Acanthamoeba: biology and increasing importance in human health

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Received 11 October 2005; revised 9 March 2006; accepted 9 March 2006.
First published online 16 May 2006.

doi:10.1111/j.1574-6976.2006.00023.x

Editor: Graham Coombs

Keywords
Acanthamoeba; encephalitis; keratitis; epidemiology; pathogenesis; disease

Abstract

Acanthamoeba is an opportunistic protozoan that is widely distributed in the environment and is well recognized to produce serious human infections, including a blinding keratitis and a fatal encephalitis. This review presents our current understanding of the burden of *Acanthamoeba* infections on human health, their pathogenesis and pathophysiology, and molecular mechanisms associated with the disease, as well as virulence traits of *Acanthamoeba* that may be targets for therapeutic interventions and/or the development of preventative measures.

Introduction

During the last two decades, *Acanthamoeba* species have become increasingly recognized as important microbes. They are now well recognized as human pathogens causing serious as well as life-threatening infections, have a potential role in ecosystems, and act as carriers and reservoirs for prokaryotes. This review describes our current understanding of these microbes. There are some excellent reviews focused on various topics in this area, which are recommended for additional study (David, 1993; Niederkorn *et al.*, 1999; Khan, 2003; Marciano-Cabral & Cabral, 2003; Schuster & Visvesvara, 2004).

Protozoa

Protozoa are the largest single-cell nonphotosynthetic animals that lack cell walls (Fig. 1). The study of protozoa, invisible to the naked eye, was initiated with the discovery of the microscope in the 1600s by Antonio van Leeuwenhoek (1632–1723). Protozoa feed by pinocytosis (engulfing liquids/particles by invagination of the plasma membrane) and/or phagocytosis (engulfing large particles, which may require specific interactions). Protozoa reproduce asexually by binary fission (parent cell mitotically divides into two daughter cells), multiple fission (parent cell divides into several daughter cells), budding and spore formation, or sexually by conjugation (two cells join, exchange nuclei and produce progeny by budding or fission) (Khan, 2006). Protozoa are among the five major classes of pathogens: intracellular parasites (viruses), prokaryotes, fungi, protozoa and multicellular pathogens. To produce disease, protozoa access their hosts via direct transmission through the oral cavity, the respiratory tract, the genitourinary tract and the skin, or by indirect transmission through insects, rodents as well as by inanimate objects such as towels, contact lenses and surgical instruments. Once the host tissue is invaded, protozoa multiply to establish themselves in the host, and this may be followed by physical damage to the host tissue or depriving it of nutrients, and/or by the induction of an excessive host immune response resulting in disease.

Discovery of pathogenic free-living amoebae

The term 'amoebae' encompasses the largest diverse group of organisms in the protists, and have been studied since the discovery of the early microscope, e.g. the largest *Amoeba proteus* (Fig. 1). Although these organisms have a common amoeboid motion, i.e. crawling-like movement, they have been classified into several different groups. These include potent parasitic organisms such as *Entamoeba* spp. that were discovered in 1873 from a patient suffering from bloody dysentery and named *Entamoeba histolytica* in 1903. Among free-living amoebae, *Naegleria* were first discovered by

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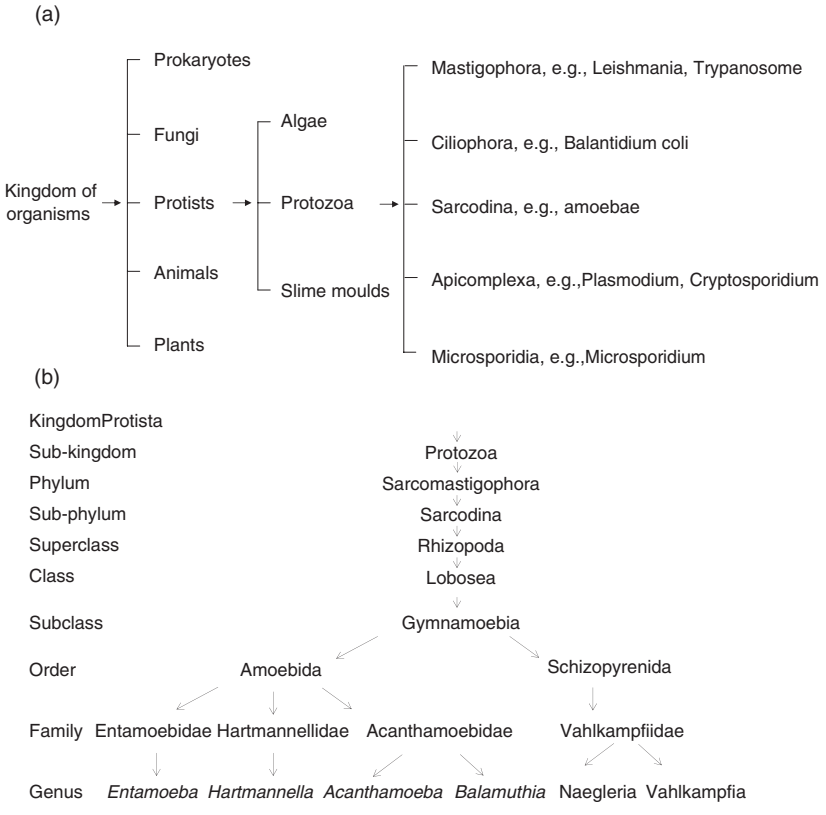


Fig. 1. The classification scheme of free-living amoebae.

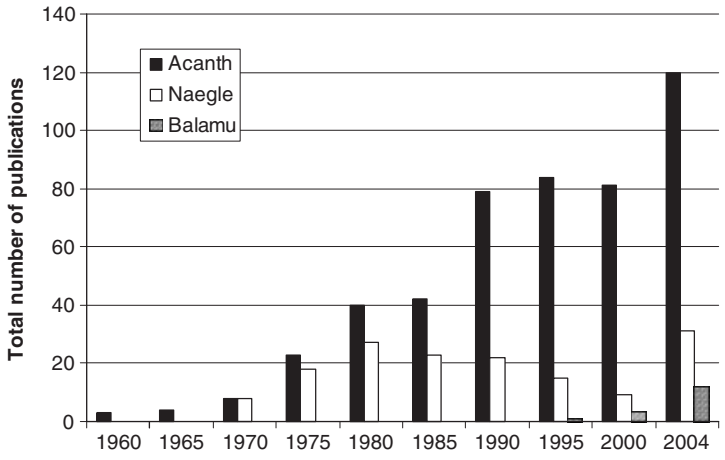


Fig. 2. The number of published articles in free-living amoebae. Data for *Acanthamoeba*, *Naegleria* and *Balamuthia* were collected from PubMed, i.e. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi.

Schardinger in 1899, who named the organism *Amoeba gruberi*. In 1912, Alexeieff suggested its genus name *Naegleria*, and much later in 1970 Carter identified *Naegleria fowleri* as the causative agent of fatal human infections (reviewed in De Jonckheere, 2002). In 1930, *Acanthamoeba* were discovered as eukaryotic cell culture contaminants and were placed in the genus *Acanthamoeba* (Castellani, 1930; Douglas, 1930; Volkonsky, 1931). *Balamuthia mandrillaris* was described relatively recently (1986) from the brain of a baboon that had died of meningoencephalitis and was described as a novel genus, i.e. *Balamuthia* (Visvesvara *et al*., 1990, 1993). Over the years, these free-living amoebae have gained increasing attention from the scientific community due to their diverse roles, in particular in causing serious and sometimes fatal human infections (Fig. 2).

Acanthamoeba spp.

Castellani (1930) discovered an amoeba in a culture of the fungus *Cryptococcus pararoseus*. These amoebae were round

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or oval in shape with diameter of 13.5–22.5 μm and exhibited the presence of pseudopodia (now known as acanthopodia). In addition, the encysted form of these amoebae exhibited double walls with an average diameter of 9–12 μm. This amoeba was placed in the genus *Hartmannella*, and named *Hartmannella castellanii*. A year later, Volkonsky (1931) subdivided the *Hartmannella* genus into three genera based on the following characteristics:

(1) *Hartmannella*: amoebae characterized by round, smooth-walled cysts.

(2) *Glaeseria*: amoebae characterized by nuclear division in the cysts.

(3) *Acanthamoeba*: amoebae characterized by the appearance of pointed spindles at mitosis, double-walled cysts and an irregular outer layer.

Singh (1950) and Singh & Das (1970) argued that the classification of amoeba by morphology, locomotion and appearance of cysts was of limited phylogenetic value and that these characteristics were not diagnostic. They concluded that the shape of the mitotic spindle was inadequate as a generic character and discarded the genus *Acanthamoeba*. In 1966, Pussard agreed with Singh (1950) that the spindle shape was an unsatisfactory feature for species differentiation but considered the distinctive morphology of the cyst to be a decisive character at the generic level and recognized the genus *Acanthamoeba*. After studying several strains of *Hartmannella* and *Acanthamoeba*, Page (1967a,b) also concluded that the shape of the spindle was a doubtful criterion for species differentiation. He considered the presence of acanthopodia and the structure of the cyst to be sufficiently distinctive to justify the generic designations of *Hartmannella* and *Acanthamoeba*. He also stated that the genus *Hartmannella* had nothing in common with *Acanthamoeba* except for a general mitotic pattern, which is a property shared with many other amoeba.

Sawyer & Griffin (1975) established the family Acanthamoebidae and Page (1988) placed *Hartmannella* in the family Hartmannellidae. The current position of *Acanthamoeba* in relation to *Hartmannella*, *Naegleria* and other free-living amoebae is shown in Fig. 1. The prefix *acanth* (Greek for spikes) was added to the term *amoebae* to indicate the presence of spine-like structures (now known as acanthopodia) on the surface of these organisms. After the initial discovery in 1930, these organisms were largely ignored for nearly the next three decades. However, in the late 1950s, they were discovered as tissue culture contaminants (Jahnes *et al*., 1957; Culbertson *et al*., 1958). Later, Culbertson *et al*. (1958, 1959) demonstrated, for the first time, the pathogenic potential of these organisms by exhibiting their ability to produce cytopathic effects on monkey kidney cells *in vitro*, and to kill laboratory animals *in vivo*. The first clearly identified *Acanthamoeba* granulomatous encephalitis (AGE) in humans was observed by Jager & Stamm (1972). The first *Acanthamoeba* keratitis cases were reported by Nagington *et al*. (1974). *Acanthamoeba* were first shown to be infected with bacteria in 1954 (Drozanski, 1956); demonstrated to harbour bacteria as endosymbionts (Proca-Ciobanu *et al*., 1975); and shown to provide a reservoir for pathogenic facultative mycobacteria (Krishna-Prasad & Gupta, 1978). *Acanthamoeba* were first linked with Legionnaires' disease by Rowbotham (1980). Since then the worldwide research interest in the field of *Acanthamoeba* has increased dramatically and continues to do so (Fig. 2).

Ecological distribution

Acanthamoeba have the ability to survive in diverse environments and have been isolated from public water supplies, swimming pools, bottled water, seawater, pond water, stagnant water, freshwater lakes, salt water lakes, river water, distilled water bottles, ventilation ducts, the water–air interface, air-conditioning units, sewage, compost, sediments, soil, beaches, vegetables, air, surgical instruments, contact lenses and their cases, and from the atmosphere (recent demonstration of *Acanthamoeba* isolation even by air sampling), indicating the ubiquitous nature of these organisms. In addition, *Acanthamoeba* have been recovered from hospitals, dialysis units, eye wash stations, human nasal cavities, pharyngeal swabs, lungs tissues, skin lesions, corneal biopsies, cerebrospinal fluid (CSF) and brain necropsies (reviewed in Khan, 2003; Marciano-Cabral & Cabral, 2003; Schuster & Visvesvara, 2004). It is not surprising that the majority of healthy individuals have been shown to possess anti-*Acanthamoeba* antibodies, indicating our common exposure to these pathogens (Cursons *et al*., 1980).

Life cycle

Acanthamoeba undergoes two stages during its life cycle: a vegetative trophozoite and a resistant cyst stage (Fig. 3). The trophozoites are normally in the range of 12–35 μm in diameter, but the size varies significantly between isolates belonging to different species/genotypes. The trophozoites exhibit spine-like structures on their surface known as acanthopodia. The acanthopodia are most likely of importance in adhesion to surfaces (biological or inert), cellular movements or capturing prey. The trophozoites normally possess a single nucleus that is approximately one-sixth the size of the trophozoite. During the trophozoite stage, *Acanthamoeba* actively feed on bacteria, algae, yeasts or small organic particles and many food vacuoles can be seen in the cytoplasm of the cell. Cell division is asexual and occurs by binary fission. For exponentially growing cells, cell division is largely occupied with G2 phase (up to 90%) and negligible G1 phase, 2–3% M phase (mitosis) and 2–3% S phase (synthesis) (Band & Mohrlok, 1973; Byers *et al*., 1990, 1991). *Acanthamoeba* can be maintained in the trophozoite

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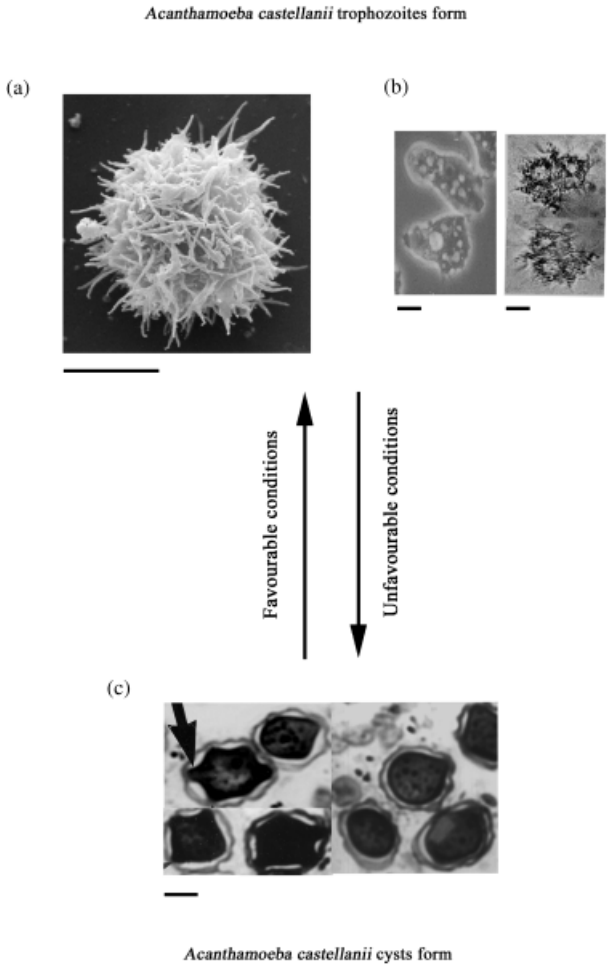


Fig. 3. The life cycle of *Acanthamoeba castellanii*. Infective form of *A. castellanii*, also known as trophozoites, as observed under (a) scanning electron microscope and (b) phase-contrast microscope. Under unfavourable conditions, trophozoites differentiate into cysts. (c) Cysts form of *A. castellanii*, characterized by double wall as indicated by arrows. Scale bar = 5 μm (published with permission from Elsevier).

stage with an abundant food supply, neutral pH, appropriate temperature (i.e. 30 °C) and osmolarity between 50–80 mOsmol. However, harsh conditions (i.e. lack of food, increased osmolarity or hypo-osmolarity, extremes in temperatures and pH) induce the transformation of trophozoites into the cyst stage. In simple terms, the trophozoite becomes metabolically inactive (minimal metabolic activity) and encloses itself within a resistant shell. More precisely, during the encystment stage, excess food, water and particulate matter is expelled and the trophozoite condenses itself into a rounded structure (i.e. precyst), which matures into a double-walled cyst with the outer wall serving only as a shell to help the parasite survive hostile conditions. Cellular levels of RNA, proteins, triacylglycerides and glycogen decline substantially during the encystment process, resulting in decreased cellular volume and dry weight (Weisman, 1976). The cyst stage is 5–20 μm in diameter but again this varies between isolates belonging to different species/genotypes. Cysts are airborne, which may help spread *Acanthamoeba* in the environment and/or carry these pathogens to the susceptible hosts. Several studies report that cysts can remain viable for several years while maintaining their pathogenicity, thus presenting a role in the transmission of *Acanthamoeba* infections (Mazur *et al.*, 1995). Cysts possess pores known as ostioles, which are used to monitor environmental changes. The trophozoites emerge from the cysts under favourable conditions leaving behind the outer shell and actively reproduce as described above, thus completing the cycle. Both the encystment and the excystment processes require active macromolecule synthesis and can be blocked by cycloheximide (a protein synthesis inhibitor).

