MINIREVIEW

Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*

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Received 8 November 2006; revised 5 February 2007; accepted 12 February 2007.
First published online 11 April 2007.

DOI:10.1111/j.1574-695X.2007.00232.x

Editor: Willem van Leeuwen

Keywords
primary amoebic meningoencephalitis; granulomatous amoebic encephalitis; amphizoic amoebae; central nervous system infection; skin infection; *Acanthamoeba* keratitis.

Abstract

Among the many genera of free-living amoebae that exist in nature, members of only four genera have an association with human disease: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri* and *Sappinia diploidea*. *Acanthamoeba* spp. and *B. mandrillaris* are opportunistic pathogens causing infections of the central nervous system, lungs, sinuses and skin, mostly in immunocompromised humans. *Balamuthia* is also associated with disease in immunocompetent children, and *Acanthamoeba* spp. cause a sight-threatening infection, *Acanthamoeba* keratitis, mostly in contact-lens wearers. Of more than 30 species of *Naegleria*, only one species, *N. fowleri*, causes an acute and fulminating meningoencephalitis in immunocompetent children and young adults. In addition to human infections, *Acanthamoeba*, *Balamuthia* and *Naegleria* can cause central nervous system infections in animals. Because only one human case of encephalitis caused by *Sappinia diploidea* is known, generalizations about the organism as an agent of disease are premature. In this review we summarize what is known of these free-living amoebae, focusing on their biology, ecology, types of disease and diagnostic methods. We also discuss the clinical profiles, mechanisms of pathogenesis, pathophysiology, immunology, antimicrobial sensitivity and molecular characteristics of these amoebae.

Introduction

Pathogenic and opportunistic free-living amoebae such as *Acanthamoeba* spp., *Balamuthia mandrillaris* and *Naegleria fowleri* are aerobic, mitochondriate, eukaryotic protists that occur world-wide and can potentially cause infections in humans and other animals (Martinez, 1985; Martinez & Visvesvara, 1997; Schuster & Visvesvara, 2004a; Visvesvara & Maguire, 2006). Because these amoebae have the ability to exist as free-living organisms in nature and only occasionally invade a host and live as parasites within host tissue, they have also been called amphizoic amoebae (Page, 1988). All three amoebae cause infections of the central nervous system (CNS). Several species of *Acanthamoeba* (e.g. *A. castellanii, A. culbertsoni, A. hatchetti, A. healyi, A. polyphaga, A. rhysodes, A. astronyxis*, and *A. divionensis*), the only known species of *Balamuthia, B. mandrillaris*, and only one species of *Naegleria, N. fowleri*, are known to cause disease. *Acanthamoeba* and *Balamuthia* cause the insidious, chronic and mostly fatal disease granulomatous amoebic encephalitis (GAE), particularly in immunocompromised or otherwise debilitated individuals. *Naegleria fowleri*, on the other hand, causes an acute, fulminant, necrotizing and hemorrhagic meningoencephalitis leading to death in healthy children and young adults with a history of recent contact with fresh water. In addition to causing CNS infections, *Acanthamoeba* also causes a vision-threatening disease, *Acanthamoeba* keratitis. Both *Acanthamoeba* and *Balamuthia* cause infections of the lungs and skin (Martinez & Visvesvara, 1997; Schuster & Visvesvara, 2004a; Visvesvara & Maguire, 2006). Recently, *Sappinia diploidea*, another free-living amoeba that normally lives in soil contaminated with faeces of elk, bison, and cattle, was identified as causing

encephalitis in an otherwise healthy young man (Gelman *et al.*, 2001), indicating that there are probably other amoebae that are capable of causing encephalitis in humans. Because most of the infections caused by these amoebae are ultimately fatal, diagnosis is often made at autopsy, even in developed countries where sophisticated diagnostic facilities are readily available. However, in Sub-Saharan Africa and Southeast Asia, where HIV/AIDS is rampant, it is quite possible that a large number of cases have gone undetected. This is for a number of reasons, among them: (i) lack of expertise needed to identify these amoebae; (ii) cultural ethos and expense that prevent autopsies; and (iii) an abundance of more prominent diseases such as HIV/AIDS, tuberculosis and malaria that consume national resources. The actual incidence of disease is therefore not really known. Table 1 provides a comparative view of the 4 amoebae dealt with in this review.

Culbertson *et al.* (1958) advanced the concept that free-living amoebae may cause human infections. During the production of the poliomyelitis vaccine they isolated an amoeba from tissue cultures thought to contain an unknown virus. They inoculated mice and cortisone-treated monkeys intracerebrally with this amoeba and demonstrated the amoebae in brain lesions of the animals that died a week later. Culbertson hypothesized that a similar infection might exist in humans. This isolate was subsequently named *Acanthamoeba culbertsoni*.

Taxonomy and classification of the small free-living pathogenic amoebae

The classical taxonomic classification divided the Protozoa into four groups: Sarcodina (amoebae), Mastigophora (flagellates), Sporozoa (most parasitic protozoa) and Infusoria (ciliates). This taxonomy has been totally abandoned by the International Society of Protozoologists for one based on modern morphological approaches, biochemical pathways, and molecular phylogenetics (Adl *et al.*, 2005). The older hierarchical systems consisting of the traditional 'kingdom', 'phylum', 'class', 'subclass', 'super-order', 'order' has been replaced by a new vocabulary. According to this new schema, the Eukaryotes have been classified into six clusters or 'Super Groups', namely Amoebozoa, Opisthokonta, Rhizaria, Archaeplastida, Chromalveolata and Excavata. The four amoebae that are dealt with in this article have been classified under two Super Groups, Amoebozoa and Excavata, as follows: (a) *Acanthamoeba* and *Balamuthia* are classified under Super Group Amoebozoa: Acanthamoebidae; (b) *Naegleria fowleri* under Super Group Excavata: Heterolobosia: Vahlkampfiidae; and (c) *Sappinia* under Super Group Amoebozoa: Flabellinea: Thecamoebidae. This schema has been proposed as the basis for future revisions (Adl *et al.*, 2005).

Acanthamoeba species

In 1913 Puschkarew isolated an amoeba from dust and named it *Amoeba polyphagus*. Page redescribed the amoeba as *Acanthamoeba polyphaga* (Puschkarew, 1913) (Page, 1967), retaining the generic designation *Acanthamoeba* as created by Volkonsky in 1931. In 1930 Castellani isolated an amoeba that occurred as a contaminant in a yeast culture plate; it was later named *Acanthamoeba castellanii*. Currently, the identities of more than 24 species of *Acanthamoeba* have been based on morphological criteria. These species are assigned to one of three distinct groups based on their morphology and cyst size. Group I consists of those species that are characterized by large amoebae with cysts that range in size from 16 to 30 μm. Group II includes by far the largest numbers of species, consisting of amoebae with cysts measuring around 18 μm or less. Group III also consists of species with cysts measuring 18 μm or less, but with subtle differences in the cyst morphology. Grouping species based on morphology, is, however, considered unreliable because of the variations in cyst morphology, a critical characteristic for species identification, that can be caused by culture conditions. Efforts were therefore made to use nonmorphological criteria such as protein and isoenzyme profiles as an adjunct to species differentiation based on morphological characteristics alone (De Jonckheere, 1983). *Acanthamoeba healyi* was described as a new species of *Acanthamoeba* based on a combination of morphological, antigenic and isoenzyme profiles by Moura *et al.* (1992). These authors used high-resolution polyacrylamide gradient gel electrophoresis to resolve complex mixtures of polypeptides of various *Acanthamoeba* spp. Based on these studies, the authors reported that species belonging to groups I, II and III could be easily distinguished from one another using the profiles of three isoenzymes: hexokinase, esterase and acid phosphatase. Furthermore, Western blot profiles developed after the separated proteins reacted with rabbit anti-*A. castellanii* serum demonstrated extensive complexity of the cellular proteins. Differences in physiological characteristics as well as those seen in protein and antigen profiles have also been used to differentiate pathogenic and nonpathogenic *Acanthamoeba* (Khan *et al.*, 2001; Walochnik *et al.*, 2004). Currently, however, molecular techniques, especially sequencing of 18S rRNA genes, are being used to understand the species complex and phylogeny of *Acanthamoeba*. Based on sequence differences, 15 genotypes (T1–T15) of *Acanthamoeba* have been established (Stothard *et al.* 1998; Horn *et al.* 1999; Gast, 2001; Hewett *et al.* 2003; Marciano-Cabral & Cabral, 2003; Khan, 2006; Köshler *et al.*, 2006).

Acanthamoeba has two stages in its life cycle: a vegetative or trophozoite stage that feeds voraciously on bacteria and detritus present in the environment and reproduces by binary fission, and a dormant but resistant cyst stage.

Table 1. Comparative characteristics of the free-living amoebae as etiological agents of amoebic encephalitis and amoebic keratitis

	Naegleria fowleri	*Acanthamoeba* spp. (encephalitis)	*Acanthamoeba spp.* (keratitis)	*Balamuthia mandrillaris*	*Sappinia diploidea**
Life cycle	Three stages: amoeba, cyst and flagellate	Two stages: amoeba and cyst		Two stages: amoeba and cyst	Two stages: amoeba and cyst
Distinctive morphological features	Vesicular nucleus; limacine movement of amoebae; flagellate stage; cyst with pores flush at the surface	Vesicular nucleus; finger-like pseudopodia projecting from surface; cyst wall with two layers and with pores		Vesicular nucleus with single or multiple nucleoli; amoeboid and 'spider-like' movements in culture; cyst wall with three layers	Presence of two abutting nuclei in amoeba and cyst stages
In vitro cultivation	Axenic, bacterized and defined media; tissue culture cells; optimal growth at 37 °C and above	Axenic, bacterized, and defined media; tissue culture cells; growth at 37 °C (CNS isolates) or c. 30 °C (keratitis isolates)		Axenic medium and tissue culture cells; optimal growth at 37 °C	Bacterized cultures
Disease	Primary amoebic meningoencephalitis (PAM)	Granulomatous amoebic encephalitis; cutaneous lesions; sinus infections	Amoebic keratitis	Granulomatous amoebic encephalitis; cutaneous lesions; sinus infections	Amoebic encephalitis; granuloma not seen
Prodromal period	Days	Weeks to months	Days	Weeks to months	Insufficient data
Epidemiology	Humans typically infected while recreating in warm fresh waters	Infection from soil, water, and air; present in hospital environment (water taps, hydrotherapy pools, air conditioning cooling towers)	Corneal trauma; contaminated lens solutions and lens cases	Infection from soil, water, and air	Present in soil, water and in air; originally identified from herbivore faeces.
Groups at risk	Children and young adults in good health	Typically, immunocompromised individuals	Mainly contact-lens wearers; low secretory IgA may contribute	Both immunocompetent (children and elderly) or immunocompromised individuals; Hispanic Americans	Insufficient data; single patient was immunocompetent
Disease at presentation	Headache, stiff neck, seizures, coma	Headache, stiff neck, behavioural changes, coma	Intense pain, photophobia, tearing	Headache, nausea, seizures, stiff neck, hydrocephalus; sinus infection; nodule formation in cutaneous infections	Headache, vomiting, photophobia, loss of consciousness; preceded by sinus infection
Clinical course	Fulminant disease; death within 1–2 weeks without treatment	Indolent subacute course; acute stage fatal in weeks	Penetration of amoebae into cornea; stromal ring due to PMN infiltrate	Indolent subacute course; once in acute stage, fatal in weeks	Patient recovered following treatment
Laboratory diagnostic methods	Amoebae present in CSF; no cysts seen in brain tissue; elevated PMNs in CSF	Cysts seen in brain tissue; IFA, IIF and PCR	Corneal scrapings or biopsy; confocal microscopy	Elevated CSF protein and pleocytic lymphocytosis, normal glucose; cysts seen in brain tissue; IFA. IIF and PCR	Binucleate amoebae in H&E-stained slides of brain tissue; PMNs and lymphocytes; immunostaining not available
Neuroimaging (CT and/or MRI)	Unremarkable; not helpful diagnostically	Presence of space-occupying or ring-enhancing lesions	Not relevant	Presence of space-occupying or ring-enhancing lesion	Mass seen in MRI; slight ring enhancement
Humoral reaction	Typically weak but titre rises with length of infection	Usually strong; protective value uncertain	IgA at corneal surface may be protective	Usually strong; protective value uncertain	Insufficient data

Table 1. Continued.

	Naegleria fowleri	*Acanthamoeba* spp. (encephalitis)	*Acanthamoeba spp.* (keratitis)	*Balamuthia mandrillaris*	*Sappinia diploidea**
Prevention	Public health monitoring of warm, fresh-water recreational sites for amoebae	Widespread in soil and water; in hospital setting, monitoring of water supply, ventilators, air conditioning units	Use of sterile lens solutions; maintaining clean lens case; avoiding swimming with contact lenses	Found in soil; preventive measures not feasible	Insufficient data; organism found in soil; preventive measures not feasible
Antimicrobial therapy	Intrathecal amphotericin B, miconazole	Pentamidine, azole compounds, flucytosine, sulfadiazine	PHMB, chlorhexidine, Brolene	Pentamidine, azithromycin, fluconazole, flucytosine,	Azithromycin, pentamidine, itraconazole, flucytosine
Prognosis	Fair if diagnosed early (within days); otherwise poor – a few patients have survived	Poor; diagnosis is often postmortem – only a few patients have survived	Excellent with early therapy	Poor; diagnosis is often postmortem – three patients have survived	Patient survived

*All information presented based on a single case.

