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# Opportunistic free-living amoebal pathogens

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**ABSTRACT**

Pathogenic free-living amoebae affecting the central nervous system are known to cause granulomatous amoebic encephalitis (GAE) or primary amoebic meningoencephalitis (PAM). Although hosts with impaired immunity are generally at a higher risk of severe disease, amoebae such as *Naegleria fowleri* and *Balamuthia mandrillaris* can instigate disease in otherwise immunocompetent individuals, whereas *Acanthamoeba* species mostly infect immunocompromised people. *Acanthamoeba* also cause a sight-threatening eye infection, mostly in contact lens wearers. Although infections due to pathogenic amoebae are considered rare, recently, these deadly amoebae were detected in water supplies in the USA. This is of particular concern, especially with global warming further exacerbating the problem. Herein, we describe the epidemiology, presentation, diagnosis, and management of free-living amoeba infections.

**KEYWORDS**

Free-living amoebae; *acanthamoeba*; *Balamuthia*; *Naegleria*; keratitis; CNS infection; encephalitis; meningoencephalitis

## Introduction

Pathogenic free-living amoebae, such as *Acanthamoeba* spp., *Naegleria fowleri* and *Balamuthia mandrillaris*, cause infection of the central nervous system (CNS) [1, 2]. The detection of brain-eating amoebae in drinking water supplies is of concern, which further indicates the severe threat posed by free-living amoebae to communities [3–6]. Furthermore, infection of the CNS with *Acanthamoeba* spp., *Naegleria fowleri* and *Balamuthia mandrillaris* almost always leads to mortality [7]. Moreover, cases of amoebic infection are underreported worldwide, because of lack of awareness and diagnostic modalities, as well as misdiagnosis, due to similarity in symptoms, of amoebic infection of CNS to other common CNS infections such as bacterial meningitis, and thus, the true burden of cases due to these amoebae is unknown [8,9].

*Acanthamoeba* spp. infect the CNS, causing granulomatous amoebic encephalitis (GAE), and can also cause a sight-threatening eye infection known as *Acanthamoeba* keratitis (AK) [10, 11, 12]. *B. mandrillaris* is known to instigate *Balamuthia* amoebic encephalitis (BAE) in the CNS and infect other organs such as the lungs and skin, in both immunocompetent and immunocompromised individuals [11]. *N. fowleri* infects the CNS, causing primary amoebic meningoencephalitis (PAM), triggering a prompt onset of disease and leading to death within days [11, 13]. Treatment of CNS infection with amoebae is complicated and hampered by the selectivity of the blood-brain barrier (BBB) that affects drug permeability into the brain. The purpose of this review is to briefly describe the epidemiology, presentation, diagnosis and management of CNS complications due to free-living amoeba and keratitis caused by pathogenic *Acanthamoeba*.

## Acanthamoeba spp

*Acanthamoeba* spp. are free-living protists that exist in a variety of environments, such as water, soil and air [14]. *Acanthamoeba* spp. can exist in two forms, namely, active trophozoites and dormant cysts, by transitioning under stressful conditions, such as starvation and desiccation, from trophozoite to cyst [15,16]. *Acanthamoeba* trophozoites, the metabolically and reproductively active form of the amoeba, is the form that the amoebae assume when under favorable conditions, such as nutrient-rich environment and appropriate pH, osmolarity and temperature [17]. *Acanthamoeba* trophozoites are irregular in shape and pseudopods are used for movement, while acanthopodia, spike-like protrusions are responsible for adhesion to inert and biological surfaces [10,18]. Based on 16S rDNA sequencing, genus *Acanthamoeba* consists of at least 22 genotypes (T1 – T22), while genotype T4 is proportionally over-represented in infections [19], only one species of *Balamuthia* genus, i.e. *B. mandrillaris*, infects humans and only one species of *Naegleria*, i.e. *N. fowleri*, is known to infect human [11]. Pathogenic *Acanthamoeba* spp. are more genetically diverse than both *B. mandrillaris* and

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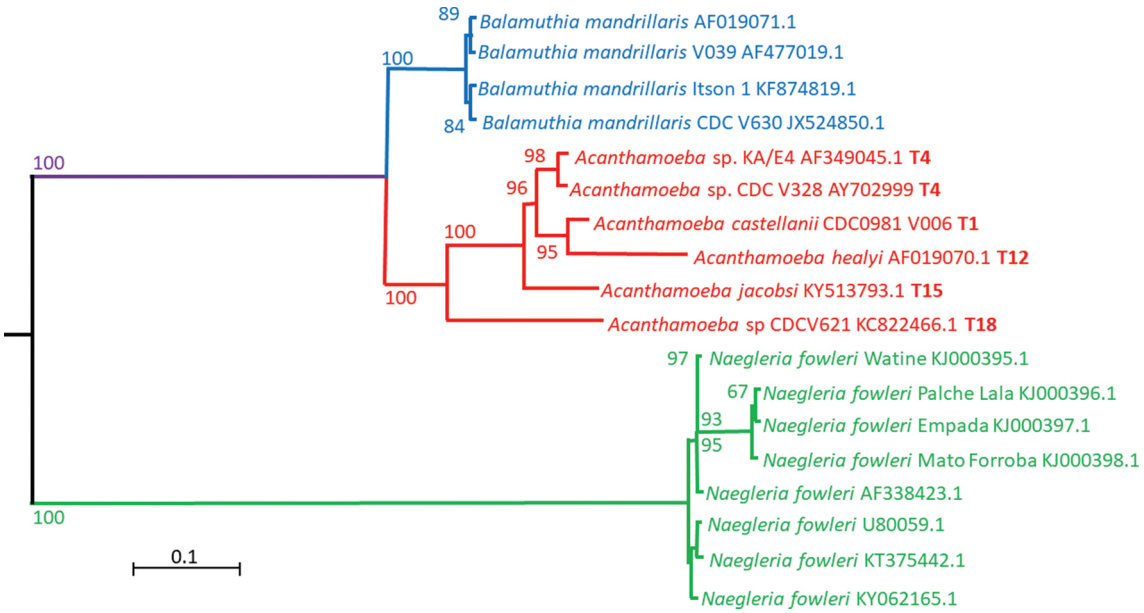


