthamoeba* strain isolated from the Swedish hospital with an 18S sequence that differed from the others was that of the ICU pool: Its 18S rDNA sequence matched 100% with *A. castellanii* ATCC 30010 (GenBank EF554328.1 (Kohsler *et al*., 2008)).

Analysis of the 18S sequences of all repeat strains isolated from each patient, confirmed that each was infected with only one strain of *Acanthamoeba*, and therefore analysis from this point forwards, was carried out using only one strain for each patient.

The 18S sequences for *Acanthamoeba* strains from the three patients and hospital samples, were aligned to each other using the alignment program for DNA, ClustalW (2.1) (Larkin M.A. *et al*., 2007), which allows single nucleotide differences to be identified. ClustalW identified sequence variation between the two *Acanthamoeba* sp. 18S sequence types from this study to be 1.2%: Corresponding to eight differences in a length of sequence 227 bp long: Six of these were three double bp omissions from the pool strain, and two were separately occurring base replacements from cytosine (C) to thymine (T) again in the pool strain.

Using 18S sequences a neighbour-joining distance tree with Kimura two-parameters correction for multiple substitutions were obtained using MEGA (5.05) software. Included in the tree are sequences from *Acanthamoeba* reference strains:

A. castellanii (50373, Neff: type species), *A. polyphaga* (30871: not associated with disease) *A. polyphaga* (Ros: associated with AK), *A. healyi* (CDC 1283:V013: associated with GAE) and *A. culbertsoni* (30171, Lilly: associated with GAE), along with the Swedish isolates from this study, and a sequence from one of the closest genera to *Acanthamoeba*, *H. vermiformis* (Costa Rica; GenBank AY680840) (Figure 7).

The tree (Figure 7) confirms the distinction between two strain types within the Swedish hospital ICU water system: Patient and hospital strains with the exception of the pool species are identical by 18S analysis; the pool strain differs by 1.5%. This tree confirms only one strain type caused the patients infection. With the Swedish hospital isolates, from the three patients and the two shower rooms, clearly cluster away from that of the differing Swedish isolate recovered from the ICU pool. The pool strain is most similar to *A. polyphaga* Ros (with 1% dissimilarity). However, all Swedish strains including the pool isolate belong to the T4 genotype, based on dissimilarity values of greater than 5%.

The ICU pool strain is found on a branch of its own, supported by a high bootstrap value of 95: This node clearly separates the T4 isolates (both *A. polyphaga* strains and *A. castellanii*) and the other Swedish hospital strains. Most species from within the T4 clade in this tree are less than 5% dissimilar from each other: The only exception is the strain that has not been associated with disease *A. polyphaga* (30871), which differs to the pool strain by more than 5% (the cut off value found within a T-group genotype) (Stothard *et al*., 1998): However its dissimilarity it less than the cut off value when compared to soil originating isolate *A. castellanii* (Neff, ATCC 50370) and AK associated isolate *A. polyphaga* Ros.

A maximum parsimony (MP) tree was chosen over one analysed by neighbour joining algorithms. As it more clearly, by associated bootstrap value shows a distinction between the T4 strains and those associated with GAE (*A. culbertsoni* and *A. healyi*). Bootstrap values of 99 are found at the node separating GAE isolates from T4 and Swedish isolates. Unsurprisingly the branch containing *Hartmannella* is clearly distinguished from that of *Acanthamoeba*, and here acts as a root for the tree.

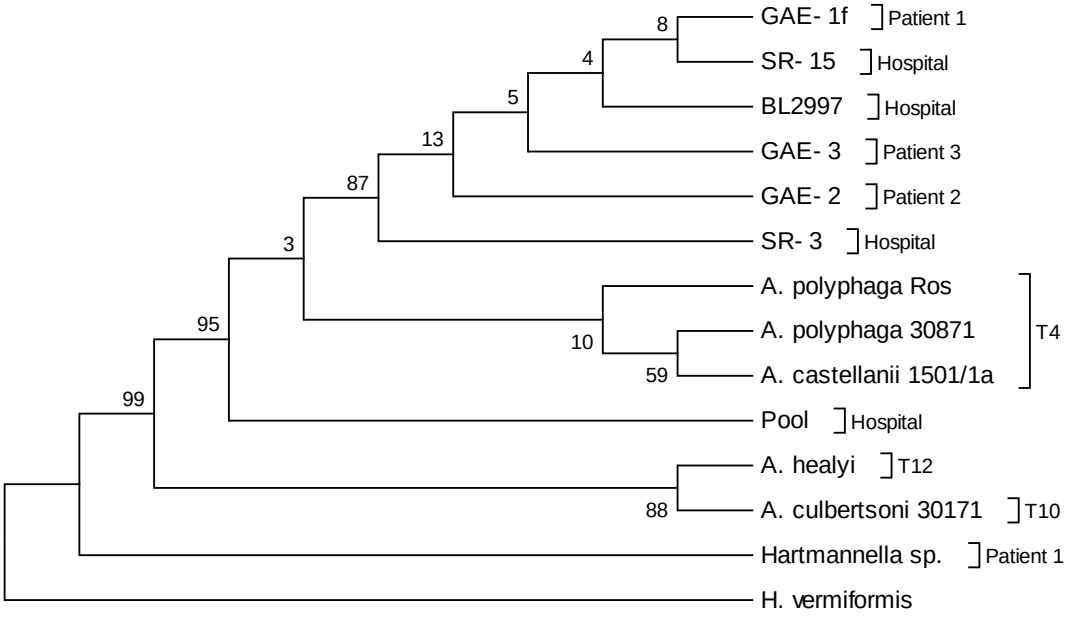

Figure 7. Maximum parsimony distance tree based on partial 18S rDNA sequences of Swedish hospital FLA with comparison species. The tree is unrooted and obtained by Kimura two-parameters correction for multiple substitutions using MEGA (5.05). Analysis is based on reference bp from 1,175 to 1,379. Designated T-groups and strain origins are shown. Bootstrap values have been included, based on 1,000 bootstrap values, and placed at the corresponding nodes.

