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Review Article

Free-Living Amebae as Opportunistic Agents of Human Disease

Govinda S. Visvesvara

Centers for Disease Control and Prevention, Roybal Campus, MS-F 66, Division of Foodborne, Waterborne & Environmental Diseases, Waterborne Diseases Prevention Branch, 1600 Clifton Road, Atlanta, GA 30333, USA
Address correspondence to Govinda S. Visvesvara, gsv1@cdc.gov

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Abstract Members of the free-living amebic genera *Acanthamoeba*, *Balamuthia*, and *Naegleria* are known to cause infections of the central nervous system (CNS) of humans and other animals. Several species of *Acanthamoeba* cause an insidious and chronic disease, granulomatous amebic encephalitis (GAE), principally in immunocompromised hosts including persons infected with HIV/AIDS. Additionally, *Acanthamoeba* spp. also causes infection of the human cornea, *Acanthamoeba* keratitis. *B. mandrillaris*, the only known species of *Balamuthia*, causes GAE in both immunocompromised and immunocompetent hosts. Both *Acanthamoeba* and *B. mandrillaris* also cause a disseminated disease including the lungs, skin, kidneys, and uterus. *N. fowleri*, on the other hand, infects immunocompetent children and young adults leading to an acute and fulminating, necrotizing primary amebic meningoencephalitis. This review describes the biology of the amebae, clinical manifestations, diagnosis including molecular identification, immunological, and epidemiological features associated with the infections caused by these amebae.

Keywords *Acanthamoeba*; *Balamuthia mandrillaris*; *Naegleria fowleri*; granulomatous amebic encephalitis; primary amebic meningoencephalitis

1 Introduction

Small free-living amebae belonging to the genera *Acanthamoeba*, *Balamuthia*, and *Naegleria* are capable of causing central nervous system (CNS) infections in humans and other animals. *Acanthamoeba* and *Naegleria* have a wide distribution in soil and water and can be readily isolated from these sources. *Balamuthia* amebae have been only recently isolated from the soil and dust and it is presumed that they are also widely distributed in nature [23,27,29,44,51,53]. The concept that *Acanthamoeba* and other small free-living amebae can cause disease in humans was put forward by the late Dr. Clyde Culbertson in the early sixties. Culbertson and colleagues, while growing poliomyelitis virus in monkey kidney cell cultures for vaccine purposes, noticed cleared areas or plaques in the control cell cultures. Upon inoculation of the culture supernatant, experimental animals died with typical symptoms of meningoencephalitis. Culbertson et al. isolated an ameba from the brains of dead and dying animals and designated it as *Acanthamoeba* Lilly A-1 strain. This isolate was subsequently named as *Acanthamoeba culbertsoni*. Culbertson and colleagues also isolated several different strains of *Acanthamoeba* from nature with varying degrees of virulence. Based on these studies, it was suggested by Culbertson that human infections due to these amebae might exist in nature [29]. Few years later, Fowler and Carter [27, 29,53] in Australia reported meningoencephalitis in an Australian boy attributed to *Acanthamoeba*, which was later identified as *Naegleria fowleri*. Currently, it is well known that several species of *Acanthamoeba*, *Balamuthia mandrillaris*, and *Naegleria fowleri* cause infections in humans and other animals [23,26,27,29,44,51,53]. In addition to these three amebae, there is just one case of amebic encephalitis caused by another free-living ameba, *Sappinia diploidea*, now redescribed as *S. pedata* [15,35].

Acanthamoeba spp. infections include granulomatous amebic encephalitis (GAE), *Acanthamoeba* keratitis (AK), disseminated infections including skin, lungs, kidneys, adrenals, and nasal abscesses. Typically, infections caused by *Acanthamoeba* occur in compromised hosts such as those with the acquired immunodeficiency syndrome (AIDS). *B. mandrillaris* causes infections in both immunocompetent and compromised hosts and occurs as GAE, cutaneous, sinus, and disseminated infections like those caused by *Acanthamoeba*. In contrast, *N. fowleri* infections occur as primary amebic meningoencephalitis (PAM) in children and young adults in apparent good health [23,27,29,44,51,53].

2 *Acanthamoeba* spp.

The life cycle of *Acanthamoeba* includes a trophic or feeding stage and a dormant cyst stage. The trophozoite

measures ca. 15–30 μm and has sluggish movement. It is characterized by the presence of thorn-like pseudopodial projections, termed acanthopodia. In nature, amebae are voracious feeders upon bacteria, a feature that makes it relatively easy to isolate and culture them in the laboratory. When food is scarce or when facing desiccation or other environmental stresses, the amebae round up and become cysts. The cyst has a double-layered cyst wall, an outer wrinkled ectocyst and an inner endocyst which can be stellate, polygonal, oval, or round. At the junction of the ecto- and endocysts are covered pores or ostioles through which the ameba exits at the time of excystation when favorable growth conditions return. Both the trophic and cyst stages possess a large nucleus with a centrally located densely staining nucleolus [26,31]. In addition to their occurrence in soil and water, *Acanthamoeba* spp. have also been recovered from hydrotherapy baths in hospitals, tap water, dental irrigation units, contact lens paraphernalia, eye wash irrigation units, home aquaria, humidifiers, heating ventilating and air conditioning units (HVAC), cooling tower effluents, and mostly any moist environment where a bacterial food source is available for growth [26]. Given their environmental ubiquity, many opportunities exist for humans to come into contact with trophic amebae or cysts. Amebae are tolerant of a wide range of osmolarities, enabling them to survive in distilled water, tissue culture media, mammalian body fluids, and sea water. Strains of *Acanthamoeba* have been isolated from tissue cultures, either as carry-overs in the original tissue explants or as laboratory contaminants, contact lens paraphernalia, corneal smears, biopsy and autopsy specimens from humans and other animals [23,26,29,51,53]. Strains isolated from human cases of *Acanthamoeba* infections generally grow optimally at 37°C, although a number of clinical human isolates grow better at ca. 30°C. The pathogenic potential and virulence of environmental isolates can be determined by mouse inoculation, using an intranasal pathway. Infection of an animal is outwardly indicated by ruffling of the fur and aimless wandering. Death of mice in 1–4 weeks following inoculation is a reliable indicator of pathogenic potential and/or virulence, though the cytopathic effect upon tissue cultures is another possible mode of assessing virulence [23,26].

