Biology and Pathogenesis of *Naegleria fowleri*

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Running Head: Brain-eating amoebae

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Summary

Naegleria fowleri is a protist pathogen that can cause lethal brain infection. Despite decades of research, the mortality rate related with primary amoebic meningoencephalitis owing to *N. fowleri* remains more than 90%. The amoebae pass through the nose to enter the central nervous system killing the host within days, making it one of the deadliest opportunistic parasites. Accordingly, we present an up to date review of the biology and pathogenesis of *N. fowleri* and discuss needs for future research against this fatal infection.

Keywords: Pathogenic amoebae; Nasal irrigation; Muslims; Diagnosis; Treatment

Content

1. Introduction

Naegleria fowleri is a protist pathogen that is extensively dispersed in the environment. *N. fowleri* is the only pathogenic species in this genus. Given the opportunity and access, *N. fowleri* can cause fatal primary amoebic meningoencephalitis (PAM). Worryingly, mortality rates concomitant with PAM remain substantially high, irrespective of modern improvements in antimicrobial chemotherapy or supportive medical care. Being a free-living amoeba, *N. fowleri* can switch phenotype depending on the environmental conditions (Fig. 1). Under favourable conditions, it exhibits a reproductively-active trophozoite stage. The trophozoite stage is considered as the infective stage. Under non-nutrient conditions; but presence of water, trophozoites switch to a transient flagellate stage allowing long distance movement, often in pursuit of nourishment. During this stage, *N. fowleri* does not reproduce or form cyst. When the environment is adverse or unfavourable, trophozoites switch into a metabolically inactive or dormant form known as the cyst form. Like the flagellate phase, the cysts are non-feeding, and non-reproductive. Only the trophozoites of *N. fowleri* can feed, reproduce, and/or become cysts. The parasites enter hosts through the nasal route, travelling via the olfactory neuroepithelia and thus gaining entry to the central nervous system with the production of PAM. The intention of this review is to present a comprehensive review of our current understanding of *N. fowleri* and to propose directions for future research. Much of our present knowledge in the biology aspects of this genus comes from other *Naegleria* species, mainly *N. gruberi*. It cannot be considered to be representative of pathogenic *N. fowleri* but covered here briefly to provide basic understanding of this genus.

2. Primary amoebic meningoencephalitis

Primary amoebic meningoencephalitis is a haemorrhagic-necrotizing meningoencephalitis, symptoms include: headache, stiff neck, fever (38.5°C–41°C), changed mental status, seizures, and coma, resulting in fatality in most cases. PAM is accompanied by strong inflammation, often made up of neutrophils, eosinophils, macrophages and lymphocytes. The incubation period from being exposed to the parasite until the development of the disease varies from one to 16 days (1). People contract the parasite through exposure to contaminated water. Following infection, *N. fowleri* infiltrate the cribiform plate and the nasal mucosa then pass along the olfactory neuroepithelial route to gain entry to the brain to produce meningoencephalitis with quick cerebral edema, leading to cerebellar herniation and death (1,2). The olfactory bulbs exhibit haemorrhage, and an inflammatory exudate, while leptomeninges are congested, diffusely hyperemic with limited infiltration (3). Focal demyelination in the white matter of the brain and spinal cord has been observed (4,5). *N. fowleri* are witnessed at the base of the brain, hypothalamus, and midbrain. Histology reveals an severe inflammatory reaction, that typically consists of neutrophils with widespread regions of lytic necrosis and manifestation of *N. fowleri* trophozoites (6). Microscopic examination reveals many amoebae in the subarachnoid and perivascular spaces (3,6) (Fig. 2).

The earliest infection of PAM was reported in 1965 in Australia (7). To date, few hundred cases of PAM have been reported worldwide. Incidentally, most cases have been described from the United States, Australia, and Europe. Trophozoites are the only form found in the lesions. *N. fowleri* overrun the olfactory bulbs following spread to the posterior areas of the brain. *N. fowleri* has been isolated from the cerebrospinal fluid (CSF) of infected patients (8). Initial symptoms include serious headache and fever (38.5 – 41°C), and then nausea, vomiting,

and signs of meningeal irritation. Notably, involvement of the olfactory lobes may lead to disturbances in the sense of smell or taste and may be prominent early in the progression of the disease, whilst visual instabilities may also ensue. The patient may experience confusion, irritability, and may behave irrationally prior to lapsing into seizures and coma (3). The disease is reffered to as “primary” to differentiate it from infection produced by other parasitic amoebae, which attack the CNS following dissemination through blood.