Feeding

Acanthamoeba feed on microorganisms present on surfaces, in diverse environments (Brown & Barker, 1999) and even at the air–water interface (Preston *et al.*, 2001). The spiny structures or acanthopodia that arise from the surface of *Acanthamoeba* trophozoites may be used to capture food particles, which usually are bacteria (Weekers *et al.*, 1993), but algae, yeast (Allen & Dawidowicz, 1990) and other protists are also grazed upon. Food uptake in *Acanthamoeba* occurs by phagocytosis and pinocytosis. Phagocytosis is a receptor-dependent process, while pinocytosis is a nonspecific process through membrane invaginations and is used to take up large volumes of solutes/food particles (Bowers & Olszewski, 1972). *Acanthamoeba* uses both specific phagocytosis and nonspecific pinocytsis for the uptake of food particles and large volumes of solutes (Bowers & Olszewski, 1972; Allen & Dawidowicz, 1990; Alsam *et al.*, 2005a). Solutes of varying molecular weights, including albumin (Mw 65 000), inulin (Mw 5000), glucose (Mw 180) and leucine (Mw 131), enter amoebae at a similar rate of 2 $\mu L\, h^{-1}$ per 10^6 cells. But how amoebae discriminate between pinocytosis and phagocytosis, why they use one or the other, and whether there are any differences in this respect between pathogenic and nonpathogenic *Acanthamoeba* remain incompletely understood (Alsam *et al.*, 2005a). Subsequent to particle uptake into a vacuole, *Acanthamoeba* exhibit the ability to distinguish vacuoles containing digestible and indigestible particles. For example, Bowers & Olszewski (1983) have shown that the fate of vacuoles within *Acanthamoeba* is dependent on the nature of particles, latex beads vs. food particles. Vacuoles containing food particles are retained and digested, whereas latex beads are exocytosed, upon presentation of new particles. Overall, these studies suggest that particle uptake in *Acanthamoeba* is a complex

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process that may play a significant role both in food uptake and in the pathogenesis of *Acanthamoeba*.

Biology

As discussed above, the *Acanthamoeba* life cycle comprises a trophozoite and a cyst stage. The trophozoite possesses a single nucleus with a prominent nucleolus. Under the microscope, an actively feeding trophozoite exhibits one or more prominent contractile vacuoles, whose function is to expel water. *Acanthamoeba* possess an extensive network of endoplasmic reticulum with ribosomes bound on the cytoplasmic surface for protein synthesis. This is followed by post-translational modifications of proteins, most notably glycosylation, in the Golgi apparatus and destined for cell membrane or for export (Byers *et al*., 1991). The trophozoite possesses large numbers of mitochondria, generating the energy required for metabolic activities involved in feeding, as well as movement, reproduction and other cellular functions. The plasma membrane is unusual in the presence of a lipophosphonoglycan, which is absent in mammalian cells (Korn *et al*., 1974), with sugars exposed on both sides of the membrane (Bowers & Korn, 1974). The cytoplasm possesses large numbers of fibrils, glycogen and lipid droplets. Actin (constituting 20% of the total protein) and myosin, together with more than 20 cytoskeletal proteins, have been isolated from trophozoites, and are responsible for cellular functions associated with movement, intracellular transport and cell division. Under optimal growth conditions, *Acanthamoeba* reproduce by binary fission. The generation time differs between isolates belonging to different species/genotypes from 8 to 24 h. The trophozoites contain cellular, nuclear and mitochondrial DNA with nuclear DNA comprising 80–85% of the total DNA. In addition, cytoplasmic nonmitochondrial DNA has been reported (Ito *et al*., 1969), but its origin is not known. Total cellular DNA ranges between 1 and 2 pg for single-cell uninucleate amoebae during the log phase (Byers *et al*., 1990). The number of nuclear chromosomes is uncertain but may be high. Measurements of nuclear DNA content (*Acanthamoeba castellanii* Neff strain, belonging to the T4 genotype) showed a total DNA content of 10^9 bp. Measurement of kinetic complexity suggests a haploid genome size of $\sim 4–5 \times 10^7$ bp (Byers *et al*., 1990). Pulse-field gel electrophoresis suggests a genome of $\sim 2.3–3.5 \times 10^7$ bp, which express more than 5000 transcripts. For comparison, the haploid genome size of *Saccharomyces* is $\sim 2 \times 10^7$ bp, and *Dictyostelium* is $\sim 5 \times 10^7$ bp (reviewed in Byers *et al*., 1990). Under harsh conditions, the trophozoites differentiate into a nondividing, double-walled resistant cyst form. Cyst walls contain cellulose (not present in the trophozoite stage) that accounts for 10% of the total dry weight of the cyst (Tomlinson & Jones, 1962). Although cyst wall composition varies between isolates belonging to different species/genotypes, the T4 isolate (*A. castellanii*) has been shown to contain 33% protein, 4–6% lipid, 35% carbohydrates (mostly cellulose), 8% ash and 20% unidentified materials (Neff & Neff, 1969).

Methods of isolation

In natural environments, *Acanthamoeba* feed on yeasts, other protozoa, bacteria, small organisms and organic particles. Any of the aforementioned can be used as growth substrates for *Acanthamoeba* in the laboratory but there are some technical problems. For example, the use of yeast and protozoa as growth substrates is problematic due to complexity in their preparations, their possible overwhelming growth and the difficulty in eradicating yeast to obtain pure axenic *Acanthamoeba* cultures. Organic substances such as glucose, proteose peptone or other substrates provide rich nutrients for unwanted organisms, i.e. yeasts, fungi, other protozoa and bacteria. To overcome these technical problems and to maximize the likelihood of *Acanthamoeba* isolation from environmental as well as clinical samples, protocols have been developed using simple plating assays as described below. Both of the following methods can be used to obtain large number of *Acanthamoeba* trophozoites for biochemical studies.

Isolation of *Acanthamoeba* using non-nutrient agar plates seeded with Gram-negative bacteria

This method has been used extensively in the isolation of *Acanthamoeba* from both environmental and clinical samples, worldwide. The basis of this method is the use of Gram-negative bacteria (*Escherichia coli* or *Enterobacter aerogenes*, formerly known as *Klebsiella aerogenes*, are most commonly used) that are seeded on the non-nutrient agar plate as food source for *Acanthamoeba*. The non-nutrient agar contains minimal nutrients and thus inhibits the growth of unwanted organisms (Khan & Paget, 2002). Briefly, non-nutrient agar plates containing 1% (w/v) Oxoid no.1 agar in Page's amoeba saline (PAS) (2.5 mM NaCl, 1 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 40 μM $CaCl_2.6H_2O$ and 20 μM $MgSO_4.7H_2O$) supplemented with 4 % (w/v) malt extract and 4 % (w/v) yeast extract are prepared, and the pH adjusted to 6.9 with KOH. Approximately 5 mL of late log phase cultures of Gram-negative bacteria (*Escherichia coli* or *Enterobacter aerogenes*) are poured onto non-nutrient agar plates and left for 5 min, after which excess culture fluid is removed and plates are left to dry before their inoculation with an environmental sample or clinical specimen. Once inoculated, plates are incubated at 30 °C and observed daily for the presence of *Acanthamoeba* trophozoites (Khan *et al*., 2001; Khan & Paget, 2002). Depending on the number of

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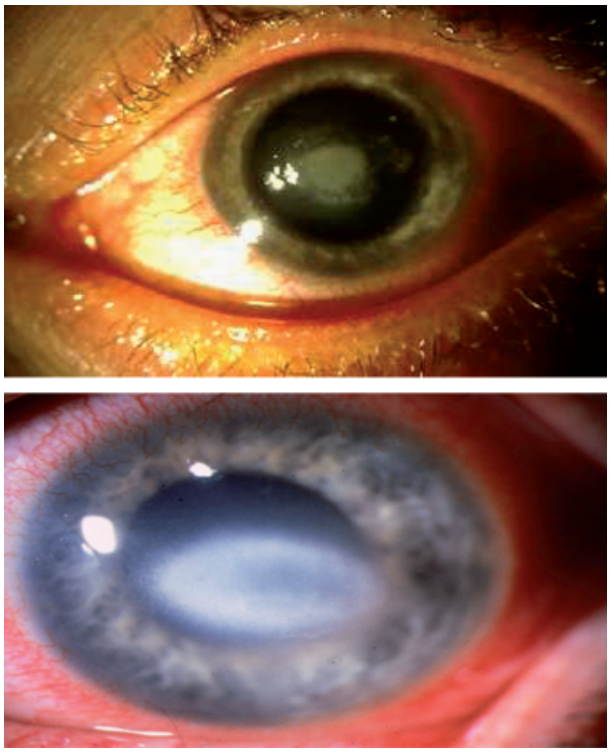


Fig. 4. *Acanthamoeba*-infected eye exhibiting the severity of the disease. Note the ulcerated epithelium and stromal infiltration exhibiting corneal opacity in acute *Acanthamoeba* keratitis (published with permission from Elsevier).

amoebae in the sample, trophozoites can be observed within a few hours (up to 12 h). However in the absence of amoebae, plates should be monitored for up to 7 days. Once bacteria are consumed, *Acanthamoeba* differentiate into characteristic cysts (Figs 3 and 4). The precise understanding of bacterial preference by *Acanthamoeba*, i.e. Gram-negative vs. Gram-positive bacteria, or why *Escherichia coli* or *Enterobacter aerogenes* are used most commonly as food substrate, and whether bacterial preferences vary between *Acanthamoeba* isolates belonging to different species/genotypes are questions for future studies.

'Axenic' cultivation of *Acanthamoeba*

Acanthamoeba can be grown 'axenically' in the absence of external live food organisms. This is typically referred to as axenic culture to indicate that no other living organisms are present. However, *Acanthamoeba* cultures may never be truly axenic as they may contain live bacteria surviving internally as endosymbionts. Under laboratory conditions, axenic growth is achieved using liquid PYG medium [proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v) and glucose 1.5% (w/v)]. Briefly, non-nutrient agar plates overlaid with bacteria are placed under UV light for 15–30 min to kill the bacterial lawn. A small piece of non-nutrient agar (stamp-sized) containing amoebic cysts is placed on plates containing these UV-killed bacteria. When amoebae begin to grow, a stamp-sized piece of the agar containing trophozoites or cysts is transferred into 10 mL of sterile PYG medium containing antibiotics, i.e. penicillin and streptomycin. The *Acanthamoeba* switch to the PYG medium as a food source, and their multiplication can be observed within several days. Once multiplying in PYG medium, *Acanthamoeba* are typically grown aerobically in tissue-culture flasks with filter caps at 30 °C in static conditions. The trophozoites adhere to the flask walls and are collected by chilling the flask for 15–30 min (at 4 °C), followed by centrifugation of the medium containing the cells.

Methods of encystment

Both xenic and axenic methods have been developed to obtain *Acanthamoeba* cysts. For xenic cultures, *Acanthamoeba* are inoculated onto non-nutrient agar plates seeded with bacteria as indicated above. Plates are incubated at 30 °C until the bacteria are cleared and trophozoites have transformed into cysts. Cysts can be scraped off the agar surface using phosphate-buffered saline (PBS) and used for assays. This resembles the most likely natural mode of encystment and can be effective, achieving up to 100% cysts. However, one major limitation may be the presence of bacterial contaminants that could hamper molecular and biochemical studies. For axenic encystment, *Acanthamoeba* are grown in PYG medium for 17–20 h. After this incubation, 8% glucose in RPMI 1640 (Invitrogen) is added to stimulate encystment. Plates are incubated at 30 °C for up to 48 h. To confirm transformation of trophozoites into cysts, sodium dodecyl sulfate (SDS, 0.5% final concentration) is added: trophozoites are SDS-sensitive and any remaining are lysed immediately upon addition of SDS, while cysts remain intact (Cordingley *et al.*, 1996; Dudley *et al.*, 2005). This method allows the simple counting of cysts using a haemocytometer and is useful in studying the process of encystment.

Storage of *Acanthamoeba*

For short-term storage, *Acanthamoeba* are maintained on non-nutrient agar plates. Plates inoculated with *Acanthamoeba* can be kept at 4 °C under moist conditions for several months or as long as plates are protected from drying out. Cysts can be reinoculated into PYG medium in the presence of antibiotics to obtain axenic cultures as described above. Alternatively, *Acanthamoeba* trophozoites can be stored as axenic cultures for long-term storage. Briefly, log-phase amoebae (actively dividing) are resuspended at a density of $3–5 \times 10^6$ parasites mL^{-1} in freezing medium (PYG containing 10% dimethylsulfoxide, DMSO) (John & John, 1994). Finally cultures are transferred to -20 °C for 24 h, followed

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by their storage at −70 °C or in liquid nitrogen indefinitely. *Acanthamoeba* cultures can be revived by thawing at 37 °C, followed by their immediate transfer to PYG medium in a T-75 flask at 30 °C. However, the inclusion of 20% fetal bovine serum in freezing medium has been shown to improve the revival viability of *Acanthamoeba* in long-term storage (John & John, 1994).

Classification of *Acanthamoeba*

Following the discovery of *Acanthamoeba*, several isolates belonging to the genus with distinct morphology were isolated and given different names based on the isolator, source or other criteria. In an attempt to organize the increasing number of isolates belonging to this genus, Pussard & Pons (1977) classified the genus based on morphological characteristics of the cysts, which were the most appropriate criteria at the time. The genus *Acanthamoeba* was classified into three groups based only on two obvious characters, i.e. cyst size and number of arms within a single cyst (Figs 3 and 4). Based on this scheme, Pussard & Pons (1977) divided the genus *Acanthamoeba* (18 species at the time) into three groups. Subsequently, the classification of Pussard and Pons gained acceptance (De Jonckheere, 1987; Page, 1988).