More than 24 species included in three different groups have been recognized in the genus *Acanthamoeba* largely based on the differences in the morphology and size of the trophozoites and cysts. Group1 includes those species that are large amebae with cysts that range in size from 16 to 30 μm (e.g., *A. astronyxis*, *A. comandoni*, *A. tubiashi*, *and A. echinulata*). Group 2 includes by far the largest numbers of species with cysts around 18 μm or less (e.g., A. castellanii, *A. polyphaga*, *A. rhysodes*, *A. hatchetti*, etc.). Group 3 consists of species with subtle differences in cyst morphology; they also measure 18 μm or less (e.g., *A. culbertsoni*, *A. royreba*, *A. lenticulata*, etc.). Until relatively recently, species were created on the basis of morphological criteria including such features as size of trophozoites, and cyst morphology. Recently, however, species identification based on morphology is considered unreliable because of variation in cyst morphology due to culture conditions. Increasingly, there is reliance upon molecular characteristics especially 18S rDNA which is more robust and evolutionarily stable and has multiple copies so it is more accurate for identifying and defining phylogenetic relationships among these organisms [48]. Currently, sequencing of the 18S rDNA is being used to differentiate isolates and to understand the phylogeny of *Acanthamoeba*. Based upon such differences, 16 genotypes (T1 to T16) of *Acanthamoeba* have been established [11,39].

Because *Acanthamoeba*, more so than other soil amebae, is often likely to harbor pathogenic bacteria such as *Legionella* spp. *Mycobacterium avium*, *Listeria monocytogenes*, *Burkholderia pseudomallei*, *Vibrio cholerae*, *Escherichia coli* serotype 0157, *Franscisella tularensis*, *Helicobacter pylori*, and *Afipia felis*, it has generated considerable interest in recent years [2,16]. According to some estimates, approximately 20–24% of clinical and environmental isolates of *Acanthamoeba* spp. harbor pathogens such as *Chlamydia*, *Chlamydophila* and approximately 5% of *Acanthamoeba* isolates harbor *Chlamydia*-like bacteria [14]. While most of this work has been based upon in vitro studies, *Acanthamoeba* infection with *Legionella*-like bacteria has been found in amebae isolated from soil samples, and *Acanthamoeba* strains harboring *M. avium* have been found to be more virulent than noninfected amebae in a mouse model of infection. Additionally, pure cultures of *A. polyphaga* have been used to isolate *L. pneumophila*, *L. anisa*, *M. masiliense* from clinical specimens such as sputum, liver, lung abscesses, and feces [26,53]. A feature common to all the prokaryotes that can develop within amebae is that they are obligate or facultative parasites of human phagocytic cells, suggesting that pathogenesis for humans may have evolved from infection of amebae and perhaps other protozoa, and that amebae may have an important role in the etiology of several bacterial diseases. Therefore, *Acanthamoeba* serving as reservoirs to these bacteria, some of which are potential pathogens of humans, has public health importance in areas as nosocomial diseases and as potential bioterrorism agents. Recently, a virus (mimivirus) about the size of a small bacterium with a 1.32 megabase genome has been discovered in *A. polyphaga* [2,16].

Acanthamoeba spp. can be readily grown and maintained in the laboratory using a variety of media. Since bacteria are their major food source, they will grow in the laboratory on non-nutrient agar covered with bacteria. They

show a preference for bacteria that are not encapsulated, the presence of a mucoid capsule inhibits phagocytosis by the amebae. Amebae feed upon the bacteria until most of the food is gone, and then encyst. Cysts remain viable for prolonged periods of time, especially if the agar plate is sealed, to prevent drying, and kept at a reduced temperature. *Acanthamoeba* can also be readily established in bacteria-free or axenic culture by harvesting amebae from a bacterized culture, washing them in distilled water or ameba 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 ameba inoculum. A medium consisting of proteose peptone-yeast extract-glucose will support axenic growth. A number of isolates from human *Acanthamoeba* infections often require calf serum for optimum growth. Several different species of *Acanthamoeba* have also been grown in a chemically defined medium [40].

Acanthamoeba granulomatous amebic encephalitis (AGAE)

Acanthamoeba granulomatous amebic encephalitis (AGAE) is an insidious, chronic infection of the central nervous system spanning from several weeks to months. AGAE occurs, most often, in humans with compromised metabolic, physiologic, or immunologic functions because of HIV/AIDS, or who are chronically ill, diabetic, have undergone organ transplantation, or are otherwise debilitated with no recent history of exposure to fresh water. Cases may occur at any time of the year. The precise portal of entry is not clearly known, but the wide dissemination of these amebae in the environment allows for many possible modes of infection. Trophic amebae and/or cysts of *Acanthamoeba* have been isolated from the nasal mucosa of healthy individuals suggesting a nasopharyngeal route as one means of invasion and it has been estimated that nasal carriage of *Acanthamoeba* may be in the range of 2% in a sampling of Australian university students to 24% in Nigerian children. Sinusitis and nasopharyngeal infections by *Acanthamoeba* have occurred in AIDS patients. Amebae may also enter the body through breaks in the skin or trauma or injury to the corneal epithelium because of a foreign body or use of a contact lens. Amebae were also found in a biopsy of a perforated gastric ulcer, suggesting that entry was via an oral route [51]. From skin lesions, amebae can be transported via hematogenous spread to other organs and organ systems of the host [23,26, 29,51,53].

Onset of AGAE is slow and is usually recognized by neurological manifestations and behavioral changes. Other symptoms include seizures, headache, and visual disturbances, stiff neck, and mental state abnormalities as well as nausea, vomiting, low-grade fever, lethargy and cerebellar ataxia, hemiparesis, seizure, and coma. Facial palsy with numbness resulting in facial asymmetry is often seen. Cerebrospinal fluid (CSF) examination of patients with AGAE has revealed lymphocytic pleocytosis with mild elevation of protein and normal or slightly decreased glucose. *Acanthamoeba*, however, is not usually found in the CSF of patients with GAE. Additionally, *Acanthamoeba* that had apparently entered from the nasopharynx through a fistula have been detected in a patient without CNS disease, and *Acanthamoeba* DNA has also been detected in the CSF [3,23,26,29,34,51,53].

Localized foci of infection can also be recognized in brain tissue by neuroimaging. Computerized tomography (CT) scans of the brain show large, low density abnormalities mimicking a single or multiple space-occupying mass. CNS of GAE cases reveal cerebral edema, encephalomalacia of cortical and basal ganglia, and multiple necrotic hemorrhagic areas. The brain stem, cerebral hemispheres, and cerebellum may show areas of hemorrhagic infarcts. Microscopic examination of CNS reveals the presence of multinucleated giant cells in the brain stem, cerebral hemispheres, cerebellum, and basal ganglion. Necrotic tissue with lipid-containing macrophages and neovascularization suggesting tumor are often seen. *Acanthamoeba* is found in either trophic or cystic stage in tissue (Figure 1). Definitive identification of amebae usually follows upon brain biopsy or at autopsy and microscopic visualization of trophic or cystic amebae in hematoxylin and eosin-stained brain tissue sections, or culturing amebae from clinical samples. In sectioned tissues, amebae can be distinguished from host cells by their prominent central nucleolus and by their location in the perivascular areas in brain tissue. Additionally, immunohistochemical techniques such as indirect immunofluorescence or immunoperoxidase are useful in the recognition of amebae in brain tissues [22, 23,26,29,51,53].