2.1. Risk factors

Being free-living amoeba, *N. fowleri* is present in rivers, freshwater lakes, canals, spas, geothermal springs, untreated domestic water supplies and swimming pools. In the majority of cases, PAM occurs in young people who have recently been exposed to or had contact with contaminated water. Notably, PAM cases go unnoticed in developing countries, among millions of other infections. For example, in countries such as Pakistan, temperatures can reach up to 50°C, whereas water temperatures are documented at 30 – 35°C, and so with persistent power cuts, millions of people go to freshwater canals, ponds, standing water etc. for “recreational activities” on a daily basis for months (Fig. 3). The occurrence of *N. fowleri* in these waters, coupled with the absence of awareness and/or control measures, along with a poor healthcare infrastructure, and unavailability of effective drugs to counter this disease is a major health risk for these communities. PAM has also been linked with ablution practice amongst religious groups. In particular, Muslims pray five times a day. Ablution is performed before every prayer, for cleansing. Performing ablution encompasses washing the hands, mouth, face, nose, ears ,arms and feet. However, when cleaning their nose, many individuals drive water forcefully up their nostrils, despite this not being a mandatory as a part of ablution. Even though ablution practices have remarkable health benefits, the process can only fulfil its purpose if the water supplies are

free of pathogenic microbes and are uncontaminated(Fig. 4) (9). Thus there is an urgent need for increased awareness so that procedures to make water safer for ritual nasal rinsing are in place. Utilizing boiled or filtered water, or indeed water that has been sanitized using the endorsed concentrations of chlorine coupled with more careful ablution (not forcing water inside the nostrils fervently) will minimize the risk in contracting this devastating disease. Additionally, in many developing countries, water scarcity is a major problem and public has to store water in tanks for days to weeks for their routine consumption which presents a major risk factor. The general public needs to be made aware of the risks related with the use of storage tanks at home and at prayer places. As a vast majority of public often carry out ablution at mosques in preparation of their prayers, it is absolutely vital that the water storage tanks are cleaned and disinfected routinely in mosques (9). Additional religious festivals such as the Kumbh Mela, where millions of Hindus meet in the Indian city of Allahabad for a ceremonial bath in the sacred Ganges River present a threat to public health in the transmission of infectious agents. In addition, nasal cleansing using neti pots is often used to provide relief to patients with sinusitis. Ayurvedic practices also include nasal irrigation, known as “jala neti,”. This comprises of inhaling water using cupped hands and consquently blowing it out, via an Aladdin’s lamp-shaped device (9). Though nasal irrigation stimulates good sinus and nasal health, it can only be effective if disinfected, filtered, or boiled water is utilized. Given the widespread use of this practice globally, a large number of PAM cases is likely missed out. Thus there is a need for increased awareness among physicians as well as the community.

3. Clinical and laboratory diagnosis

Patients exhibiting CNS symptoms together with a history of swimming or use/exposure to contaminated water for nasal cleansing should be suspected of PAM. The computed