Group 1: Four species were placed in this group including *A. astronyxis*, *A. comandoni*, *A. echinulata* and *A. tubiashi*. These species exhibit large trophozoites, while in the cyst forms ectocyst and endocyst are widely separated and exhibit the following properties:

(1) fewer than six arms with average diameter of cysts ≥18 μm – *A. astronyxis*;.
(2) 6–10 arms and average diameter of cysts ≥25.6 μm – *A. comandoni*;
(3) 12–14 arms and average diameter of cysts ≥25 μm – *A. echinulata*;
(4) average diameter of cysts ≥22.6 μm – *A. tubiashi*.

Group 2: This group included 11 species, which are the most widespread and commonly isolated *Acanthamoeba*. Ectocyst and endocyst may be close together or widely separated. Ectocysts may be thick, thin and endocysts may be polygonal, triangular or round with a mean diameter of less than 18 μm. The species included in this group were *A. mauritaniensis*, *A. castellanii*, *A. polyphaga*, *A. quina*, *A. divionensis*, *A. triangularis*, *A. lugdunensis*, *A. griffini*, *A. rhysodes*, *A. paradivionensis* and *A. hatchetti*.

Group 3: Five species were included in this group, *A. palestinensis*, *A. culbertsoni*, *A. royreba*, *A. lenticulata* and *A. pustulosa*. Ectocysts in this group are thin and endocysts may have 3–5 gentle corners with the mean cyst diameter of <18 μm.

Later, *A. tubiashi* in group 1 and *A. hatchetti* in group 2 were added by Visvesvara (1991).

From above it is obvious that identification of the various species of *Acanthamoeba* based on morphological features alone is problematic. In addition, several studies have demonstrated inconsistencies and/or variations in cyst morphology of the same isolate/strain. For example, Sawyer (1971) discovered that the ionic strength of the growth medium could alter the shape of cyst walls, thus substantially reducing the reliability of cyst morphology as a taxonomic characteristic. Furthermore, this scheme had limited value in associating pathogenesis with a named species. For example, several studies demonstrated that strains/isolates within *A. castellanii* can be virulent, weakly virulent or avirulent. This discrepancy in assigning an unambiguous role to a given species presented a clear but urgent need to reclassify the genus. The discovery of advanced molecular techniques led to the pioneering work of the late Dr. T. Byers (Ohio University, USA) in the classification of the genus *Acanthamoeba* based on rRNA gene sequences. Because life evolved in the sea, most likely through self-replicating RNA as the genetic material or as a common ancestor and evolved into diverse forms, it is reasonable to study the evolutionary relationships through such molecules, i.e. rRNA. In addition, this is a highly precise, reliable and informative scheme. Each base presents a single character providing an accurate and diverse systematic. Based on rRNA sequences, the genus *Acanthamoeba* is divided into 15 different genotypes (T1–T15; Table 1) (Schuster & Visvesvara, 2004). Each genotype exhibits 5% or more sequence divergence between different genotypes. Note that in a recent study, Maghsood *et al.* (2005) proposed

Table 1. Known *Acanthamoeba* genotypes and their associations with human diseases, i.e. keratitis and granulomatous encephalitis

Acanthamoeba genotypes	Human disease association
T1	Encephalitis
†T2a	Keratitis
†T2b–ccap1501/3c-a-like sequences	NA
T3	Keratitis
T4*	Encephalitis, keratitis
T5	NA
T6	Keratitis
T7	NA
T8	NA
T9	NA
T10	Encephalitis
T11	Keratitis
T12	Encephalitis
T13	NA
T14	NA
T15	NA

*This genotype has been most associated with both diseases.
†Basis of T2 division into T2a and T2b has been proposed by Maghsood *et al.* (2005)
NA, no disease association has been found yet.

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to subdivide T2 into further two groups, i.e. T2a and T2b. This is due to a sequence dissimilarity of 4.9% between these two groups, which is very close to the current cut-off limit of 5% between different genotypes. This should help to differentiate pathogenic and nonpathogenic isolates within this genotype. With the clear advantage of rRNA gene sequences over morphology-based classification, an attempt is made to refer to the genotype rather than species name wherever possible in this review. Based on this classification scheme, the majority of human infections due to *Acanthamoeba* have been associated with the T4 genotype. For example, more than 90% of keratitis cases have been linked with this genotype. Similarly, T4 has been the major genotype associated with nonkeratitis infections such as AGE and cutaneous infections. Moreover, recent findings suggest that the abundance of T4 isolates in human infections is most likely due to their greater virulence and/or properties that enhance their transmissibility as well as their decreased susceptibility to chemotherapeutic agents (Maghsood *et al*., 2005). Future studies will identify virulence traits and genetic markers limited only to certain genotypes, which may help clarify these issues. A current list of genotypes and their association with human infections is presented in Table 1. With the increasing research interests in the field of *Acanthamoeba* and the worldwide availability of advanced molecular techniques, undoubtedly additional genotypes will be identified. These studies will help to clarify the role of *Acanthamoeba* in the ecosystem, bacterial symbiosis, as well as in causing primary and secondary human infections.

Human infections

Acanthamoeba cause two well-recognized diseases that are major problems in human health: a rare AGE involving the central nervous system (CNS) that is limited typically to immunocompromised patients and almost always results in death, and a painful keratitis that can result in blindness.

Acanthamoeba keratitis (from contact lens to cornea)

First discovered by Nagington *et al*. (1974) in the UK, *Acanthamoeba* keratitis has been recognized as a significant ocular microbial infection. A key predisposing factor in *Acanthamoeba* keratitis is the use of contact lenses exposed to contaminated water, but the precise mechanisms associated with this process are not fully understood. Overall this is a multifactorial process that involves (1) contact lens wear for extended periods of time, (2) lack of personal hygiene, (3) inappropriate cleaning of contact lenses, (4) biofilm formation on contact lenses and (5) exposure to contaminated water. For example, Beattie *et al*. (2003) have shown that *Acanthamoeba* exhibit higher binding to used contact lenses as compared with unworn contact lenses. Tests on used contact lenses showed the presence of saccharides including mannose, glucose, galactose, fucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, *N*-acetyl neuraminic acid (sialic acid), and proteins, glycoproteins, lipids, mucins, polysaccharides, calcium, iron, silica, magnesium, sodium, lactoferrin, lysozyme and immunoglobin (Ig) molecules (Tripathi & Tripathi, 1984; Gudmundsson *et al*., 1985; Klotz *et al*., 1987) on the surface of contact lenses after only 30 min of contact lenses being worn. These may act as receptors for *Acanthamoeba* trophozoites and/or enhance the ability of parasites to bind to contact lenses. For example, *Acanthamoeba* expresses a mannose binding protein (MBP) on its surface, which specifically binds to mannose residues. This may explain the ability of *Acanthamoeba* to exhibit higher binding to used rather than unworn contact lenses (Beattie *et al*., 2003). Alternatively, biofilm formation on contact lenses may provide increased affinity for *Acanthamoeba*. This is shown by increased *Acanthamoeba* binding to biofilm-coated lenses as opposed to contact lenses without biofilms (Simmons *et al*., 1998; Tomlinson *et al*., 2000; Beattie *et al*., 2003). In addition, biofilms may enhance *Acanthamoeba* persistence during contact lens storage/cleaning as well as providing nutrients for *Acanthamoeba*. Once an *Acanthamoeba*-contaminated lens is placed over the cornea, parasites invade the cornea. *Acanthamoeba* transmission onto the cornea is dependent on the virulence of *Acanthamoeba* (discussed below) and physiological status of the cornea. For example, several studies showed that corneal trauma is a prerequisite in *Acanthamoeba* keratitis *in vivo*, and animals with intact corneas (i.e. epithelial cells) do not develop this infection (Niederkorn *et al*., 1999). Nevertheless, corneal trauma followed by exposure to contaminated water, soil or other vector (inert objects or biological surfaces such as unclean hands) is sufficient, resulting in *Acanthamoeba* keratitis and is the most likely cause of *Acanthamoeba* keratitis in noncontact lens wearers (Sharma *et al*., 1990; Chang & Soong, 1991). The requirement of corneal trauma can be explained by the fact that the expression of *Acanthamoeba*-reactive glycoprotein(s) on damaged corneas is 1.8 times higher than on healthy corneas, suggesting that corneal injury contributes to *Acanthamoeba* infection (Jaison *et al*., 1998). Future studies will determine whether corneal injury simply exposes mannose-containing glycoprotein(s), providing additional binding sites for *Acanthamoeba*, or whether the expression of mannose-glycoprotein(s) is generally higher on the healing corneal epithelial cells. It is important to note that *Acanthamoeba* must be present in the trophozoite stage to bind to human corneal epithelial cells. Recent studies have shown that *Acanthamoeba* cysts do not bind to human corneal epithelial cells, indicating that cysts are a noninfective stage (Dudley *et al*., 2005; Garate *et al*., 2006).

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Epidemiology

Originally thought to be a rare infection, *Acanthamoeba* keratitis has become increasingly recognized as important in human health. This is due to increased awareness and the availability of diagnostic methods. Over the last few decades, it has become clear that contact lens users are at increased risk of corneal infections. For example, contact lens wearers are 80-fold more likely to contract corneal infection than those who do not (Dart *et al*., 1991; Alvord *et al*., 1998). The incidence rate of microbial keratitis in users of extended-wear contact lenses is determined at 20.9 per 10 000 wearers per annum in the USA (Poggio *et al*., 1989). Similar findings have been reported from Sweden (Nilsson & Montan, 1994), Scotland (Seal *et al*., 1999) and the Netherlands (Cheng *et al*., 1999). At present there are approximately 70 million people throughout the world wearing contact lenses (Barr, 1998) and, with their wider potential application beyond vision correction such as UV protection and cosmetic purposes, this number will undoubtedly rise. With an increasing number of people wearing contact lenses, it is important to assess any associated risks, and to make both existing and new users aware. Among other microbial agents, bacteria including *Pseudomonas* and *Staphylococcus* and protozoa including *Acanthamoeba* are the major causes of corneal infections in contact lens users (Giese & Weissman, 2002). The incidence rate of *Acanthamoeba* keratitis varies between different geographical locations. For example, in Hong Kong, an incidence rate of 0.33 per 10 000 contact lens wearers is reported, 0.05 per 10 000 in Holland, 0.01 per 10 000 in the USA (Stehr-Green *et al*., 1989), 0.19 per 10 000 in England (Radford *et al*., 2002) and 1.49 per 10 000 in Scotland (Seal *et al*., 1999; Lam *et al*., 2002). However, these variations do not reflect the geographical distribution of *Acanthamoeba*, and are most likely due to extended wear of soft contact lenses, lack of awareness of the potential risks associated with wearing contact lenses, enhanced detection and/or local conditions that promote growth of pathogenic amoebae only, e.g. water hardness or salinity.

Pathophysiology

The onset of symptoms can take from a few days to several weeks, depending on the inoculum size of *Acanthamoeba* and/or the extent of corneal trauma. During the course of infection, symptoms may vary depending on the clinical management of the disease. Most commonly *Acanthamoeba* keratitis is associated with considerable production of tears, epithelial defects and photophobia, which leads to inflammation with redness, stromal infiltration, oedema, stromal opacity together with excruciating pain due to radial neuritis (with suicidal pain), epithelial loss and stromal abscess formation with vision-threatening consequences (Fig. 4). Other symptoms may involve scattered subepithelial infiltrates, anterior uveitis, stromal perforation and the presence of scleral inflammation. Secondary infection due to bacteria may additionally complicate the clinical management of the disease. Glaucoma is commonly reported, and occasionally posterior segment signs such as nerve oedema, optic atrophy and retinal detachment are observed. In untreated eyes, blindness may eventually result as the necrotic region spreads inwards (Niederkorn *et al*., 1999).

Clinical diagnosis

The clinical diagnosis of *Acanthamoeba* keratitis includes both clinical syndromes and/or demonstration of the presence of amoebae (Martinez & Visvesvara, 1991). In the majority of cases, this infection is misdiagnosed as Herpes simplex virus or adenovirus infection. The clinical symptoms are indicated above, but the use of contact lenses by the patient, together with excruciating pain, is strongly indicative of *Acanthamoeba* keratitis. The confirmatory evidence comes from the isolation of *Acanthamoeba* from either the contact lens case or the corneal biopsy. To this end, several methods are available. For example, light microscopy has been used for rapid identification of *Acanthamoeba* on contact lenses, in lens case solution or in corneal biopsy specimens (Epstein *et al*., 1986). Winchester *et al*. (1995) demonstrated the use of noninvasive confocal microscopy to aid in the diagnosis of *Acanthamoeba* keratitis. Confocal microscopy has the advantage over conventional optical microscopy that it can image layers within the substance of a specimen of substantial thickness, so it is effective in imaging the cornea. Such microscopic identification based on morphological characteristics requires skill and the use of robust keys for identification. Examiners must have familiarity with the morphological characteristics of *Acanthamoeba* species otherwise diagnosis may require histological examination of material obtained by corneal biopsy or keratoplasty. In addition, PCR-based methods using the 18S rRNA gene have been developed for the rapid detection of *Acanthamoeba*. This method is highly specific and can detect fewer than five cells (Lehmann *et al*., 1998; Khan *et al*., 2001; Schroeder *et al*., 2001). Despite the development of microscopic and molecular-based approaches, cultivation of *Acanthamoeba* from corneal biopsy specimens or from contact lenses or lens cases remains the most widely used assay in the clinical settings because it is simple, inexpensive and there is no loss of cells during centrifugation/washing steps. In addition, this method provides large numbers of *Acanthamoeba*, which could be used for typing, sequencing, epidemiological studies or pathogenicity assays. Briefly, specimens (contact lenses or corneal biopsy specimens) are inoculated onto non-nutrient agar plates seeded with Gram-negative bacteria. Plates are incubated at 30 °C and observed daily for the presence of amoebae as described above (Khan

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& Paget, 2002). *Acanthamoeba* can be identified at the genus level, based on the morphological characteristics of trophozoites and cysts using phase-contrast microscope (Fig. 5) or PCR-based assays as described above.

Host susceptibility

Previous studies have demonstrated clearly the host specificity in *Acanthamoeba* keratitis. For example, successful *Acanthamoeba* keratitis models that mimic the human form of disease were only produced in pigs and Chinese hamsters but not in rats, mice or rabbits, suggesting that expression of specific molecular determinants may be limited to certain mammalian species (reviewed in Niederkorn *et al*., 1999). Even in susceptible species, corneal injury is a prerequisite for *Acanthamoeba* keratitis, and animals that have intact epithelial layers do not develop *Acanthamoeba* keratitis (Niederkorn *et al*., 1999). The importance of corneal injury is demonstrated further by reports that injury to the surface of the cornea, even with a splash of *Acanthamoeba*-contaminated water, can lead to *Acanthamoeba* keratitis in individuals who do not wear contact lenses (Sharma *et al*., 1990; Chang & Soong, 1991). It has been shown that the expression of *Acanthamoeba*-reactive glycoproteins(s) on surface-damaged corneal epithelial cells is significantly higher than on the surface of normal corneal epithelial cells, suggesting that corneal injury contributes markedly to *Acanthamoeba* keratitis (Jaison *et al*., 1998). In addition, some individuals may lack antiacanthamoebic defence determinants in tear film (discussed below) or exhibit corneal properties at both the surface and the molecular level, which could render the cornea more susceptible to *Acanthamoeba* keratitis.