3.3.2.2 Cox*1/2* phylogenetic analysis

The BLAST analysis was repeated for the Swedish hospital strains, but this time using an alternative sequence, that of the mitochondrial *cox1/2* gene. Results follow their 18S sequences, and confirm all Swedish strains except one, match each other, and in the case of *cox1/2*, share 89% similarity with *A. castellanii* (GenBank U12386.1). Again as with 18S sequence analysis, the differing strain was found to be that of the ICU pool, which only shared 88% sequence homology with *A. castellanii* (GenBank U12386.1).

Sequence variation between the aligned *cox1/2* sequences of the ICU pool strain, and each of those from the three patients and hospital water system, was 4%.

An MP tree was constructed with *cox1/2* sequences (Figure 8), and included the Swedish strains from this study, with the exception of the post-treatment strain BL2997, and reference strains: *A. castellanii* (50373, Neff: type species), *A. polyphaga* (30871: not associated with disease) *A. polyphaga* (Ros: associated with AK), and *A. culbertsoni* (30171, Lilly: associated with GAE) (Figure 8). As with 18S analysis, two genotypes were represented here from samples collected from the Swedish hospital ICU. When comparing MP analysis to NJ (Figure 9), the MP tree contains three nodes with bootstrap values of over 90, while the NJ tree has three nodes over 75, and one over 90. It is generally considered that bootstrap values of greater than 70 are evidence to support the distinction of clades. Although the order of strains is reversed between the two algorithms, the grouping of taxa remains the same.

Phylogenetic analysis using *cox1/2* compared to 18S sequence data provides more differentiation between the species, where strains are not clumped together into large genotypes (Figures 8 and 9).

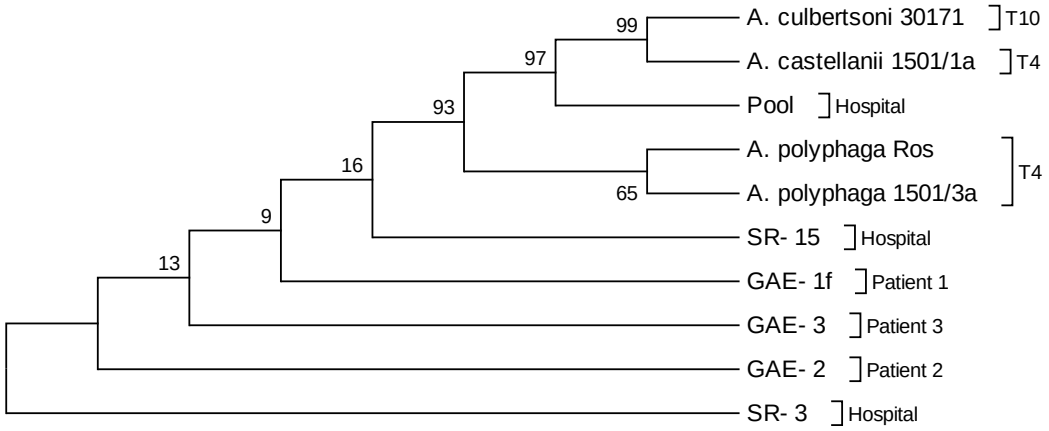

Figure 8. Maximum parsimony tree based on partial mitochondrial *cox1/2* sequences of *Acanthamoeba* spp. The tree is unrooted and obtained by Kimura two-parameters correction for multiple substitutions using MEGA (5.05). The tree is based on reference bp 8,002 to 8,566. Designated T-groups and origin of strains are shown. Bootstrap values have been included, based on 1,000 bootstrap values, and placed at the corresponding nodes.

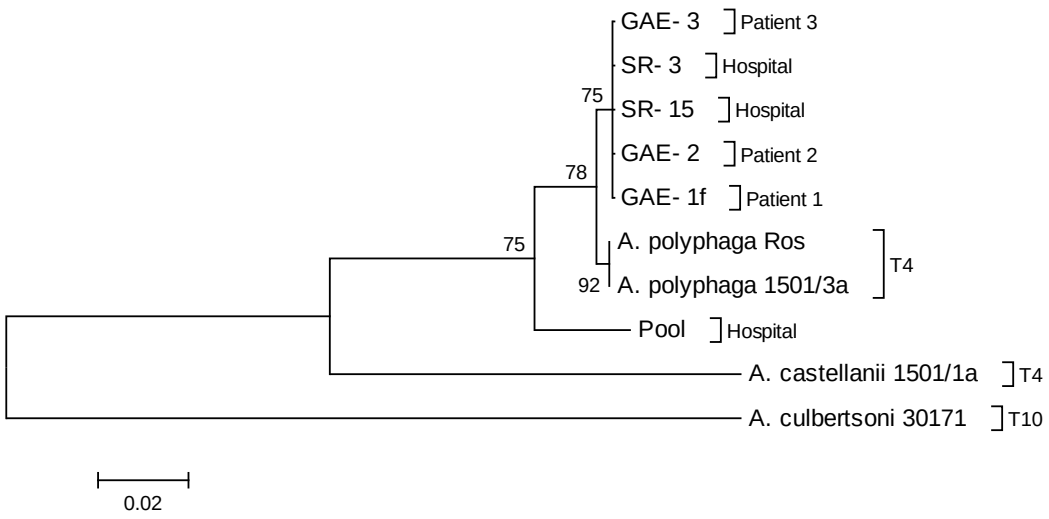

Figure 9. Maximum parsimony tree based on partial mitochondrial *cox1/2* sequences of *Acanthamoeba* spp. The tree is unrooted and obtained by Kimura two-parameters correction for multiple substitutions using MEGA (5.05). The tree is based on reference bp 8,002 to 8,566. Designated T-groups and origin of strains are shown. Bootstrap values have been included, based on 1,000 bootstrap values, and placed at the corresponding nodes. The scale bar represents the corrected number of nucleotide substitutions per base using Kimura method.