tomographic scan (CT) reveal involvement of the CNS, as cisternae, around and above the midbrain and the subarachnoid spaces are eliminated on pre-contrast CT. Noticeable augmentation is seen after intravenous contrast medium administration. Whereas the ventricular size is usual, the sulci and adjacent gray matter are also intensely enhanced, (10). The definitive diagnosis of PAM involves CSF findings, i.e., presence of amoebae in the CSF. In the majority of cases, motile trophozoites are observed in CSF by wet mount. Brief centrifugation of the CSF at 5,000 x g for 5 min is helpful to concentrate amoebae. In addition to microscopy, immunofluorescence assay (IF) (11-13), enzyme-linked immunosorbent assay (ELISA) (14), flow cytometry (15), and PCR-based assays have been developed. Assays should be employed on both CSF and nasal exudates. Apart from the presence of amoebae, CSF findings in PAM are comparable to bacterial meningitis. For example, the red blood cell count in CSF increases several fold from 250 cells per mm^3 in the early stage to 25,000 cells per mm^3 in the late stage. Similarly, the white blood cell count is raised, with a polymorphonuclear leukocyte predominance, with a range of 300 cells per mm^3 to as high as 26,000 cells per mm^3. The CSF pressure is typically raised (300 – 600 mm H_2O). The protein concentration can range from 100 mg per 100 mL to 1000 mg per 100 mL, while glucose might be 10 mg per 100 mL or less (16). Endeavours ought to be made to culture the amoebae from the CSF. A few drops of CSF should be transferred to a non-nutrient agar plate seeded with bacteria and amoebae growth should observed daily for up to seven days. Amoebae appear as the trophozoite form within 1 – 2 days. *N. fowleri* can be differentiated from other pathogenic amoebae using enflagellation experiment by mixing one drop of amoebae culture or sedimented CSF and one mL of distilled water for 1 – 2 h with periodic observation for the presence of flagellates, however the molecular methods remain the method of choice (17). An indirect immunofluorescence assay (IIF) for the

recognition of *N. fowleri* antigen in paraffin-embedded brain tissue slide is routinely performed at Centers for Disease Control. Additionally, PCR-based assays have been established for the sensitive, rapid, and precise identification of *N. fowleri* in clinical samples, and cultured amoebae from patients and the environment (14, 18-35) (Table 1).

Overall, patients exhibiting symptoms involving the CNS and similar to bacterial meningitis, but having negative CSF Gram stain and a recent history of swimming and/or contact with nasal freshwater, should be suspected of PAM. If active amoebae are not observed, the nasal exudates and CSF should be subjected to amoeba identification using ELISA, IF, and PCR assays for confirmation.

4. Chemotherapeutic strategies

The fatality rate associated with PAM is more than 90%. There only are 5 documented survivors of PAM, who received combination drugs. All treatments included amphotericin B, which affects membrane integrity (Table 2) (11, 36-40). At present, the U.S. Centers for Disease Control and Prevention (CDC) recommends that patients suspected of PAM should be given the following combination of drugs: deoxycholate amphotericin B intravenously (IV) and intrathecally (IT), an azole drug such as fluconazole IV or orally (PO), azithromycin IV or PO, rifampin IV or PO, miltefosine PO, and dexamethasone IV (Table 2). While *N. fowleri* is sensitive to amphotericin B, the minimum inhibitory concentration (MIC) of amphotericin B against *N. fowleri* is variable, among different isolates and may range from 0.026 – 0.078 mg per mL (41-43). However, clinical use of amphotericin B is limited due to its toxicity, including acute infusion-related reactions and dose-related nephrotoxicity. New formulations of amphotericin B have been introduced with improved toxicity profiles. For example, the MIC for deoxycholate amphotericin B was 0.1 µg/mL, while that of liposomal amphotericin was 10 times

higher at 1 µg/mL suggesting that deoxycholate amphotericin B should be preffered over liposomal or lipid complex formulation.

Among other drugs of the azole class, ketoconazole showed the lowest MIC (out of ketoconazole, fluconazole, and itraconazole tested). Voriconazole exhibits inhibitory and amoebacidal effects. Both miconazole and fluconazole have been used as part of successful treatment regimens, although none of the drugs tested are as active as amphotericin B (36, 37, 39-46). Recently, it is shown that amphotericin B and azithromycin exhibit synergistic effects, indicating that the combined use of these two drugs may result in successful prognosis (39, 46, 47).

Miltefosine is an anticancer agent, that showed potent activity against *N. fowleri in vitro* and *in vivo* effects (46, 48, 49). Notably, miltefosine can cross the blood-brain barrier and concentrate in brain tissue, thus making this a useful drug against PAM. Recently, the CDC has recommended its use in the USA under single-patient emergency Investigational New Drug (IND) protocols. It was first given to a PAM patient in 2010 in combination with amphotericin B, azithromycin, fluconazole, and rifampin; however, the drug was not administered to the patient until 5 days after patient's initial presentation to hospital and the patient did not survive (40). In 2013, the U.S. CDC has been able to procure a supply of miltefosine under an expanded access IND allowing for rapid deployment to PAM patients in the U.S. In 2013, two U.S. PAM patients received miltefosine, together with amphotericin B, azithromycin, fluconazole, and rifampin, shortly after their hospital admissions, which resulted in successful treatment of PAM (39, 40).