Risk factors

As indicated above, the major risk factor for *Acanthamoeba* keratitis is poor hygiene in the use of contact lenses (Fig. 6). In support of this statement, more than 85% of *Acanthamoeba* keratitis cases occur in wearers of contact lenses. This is associated with individual behaviour. For example, *Acanthamoeba* keratitis has been associated frequently with young males (Niederkorn *et al*., 1999), which could be due to their poor personal hygiene, poor handling and care of their lenses or lens storage cases, and noncompliance with disinfection procedures such as using home-made saline (Brennan, 2002). Contact lenses that have been scratched or fragmented through mishandling should not be used. Additional factors are swimming or washing eyes while wearing contact lenses, and the use of chlorine-based disinfectants for contact lens cleaning because *Acanthamoeba* are highly resistant to chlorine (Radford *et al*., 1995, 1998; Seal *et al*., 1999). In addition, *Acanthamoeba* exhibit significantly higher binding to silicone hydrogel contact lenses than to the conventional hydrogel contact lenses (Beattie *et al*., 2003),

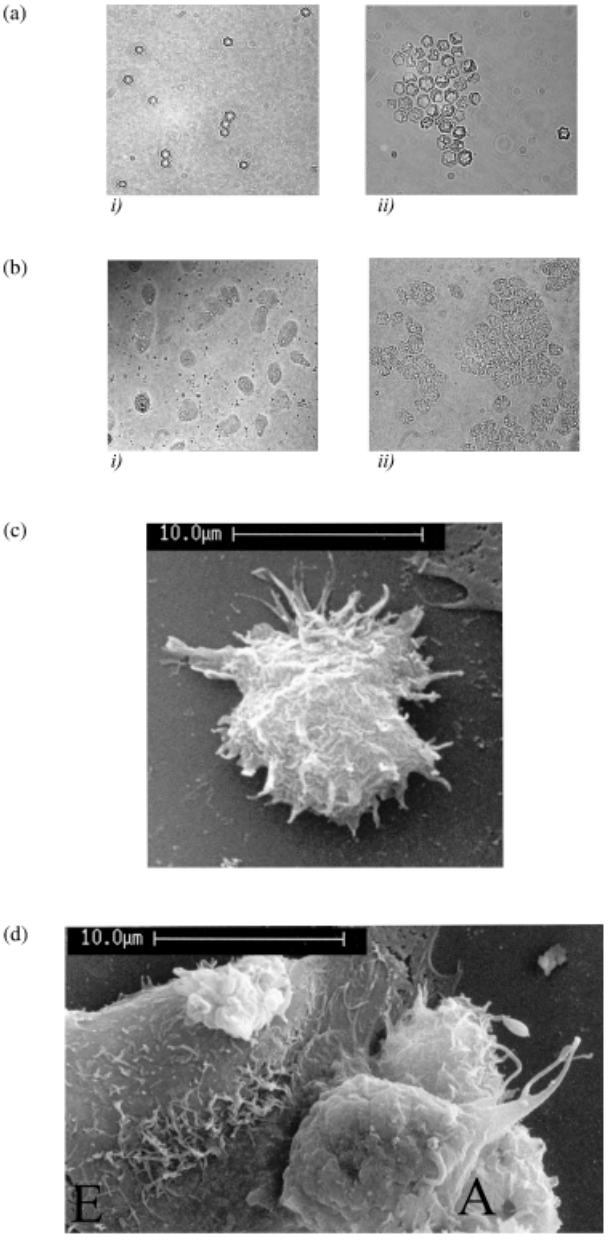


Fig. 5. (a) *Acanthamoeba* cysts under phase-contrast microscope. (i) Non-nutrient agar plates exhibiting *Acanthamoeba* cysts. (ii) *Acanthamoeba* cysts were collected from non-nutrient agar plates using PBS and observed under the phase-contrast microscope. Note cysts formed clusters in PBS; ×400. (b) *Acanthamoeba* trophozoites on non-nutrient agar plates observed under phase-contrast microscope. Note the characteristic contract vacuole in *Acanthamoeba* trophozoites; ×400. (c) *Acanthamoeba* trophozoite binding to glass cover slips observed under scanning electron microscope. Note the large number of acanthopodia on the surface of *A. castellanii* trophozoites belonging to T4 genotype. (d) *Acanthamoeba* binding to corneal epithelial cells. *Acanthamoeba castellanii* (T4 isolate) were incubated with corneal epithelial cells, followed by several washes and observed under scanning electron microscope. Note that parasites were able to exhibit binding to the host cells and binding was mediated by acanthopodia. A, amoeba, E, corneal epithelial cell. Scale bar = 10 μm.

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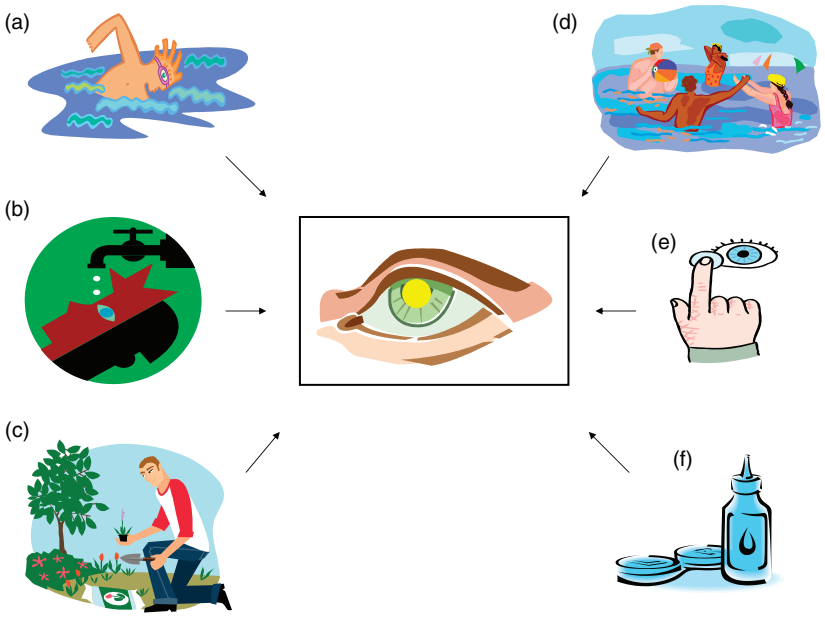


Fig. 6. The risk factors contributing to *Acanthamoeba* keratitis: (a) swimming, especially while wearing contact lenses; (b) washing eyes during or immediately after contact lens wear; (c) working with soil and rubbing eyes; (d) water-related activities (splashing water), especially during or immediately after contact lens wear; (e) handling contact lenses without proper hand washing; (f) use of home-made saline (or even chlorine-based disinfectants) for contact lens cleaning.

suggesting that polymer characteristics of the lens or surface treatment procedures may increase the risk of *Acanthamoeba* keratitis. Thus, extended wear of lenses without proper maintenance and recommended replacement, together with lens type, can be important risk factors for *Acanthamoeba* keratitis.

Overall, these characteristics suggest that although the intact cornea is highly resistant to *Acanthamoeba* infection, corneal trauma (microscopic defects) followed by exposure to contaminated water (during swimming, eye washing, water splash), dust, vegetable matter or any foreign particle are important risk factors associated with *Acanthamoeba* keratitis. Because *Acanthamoeba* is ubiquitously present in water, air, at the water–air interface and soil (Preston *et al.*, 2001; Khan, 2003; Marciano-Cabral & Cabral, 2003), susceptible hosts should be warned of the risks associated with the wearing of contact lenses while swimming or bathing/washing, cleaning lenses with home-made saline, etc. Proper cleaning of contact lenses is crucial in preventing this devastating infection. Chlorine-based cleaning solutions should not be used because *Acanthamoeba* are highly resistant to chlorine, but a two-step hydrogen peroxide system at a concentration of 3% is highly effective against cysts and trophozoites (Beattie *et al.*, 2002). In addition, strategies and/or control measures to reduce the formation of biofilms and the build-up of carbohydrate moieties on contact lenses should help to prevent infection (Table 2).

Treatment

Acanthamoeba keratitis is a difficult infection to treat. Early diagnosis followed by aggressive treatment is essential for a successful prognosis (Perez-Santonja *et al.*, 2003). The recommended treatment regimen includes a biguanide (0.02 % polyhexamethylene biguanide, PHMB, or 0.02% chlorhexidine digluconate, CHX) together with a diamidine (0.1% propamidine isethionate, also known as Brolene or 0.1% hexamidine, also known as Desomedine). If bacteria are also associated and/or suspected with the infection, addition use of antibiotics, i.e. neomycin or chloramphenicol, is recommended. The initial treatment involves hourly topical application of drugs day and night for 2–3 days, followed by hourly topical application during the day for a further 3–4 days. Subsequently, application is reduced to 2-hourly application during the day for up to a month. This is followed by application six times a day for the next several months for up to a year, clearly presenting appreciable social and economic burdens resulting from this infection. Persistent inflammation and severe pain may be managed by topical application of steroids such as 0.1% dexamethasone together with pain killers. However, it should be noted that dexamethasone causes a significant increase in the proliferation of amoebae numbers and increases severity in *Acanthamoeba* keratitis in an *in vivo* model (McClellan *et al.*, 2001). Thus, prolonged application of steroids should be carried out with care. This aggressive, complicated and prolonged management is required because of the ability of *Acanthamoeba* to adapt rapidly to harsh conditions and to switch to the resistant cyst form, and because of the lack of available methods for the targeted killing of both trophozoites and cysts. The presence of antibiotics (neomycin or chloramphenicol) limits possible bacterial infection or, at the very least, eliminates the food source for *Acanthamoeba*. As a last resort a keratoplasty may be indicated, especially in drug-

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Table 2. Risk factors associated with *Acanthamoeba* infections

No.	Risk factors associated with *Acanthamoeba* keratitis
1	Handling of contact lenses (CL) with unclean hands
2	Washing CL with home-made saline/tap water
3	CL wear for more than recommended times
4	CL wear during swimming
5	Washing eyes and/or swimming with corneal trauma – splashing eyes with contaminated water
6	Reusing CL without proper cleaning
7	Incubating CL in disinfectants for less than recommended times
8	Chlorine-based disinfectants are less effective in killing *Acanthamoeba*
No.	Risk factors associated with *Acanthamoeba* encephalitis
1	HIV/AIDS patient activities that may result in skin cut/bruises followed by exposure to contaminated soil/water
2	Individuals with lymphoproliferative disorders, haematological disorders, diabetes mellitus, pneumonitis, renal failure, liver cirrhosis, rhinitis, pharyngitis, gammalobulinaemia, pregnancy, systemic lupus erythematosus, glucose 6-phosphate deficiency, tuberculosis are at risk
3	Alcohol misuse
4	Organ/tissue transplantation with immunosuppressive therapy
5	Excessive use of steroids or antibiotics

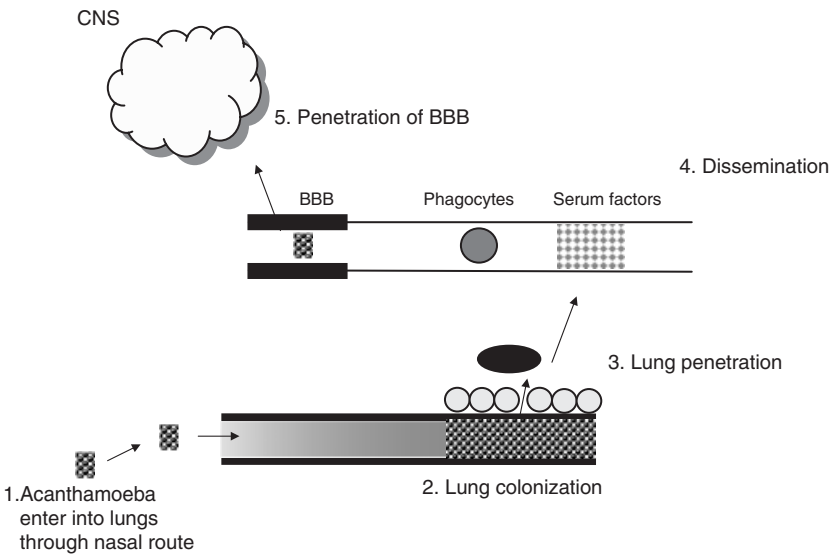


Fig. 7. The model of *Acanthamoeba* granulomatous encephalitis. *Acanthamoeba* are thought to enter lungs via the nasal route. Next, amoebae traverse the lungs into the bloodstream, followed by haematogenous spread. Finally, *Acanthamoeba* cross the blood–brain barrier and enter into the central nervous system (CNS) to produce disease. It is noteworthy that *Acanthamoeba* may bypass the lower respiratory tract and directly enter into the bloodstream via skin lesions. The olfactory neuroepithelium may provide an alternative route of entry into the CNS.

resistant cases. In the case of penetrating keratoplasty to achieve rehabilitation from corneal scaring, topical treatment with the above is essential as a first measure. Rejection can occur but is rare. Recurrence of infection does occur even though it is recommended that topical treatment continues for up to a year postoperatively as cysts may survive in the acceptor cornea.

AGE (from the environment to the CNS)

AGE is a rare infection but almost always proves fatal. The mechanisms associated with its pathogenesis are unclear, but the pathophysiological complications involving the CNS most likely include induction of the proinflammatory responses, invasion of the blood–brain barrier and the connective tissue, and neuronal damage leading to brain dysfunction. Routes of entry include the lower respiratory tract, leading to amoebae invasion of the intravascular space, followed by haematogenous spread. Skin lesions may provide direct amoebae entry into the bloodstream, thus bypassing the lower respiratory tract (Fig. 7). Amoebae entry into the CNS most likely occurs at the sites of the blood–brain barrier (Martinez, 1985, 1991). The cutaneous and respiratory infections can last for months but the involvement of the CNS can result in fatal consequences within days or weeks. In addition, olfactory neuroepithelium provides another route of entry into the CNS and has been studied in experimental models (Martinez, 1991; Martinez & Visvesvara, 1997) (Fig. 7).