**Figure 1.** A PhyML phylogenetic tree (GTR model) based on the 18S ribosomal gene of the three pathogens discussed in this study. Sequences obtained from genbank (https://www.ncbi.nlm.nih.gov/genbank/) were aligned using ClustalW version 2 [179] and the trees calculated using seaview version 4 [189]. The tree was rooted with *Giardia intestinalis* (AF473852.1) and *Giardia ardeae* (Z17210.1). Bootstrap analysis was performed using 1000 pseudo-replicates. The scale bar shows the evolutionary distance for the nucleotide substitutions per site.

*N. fowleri,* perhaps suggesting a more recent evolution of the latter two species (Figure 1). Whereas pathogenicity is limited to a single species in both *Balamuthia* and *Naegleria*, many genotypes, broadly equivalent to species, show human pathogenicity in *Acanthamoeba*. It has been proposed that *N. fowleri* evolved from *N. lovaniensis* and has spread throughout the world with the winds and minor differences detected in the ITS1 region allow the progression of the natural spread of *Naegleria fowleri* to be deduced [20]. This distribution indicates a relatively recent series of events since this contravenes the 'everything is everywhere' concept [21], which is generally found to be applicable to protists and seems to hold true for *Acanthamoeba* [22].

Intracellular components of *Acanthamoeba* trophozoites, enclosed within the plasma membrane, include mitochondria, nucleus, Golgi complex, endoplasmic reticulum and vacuoles [23]. *Acanthamoeba* trophozoites (15–45 µm) transform into a more compact dormant cyst stage (10–25 µm) when exposed to unfavorable conditions, including extreme temperatures or pH, high salination or osmolarity and lack of nutrients or drought [24]. *Acanthamoeba* cysts are round and are surrounded by a capsule, consisting of an inner endocyst and an outer ectocyst, made up primarily of cellulose and consolidated by binding of cellulose-binding lectins to glycopolymers [14,24–26]. *Acanthamoeba* cysts may survive in the environment for prolonged periods of more than 20 years without losing their virulence [27] and resist extreme physiological, chemical and radiological conditions [24]. *Acanthamoeba* spp. can infect the CNS, causing GAE, and the eyes, leading to a devastating infection known as AK, and may cause infections secondary to other (mainly bacterial) infections that have disrupted tissues, especially the skin in immunocompromised individuals [28, 29, 13]. It is widely recognized that the route of entry for *Acanthamoeba* involves the respiratory tract, leading to invasion of the alveolar blood vessels, followed by hematogenous spread and that the blood-brain barrier is where entry into the CNS most likely occurs [11].

## Epidemiology

Due to increased awareness, AK, originally considered a rare infection, has been progressively considered as important in human health [14]. The incidence rate of AK varies worldwide, and some indicative rates are up to 3.3 per 1000 contact lens wearers in Hong Kong, up to 0.5 per 1000 in Holland, up to 0.1 per 1000 in the USA, up to 14.9 per 1000 in Scotland and up to 1.9 per 1000 in England [30–32]. A recent study revealed that out of 129 patients, 55 patients (38.4%) had infections in their left eyes, 60 (41.9%) had infections in their right eyes and 14 (9.7%) were suffering in both eyes [33]. Moreover, another study revealed that out of 245 patients, 243 patients suffered from infection in one eye and 2 suffered in both eyes [34]. A ten-year survey revealed that the most common age (75.5%) for patients suffering from AK was between 15 to 25 years [35]. Other studies found that out of 245 cases, 48.2% (118 cases) were between 40 amd 59 years old, 28.6% (70 cases) were under 40 years

and 23.3% (57 cases) were over 60 years [34]. A study, comprising 194 cases, also revealed that 'bad outcomes', defined as having keratoplasty, corneal perforation, ocular surgeries, more than 10.5 months of antiamoebic treatments or degradation of vision from normal to 20/80, were more frequent (66%) in older, 35 to 76 years old, as compared to younger individuals, 15 to 34 years old, irrespective of gender [36]. While statistics are readily available for AK, it is difficult to ascertain the incidence of GAE due to lack of proper monitoring or healthcare systems, limited diagnostic expertise and low autopsy rates [37]. The disease was found to occur mostly in men and at ages between 20 years and 40 years [38]. Only 2–3% survival rates are observed in the cases reported in the literature and the approximate mortality rate was calculated to be 1.57 GAE deaths per 10,000 HIV/AIDS deaths in the USA, for HIV/AIDS patients [39].