3.3.3 Pathogenicity assays

3.3.3.1 Amoebicidal activity of human serum

Normal Human Serum (NHS) exhibited amoebicidal activity against trophozoites of all seven strains of *Acanthamoeba*. Observations were made by microscopy. Following 5 and 10-minute incubation trophozoites appeared live and viable. After 20 minutes of incubation with the sera, cytopathogenic changes had begun and trophozoites appeared distressed and rounded. A pellet of killed *Acanthamoeba* had formed in all test wells, between 70-80 minutes of incubation (Figure 10 A-C, and Table 8). Controls were as expected, with trophozoites incubated for the same period of time in heat-inactivated NHS remaining viable.

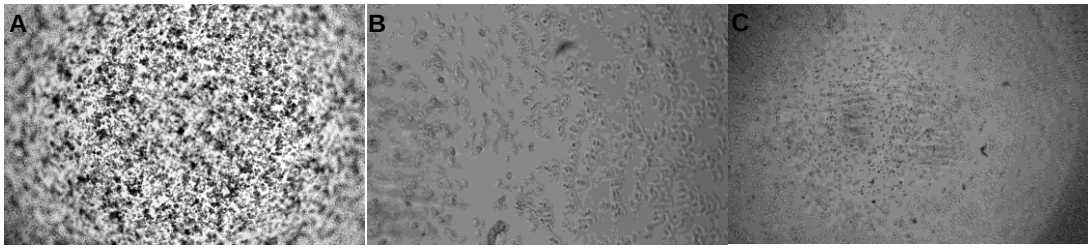

Figure 10. Activity of Normal Human Serum against *Acanthamoeba* trophozoites. A, effect of complement from NHS on *Acanthamoeba* trophozoites; B, control,

Acanthamoeba incubated with heat-inactivated NHS; C, pellet formed in the round bottomed well after lysis of *Acanthamoeba* trophozoites.

3.3.3.2 Tolerance of increased temperature and osmolarity

All *Acanthamoeba* isolates were able to continue trophic growth on NNA *E. coli*- seeded plates at both 32°C and 37°C. However, when challenged in conditions of high sugar alcohol (1M mannitol), no strains of *Acanthamoeba* were able to maintain trophic growth, at either temperature tested. Controls were as expected.

Cysticidal affect of high osmolarity (1M mannitol) was examined, with agar slices cut from the incubated mannitol plates with trophozoites, and transferred onto fresh NNA *E. coli*-seeded plates without mannitol (inverted). Trophic growth of all strains resumed within 72 hours, and the leading edge was visible emerging from the upturned slice. Although trophozoites were unable to survive in conditions of high osmolarity, cysts were formed in the presence of the mannitol, and remained viable and able to resume trophic growth once conditions became more favourable (data not shown).

3.3.3.3 Protease secretion assays

Non-stained lanes of the SDS-PAGE confirm areas of gelatine digestion, and extracellular protease activity. Zymography with ACM has shown all *Acanthamoeba* assayed have the capacity to produce proteases (Figure 11). Lanes associated with the Swedish isolates (1-3, 5 and 6) show a distinction from that of AK causing *A. polyphaga* Ros (4). Controls were as expected, confirming proteases are not present in sterile ACM.

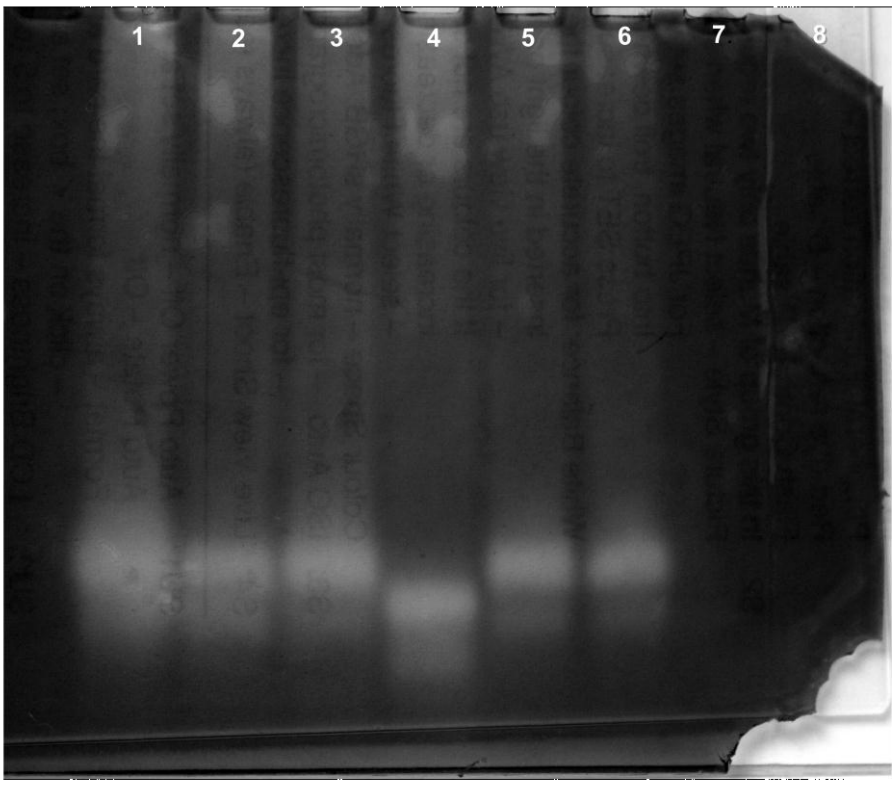

Figure 11. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) containing gelatine, to observe the presence of extracellular proteases in *Acanthamoeba* culture medium (ACM). **Lane 1**, ICU pool; **2**, Shower room **3**; 3, Shower room 15; **4**, *A. polyphaga* Ros; **5**, Patient 3; **6**, Patient 2; **7**, Left blank; **8**, negative control, containing sterile culture media.