Epidemiology

The epidemiology of AGE is rather confusing. The fact that this infection is normally secondary makes diagnosis

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difficult and thus contributes to our inability to assess the actual number of AGE infections. The number of AGE cases in HIV patients, although not completely accurate, may indicate the real burden of this infection. This has only been made possible by the pioneering work of G. S. Visvesvara (CDC, USA) and the late Dr. A. J. Martinez (University of Pittsburgh School of Medicine, USA). In the USA, there were approximately 350 000 deaths due to HIV/AIDS during 1981–1996 with the highest mortality during the mid 1990s: 49 000 in 1994 and 50 000 in 1995, which declined to 39 000 in 1996 (Heath *et al*., 1998; Center for Disease Control, http://www.cdc.gov). Over a similar period, the number of AGE deaths in HIV/AIDS patients was approximately 55 (Martinez & Visvesvara, 1997). Thus, the approximate rate can be calculated as 1.57 AGE deaths per 10 000 HIV/AIDS deaths in the USA, even though the number of AGE infections may be much higher in countries with warmer climates due to increased ubiquity and/or increased outdoor activities. At present, the estimated worldwide number of HIV/AIDS patients is a massive 40–45 million (as of 2005) and continues to rise sharply. Hypothetically, this figure represents the number of AGE-susceptible hosts. If this is so, why are there not a large number of AGE infections? There could be several explanations. At least for the USA, the number of deaths due to HIV/AIDS has been declining since the late 1990s, i.e. 22 000 deaths in 1997 and 18 000 deaths in 2003 in the USA (Heath *et al*., 1998; CDC), thus reducing the number of AGE-susceptible hosts. This decline in HIV/AIDS deaths in the USA is attributed to early diagnosis followed by the introduction of novel antiretroviral therapies, i.e. highly active antiretroviral therapy (HAART), which was first introduced in 1996. As well as improving AIDS symptoms, HAART has protective effects against *Acanthamoeba* and other opportunistic pathogens (Seijo Martinez *et al*., 2000; Carter *et al*., 2004; Pozio & Morales, 2005). However, these therapies are not available to the majority of HIV/AIDS patients in less developed or developing countries in other parts of the world. Thus, the approximate rate of 1.57 AGE infections per 10 000 HIV/AIDS deaths in such countries may provide only a minimum estimate of the burden of AGE infections. The fact that AGE cases are not being reported in developing countries (especially in Africa) is due to a lack of expertise, reporting problems, lack of proper monitoring and the lack of proper healthcare systems.

Of interest, there were five million new reported cases of HIV/AIDS in 2003 alone (approximately 14 000 infections per day), while 3 million deaths occurred due to HIV/AIDS-related diseases (approximately 8500 deaths per day), mostly in Africa (even though there has not been a single reported case of AGE in Africa). And applying 1.57 AGE deaths per 10 000 HIV/AIDS deaths, the total number of AGE infections in 2003 can be estimated at approximately 471. Although this number is significantly less than the three million deaths in total, AGE is certainly a contributing factor in AIDS-related deaths, and needs continued attention. In addition, other infections such as diabetes, malignancies, malnutrition, alcoholism or a compromised immune system due to immunosuppressive therapy or other complications may all contribute to AGE infections.

Pathophysiology of AGE

The clinical symptoms may resemble viral, bacterial or tuberculosis meningitis: headache, fever, behavioural changes, hemiparesis, lethargy, stiff neck, aphasia, ataxia, vomiting, nausea, cranial nerve palsies, increased intracranial pressure, seizures and death. These are due to haemorrhagic necrotizing lesions with severe meningeal irritation and encephalitis (Martinez, 1985, 1991). Patients with respiratory infections, skin ulcerations or brain abscesses should be strongly suspected for infections due to free-living amoebae. Post-mortem examination often shows severe oedema and haemorrhagic necrosis. It is not known whether this necrotic phase is caused by actively feeding trophozoites or inflammatory processes such as the release of cytokines. The lesions due to AGE are most numerous in the basal ganglia, midbrain, brainstem and cerebral hemiparesis, with characteristic lesions in the CNS parenchyma resulting in chronic granulomatous encephalitis. A granulomatous response may be absent or minimal in patients with a severely impaired immune system, which is interpreted as impairment of the cellular immune response (Martinez *et al*., 2000). The affected tissues other than the CNS may include subcutaneous tissue, skin, liver, lungs, kidneys, adrenals, pancreas, prostate, lymph nodes and bone marrow.

Diagnosis of AGE

Because of the rarity of the disease and complicated symptoms, which are common to other pathogens causing CNS infections, the diagnosis of AGE is problematical. The symptoms are similar to other CNS pathogens including virus, bacteria and fungi. This makes diagnosis of AGE problematic and requires a high underlying suspicion that it is the cause, which requires expertise. Brain image analyses using computed tomography (CT) or magnetic resonance imaging (MRI) scans may show multifocal areas of signal intensities or lesions, indicating brain abscess or tumours suggestive of CNS defects. CSF findings, although not confirmatory of AGE, are of value in diagnosing CNS involvement. Pleocytosis with lymphocytic predominance is an important characteristic with elevated numbers of polymorphonuclear leukocytes, increased protein concentrations, decreased glucose concentrations and minimal cloudiness (Marciano-Cabral & Cabral, 2003). The absence of viral and bacterial pathogens should be strongly suspected of AGE. Due to the

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low density of parasites, the detection of a host immune response should be of primary importance. The demonstration of high levels of *Acanthamoeba*-specific antibodies in the patient's serum may provide a useful and straightforward method for suspected AGE infection. This is performed using indirect immunofluorescence (IIF) assays. Serial dilutions of the patient's serum are incubated with fixed amoebae-coated slides (preferably T1, T4, T12 isolates as they have been shown to cause AGE infections), followed by incubation with fluorescein isothiocyanate (FITC)-labelled antihuman antibody and visualization under a fluorescence microscope. It is important to remember that the levels of anti-*Acanthamoeba* antibodies in normal populations may be in the range 1 : 20–1 : 60 (Cursons *et al.*, 1980; Cerva, 1989). However, patients with a severely impaired immune system may not develop a high titre, and thus other clinical findings should be taken into account for correct diagnosis.

Confirmatory evidence comes from direct microscopic observation of amoebae in the CSF (after centrifugation at low speed) or in the brain biopsy but requires familiarity of morphological characters. Giemsa-Wright, acridine orange or calcofluor white staining may facilitate morphologically based positive identification of these amoebae. The lack of familiarity with morphological characteristics of amoebae may require immunohistochemical studies using antisera made against *Acanthamoeba* to identify the aetiological agent, which should aid in the clinical diagnosis of AGE. In addition, it is helpful to inoculate a few drops of the CSF and/or brain biopsy for amoebae culturing as described previously. *Acanthamoeba* feed on bacteria as a food source, and depending on the number of amoebae in the specimen, trophozoites can be observed within a few hours (up to 12 h). However, in the absence of amoebae, plates should be monitored for up to 7 days (Khan & Paget, 2002; Khan *et al.*, 2002). This method is particularly useful if problems are encountered in differentiating *Acanthamoeba* from monocytes, polymorphonuclear leukocytes and macrophages. As discussed, PCR-based methods have been developed but microscopy and plating-based analysis remain the methods of choice.

Host susceptibility

AGE is a rare disease that occurs mostly in immunocompromised or debilitated patients due to HIV infection, diabetes, immunosuppressive therapy, malignancies, malnutrition and alcoholism, and usually occurs as a secondary infection. This is due to the inability of *Acanthamoeba* to evade the immune system of immunocompetent individuals. Indeed, healthy human serum exhibits amoebacidal activities by activating the alternative complement pathway. Of interest, protozoan parasites with the ability to evade the host immune system possess sialic acid on the surface of their plasma membranes blocking alternative pathway convertase or by expressing a special coat or capsule. For example, expression of variable surface glycoprotein (VSG) on the surface of African *Trypanosoma* cover underlying components of the plasma membrane, thus preventing activation of the alternative complement pathway (Ferrante & Allison, 1983). However, the plasma membrane of *Acanthamoeba* lacks sialic acid (Korn & Olivecrona, 1971) or any protective coat or capsule (Bowers & Korn, 1968) and thus the amoebae are exposed to complement-mediated attack in an antibody-independent pathway (Ferrante & Rowan-Kelly, 1983). In addition, the presence of anti-*Acanthamoeba* antibodies in normal populations provides additional protection against these opportunistic pathogens (Cursons *et al.*, 1980). Overall, complement pathways and the antibodies together with neutrophils and macrophages show potent amoebalytic activities, thus suppressing infection. The conclusion from these findings is that a debilitated immune status of the host is usually a prerequisite in AGE, but the core basis of host susceptibility in contracting AGE requires further study as it may involve other factors such as host ethnicity (i.e. genetic basis of the host) or the inability of the host to induce a specific immune response against these pathogens. Interestingly, in a study by Chappell *et al.* (2001), Hispanic subjects were 14.5 times less likely to be seropositive against a T4 isolate than Caucasians. But whether Hispanics may be more susceptible to *Acanthamoeba* (T4 genotype) infections remains to be determined. Future studies will identify the precise host factors that play an important role in controlling this fatal infection, and may help develop therapeutic interventions for susceptible hosts.

Risk factors

AGE is normally a secondary infection to other primary diseases. Almost all reported cases have occurred in immunocompromised patients due to HIV (AIDS patients), and/or in individuals with lymphoproliferative disorders, haematological disorders, diabetes mellitus, pneumonitis, renal failure, liver cirrhosis, rhinitis, pharyngitis, gammaglobulinaemia, systemic lupus erythematosus, glucose 6-phosphate deficiency, tuberculosis and chronic alcoholism, pregnant women, malnourished individuals, those with chronic illness or otherwise debilitated or those undergoing radiotherapy. Patients undergoing organ/tissue transplantation with immunosuppressive therapy, steroids and excessive antibiotics are also at risk (Table 2). The risk factors for patients suffering from the above diseases include exposure to contaminated water such as in swimming pools, on beaches, or in garden soil.

Treatment

For AGE, there are no recommended treatments and the majority of cases due to AGE are identified post-mortem.

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This is due to low sensitivity of *Acanthamoeba* to many antiamoebic agents but more importantly the inability of these compounds to cross the blood–brain barrier into the CNS. Current therapeutic agents include a combination of ketoconazole, fluconazole, sulfadiazine, pentamidine isethionate, amphotericin B, azithromycin, itraconazole or rifampin that may be effective against CNS infections due to free-living amoebae, but have severe side-effects. Recent studies have suggested that alkylphosphocholine compounds, such as hexadecylphosphocholine, exhibit anti-*Acanthamoeba* properties as well as the ability to cross the blood–brain barrier and may thus have value in the treatment of AGE (Kotting *et al.*, 1992; Walochnik *et al.*, 2002). Further studies are needed to determine their precise mode of action on *Acanthamoeba*, to develop methods of application and, more importantly, to assess the success of these compounds *in vivo*. Even with treatment, survivors may develop disability such as hearing loss and vision impairment.

Cutaneous acanthamebiasis

Other infections due to *Acanthamoeba* involve nasopharyngeal and, more commonly, cutaneous infections. The cutaneous infections are characterized by nodules and skin ulcerations and demonstrate *Acanthamoeba* trophozoites and cysts. In healthy individuals, these infections are very rare and are self-limiting. However, in immunocompromised patients, this provides a route of entry into the bloodstream, followed by the haematogenous spread to different tissues, which may lead to fatal consequences. Involvement of the CNS leads to death within weeks (Torno *et al.*, 2000). Both AGE and cutaneous infections can occur in combination or independent of each other. The direct demonstration of amoebae in biopsy using calcofluor staining, IIF or PCR-based assays, or isolation of amoebae from the clinical specimen using plating assays, provides positive diagnosis as described above. There is no recommended treatment, but topical application of itraconazole, 5-fluorocytosine, ketoconazole and chlorohexidine may be of value.

Pathogenesis

As indicated above, the ability of *Acanthamoeba* to produce *Acanthamoeba* keratitis is not related merely to their exposure to the eye but due to the virulence and tissue specificities. For example, Gray *et al.* (1995) tested the storage cases of 101 asymptomatic daily contact lens wearers for the presence of microbes and found that 81% were contaminated with bacteria, fungi and protozoa including *Acanthamoeba*. The occurrence of fungi was higher than protozoa, 24 vs. 20% respectively, and significantly higher than *Acanthamoeba*, 24 vs. 8%, respectively. Even though fungi rarely cause corneal infections in wearers of contact lenses, this provides further supporting evidence for the proposition that keratitis-causing microbes possess specific virulence properties enabling them to become potential ocular pathogens. Of interest, 75% of individuals in this study used hydrogen peroxide as a disinfectant for their lenses, indicating the ability of microbes to resist and survive the many available disinfection methods for cleaning contact lenses. Indeed, the organisms identified as contaminants of the cases exhibit catalase activity, an enzyme that breaks down hydrogen peroxide to oxygen and water (Gray *et al.*, 1995). The pathogenesis of *Acanthamoeba* is highly complex and involves several determinants working in concert to produce disease. The sequence of events, at least for *Acanthamoeba* keratitis, involves breaching of the surface epithelium, keratocyte depletion by *Acanthamoeba*, stromal necrosis and induction of an intense inflammatory response (Garner, 1993; Vemuganti *et al.*, 2004). For simplicity, in the following account these factors are described separately as contact-dependent and contact-independent mechanisms (Fig. 8).

Contact-dependent factors

The ability of the amoebae to bind to host cells is the first crucial step in the pathogenesis of *Acanthamoeba* infections. This leads to secondary events such as interference with host intracellular signalling pathways, toxin secretions and the ability to phagocytose host cells, ultimately leading to cell death.

MBP

Morton *et al.* (1991) showed that binding of *Acanthamoeba* to corneal epithelial cells of rabbit is mediated by amoeba adhesin, i.e. MBP, expressed on the surface of the parasite. The role of the MBP was subsequently established with the discovery that it is important in parasite binding to various cell types including rabbit corneal epithelial cells (Morton *et al.*, 1991; Yang *et al.*, 1997), pig corneal epithelium (van Klink *et al.*, 1992), Chinese hamster corneal epithelium (van Klink *et al.*, 1993), human corneal fibroblasts (Badenoch *et al.*, 1994), rat microglial cells (Shin *et al.*, 2001) and human corneal epithelial cells (Sissons *et al.*, 2004a). Alsam *et al.* (2003) extended these to include human brain microvascular endothelial cells, suggesting a possible role of MBP in AGE. It is not clear whether there are other more specific mechanisms of amoebic binding to host cells, but at least the initial binding seems to be dependent on the expression of MBP and its binding to mannose-containing glycoproteins on the surface of the host cell. Indeed, amoebae will bind even to mannose-coated tissue culture plates (Yang *et al.*, 1997). The significance of MBP is further shown by the observation that it is expressed only during the infective

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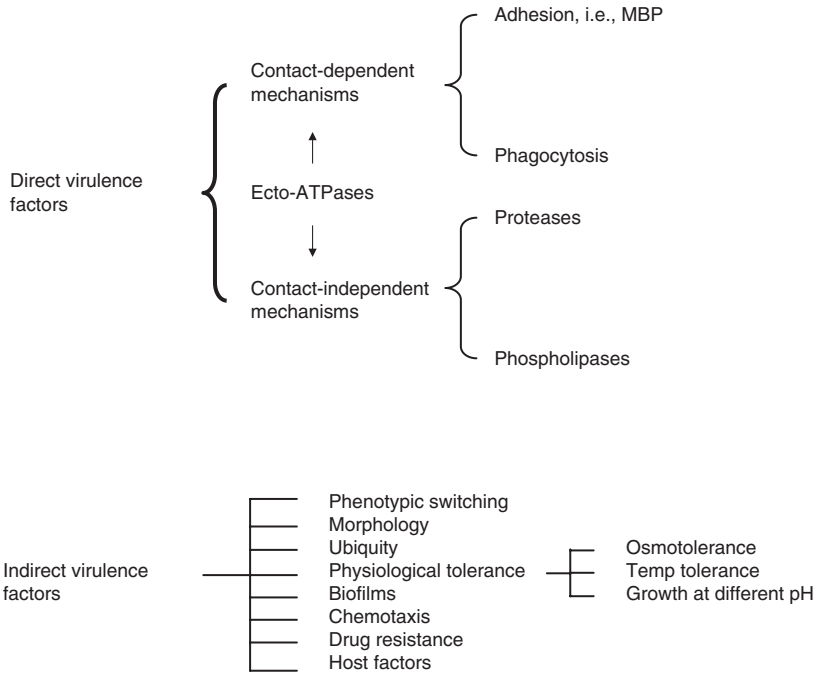


Fig. 8. Direct and indirect virulence factors that contribute to *Acanthamoeba* infections.

trophozoite stage of *Acanthamoeba* and that cysts lack MBP, and therefore cannot bind to the host cells (Dudley *et al*., 2005; Garate *et al*., 2006). Garate *et al*. (2004) have identified an *mbp* gene in *Acanthamoeba* containing 6 exons and 5 introns that spans 3.6 kb. The 2.5-kb cDNA codes for an 833-amino acid (aa) precursor protein with a signal sequence (residues 1–21 aa), an N-terminal extracellular domain (residues 22–733 aa) with five *N*- and three *O*-glycosylation sites, a transmembrane domain (residues 734–755 aa), and a C-terminal intracellular domain (residues 756–833 aa) (Garate *et al*., 2004). An understanding of the precise events in MBP binding to mannose-containing glycoprotein and/or additional *Acanthamoeba* determinants secondary to MBP should be a focus for future studies. Of interest in this context is a recently identified laminin-binding protein from *Acanthamoeba* (Hong *et al*., 2004): laminin is a major mannosylated glycoprotein constituent of the extracellular matrix (ECM) and the basement membrane of the host cells.