## Diagnosis

Due to its rarity and similarity of its symptoms, including fever, headache, hemiparesis, nausea, seizures, cranial nerve palsies, stiff neck, personality changes, depressed level of consciousness and coma [40], to other pathogens of the CNS such as fungi, virus and bacteria, GAE diagnosis is problematic and is linked to suspicion of amoebic infection, which, in turn, depends on expertise [41]. Following indication of brain defects such as lesions, detected through magnetic resonance imaging (MRI) or computed tomography (CT) scans, detection of pleocytosis with increased polymorphonuclear leukocytes, diminished glucose concentrations and enhanced protein concentrations in the cerebrospinal fluid (CSF) might indicate GAE [41]. Suspicion of GAE should be further strengthened by the absence of viral, fungal and bacterial pathogens and may be assessed by detecting elevated levels of *Acanthamoeba*-specific antibodies using indirect immunofluorescence (IIF) assays, by incubating patient's serum on fixed amoeba-coated slides and fluorescein isothiocyanate (FITC)-labeled antibody [14]. Similarly, AK is difficult to diagnose and may be commonly misdiagnosed as adenovirus and Herpes Simplex virus infections [14]. To confirm amoebic infection, for both AK and GAE, materials from debridement, corneal scrap, corneal biopsy, corneal smear, contact lenses solutions, contact lens, CSF or brain biopsy can be observed using light or confocal microscopy [14, 34, 40]. Despite not being widely used in diagnosis, staining with Wright Giemsa (trophozoites appearing as purple bodies), Trichrome (trophozoites appear green and red, while cysts walls appear green) and Calcofluor white (trophozoites emit red, while cysts walls emits green fluorescence under ultraviolet illumination) are some example of stains that can be used to further improve the microscopic detection of *Acanthamoeba* spp [33,40,42]. Fluorescence microscopy can be used, by incubating the patient specimens with anti-*Acanthamoeba* spp. monoclonal antibodies followed by a secondary antibody with a fluorescent marker [40]. Moreover, transmission electron microscopy can be utilized to identify and differentiate *Acanthamoeba* spp. from host cells and other amoebae [40]. Beside microscopic techniques, molecular techniques, such as polymerase chain reaction (PCR), have been employed for the detection of *Acanthamoeba* spp [14,40,43]. The higher sensitivity (94%) of PCR as a diagnostic tool for *Acanthamoeba* spp. as compared to morphological detection (microscopic examination (33%) or culture (7%)) has been reported [44]. Specific rRNA genes or mitochondrial DNA used to detect *Acanthamoeba* spp. from patient samples using PCR and real-time PCR has been used to differentiate *Acanthamoeba* spp. from other amoebae [40,45–47]. Despite microscopic and molecular-based approaches, the most widely used technique is the cultivation of *Acanthamoeba* spp. from patient samples due to its simplicity, low cost and low number of cells required [14,48]. This is done by incubating the samples on 1.5% non-nutrient agar plates covered with Gram-negative bacteria at 30$^{O}$C and observing the plates for the presence of *Acanthamoeba* spp [11,14,49–52].

## Management

While a plethora of compounds have shown *in vitro* anti-*Acanthamoeba* spp. activity, a limited number of compounds have shown effects clinically in the treatment of GAE, mainly due to inability of compounds to cross the BBB [14]. Usually, using a cocktail of drugs, a hit and miss approach is used in the treatment of GAE and as such, ketoconazole (inhibits ergosterol synthesis) [53–55], amphotericin B (acts on ergosterol and generates disruption of membrane integrity) [56], fluconazole (inhibits ergosterol synthesis) [53,57], 5-fluorocytosine (inhibits synthesis of RNA and DNA) [55], trimethoprim-sulfamethoxazole (folate biosynthesis inhibitors) [53,54,57,58], voriconazole (inhibits ergosterol synthesis) [56, 180], rifampin (inhibits RNA transcription by acting on RNA polymerase) [53,54], miltefosine (inhibits cytochrome c oxidase, interacts with lipids and causes apoptosis-like cell death) [57, 58, 180], pentamidine (inhibits the synthesis of DNA, RNA, phospholipids and proteins) [58] and amikacin (inhibits mRNA binding) [180] are compounds that have demonstrated efficacy in reducing mortality associated with the disease [13]. The majority of drugs used in the treatment of *Acanthamoeba* spp. infections are highly toxic to human keratocytes and the required treatment duration is long and may last up to six months [176].

A 32-year-old male was diagnosed with GAE after hematopoietic stem-cell transplant [59]. He was treated with a regimen consisting of pentamidine isethionate (21 days), co-trimoxazole and azithromycin (28 days), metronidazole (60 days), fluconazole (120 days) and miltefosine (150 days) [59]. The treatment resulted in a successful outcome with continuous improvement in the patient condition [59]. Similarly, five months following an orthotopic heart transplantation, a 60-year-old woman was diagnosed with GAE [60]. Following consultation with CDC, she was treated with a regimen consisting of flucytosine, fluconazole and miltefosine for six months [60]. After six months of treatment, brain MRI demonstrated that the opacities have been cleared [60]. In another case, a 38-year-old male was diagnosed with AIDS and a ring-enhancing central nervous system lesion was revealed by brain imaging, which was later found to be caused by *Acanthamoeba* spp [61]. A regimen consisting of fluconazole, flucytosine, miltefosine and trimethoprim-sulfamethoxazole was administered for seven months [61]. The patient remained asymptomatic 5 months after discontinuation of antiamoebic treatment [61].

Despite advances in supportive care against infectious diseases, the mortality rate due to GAE remains alarmingly high and therefore, efforts toward developing repurposed, novel or improved drugs are ongoing [62]. Studies showed that food and drug administration (FDA)-approved drugs Amlodipine, a calcium channel blocker, prochlorperazine, a potassium channel blocker, loperamide, another calcium channel blocker, guanabenz, an adrenergic receptor blocker, and digoxin, inhibitor of the transport of potassium and sodium across cell membranes, possess anti-*Acanthamoeba* spp. activity [17,63,64]. Polyhexamethylene biguanide expressed both amoebicidal and cysticidal activities against *Acanthamoeba* spp. while exhibiting limited cytotoxicity against the eye surface [65,66], while chlorhexidine also showed both amoebicidal and cysticidal activities against *Acanthamoeba* spp., by interacting with the surface proteins inducing cellular damage [67]. A study in which the ability of chlorhexidine and polyhexamethylene biguanide to treat AK was compared concluded that the overall outcome was similar for both drugs [68]. Alexidine was shown to exhibit amoebicidal and cysticidal effects at 10 µg/mL and 100 µg/mL, respectively [69], acriflavine hydrochloride and proflavine were shown to exhibit amoebicidal activity against *Acanthamoeba* trophozoites at concentrations of 100 µg/mL and higher [70], while polymyxin B and E expressed an amoebicidal effect *in vitro* at concentrations of 50 µg/mL [70] and 62.5 µg/mL [71], respectively. Recently, staurosporine, isolated from a strain of *Streptomyces sanyensis*, was shown to be effective against both trophozoites and cysts of *Acanthamoeba* spp. by activating programmed cell death via the mitochondrial pathway [72].