3.3.3.4 Cytopathogenicity of *Acanthamoeba*

In the cytopathogenicity assays, within 96 hours all *Acanthamoeba* species had produced varying degrees of cytopathic effect on the HeLa cell monolayer (Table 8). Of all the strains tested the non-pathogenic *A. polyphaga* 1501/3a (30871) and Shower room 3 isolate, equally resulted in the lowest level of` HeLa cell monolayer degradation. Obvious cleared areas within the monolayer were present, containing trophic amoebae. Extensive degradation to the monolayer occurred in

wells containing the AK causing strain *A. polyphaga* Ros, again with numerous trophic cells. While isolates derived from Patient two, ICU pool, and Shower room 15, all severely destroyed the HeLa cell monolayer with assays of each containing an abundance of large amoebae full of vacuoles confirming digestion of food particles. The strain isolated from Patient three caused the most epitheloid monolayer degradation and resulted in the highest abundance of large digesting trophozoites. Control assays were maintained for comparison, and both that with sterile culture media and ¼ strength Ringer's solution contained an intact fully confluent HeLa cell monolayer.

Summaries of *Acanthamoeba* sp. pathogenicity characteristics are shown in Table 8. Based on results obtained in this study, all strains that were tested are shown to have at least the potential to be pathogenic. However, because the shower room 3 isolate and *A. polyphaga* 1501/3a (30871) exhibited reduced rate of cytopathic activity towards a monolayer of HeLa cells they could be considered less pathogenic compared with the known Swedish GAE isolates and AK isolate *A. polyphaga* Ros.

Table 8. Pathogenicity characteristics of *Acanthamoeba* sp. collected from an outbreak of GAE with a single ward of a Swedish hospital, and compared with an AK isolate and a non-pathogen.

Strain	**Growth**			**Protease**	**Cytopathic**	**Complement**	**T group**
	32°C	**37°C**	**1M mannitol**	**secretion**	**activity**	**lysis / min**	
Patient 2	●●	●●	-	Yes	+++	< 80	T4
Patient 3	●●	●●	-	Yes	+++	< 80	T4
Shower room 3	●●	●●	-	Yes	+	< 80	T4
Shower room 15	●●	●●	-	Yes	+++	< 80	T4
ICU Pool	●●	●	-	Yes	+++	< 80	T4
A. polyphaga Ros	●●	●●	-	Yes	++	< 80	T4
A. polyphaga 1501/3a (30871)	●●	●●	-	Yes	+	< 80	T4

●●: Good growth; ●: Growth; +: 0-25% of monolayer destroyed; ++: 25-50% of monolayer destroyed; +++: 50-75% of monolayer destroyed. -: None.

3.3.4 Drug sensitivity assays

An array of antimicrobial compounds was tested on trophozoites and cysts of the *Acanthamoeba* strains (Table 9 and 10).

Against trophozoites, sensitivity assays to determine the Minimum Trophozoite Amoebicidal Concentration (MTAC) were completed (Table 9). PHMB exhibited the best antimicrobial activity with average an MTAC of 2.9 μg/ml, followed by miltefosine with an MTAC of 11.7 μg/ml, and hexamidine with an average MTAC of 23.4 μg/ml. Wide ranges of effective MTAC values were observed with the therapeutic agents, natamycin and voriconazole. The average MTAC of natamycin to exhibit antimicrobial activity was 187.5 μg/ml. Voriconazole showed the highest MTAC, exhibiting antimicrobial activity between a range of 3.9 - > 125 μg/ml. However the only strain to be killed by voriconazole within the tested concentration range was AK isolate *A. polyphaga* Ros, with a MTAC of 3.9 μg/ml, for all other strains against voriconazole, MTAC values were greater than 125 μg/ml, and above the range tested (data not shown).

The minimum cysticidal concentration (MCC) was defined as the lowest concentration of test compound that resulted in no excystment and trophozoite replication after 48 hours of exposure. Against cysts miltefosine, voriconazole and natamycin showed little activity, with MCC levels undetected at concentrations greater than 500 μg/ml. Hexamidine and PHMB were the only therapeutic agents to show antimicrobial activity towards cysts, within the concentration range tested. Hexamidine resulted in antimicrobial activity towards 43% of the strains (3/7), causing a detrimental effect to isolates from Patient 3, MCC level of 62.5 μg/ml, as well as the pool and shower room 15 isolates, both with MCC levels of 1.9 μg/ml.

The compound to show the most activity against cysts was the disinfectant PHMB, with MCC levels of 1.9 μg/ml for 100% of strains (Table 10).

Table 9. *In vitro* sensitivities of five antimicrobial compounds against several strains of *Acanthamoeba* trophozoites and cysts.

	MTAC µg/ml[a]		**MCC µg/ml[a]**	
	Mean	*Range*	*Mean*	*Range*
Miltefosine	11.7	7.8 - 15.6	>500	>500
Voriconazole	>125	3.9 - >125	>500	>500
Natamycin	187.5	125 – 250	>500	>500
Hexamidine	23.4	15.6 – 31.25	>500	1.9 - >500
PHMB	2.9	1.9 – 3.9	1.9	1.9

MTAC: Minimum Trophozoite Amoebicidal Concentration; MCC: Minimum Cysticidal Concentration; PHMB: Polyhexamethylene biguanide; [a]: n = 7.

Table 10. *Acanthamoeba in vitro* Minimum Cysticidal Concentrations (MCC), against five antimicrobial compounds.