In cases where dissemination has occurred, trophozoites and cysts of *Acanthamoeba* are usually found in the CNS tissues, pulmonary parenchyma, maxillary sinus, prostate, adrenals, and skin lesions. The amebae are present within the perivascular area so the brain can be distinguished from the host cells by their prominent central nucleolus. Since *Balamuthia* also produces cysts in the brain tissue, identification of the genus is based on immunochemical analysis, electron microscopy, and molecular techniques.

Some patients, especially those with HIV/AIDS, develop skin lesions, abscesses, or erythematous nodules on the chest, arms, and legs. These nodules are usually firm and nontender but sometimes may become ulcerated and purulent [22,23,26,29,51,53].

In addition to their involvement in human disease, *Acanthamoeba* spp. also cause infections of the CNS of

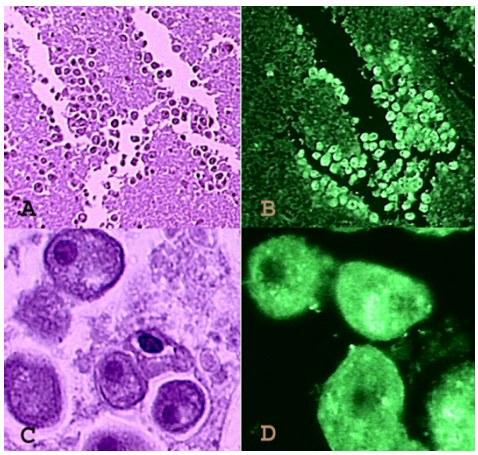

Figure 1: CNS section of a patient infected with *Acanthamoeba*. (A) Low-power view of a CNS section showing multitudes of amebae, H&E stain, X100; (B) amebae from the same area as in A reacted with rabbit anti-*Acanthamoeba* serum showing intense reaction with the serum, X100; (C) a higher magnification (X1,000) showing the characteristic nuclear morphology of the trophozoites; (D) reactivity of the amebae with the rabbit anti-*Acanthamoeba* serum. No such reaction seen with heterologous sera.

animals including gorillas, baboons, gibbons, monkeys, dogs, ovines, bovines, horses, kangaroos, and have also been isolated from birds, reptiles, amphibians, fishes, and even invertebrates [22,23,29,51,53]. It has been shown by the analysis of SSU rRNA gene sequences that several *Acanthamoeba* isolates from fish, reptiles, birds, and those isolated from *Acanthamoeb*a keratitis cases belong to the same genotype T4, suggesting that features that enable these amebae to infect animals may also help them to infect humans [50].

Much of the damage is done by *Acanthamoeba* trophozoites in the course of infections that are probably the result of several different pathogenic mechanisms including phagocytosis of host cells. *Acanthamoeba* may develop specialized feeding cups like those found in *N. fowleri* amebae (see below) which may help nibble away bits and pieces of host tissue thereby destroying host cells, another mechanism involved in damaging host tissues. Several studies have described that enzymes produced and secreted by *Acanthamoeba* may facilitate spread of amebae by opening avenues for invasion and providing nutrients in the form of lysed host cells components. Subsequently, depending upon the species and strains studied, *Acanthamoeba* may produce serine and cysteine proteinases, metalloproteinases, plasminogen activators, and may exhibit chemotactic responses. It has been demonstrated that a serine proteinase produced by a pathogenic isolate of *A. healyi* might aid in complement destruction and degradation of human IgA antibody. Other more recent studies have shown that the initial process of invasion occurs when a 136-kDa mannose-binding protein (MBP), a lectin, expressed on the surface of the ameba adheres to mannose glycoproteins on the surface of the epithelial cells and is therefore central to the pathogenic potential of *Acanthamoeba* [10,32].

Host response

Since acanthamoebae are ubiquitous in nature, humans are exposed to them and may produce antibodies to these amebae. Antibodies to *Acanthamoeba* have been found in healthy soldiers as well as in hospitalized patients in the former Czechoslovakia, adults and children from New Zealand, and patients hospitalized for respiratory problems. Tests such as indirect fluorescent antibody (IFA) and enzyme immunoassay (EIA) have been developed to detect antibody to *Acanthamoeba* in sera of patients as well as infected individuals [23,26,51,53]. According to one study, Hispanics are 14.5 times less likely to develop antibodies to *Acanthamoeba*, especially *A. polyphaga*, than Caucasians [9]. Whether this natural antibody results in protective immunity against infection is not known. Much of the information about the host immune response to *Acanthamoeba* is from in vitro and mouse experimental studies. The alternative complement pathway and antibody formation are both important defense mechanisms, activating neutrophils to destroy invading amebae. Once stimulated, neutrophils release liposomal enzymes and reactive oxygen intermediates, including hypochlorite and hydrogen peroxide that promote destruction of amebae. More recently, Marciano-Cabral et al. (2000) demonstrated the role of macrophages and brain microglia cells—a specialized macrophage cell-type—in *in vitro* killing of *Acanthamoeba*. They noted that microglial cells produce a variety of interleukins (IL-1 α or IL-1 β) and tumor necrosis factor when cultured with *Acanthamoeba*. In an earlier study from the same laboratory, although *Acanthamoeba* activated the complement pathway, it was shown to be resistant to complement-mediated lysis [26]. Different conclusions in these studies can reflect upon a number of factors including species and strains of *Acanthamoeba* used, as well as the growth conditions employed.

Acanthamoeba keratitis

Acanthamoeba keratitis (AK) is an acute localized infection involving the cornea. Unlike GAE, it occurs in immunocompetent individuals following corneal trauma or, more commonly, as the result of poor hygiene in the care of contact lenses or contact lens cases [45]. Wearers of contact lenses are a high risk group for *Acanthamoeba* keratitis. The majority of cases are due to the use of nonsterile tap water in preparation of contact lens solutions [47]. Amebae proliferate in the storage case contaminated with bacteria resulting

in the transfer of amebae from the case to the lens surface and, ultimately, to the corneal surface, where it is difficult to eradicate them with antimicrobial treatment. In the presence of an antimicrobial agent, trophic amebae encyst and survive with the course of therapy. No case of GAE has ever been reported to be developed from a case of amebic keratitis, although a case of uveitis was associated with fatal GAE [19]. Amebae isolated from keratitis infections generally have lower temperature optima than that of GAE isolates, consistent with their superficial location. Among the species that have been implicated as etiological agents are *A. castellanii*, *A. polyphaga*, *A. rhysodes*, *A. culbertsoni*, and *A. hatchetti*.