ADEM, acute disseminated encephalomyelitis; CFS, cerebrospinal fluid; CNS, central nervous system; CT, computerized tomography; IFA, immunofluorescent antibody staining; IIF, indirect immunofluorescent staining of tissue sections; MRI, magnetic resonance imaging; PCR, polymerase chain reaction; PHMB, polyhexamethylene biguanide; PMN, polymorphonuclear leukocyte.

A unique and characteristic feature of *Acanthamoeba* spp. is the presence of fine, tapering, thorn-like acanthopodia that arise from the surface of the body. The trophozoites range in size from 15 to 50 μm depending upon the species. They are uninucleate, and the nucleus has a centrally placed, large, densely staining nucleolus. The cytoplasm is finely granular and contains numerous mitochondria, ribosomes, food vacuoles, and a contractile vacuole. When food becomes scarce, or when it is facing desiccation or other environmental stresses, the amoebae round up and encyst. Cysts are double-walled and range in size from 10 to 25 μm. The outer cyst wall, the ectocyst, is wrinkled with folds and ripples and contains protein and lipid. The inner cyst wall, the endocyst, contains cellulose and hence is Periodic acid-Schiff (PAS)-positive. The endocyst varies in shape: it may be stellate, polygonal, oval, or spherical. Pores or ostioles that are covered by convex–concave plugs or opercula are present at the junction of the ectocyst and the endocyst. Cysts are uninucleate and possess a centrally placed dense nucleolus (Fig. 1a and b). Upon return to favourable growth conditions, the dormant amoeba is activated to leave the cyst by dislodging the operculum and reverting to a trophic form (Page, 1967). In either the trophic or the cyst stage these organisms have a wide distribution in nature, and it is virtually impossible not to isolate members of this genus from soil, water and other samples.

Acanthamoeba spp. are ubiquitous and occur worldwide. They have been isolated from soil, fresh and brackish waters, bottled mineral water, cooling towers of electric and nuclear power plants, heating, ventilating and air conditioning units, humidifiers, Jacuzzi tubs, hydrotherapy pools in hospitals, dental irrigation units, dialysis machines, dust in the air, bacterial, fungal and mammalian cell cultures, contact-lens paraphernalia, ear discharge, pulmonary secretions, swabs obtained from nasopharyngeal mucosa of patients with respiratory complaints as well as of healthy individuals, maxillary sinus, mandibular autografts, and stool samples. In addition, several *Acanthamoeba* species have been isolated from the brain, lungs, skin, and cornea of infected individuals. *Acanthamoeba* spp. are tolerant of a wide range of osmolarity, enabling them to survive in distilled water, tissue culture media, mammalian body fluids, and sea water (Martinez, 1985; Martinez & Visvesvara, 1997; Schuster & Visvesvara, 2004a; Visvesvara & Maguire, 2006).

Acanthamoeba spp. have recently received much attention because they are known to serve as hosts for bacterial pathogens such as *Legionella* spp., and potentially for a large number of pathogenic bacteria including *Francisella tularensis*, *Mycobacterium avium, Burkholderia spp.*, *Vibrio cholerae*, *Listeria monocytogenes*, *Helicobacter pylori*, *Afipia felis* and *Escherichia coli* serotype O157 (Greub & Raoult, 2004; Berger *et al.*, 2006). Although *Legionella* has complex growth

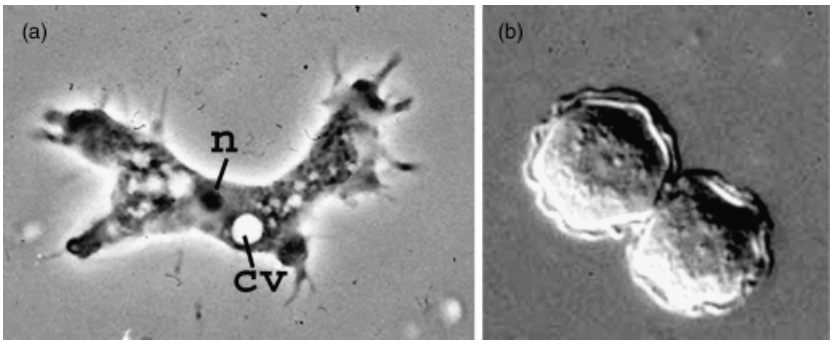

Fig. 1. *Acanthamoeba castellanii*, trophozoite (a) and cysts (b): n, nucleus; cv, contractile vacuole. Both images at × 1000.

requirements, it survives in nutritionally deficient environments, perhaps as a result of an association with amoebae or other protists. *Legionella* is an intracellular parasite of human mononuclear phagocytes and, in the course of infection, destroys its host cells. It apparently does the same to *Acanthamoeba*, proliferating within phagosomes after uptake and causing lysis of amoebae (Gao & Kwaik, 2000). Approximately 20–24% of clinical and environmental isolates of *Acanthamoeba* harbour intracellular bacteria (Fritsche *et al*., 2000). While most of these reports have been based on *in vitro* studies, *Acanthamoeba* infected with *Legionella*-like bacteria has been isolated from soil samples (Newsome *et al*., 1998). It has also been shown that *Acanthamoeba* harbouring *M. avium* were more virulent than noninfected amoebae in a mouse model of infection (Cirillo *et al*., 1997). Furthermore, pure cultures of *Acanthamoeba polyphaga* have been used to isolate *L. pneumophila* (Rowbotham, 1998), *L. anisa* (La Scola *et al*., 2001), and recently *Mycobacterium massiliense* (Adékambi *et al*., 2004) from human clinical specimens such as sputum, liver and lung abscesses, and even human faeces. Obligate intracellular pathogens such as *Chlamydia, Chlamydophila*, and *Chlamydia*-like bacteria have been found in *c*. 5% of *Acanthamoeba* isolates, and *Chlamydophila pneumophila*, a respiratory pathogen, can survive and grow within *Acanthamoeba* (Fritsche *et al*., 2000). Moreover, *Acanthamoeba* has also been known to harbour echo virus (Schuster & Visvesvara, 2004a). Recently, a mimivirus, a virus about the size of a small bacterium with a 1.2-megabase genome, was discovered in *A. polyphaga* (Raoult *et al*., 2004). Whether endocytobiont-bearing *Acanthamoeba* strains can serve as environmental reservoirs for these bacteria and viruses, some of which are potential pathogens for humans, is unknown. A feature common to many of the prokaryotes that can develop within amoebae is that they are obligate or facultative parasites of human phagocytic cells, suggesting that pathogenesis for humans may have evolved from infection of amoebae and perhaps other protists, and that amoebae may have an important role in the etiology of certain bacterial diseases in the community and in a nosocomial setting (Schuster & Visvesvara, 2004a).

Cultivation

Acanthamoeba spp. can be easily cultivated in the laboratory on non-nutrient agar plates coated with bacteria such as *Escherichia coli* or *Enterobacter aerogenes*. They show a preference for bacteria that are not encapsulated or pigmented: the mucoid capsule inhibits phagocytosis by the amoebae, and bacterial pigments are often toxic. The amoebae will feed on edible bacteria, multiply, and completely cover the surface of the plates within a few days. Abundant growth occurs in a dilute peptone–yeast extract–glucose medium (at concentrations of, for example, 0.05, 0.05, 0.1% w/v, respectively), solidified with agar (1.5%) and seeded with bacteria. A suitable bacterial source can be applied to the agar surface, either as a streak or spread as a lawn, and the amoebae inoculated onto the agar surface. At an early growth stage, amoebae produce plaque-like clearings in the bacterial lawn, ultimately encysting after most of the bacteria have been consumed. Cysts remain viable for prolonged periods of time, especially if the agar plate is sealed to prevent drying and kept at a reduced temperature. They can be maintained in the laboratory indefinitely by periodically cutting out a small piece of agar containing trophozoites and/or cysts and transplanting it onto a fresh agar plate coated with bacteria as before. The use of non-nutrient agar covered with a bacterial lawn is the recommended technique for isolation of amoebae both from clinical specimens such as brain tissue and corneal scrapings, and from environmental soil and water samples. *Acanthamoeba* can also be readily established in bacteria-free or axenic culture by harvesting amoebae from a bacterized culture, washing them by centrifugation in distilled water or saline to eliminate most of the bacteria, and then inoculating the washed cells to a suitable growth medium containing antibiotics. A combination of penicillin – streptomycin or gentamicin is effective in inhibiting or killing any residual bacteria present in the amoeba inoculum. An enriched medium with higher concentrations of proteose peptone – yeast extract – glucose has been successfully used to grow these amoebae. A number of the isolates from human *Acanthamoeba* infections appear to require further medium

enrichment with calf serum, and some strains are refractory to bacteria-free growth. In addition, *Acanthamoeba* spp. can be cultured on mammalian cell cultures as well as on a chemically defined medium (Schuster, 2002).

Granulomatous amoebic encephalitis (GAE): clinical and laboratory diagnosis

GAE, as an opportunistic disease, affects hosts whose metabolic, physiological, or immunological integrity are compromised, and hence cases may occur at any time of the year with no pattern of seasonal occurrence. Several species of *Acanthamoeba* (*A. culbertsoni, A. castellanii, A. polyphaga, A. astronyxis, A. healyi* and *A. divionensis*) have been known to cause GAE, primarily in patients with HIV/AIDS or who are chronically ill, diabetic, have undergone organ transplantation or are otherwise debilitated with no recent history of exposure to recreational freshwater. A few cases, however, have been described from individuals with no obvious signs of immunodepression. Onset of GAE is slow and insidious and develops as a chronic disease spanning from several weeks to months (Martinez & Visvesvara, 1997; Schuster & Visvesvara, 2004a; Visvesvara & Maguire, 2006). The usual features of GAE consist of headache, stiff neck, and mental-state abnormalities, as well as nausea, vomiting, low-grade fever, lethargy, cerebellar ataxia, visual disturbances, hemiparesis, seizures and coma. Facial palsy with numbness resulting in facial asymmetry is often seen. Cerebral hemispheres are usually the most heavily affected CNS tissue. They are often edematous, with extensive hemorrhagic necrosis involving the temporal, parietal, and occipital lobes. Computerized tomography (CT) scans of the brain show large, low-density abnormalities mimicking a single or multiple space-occupying mass. Magnetic resonance imaging (MRI) with enhancements shows multiple, ring-enhancing lesions in the brain (Fig. 2a) (Shirwadkar *et al.*, 2006). *Acanthamoeba* spp. also cause infections of the CNS of animals, including gorillas, monkeys, dogs, ovines, bovines, horses, and kangaroos as well as birds, reptiles, amphibians, fishes and even invertebrates (Visvesvara & Stehr-Green, 1990; Dyková *et al.*, 1999; Visvesvara & Maguire, 2006). Recently, an *Acanthamoeba* that was isolated from the liver of a toucan and grew at 44 °C was typed as a member of the T4 genotype, a group that contains most of the human pathogenic genotypes (Visvesvara *et al.*, 2007). These studies, based on sequencing of the small subunit rRNA gene, have shown that several *Acanthamoeba* isolates from fish, reptiles and those associated with human *Acanthamoeba* keratitis infections belong to the same T4 phylogenetic grouping, suggesting that features that enable these amoebae to infect animals may also help them to infect humans (Visvesvara *et al.*, 2007).

Acanthamoeba spp. infecting the CNS are not readily found in the cerebrospinal fluid (CSF), although they have been isolated from the CSF in a few cases. In one case *Acanthamoeba* was isolated from the CSF of a Nigerian patient and on two occasions from Indian patients (Visvesvara & Stehr-Green, 1990). *Acanthamoeba* that had apparently entered from the nasopharynx through a fistula have

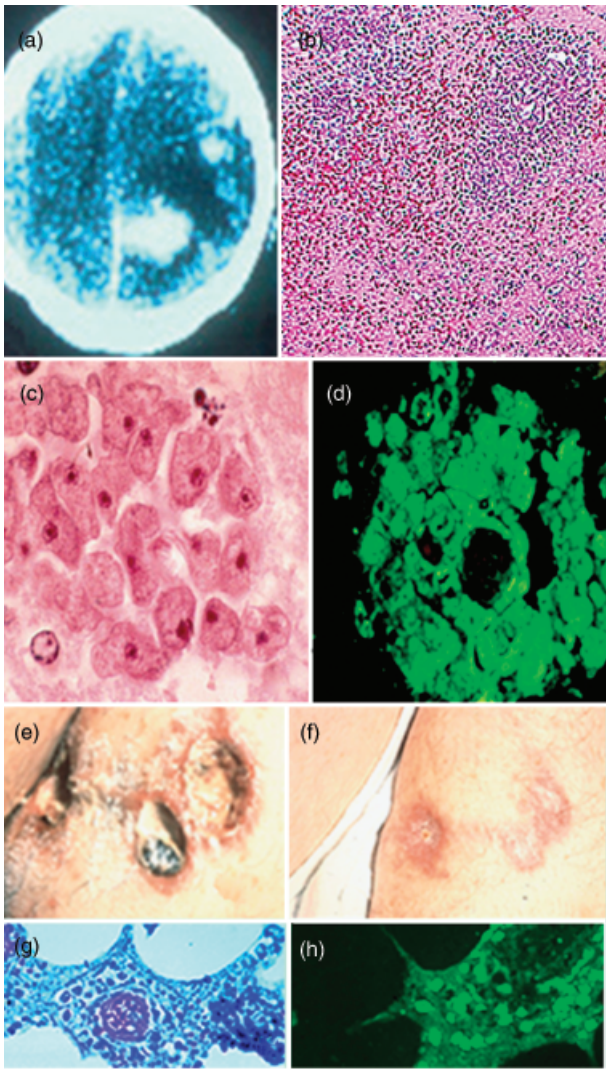

Fig. 2. Pathological features of encephalitis and skin infections caused by *Acanthamoeba* spp. (a) CT scan of a patient with *Acanthamoeba* GAE showing a space-occupying mass. (b) A section of the brain depicting inflammatory exudate consisting of polymorphonuclear leukocytes, lymphocytes and red blood cells with scattered *Acanthamoeba* trophozoites (×100). (c) A higher magnification (×1000) of an area of the cortical region with a cluster of *Acanthamoeba* trophozoites. (d) A fluorescing (bright green) cluster of *Acanthamoeba* trophozoites in an area similar to the one shown in (c) that reacted with anti-*A. castellanii* serum in the immunofluorescence test. (e) Skin ulcer caused by *Acanthamoeba* in an immunocompromised patient. (f) Skin ulcer that completely healed after several months of treatment as described in the text. (g) A hematoxylin-eosin-stained section of the skin ulcer with *Acanthamoeba* trophozoites and cysts. (h) The same area as in (g) stained with anti-*A. castellanii* serum. The amoebae emit an intense green fluorescence.

been detected in the CSF of a patient without CNS disease (Petry *et al.*, 2006). CSF examination in general reveals lymphocytic pleocytosis with mild elevation of protein and normal or slightly depressed glucose.