Other candidate drugs that have shown anti-*N. fowleri* effects but not tested clinically, include antipsychotic drugs, i.e., trifluoperazine and chlorpromazine (49-51), acrolein, a

metabolic product of cyclophosphamide (52), artemisinin (53, 54), cannabinoids (55), mono- and di-amidino derivatives (56), synthetic antimicrobial peptide, tritrypticin (57), and corifungin, a water-soluble polyene macrolide (58). Of note, corifungin resulted in 100% survival of mice for 17 days post-infection.

In addition to antimicrobial chemotherapy, there is a need to manange PAM patients effectively. The overwhelming inflammation of the brain leading to cerebral edema, along with raised eminent intracranial pressure and consequent herniation of the brain often leads to the death of PAM patients. Among five documented survivors, four patients received dexamethasone to reduce the intracranial pressure. The management of one of the PAM patients' included drainage of CSF via an external ventricular drain, hyperosmolar therapy with mannitol and 3% saline, moderate hyperventilation (goal P_aCO2: 30 – 35 mm Hg), and induced hypothermia (32 – 34°C; Linam et al. 2015). Largely, these findings indicate that in addition to antimicrobial chemotherapy, PAM patients should have their intracranial pressure closely monitored.

5. Pathogenesis

In vivo, *ex vivo* and *in vitro* models have been developed to study molecular mechanisms associated with *N. fowleri* pathogenesis. *In vivo*, mice are inoculated intranasally with *N. fowleri* that results in high mortality rate. The susceptibility of mice is influenced by weight (mice weighing less than 15 g are more sensitive), and age (younger mice are more sensitive) (59, 60). Following infection, *N. fowleri* are observed in mucous layer of the olfactory epithelium within 8 h post-infection (61) and infected mice exhibit focal inflammation with the presence of *N. fowleri* in the submucosal nerve plexus, olfactory nerves penetrating the cribriform plate, and the olfactory bulb of the brain within 24 h post-infection. Following 96 h, the inflammatory

response, primarily in the form of neutrophil polymorphs, is severe in the olfactory bulb and the brain, with tissue damage (62-65). Numerous amoebae are seen interspersed with the degenerating neurones, glial processes, and neutrophil polymorphs with major concentrations in the perivascular regions and in the lumina of blood vessels (66). When *N. fowleri* are incubated with host cells *in vitro*, host cells show cell shrinkage, cell damage, invasion and destruction via phagocytic processes (63). Using *ex vivo* model, Gianinazzi *et al.*, (67) showed that infection of organotypic slice cultures from rat brain with amoebae is comparable to findings with *in vivo* infection, suggesting its usefulness in the study of *N. fowleri* pathogenesis. For simplicity, the pathogenicity of *N. fowleri* is divided into contact-mediated and contact-independent mechanisms (Fig. 5).

5.1. Contact-dependent mechansims

Adhesion is a primary step in *N. fowleri*-mediated host cell damage. The capability of *N. fowleri* to bind to nasal mucosa, locomotion, and chemotactic response to nerve cell components play a substantial role in disease progression (68-70). Binding is mediated by adhesins expressed on the surface of *N. fowleri*. Two integrin-like proteins, co-localized to the focal adhesion-like structures, have been described in *N. fowleri*. In support, anti-integrin antibody reduced binding of *N. fowleri* to extracellular matrix (ECM) (71). A fibronectin binding protein of 60 kDa is described, which is important for *N. fowleri*-mediated host cells cytotoxicity. Protein kinase C activity is shown in *N. fowleri* extracts that affects binding to and cytotoxicity of host cells (70). *N. fowleri* is shown to induce reactive oxygen species (ROS) in host cells, resulting in host cell damage (72). Following binding, phagocytosis and amoebastomes are involved in *N. fowleri*-mediated host cell damage via piecemeal consumption of target cells mediated by a sucker apparatus protruding from the surface of *N. fowleri* (73-75). These processes are actin-dependent

and involve polymerization of monomeric G-actin into filamentous F-actin. A *nfa1* gene of 360 bp has been identified that encodes Nfa1 protein (13.1 kDa), expressed on the pseudopodia (76). Anti-Nfa1 antibody or gene silencing of *nfa1* reduces *N. fowleri*-induced host cell cytotoxicity (77-82). Overall, the expression of Nfa1 appears to be important for *N. fowleri* pathogenesis. In addition, surface membrane-enriched fractions of *N. fowleri* contain potent cytolytic activity unaffected by treatment at high temperature (83). Later studies identified a 17 kDa membrane protein, Mp2CL5, expressed in *N. fowleri* but not in non-pathogenic species, suggesting that it may play a role in the pathogenicity of *N. fowleri* (84, 85).