Amebae adhere to the corneal surface, perhaps aided by specific receptors that enable them to bind to the corneal epithelium and by the presence of calcium ions [10,32]. Once attached, they infiltrate the stroma and cause tissue necrosis. Symptoms of infection include severe pain, lacrimation, photosensitivity, and the appearance of ring infiltrates. Corneal transplants have been used to repair corneal damage and eliminate infection; nucleation has been employed where treatment has failed. Typically, only one eye is involved; however, bilateral keratitis has also been reported. AK may be confused with viral keratitis resulting in delay in correct diagnosis and thus delay in initiating appropriate therapy to eliminate the amebae. Definitive diagnosis is based on visualization of amebae upon microscopic examination of corneal scrapings or biopsies or their cultivation from affected tissue. Confocal microscopy has been used as an aid in the diagnosis of *Acanthamoeba* keratitis [33]. Molecular techniques, such as PCR and real-time PCR, have also been used to identify *Acanthamoeba* in the CSF, brain, and corneal tissue as well as tear fluid and genotype the ameba involved in the infection [23,26, 53]. Based on 18S rDNA sequencing, a majority of the isolates obtained from the environment, keratitis patients, CSF, brain, lungs, skin, and nasal passages, and other non-AK sources have been identified as belonging to genotype T4. Recently, a multiplex real-time PCR assay has been developed that can detect not only *Acanthamoeba* but also *Balamuthia* and *N. fowleri* in clinical specimens [36]. In the United States, AK was recognized for the first time in 1973 in a south Texas rancher and *A. polyphaga* was isolated from corneal scrapings and biopsy specimens. A number of cases occurred sporadically in different parts of the country from 1973 to 1984 and a jump in the number of cases was observed in 1985. A case control study conducted by CDC at that time revealed that a major risk factor was the use of contact lenses and the use of nonsterile home-made saline [47]. Recently, another dramatic increase in AK cases in the Chicago, Illinois area occurred [20] and CDC conducted an investigation in February 2007 and found a national increase in the AK cases from 2004 through 2006 [49]. This increase was associated with the use of Advanced Medical Optics Complete® MoisturePlus™ multipurpose contact lens solution leading to an international recall by the manufacturer [20,49]. In vitro testing suggested that this solution was inefficient in neutralizing cysts of *Acanthamoeba* belonging to three different species [18].

Antimicrobial therapy

Use of antimicrobial agents against GAE has not been particularly successful, in large part because of several factors: (i) lack of clear-cut symptoms, (ii) lack of reliable noninvasive diagnostic tests and lack of knowledge of the care-givers. Diagnosis is, more often than not, at postmortem. In some cases, however, both CNS and cutaneous infections have been successfully treated. In these cases no single drug has been effective in clearing an infection and combination of pharmaceuticals including pentamidine isethionate, sulfadiazine, 5-fluorocytosine, fluconazole, and itraconazole has often been employed [23,26,53]. Topical applications of chlorhexidine and ketoconazole cream have been effective in treatment of skin nodule infections without CNS involvement [23,26,53]. Recently, voriconazole, a triazole compound, was used to clear cutaneous infection in a patient recovering from lung transplantation [55]. This was possible because of in vitro testing carried out on four clinical isolates that revealed it to be inhibitory even at a concentration of 5 μg/mL [42]. Another drug, miltefosine, a hexadecylphosphocholine, has also shown to have amebicidal activity [42,56]. Miltefosine has been successfully used to treat a GAE patient in Austria [1]. In many cases, however, therapy had to be discontinued because of toxicity [23,26,53].

Treatment of *Acanthamoeba* keratitis has been more successful than that of GAE. A variety of drugs have been used in treatment, including chlorhexidine, polyhexamethylene biguanide (PHMB), propamidine isethionate, dibromopropamidine isethionate, neomycin, paromomycin, polymyxin B, clotrimazole, ketoconazole, miconazole, itraconazole [23,26,45,51,53]. Brolene, a commercially available eye medication (in Great Britain) containing propamidine isethionate and dibromopropamidine isethionate, was found to be effective in treatment of *Acanthamoeba* infections but it may be accompanied by drug toxicity and resistance. Used in conjunction with propamidine and other antimicrobials, dimethylsulfoxide was found by in vitro testing to enhance the uptake of antimicrobials into cysts. As is the case for GAE infections, treatment of amebic keratitis often employs a combination of drugs. Recently, however, significant medical cure has been achieved with the application of either PHMB or chlorhexidine gluconate with or without Brolene® [45]. When medical treatment failed debridement and/or penetrating keratoplasty have been

used with good results in some cases. Currently, the drugs of choice for *Acanthamoeba* keratitis are chlorhexidine gluconate, PHMB, and propamidine isethionate (Brolene).

3 *Naegleria fowleri*

N. fowleri causes an acute, fulminating, and hemorrhagic infection, primary amebic meningoencephalitis (PAM) with abrupt onset in previously healthy children and young adults with a history of swimming in fresh water lakes, streams, or pools, or washing in tap water containing the amebae about a week prior to the onset of symptoms. Amebae are aspirated into the nasal passages and, after attaching to the nasal mucosa, migrate across the cribriform plate to the brain via the olfactory nerves, causing extensive damage to the frontal lobes of the brain. The first recognized case of PAM was reported in Australia in 1965 but was attributed to *Acanthamoeba*. The first case of *N. fowleri* infection in the US was identified in Florida in 1966 and the term primary amebic meningoencephalitis was coined to describe the disease. The striking feature of PAM is the rapid onset of symptoms following exposure to fresh water, within as little as 24 hours. The disease progresses rapidly and death usually occurs within a week or 10 days. With perhaps a few exceptions all PAM cases reported in literature have been fatal. Diagnosis of PAM can be made by microscopic examination of freshly collected CSF. Amebae, if present, can be identified by their active movement. They can be cultured from samples of CSF or postmortem by placing macerated brain tissue into growth medium or inoculating the brain tissue into tissue culture monolayers [27,29,51,53].

Biology

The genus *Naegleria* consists of more than 40 species and *N. fowleri* is the only species that is known to infect humans. It is also referred to as an ameboflagellate because it has a transitory, pear-shaped, nondividing, nonfeeding flagellate stage in addition to ameboid and cyst stages. The ameboid form is the feeding and dividing stage in the life cycle, but the ameba has the potential of transforming into a flagellate when conditions are appropriate, as when the soil habitat is diluted by rain water. In the laboratory, this transformation can be induced by washing trophic amebae in distilled water. The flagellate stage is transitory and soon reverts to the ameboid form.