Pathophysiology of *Acanthamoeba* infections

Examination of the autopsied brain reveals cerebral edema, areas of cortical and basal ganglia softening, and multiple necrotic and hemorrhagic areas of CNS tissues (Fig. 2b). The brainstem, cerebral hemispheres and cerebellum may show areas of hemorrhagic infarcts. Histological examination reveals the presence of multinucleated giant cells in the cerebral hemispheres, brain stem, mid-brain, cerebellum, and basal ganglion. Necrotic tissue with lipid-containing macrophages and neovascularization suggesting a tumour is often seen. Amoebic trophozoites and cysts are usually scattered throughout the tissue. Many blood vessels are thrombotic with fibrinoid necrosis and cuffed by polymorphonuclear leukocytes, amoebic trophozoites, and cysts (Fig. 2c) that can be clearly identified by immunohistochemical techniques (Fig. 2d). Multinucleated giant cells forming granulomas may be seen in immunocompetent patients but less often in immunocompromised patients. Some patients, especially those with HIV/AIDS, develop chronic ulcerative skin lesions, abscesses, or erythematous nodules (Martinez & Visvesvara, 1997; Schuster & Visvesvara, 2004a; Visvesvara & Maguire, 2006), especially of the chest and limbs. These nodules are usually firm and nontender but sometimes they become ulcerated and purulent. Furthermore, reports of several cases with multiple skin ulcers (Fig. 2e and f) but without dissemination to the CNS also exist (Visvesvara & Stehr-Green, 1990; Slater *et al.*, 1994).

Amoebic trophozoites and cysts are found within the CNS tissues, pulmonary parenchyma, prostate, and skin lesions (Fig. 2g). The amoebae can be distinguished from host cells by their prominent central nucleolus and by their location in the perivascular areas in brain tissue (Fig. 2c). Definitive identification of the amoebic genus is based on the visualization of trophic or cystic stages in immunofluorescence-stained brain tissue sections (Fig. 2d and h), because *Balamuthia* also produces cysts in the brain tissue of infected individuals.

The prodromal period is unknown and several weeks or months may elapse following infection before the disease becomes apparent. Because of the time delay, the precise portal of entry is not clearly known, but the wide dissemination of these amoebae in the environment allows for many possible contacts and modes of infection. Trophic amoebae and/or cysts of *Acanthamoeba* have been isolated from the nasal mucosa of healthy individuals, suggesting a nasopharyngeal route as one means of invasion. Amoebae may also enter the body through breaks in the skin, resulting in hematogenous dissemination to the lungs and brain, or by inhalation of amoebic cysts (Visvesvara & Maguire, 2006). A skin biopsy may demonstrate amoebic trophozoites and cysts (Fig. 2g and h).

Immunology

Because *Acanthamoeba* are ubiquitous in nature, contact of humans and other animals with trophic amoebae, cysts or their antigens elicits antibodies to these amoebae in sera. Whether this natural antibody results in protective immunity against infection is unknown. Previous studies have shown that antibodies to *Acanthamoeba* exist in sera of healthy soldiers as well as of hospitalized patients in Czechoslovakia, adults and children from New Zealand, and patients hospitalized for respiratory problems (Schuster & Visvesvara, 2004a). Antibodies to *Acanthamoeba* have also been demonstrated in patients who developed GAE and/or skin infections (Martinez, 1985; Martinez & Visvesvara, 1997; Schuster & Visvesvara, 2004a; Visvesvara & Maguire, 2006). For example, a Nigerian patient from whose CSF *A. rhysodes* was repeatedly isolated exhibited an increase in titre from 1 : 256 to 1 : 1024 against the amoeba in serum samples collected 16 months apart. An antibody titre for *A. castellanii* of 1 : 512 was detected in an immunocompromised patient with systemic lupus erythematosus who developed *Acanthamoeba* encephalitis. High titres (1:256) of antibody to *A. castellanii* were also demonstrated in an acute serum sample from a renal transplant patient with cutaneous acanthamoebiasis who had been taking immunosuppressive medications (Slater *et al.*, 1994). It is interesting to note that complement-fixing antibody to *A. culbertsoni* was demonstrated in two of 1000 serum samples collected randomly in the United States. One of the serum samples, in particular, was from a patient with an old brain infarct. The initial serum sample had a complement fixation titre of 1:8 and this rose to 1:16 and 1:64 in subsequent serum samples taken 1 and 2 months, respectively, after the initial sample was collected. The patient later died of cerebral hemorrhage, and amoebae were demonstrated in the brain sections, but unfortunately the exact identity of the amoeba was not determined (Visvesvara & Stehr-Green, 1990). A low antibody titre of 1:16 to *Acanthamoeba* was reported in an encephalitic patient with systemic lupus, although on autopsy amoebae were detected by immunoperoxidase staining using rabbit anti-*Acanthamoeba* serum; *A. polyphaga* was also isolated from brain tissue (Bloch & Schuster, 2005). The low antibody titre was presumably a result of the use of high-dose anti-inflammatory agents in treating the disease. According to another limited study, Hispanics are 14.5 times less likely to develop antibodies to *Acanthamoeba,* especially *A. polyphaga,* than Caucasians (Chappell *et al.*, 2001).

In vitro studies have demonstrated that *Acanthamoeba* activates the alternative complement pathway, which is independent of antibody activation. In an immunocompetent host, immunogloblins and complement components promote recognition of these amoebae by neutrophils, macrophages and probably lymphocytes. The alternative complement pathway and antibody formation, two important defence mechanisms, stimulate neutrophils that release lysosomal enzymes and reactive oxygen intermediates, including hypochlorite and hydrogen peroxide, which in turn promote the destruction of amoebae. Although the specific macrophage factors responsible for killing *Acanthamoeba* are not yet clearly known, immunomodulator-activated macrophages are able to phagocytize and destroy amoebae (Marciano-Cabral & Cabral, 2003). However, in some of the reported cases of GAE caused by *Acanthamoeba*, a typical granulomatous reaction, which is an indicator of cell-mediated immunity, is not seen. The amoeba itself may induce macrophage toxicity, which is an important factor in producing granulomatous reaction (Martinez, 1985).

Mechanisms of pathogenesis

Much of the damage done by *Acanthamoeba* trophozoites in the course of corneal or brain infections is probably the result of several different pathogenic mechanisms. It was believed that *Acanthamoeba* lacked the specialized feeding cups (amoebastomes) that are found in *Naegleria* amoebae (see below), although phagocytosis of host cells is undoubtedly involved in damage to host tissues. Pettit *et al.* (1996) showed by scanning and transmission electron microscopy, however, that several species of *Acanthamoeba* do indeed produce food cups while ingesting rat B103 neuroblastoma cells, and subsequently channel the ingested material into intracytoplasmic food vacuoles. More recent studies have confirmed this by showing that *Acanthamoeba* undergoes certain morphological changes when associated with hamster cornea *in vitro*, and produces amoebastome-like (food cup) surface structures that ingest detached epithelial cells and thus aid in phagocytosis (Omaña-Molina *et al.*, 2004). Several studies have described enzymes secreted by *Acanthamoeba*, which may facilitate the spread of amoebae by opening avenues for invasion, and providing nutrients in the form of lysed host cells. In one of the earliest studies it was shown that the pathogenic species *A. culbertsoni* excretes more phospholipase enzyme into the culture medium than does the nonpathogenic *A. rhysodes*. Moreover, small amounts (5 $\mu g\,mL^{-1}$) of the crude enzyme preparation from *A. culbertsoni* culture supernatant produced cytopathic effects on cell cultures, whereas a similar preparation from *A. rhysodes* did not (Visvesvara & Balamuth, 1975). In another study, serine protease activity was found in pathogenic isolates but to a lesser extent or not at all in nonpathogenic isolates (Khan, 2006). Subsequently, a number of other studies have shown that acanthamoebae, depending on the species and strains studied, produce serine and cysteine proteinases, metalloproteinases, plasminogen activators and exhibit chemotactic responses to corneal endothelial extracts (Sissons *et al.*, 2006).

In vitro studies using rabbit corneal epithelium have shown that mannose, but not other sugars, inhibits the binding of *Acanthamoeba* trophozoites to the epithelial cells. In addition, more recent studies using *Acanthamoeba* isolates from keratitis cases have shown that the initial process of invasion occurs when a 136-kDa mannose-binding protein (MBP) expressed on the surface of the amoeba adheres to mannose glycoproteins on the surface of the epithelial cells (Clarke & Niederkorn, 2006; Garate *et al*, 2006b). The various proteinases then kick in and facilitate the invasion and destruction of the corneal stroma. The cytopathic effect (CPE) that is seen *in vitro* when *Acanthamoeba* is inoculated into tissue culture monolayers is probably the result of the activity of these proteinases. It is MBP that mediates the adhesion of the amoeba to corneal epithelial cells and is central to the pathogenic potential of *Acanthamoeba*. This lectin consists of a large extracellular domain, a single transmembrane domain, and a short cytoplasmic domain that is located at the C-terminus. An anti-MBP-immunoglobulin A (IgA) produced by oral immunization of Chinese hamsters was detected in the tear fluid, and this antibody protected the animals from *Acanthamoeba* keratitis (Garate *et al.*, 2006a). In another study, the blood–brain barrier permeability was increased by more than 45% by *A. castellanii*, an encephalitis isolate belonging to the T1 genotype, or by the conditioned medium (Alsam *et al.*, 2005). This activity was blocked by phenylmethyl sulfonyl fluoride (PMSF), identifying it as a serine protease. A recent paper has described the presence of a serine proteinase gene in a highly pathogenic keratitis isolate (*A. hatchetti*, genotype T6). This gene expresses serine proteinases with a molecular weight (MW) of 33 kDa, as in *Acanthamoeba* T4 and T12 genotypes, that are blocked by PMSF, and a second proteinase with a MW of 20 kDa that is not completely inhibited by PMSF (Blaschitz *et al.*, 2006). Two major proteases with MWs of *c.* 150 and 130 kDa have also been described (Sissons *et al.*, 2006). The 150-kDa protease was inhibited by PMSF, identifying it as a serine protease, and the 130-kDa protease was inhibited by 1,10-phenanthroline, indicating that it is a metalloprotease. These proteases may digest extracellular matrix and thus may play a crucial role in the invasion of brain tissue. In another study, it was suggested that a serine proteinase produced by a GAE isolate of *A. healyi* might aid in complement destruction and degradation of human IgA antibody (Clarke & Niederkorn, 2006). *In vivo* experiments indicate that even large numbers of *Acanthamoeba* trophozoites when injected into the eye of

Chinese hamsters are eliminated within 15 days because of an intense neutrophilic infiltrate (Clarke & Niederkorn, 2006).

Based on *in vitro* studies it has been shown that macrophages and brain microglial cells play a role in the killing of *Acanthamoeba* and that the microglial cells produce a variety of interleukins (IL-1a and b, and tumor necrosis factor) when cultured with *Acanthamoeba*. The macrophage-mediated killing is probably contact-dependant (Clarke & Niederkorn, 2006).

Acanthamoeba keratitis: clinical and laboratory diagnosis

Acanthamoeba keratitis (AK) is a painful vision-threatening infection caused by these amoebae. If the infection is not treated promptly, it may lead to ulceration of the cornea, loss of visual acuity, and eventually blindness and enucleation (Jones *et al.*, 1975; Seal, 2003). AK is associated with trauma to the cornea or contact-lens wear and the use of amoeba-contaminated saline. Minor erosion of the corneal epithelium may occur while wearing hard or soft contact lenses, and the subsequent use of contaminated saline solution is the major risk factor for *Acanthamoeba* keratitis. AK is characterized by inflammation of the cornea, severe ocular pain and photophobia, a characteristic 360° or paracentral stromal ring infiltrate, recurrent breakdown of corneal epithelium, and a corneal lesion refractory to the commonly used antibiotics (Fig. 3a and b). Typically, only one eye is involved; however, bilateral keratitis has also been reported. Unlike GAE, it occurs in immunocompetent individuals following corneal trauma or more commonly as a result of poor hygiene in the care of contact lenses or contact-lens cases. No case of GAE has been reported to have developed from a case of AK, although a case of uveitis was associated with fatal GAE. The condition may be confused with viral or fungal keratitis and is often misdiagnosed as dendritic keratitis resulting from the herpes simplex virus, with a resultant delay in initiating the appropriate therapy to eliminate the amoebae. A nonhealing corneal ulcer is often the first clue that *Acanthamoeba* keratitis may be the problem.