Strain	**MCC µg/ml**				
	Miltefosine	**Voriconazole**	**Natamycin**	**Hexamidine**	**PHMB**
Patient 2	>500	>500	>500	>500	1.9
Patient 3	>500	>500	>500	62.5	1.9
Shower room 3	>500	>500	>500	>500	1.9
Shower room 15	>500	>500	>500	1.9	1.9
ICU Pool	>500	>500	>500	1.9	1.9
A. polyphaga	>500	>500	>500	>500	1.9

Ros					
A. polyphaga	>500	>500	>500	>500	1.9
1501/3a					
(30871)					

PHMB: Polyhexamethylene biguanide

3.4 Discussion

3.4.1 Molecular

Both clinical and hospital samples were analysed for FLA using culturing methods. Where upon recovery, all amoebae were morphologically classified to genus level. All recovered *Acanthamoeba* strains were established axenic culture, but despite continuous attempts the *Hartmannella* sp. could not be maintained this way. Few publications in the literature suggests it is possible to maintain *Hartmannella vermiformis* by axenic culture in modified PYNFH medium (Fields *et al*., 1990): One group compared several *Hartmannella* strains in their study but only *H. vermiformis* (ATCC 50237), was maintained by axenic techniques, the other two were cultured in the presence of live, and heat-killed *E. coli* (Kuiper *et al*., 2006).

The use of culturing techniques obtained a 73% (16 of 22) recovery of *Acanthamoeba* from the samples. In general it is relatively rare to recover viable *Acanthamoeba* from CSF samples (Callicott, 1968, Sharma *et al.*, 1993, Singhal *et al.*, 2001). More often, fluid specimens test PCR positive for *Acanthamoeba*, when they have been culture negative. Here, one culture negative sample, tested positive for *Acanthamoeba* by PCR using a direct DNA extraction method. With the use of PCR techniques, 77% (17 of 22) of samples tested *Acanthamoeba* positive, highlighting the sensitivity of PCR, and its ability to detect *Acanthamoeba* where culturing methods failed. PCR requires the survival of DNA rather than the numerous intact viable cells that must be present for culturing to be successful. Although the 18S PCR is a sensitive technique (Ledee *et al*., 1996) because the gene is present in such abundance within the cells, for further examination of the strains and downstream assays such as pathogenicity tests, culturing of the isolates is required.

Morphological classification of *Acanthamoeba* beyond genus level is extremely difficult and can be subjective. The sensitive nature of PCR and the ability to use it to classify strains into clades such as T-groups highlights the valuable of molecular typing, and how it should be used in conjunction with original culture and microscopic diagnostic techniques.

A previously designed PCR primer pair was used to amplify a highly variable diagnostic region of the SSU 18S rDNA (204 bp) (Schroeder *et al*., 2001), in addition to a pair designed for this study: Which amplifies a 564 bp segment of the mitochondrial *cox1/2* gene, modified from (Kilvington *et al*., 2004). Both genes are attractive targets for species differentiation within *Acanthamoeba*.

The findings from molecular analysis carried out during this study show an unprecedented case of three patients from the same ward within a Swedish hospital, all being infected with a single strain of *Acanthamoeba*: this was confirmed by identifying all isolates to have identical 18S gene sequences, as well as identical *cox1/2* sequences, by 18S could be typed into T4. A second strain was also identified from the Swedish hospitals' water system, originating from the pool: The isolate was also found to belong to T4, but have different 18S and *cox1/2* sequences, from the others. Until now approximately 150 cases of GAE caused by *Acanthamoeba* have been described worldwide, with T4 species as the predominate group associated with both GAE and AK, followed by T1, T2, T5, T10 and T12 (Lackner *et al.*, 2010, Walochnik *et al.*, 2008).

A related amoeba from the genus *Hartmannella* was also isolated from the Swedish hospital sample set, surprisingly originating from a patient. So far *Hartmannella* has not reliably been reported as the cause of a fatal disease in humans (Schuster and Visvesvara, 2004), but early reports within the literature

often referred to *Acanthamoeba* as *Hartmannella*, and used the genus names interchangeably (Cleland *et al.*, 1982, Jager and Stamm, 1972). Here, *Hartmannella* has been associated with human disease, but most likely as an opportunistic secondary species, as *Acanthamoeba* was also isolated from the same patient.

The interactions that occur between amoebae are a relatively unexplored area, but could perhaps begin to explain why *Hartmannella* has only been found in humans as a co-infection with *Acanthamoeba.* In the case of *B. mandrillaris*, cells have been found to feed on *Acanthamoeba* sp. (personal communication, Dr W. Heaselgrave). While a previous study identified a *Hartmannella* sp. in association with a human infection, and determined it was unlikely to be the cause of the infection, but rather an opportunistic coloniser which may have worsened the disease (Centeno *et al*., 1996).

Free-living amoebae are increasingly being recovered from hospital systems, unsurprising given the ubiquitous nature of *Acanthamoeba* (Carlesso *et al.*, 2010, Thomas *et al.*, 2006): Most likely caused by increased frequency of testing and improvements in techniques, arisen from a better understanding of the need to keep opportunistic pathogens away from immune compromised hospital patients. Microbial biodiversity within hospital water systems' is an area of concern given the association of FLA with amoeba-resisting bacteria (ARB) and viruses that have the potential to cause infections in humans (Centeno *et al*., 1996). To rid a water system of FLA is almost impossible, amoebae including *Hartmannella* sp. have even been isolated from heavily cleaned hospital water systems (Thomas *et al*., 2006). The Swedish hospital did however successfully rid their ICU water system of *Acanthamoeba* by super chlorinating the system; unfortunately this was only maintained for a limited period. Retesting approximately 1-year post treatment,

showed *Acanthamoeba* of the same genotype and cox1/*2* sequence to be present again.