Host intracellular signalling in response to *Acanthamoeba*

The initial binding of *Acanthamoeba* to the surface of host cells interferes with the host intracellular signalling pathways. Several studies have shown that *Acanthamoeba* induces apoptosis in host cells (Alizadeh *et al*., 1994; Sissons *et al*., 2005a). Apoptosis, or programmed cell death, is known to be dependent on the host cell's own signalling pathways, involving Ca^{2+} responses (Taylor *et al*., 1995). Previous studies have shown that increases in cytosolic levels of Ca^{2+} in response to *Acanthamoeba* metabolites are dependent on transmembrane influx of extracellular Ca^{2+} (Mattana *et al*., 1997). Among other roles, the changes in the levels of intracellular Ca^{2+} exert effects on the cytoskeletal structure, induce morphological changes, or alter the permeability of the plasma membrane, finally leading to target cell death within minutes. An understanding of the complex intracellular signalling pathways is crucial to identify targets for therapeutic interventions. There are more than 10 000 signalling molecules in a single host cell at any one time, so the identification of key molecules and how they interact in response to *Acanthamoeba* leading to a functional outcome is clearly a challenge. However, it most likely involves events both at the transcriptional and the post-translational level. It is well established that proteins that regulate cell fate require tyrosine as well as serine/threonine phosphorylations for intracellular signalling. To this end, recent studies have shown that *Acanthamoeba* upregulates or downregulates the expression of a number of genes important for regulating the cell cycle (Sissons *et al*., 2004a). Overall, it is shown that *Acanthamoeba* upregulates the expression of genes such as GADD45A and p130 Rb, associated with cell cycle arrest, as well as inhibiting the expression of other genes, such as those for cyclins F, G1 and cyclin-dependent kinase-6 that encode proteins important for cell cycle progression. The overall response of these events is shown to be arrest of the host cell cycle (Fig. 9). This is further supported by the dephosphorylation of retinoblastoma protein (pRb). In the unphosphorylated form, pRb remains

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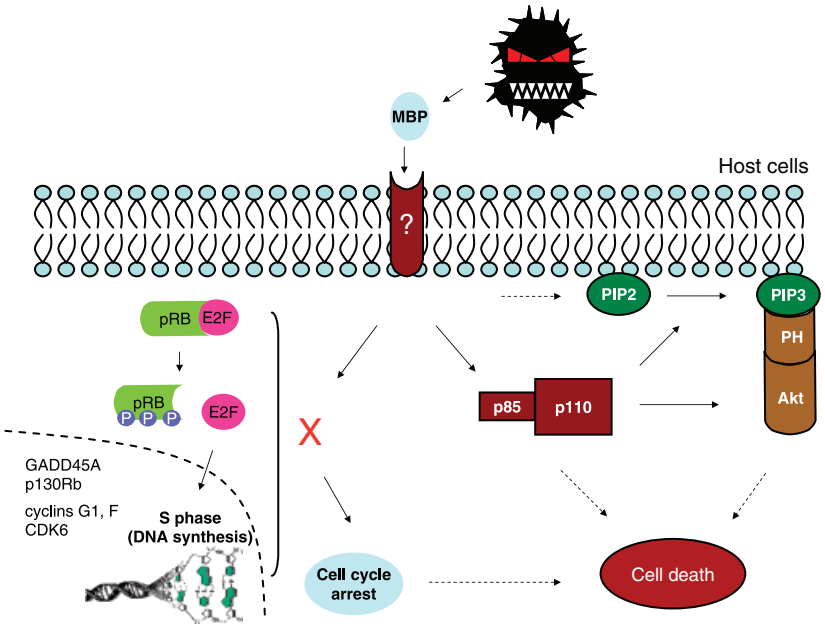


Fig. 9. Host intracellular signalling in response to *Acanthamoeba*. Note that *Acanthamoeba* induce cell cycle arrest in the host cells by (i) altering expression of genes as well as by (ii) modulating retinoblastoma protein phosphorylations. *Acanthamoeba* have also been shown to induce host cell death via phosphatidylinositol 3-kinase.

bound to E2F transcription factors (required for DNA synthesis) in the cytoplasm and inhibits E2F translocation into the nucleus. However, when phosphorylated by cyclin-dependent kinases (CDKs), pRb undergoes a conformational change resulting in E2F–pRb complex dissociation. The released E2F translocates into the nucleus and initiates DNA synthesis for the S-phase (Fig. 9). Thus, pRb is a potent inhibitor of G1/S cell cycle progression. Recent studies have shown that *Acanthamoeba* inhibits pRb phosphorylations in human corneal epithelial cells as well as in human brain microvascular endothelial cells, indicating that amoebae induce cell cycle arrest in host cells. Other studies have shown that *Acanthamoeba* induces apoptosis in host cells, but whether these events are independent of each other, or whether cell cycle arrest is a primary event that leads subsequently to apoptosis, is not clear. However, recent studies have shown that *Acanthamoeba*-mediated host cell death is dependent on activation of phosphatidylinositol 3-kinase (PI3 K) (Fig. 9). This was confirmed using LY294002, a specific PI3 K inhibitor as well as using host cells expressing mutant p85, i.e. a regulatory subunit of PI3 K (dominant negative PI3 K). PI3 K has been known traditionally to be important for cell survival pathways, so these results are not surprising. For example, Thyrell *et al.* (2004) have shown that interferon-alpha (IFNα) induces PI3K-mediated apoptosis in myeloma cells without Akt phosphorylations. It was further shown that downstream effectors of PI3K-mediated apoptosis involve activation of proapoptotic molecules, Bak and Bax, loss of mitochondrial membrane potential and release of cytochrome *c*: all well-known mediators of apoptosis. Similar mechanisms may exist in *Acanthamoeba*-mediated host cell death.

Phagocytosis

Adhesion of *Acanthamoeba* to the host cells leads to secondary processes such as phagocytosis or secretion of toxins (discussed later). Phagocytosis is an actin-dependent process involving polymerization of monomeric G-actin into filamentous F-actin. The ecological significance of *Acanthamoeba* phagocytosis is in the uptake of food particles such as bacteria, plasmids or fungal cells. The ability of *Acanthamoeba* to form food cups, or amoebastomes during incubations with host cells suggests they have a role in *Acanthamoeba* pathogenesis (Pettit *et al.*, 1996; Khan, 2001). In support of this, it is has been shown that cytochalasin D (a toxin that blocks actin polymerization) inhibits *Acanthamoeba*-mediated host cell death, confirming that actin-mediated cytoskeletal rearrangements play an important role in *Acanthamoeba* phagocytosis (Taylor *et al.*, 1995; Niederkorn *et al.*, 1999). The ability of *Acanthamoeba* to phagocytose is an intracellular signalling-dependent process. Genistein (a protein tyrosine kinase inhibitor) inhibits while sodium orthovanadate (protein tyrosine phosphatase inhibitor) enhances *Acanthamoeba* phagocytosis, indicating that a tyrosine kinase-induced actin polymerization signal is important in *Acanthamoeba* phagocytosis (Alsam *et al.*, 2005a). Rho GTPases are the major regulators of the actin cytoskeleton, which link external signals to the cytoskeleton (Mackay & Hall, 1998). As their name indicates, Rho GTPases bind and hydrolyse GTP and, in the process, stimulate pathways that induce specific cytoskeletal rearrangements resulting in distinct phenotypes (Mounier *et al.*, 1999). There are three well-studied pathways: the RhoA pathway, leading to stress fibre formation; Rac1

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activation, triggering lamellipodia formation; and Cdc42 activation, promoting filopodia formation (Mackay & Hall, 1998). Recent studies have shown that the Rho kinase inhibitor, Y27632, partially blocks *Acanthamoeba* phagocytosis. Y27632 blocks stress fibre formation by inhibiting myosin light chain phosphorylation and cofilin phosphorylations but is independent of profilin pathway. Overall, these findings suggested that, in addition to the RhoA pathway, the Rac1 and Cdc42 pathways may also be involved in *Acanthamoeba* phagocytosis. This is further supported with the finding that LY294002, a specific inhibitor of PI3K, inhibits *Acanthamoeba* phagocytosis. PI3K is shown to be involved in Rac1-dependent lamellipodia formation (Wennström *et al.*, 1994) and Cdc42-dependent cytoskeletal changes (Jimenez *et al.*, 2000). Future research into the identification of additional molecules/pathways, how various intracellular signalling pathways interact and/or whether they are independent of each other will enhance our understanding of *Acanthamoeba* phagocytosis, and should be of value in the development of therapeutic interventions. Of interest, mannose but not other saccharides, inhibits *Acanthamoeba* phagocytosis, indicating that *Acanthamoeba* phagocytosis is a receptor-dependent process, and *Acanthamoeba* adhesin (or MBP) is involved in its ability to phagocytose (Allen & Dawidowicz, 1990; Alsam *et al.*, 2005a). Overall, these findings suggest that MBP is crucial in *Acanthamoeba* binding to the target cells as well as its phagocytotic processes.

Ecto-ATPases

Ecto-ATPases are glycoproteins present in plasma membranes that have their active sites facing the external medium rather than the cytoplasm. Ecto-ATPases hydrolyse extracellular ATP and other nucleoside triphosphates (Sissons *et al.*, 2004b). The resultant ADP can have toxic effects on the host cells. For example, Mattana *et al.* (2002) have shown that ADP released by *Acanthamoeba* binds to $P2y_2$ purinergic receptors on the host cells, causing an increase in intracellular Ca^{2+}, inducing caspase-3 activation and finally resulting in apoptosis. A P2 receptor antagonist, suramin, inhibits *Acanthamoeba*-mediated host cell death (Mattana *et al.*, 2002; Sissons *et al.*, 2004b), suggesting that ecto-ATPases play an important role in *Acanthamoeba* pathogenesis, in this case by a contact-independent mechanism. Furthermore, clinical isolates exhibit higher ecto-ATPase activities compared with environmental isolates. Ecto-ATPase activity is significantly increased in the presence of mannose but not by other sugars. Interestingly, weak- and/or nonpathogenic *Acanthamoeba* (*A. astronyxis* belonging to the T7 genotype) show no differences in ecto-ATPase activities in the presence of mannose. Also, *A. astronyxis* do not bind to the host cells (Alsam *et al.*, 2003). Taken together, these findings suggest that the engagement of *Acanthamoeba* adhesin (i.e. MBP) enhances ecto-ATPase activities and thus ecto-ATPases may also play a role in *Acanthamoeba* pathogenesis but this time in a contact-dependent mechanism. How MBP is associated with ecto-ATPase activities is not clear. Of interest, several ecto-ATPases of approximate molecular weights of 62, 100, 218, 272 and > 300 kDa have been described in *Acanthamoeba* (Sissons *et al.*, 2004b). The differences in ecto-ATPases have been attributed to strain differences. Future research will elucidate their function in *Acanthamoeba* biology, and investigate their precise role in contact-dependent and contact-independent mechanisms of *Acanthamoeba* pathogenesis, and their usefulness as diagnostic targets for genotype differentiation.

Contact-independent factors

To produce damage to the host cell and/or tissue migration, the majority of pathogens rely upon their ability to produce hydrolytic enzymes. These enzymes may be constitutive, which are required for routine cellular functions, or inducible, which are produced under specific conditions, for example upon contact with the target cells. These enzymes can have devastating effects on host cells by causing membrane dysfunction or physical disruptions. Cell membranes are made of proteins and lipids, and *Acanthamoeba* are known to produce two types of hydrolytic enzymes: proteases, which hydrolyse peptide bonds; and phospholipases, which hydrolyse phospholipids.

Proteases

Proteases are well-known virulence factors in the majority of viral, bacterial, protozoan and multicellular pathogens. These enzymes hydrolyse peptide bonds and thus exhibit the ability to degrade various substrates. *Acanthamoeba* secrete large amounts of proteases. Interestingly, both clinical and nonclinical isolates of *Acanthamoeba* exhibit protease activities, but larger amounts are observed with the former. This suggests that the principal physiological role of proteases is to degrade the substrate for feeding purposes. This is consistent with the idea that *Acanthamoeba* are primarily free-living environmental organisms and that their role in human/animal infections is secondary or opportunistic. There are four major types of proteases: aspartic, cysteine, serine and metalloproteases, and so far *Acanthamoeba* are known to produce all but aspartic protease activities, as described below.

All *Acanthamoeba* isolates tested to date exhibit proteolytic activities and serine proteases seem to be the most abundant in almost all genotypes. For example, Hadas & Mazur (1993) demonstrated the presence of a 35-kDa serine

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protease in *Acanthamoeba* isolates (some most likely belonging to the T4 genotype). Other studies using T4 isolates have identified extracellular serine proteases of approximate molecular weights of 36, 49 and 66 kDa (Mitro *et al*., 1994), 107 kDa (Khan *et al*., 2000), and 55, 97 and 230 kDa (Cao *et al*., 1998), but other studies show the presence of serine proteases of 27, 47, 60, 75, 100 and > 110 kDa (Alfieri *et al*., 2000). Furthermore, Mitra *et al*. (1995) demonstrated a 40-kDa serine protease in *Acanthamoeba* belonging to the T4 genotype. This protease was shown to activate plasminogen, whose physiological function is to degrade ECM components. This diversity of serine proteases within a single genotype of *Acanthamoeba* may be due to strain differences, differences in their virulence, culture under diverse conditions or in assay methods. Kong *et al*. (2000) demonstrated a 33-kDa serine protease from *A. healyi* (T12 isolate), which demonstrated degradation of type I and IV collagen and fibronectin, which are the main components of the ECM, as well as fibrinogen, IgG, IgA, albumin and haemoglobin. These properties of serine proteases most likely facilitate *Acanthamoeba* invasion of corneal stroma and lead to secondary reactions such as oedema, necrosis and inflammatory responses. Other studies have identified serine proteases with approximate molecular weights of 42 kDa (Cho *et al*., 2000) and 12 kDa that degrade immunoglobulins, protease inhibitors and interleukin-1 (Na *et al*., 2001, 2002), but their genotypes are not known. A direct functional role of serine proteases in *Acanthamoeba* infection is indicated by the observations that intrastromal injections of *Acanthamoeba* conditioned medium produces corneal lesions *in vivo* similar to those observed in *Acanthamoeba* keratitis patients, and this effect is inhibited by PMSF, a serine protease inhibitor (He *et al*., 1990; Na *et al*., 2001).