5.2. Contact-independent mechanisms

A 66 kDa membrane-bound cytolytic pore-forming protein, N-PFP is expressed by *N. fowleri* (86). It depolarizes the membrane potential, affecting membrane integrity (86). Two pore-forming polypeptides, *Naegleria*pores A and B, have been identified from *N. fowleri*. Both polypeptides have comparable structural properties with antimicrobial and cytolytic polypeptides of amoebapores of *E. histolytica* and of cytotoxic natural killer and T cells (87-89).

Phospholipase A, phospholipase A_2, phospholipase C, sphingomyelinase, neuroaminidase, elastase, and proteolytic enzyme activities are observed in cell-free lysate (90,-92). In PAM patients, extensive demyelinization is observed in the white matter, which is likely the result of phospholipases (4). Amoebae degrade sphingomyeline with liberation of choline, sphingosine and fatty acids. It is further confirmed that *N. fowleri* releases phospholipases, lysophospholipase and sphingomyelinase, as well as factors that cause damage to the lipid-rich cytoplasmic membrane of cells and demyelinizes nerve tissue (92, 93).

N. fowleri exhibit extracellular proteolytic activity (92, 94) that degrade zonula occludens-1 (ZO-1) and claudin-1 proteins but not occludin (95). The optimal protease activity is

observed at pH 7.0 and 35°C and activity can be inhibited mainly using cysteine proteases inhibitors, while serine protease activity is also observed (94). Additionally, acid proteinase, *N*-acetylglucosaminidase, acid phosphatase, 5'-nucleotidase, aspartate aminotransferase, alpha-D-glucosidase, and aminopeptidase activities have been shown (83). Among these, acid proteinase, *N*-acetylglucosaminidase, and acid phosphatase are connected with cytoplasmic granules that are similar to lysosomes; 5'-nucleotidase is associated with surface membrane; aspartate aminotransferase is related with mitochondria; and alpha-D-glucosidase and an aminopeptidase is connected with surface membrane as well as lysosomal particles (83). In addition, cysteine proteases, lipases, sphingomyelinase, elastase, cathepsin B-like proteases, beta-glucosidase, beta-galactosidase, beta-fucosidase, alpha-mannosidase, hexosaminidase, arylsulfatase A, and beta-glucuronidase, sphingomyelinase, neuraminidase, or arylsulfatase B, phospholipases, lysophospholipases, sphingomyelinases, neuraminidase, electrondense granules (small cytoplasmic components endowed with proteolytic activities), peroxiredoxin, thrombin receptor have been described, which may play a role in *N. fowleri* pathogenesis (92, 96-100). A 30 kDa cysteine protease has been described in *N. fowleri* that degrades ECM and produces host cell toxicity. In addition, cysteine proteases of approx.. molecular weights of 58 kDa, 128 kDa, and 170 kDa are detected in *N. fowleri* (101, 102). Two cathepsin B and cathepsin B-like cysteine protease genes, cathepsin B (*nfcpb*) and cathepsin B-like (*nfcpb-L*) with 38.4 and 34 kDa molecular weight have been described that degrade IgA, IgG, IgM, collagen, fibronectin, hemoglobin, and albumin (103).