More than 30 cases of AK were identified recently from the Chicago (Illinois) area alone (Joslin *et al.*, 2006). It is estimated that as of August 2006 more than 5000 cases of AK have occurred in the United States. Because AK is not a reportable disease in the United States, the actual number is not known and may be even higher. Large numbers of cases have also been reported from the United Kingdom and India (Sharma *et al.*, 2000; Seal, 2003). Wearers of contact lenses are a high-risk group for AK, especially in the United Kingdom and the United States. However, in India corneal trauma is the usual cause of AK (Sharma *et al.*, 2000). The majority of cases in contact-lens users result from the use of nonsterile tap water in the preparation of contact-lens solutions, and/or failure to keep lens storage cases clean. Introduced into the eye by a contaminated contact lens, amoebae adhere to the corneal surface, perhaps aided by specific receptors such as the mannose receptor (see above) that enable them to bind to corneal epithelium and by the presence of calcium ions. Once attached, the amoebae infiltrate the stroma and cause tissue necrosis (Clarke & Niederkorn, 2006).

The first case of AK in the United States occurred in 1973 in a south Texas rancher following trauma to his right eye (Jones *et al.*, 1975). Both trophozoites and cyst stages of *A. polyphaga* were demonstrated in corneal sections and were repeatedly cultured from corneal scrapings and biopsy specimens. Between 1973 and 1986, 208 cases were diagnosed and reported to the Centers for Disease Control and

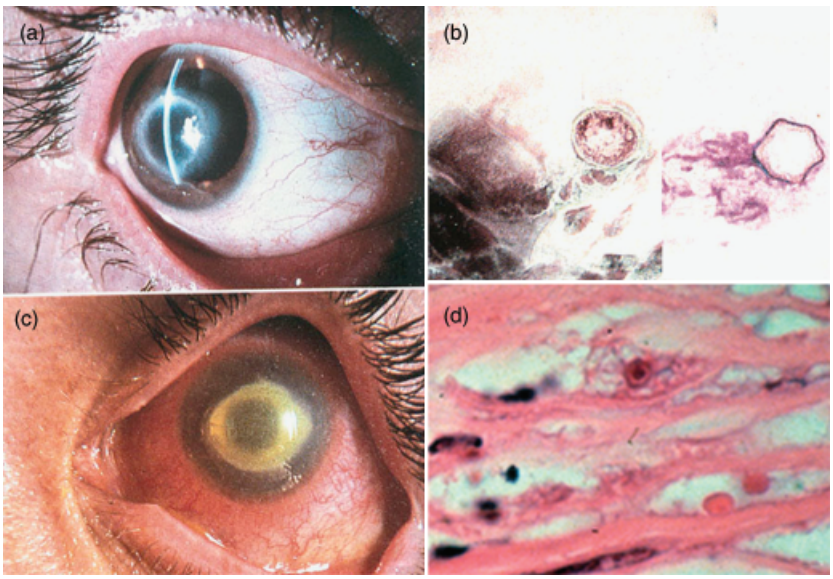

Fig. 3. *Acanthamoeba* keratitis. (a) Slit-lamp examination of a patient's eye showing a 360° infiltrate, a characteristic feature of the disease. (b) A patient's eye depicting the perforation of the cornea. (c) A corneal scraping stained with trichrome shows the presence of an *Acanthamoeba* trophozoite and a cyst, ×1000. (d) A hematoxylin-eosin-stained section of debrided corneal tissue showing a trophozoite, ×1000.

Prevention (Visvesvara & Stehr-Green, 1990). The number of cases increased gradually between 1973 and 1984, and a dramatic increase began in 1985, reflecting the increased popularity of contact lenses. An in-depth epidemiological and case-control study revealed that a major risk factor was the use of contact lenses, predominantly daily-wear or extended-wear soft lenses, and that patients with AK were significantly more likely than controls to use homemade saline solution instead of commercially prepared saline (78% and 30%, respectively). Likewise, AK victims disinfected their lenses less frequently than recommended by the lens manufacturers (72% and 32%, respectively), and were more likely to wear their lenses while swimming (63% and 30%, respectively). In the early stages of AK the anterior cornea is destroyed by the invading *Acanthamoeba* trophozoites. Amoebic trophozoites and cysts are seen infiltrated between the lamellae of the cornea. Infiltration of inflammatory cells consisting primarily of polymorphonuclear leukocytes into the superficial and middle layers of the corneal stroma is also commonly seen. During later stages of the disease process, AK is characterized by ulceration, descemetocele formation, and perforation of the cornea. *Acanthamoeba* trophozoites and especially cysts can be recovered from corneal scrapings or biopsy. Definitive diagnosis is based on visualization of amoebae upon microscopic examination of corneal scrapings or biopsies or on their cultivation from affected tissues (Fig. 3c and d). More recently, confocal microscopy has been used as an aid in the diagnosis of AK (Joslin *et al.*, 2006; Parmar *et al.*, 2006). Amoebae isolated from keratitis infections generally have lower temperature optima than GAE isolates, consistent with their superficial location. Immunoblotting was used to test sera from keratitis patients as well as from non-AK patients for anti-*Acanthamoeba* antibodies. Significantly, sera from keratitis patients had low levels of IgA, a secretory antibody, indicating perhaps greater susceptibility to AK (Clarke & Niederkorn, 2006). In an earlier study, immunofluorescent and precipitin antibodies to *Acanthamoeba* were demonstrated in patients with AK (Jones *et al.*, 1975; Clarke & Niederkorn, 2006).

Molecular characterization

Molecular techniques are increasingly being used in the identification of the genotype of *Acanthamoeba* as well as in the identification of the amoeba in corneal specimens, including the corneal epithelium and tear fluid. Conventional PCR protocols have been used in detecting *Acanthamoeba* in corneal scrapings obtained from AK cases as well as from GAE cases and the environment (Schuster & Visvesvara, 2004a; Booton *et al.*, 2005). Based on 18S rRNA gene sequencing, most of the *Acanthamoeba* isolates, both keratitis and non-keratitis (non-AK) sources, have been typed as the T4 genotype. Booton *et al.* (2005) reported that, out of a total of 249 isolates examined, 72% (179/249) were typed as genotype T4. Of these 179 isolates identified as T4, 53% came from the environment, 94.3% from keratitis patients, and 79.3% from non-AK sources. The non-AK isolates were isolated from CSF, brain, nasal passages, lungs and skin. A few *Acanthamoeba* isolates from the brain have been typed as belonging to rare genotypes (T1, T10, and T12), and at least two of these rare genotypes (T10 and T12) have yet to be isolated from the environment (Booton *et al.*, 2005). Moreover, of the few non-T4 keratitis isolates, three isolates from Iran have been typed as T2 and 2 as T3, one each from the UK as T3 and T12, and one isolate from Austria as T6 (Magshood *et al.*, 2005; Blaschitz *et al.*, 2006). Research is underway to differentiate more precisely the group II and/or sequence type T4 by examining variability within the internal transcribed spacer 1 (ITS1) sequence, and analysis of GC contents and microsatellites (Köshler *et al.*, 2006). A real-time PCR assay for the detection of *Acanthamoeba* in keratitis patients has also been developed. This is a TaqMan assay specific for *Acanthamoeba* species (Riviére *et al.*, 2006). However, this assay can identify only genotypes T1 and T4 and lacks the ability to identify the two other genotypes T7 and T10 known to cause GAE. A multiplex real-time PCR assay has recently been developed that can detect not only several genotypes of *Acanthamoeba* but also *Balamuthia* and *N. fowleri* in human specimens (Qvarnstrom *et al.*, 2006). The multiplex assay is of value for the simultaneous detection of all three pathogenic free-living amoebae in clinical specimens as well as in environmental samples.

During the last few years a practical and rapidly evolving method, matrix-assisted laser desorption-ionization time-of-flight MS (MALDI-TOF MS), has been used not only to identify protists but also to tabulate strain differences based on characteristic protein patterns (biomarkers) (Moura *et al.*, 2003a). Using MALDI-TOF, at least 20 biomarker fingerprints of *Acanthamoeba*, *Balamuthia* and *N. fowleri* with molecular masses ranging from 2 to 14 kDa have been detected (Fig. 4). Because the entire procedure can be performed in 15 min, it too may be extremely useful in the rapid identification of these amoebae in biological samples (Moura *et al.*, 2003b).

Therapy and prognosis: GAE

Treatment of GAE is problematic because of the lack of clear-cut symptoms, the lack of a good reliable diagnostic test, and the fact that diagnosis is often made postmortem. However, several patients with GAE caused by *Acanthamoeba* spp., as well as some with *Acanthamoeba* cutaneous infection without CNS involvement, have been successfully treated with a combination of pentamidine isethionate, sulfadiazine, flucytosine, and fluconazole or itraconazole. For *Acanthamoeba* cutaneous infection without CNS involvement, topical

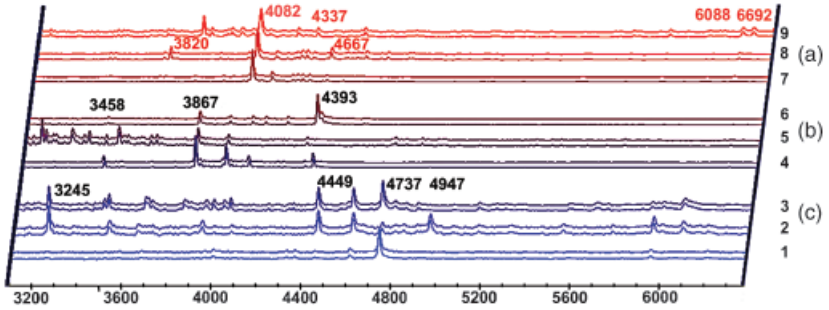

Fig. 4. SpecXMultiViewer depicting MALDI-TOF mass spectra in the range of 3000 – 8000 Da of whole cells of (a) three strains of *Acanthamoeba*, (b) three strains of *Balamuthia*, and (c) three human isolates of *Naegleria fowleri*. Each figure is the average of 20 accumulated spectra acquired with 10^6 whole cells per well in a matrix. A key set of peaks was consistently seen in all wells. Spectra can be easily differentiated for each genus, and differences between isolates are evident among the studied species.

applications of chlorhexidine gluconate and ketoconazole cream in addition to the above-noted antimicrobials have resulted in therapeutic success. In many cases, however, therapy had to be discontinued because of undesirable side effects of the medications (Seijo Martinez *et al.*, 2000; Schuster & Visvesvara, 2004b). A combination of factors – late diagnosis, suboptimal efficacy of antimicrobial therapy, and problems inherent to the immunocompromised host – make for a poor prognosis for GAE patients.

Therapy and prognosis: AK

Treatment of *Acanthamoeba* keratitis has been fairly successful. A variety of drugs have been used, including chlorhexidine, polyhexamethylene biguanide, propamidine isethionate, dibromopropamidine isethionate, neomycin, paromomycin, polymyxin B, clotrimazole, ketoconazole, miconazole, and itraconazole (Schuster & Visvesvara, 2004b). Brolene, a commercially available eye medication (in Great Britain) containing propamidine isethionate and dibromopropamidine isethionate, was found to be effective in the treatment of *Acanthamoeba* infections but may be accompanied by drug toxicity and development of resistance. A number of compounds, including a variety of diamidine compounds, synthetic maganins combined with silver nitrate, imidazole and triazole compounds, azithromycin, phenothiazines and povidone-iodine have been screened *in vitro* for efficacy against *Acanthamoeba* spp. Recently, voriconazole, a triazole compound, was tested on four clinical isolates of *Acanthamoeba* and was found to be inhibitory even at a concentration of 5 $\mu g\,mL^{-1}$. However, the amoebae recovered from the effects of the drug upon transfer to a drug-free medium after a week or more (Schuster *et al.*, 2006a). Another drug, miltefosine, a hexadecylphosphocholine, has also been shown to have amoebacidal potential (Walochnik *et al.*, 2002; Schuster *et al.*, 2006a). Significantly, medical cure has been achieved with the application of either polyhexamethylene biguanide (PHMB) or chlorhexidine gluconate with or without Brolene (Seal, 2003; Schuster & Visvesvara, 2004b). When medical treatment failed, a combination of debridement and penetrating keratoplasty has been used with good results in some cases. Currently, the drugs of choice for AK are chlorhexidine gluconate, PHMB and Brolene, and they have greatly improved the prognosis for AK sufferers (Seal, 2003).

Prevention and control

Because GAE caused by *Acanthamoeba* spp. occurs in hosts with weakened immune functions, no clearly defined methods exist for the prevention of infection with these amoebae. In the case of AK, however, because contact lenses and lens-care solutions are well-known risk factors, educating lens wearers regarding the proper care of contact lenses (and contact-lens cases) is important for the prevention of infection. Moreover, lens wearers should be instructed not to wear contact lenses during swimming, performing water-sport activities, or relaxing in a hot tub or Jacuzzi.