Among cysteine proteases, although data are limited, a 65-kDa extracellular cysteine protease is reported in *Acanthamoeba* isolates belonging to the T4 genotype as well as 43-, 70- and 130-kDa cysteine proteases (Hadas & Mazur, 1993; Alfieri *et al*., 2000). It is worth noting that in these studies 65 kDa and 70 kDa may refer to the same protease as the difference may have been due to variation in protease mobility in the substrate gels. Recently, Hong *et al*. (2002) identified a 24-kDa cysteine protease (most likely intracellular) from an *Acanthamoeba* isolate belonging to the T12 genotype. The physiological roles of cysteine proteases remain to be identified.

In addition to serine and cysteine proteases, there is evidence for metalloprotease activity in *Acanthamoeba* (Mitro *et al*., 1994; Alfieri *et al*., 2000). Cao *et al*. (1998) showed a 80-kDa metalloprotease, but its origin (whether *Acanthamoeba* or the host cells) is not known. Recent studies have identified a 150-kDa extracellular metalloprotease from *Acanthamoeba* isolated from an AGE patient (belonging to the T1 genotype) (Alsam *et al*., 2005b). This metalloprotease exhibited properties of ECM degradation, as evidenced by its activity against collagen I and III (major components of collagenous ECM), elastin (elastic fibrils of ECM), plasminogen (involved in proteolytic degradation of ECM), as well as degradation of casein, gelatin and haemoglobin, suggesting a role both in AGE and *Acanthamoeba* keratitis infections (Sissons *et al*., 2005b).

Overall, these studies have shown that amoebae exhibit diverse proteases and elastases (Ferrante & Bates, 1988), which could play important roles in *Acanthamoeba* infections. However, their precise modes of action at the molecular level are only beginning to emerge. That some of the above proteases are secreted only by clinical isolates may indicate their role as potent virulence factors and/or diagnostic targets. Future studies in the role of proteases as vaccine targets, search for novel inhibitors by screening of chemical libraries, or rational development of drugs based on structural studies should enhance our ability to targets these pathogens.

Phospholipases

Phospholipases are a diverse group of enzymes that hydrolyse the ester linkage in glycerophospholipids and can cause membrane dysfunction. The five major phospholipases are A1, A2, B, C and D, and each has the ability to cleave a specific ester bond in the substrate of the target membrane. All phospholipases are present in multiple forms. Our knowledge of phospholipases in the virulence of *Acanthamoeba* is fragmented, but several studies have shown the presence of phospholipase activities in *Acanthamoeba* (Victoria & Korn, 1975a,b; Cursons *et al*., 1978). Cursons *et al*. (1978) were the first to demonstrate phospholipase A in *Acanthamoeba* and suggested their role in AGE infection *in vivo*. Because phospholipases cleave phospholipids, their possible role in membrane disruptions, penetration of the host cells and cell lysis should be focal points for future studies. Other actions of phospholipases may involve interference with intracellular signalling pathways. For example, phospholipases generate lipids and lipid-derived products that act as second messengers (Dennis *et al*., 1991; Serhan *et al*., 1996). Oishi *et al*. (1988) showed that lysophospholipids, end-byproducts of phospholipase B, induce activation of protein kinase C, which has diverse functions in host cell signalling pathways. Phospholipase C of *Clostridium perfringens* induces expression of interleukin-8 synthesis in endothelial cells (Bryant & Stevens, 1996; Bunting *et al*., 1997). Overall, these studies suggest that *Acanthamoeba* phospholipases and/or lysophospholipases may play a direct role in causing host cell damage or affect other cellular functions such as the induction of inflammatory responses and thus facilitate the virulence of *Acanthamoeba*, but this remains to be fully established. More studies are needed to identify and

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characterize *Acanthamoeba* phospholipases and to determine their potential role for therapeutic intervention. This is not a novel concept: earlier studies have shown that phospholipase C from *C. perfringens* induces protection against *C. perfringens*-mediated gas gangrene (Kameyama *et al.*, 1975). In addition, the targeting of phospholipases using synthetic inhibitor compounds has been shown to have the potential to prevent *Candida* infections (Hanel *et al.*, 1995). Antibodies produced against *Acanthamoeba* phospholipases may also be of potential value in the development of sensitive and specific diagnostic assays.

Indirect virulence factors

The ability of *Acanthamoeba* to produce human diseases is a multifactorial process and is, among other factors, dependent on its ability to survive outside its mammalian host for various times and under diverse environmental conditions (high osmolarity, varying temperatures, food deprivation, resistance to chemotherapeutic drugs). The ability of *Acanthamoeba* to overcome such conditions can be considered a contributory factor towards disease and as indirect virulence factors (Fig. 8).

Morphology

The infective forms of *Acanthamoeba* or trophozoites do not have a distinct morphology. However, they do possess spine-like structures known as acanthopodia on their surface, which may play a key role in the pathogenesis of *Acanthamoeba* infections by modulating binding of pathogenic *Acanthamoeba* to corneal epithelial cells (Khan, 2001) (Fig. 5). It would not be surprising if MBP, which is involved in binding of amoebae to host cells, is localized on acanthopodia. In addition, their amoeboid motion resembles that of macrophages/neutrophils. From this, it can be speculated that *Acanthamoeba* may use mechanisms similar to leukocytes to traverse the biological barriers such as the blood–brain barrier.

Temperature tolerance and osmotolerance

Upon contact with tear film and corneal epithelial cells, *Acanthamoeba* are exposed to high osmolarity (tear salinity) as well as high temperatures. For successful transmission, amoebae must withstand these burdens and exhibit growth. Growth at high temperature and high osmolarity are the hallmarks of pathogenic *Acanthamoeba* (De Jonckheere, 1983; Walochnik *et al.*, 2000; Khan *et al.*, 2001, 2002). These studies have shown that the ability of *Acanthamoeba* to grow at high temperature and high osmolarity correlate with the pathogenicity of *Acanthamoeba* isolates. However, the precise mechanisms by which pathogenic *Acanthamoeba* adapt to higher temperatures and maintain their metabolic activities remain entirely unknown. Interestingly, temperature tolerance studies in *Candida neoformans* have identified the Ca^{2+}-dependent protein phosphatase calcineurin as a requirement for its growth at 37 °C (Odom *et al.*, 1997a, b). Furthermore, *C. neoformans* strains in which the calcineurin gene has been disrupted are avirulent in a model of cryptococcal meningitis *in vivo*. These studies might serve as a basis for research into determining the physiological properties of *Acanthamoeba*.

Growth at different pH

Pathogenic *Acanthamoeba* can grow at pH ranging from 4 to 12 (my unpublished data), which gives it the potential to colonize several niches. For example, the ability of *Candida albicans* to grow at diverse pH is crucial for its virulence (Davis *et al.*, 2000) and two pH-regulating genes, *PHR1* (expressed at neutral and basic pH) and *PHR2* (expressed at acid pH) have been identified. Deletion of *PHR2* results in the loss of virulence, while deletion of *PHR1* results in reduced virulence in a systemic model (De Bernardis *et al.*, 1998). The clinical significance of the ability of *Acanthamoeba* to exhibit growth at different pH remains to be determined.

Phenotypic switching

Phenotypic switching in *Acanthamoeba* is the ability to differentiate into a morphologically distinct dormant cyst form or a vegetative trophozoite form. This is a reversible change dependent on environmental conditions (Fig. 3). Cysts are resistant to various antimicrobial agents and adverse conditions such as extreme temperature, pH, osmolarity and desiccation, and they can be airborne (Weisman, 1976; Byers, 1979; Cordingley *et al.*, 1996; Turner *et al.*, 2000): all of which presents a major problem in chemotherapy because their persistence may lead to recurrence of the disease. Furthermore, *Acanthamoeba* cysts can survive for several years while maintaining pathogenicity (Mazur *et al.*, 1995). These characteristics suggest that the primary functions of cysts lie in withstanding adverse conditions and in the spread of amoebae throughout the environment. In addition, this may represent the ability of *Acanthamoeba* to alternate expression of surface proteins/glycoproteins, in response to changing environments and/or immune surveillance. Overall, phenotypic switching represents a major factor in the transmission of *Acanthamoeba* infections; however, the underlying molecular mechanisms in these processes remain to be elucidated. At present it is not clear whether *Acanthamoeba* shows antigenic variations, and their possible involvement in phenotypic switching should be investigated in future studies.

Drug resistance

Current treatment for *Acanthamoeba* keratitis involves topical application of mixtures of drugs including CHX, PHMB,

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neomycin and propamidine isethionate as indicated above. These drugs have been shown to be most effective in killing *Acanthamoeba* trophozoites (Wright *et al*., 1985; Cohen *et al*., 1987; Moore & McCulley, 1989; Larkin *et al*., 1992; Hay *et al*., 1994; Seal *et al*., 1995; Russell & Chopra, 1996; Murdoch *et al*., 1998; Lim *et al*., 2000; Turner *et al*., 2000; Lloyd *et al*., 2001). Both CHX and PHMB are 'membrane-acting' cationic biocides. At alkaline pH, surface proteins of *Acanthamoeba* are negatively charged, interacting rapidly with these cationic biocides and inducing structural and permeability changes in the cell membrane, leading to leakage of ions, water and other cytoplasmic components, and resulting in cellular damage (Perrine *et al*., 1995). Drugs such as propamidine isethionate belong to the diamidine family and are effective inhibitors of DNA synthesis (Duguid *et al*., 1997). Of concern, several studies have recently shown the increasing resistance of *Acanthamoeba* to antimicrobial chemotherapy; however, the mechanisms of such drug resistance in *Acanthamoeba* remain incompletely understood (Ficker *et al*., 1990; Larkin *et al*., 1992; Murdoch *et al*., 1998; Lim *et al*., 2000; Lloyd *et al*., 2001). One intriguing report was made by Ficker *et al*. (1990), who observed the development of propamidine resistance during the course of therapy for *Acanthamoeba* keratitis, which led to recurrence of the infection. This may be due to the fact that although propamidine isethionate inhibits DNA synthesis (Johnson & Thomas, 2002), at presently recommended concentrations it may not be active against cyst forms of *Acanthamoeba* due to their reproductive inactivity and/or very limited metabolic activity. Also, it is likely that the double-walled structure of *Acanthamoeba* cysts, comprising an inner endocyst and an outer ectocyst (33% protein, 4–6% lipids and 35% carbohydrates, mostly cellulose), provides a physical barrier against chemotherapeutic agents (Neff & Neff, 1969; Turner *et al*., 2000). Additionally, the dangers of increased selection pressure induced by continuous drug exposure should not be ignored. Precise understanding of these mechanisms is crucial for the development of much needed drugs against this serious infection.

Ubiquity

Acanthamoeba have been found in diverse environments, from drinking water to distilled water wash bottles, so it is not surprising that humans encounter and interact regularly with these organisms, as is evidenced by the fact that most (100% of the population in some areas) individuals tested possess *Acanthamoeba* antibodies. This clearly suggests that these are one of the most ubiquitous protozoans and often come into contact with humans. This provides amoebae with a wider access to approach the limited susceptible hosts.

Biofilms

Biofilms are known to play an important role in the pathogenesis of *Acanthamoeba* keratitis. Biofilms are microbially derived sessile communities, which can be formed in aqueous environments as well as on any materials and medical devices including intravenous catheters, contact lenses, scleral buckles, suture material and intraocular lenses (Zegans *et al*., 2002). With contact lenses, biofilms are formed through contamination of the storage case. Once established, biofilms provide attractive niches for *Acanthamoeba*, by fulfilling their nutritional requirements as well as providing resistance to disinfectants. For example, Beattie *et al*. (2003) have shown that *Acanthamoeba* exhibit significantly higher binding to used and *Pseudomonas* biofilm-coated hydrogel lenses compared with unworn contact lenses. In addition, the abundant nutrient provided by the biofilm encourages transformation of *Acanthamoeba* into the vegetative, infective trophozoite form, and it is important to remember that binding of *Acanthamoeba* to human corneal epithelial cells most likely occurs during the trophozoite stage as cysts exhibit no and/or minimal binding (Dudley *et al*., 2005; Garate *et al*., 2006). Overall, these findings suggest that biofilms play an important role in *Acanthamoeba* keratitis in wearers of contact lenses and preventing their formation is an important preventative strategy.

Host factors

The factors that enable *Acanthamoeba* to produce disease are not limited solely to the parasite, but most likely involve host determinants. Evidence for this comes from recent studies in the UK, Japan and New Zealand, which suggest that storage cases of contact lenses of 400–800 per 10 000 asymptomatic wearers are contaminated with *Acanthamoeba* (Larkin *et al*., 1990; Devonshire *et al*., 1993; Watanabe *et al*., 1994; Gray *et al*., 1995). This number is remarkably high compared with the incidence of *Acanthamoeba* keratitis in wearers of contact lenses, which is 0.01–1.49 per 10 000. These findings suggest that factors such as host susceptibility, tissue specificity, tear factors, sIgA and corneal trauma, as well as environmental factors such as osmolarity may be important in initiating *Acanthamoeba* infections. In addition, malnutrition, mental stress, age, metabolic factors and other primary diseases may play a role in the pathogenesis of *Acanthamoeba* infections.

However, the extent to which such host factors contribute to the outcome of *Acanthamoeba* infections is unclear because host factors are more complex and difficult to study than those of the parasite. For example, in bacterial infections such as salmonella, the genetic constitution of the host determines susceptibility (Harrington & Hormaeche, 1986; Fleiszig *et al*., 1994). These studies with *Salmonella* were possible only because transgenic animals were available.

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Immune response

Despite the ubiquitous presence of these organisms in diverse environments, the number of infections due to *Acanthamoeba* has remained very low. This is due to the fact that *Acanthamoeba* are opportunistic pathogens and their ability to produce diseases is dependent on host susceptibility (e.g. immunocompromised patients or contact lens wearers), environmental conditions (personal hygiene, exposure to contaminated water/soil) and their own virulence. For AGE, in normal circumstances, a competent immune system is sufficient to control these pathogens. But due to the complexity of the immune system, the precise factors that contribute to host resistance and the associated mechanisms remain unclear. By contrast, *Acanthamoeba* keratitis can occur in normal individuals, although oral immunization can prevent this infection. This suggests that the immune system still plays an important role in this condition. Although there are obvious similarities in the immune response to AGE and to *Acanthamoeba* keratitis, they are described separately for simplicity.