In nature, the amebae feed on bacteria and reproduce by binary fission just like *Acanthamoeba* and other free-living ameba species. The trophic amebae exhibit rapid sinusoidal movement by the formation of an eruptive anterior lobopodium. The posterior end or the uroid is sticky and often has several trailing filaments to which bacteria may adhere. The trophozoite measures 10 to 25 μm and is characterized by a single nucleus with a prominent, centrally placed nucleolus that stains densely with chromatic dyes. The nuclear division is of promitotic pattern wherein the nuclear envelope remaining intact throughout mitosis. The cytoplasm contains numerous mitochondria, ribosomes, food vacuole, and a contractile vacuole. Like the trophic ameba, the flagellate has a single nucleus with a large nucleolus and usually has two anterior flagella, but three or four flagella may also be seen occasionally. The flagellate does not have a cytostome and hence cannot feed. It ranges in length from 10 to 16 μm. During adverse conditions when food supply becomes scarce or its habitat dries, the trophozoite transforms into a resistant cyst that can survive environmental stress (e.g. desiccation), although it is more vulnerable than the cyst of *Acanthamoeba*. The *N. fowleri* cyst is uninucleate, measures 8 to 12 μm, and is usually spherical and double-walled with a thick endocyst and a closely apposed thinner ectocyst. The wall has pores flush with its surface that may not be readily seen [27,31].

N. fowleri can also be established in axenic culture by washing amebae from a bacterized culture by centrifugation and transferring them to an antibiotic-containing medium, omitting the antibiotic once the bacteria have been eliminated. It can also be grown in a chemically defined medium for pathogenic *N. fowleri* [27,40].

Although widely distributed in soil and water, *N. fowleri* is not as common as *Acanthamoeba*. *N. fowleri* is present worldwide and has been isolated from fresh and warm water lakes, streams, spas, heated but unchlorinated swimming pools, hot springs, hydrotherapy and remedial pools, aquaria, sewage, and even from nasal passages and throats of healthy individuals. *Naegleria* amebae have not been recovered from sea water, and they rarely occur as tissue culture contaminants, suggesting sensitivity to elevated osmolarites. *N. fowleri* is thermophilic and can tolerate temperatures of up to 45°C. Therefore, these amebae proliferate during summer months when the ambient temperature is likely to be high. Typically, cases of PAM occur during hot summer months when the confluence of large numbers of people engaged in swimming, diving, and water skiing in lakes, ponds, and inadequately chlorinated swimming pools, and other warm fresh water bodies encounter the amebae [27,29,51,53,58].

The laboratory mouse is an ideal animal model to study PAM. Mice can be inoculated intranasally or intracerebrally with a suspension of amebae and most of them die within a week. Adult mice are less likely to become infected than young ones. Pathogenic isolates from PAM patients tend to loose their virulence with repeated subculturing [27]. Virulence, however, can be restored or enhanced by animal passage or even by culturing amebae on tissue culture monolayers [27,29,51,53].

Diagnosis

PAM can be mistaken for pyogenic or bacterial or viral meningitis because of a lack of distinctive symptoms or clinical features. The CSF may have relatively high pressure, low to normal glucose, and high protein concentration. The CSF is pleocytotic with a preponderance of polymorphonuclear leukocytes (PMN) early in the course of the disease, but no bacteria. A wet mount examination of the CSF may reveal the presence of actively moving amebae and a Giemsa or trichrome stains of CSF smears will be necessary to identify the characteristic nuclear morphology of the amebae so that they can be differentiated from the host cells [27,29,51,53]. A recently developed real-time, multiplex PCR assay can identify the DNA in CSF of all three pathogenic amebae, *Acanthamoeba*, *Balamuthia mandrillaris*, and *Naegleria fowleri*, known to cause infections. This usually can be accomplished within 5 h from the time the specimen arrives in the laboratory, an important factor for a quick diagnosis since most patients are treated initially with antibacterial drugs that are ineffective against *N. fowleri* [36].

A sudden onset of bifrontal or bitemporal headaches, high fever, stiff neck, nausea, vomiting, irritability, and restlessness usually presents the initial symptoms of PAM. Later, symptoms may include photophobia, diplopia, lethargy, confusion, bizarre behavior, seizures, and coma, preceding death. The disease progresses rapidly, and owing to the delay in making a correct diagnosis, the prognosis for the patient is poor.

Since *N. fowleri* amebae gain access to the nasal mucosa and migrate across the cribriform plate to the brain via the olfactory nerves, extensive damage is caused to the olfactory lobes leading to hemorrhagic necrosis and purulent exudates. The leptomeninges (the arachnoid and pia mater) are severely congested, diffusely hyperemic, and opaque. Most lesions are found in and around the base of the orbitofrontal and temporal lobes, base of the brain, hypothalamus, midbrain, pons, medulla oblongata, and upper portion of the spinal cord [27,29,51,53].

Microscopic examination of the cerebral hemispheres, brain stem, cerebellum, and upper portion of the spinal cord reveals fibrino-purulent leptomeningeal exudates containing predominantly PMNs and few eosinophils, macrophages, and some lymphocytes. Large numbers of amebic trophozoites are seen within edematous and necrotic neural tissue (Figure 2). Amebae can be recognized by their large, densely staining nucleoli in Virchow-Robin spaces usually around blood vessels with no inflammatory response. Cysts are not found. The amebae can be specifically identified by immunohistochemical methods using polyclonal or monoclonal antibodies (Figure 2). *N. fowleri* can be cultured from samples of CSF or from the brain tissue obtained at autopsy. With few exceptions, all cases of PAM reported in the literature have been fatal.

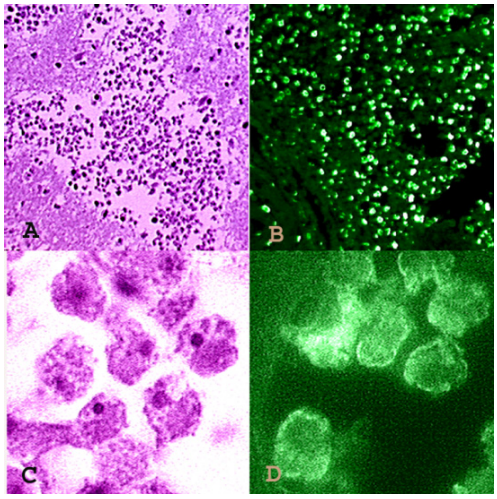

Figure 2: CNS section of a patient who died of primary amebic meningoencephalitis due to *Naegleria fowleri*. (A) The normal architecture of the brain is disrupted. Note the numerous amebae, H&E stain, X100; (B) amebae from the same area as in A reacted with rabbit anti-*Naegleria fowleri* serum showing intense reaction with the serum, X100; (C) higher magnification (X1,000) showing numerous amebic trophozoites but no cysts; (D) reactivity of the amebae with the rabbit anti-*Naegleria fowleri* serum. No reactivity was seen with heterologous sera.

Molecular methods

Naegleria species are phenotypically alike and hence making specific microscopic identification of *N. fowleri* is difficult. Molecular techniques, such as PCR and nested PCR assays, for the specific identification of *N. fowleri* in cultured amebae from patients and the environment as well as *N. fowleri* DNA in the environment, have been developed [27, 29,51,53]. 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 the clinical isolates, it has been shown that two strains of *N. fowleri*, isolated from two PAM patients who visited the same hot spring in southern California but at different times, belonged to the same type II genotype [59]. A real-time multiplex PCR can identify *N. fowleri* DNA in the CSF and brain tissue samples of infected PAM patients antemortem. Recent experiments indicate that this test [36] identifies all three genotypes known to occur in the US. 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. This technique distinguishes several *Naegleria* species in environmental samples [37].