Balamuthia mandrillaris

Over the years, reports have appeared in the literature of encephalitis caused by amoebae that produce cysts in the brain. Although these amoebae could not be definitively identified as *Acanthamoeba* or *N. fowleri* by immunofluorescent staining, they were considered to be *Acanthamoeba* simply because cysts were seen in the brain sections. In 1986, however, an amoeba that was morphologically different from *Acanthamoeba* was isolated from the brain of a pregnant mandrill baboon. This amoeba also produced cysts in the brain and was initially described as a leptomyxid amoeba (because of the resemblance to soil amoebae included under the family Leptomyxidae), but in 1993 it was recognized as being different from the leptomyxids and was designated as a new genus and species, namely *Balamuthia mandrillaris* (Visvesvara *et al.*, 1990, 1993). An antibody raised in rabbits against this amoeba, when used in the indirect immunofluorescent (IIF) staining assay, not only reacted with the amoebae in the brain sections of the mandrill but also reacted with the amoebae in brain sections of all those cases that had tested negative with the anti-*Acanthamoeba* and *N. fowleri* antibodies, thus enabling the specific identification of the etiological cause of these cases. To date, *B. mandrillaris* is the only known species belonging to the genus *Balamuthia* and it causes GAE both in humans and in other animals. Balamuthiasis is

Fig. 5. *Balamuthia mandrillaris* trophozoite (a), and cyst (b). Both images at × 850.

similar to GAE caused by *Acanthamoeba,* and occurs in immunocompromised hosts, including HIV/AIDS patients and intravenous drug users; it has also been reported from immunocompetent individuals, especially young children and older individuals. The disease has a chronic, subacute phase that develops over a period of time spanning from 2 weeks to 2 years. *Balamuthia mandrillaris* is also known to cause skin infection, similar to that caused by *Acanthamoeba*. Cases of *Balamuthia* GAE may occur at any time of the year and therefore have no relation to seasonal changes (Martinez & Visvesvara, 2001; Visvesvara & Maguire, 2006). *Balamuthia* GAE infections have been recorded in a variety of mammalian species (Rideout *et al*., 1997; Schuster & Visvesvara, 2004a).

Balamuthia mandrillaris, like *Acanthamoeba*, has only two life-cycle stages, namely the trophozoite and the cyst (Fig. 5a and b). The trophozoite is pleomorphic and measures from 12 to 60 μm (mean of 30 μm). The trophic amoebae are usually uninucleate, although binucleate forms are occasionally seen. The nucleus contains a large, centrally placed, dense nucleolus; occasionally, however, amoebae with two or three nucleolar bodies have been seen, especially in infected tissues. Cysts are also uninucleate, are more or less spherical, and range in size from 12 to 30 μm (mean of 15 μm). Cysts, when examined with a light microscope, appear to be double-walled, the outer wall being wavy and the inner wall round, and pores are not seen in the wall. Ultrastructurally, however, the cyst wall has three layers: an outer thin and irregular ectocyst, an inner thick endocyst, and a middle amorphous fibrillar mesocyst (Visvesvara *et al*., 1993).

Cultivation

Balamuthia is a soil amoeba. Unlike *Acanthamoeba*, it cannot be cultured on agar plates coated with bacteria. Although *B. mandrillaris* has recently been isolated from the environment, its food source in nature is not clearly known (Schuster *et al*., 2003). *In vitro*, *Balamuthia* can feed on small amoebae (*Acanthamoeba* and *Naegleria*) and may do so in nature. Isolation from soil is further complicated by the relatively long generation times (>20 h) of *Balamuthia* isolates *in vitro*. Many other organisms (protozoa and fungi) in the environmental samples overgrow potential *Balamuthia* isolates. It can be isolated from human or animal tissue and grown in the laboratory using mammalian cell cultures such as monkey kidney (E6), human lung fibroblasts and human brain microvascular endothelial cells. The amoebae feed on the cell monolayer, decimating it within several days. It has also been grown axenically in a cell-free medium. Doubling times for three different clinical isolates ranged from 21 to 28 h in this medium. In culture, the organism is sensitive to changes in osmolarity and does not readily tolerate either hypo- or hyperosmotic conditions (Schuster, 2002). In a recent study, Matin *et al*. (2006) studied the interactions of *B. mandrillaris* with monkey kidney fibroblast-like cells (COS-7), human brain microvascular endothelial cells (HBMEC), *Acanthamoeba*, and *Escherichia coli*. According to the study, optimal growth of *Balamuthia* occurred on HBMEC as compared with COS-7 cells. *Balamuthia*, however, did not grow on *E. coli*, but when associated with *Acanthamoeba* trophozoites, *B. mandrillaris* caused partial damage to *Acanthamoeba*. The *Acanthamoeba*, however, encysted, and *Balamuthia* did not ingest *Acanthamoeba* cysts.

Diagnosis: clinical and laboratory methods

Like *Acanthamoeba* spp., *B. mandrillaris* is not readily recovered from the CSF. Only in one case has it been isolated from the CSF obtained at autopsy (Jayasekera *et al*., 2004). CSF examination in general reveals lymphocytic pleocytosis with mild to severe elevation ($\geq 1000\ \mathrm{mg\,dL^{-1}}$) of protein and normal or low glucose. Brain or skin biopsies are an important diagnostic procedure; both *Acanthamoeba* spp. and *B. mandrillaris* have been identified antemortem in brain biopsies from several patients. A few patients have survived the infection because of treatment with a combination of antimicrobials. In a large number of cases, however, final diagnosis was made only at autopsy. In general, *Acanthamoeba* spp. and *B. mandrillaris* are difficult to differentiate in tissue sections by light microscopy because of their similar morphology. However, they can be differentiated by immunofluorescence analysis of the tissue

sections using rabbit anti-*Acanthamoeba* or anti-*B. mandrillaris* sera. Transmission electron microscopy can also be performed on glutaraldehyde-fixed and plastic-embedded (Epon) specimens or by deparaffinizing formalin-fixed autopsy tissues followed by fixation in Karnovsky's fluid and embedment in Epon. Ultrastructure demonstrates the characteristic features of trophozoites (one to several nucleolar masses) and cysts (triple wall) and thus is helpful in the identification of the specific amoeba. Clinical specimens for culture should be processed as soon as possible. Freezing tissue samples may cause destruction of trophic amoebae, although cysts may survive the freezing process and *in vitro* cultures of several isolates have been established from frozen brain specimens. Because *Balamuthia* will not grow on bacteria-coated agar plates, biopsy specimens should be inoculated onto monolayers of mammalian cell cultures (Visvesvara *et al*., 1990, 1993; Rideout *et al*., 1997).

Mechanisms of pathogenesis

Little information is available on the pathogenic mechanisms of *B. mandrillaris*. Because the amoeba has the capability of invading the human and animal CNS it can be expected to possess certain enzymes, similar to those in *Acanthamoeba,* that will help in the process. Although enzymatic activity has been visualized by reacting the electrophoretically separated *Balamuthia* proteins with appropriate substrates and staining agents, it is not clear whether these enzymes have any activity with reference to pathogenicity (Visvesvara *et al*., 2001). Recent studies have indicated that *Balamuthia* induces human brain microvascular endothelial cells to release a pleiotropic cytokine, IL-6, which is known to play a role in initiating early inflammatory response (Jayasekera *et al*., 2005). Furthermore, metalloprotease activity, which may play an important role in the degradation of the extracellular matrix and thus in CNS pathology, has been shown in two isolates of *Balamuthia* (Matin *et al*, 2006). In an even more recent study it was shown that *B. mandrillaris* interacts with the extracellular matrix (ECM) proteins such as collagen-1, fibronectin and laminin-1, which are usually present in host connective tissue (Rocha-Azevedo *et al*., 2007). The study found that the binding activity of the amoebae on laminin was greater than that on collagen or fibronectin, and that this binding could be abolished by sialic acid. The study also showed, by scanning electron microscopy, that binding to ECM is associated with changes in the shape of the trophic amoebae and production of surface projections or food cups reminiscent of those seen with *N. fowleri* and *Acanthamoeba* spp.

Molecular characterization

Molecular techniques are currently being used not only to identify these amoebae but also to gain an understanding of the phylogeny and epidemiology of the genus *Balamuthia* (Booton *et al*., 2003a, b). A PCR probe consisting of a primer-pair specific for *Balamuthia* has been developed from sequence data of mitochondrial 16S rRNA genes. Using this probe, the DNA of a clinical isolate obtained from the brain of a child was found to be identical to that of an amoeba isolated from flowerpot soil in the home of the child (Schuster *et al*., 2003). PCR was also used to confirm *Balamuthia* GAE in a Portuguese boy (Tavares *et al*., 2006) and to corroborate immunofluorescent antibody diagnoses in clinical serum samples (Yagi *et al*., 2005). It has also been applied retrospectively to confirm *Balamuthia* infections in archived slide specimens from DNA extracted from tissue sections that were previously formalin-fixed and paraffin-embedded (Yagi *et al*., 2005). A real-time multiplex PCR assay has been developed that can specifically identify *Balamuthia* DNA in human CSF and brain tissue in about 5 h, which can contribute to more timely therapy (Qvarnstrom *et al*., 2006). Nevertheless, the key to timely diagnosis of balamuthiasis is a high degree of suspicion on the part of the attending physician, and familiarity by the pathologist with the appearance of the amoebae in brain and skin tissue sections.

Pathophysiology of *Balamuthia* infections

The disease profile and pathology of balamuthiasis are similar to those of *Acanthamoeba*. The amoebae are found in similar locations in the brain (Fig. 6a–d), causing hemorrhagic necrosis in the midbrain, thalamus, brainstem, and cerebellum. Unlike most cases of *Acanthamoeba* GAE, *Balamuthia* cases have occurred in children and older individuals with no known immunodeficiency (Schuster *et al*., 2004; Visvesvara & Maguire, 2006). Patients with *Balamuthia* GAE have presented with facial skin lesions and/or rhinitis with infections of the sinus cavities, or otitis media. Painless skin lesions appearing as plaques a few millimetres thick and one to several centimetres wide are seen in a large number of Peruvian patients. Most patients generally have a single lesion, but multiple lesions are also seen. The lesions are commonly seen in the centre of the face but occasionally they may occur on the trunk, and hands and feet (Bravo *et al*., 2006). In the Peruvian patients, as a rule, skin lesions precede CNS involvement.

The CNS symptoms manifest initially as headache and photophobia and later include nausea and vomiting, fever, myalgia, weight loss, and seizures. The time between the appearance of skin lesions and the onset of neurological symptoms may range from 1 month to about 2 years. A unique Peruvian case, however, did not progress to CNS involvement, although a skin biopsy from an ulcer on the face showed the presence of cysts. This Peruvian patient appeared to have spontaneously recovered without treatment. At a follow-up examination 8 years later the patient's

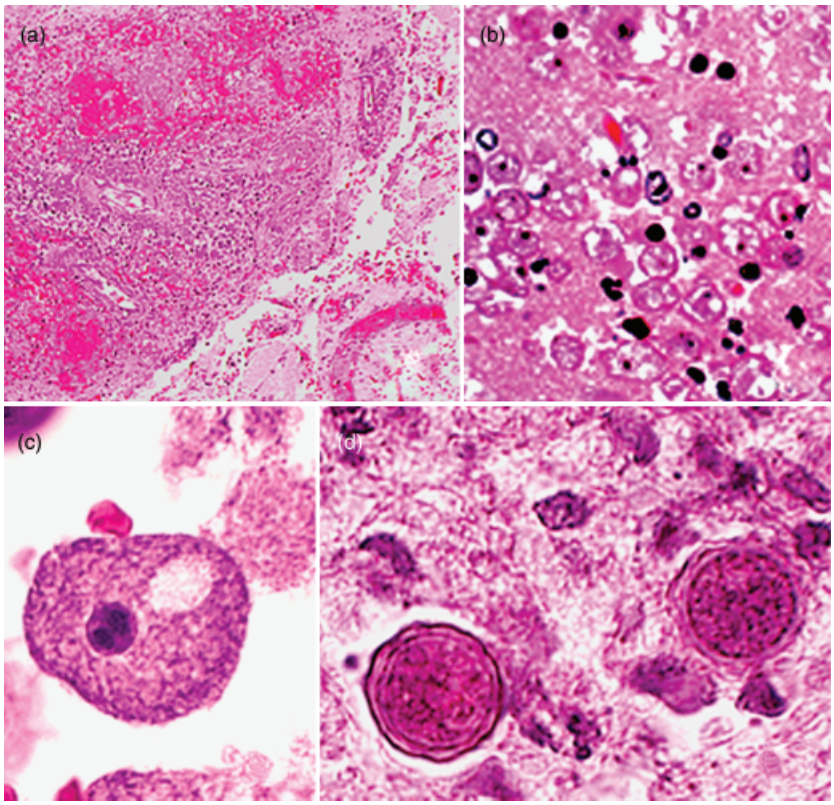

Fig. 6. Pathological features of *Balamuthia mandrillaris* GAE. (a) A section of the brain depicting inflammatory exudate consisting of polymorphonuclear leukocytes, lymphocytes and red blood cells along with scattered *Balamuthia* trophozoites and cysts (×100). (b) A higher magnification (×250) of an area of the cortical region with *Balamuthia* trophozoites interspersed in the brain tissue. (c) A high-power view of an area similar to the one seen in (b) showing a trophozoite with multiple nucleoli in the nucleus, a feature that is characteristic of *Balamuthia* trophozoites (×1000). (d) A high magnification image showing two cysts in a brain section (×1000).

skin lesion had healed and he appeared to be well and without any symptoms (Bravo *et al.*, 2006). As in acanthamoebiasis, the disease is of a chronic nature, developing slowly and insidiously. CSF findings can be informative: elevated protein, pleocytosis, low or normal glucose. CT and MRI scans of the head have been used and are important radiological tests. However, these scans only reveal the presence of single or multiple heterogeneous, hypodense, nonenhancing, 'space-occupying lesions', which may mimic a brain abscess, brain tumour, or intracerebral hematoma, and, hence, may not specifically identify the opportunistic amoebae. In North America, most balamuthiasis cases present with CNS symptoms, and some balamuthiasis patients have been erroneously diagnosed as having neurotuberculosis or neurocysticercosis, thus delaying initiation of appropriate antimicrobial therapy. In South America, because many of the balamuthiasis cases present with skin lesions, the differential diagnoses include leishmaniasis, sporotrichosis, lupus vulgaris and Wegener's granulomatosis (Bravo *et al.*, 2006).