*Acanthamoeba* spp. synthesizes ergosterol *de novo* from acetate, which is used in the membrane of the *Acanthamoeba* spp., while human cells possess cholesterol, which makes ergosterol a potential target against *Acanthamoeba* spp [73]. Various compounds that can target ergosterol, including ketoconazole, clotrimazole, miconazole, voriconazole, fluconazole and amphotericin B, have shown effectiveness against the amoeba [29]. Moreover, the cationic steroid antibiotic (CSA)-13, which is a ceraginin known to act by disrupting the cell membrane, was evaluated against *Acanthamoeba* spp. *in vitro* at a concentration of 25 mg/mL [74].

Also, propamidine isethionate was revealed to be effective *in vitro* against *Acanthamoeba* spp., showing amoebicidal activity at 1.95–10 µg/mL and cysticidal at 31.25–125 µg/mL [71,75]. Moreover, it was recently demonstrated that preserved propamidine (containing benzalkonium chloride) showed improved antiamoebic activities against both cysts and trophozoites of *Acanthamoeba* spp [76].

Bacterial and eukaryotic microbial cells synthesize folate, while mammalian cells do not, for the synthesis of nucleic acid, hence making the synthesis of folate a target. When the effect of folate inhibitors on *Acanthamoeba* spp. was studied *in vitro,* it was reported that 5-fluorouracil and methotrexate exhibited amoebistatic activity at concentrations of 1.97 µg/mL and 2.45 µg/mL respectively, while pyrimethamine and metoprine exhibited amoebistatic activity at concentrations of 100 µg/mL and 50 µg/mL, respectively [77]. Rifampicin has been reported to possess an amoebicidal effect at a concentration higher than 32 µg/mL against *Acanthamoeba* spp. *in vitro* [78], while macrolide compounds, such as corifungin, rokitamycin and spiramycin [70,75,79,80], and aminoglycosides, including paromomycin, neomycin and N-chlorotaurine [70,71,81,82], have also shown anti-*Acanthamoeba* spp. activities. Trifluoperazine and chlorpromazine were shown to be more effective than ketoconazole, pentamidine, amphotericin B and miconazole when assessed against *Acanthamoeba* spp [78]. and when evaluated against *Acanthamoeba* spp. *in vitro*, artesunate was shown to exhibit amoebistatic activity that was dose dependent, increasing from 54% at 50 mg/mL to 93.2% at 100 mg/mL [83]. Recently, histone deacetylase inhibitors MPK472 and KSK64 showed both cysticidal and amoebicidal activities at 10 µM [84].

Recent studies have demonstrated that efficacy of compounds against *Acanthamoeba* spp. can be enhanced by synthesizing drug-conjugated nanoparticles through conjugation with metals, such as silver or gold [63]. Also, another recent study described the potent anti-*Acanthamoeba* spp. activity of

a nanodrug consisting of iron oxide nanoparticles loaded with amphotericin B and conjugated with isoniazid [85]. Moreover, small interfering RNA molecules (siRNAs) have been shown to be able to improve the treatment of AK when used in combination with chlorhexidine [86]. Furthermore, aminoglycoside G418 was shown to cause programmed cell death in *Acanthamoeba* spp [87]. Another recent study depicted that ion transporters may be involved in the sensory perception of *A. castellanii,* suggesting their value as potential therapeutic targets to block cellular differentiation, which is one of the reasons that these amoebae infections are difficult to treat [88].

However, despite having shown promising results against *Acanthamoeba* trophozoites and cysts *in vitro*, the effects of these many of the compounds *in vivo* and in the clinical setting in cases of GAE are yet to be investigated. There are necessary to evaluate the actual effectiveness of the compounds in cases of GAE.