Mechanisms of pathogenesis

N. fowleri produces sucker-like appendages or amebostomes [27,29,51,53] and nibbles away at the cells and tissues. Others (not necessarily mutually exclusive) studies have reported: (1) phospholipase A and B activity or a cytolytic factor causing destruction of cell membranes, (2) neuraminidase or elastase activity facilitating destruction of tissue culture cells, (3) presence of a perforin-like, pore-forming protein that lyses target cells, and (4) the presence within *N. fowleri* amebae of a cytopathic protein that triggers the apotosis pathway in susceptible tissue culture cells [27,29,51,53].

Host response

Given the rapid onset and progression of PAM in humans, there is little opportunity for an effective humoral response to develop against the amebae. Seidel et al. [46] in reporting on the 9-year-old who survived PAM, found anti-*Naegleria* antibody of the IgM class at 7, 10, and 42 days at a titer of 1:4096. Furthermore, the antibodies persisted through 4 years [53]. Marciano-Cabral et al. [28] have demonstrated that large numbers of humans are exposed to *Naegleria* from screening human sera for antibodies against the ameba. Using agglutination of paraformaldehyde-fixed *N. fowleri* amebae by human serum samples as a criterion, differences were observed in agglutinating titers of individuals from two different geographic locations (Virginia and Pennsylvania) in the United States. The authors speculated that regional differences in titer reflected the degree of environmental exposure to amebae. Additionally, sera collected from several individuals with a history of extensive swimming in fresh water lakes in the southeastern US as well as in California also revealed IgM antibodies to *N. fowleri*. These antibodies are particularly well developed to antigens of approximately 190, 66, 30, and 14 kDa. Whether these antibodies have protective activity is not clear. Clearly, humans are exposed to *Naegleria*, if not to the pathogenic species perhaps to the nonpathogneic *N. gruberi* and/or *N. lovaniensis*, through swimming, inhalation of dust and aerosols arising from water taps and humidifiers [27–29, 51,53]. Therefore, it can be concluded that both humans and other animals are exposed to *Naegleria* amebae and develop antibodies against them. Whether these antibodies are protective remains unclear.

Antimicrobial treatment

There is a paucity of effective antimicrobial agents for treating PAM. *N. fowleri* is highly sensitive to amphotericin B, and this has been the drug of choice in treating PAM cases. Among the few cases reported to have survived PAM, only one well-documented case, a 9-year-old female, was rapidly diagnosed and treated early in the course of infection with intravenous and intrathecal amphotericin B, miconazole, and oral rifampin [46]. The authors suggested, based on in vitro testing, that amphotericin B and miconazole had a synergistic action, while rifampin was without effect on the amebae. 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. Phenothiazine compounds (chlorpromazine and trifluoperazine), because of their ability to accumulate in the central nervous system, were tested in vitro against pathogenic *Naegleria* with good evidence of growth inhibition. Azithromycin, a macrolide antimicrobial, has been shown to be effective against *N. fowleri* both in vitro and in vivo (mouse model of disease. *N. fowleri* was also susceptible to the triazole, voriconazole; low concentrations ($\leq$ 10 μg/ml) were amebastatic while concentrations $\geq$ 10 μg/ml were amebicidal [27,29,42,51,53].

4 *Balamuthia mandrillaris*

Biology

There had been reports in the literature over the years of encephalitis caused by amebae that were neither *Acanthamoeba* nor *Naegleria* by immunofluorescence staining. Ultimately, the isolation of amebae from the brain of a mandrill baboon that died in the San Diego Wildlife Park enabled infections of these non-*Acanthamoeba*-*Naegleria* cases to be diagnosed [52]. The ameba is larger (12–60 μm) than either *Acanthamoeba* or *Naegleria* and resembled in many of its features the leptomyxid amebae included in the genus *Leptomyxa*. In 1993, it was recognized as being different from the leptomyxids and was designated as a new genus and species, *Balamuthia mandrillaris* [54]. An antibody produced in rabbits against this amebae reacted strongly, not only with the amebae in the brain sections of the mandrill but also with the amebae in the brain sections of all those cases that had tested negative with the anti-*Acanthamoeba* and anti-*N. fowleri* sera in the IIF test, indicating that *B. mandrillaris* had caused those infections. With the identification of the baboon ameba, human cases were soon discovered and additional strains of the ameba were isolated [44,51,53,54].

To date, *B. mandrillaris* is the only known species belonging to the genus *Balamuthia* and causes GAE in both humans and other animals. The disease it causes is similar to GAE caused by *Acanthamoeba* and develops in immunocompromised hosts, including AIDS patients and intravenous drug users. But it has also been reported from immunocompetent individuals where, in addition to the typical GAE, it infects the sinus cavities and crosses into the brain. The disease is chronic, developing over a period of

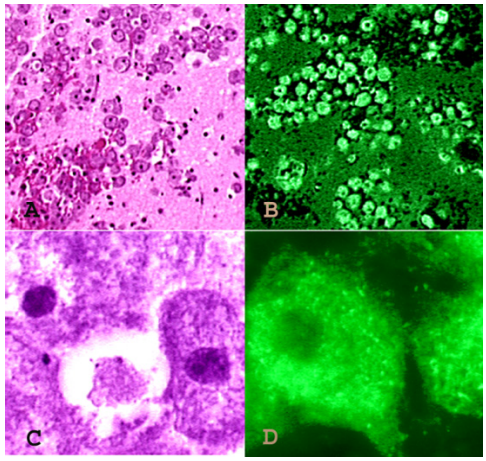

Figure 3: CNS section of a patient who died of *Balamuthia mandrillaris* GAE. (A) Low-power view; note the numerous amebae in pockets, H&E stain, X100; (B) amebae from the same area as in A reacted with rabbit anti-*Balamuthia mandrillaris* serum showing intense reaction with the serum; (C) higher magnification (X1,000) of the same section showing two trophozoites with multiple nucleoli, a feature characteristic of *Balamuthia mandrillaris*; (D) reactivity of the amebae with the rabbit anti-*Balamuthia mandrillaris* serum. No reactivity was seen with heterologous sera.

time from about 2 weeks to 2 years. Recently, however, two clusters of transmission of *B. mandrillaris* infection through solid organ transplantation were reported [7,8]. Humans over a wide age range from few months to > 50 years have developed infections [44,51,53].