Immunology

Antibodies to *B. mandrillaris* have been demonstrated in sera from patients with balamuthiasis as well as from healthy individuals using immunofluorescence and flow cytometry techniques. Antibodies of the IgG and IgM classes were detected with titres ranging from 1:64 to 1:256 in South Australia (Huang *et al.*, 1999). In another study, about 3% of *c.* 225 serum samples collected from patients hospitalized with encephalitis in California had titres of 1:128–1:256 for anti-*B. mandrillaris* antibody by immunofluorescence antibody (IFA) testing (Schuster *et al.*, 2006b). The Californian patients were subsequently confirmed as being infected with *B. mandrillaris* by specific immunostaining of biopsy sections using rabbit anti-*B. mandrillaris* serum and by PCR. In addition, about 10% of this series of patients had titres of 1:32 or greater, suggesting a humoral immune response to these amoebae or, possibly, cross-reactivity with other antigens (Schuster *et al.*, 2006b). Anti-*B. mandrillaris* titres of 1:128 in the acute phase that dropped to 1:64 in the convalescent serum stage were found in two Californian patients who survived balamuthiasis (Deetz *et al.*, 2003).

Balamuthia GAE has also been reported in a variety of animals including gorillas, baboons, gibbons, monkeys, horses, sheep and dogs (Rideout *et al.*, 1997; Visvesvara & Maguire, 2006). An animal model using severe combined immunodeficient (SCID) mice has been developed to study *Balamuthia* GAE (Janitschke *et al.*, 1996). Kiderlen & Laube (2004) showed that, when immunodeficient mice were infected intranasally with *B. mandrillaris*, the amoebae

could adhere to the nasal epithelium, migrate along the olfactory nerves, traverse the cribriform plate of the ethmoid bone, and subsequently invade the brain, which is similar to the invasion pathway described for *N. fowleri* (Kiderlen & Laube, 2004). Recent experiments, however, indicate that *Balamuthia* amoebae migrate to the CNS even after oral infection of both immunocompetent and immunodeficient mice, and *Balamuthia* antigen but not organisms have been found in mouse faecal pellets (Kiderlen *et al*., 2007).

Therapy and prognosis

Because most of the cases of *Balamuthia* GAE have presented with no clear-cut clinical profile, they have been treated empirically with steroids as well as with antibacterial, antifungal and antiviral agents with almost no effect upon the course of the infection. Anti-inflammatory steroids that were administered may have actually facilitated spread of the infection by suppressing the inflammatory response. Two patients from California, a 60-year-old man, and a 6-year-old girl, in addition to a 70-year-old woman from New York survived balamuthiasis after treatment with a combination of pentamidine isethionate, sulfadiazine, clarithromycin, fluconazole, and flucytosine (5-fluorocytosine) (Deetz *et al*., 2003; Jung *et al*., 2003). In the case of the Peruvian balamuthiasis patients with cutaneous lesions noted above, one recovered without any treatment and two others became well after prolonged therapy with albendazole and itraconazole. One of the patients had a large lesion on the chest wall that was surgically removed. Surgical excision of the lesion may have reduced the parasite load, thus aiding the recovery process (Bravo *et al*., 2006). The use of multiple antimicrobials in treatment makes it difficult to single out one or more of the drugs that might be the basis for optimal therapy. Furthermore, drugs may show synergistic activities *in vivo* that are not seen in *in vitro* testing.

In vitro studies have shown that pentamidine and propamidine isethionates at a concentration of $1\,\mu g\,mL^{-1}$ inhibit the growth of amoebae by 82% and 80%, respectively. The drugs, however, were amoebastatic but not amoebacidal. (It should be noted that a one-time dose of a drug *in vitro* is different from the repeated dosage typical in a clinical setting.) Among other drugs tested with little or no activity were macrolide antibiotics, azole compounds, gramicidin, polymyxin B, trimethoprim, sulfamethoxazole, and a combination of trimethoprim-sulfamethoxazole as well as amphotericin B (Schuster & Visvesvara, 2004b). Recent information based on *in vitro* data has shown that miltefosine at a concentration $> 40\,\mu M$ lysed the amoebae. Voriconazole, however, had virtually no effect on *Balamuthia* (Schuster *et al*., 2006a). Given the problems with the diagnosis of infection and the lack of effective antimicrobial agents, the prognosis for patients is poor.

Epidemiology

Specific information on the source of infections with *Balamuthia* is scarce. Gardening, playing with dirt, or inhalation of cysts in soil carried on the wind may have been the source of infections because *Balamuthia* has been isolated from soil (Schuster *et al*., 2003; Dunnebacke *et al*., 2004). The amoeba may enter the body through a break in the skin, as probably happened in the case of the 60-year-old man (described above) who had been digging in his yard (Deetz *et al*., 2003). Once it gains entry into the body it is likely to spread hematogenously to the brain. It is possible, of course, that water might serve as a vehicle for transmission, but no human case has been attributed to swimming or other water activities. There are two reports of balamuthiasis in dogs, one from California and the other from Australia; the Californian canine had been receiving prednisone and was immunosuppressed, and the other animal had lymphoma. Both animals swam in stagnant ponds prior to developing the disease, suggesting water as the source of infection (Foreman *et al*., 2004; Finnin *et al*., 2007).

A recent paper deals with *Balamuthia* infection in Hispanic Americans (Schuster *et al*., 2004): a retrospective analysis of GAE cases in California revealed that 73% of *Balamuthia* GAE cases in California occurred in Hispanic Americans, although the population of Hispanic Americans in California, as of 2005, was 32%. In the United States, Hispanic Americans constitute 12.5% of the population but represent *c*. 50% of all cases of balamuthiasis. One possible explanation for the over-representation of Hispanic Americans is that they are more likely to reside in agrarian settings with increased exposure to soil and water, further supporting an association of infection with soil and/or water containing trophic amoebae or cysts (Schuster *et al*., 2004). An alternative explanation is genetic predisposition based on as yet undetermined factors that may play a role. Are Hispanics less likely to produce antibodies to *Balamuthia*, as appears to be the case with exposure to *Acanthamoeba*?

It is only recently that it has been shown that *Balamuthia* may act as hosts for pathogenic microorganisms such as *Legionella*, as does *Acanthamoeba* (Shadrach *et al*., 2005). In addition, Michel *et al*. (2005) have shown that *B. mandrillaris* (strain CDC:V039) supported the intracellular growth of *Simkania negevensis*, a *Chlamydia*-like bacterium, but lost the ability to form cysts. Furthermore, the amoebae periodically shed the elementary bodies (EBs), the infectious stage of *S. negevensis*, thus facilitating mass production of these EBs.

Prevention and control

Cases of balamuthiasis, like those of acanthamoebiasis, have occurred in hosts with weakened immune systems and presently no clearly defined methods are available for the prevention of infection with these amoebae. Acute CNS

disease presents with symptoms that require immediate attention, while recalcitrant sinus and cutaneous infections are treated with antimicrobials. Many of the more recent cases have occurred in apparently immunocompetent children, suggesting that subtle immunological deficiencies may be involved.

Naegleria fowleri

Naegleria fowleri is an amoeboflagellate, as it has a transitory, pear-shaped flagellate stage along with amoeboid trophozoite and resistant cyst stages in its life cycle (Fig. 7a–c). Although the cyst is resistant to environmental stress (e.g. desiccation) it is more fragile than cysts of *Acanthamoeba*. The trophozoite moves rapidly by producing hemispherical bulges, lobopodia, at the anterior end and exhibits active sinusoid/limacine locomotion. It measures from 10 to 25 μm, can be maintained on a diet of Gram-negative bacteria *in vitro*, and reproduces by binary fission. It has a single vesicular nucleus with a prominent, centrally placed nucleolus that stains densely with chromatic dyes. The cytoplasm shows a sharp distinction between ectoplasms and endoplasms; the latter contains numerous mitochondria, ribosomes, food vacuoles, and, defining the posterior end (uroid), the contractile vacuole and trailing protoplasmic filaments**.** The trophozoite transforms into a flagellate stage, usually with two flagella, when the ionic concentration of the milieu changes. In the laboratory, this transformation can be induced by washing trophic amoebae in distilled water or dilute saline. The temporary, pear-shaped flagellate stage neither divides nor feeds, ranges in length from 10 to 16 μm, and usually reverts to the amoeboid form within an hour or less. The trophozoite transforms into the resistant cyst when the food supply diminishes and/or growth conditions become adverse. The cyst is usually spherical and double-walled with a thick endocyst and a closely apposed thin ectocyst. The cyst wall has pores, but the pores are difficult to see with the light microscope because they are flush with the cyst wall. Both the flagellate and cyst stages also possess a single nucleus with a prominent nucleolus (Schuster & Visvesvara, 2004a).

Naegleria fowleri occurs worldwide and has been isolated from soil and fresh water. More than 30 species of *Naegleria* have been described based on the sequence of the SS rRNA gene (De Jonckheere, 2004). Only one species, *N. fowleri*, is known to cause infection, although two other species, *N. australiensis* and *N. italica*, can cause infection in mice after intranasal or intracerebral inoculation. These species have never been identified in human infections. *Naegleria fowleri* is thermophilic and can tolerate temperatures of up to 45 °C. Therefore, these amoebae proliferate during warmer months of the year when the ambient temperature is likely to be high. Infections occur in children and young adults – age groups that are more energetic in aquatic activities and thus are likely to come into contact with amoebae in water. Temperature tolerance by itself, however, does not equate with pathogenesis because there are other thermophilic *Naegleria* amoebae (*N. lovaniensis*) that are nonpathogenic.

Cultivation

In nature the amoebae feed upon bacteria and have been isolated from fresh-water pools, puddles, lakes, rivers, swimming pools, hot springs, thermally polluted effluents of power plants, hydrotherapy pools, aquaria, sewage, irrigation canals, and even from the nasal passages and throats of healthy individuals, using non-nutrient agar spread with bacteria. They also are readily isolated from clinical specimens such as brain tissue and CSF. However, *Naegleria* amoebae have not been recovered from seawater, as a result of sensitivity to elevated levels of osmolarity. The amoeba grows well on monolayers of E6 and HLF cell cultures, destroying the confluent layers within 2–3 days. Like *Acanthamoeba* and *Balamuthia, N. fowleri* can be grown in a cell-free axenic medium as well as in a chemically defined medium (Schuster, 2002).

Naegleria fowleri, like *Acanthamoeba*, can support the growth of intracellular bacteria such as *Legionella pneumophila*. However, reports of environmental or clinical isolates of *N. fowleri* amoebae harbouring endocytobionts do not exist, although, a nonpathogenic strain of *N. clarki* and other *Naegleria* species have been described to harbour endocytobionts (Walochnik *et al.*, 2005). The absence of environmental strains with endocytobionts is probably a result in part of the greater sensitivity of *N. fowleri* to

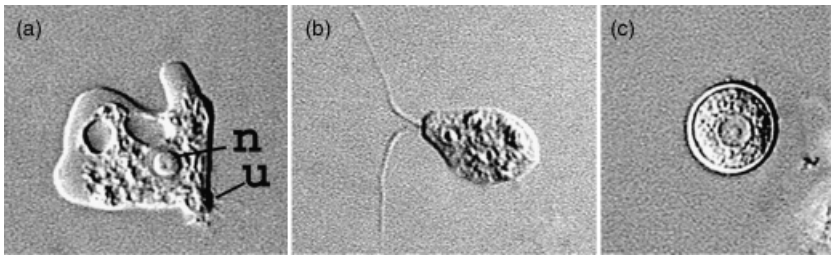

Fig. 7. *Naegleria fowleri*: (a) trophozoite, (b) a flagellate, and (c) a cyst. All images at × 1000.

environmental stress and of their tendency to lyse more readily than *Acanthamoeba* (Schuster & Visvesvara, 2004a).

Primary amoebic meningoencephalitis (PAM)

Naegleria fowleri causes an acute, fulminating hemorrhagic meningoencephalitis principally in healthy children and young adults with a history of recent exposure to warm fresh water. Because PAM occurs primarily in healthy individuals, the organism is not regarded as an opportunistic amoeba, as are *Acanthamoeba* and *Balamuthia,* but as a pathogen. The typical cases of PAM occur in the hot summer months when large numbers of people engage in recreational aquatic activities in fresh-water bodies such as lakes, ponds and swimming pools that may harbour these amoebae. The striking feature of PAM is the rapid onset of symptoms following exposure. The disease progresses rapidly, and, without prompt diagnosis and intervention, death usually occurs within a week or less. Although retrospective examination of brain tissue indicates that PAM caused by *N. fowleri* occurred as far back as 1901, it was first described by Fowler and Carter from Australia in 1965 but attributed to *Acanthamoeba*. Butt described the first case caused by *N. fowleri* from the United States in 1966 and coined the term primary meningoencephalitis (Martinez, 1985; Visvesvara & Maguire, 2006).