### *Balamuthia mandrillaris*

*B. mandrillaris* are free-living protists that exist in an array of environments, such as water, soil and dust [89, 176; 90]. Also, similar to *Acanthamoeba* spp., *B. mandrillaris* exist in two stages, an active trophozoite and a dormant cyst stage. *B. mandrillaris* exist in the trophozoite form when exposed to favorable conditions, such as adequate pH, osmolarity and temperature and nutrient-rich environment. On the other hand, *B. mandrillaris* trophozoites, usually measuring around 15 µm to 60 µm in diameter, possess a distinctive irregular branching structure, a nucleus with varying numbers of nucleolus and other organelles including endoplasmic reticulum and mitochondria [91,92]. Binary fission, a form of mitosis whereby the amoebae and nucleus divide to form daughter cells, is the reproduction method employed by *B. mandrillaris* [91]. *B. mandrillaris* convert into a dormant and resistant cyst stage when exposed to harsh conditions, such as lack of food, excess of waste products, overcrowding of cells and extreme pH, temperatures and osmolarity [183]. The cysts of *B. mandrillaris* are approximately 10 µm to 30 µm, spherical and uninucleate and are surrounded by a cyst wall consisting of three layers, namely, an ectocyst, a thin irregular outer wall, a mesocyst, a fibrillar middle layer, and an endocyst, a thick inner wall [91,]. Cysts wall were found to contain mannose, glucose and trace amounts of galactose and linkage analysis revealed carbohydrates with linear and branching saccharides and the presence of cellulose [93]. *B. mandrillaris* cysts are resistant to temperatures of up to 70°C, repeated freeze–thawing (5 times), 0.5% SDS 200mJ ultraviolet irradiation $cm^2$ and 25 ppm chlorine [94]. Under favorable conditions, such as neutral pH, moderate temperature (30 to 37°C) and availability of nutrients, cysts may differentiate into the active trophozoite stage [91]. *B. mandrillaris* may cause BAE and infect other organs such as lungs and skin, in both immunocompetent and immunocompromised individuals [11]. BAE is a chronic disease lasting between 3 and 24 months that almost always ends in fatality. It has been found in immunocompetent individuals, unlike GAE caused by *Acanthamoeba* spp [11,91,95]. Of note, reports have indicated the development of BAE in patients who have undertaken organ transplants, indicating that *B. mandrillaris* may be transmitted through donors [96]. While amoeba penetration of the olfactory neuroepithelium via the nasal route has been suggested, the more commonly recognized route is hematogenous dissemination, following entry of *Balamuthia* through the respiratory tract or skin and entry into the CNS achieved at the blood-brain barrier [91].

## Epidemiology

The range of ages of patients suffering from BAE are large, such as 4 months to 91 years [97], 1 to 89 years [98] and 1.5 to 72 years [99], suggesting that BAE affects people of virtually all ages. Reports also show that BAE affects more males as compared to females [97–99]. Moreover, as opposed to GAE by *Acanthamoeba* spp., where the disease usually occurs in immunocompromised individuals, BAE mainly affects immunocompetent individuals [100].

## Diagnosis

Symptoms such as abdominal pain, headache, hallucinations, fever, nausea, skin lesions, irritability, stiff neck, seizures, hemiparesis, photophobia, breathing difficulties, weight loss, sleep disturbance and slurred speech would indicate BAE [101]. Symptoms indicating BAE should lead to CT scans and MRI imaging that, in cases of BAE, would reveal ring-enhancing lesions [102–108]. Microscopic analysis of biopsies and CSF, usually extracted upon confirmation of CNS infection, may contain *B. mandrillaris* that can be seen more easily when using stains, including calcofluor white and Giemsa stain, but due to requirement of expert knowledge on morphology for diagnosis of *B. mandrillaris*, it is rarely used [91]. The method of choice for identification of *B. mandrillaris* is immunofluorescence assays [95,102–111]. Suspicion of BAE should be further strengthened by the absence of viral, fungal and bacterial pathogens and may be assessed by detecting elevated levels of *B. mandrillaris*-specific antibodies using indirect immunofluorescence (IIF) assays, by incubating patient's serum with *B. mandrillaris*, followed by fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies and then

quantified using flow cytometry or fluorescence microscope [112,113]. PCR is also used for identifying *B. mandrillaris* and sensitive primers specific to *B. mandrillaris* mitochondrial 16S rRNA gene have been established [109–111,114,115]. Matrix-assisted laser desorption-ionization time-of-flight MS (MALDI-TOF MS) can allow identification of *B. mandrillaris* within 15 min, by identifying the characteristic patterns of protein of *B. mandrillaris* [11]. Other techniques that can be used to detect the amoebae include metagenomics, the use of sequencing technology, such as next-generation sequencing (NGS), and bioinformatics [116].

## Management

No effective drug currently exists for the treatment of BAE [117] and usually, a cocktail of drugs, using a hit and miss approach, is used in the treatment of BAE, similar to GAE; as such, artesunate (inhibits membrane glutathione S transferase), itraconazole (inhibits biosynthesis of ergosterol), metronidazole (inhibits synthesis of nucleic acid), 5-fluorocytosine, amphotericin B, fluconazole, pentamidine, trimethoprim-sulfamethoxazole, trifluoperazine (blocks central dopamine receptors), sulfadiazine (inhibits synthesis of folic acid), azithromycin, albendazole (inhibits polymerization of tubulin), clarithromycin (inhibits synthesis of proteins), ketoconazole, miltefosine, flucytosine (inhibits DNA and RNA synthesis) and thioridazine are the drugs that have been used in successful treatment of *B. mandrillaris* [95,105,110,118].

A 26-year-old Hispanic male was diagnosed with BAE and was treated with a regimen including miltefosine, azithromycin, trimethoprim-sulfamethoxazole, flucytosine, fluconazole, sulfadiazine, metronidazole, voriconazole, clarithromycin, pentamidine, albendazole, dexamethasone and amphotericin B [119]. Treatment with miltefosine continued for 3 weeks after stopping the regimen, while treatment with azithromycin and fluconazole continued for an additional 87 weeks and trimethoprim-sulfamethoxazole was continued for 39 weeks [119]. After 2 years, MRI imaging demonstrated that no signs of disease can be observed [119]. A 4-year-old girl was diagnosed with BAE and was treated with an initial regimen, consisting of flucytosine, fluconazole, azithromycin, pentamidine and sulfadiazine, to which miltefosine was later added and pentamidine was removed [181]. The condition of the patient gradually improved and was discharged but still given azithromycin, fluconazole and miltefosine after discharge [181]. MRI demonstrated a gradual reduction in the size of the lesions and no neurological signs or difficulties were reported following discharge [188]. Similarly, a 2-year-old boy was diagnosed with BAE and with MRI, showing mild ventricular enlargement, persistent parenchymal lesions and inflammation of the basilar cisterns [118]. He was treated with pentamidine, fluconazole, flucytosine, sulfadiazine, clarithromycin and thioridazine and gradually improved over 2 months of treatment [118]. The number and size of ring-enhancing lesions in the brain decreased as shown by subsequent brain MRI and no evidence of disease was observed in the patient after 22 months [118].