Acanthamoeba keratitis and the immune response

Because the normal cornea is avascular, many primary host defences are provided by the eyelids and secreted tear film. The tear fluid produced by the lacrimal system, together with constant eye lid movement, provides the first line of defence in *Acanthamoeba* keratitis. The tear film contains lysozyme, lactoferrin, β-lysins, sIgA, prostoglandins, and other compounds with anti-microbial and immunological properties (Nassif, 1996; Qu & Leher, 1998). Of the three distinct layers in the tear film (i.e. the oil layer, the aqueous layer and the mucous layer), the aqueous layer is the source of compounds with antimicrobial properties such as nonspecific anti-microbial lysozyme, lactoferrin and specific sIgA. A role for sIgA in *Acanthamoeba* keratitis is indicated by the findings that *Acanthamoeba* keratitis patients show decreased levels of sIgA, and specific anti-amoebic sIgA inhibits *Acanthamoeba* binding to corneal epithelial cells, suggesting that sIgA plays an important role normally in alleviating this infection (Leher *et al*., 1999; Alizadeh *et al*., 2001; Walochnik *et al*., 2001). The mechanisms of how binding is inhibited are unclear, but could involve interference with the amoeba adhesins and the associated pathways. Overall, the tear film in conjunction with the blink reflex is highly effective in blocking microbial access to corneal epithelial cells and expelling amoebae from the surface of the eye and carrying them to the conjunctiva. Although the cornea is protected by only limited immune mechanisms, the conjunctiva is highly vascular with lymphoid tissue and contains mainly IgA-producing plasma cells, T-lymphocytes, natural killer cells and macrophages, which are highly effective in clearing *Acanthamoeba* and stimulating humoral and T-cell responses. For example, conjunctival macrophage depletion exacerbates *Acanthamoeba* keratitis symptoms *in vivo*, and increases the rate of infection to 100% (van Klink *et al*., 1996). Thus, in order to proceed with infection, *Acanthamoeba* must remain at the corneal surface and invade into the stromal tissue. Of interest, the tear film in wearers of contact lenses differs in terms of volume and make up, and it seems that use of extended contact lens wear alters the levels of inflammatory mediators in tears and contributes to increased inflammation (Thakur & Willcox, 2000). Other major changes are exclusion of atmospheric oxygen and thinning of the basal tear film. It has been proposed that hypoxia is responsible for the metabolic compromise of the cornea and also leads to less resistance to microbial infection (Weisman & Mondino, 2002). This indicates that the cornea is a stressed environment in the presence of a contact lens, and this affects components of the tear film, thereby making invasion by the amoebae more likely. In addition, the highly virulent strains of *Acanthamoeba* evade these primary defences to traverse the cornea and invade the stroma. Once in the stroma, *Acanthamoeba* secrete proteolytic enzymes, causing stromal degradation and leading to macrophages/neutrophil infiltration, which modulate B- and T-lymphocyte activity to clear *Acanthamoeba*. The macrophages provide defence against *Acanthamoeba* keratitis directly by clearing amoebae and by inducing an inflammatory response, in particular secretion of macrophage inflammatory protein 2 (MIP-2). This results in the recruitment of other immune cells such as neutrophils, which are highly potent in destroying *Acanthamoeba* in a myeloperoxidase-dependent manner (Hurt *et al*., 2001, 2003). Overall, these studies suggest that upon entry into the eye, *Acanthamoeba* have to deal with normal tear film containing the nonspecific antimicrobial compounds together with the sweeping action of the eyelids as well as specific immunoglobulins and cell-mediated immunity.

AGE and the immune response

As AGE is a rare infection, much of our understanding of its immunopathogenesis comes from studies using animals. The following account highlights some of the findings arising during the last decade. Complement is the first line of powerful defence against invading pathogens. Complement activation is achieved by: the classical pathway (activated by specific antibodies attached to *Acanthamoeba* surface); alternative pathways (activated by opsonization); or mannose-binding lectin pathways (activated directly by components of the surface composition of the pathogen). The ultimate effects are: to induce the deposition of complement proteins, leading to opsonization of amoebae followed by their uptake by phagocytes; or the formation of the

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membrane attack complex (MAC) resulting in the target cell death.

In support of MAC attack, previous studies have shown that normal human serum exhibits complement-mediated lysis in *Acanthamoeba*, resulting in up to 100% amoebae death (Ferrante & Rowan-Kelly, 1983; Ferrante, 1991; Stewart *et al*., 1992). This is due to the fact that the plasma membranes of *Acanthamoeba* lack sialic acid (Korn & Olivecrona, 1971) or any protective coat or capsule (Bowers & Korn, 1968) and thus *Acanthamoeba* are exposed to complement-mediated killing (Ferrante & Rowan-Kelly, 1983). However, recent studies have shown that a subpopulation of the virulent strains of *Acanthamoeba* are resistant to complement-mediated lysis (Toney & Marciano-Cabral, 1998; Sissons *et al*., 2005c). But complement pathway in the presence of phagocytes (macrophages/neutrophils) is highly effective in clearing *Acanthamoeba* (Stewart *et al*., 1994). Overall, complement pathways and the antibodies together with neutrophils and macrophages show potent amoebalytic activities, thus suppressing the infection by clearing amoebae (Marciano-Cabral & Toney, 1998; Toney & Marciano-Cabral, 1998). It is important to note that although macrophages/neutrophils from naïve animals are able to destroy *Acanthamoeba*, cells from immune animals exhibit significantly increased amoebalytic activities. The macrophage-mediated killing is contact-dependent and is inhibited with cytochalasin D, i.e. an actin polymerization inhibitor (van Klink *et al*., 1997). This is further confirmed with the findings that conditioned medium obtained from macrophages after treatment with lipopolysaccharide and interferon-gamma had no effects on *Acanthamoeba* (Marciano-Cabral & Toney, 1998). Similarly, the neutrophils exhibit potent amoebacidal effects. Neutrophil-mediated killing is significantly increased in the presence of anti-acanthamoebic antibodies (Stewart *et al*., 1994). These interactions also stimulate secretion of proinflammatory cytokines including interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α (Marciano-Cabral & Toney, 1998; Toney & Marciano-Cabral, 1998). Other studies in mice have shown significant increased natural killer cell (NK) activities in *Acanthamoeba*-infected animals suggesting that NK cells may also play a role in protective immunity (Kim *et al*., 1993). The fact that up to 100% of individuals may possess anti-*Acanthamoeba* antibodies suggests that immunocompetent individuals exhibit *Acanthamoeba*-specific T-cell responses (in particular CD4 T-cells) (Cursons *et al*., 1980; Tanaka *et al*., 1994). Based on these findings, it is clear that debilitated immune status of the host is usually a prerequisite in AGE but the core basis of host susceptibility in contracting AGE is not fully understood and may also involve the host ethnic origin (i.e. genetic basis of the host) or the inability of the host to induce specific immune response against these pathogens. Of interest, both *Acanthamoeba* cysts and trophozoites are immunogenic, and immune sera from animals infected with *Acanthamoeba* trophozoites cross-react with cysts, suggesting that some of the antigenic epitopes are retained during the encystment process (McClellan *et al*., 2002). Future studies will identify the precise host factors, which play an important role in controlling this fatal infection and may help to develop therapeutic interventions in the susceptible hosts.

Acanthamoeba and bacteria interactions

Acanthamoeba were first shown to be infected and lysed by bacteria in 1954 (Drozanski, 1956) and to harbour bacteria as endosymbionts in 1975 (Proca-Ciobanu *et al*., 1975). Later studies revealed that *Acanthamoeba* act as reservoirs for pathogenic facultative mycobacteria (Krishna-Prasad & Gupta, 1978). *Acanthamoeba* have been shown also to harbour virulent *Legionella* spp. associated with Legionnaires' disease (Rowbotham, 1980). At the same time, it is well established that *Acanthamoeba* consume bacteria in the environment, so the interactions of *Acanthamoeba* and bacteria are highly complex and dependent on virulence of the amoebae, virulence of the bacteria and environmental conditions. The outcome of these convoluted interactions may be beneficial to *Acanthamoeba* or to bacteria or may result in the development of a symbiotic relationship. Adding to this complexity, *Acanthamoeba* are known to interact with various Gram-positive and Gram-negative bacteria, resulting in a range of outcomes. A complete understanding of *Acanthamoeba*–bacteria interactions is beyond the scope of this review and for further information readers are referred to Greub & Raoult (2004). For simplicity and for researchers new to this area, these interactions are discussed in three sections below.

Acanthamoeba as bacterial predators

This group includes bacteria that are used as food sources for *Acanthamoeba*. Bacteria are taken up by phagocytosis, followed by their lysis in phagolysosomes. Although *Acanthamoeba* consume both Gram-positive and Gram-negative bacteria, they preferentially graze on Gram-negative bacteria, which are used widely as a food source in the isolation of *Acanthamoeba* (Bottone *et al*., 1994). However, the ability of *Acanthamoeba* to consume bacteria is dependent on the virulence properties of bacteria and the environmental conditions. For example, recent studies have shown that in the absence of nutrients, virulent strains of *Escherichia coli* K1 invade *Acanthamoeba* and remain viable intracellularly. And upon the availability of nutrients, K1 escapes *Acanthamoeba*, grows exponentially and lyses the host amoebae (Alsam *et al*., 2005c). By contrast, in the absence of nutrients the avirulent strains of *Escherichia coli* K12 are phagocytosed (instead of them invading) by

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Acanthamoeba and are killed (Alsam *et al*., 2005c). This demonstrates clear differences in the ability of *Acanthamoeba* to interact with *Escherichia coli* that are dependent on the virulence properties of the *Escherichia coli* and the environmental conditions. Future studies should examine the basis of these differences as well as how bacteria are taken up (invasion vs. phagocytosis) and the molecular mechanisms involved.

Acanthamoeba as bacterial reservoirs

The ability of *Acanthamoeba* to act as a bacterial reservoir has gained much attention (Greub & Raoult, 2004), because the majority of the bacteria involved are human pathogens. These include *Legionella pneumophila* (causative agent of Legionnaires' disease) (Rowbotham, 1980); *Escherichia coli* O157 (causative agent of diarrhoea) (Barker *et al*., 1999); *Coxiella burnetii* (causative agent of Q fever) (La Scola & Raoult, 2001); *Pseudomonas aeruginosa* (causative agent of keratitis) (Michel *et al*., 1995); *Vibrio cholerae* (causative agent of cholera) (Thom *et al*., 1992); *Helicobacter pylori* (causative agent of gastric ulcers) (Winiecka-Krusnell *et al*., 2002); *Simkania negevensis* (causative agent of pneumonia) (Kahane *et al*., 2001); *Listeria monocytogenes* (causative agent of listeriosis) (Ly & Muller, 1990); and *Mycobacterium avium* (causative agent of respiratory diseases) (Krishna-Prasad & Gupta, 1978; Steinert *et al*., 1998). This property of *Acanthamoeba* is particularly important as these bacterial pathogens not only survive intracellularly but multiply within them. This allows bacteria to transmit throughout the environment, evade host defences and/or chemotherapeutic drugs, and reproduce in sufficient numbers to produce disease. Upon favourable conditions, the increasing bacterial densities lyse their host amoebae and infect new amoebae and/or produce disease. In the long term, these *Acanthamoeba*–bacteria interactions can be considered as 'parasitic' as they result usually in the death of the amoeba. It is important to recognize that the virulence determinants responsible for bacterial invasion of *Acanthamoeba*, their intracellular survival via inhibition of formation of phagolysosomes or growth at acidic pH, and their escape from *Acanthamoeba* vary between different bacteria.

Acanthamoeba as bacterial Trojan horse

One of the key requirements for many bacterial pathogens is to survive harsh environments during transmission from one host to another. Once bacteria invade host tissue such as nasal mucosa, lung epithelial cells or gut mucosa to produce disease, they must resist the innate defences as well as cross biological barriers. To this end, *Acanthamoeba* may act as a 'Trojan horse' for bacteria. The term 'Trojan horse' is used to describe bacterial presence inside *Acanthamoeba* as opposed to 'carrier', which may be mere attachment/adsorption to the surface. Recent studies have shown that *Burkholderia cepacia* (a causative agent of lung infection) remains viable within *Acanthamoeba* but does not multiply (Marolda *et al*., 1999; Landers *et al*., 2000). Essig *et al*. (1997) have shown similar findings using *Chlamydophila pneumoniae* (causative agent of respiratory disease). This property has also been observed with other bacterial pathogens, as discussed above. Overall, these findings suggest that *Acanthamoeba* facilitate bacterial transmission and/or provide protection against the human immune system. In support of this, Cirillo *et al*. (1997) showed increased *Mycobacterium avium* colonization of mice, when inoculated in the presence of amoebae. The ability of *Acanthamoeba* to resist harsh conditions (such as extreme temperatures, pH and osmolarity), especially during their cyst stage, suggest their usefulness as bacterial vectors. In particular, *Acanthamoeba* cysts are notoriously resistant to chlorine (a key and sometimes the only compound used in cleaning water systems). This poses clear challenges in eradicating bacterial pathogens from public water supplies, especially in developing countries. In addition, *Acanthamoeba*–bacteria interactions also affect bacterial virulence. For example, *L. pneumophila* grown within *Acanthamoeba* exhibited increased motility, virulence and drug resistance compared with axenically grown *Legionella* (Greub & Raoult, 2002).

Conclusions

Studies of *Acanthamoeba* have grown exponentially. These organisms have gained attention from the broad scientific community studying cellular microbiology, environmental biology, physiology, cellular interactions, molecular biology and biochemistry. This is due to their versatile roles in ecosystems and their ability to capture prey by phagocytosis (similar to macrophages), act as vectors, reservoirs and Trojan horses for bacterial pathogens, and to produce serious human infections such as blinding keratitis and fatal encephalitis. In addition, this unicellular organism has been used extensively to understand the molecular biology of motility. The ability of *Acanthamoeba* to switch phenotypes makes it an attractive model for the study of cellular differentiation processes. Moreover, the increasing numbers of HIV/AIDS patients and contact lens wearers, and warmer climates will add to the increasing burden of *Acanthamoeba* infections. Our understanding of *Acanthamoeba* pathogenesis at both the molecular and the cellular level, as well as its ability to transmit, adapt to diverse conditions, overcome host barriers and emerge as infective trophozoites will provide targets for therapeutic interventions. The availability of the *Acanthamoeba* genome, together with the recently developed transfection assays (Yin & Henney, 1997; Peng *et al*., 2005), and RNA interference methods (Lorenzo-Morales *et al*., 2005), will undoubtedly increase

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the pace of our understanding of this complex but fascinating organism.

Acknowledgements

I am grateful to Selwa Alsam, James Sissons, Samantha Jayasekera, Ricky Dudley and Abdul Matin, School of Biological and Chemical Sciences, Birkbeck, University of London, UK; Kwang Sik Kim, Division of Infectious Diseases, Johns Hopkins University School of Medicine, Baltimore, MD, USA; Ed Jarroll, Northeastern University, Boston, MA, USA; Sim Webb, University of East Anglia, Norwich, England, UK; Amir Maghsood, Department of Medical Parasitology & Mycology, School of Public Health and Institute of Health Research, Tehran University of Medical Sciences, Tehran, Iran, for assistance, Joe Harrison, Kemnal Technology College, Kent, UK, for technical support and Graham Goldsworthy, School of Biological and Chemical Sciences, Birkbeck College, for critical review of the manuscript. This work was supported by grants from the Faculty Research Fund, Central Research Fund, University of London, The Nuffield Foundation, The Royal Society and The British Council for Prevention of Blindness.

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