3.4.2 Pathogenicity

Identification to a genus level is all that is required to diagnose Acanthamebiasis. However by determining the pathogenicity of the isolate, and its sensitivity to antimicrobial compounds, the potential to provide substantial benefit to disease prognosis and the patient's outcome is gained. Microscopy and identification based on morphological characteristics alone are not sufficient to determine the pathogenicity of *Acanthamoeba* sp.. However, studies have shown a series of pathogenicity traits can be tested for, to distinguishing pathogenic from non-pathogenic isolates (Khan *et al.*, 2001, Khan *et al.*, 2002, Khan *et al.*, 2000).

Thermotolerance is an indicator of pathogenicity, (Khan *et al*., 2001, Khan *et al*., 2002, Walochnik *et al*., 2000), and *Acanthamoeba* must be able to withstand the internal temperature of the human body, at 37°C to cause GAE. All the Swedish isolates were thermotolerant, and grew at 32°C and 37°C, although in the case of the pool isolate its growth rate was slightly slower by comparison. Additionally both *A. polyphaga* species were also thermotolerant, surviving not only 32°C but also 37°C. Survival at 32°C is not surprising given that *A. polyphaga* (Ros) is an AK causing species, originally isolated from a human cornea (Hughes and Kilvington, 2001). However based on assays completed here, it appears to have the potential to survive within a human body. *Acanthamoeba polyphaga* (30871) has previously been shown to grow at 37°C, further supporting previous evidence to suggest that the species should be considered pathogenic (Khan *et al*., 2002, Stothard *et al*., 1998).

Osmotolerance is another indicator of pathogenicity, and amoebae that have the capacity to grow on agar with a higher osmolarity (1M mannitol) are more likely to be pathogenic and be able to withstand the human body. Here only *A. culbertsoni* (30171) the control, exhibited osmotolerance and grew on mannitol-containing plates. Although all the Swedish isolates were able to form viable cysts that could resume trophic growth once osmotic pressure had been removed. Previously, evidence has shown *A. polyphaga* (30871) to exhibit osmotolerance (Khan *et al*., 2002), yet here the strain was unable to grow on mannitol-containing plates. When considering osmolarity in relation to pathogenicity characteristics, epidemiological information for given isolates must be taken into consideration. Osmotolerant *A. griffini* (1501/4) was classified as a weak pathogen, despite never having been associated with disease, its ability to withstand increased osmotic environments is more likely to attributed to having originated from the sea around the USA coast (Khan *et al*., 2001). The exact reasons why these strains did not grow on mannitol-NNA plates is unclear, but despite this all other tests suggest they were pathogenic.

To cause infection, *Acanthamoeba* have to infiltrate the human body's defence system. In ocular infections amoebae must cross the stromal layer of the cornea, and the blood-brain barrier in encephalitis. Very little is known about the exact mechanism of how the pathogenic cascade is achieved, but for invasion to take place, interactions between the amoebae and host cells must occur, most likely involving the host extracellular matrix (da Rocha-Azevedo *et al*., 2010). It has been shown that secreted amoebic proteases have the ability to degrade components of the host extracellular matrix, such as collagens I, III, and IV, elastin, fibronectin and laminin (He *et al.*, 1990, Hurt *et al.*, 2003, Na *et al.*, 2001, Sissons *et al.*, 2006). By

inhibiting proteases, the capabilities of *Acanthamoeba* to invade collagen and BD Matrigel (basement membrane matrix) are reduced (da Rocha-Azevedo *et al*., 2010). Here all isolates including the Swedish water strains produced extracellular proteases. The pathogenesis of *Acanthamoeba* infections is highly complex and multifaceted involving proteolytic activity. Proteases are degradative enzymes that are vital for migration and tissue invasion. All *Acanthamoeba* isolates tested to date, including pathogenic and non-pathogenic produce extracellular proteases, to catalyse the hydrolysis of large proteins into smaller molecules ready for absorption by the cell (Khan, 2009).

Acanthamoeba are free-living organisms, which happen to be opportunistic pathogens with a secondary ability of being able to cause infection. As all isolates tested here produce extracellular proteases, it was not unexpected to observe that they all too exhibit cytopathic effects on epitheloid cells when incubated together. However, subtle differences were found between some of the strains, when compared to each other. The length of time taken to reach the same point of destruction varied amongst the strains: With isolates from shower room 3, and *A. polyphaga* (30871) causing destruction to the HeLa cell monolayer at a slow rate, which was only marginally slower than the AK isolate *A. polyphaga* (Ros).

The innate resistance factor complement is the host's powerful first line of defence against invading *Acanthamoeba*. Although many microorganisms are susceptible to complement lysis, some are better suited to withstand its lytic activity than others (Jokiranta *et al*., 1995), which includes the highly pathogenic species *A. culbertsoni* (Toney and Marciano-Cabral, 1998). Resistance to complement by some *Acanthamoeba* strains seems to show some correlation to pathogenicity, although reports have shown both pathogenic and non-pathogenic strains are

susceptible to lysis by complement (Ferrante, 1991, Ferrante and Rowan-Kelly, 1983, Pumidonming *et al.*, 2011).

Ultimately *in vitro* analyses have shown no single Swedish hospital strain to be any greatly more virulent than any of the others, and in fact they have comparable pathogenicity traits to *A. polyphaga* Ros and *A. polyphaga* 30871. For an *Acanthamoeba* infection to take hold, multiple virulence factors must be met. Which accounts for the rarity of the disease despite the ubiquitous nature of *Acanthamoeba* within the environment. Never before has an outbreak of GAE been documented, suggesting extraordinary circumstances had occurred within the Swedish hospital involving and/or the amoebae or patients. Pathogenicity assays have confirmed that all the Swedish hospital isolates are virulent but perhaps the line between pathogenic and non-pathogenic is not so clearly defined, and because all strains have the ability to produce extracellular proteases (Khan, 2009) for example, they also have an innate capacity to cause disease, providing predisposing factors allow.