Despite being used in the treatment of BAE, drugs such as clarithromycin and pentamidine have been reported to have high toxicity [119]. The high mortality rate due to BAE warrants efforts toward developing repurposed, novel or improved drugs and as such, several compounds have been investigated against *B. mandrillaris in vitro*. Propamidine isethionate was shown to be able to exhibit antiamoebic activities against *B. mandrillaris* [120]. Cycloheximide can inhibit encystation and cytopathogenicity of *B. mandrillaris* [121]. Amlodipine showed antiamoebic activity against *B. mandrillaris* [122], while polymyxin B expressed antiamoebic activity against the amoebae at 1 µg/mL [120]. Apomorphine, demethoxycurcumin and haloperidol demonstrated antiamoebic activity against *B. mandrillaris* [122]. Gramicidin revealed antiamoebic activity against *B. mandrillaris* at 10 µg/mL [120], while resveratrol, loperamide, prochlorperazine and procyclidine were shown to cause irreversible damage to *B. mandrillaris* [122]. Diminazene aceturate has also been tested against both trophozoites and cysts of *B. mandrillaris* and showed better efficacy than amphotericin B, ciclopirox olamine, miltefosine, natamycin, paromomycin sulfate, pentamidine isethionate, protriptyline hydrochloride, spiramycin, sulconazole nitrate and telithromycin against both trophozoites and cysts [123]. Artemisinin and cytochalasin D inhibited encystation and inhibited cytopathogenicity of *B. mandrillaris* [121]. Nitroxoline was shown to be amoebicidal with an $IC_{50}$ of 2.84 µM and cysticidal with an $IC_{50}$ of 15.48 µM and at 35 µM, nitroxoline protected human brain tissue from the amoebae [124].

Recently, it has been revealed that quinazolinones have antiamoebic activities against *B. mandrillaris* while also reducing the cytopathogenicity of the amoebae [125]. Moreover, amoebicidal and amoebistatic activities of benzimidazoles, thiazoles, indazoles, indoles and tetrazoles against *B. mandrillaris* have also been reported [126,127]. Furthermore, curcumin was shown to have antiamoebic activities against *B. mandrillaris* [128]. Of interest, efficacy of compounds against *B. mandrillaris* can be enhanced by formation of drug-conjugated nanoparticles through conjugation with metals such as silver or gold [125–128].

## *Naegleria fowleri*

*N. fowleri*, widely dispersed in nature, are free-living protists that exist in a variety of environments, such as water, soil and dust [129,130]. While *Acanthamoeba* spp. and *B. mandrillaris* exist in two stages, *N. fowleri* exist in three forms, active trophozoites, motile

flagellates and dormant cysts. *N. fowleri* exist in the trophozoite stage, the infectious stage, under favorable conditions, such as adequate pH, osmolarity and temperature and nutrient-rich environment. *N. fowleri* trophozoites exhibit food cups, known as amoebastomes, that vary in size and numbers and are responsible for ingestion and attachment [131]. Some of the organelles found in the cytoplasm of the trophozoites include loosely organized endoplasmic reticulum, vesicular nucleus and mitochondria [132]. When in nutrient-poor aqueous environments, trophozoites convert into flagellates to allow long distance movement in search of nutrition. While the general ultrastructure of trophozoites and flagellates is similar, flagellates contain flagella that allow movement [132]. *N. fowleri* flagellates do not reproduce or form cysts [133]. When subjected to harsh conditions, such as excess of waste products, lack of food, overcrowding of cells and extreme pH, temperatures and osmolarity, *N. fowleri* trophozoites convert into a resistant, nonfeeding and nonreproductive cyst stage. A mature cyst wall consists of a thick inner and a thin outer component and some of the organelles found in cysts include endoplasmic reticulum, nucleus and mitochondria [134]. Little is known about the composition of cysts of *N. fowleri,* but it has been shown that enolase is one of the proteins expressed in *N. fowleri* cysts [135]. Following infection through exposure with contaminated water, *N. fowleri* infiltrate the cribriform plate and the nasal mucosa and then pass along the olfactory neuroepithelial route to gain entry to the brain [136].

## Epidemiology

PAM is a disease lasting an average of 4 days [137]. While *N. fowleri* is widespread in nature, the highest number of cases was reported in the United States (41%), Pakistan (11%) and Mexico (9%) [8,138]. Reports have demonstrated that patients of all ages were affected by PAM and that the mean and median age of patients suffering from PAM were around 29 years [137] and 14 years [138], respectively. Reports also show that PAM occurs mainly in males as compared to females [137,138]. The commonly reported exposure linked to PAM was swimming/diving (58%), bathing (16%), water sports (10%) and nasal irrigation (9%) while water sources were ponds/lakes/ reservoirs (45%), swimming pools (13%), ditches/canals/ puddles (12%) and tap water (12%) [138]. Interestingly, *N. fowleri* was recently detected in tap water in Texas USA [139] and, in August 2021, a seven-year-old boy died following *N. fowleri* infection in California [140].