N. fowleri produce nitric oxide *in vitro*, that share epitopes with the mammalian nitric oxide synthase, suggesting that nitric oxide may participate in *N. fowleri* pathogenesis (104). *N. fowleri* exhibit hemolytic activity. The hemolytic activity is membrane-associated, and

unaffected by a high salt concentration, chelating agents, and pH extremes (83). More recently, a heat shock protein 70 (HSP70) has been identified. The Nf-cHSP70 is contained in the cytoplasm, pseudopodia, and phagocytic food-cups. The inhibition of synthesis of Nf-cHSP70 inhibits *N. fowleri* proliferation as well as reducing host cell cytotoxicity. These results suggest that Nf-cHSP70 may play an significant role in stimulating the proliferation and in the regulation of the host's immune system (85, 105). Among other potential pathogenicity factors include, (i) cyclophilin that is overexpressed in highly pathogenic *N. fowleri*, (ii) apoptosis-linked gene-2-interacting protein X1 (AIP1), a regulator of endosomal sorting of cellular material between organelles (85). Golgi-localized transmembrane protein HID-1 is involved in vesicular exocytosis by preventing the mis-sorting of peptides to lysosomes for degradation (106, 107). Both AIP1 and HID-1 are thought to be useful as *N. fowleri* pathogenicity factors, potentially acting to regulate vesicular trafficking in the amoeba (85). Other factors include, (iii) Ras-related protein Rab-1, which may be involved in vesicular trafficking and, thus, in the phagocytosis of target cells, (iv) myosin II heavy chain as well as myosin Ie (likely involved in phagocytic processes), (v) Villin-1 protein, likely involved in actin-dependent pathogenic processes may also be important for *N. fowleri* pathogenesis (85).

6. The host-damage response to *N. fowleri*

The fact that immunization of mice with *N. gruberi* affords protection against subsequent challenge with *N. fowleri*, suggests the role of immune response in PAM. Protection can also be transferred in mice by immune serum (3). At early satges of infection, the host secretes mucus that traps amoebae. The exposure to *N. fowleri* induces activation of innate defence, such as mucin secretion (MUC5AC) and inflammation (IL-8 and IL-1 beta) in respiratory epithelial cells, via ROS production (108). Mucins inhibit binding of *N. fowleri* to cells and inhibit

cytotoxicity *in vitro* and *in vivo*. However, few *N. fowleri* penetrate the epithelium and induce an inflammatory reaction (62, 109). Eosinophils and neutrophils surrounding *N. fowleri* are observed during later stages of infection. The inflammation increases with time, with a major neutrophil response. To determine whether inflammation plays a role in tissue damage, CD38-/- knockout mice with deficiencies in chemotaxis were compared with the parent strain, C57BL/6J were used. It was shown that inflammation and mortality are delayed in knockout mice, suggesting that the host inflammatory response and polymorphonuclear cell lysis contribute to the CNS tissue damage (109). *In vitro*, microglial cells exposed to *N. fowleri* lysates induce pro-inflammatory cytokine release, including TNF-α, IL-6, and IL-1β (110). Likewise, astrocytes exposed to amoebae lysates lead to expression of IL-8, IL-1 beta, and IL-6 in a ERK, JNK and p38 MAPKs-dependent manner (111, 112).

In vitro studies show that neutrophils do not kill *N. fowleri in vitro*, unless pre-exposed to conditioned medium (CM) from Phytohaemagglutinin (PHA)-stimulated mononuclear leukocytes. Additionally, the presence of antibody or complement augment *N. fowleri* killing by CM modified neutrophils. Similarly, neutrophils from *N. fowleri*-immunized mice are adept of killing *N. fowleri*. Neutrophils surround *N. fowleri* prior to killing, through contact-dependent and contact-independent mechanisms described above. A single neutrophil is unable to phagocytose an entire *N. fowleri*, several neutrophils are able to rupture *N. fowleri* by pinching off and engulfing portions of it (3, 113, 114). TNF-α stimulates the adherence of neutrophils to *N. fowleri*, followed by destruction of *N. fowleri*. Notably, TNF-α augment the neutrophil activity by enhancing oxygen radical production. Neutrophils which lack myeloperoxidase but have a normal oxygen-dependent respiratory burst do not possess amoebicidal properties. Arginine, a scavenger of hypochlorite, reduces ability of neutrophils to kill *N. fowleri*. Catalase

inhibits cidal activity of neutrophils, suggesting that TNF-α-mediates destruction of amoebae by neutrophils (115-117). Treatment of immunized mice with monoclonal antibody NIMP-R10, that causes selective neutrophil depletion led to increased mortality when challenged with *N. fowleri*. In contrast, immunized mice without antibody treatment show more than 95% survival, together with significant neutrophil response in nasal mucosa and olfactory lobes (118).