B. mandrillaris is also known to cause infections of the skin and maxillary sinus and disseminates into a wide variety of organs including the lungs, kidney, and uterus, similar to that caused by *Acanthamoeba*. *Balamuthia* GAE, like *Acanthamoeba* GAE may occur at any time of the year and, therefore, has no relation to seasonal changes [44,51,53].

B. mandrillaris, like *Acanthamoeba*, has only two stages in its life cycle (Figure 3). The trophozoite is pleomorphic and measures 12 to 60 μm with a mean of about 30 μm. The trophozoites are usually uninucleate, but binucleate forms are occasionally seen. The nucleus contains a large, centrally located, densely staining nucleolus although occasionally trophozoites with two or three nucleolar bodies are seen, especially in infected tissues. Cyst is also uninucleate, more or less spherical, and ranges in size from 12 to 30 μm with a mean of 15 μm. Under the light microscope, cysts appear to be double walled, with a wavy outer wall and a round inner wall. Ultrastructurally, however, the cysts are tripartite with an outer thin and irregular ectocyst, an inner thick endocyst, and a middle amorphous fibrillar mesocyst (Figure 3) [52, 54].

Cultivation

The ameba grows well and abundantly when supplied with a diet of tissue culture cells including monkey kidney, human lung fibroblasts, rat glial cells, human brain microvascular cells (HBMEC) etc. The amebae feed on the cell monolayer, decimating it within several days. *B. mandrillaris*, unlike *Acanthamoeba* and *N. fowleri* cannot be grown on bacteria-coated agar plates. It has however been isolated from the environment and it is believed that it feeds on small amebae that are present in nature as it has been shown that it can feed on *Acanthamoeba* and *Naegleria* in vitro in the laboratory but did not ingest *Acanthamoeba* cysts [44,53]. It can be isolated from human and animal tissue using mammalian cell cultures listed above. *Balamuthia* can also be grown axenically in a complex cell-free medium containing fetal bovine serum [40].

Diagnosis

CSF examination in general reveals lymphocytic pleocytosis, increase in protein, and normal or decreased glucose levels. Although *B. mandrillaris* has been isolated antemortem from brain biopsies obtained from patients and also from a CSF obtained at postmortem final diagnosis as *Balamuthia* GAE was made only at autopsy [44,53]. Since both *Acanthamoeba* and *B. mandrillaris* produce cysts in tissue and cysts look similar in sectioned tissues at light microscopy, it is difficult to specifically identify the genus and species. In few cases *B. mandrillaris* can be identified based on the presence of multiple nucleoli within its nucleus. They can also be differentiated from *Acanthamoeba* by immunohistochemical analysis of the tissue sections using rabbit anti-*Acanthamoeba* and anti-*B. mandrillaris* sera and by transmission electron microscopy and molecular methods [44, 52–54].

Pathology

There is a pronounced similarity between *Balamuthia* encephalitis and that of *Acanthamoeba*. The amebae are found in similar locations in the brain causing hemorrhagic necrosis in the midbrain, thalamus, brainstem, and cerebellum [44,52–54]. Cases have occurred in children as well as older persons. Most of the young who have developed the disease had no obvious risk factors and have reportedly been in good health. Children developing *Balamuthia* GAE had facial skin lesions and/or rhinitis with infections of the sinus cavities or otitis media. Symptoms in children and adults have included headache, nausea and vomiting, fever, myalgia, weight loss, and seizures. Cutaneous ulcers or lesions may appear initially followed by neurological symptoms as amebae invade the CNS. In a number of Peruvian patients, painless skin lesions appear as plaques a few millimeters thick and one tom several centimeters wide. The lesions may occur in the center of the face and on the hands, feet, trunk accompanied by rhinitis before CNS involvement [6,44]. The duration of symptoms has ranged

from several days to 2 years, and the period from onset of symptoms to time of death has ranged from about one week to $>$ 2 months. Like its *Acanthamoeba* counterpart, the disease is of a chronic nature, developing slowly and insidiously.

Initial CT scans may be unremarkable. Later on when the infectious process has advanced CT and MRI may indicate hemorrhage within the lesions, and angiography may demonstrate occluded blood vessels corresponding to areas of infarction. The brain scans may also show "space occupying mass" which may mimic a brain abscess, brain tumor, or intracerebral hematoma. Many GAE cases have been erroneously diagnosed as neurotuberculosis or neurocysticercosis [44,53].

At autopsy the brain appears to be edematous with multiple areas of meningeal softening. Inflammation is seen in the brain stem, cerebral hemispheres, and cerebellum. The hemorrhagic necrosis and inflammatory infiltrates consist of neutrophils, mononuclear cells, and multinucleated giant cells interspersed with *Balamuthia* trophozoites and cysts (Figure 3).

The disease has also been reported in animals, particularly those in zoological parks. Several of the cases have been in gorillas, gibbons, monkeys, horses, sheep and dogs [13,44,51]. An animal model for the study of *Balamuthia* GAE has made use of the SCID (Severe Combined Immuno Deficient) mouse, with a 70% mortality following intranasal inoculation of SCID animals compared with 10% mortality in immunocompetent BALB/c mice [17]. In a more recent experiment, it was shown that *Balamuthia* amebae migrate to the CNS after oral infection of both immunocompetent and immunodeficient mice and *Balamuthia* antigen but no organisms have been found in mouse fecal pellets [24].

Mechanisms of pathogenesis

Balamuthia probably invades human tissue by ingesting small pieces of host tissue and producing proteolytic enzymes that degrade ghost tissues. It may also induce host cells to release IL-6 that plays a role in initiating early inflammatory response [44]. More recently, however, it has been shown that *B. mandrillaris* interacts with the host connective tissue containing extracellular matrix (ECM) proteins such as collagen -1, fibronectin, and laminin-1 [38]. It has been shown by scanning electron microscopy that surface projections or food cups are produced by *Balamuthia* when it binds to ECM [38].

Molecular characterization

Recent evidence based upon sequencing of 16SSU rDNA suggests that *B. mandrillaris* is phylogenetically related to *Acanthamoeba* [4]. PCR probes have been developed to identify *Balamuthia* in clinical specimens. A real-time, multiplex PCR assay is routinely used at CDC to identify *Balamuthia* DNA in human CSF and brain tissue [36]. Molecular techniques are used to identify *Balamuthia* not only in clinical specimens but also in the epidemiology of balamuthiasis. For example, a *Balamuthia*-specific primer-pair using sequence data from the mitochondrial 16S rRNA gene has been used to match *Balamuthia* DNA isolated from the brain of an infected child with the DNA of ameba isolated from the flower potting soil in the child's home [5]. It has also been shown that PCR can retrospectively confirm *Balamuthia* infections in archived slide specimens fixed in formalin and embedded in paraffin [57]. Sequencing of the 18S r DNA gene and the 16Sr DNA has shown that there is very little variation and that infections caused by *Balamuthia* are due to a single species [4].