To provide a fuller picture of virulence, several other pathogenicity assays could have been included, such as longevity tests observing cyst endurance in storage over an extended period of time. As well as alternative extracellular proteases assays, identifying their presence: Invasion/migration assays can establish if the trophozoites have the ability to pass through a layer of gelatine collagen, or BD Matrigel (basement membrane matrix) (da Rocha-Azevedo *et al*., 2010, Hurt *et al*., 2003) to a lower chamber containing nutrients. Proteases can be classified into six major groups including, serine, aspartic, cysteine, metalloproteases, threonine, and glutamic acid (Khan, 2009), determination of the type and also quantity produced by each strain, will improve the understanding its pathogenicity potential.

A full examination of the Swedish hospital system should also be carried out, identifying if the system supports large numbers/types of microorganisms making it an ideal home to harbour pathogenic FLA.

3.4.3 Antimicrobial sensitivity

There is no current effective treatment for GAE or disseminated acanthamebiasis, and very few people have ever responded favourably to therapy. For antimicrobials to be successful in treating GAE, they like the amoebae must be able to cross the blood-brain barrier, and enter the brain and CSF. Most acanthamoeba infections, are treated with antimicrobial combinations, and of those, success has been achieved with intravenous pentamidine isethionate in combination with topical chlorhexidine and ketoconazole administered to a transplant patient who contracted disseminated infection (Slater *et al*., 1994): An HIV/AIDS patient, received fluconazole and sulfadiazine combined with surgical excision of the brain lesion (Seijo Martinez *et al*., 2000): While a previously healthy paediatric, received ketoconazole and had a total excision of the infected area (Ofori-Kwakye *et al*., 1986): Combination therapy with oral miltefosine and amikacin were used to successfully treat a patient with GAE in Austria (Walochnik *et al*., 2008). Another successful outcome arose from a 17-year-old immunocompromised patient who presented with acute purulent meningoencephalitis, and was treated with a combination of meropenem, linezolid, moxifloacin, and fluconazole (Lackner *et al*., 2010). The strategy of combining antimicrobial compounds may result in beneficial synergistic effects, and additionally avoid any potential resistance patterns. Particularly as *in vitro* sensitivity testing of clinical isolates results in strain and species differences, highlighting that no single compound can be assumed effective

against all amoebae (Schuster and Visvesvara, 1998). Further compounded by the poor correlation observed between *in vitro* MCC findings and patient response to those therapeutic agents (Elder *et al.*, 1994, Kilvington *et al.*, 2002).

Unfortunately as access was not granted to any patient clinical information from the Swedish outbreak, sensitivity assays were designed using a range of antimicrobial compounds, with the intention of hopefully testing the strains *in vitro* against a compound that they were exposed to during the course of treatment: If not the actual compound, at least one belonging to the same family.

For this study *in vitro* cyst sensitivity testing, identified all strains could resist cidal effects of miltefosine, voriconazole and natamycin at concentrations lower than 500 μg/ml. Hexamidine also showed little effect towards cysts, at concentrations lower than 500 μg/ml for four of the strains (Patient 2, Shower room 3, *A. polyphaga* Ros, and *A. polyphaga* 30871): Whilst being cysticidal to Patient 3's isolate at concentrations above 62.5 μg/ml, and to Shower room 15 and the Pool isolates at greater than 1.9 μg/ml. However good cysticidal activity against all strains was detected in assays with PHMB (>1.9 μg/ml).

Polyhexamethylene biguanide (PHMB) is an antiseptic in the same family as chlorhexidine but is not as cytotoxic. It is used to good effect as a treatment of AK, where it is administered as a topical solution of 0.02%, either alone or in combination therapy. As it has a low mammalian toxicity, it can therefore be applied over long periods of time. Its structure is a positively charged polymer, with low surface tension, that against *Acanthamoeba* targets the plasma membrane by binding with the acidic phospholipids. As PHMB is a disinfectant it is only suitable for topical application, and despite having good cysticidal effect on many strains of *Acanthamoeba* it cannot be used to treat systemic infections. Several AK cases have

been documented, reporting no response to treatment with PHMB, even though isolated amoebae were found to be sensitive to the compound in *in vitro* assays (Elder *et al.*, 1994, Murdoch *et al.*, 1998, Perez-Santonja *et al.*, 2003). Clearly inconsistencies between *in vitro* sensitivity profiles and management of individual cases of acanthamebiasis can occur. *In vitro* drug sensitivity assays are undoubtedly important in the development of potential new compounds to treat acanthamoeba infections, but such assays clearly have limitations: Can any effects occurring within the carefully controlled environment of a microtitre well fully represent the human body? Effects of the antimicrobials on the amoebae may be altered by host cellular and humoral responses, potentially affecting the course of the disease.

This study reported some cysticidal effect caused by the antimicrobial agent, hexamidine di-isethionate (Desomedine): The compound is a homologue of propamidine but has been shown in some cases to have greater cysticidal activity (Brasseur *et al*., 1994, Perrine *et al*., 1995), and disputed more recently (Seal, 2003). The diamidine group, contains many homologues including not only propamidine but, butamidine, heptamidine, hexamidine, octamidine, pentamidine and nonamidine (Perrine *et al*., 1995). The antimicrobial properties of the diamidines' against *Acanthamoeba* have been found to be proportional to the length of the alkyl chain of the diamidine molecule, linked to its lipophilicity and ability to penetrate the amoeba cell membrane. Which causes structural changes to the plasma membrane leading to alterations of cell permeability (Perrine *et al*., 1995). Currently the recommended treatment regime for AK includes an aromatic diamidine, either hexamidine (Desomedine) or propamidine isethionate (Brolene) at 0.1% (1 mg/ml),

in combination with a disinfecting biguanide either 0.02% PHMB or chlorohexidine digluconate (Khan, 2009).