Activated macrophages exhibit *N. fowleri* killing by producing nitric oxide in an arginine-dependent cytolytic mechanism and non-oxidative mediators including TNF-α and IL-1 and possibly other factors. Notably, TNF-α alone, or in combination with IL-1 is neither amoebicidal nor amoebistatic (119, 120). Amoebicidal activity of the CM from activated macrophages can be recovered by ammonium sulfate precipitation, while heat treatment inactivates, suggesting their proteinaceous nature (121). Similarly, amoebicidal activity of natural killer cells increases during the course of infection, likely due to other anti-ameobic factors that enhance amoebae killing.

Given that antibodies augment host cell-mediated amoebae killing, several surveys were conducted to detect normal exposure to amoebae. *N. fowleri* antibody is detected in normal human sera and saliva with titres ranging from 1:5 to 1:20. The antibodies belong to IgA, IgG, and IgM (3, 122-125). *In vitro* studies show that sIgA are capable of inhibiting *N. fowleri* binding to collagen type I (126), as well as blocking proliferation of *N. fowleri*, cytotoxicity of amoebae against host cells *in vitro* and *in vivo*, suggesting that antibodies weaken the virulence of *N. fowleri* (127, 128).

Fresh adult human serum exhibit amoebicidal effects, while *N. fowleri* proliferation is inhibited by incubation in culture medium complemented with 10% fresh human serum. Heat inactivation (56°C, 30 min) of serum abolished lytic and inhibitory effects, and suggested the

role of complement via the alternative pathway. The presence of anti-*N. fowleri* antibody enhance amoebicidal effects (129-131).

6.1. Immune evasion

The nasal mucosa is the first line of defence. To overcome this barrier, *N. fowleri* is shown to possess mucinolytic activity. A 37 kDa protein with mucinolytic activity has been identified in *N. fowleri* that can be inhibited by cysteine protease inhibitors, suggesting that amoebae evade mucus by degrading mucins via proteases (109). Post-infection, there is an impairment of the blastogenic response of splenocytes to *N. fowleri* (132). Lymphoblastic transformation induced by T-cell mitogen is reduced in comparison to the uninfected control mice. The blastogenic response to B-cell mitogen remains depressed in the infected mice up to 14 days post-infection, suggesting that there is a suppression of cell-mediated immunity during infection (133). Although, weakly pathogenic and non-pathogenic amoebae are lysed by complement, but virulent *N. fowleri* are resistant to complement-mediated lysis (134). The chelation of extracellular calcium enhanced complement-mediated *N. fowleri* lysis, suggesting that Ca^{2+} ions impact complement resistance in *N. fowleri* (135). *N. fowleri* resist complement damage via expression of complement-regulatory proteins, and shedding of membrane attack complex (C5b-C9) on vesicles (136, 137). Neither a repair process involving *de novo* protein synthesis nor a complement-inactivating protease is involved in increased resistance of amoebae to complement-mediated lysis (138), except the likely expression of complement-regulatory proteins. To this end, *N. fowleri* express a “CD59-like” surface protein (18 kDa) that may play a role in complement resistance and possibly protect amoebae against action of pore-forming proteins (139, 140). When exposed to normal human serum, membrane blebbing is observed on the surface of complement-resistant *N. fowleri* that likely represents shedding of the attack

complex. This is further supported by treatment of complement-resistant *N. fowleri* with cytochalasin D or cytochalasin B, which increased amoebae susceptibility to complement damage (137). Enzymatic removal of surface components from *N. fowleri* with phosphatidylinositol-specific phospholipase C or with endoglycosidase H increased the susceptibility of amoebae to complement-mediated lysis (136). Following exposure to serum, activation of protein kinases is observed including serine/threonine or tyrosine kinases, involved in complement resistance (141). Blocking activation of protein kinases makes *N. fowleri* susceptible to complement lysis.

The ability of *N. fowleri* to internalize surface-bound antibody enable them to evade the host's immune defences (3). Although sIgA antibodies are capable of inhibiting *N. fowleri* binding, amoebae are capable of eradicating antigen-antibody complex on the surface through capping and internalizing surface-bound antibody (126, 142).