PAM: clinical and laboratory diagnosis

PAM should be suspected in young adults and children with acute neurological symptoms as described below and recent exposure to fresh water. The time from initial contact (swimming, diving, water skiing, or simply immersing head in water) to onset of illness is usually 5–7 days, and may even be as short at 24 h. Because there are no distinctive clinical features that differentiate PAM from acute pyogenic or bacterial meningoencephalitis it is imperative that the attending physician obtains information regarding the patient's contact with fresh water, including hot springs, during the past week. The earliest symptoms are sudden onset of bifrontal or bitemporal headaches, high fever, nuchal rigidity, followed by nausea, vomiting, irritability and restlessness. Nuchal rigidity usually occurs with positive Kernig and Brudzinski signs. Photophobia may occur late in the clinical course, followed by neurological abnormalities, including lethargy, seizures, confusion, coma, diplopia or bizarre behaviour, leading to death within a week. Cranial nerve palsies (third, fourth, and sixth cranial nerves) may indicate brain edema and herniation. Intracranial pressure is usually raised to 600 mm H_2O or higher. Cardiac rhythm abnormalities and myocardial necrosis have been found in some cases (Martinez, 1985).

CSF may vary in colour from greyish to yellowish-white, and may be tinged red with a few red cells (250 mm^{-3}) in the early stages of disease. However, as the disease progresses the red blood cell number increases to as high as 24 600 mm^{-3}. The white blood cell count, predominantly polymorphonuclear leukocytes (PMN), also may vary from 300 cells mm^{-3} to as high as 26 000 mm^{-3}. No bacteria are seen. The CSF pressure is usually elevated (300–600 mm H_2O). The protein concentration may range from 100 mg per 100 mL to 1000 mg per 100 mL, and glucose may be 10 mg/100 mL or lower (Martinez, 1985; Visvesvara & Maguire, 2006). A wet-mount of the CSF should be examined immediately after collection under a microscope, preferably equipped with phase-contrast optics, for the presence of actively moving trophozoites. Smears of CSF should be stained with Giemsa or Wright stains to identify the trophozoite, if present. The amoeba can be clearly differentiated from host cells by the nucleus with its centrally placed large nucleolus (Fig. 8a). Gram stain is not useful. The cause of death is usually increased intracranial pressure with brain herniation, leading to cardiopulmonary arrest and pulmonary edema (Martinez, 1985; Visvesvara & Maguire, 2006).

Pathophysiology of *N. fowleri* infections

The cerebral hemispheres are usually soft, markedly swollen, edematous and severely congested. The leptomeninges (arachnoid and pia mater) are severely congested, diffusely hyperemic and opaque, with limited purulent exudate within sulci, the base of the brain, brainstem and cerebellum. The olfactory bulbs are characterized by hemorrhagic necrosis and are usually surrounded by purulent exudate. The cortex also shows numerous superficial hemorrhagic areas. Most of the lesions are found in and around the base of the orbitofrontal and temporal lobes, base of the brain, hypothalamus, midbrain, pons, medulla oblongata, and the upper portion of the spinal cord. CT scans may show obliteration of the cisternae around the midbrain and the subarachnoid space over the cerebral hemispheres. Marked diffuse enhancement in these regions may be seen after administration of intravenous contrast medium (Martinez, 1985; Visvesvara & Maguire, 2006).

Microscopically, the cerebral hemispheres, brain stem, cerebellum, and upper portion of the spinal cord are filled with fibrino-purulent leptomeningeal exudate containing predominantly PMNs, a few eosinophils, a few macrophages and some lymphocytes. Large numbers of amoebic trophozoites, usually in pockets, without the presence of PMNs, are seen within edematous and necrotic neural tissue (Fig. 8b and c). Trophic amoebae are also seen deep in Virchow–Robin spaces, usually around blood vessels with no inflammatory response. Amoebae ranging in size from 8

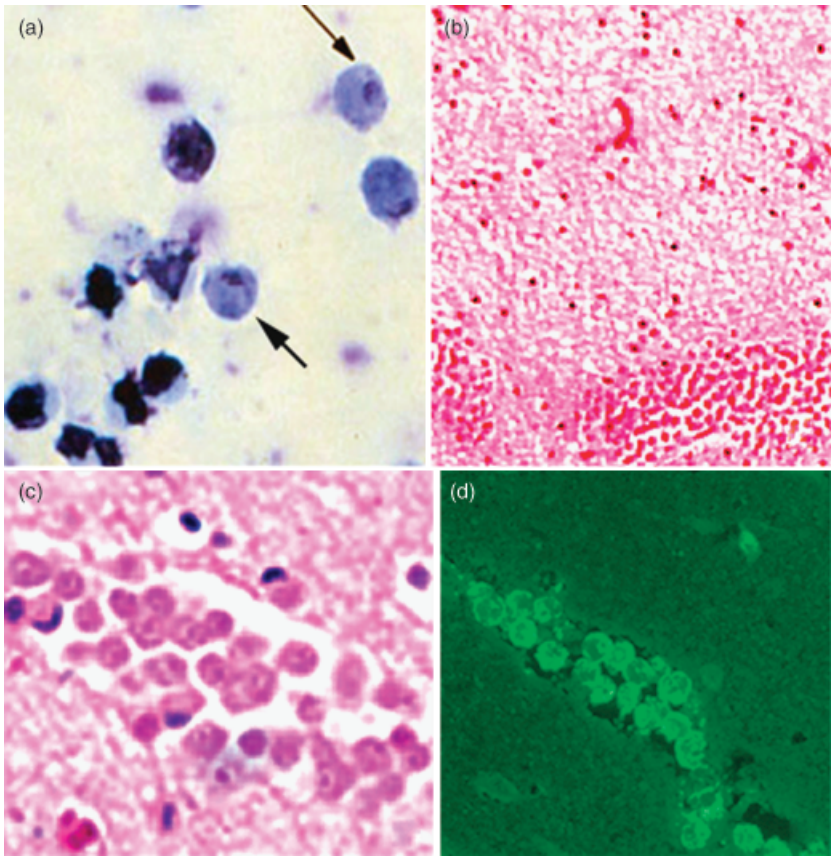

Fig. 8. Pathological features of PAM caused by *Naegleria fowleri*. (a) A CSF smear stained with Giemsa stain. Note amoebae at arrows (×1000). (b) A section through the cerebellum showing extensive inflammation. (c) An area of the cerebellum showing extensive destruction of the brain architecture with large numbers of amoebae in the perivascular area (×1000). (d) A section, similar to the one shown in (c), but reacted with anti-*N. fowleri* serum. Note the brightly staining *N. fowleri* trophozoites.

to 12 μm are recognizable by the presence of a large nucleus with a centrally located, deeply staining large nucleolus (Fig. 8c), and they can be specifically identified as *N. fowleri* by polyclonal or monoclonal antibody staining (Fig. 8d). Amoebic cysts are conspicuously absent. *Naegleria fowleri* amoebae proliferate in brain tissue, meninges, and CSF. The amoebae can be cultured from samples of CSF or from brain tissue obtained at postmortem by placing macerated brain tissue onto an agar plate coated with bacteria or into axenic growth medium, or by inoculating the brain tissue onto tissue culture monolayers. With perhaps a few exceptions (see below), all cases of PAM reported in the literature have been fatal (Seidel *et al*., 1982; Martinez & Visvesvara, 1997; Schuster & Visvesvara, 2004a; Visvesvara & Maguire, 2006).

Mechanisms of Pathogenesis

The portal of entry into the CNS is the olfactory neuroepithelium. It is believed that the sustentacular cells lining the olfactory neuroepithelium phagocytose the amoebae that enter the nasal passages of the victims while indulging in aquatic activities. The amoebic trophozoites pass through the sieve-like cribriform plate and penetrate into the subarachnoid space and continue on to the brain parenchyma. The incubation period of PAM varies depending on the size of the inoculum, and on the virulence of the particular strain of infecting amoebae. *Naegleria fowleri* when inoculated into tissue culture cells destroy the cell monolayer by causing a cytopathic effect or CPE. The amoebae produce sucker-like appendages or amebostomes that 'nibble' away at the tissue culture cells. Other possible factors involve the production of: (i) phospholipase A and B activity or a cytolytic factor causing destruction of cell membranes; (ii) neuraminidase or elastase activity facilitating destruction of tissue culture cells; (iii) a perforin-like, pore-forming protein that lyses target cells; and (iv) the presence within *Naegleria* amoebae of a cytopathic protein that triggers the apoptosis pathway in susceptible tissue culture cells (Schuster & Visvesvara, 2004a).

PAM has been duplicated in the laboratory by inoculating mice intranasally or intracerebrally with a suspension of amoebae. Mice develop the disease and usually die within a week, depending on the virulence of the strain of *N. fowleri*. It is well known that continuous cultivation in an axenic medium reduces the virulence of amoebae, but virulence can be restored by passaging the amoebae through mouse brain. According to one study, mice inoculated with

N. fowleri evoked an IgG response that apparently conferred protection upon challenge with virulent amoebae. Domesticated as well as wild mammals, including raccoons, muskrats, squirrels and rabbits, develop antibodies to *Naegleria* amoebae. Furthermore, sera from some wild animals (raccoons, muskrats, squirrels, bull frogs, but not toads and box turtles) possessed amoebacidal activity that was destroyed upon heating, suggesting a role for complement in the process. Close contact with soil and water makes it likely that PAM occurs in such animals (Schuster & Visvesvara, 2004a). Recently, PAM has been diagnosed in the South American tapir and domestic cattle (Lozano-Alarcon *et al.*, 1997; Daft *et al.*, 2005).

Immunology

Because most PAM patients die soon after infection there is insufficient time to mount a detectable immune response – hence the limited usefulness of serological tests in the diagnosis of PAM. Previous attempts to detect an antibody response to *N. fowleri* using IFA have therefore been largely unsuccessful (Schuster & Visvesvara, 2004a). However, Seidel *et al.* (1982) documented a specific antibody response to *N. fowleri* in a Californian patient who recovered from this disease. A titre of 1 : 4096 was demonstrated by IFA in the serum samples collected 7, 10 and 42 days after admission to hospital, and the elevated antibody titres persisted even after 4 years. Based on immunoblot studies conducted at Centers for Disease Control and Prevention (CDC), IgM was the principal class of antibody generated by this patient as well as by three others who contracted PAM (Fig. 9). In addition, the CDC study indicated that sera collected from several individuals with a history of extensive swimming in fresh-water lakes in the southeastern United States as well as in California also exhibit IgM antibodies to *N. fowleri*. These antibodies are particularly well developed to *N. fowleri* antigens of *c.* 190, 66, 30 and 14 kDa. Whether these antibodies have any protective activity is not clear at this time (Visvesvara *et al.*, 2007). A previous study that used an agglutination test on serum specimens obtained from humans in North Carolina, Pennsylvania, and Virginia demonstrated antibody specificity for the surfaces of particular *Naegleria* spp., and people in certain geographic areas are exposed to these antigens more extensively than are people from other geographical areas. The study showed that sera from individuals residing in the southeastern United States had significantly greater agglutinating ability than did sera obtained from Pennsylvania, a northeastern state. The study showed that the agglutinating antibody is of the IgM class (Marciano-Cabral *et al.*, 1987). Summarizing several published studies: (i) > 80% of hospitalized patients in the United States (Tennessee) had IgG and IgM antibodies ranging from 1:20 to 1:640 to *N. fowleri* and

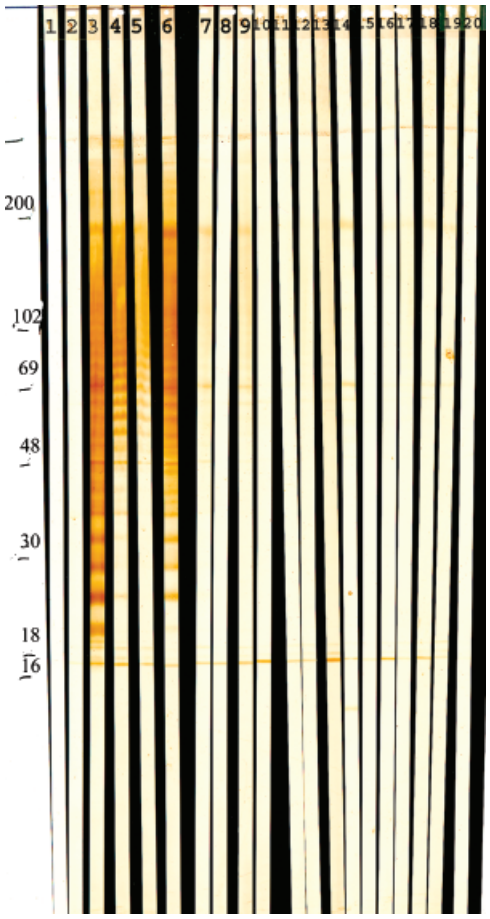

Fig. 9. An immunoblot showing multiple IgM bands in serum from two patients infected with *Naegleria fowleri* and from a number of healthy swimmers in California and Georgia (United States). Lane 1, PBS; lane 3, acute serum sample obtained from a California patient who survived the infection; lanes 4 and 5, serum samples taken 6 months and 4 years later from the same Californian patient; lane 6, acute serum sample from a South Carolina patient; lanes 2 and 7–20 serum samples obtained from Californian and Georgian swimmers. The blots for PAM patients show prominent bands representing IgM antibodies as typical of recent infections. With recovery (lanes 4 and 5), the IgM titres wane. Faint IgM bands appear in the non-infected swimmers, indicating exposure to *N. fowleri* antigens but with a weak humoral response.