Here, hexamidine against cysts showed variable amoebicidal results: with very weak cytotoxic effects against four strains >500 μg/ml; limited towards another at 62.5 μg/ml, with strong activity shown against two strains, at 1.9 μg/ml. Variability was also shown against trophozoites, with an average MTAC of 23.4 μg/ml, with a range of 15.6 – 31.25 μg/ml. Such variability in the effectiveness of one agent reflects the suggestion that natural variation in *Acanthamoeba* species accounts for much of the variation found in sensitivity assays to determine cidal effects caused by antimicrobials (Elder *et al.*, 1994, Khan, 2009).

In all cases by *in vitro* assays, trophozoites were more susceptible to therapeutic compounds than cysts, as has been previously reported (Elder *et al.*, 1994, Jones *et al.*, 1975, Walochnik *et al.*, 2009). As with cysts assays, PHMB showed the most activity against strains, with average MTAC values of 1.9 μg/ml. Next best amoebicidal activity towards the strains was shown by the promising compound Miltefosine (Impavido®, hexadecylphosphochlorine): An alkylphosphocholine anticancer agent that appears to hold necessary therapeutic properties for the treatment of AK (Walochnik *et al*., 2009), and GAE (Walochnik *et al*., 2008). Although the mode of action is unknown, miltefosine appears to accumulate in the cell membrane causing structural disruption and affecting cell metabolism (Eibl and Unger, 1990, Walochnik *et al.*, 2009). Recently it has been used for the treatment of disseminated *Acanthamoeba* infections, and is also a potential antimicrobial agent for treatment of AK and GAE (Mrva *et al*., 2011). Miltefosine has been shown to be effective against clinical and environmental

isolates of *Acanthamoeba*, starting at concentrations of 2 μg/ml (~5 μM) (Schuster *et al.*, 2006a, Walochnik *et al.*, 2002), and 25.5 μg/ml (62.5 μM) (Mrva, M 2011): Here assays showed amoebicidal effects at 11.7 μg/ml, between the strong and weak amoebicidal activity previously shown.

Natamycin (Natacyn®, 5% (50 mg/ml)) is a macrolide polyene antifungal agent used to treat fungal eye infections. The macrolide antibiotic group is large, containing many agents including amphotericin B, erythromycin, azithromycin and clarithromycin. The macrolides have broad-spectrum activities against a range of microorganisms (Mattana *et al*., 2004), and have been used to treat GAE, with both successful outcomes (Nachega *et al.*, 2005, Walia *et al.*, 2007), and failure (Kuashal *et al*., 2008). The precise mode of action of the macrolides within *Acanthamoeba* is not fully understood but thought to involve the plasma membrane and ergosterol (Raederstorff and Rohmer, 1985), altering cell permeability (Mattana *et al*., 2004).

Antifungal agents, natamycin (>500 μg/ml), and voriconazole (>500 μg/ml) showed only weak amoebicidal effects against cysts, but higher cytotoxicity against trophozoites was attained, natamycin (187.5 μg/ml) and voriconazole (>125 μg/ml). Voriconazole belongs to the azole group, and includes the homolog compounds, clotrimazole, miconazole, ketoconazole, and fluconazole. It has a strong inhibitory effect upon *Acanthamoeba* trophozoites but not cysts (Schuster *et al*., 2006a), with a mode of action that is similar to that of natamycin (Sanati *et al*., 1997).

Through the use of *in vitro* sensitivity assays, several pharmaceutical agents have been shown to have antimicrobial properties towards *Acanthamoeba* cysts and trophozoites, but no single compound has proven effective against all isolates of the genus: Reflecting the high natural variability within the species. This only goes to

highlight the importance of carrying out drug sensitivity tests for every case of AK and GAE diagnosed. Which would not only help identify the best choice compound, but also help discount any that are potentially ineffective. The Amoebae Laboratory, at the University of Leicester now routinely carries out such sensitivity testing for the Leicester Royal Infirmary, and other UK hospitals. Their experience has shown clinical strains of AK usually show sensitivity to biguanides, but sensitivity to all other drug compounds in particular diamidines, is very variable (personal communication Dr W. Heaselgrave). So it is important to identify the effective treatments quickly to improve patient prognosis. However, cases that fail to respond to medical therapy do occur despite the apparent *in vitro* sensitivity to compounds such as PHMB (Elder *et al.*, 1994, Murdoch *et al.*, 1998, Perez-Santonja *et al.*, 2003). The cause of this is unknown, but for these patients prognosis is poor, as they remain culture positive and have to undergo repeated penetrating keratoplasty. With *Acanthamoeba* infections, prompt instigation of treatment is necessary for good prognosis. Therefore sensitivity testing is important to ensure therapy stands a chance of being effective and is not a waste of time.

3.4.1 Conclusion

Epidemiological implications and a better understanding of the course of infection may occur as a result of speciation of the isolate involved in acanthamebiasis. The value of the sequencing systems has been clearly shown here, by allowing strain differentiation. Severely debilitated patients on a paediatric ICU, within a Swedish hospital fell ill with symptoms of GAE. Clinical samples were collected from the three patients and any water-associated places that the patients

had access to. Epidemiological typing was carried out, with CSF samples cultured for FLA, confirming the patients were all infected with T4 *Acanthamoeba* isolates.

In addition to the *Acanthamoeba*, one patient had also contracted an extremely rare associated infection with a species of *Hartmannella* as well.

The combined use of two molecular genotyping systems both 18S and *cox1/2*, confirmed the infection had originated from the water system within the hospital and the patients were all infected with the same strain. The T-group and *cox1/2* systems both proved to be independently rapid, sensitive and highly effective tools for use within epidemiological studies.

Pathogenicity and antimicrobial sensitivity assays confirmed the isolates originating from the Swedish hospital were all virulent, but no more pathogenic than expected given that they were associated with an unprecedented outbreak of GAE.

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