## Diagnosis

Initial symptoms such as headache, fever, nausea and fatigue and symptoms exhibited in the later stages, including altered nuchal rigidity, mental status, extremity weakness, seizures, coma, photophobia, drowsiness, blurred vision, abnormal gait, cranial nerve abnormalities and sensory abnormalities [138] together with a history of exposure to contaminated water, are indicative of PAM [13]. CT scan can indicate the involvement of the CNS and is usually followed extraction of CSF or brain biopsy [12]. Microscopic examination of samples may reveal *N. fowleri* due to its characteristic food cups and flagellate form [141,142]. Stains, such as Wright-Giemsa and Gram stain, are valuable in the microscopic identification of *N. fowleri* [143]. Inoculating samples onto bacteria-coated non-nutrient agar is a simple method with low cost and low cell number requirement for the detection of *N. fowleri*. Immunofluorescence staining, enzyme-linked immunosorbent assay, immune phosphate staining, flow cytometry and PCR assays can also be used in the detection of *N. fowleri* [136,141,144]. Due to its sensitivity and rapidity, PCR is the method of choice for detection and identification of *N. fowleri* [141]. NGS has also been utilized for the identification of *N. fowleri* [145].

While not tested in clinical setting, another method is the recognition through 'untargeted metabolomics methods,' which involves analyzing cell metabolites through liquid chromatography-mass spectrometry and then comparing results with available libraries and analyzing peaks through bioinformatics software for identification of proteins specific to *N. fowleri* [146,147].

## Management

A cocktail of drugs using a hit and miss approach is used in the treatment of PAM, similar to GAE and BAE, and as such, sulfisoxazole (competitive inhibitor of dihydropteroate synthetase), ornidazole and miconazole (inhibit biosynthesis of ergosterol), chloramphenicol (inhibits protein synthesis), ceftriaxone and dexamethasone (prevent inflammation), azithromycin (interferes with their protein synthesis), amphotericin B, miltefosine, rifampin and fluconazole are some of the drugs that have been used in successful treatment of PAM [148–153].

A 12-year-old girl was diagnosed with PAM, a regimen consisting of azithromycin, amphotericin, fluconazole, rifampin and dexamethasone was initiated and miltefosine was then added to the regimen following suggestion from CDC [154]. Intraventricular shunt and controlled hypothermia

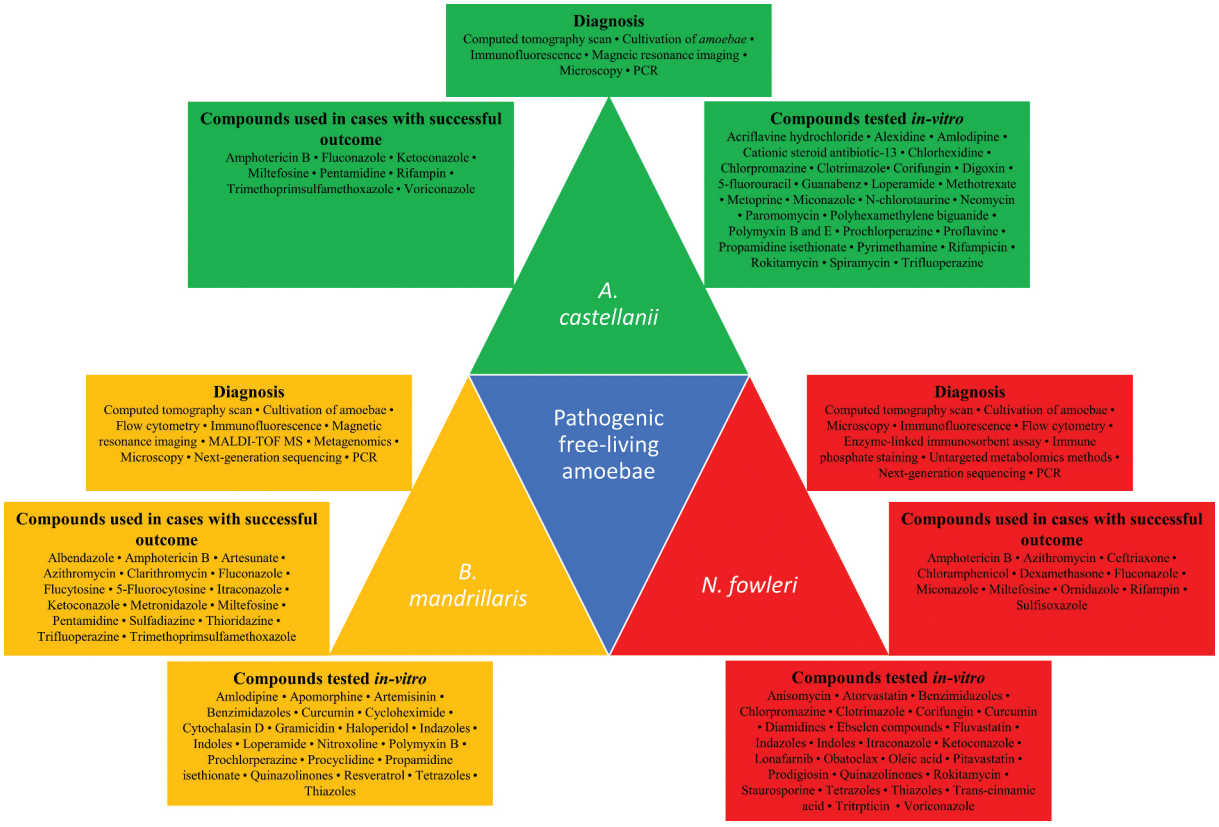


**Figure 2.** Diagnosis and management of brain-eating amoebae. Diagnostic tools and techniques used in the detection of *A. castellanii, N. fowleri* and *B. mandrillaris* are depicted. The compounds/drugs utilized in cases with successful outcomes and those tested *in vitro* against the three amoebae are also portrayed.

was also used as treatment [154]. The patient recovered as demonstrated by brain imaging and negative PCR results [154]. Another 12-year-old girl was diagnosed with PAM and a regimen consisting of amphotericin B, fluconazole, rifampin, azithromycin, dexamethasone and miltefosine [150]. Induced hypothermia (32°C–34°C) was also used in the management of PAM [150]. The patient was discharged after 55 days of hospitalization and had normal levels of functioning and no deficits after 6 months [150]. A 73-year-old male was diagnosed with PAM and was treated with amphotericin B and rifampicin [155]. This treatment led to a positive outcome and after 4 weeks of treatment, CSF examination was normal and no neurological deficit was observed [155].