N. lovaniensis; (ii) serum specimens collected in Virginia possessed age-dependent agglutinating antibodies of IgM class to *N. fowleri*; (iii) antibodies to pathogenic and non-pathogenic *Naegleria* ranging in titres from 1:2 to 1:120 were demonstrated in healthy individuals in New Zealand, and it was shown that the antibodies belonged mainly to the IgM and IgG classes but failed to neutralize *N. fowleri*; (iv) mice immunized intraperitoneally with live *N. fowleri* were more resistant to subsequent intranasal challenge; and (v) protective immunity to *N. fowleri* can be transferred to syngenic mice by immune serum but not by immune spleen cells. Furthermore, multiple doses of *N. fowleri* culture

supernatants were superior to multiple doses of *N. fowleri* lysate in affording protection to mice. The protective activity of the culture supernatants resided primarily in a 200 000-MW fraction that is released to the medium.

Molecular techniques

Biochemical techniques such as isoenzyme analysis have been developed for the specific identification of *N. fowleri* amoebae cultured from the CSF and brain specimens of patients as well as from the environment (water and soil) (Visvesvara & Healy, 1980; De Jonckheere, 1982; Moss *et al.*, 1988). Furthermore, monoclonal antibodies (MAb) that can specifically identify *N. fowleri* in the CSF have also been developed (Visvesvara *et al.*, 1987). More recently, molecular techniques such as PCR and nested PCR assays for the specific identification of *N. fowleri* in cultured amoebae from patients and the environment as well as of *N. fowleri* DNA in the environment have been developed (Réveiller *et al.*, 2002; Marciano-Cabral *et al.*, 2003; Zhou *et al.*, 2003; Cogo *et al.*, 2004; De Jonckheere, 2004). Sequencing of the 5.8S rRNA gene and the internal transcribed spacers 1 and 2 (ITS1 and ITS2) of *N. fowleri* has shown that specific genotypes can be distinguished. Based on the sequencing of the ITS of clinical isolates it was shown that two strains of *N. fowleri*, isolated from two PAM patients who visited the same hot spring in California but at different times, belonged to the same type II genotype (Zhou *et al.*, 2003). A recently developed real-time multiplex PCR assay identifies *N. fowleri* DNA in the CSF and brain tissue samples obtained from PAM patients antemortem. This test identifies all three genotypes known to be present in the United States. The time taken to identify *N. fowleri* from the time the specimen arrives in the laboratory is about 5 h, and can be cut down to about 2 h if the specimen is CSF, because no time is lost controlling for inhibition. Such fast turn-around times will be invaluable in appropriately treating the patient, because most patients are treated with antibacterial drugs that are ineffective against *N. fowleri* (Qvarnstrom *et al.*, 2006). A new sensitive, rapid and discriminating technique that uses a single primer set and the DNA intercalating dye SYT09 for real-time PCR and melting-curve analysis of the 5.8S rRNA gene flanking the ITS has been developed that distinguishes several *Naegleria* species in environmental samples (Robinson *et al.*, 2006). The new MALDI-TOF-MS technique mentioned earlier (Moura *et al.*, 2003b) can also specifically identify *Naegleria* amoebae (Fig. 4).

Therapy and prognosis

Few patients have survived PAM. One of these survivors, a Californian girl, was aggressively treated with intravenous and intrathecal amphotericin B, intravenous and intrathecal miconazole, and oral rifampin (Seidel *et al.*, 1982). Over a 4-year follow-up, she remained completely healthy and free of any neurological deficits. It was believed that amphotericin B and miconazole had a synergistic effect but that rifampin was without effect on the amoebae. Based on *in vitro* testing and *in vivo* mouse studies, amphotericin B was reported to be more effective against *Naegleria* than amphotericin B methyl ester, a water-soluble form of the drug. *In vitro* studies of phenothiazine compounds (chlorpromazine and trifluoperazine), which can accumulate in the CNS, were found to have inhibitory effects on *N. fowleri* (Schuster & Visvesvara, 2004b). Azithromycin, a macrolide antimicrobial, has been shown to be effective against *Naegleria* both *in vitro* and *in vivo* (mouse model of disease) (Goswick & Brenner, 2003). Other macrolides (erythromycin, clarithromycin) are less effective. *Naegleria fowleri* was sensitive to the triazole compound voriconazole; low concentrations ($\leq 10\,\mu g\,mL^{-1}$) were amoebastatic, while concentrations $\geq 10\,\mu g\,mL^{-1}$ were amoebacidal (Schuster *et al.*, 2006a, b).

Prevention and control

Naegleria fowleri is a thermophilic amoeba and hence proliferates in water when the ambient temperature increases above 30 °C. With the anticipated temperature increase resulting from global warming, it is possible that cases of *N. fowleri* PAM may be seen even in countries where it had previously not been recorded (Cogo *et al.*, 2004). Because *N. fowleri* is susceptible to chlorine in water (one part per million), amoeba proliferation can be controlled by adequate chlorination of heavily used swimming pools, especially during summer months. However, it is not possible to chlorinate natural bodies of water such as lakes, ponds and streams, where *N. fowleri* may proliferate. Sunlight and the presence of organic matter in swimming pools can reduce the efficacy of chlorine. In high-risk areas, monitoring of recreational waters for *N. fowleri* amoebae should be considered by local public health authorities and appropriate warnings posted, particularly during the hot summer months. Warning children not to immerse their heads in suspect waters is judicious. In both Australia and France, where swimming pools and thermal effluents from nuclear power plants, respectively, are possible sources of infection, such monitoring of water is routine (Schuster & Visvesvara, 2004a).

If there is a single source of infection, such as a popular swimming area, a minor outbreak of PAM may occur over a period of time. Sixteen deaths arising from PAM over a 3-year period were retrospectively traced to a swimming pool in Czechoslovakia with low free chlorine concentration (Schuster & Visvesvara, 2004a). Two children in Arizona playing in a wading pool filled from the domestic water supply developed PAM (Okuda *et al.*, 2004). *Naegleria fowleri* was identified in the water by nested PCR analysis

(Marciano-Cabral *et al*., 2003). This was the first time that a domestic water supply had been implicated as the source of *N. fowleri* in the United States. Domestic water supply, carried overland in pipes warmed by the sun, had also figured as the source of infection in Australia through nasal aspiration. Drinking amoeba-containing water has never been known to cause PAM. Because of a cluster of cases of PAM involving children in South Australia, the South Australia High Commission established the amoeba monitoring program, which routinely determines residual chlorine levels and the total coliform counts to anticipate conditions favourable for the growth of *N. fowleri*. They also conducted safety campaigns to educate the public in order to minimize the incidence of PAM (Martinez, 1985).

Sappinia diploidea

Gelman *et al*. (2001) reported the first and only case of amoebic encephalitis caused by *Sappinia diploidea*, in a 38-year-old previously healthy, immunocompetent male. The patient lost consciousness for about 45 min, and developed nausea and vomiting followed by bifrontal headache, photophobia, and blurry vision for 2–3 days. He had a sinus infection prior to his infection. MRI showed a solitary 2-cm mass in the posterior left temporal lobe. The excised mass on sectioning showed necrotizing hemorrhagic inflammation containing amoebic organisms. The characteristic feature of the amoeba was the presence of two tightly apposed nuclei. Cysts were not seen in the brain tissue (Fig. 10a). *Sappinia diploidea* had never before been implicated in human or animal disease, although it had been isolated from soil, fresh water, forest litter, mammalian faeces and the rectum of lizard. It has been described from Europe, North America, Egypt, the Middle East, the West Indies, and Japan. Both trophozoite and cyst stages are binucleate. The trophozoite measures 40–80 μm, is ovoid or oblong, and appears to be flattened with occasional wrinkles on the surface. The cytoplasm contains the contractile vacuole and food vacuoles (Figs. 10b and c). The mature cyst is round and measures 15–30 μm. *Sappinia diploidea* can be cultivated on non-nutrient agar plate coated with bacteria.

Conclusions

Encephalitis caused by the free-living amoebae *Acanthamoeba*, *Balamuthia* and *Naegleria* is relatively rare. The amoebic encephalitides are of concern, however, since mortality is high and children are often victims. The high mortality is in large part the result of clinicians' lack of familiarity with these amoebic diseases, delay in diagnosis, and the lack of optimal antimicrobial therapy. Most of the cases of amoebic encephalitides reported in the literature have been from countries where a well-developed medical establishment is available for identifying these cases. It can be assumed that many more cases occur on a global scale – in humans as well as animals – which go undiagnosed and unreported.

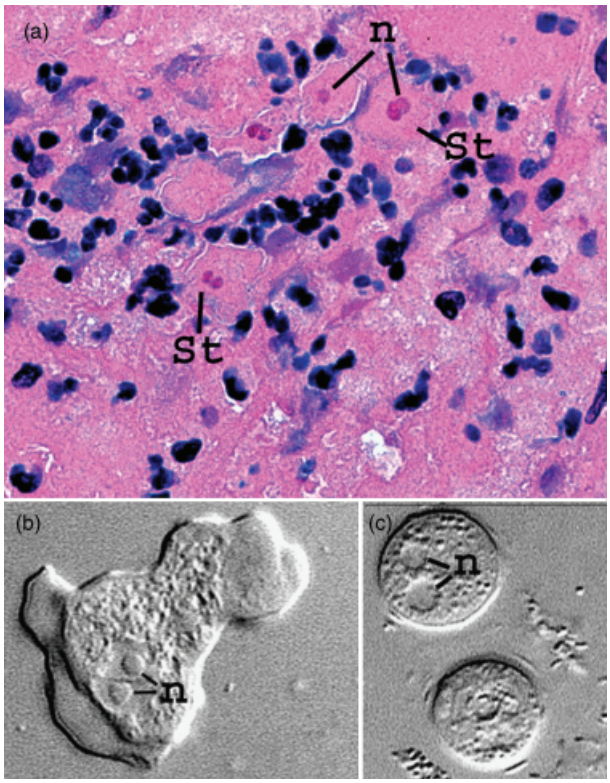

Fig. 10. *Sappinia diploidea*. (a) A section through an abscess excised from the brain of a man infected with *S. diploidea:* (st) *Sappinia* trophozoite. The heavily stained cells are polymorphonuclear leukocytes: (*n*) characteristic double nuclei of amoebae. Hematoxylin-eosin-stained preparation (×500). (b) *Sappinia diploidea* trophozoite from *in vitro* culture, differential-interference micrograph. The stiff pellicle (cell membrane) of *Sappinea* sharply demarcates the ectoplasm and endoplasm: (*n*) closely apposed nuclei, each with a prominent nucleolus (×1000). (c) Differential-interference micrograph of two cysts. One of the cysts shows two nuclei (*n*), although the nucleoli cannot be seen. The cyst wall is smooth and lacks pores (×1000).

In addition to its role as an encephalitis agent, *Acanthamoeba* is proving to be a versatile threat. Amoebic keratitis, caused by *Acanthamoeba*, affects mainly the wearers of contact lenses. Thousands have been affected worldwide by this sight-threatening disease, many times more than those affected by the encephalitides, but at least now there are effective drugs for treating keratitis. *Acanthamoeba* also can serve as hosts for endocytobionts including *Legionella, Parachlamydia* and mimivirus (Berger *et al*., 2006). These endocytobionts are potential agents of legionellosis and Pontiac fever as well as of nosocomial pneumonia, diseases that are manifestations of the 'sick building syndrome', in which amoebae and their endocytobionts may multiply in ventilation and cooling systems and the water supply of office buildings and hospitals.

Although steady progress has been made in understanding the biology of these protists and their detection in human tissue, our knowledge of virulence factors and mechanisms of pathogenesis remains imperfect. Another major area that needs investigating is host susceptibility factors that may contribute to the expression of the pathogenic mechanisms of the amoebae. What factors single out a handful of naegleriasis patients among the thousands that have used, for example, a swimming pool or hot spring? Why are there so few cases of balamuthiasis when human contact with soil is inescapable?

It is worth noting that the amoebic diseases described in this review have been recognized only within the past 40 years. Retrospective diagnoses have been made from pathology specimens and hospital records of cases going back to the start of the 20th century; thus one might wonder why it took so long to identify these diseases. Human progress has facilitated their occurrence in the population: the popularity of contact lenses, availability of immunosuppressive drugs that enable organ transplantation, medications that prolong human life to a point at which the immune system is less able to deal with amoebic and other infections, and energy-saving measures that seal off the interior of a building making the occupants vulnerable to amoebic endocytobionts in the ventilation system. Paralleling these aspects of progress is the development of sophisticated diagnostic techniques and their application for the diagnoses of amoebic infections: genomic studies of amoebae, conventional and real-time PCR, high-resolution electrophoresis, enzyme immunoassay, mass spectrometry, immunostaining, and neuroimaging. Recognition of *Sappinia* in the etiology of encephalitis suggests that there are other such quirky amoebic agents of disease waiting to be identified.

Acknowledgements

The authors are indebted to Drs Cathy A. Slater and Anthony A. Gaspari for Fig. 2e and f; the late Dr Frederick H. Theodore for Fig. 3a and c; Dr Rolf Michel for cultures of *Sappinia diploidea*; Dr Thomas Nerad for helpful discussions on the taxonomy of protists; Drs John R. Barr and Adrian R. Woolfitt for help with mass spectrometry; Marianna Wilson for help with the Western blot; and Rama Sriram for expert technical assistance.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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