Immunology

Since GAE due to *Balamuthia* is a chronic infection, it is logical to expect an antibody response. Indirect immunofluorescence (IFA) and flow cytometry techniques have been used to identify antibodies to *Balamuthia* in the sera of healthy persons as well as patients with *Balamuthia* GAE [44,51,53]. According to these studies, titers ranged from $1:64$ to $1:256$, and antibodies belonged to the IgG and IgM classes. Neonatal serum samples had lower titers ($1:4$), but titers rose to adult levels in the 1–5 year age group. Schuster et al. examined serum samples from encephalitis patients as a means of detecting *Balamuthia* GAE. Although no cases of GAE were detected in their study, about 10% of the patient population had titers of or greater than $1:64$, suggesting that individuals do have a humoral response to these amebae [43]. The presence of such antibody titers in humans and animals is probably due to exposure to these amebae in the environment as they are presumed to be ubiquitous. According to a recent study, West Africans belonging to traditional farming/hunting communities had high-titered antibodies to *B. mandrillaris* and these authors believe that the West Africans probably had constant contact with amebae antigenically similar to *B. mandrillaris* and therefore developed high titers to *B. mandrillaris*. According to them, such high titers might stem from actual infection with *Balamuthia* that were successfully overcome indicating that not all infections with *B. mandrillaris* are lethal and that not all *B. mandrillaris* are pathogenic [25].

Antimicrobial therapy

Most of the individuals who were diagnosed as having the disease had been treated empirically with antibacterial, antifungal, or antiviral agents but these had minimal effect upon progression of the disease. In many cases, anti-inflammatory steroids have been administered which may

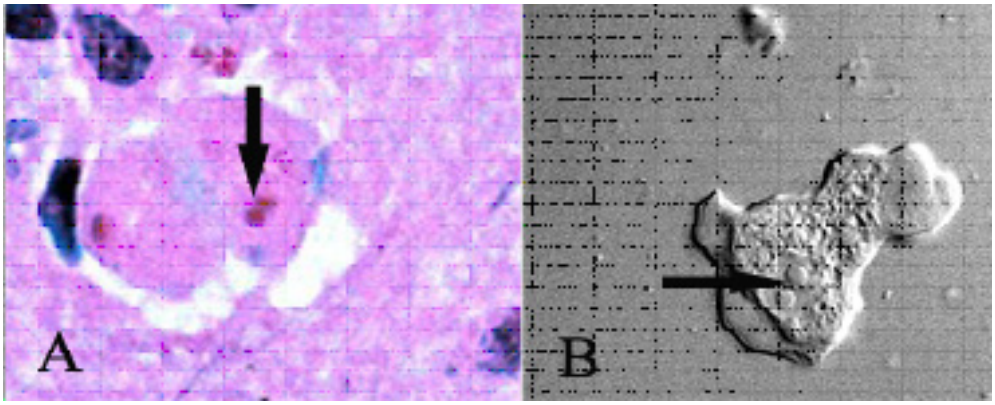

Figure 4: CNS section of the patient who survived infection with *Sappinia pedata* encephalitis (A), X1,000. Note the binucleate ameba (at arrow). (B) *Sappinia pedata* trophozoite from culture exhibiting two nuclei apposed to each other (arrow), X1,000.

have actually enabled spread of the infection. Recently, however, three patients, an ∼60-year-old patient from California, a 6-year-old girl, also from California, and a 70-year-old female from New York, survived the infection after treatment with a combination of pentamidine isethionate, sulfadiazine, azithromycin/clarithromycin, fluconazole, and 5-fluorocytosine [12,21]. Currently, several patients with balamuthiasis are being treated with the above regimen as well as miltefosine [7,8], which has been found to have strong inhibitory effects in vitro on *Balamuthia* [42] and has been used successfully to cure this infection [30]. Other survivors include two Peruvian patients with cutaneous lesions who became well after prolonged therapy with albendazole and itraconazole [6]. *In vitro* studies have shown that pentamidine and propamidine isethionate at a concentration of 1 μg/ml inhibit growth of amebae by 82% and 80%, respectively, see [44,53]. The drugs, however, were amebastatic but not amebacidal. Amphotericin B, effective against *Naegleria*, had little effect upon *Balamuthia*. Among other drugs tested were macrolides antibiotics, azole compounds, gramicidin, polymyxin B, trimethoprim, sulfamethoxazole, and a combination of trimethoprim-sulfamethoxazole [53]. Given the problems with diagnosis of infection and the lack of effective antimicrobial agents, the prognosis is poor.

Epidemiology

Little is known about the source of these infections. Several individuals who have developed the disease had contact with soil in one way or another: contamination of a wound while gardening or inhalation of dust containing *Balamuthia* cysts may allow the ameba to enter the body and spread to the CNS via a hematogenous route to the brain. It is possible, of course, that water might serve as a vehicle for transmission as in the case of *Naegleria* PAM, but no case has been attributed to swimming or other water activities although two dogs contracted *Balamuthia* GAE after exposure to water [13]. A recent paper dealing with *Balamuthia* infection in Hispanic Americans suggests possible genetic predisposition based on as yet undetermined factors [41].

5 *Sappinia*

Sappinia pedata, previously described as *S. diploidea*, has caused single case of encephalitis in an immunocompetent male who developed headache, seizure, blurred vision, photophobia, and vomiting [15,35]. MRI revealed a single space-occupying lesion that was surgically removed. Trophic amebae but no cysts were seen in the excised brain tissue (Figure 4). The patient was also treated empirically with azithromycin, pentamidine isethionate, itraconazole, and flucytosine and recovered with no neurological sequleae. Members of the genus *Sappinia* have been previously isolated from feces of humans, elk, bison, and cattle. The amebae are large measuring $> 40\,\mu$m and are characterized by the presence of two nuclei tightly apposed to one another. Cysts are also binucleate and can survive adverse conditions in the environment. A recently developed real-time PCR using PCR primers and TaqMan probe detects both species of *Sappinia* but not other free-living amebae including *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri* or human DNA. The oligonucleotides designed for this purpose were SappF1576 (5'-TCT GGT CGC AAG GCT GAA AC-3'), SappR1736 (5'-GCA CCA CCA CCC TTG AAA TC-3'), and SappP1705 (HEX-5'-TGT CAA TCT GTC AAT CCT CGT CAA GTC T-BHQ-3') [35].

6 Conclusions

Encephalitis caused by the free-living amebae *Acanthamoeba*, *Naegleria*, and *Balamuthia*, although not a major public health problem, is still a concern because it causes

high mortality, especially in children. These infections are difficult to diagnose and it is likely that many cases have gone undetected. A combination of limited resources and lack of diagnostic expertise contribute to the lack of knowledge of the actual incidence of these infections.

Disclaimer

The findings and conclusions in this paper are those of the author and do not necessarily represent the views of the Department of Health and Human Services or the Centers for Disease Control and Prevention.

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