Amphotericin B, one of the primary drugs of choice in the treatment of PAM, has been associated with multiple side effects, including use-limiting renal toxicity [156]. Hence, development of treatments against *N. fowleri* is still ongoing and in this effort, several compounds have been investigated against the amoeba *in vitro*. Azole agents, such as voriconazole, itraconazole, clotrimazole and ketoconazole, are antifungal agents that are effective against *N. fowleri* and ketoconazole was reported to be as effective as amphotericin B against *N. fowleri* [157,158]. Chlorpromazine and rokitamycin were revealed to be active against *N. fowleri*, both *in vitro* and *in vivo*, since at 12.5 µg/mL and 6.25 µg/mL, respectively, they completely inhibited the growth of the parasite [157]. Corifungin gave rise to 100% survival rate in mouse models infected with *N. fowleri,* indicating its efficacy against the parasite, which is thought to be related to affecting the mitochondria [159]. Tritrpticin showed antiamoebic activities at 100 µg/mL [158]., while secondary metabolites of *Larrea tridentata* were also shown to be effective against *N. fowleri* [160]. Diamidines, having the capability to cross the BBB, were shown to possess antiamoebic activities against *N. fowleri* [161] and ebselen compounds, which can also cross the BBB, were shown to possess antiamoebic activities against *N. fowleri* [162]. Recently, HMG-CoA reductase inhibitors were revealed as drug leads against *N. fowleri*, since pitavastatin, an inhibitor of HMG Co-A reductase, was able to kill 80% of trophozoites within 16hrs [163] and farnesyltransferase inhibitor lonafarnib showed activity against *N. fowleri* with $EC_{50}$ of 1.5 µM [164]. Also, fluvastatin and atorvastatin were shown to cause programmed cell death in *N. fowleri* by analyzing cell membrane damage, condensed chromatin, ROS generation and mitochondrial membrane potential [165]. Moreover, staurosporine, an indolocarbazole from *Streptomyces sanyensis*, was shown to induce programmed cell death in *N. fowleri*

with a low $IC_{50}$ of 0.08 μM [166]. Anisomycin, prodigiosin and obatoclax are also compounds that have been shown to possess activity against *N. fowleri* at low micromolar concentrations [167]. Importantly, the antiparasitic agent, miltefosine, has been shown to be effective in the successful treatment and it has been recommended by the Centers for Disease Control and Prevention against PAM [168].

Of note, it has been demonstrated that quinazolinones have antiamoebic activities against *N. fowleri* while also reducing the cytopathogenicity of the amoebae [125]. Moreover, amoebicidal and amoebistatic activities of benzimidazoles, thiazoles, indazoles, indoles and tetrazoles against *N. fowleri* have also been reported [126,127]. Furthermore, curcumin was shown to have amoebicidal activities against *N. fowleri* [128]. Also, trans-cinnamic acid and oleic acid were also shown to possess antiamoebic activities against *N. fowleri* [169,170]. Of interest, efficacy of drugs against *N. fowleri* can be enhanced by formation of drug-conjugated nanoparticles through conjugation with metals such as silver or gold [125–128,169,170]. In addition, anti-*N. fowleri* activity has shown hesperidin conjugated with silver nanoparticles [171]

## Concluding remarks

Infections due to brain eating amoebae are on the rise and the discovery of brain-eating amoebae in drinking water supplies highlights the threat that these amoebae pose [3–6,172]. Infections due to pathogenic free-living amoebae are lethal, albeit they are rare, and thus, pharmaceutical companies are not eager to invest funding in developing novel therapies. Nevertheless, with the emergence of global warming and nature of these amoebae being thermophilic, it is logical to suggest that infectious diseases such as those caused by free-living amoebae will be escalating [8,173].

Several diagnostic techniques have been developed for *Acanthamoeba* spp., *B. mandrillaris* and *N. fowleri*, including CT scan, flow cytometry, immunohistochemistry, microscopic analysis, PCR assays, cell staining, immunofluorescence staining, enzyme-linked immunosorbent assay, NGS and liquid chromatography-mass spectrometry (Figure 2). However, less invasive, faster and more sensitive diagnostic techniques should be established. Infection of the CNS by free-living amoebae is a dangerous infection with extremely high mortality rates that warrant the development of novel treatment options.

Currently, there are no standardized drugs to treat these infections and clinicians rely on a combination of therapies comprising antibiotic, anticancer, antifungal and anti-inflammatory drugs for therapy. Comprehensive research is essential over forthcoming years to determine the translation value of *in vitro*/early-stage drug leads and urgent collaborations between academia, the pharmaceutical industry and water companies are necessitated [174–178,182].

While several novel compounds have been investigated against the amoebae, conjugation of compounds with metal to form nanoparticles has been shown to be an effective strategy to improve efficacy of drugs against *Acanthamoeba* spp., *B. mandrillaris* and *N. fowleri*. However, the efficacy of drug-metal conjugated nanoparticles is yet to be investigated *in vivo* and their mechanism of actions is not completely understood. Recently, delivery of drugs via the intranasal route, leading to effective concentrations of drugs being delivered into the brain, has been suggested and may be a possible solution for successful treatment of brain infections, which is greatly hampered by the inability of existing drugs to cross the BBB [12, 133].

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NAK and RS conceived the idea. MRM reviewed the literature under the guidance of SM and RS. MRM prepared the first draft of the manuscript. NAK and RS corrected the manuscript. All authors read and approved the final manuscript.

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