6.2. Immunization

Intravenous, intranasal, subcutaneous, and intraperitoneal immunization of mice with formalin/formaldehyde-fixed *N. gruberi* affords protection against subsequent intranasal challenge with *N. fowleri*, albeit intravenous inoculation provides greater protection (3). Mice immunized with intact *N. fowleri* show better survival rate on challenge than those immunized with cell fragments, and *N. gruberi* is a better immunogen than *N. fowleri* (143-146). Cry1Ac protoxin protein, produced by the bacterium *Bacillus thuringiensis*, is a useful mucosal adjuvant (62). For example, *N. fowleri* lysates co-administered with Cry1Ac induces 100% protection against subsequent challenge with *N. fowleri* (62). The protection is mediated through increased metaplasia in the olfactory epithelium, allowing for secretion of IgA (147), as well as increased activated lymphocytes, and augmenting Th-1 and Th-2 type immune response in STAT6-

dependent manner (148). Cholera toxin is also shown to be useful adjuvant. Immunization with *N. fowleri* lysates plus Cholera toxin offers 100% protection (survival up to 100%) against subsequent challenge with *N. fowleri* (149). Immunization with amoebae culture supernatant produced a survival rate of 67 - 78%. Fractionation showed that high molecular weight fraction (>200 kDa) provided greater protection. Again, the degree of protection is related to levels of anti-*Naegleria* antibodies. Histological findings show that this protection is expressed at the nasal mucosa, and possibly results from the combined effects of polymorphonuclear leukocyte-mediated killing of *N. fowleri* and mechanical elimination of amoebae by extensive shedding of necrotic epithelium (118, 150).

As the majority of cases prove fatal, intracisternal passive immune therapy in rabbits with PAM has been tested. The results showed that passive intracisternal treatment using anti-*N. fowleri* immune serum or its immunoglobulin G fraction, or the monoclonal antibody provides consistent, albeit temporary protective effects. The protective effect is heat-resistant to 56°C, suggesting that passive intracisternal antibody treatment may assist as an adjunctive therapy in PAM (159). More recently, rNfa1 has been tested as a potential vaccine candidate. Mice immunized intra-peritoneally or intra-nasally with rNfa1 protein develop specific IgG, IgA and IgE antibodies. High levels of the Th1 cytokine, IF-γ, and the regulatory cytokine, IL-10, are also induced, associated with prolonged survival time of mice (110). Later studies tested *nfa1* DNA vaccination in mice. Mice vaccinated intranasally with viral particles of *nfa1* exhibit high levels of IgG together with higher IL-4 and IF-γ production compared with the control groups, suggesting a Th1/Th2 mixed-type immune response, associated with significantly higher survival rates of up to 90%. These findings showed that *nfa1* vaccination can provide protective immunity (111, 112).

7. Cell Biology and Species determination

N. fowleri was discovered by Fowler and Carter in Australia (7) as the causative agent of PAM, and named in honour of Dr Malcom Fowler who first recognized the disease. Among the genus *Naegleria*, this is the only pathogenic species to be isolated from PAM patients. It has three stages in its life cycle, an amoeboid stage, a flagellate stage, and a cyst stage. The flagellate stage is transient, that is non-feeding, non-reproductive, and non-encysting stage. Upon favourable conditions, cyst stage excysts into amoeboid stage. The three forms and their inter-conversion make this protist an excellent model to study cellular differentiation processes.

The amoeboid stage is characterized by a large nucleus with nucleolus, numerous mitochondria, food vacuoles, a contractile vacuole, endoplasmic reticulum enclosed within a plasma membrane, ribosomes, membrane-bound cytoplasmic organelles (152-154). The amoeboid stage is long and slender (8-15 µm) and move by forming one or more lobose pseudopodia (153-155). Cysts are spherical, with a smooth single layer, often clumped closely together, and 7-12 µm in diameter. Ultrastructure examination reveals an average of one to two mucoid-plugged pores per cyst, through which trophozoite emerges (3). The flagellate has an elongate, pear-shaped body, usually possessing two flagella of equal length, a nucleus in the narrower anterior region, and no cytostome. There is a distinct nuclear membrane and nucleolus, vacuoles and cytoplasmic inclusions, mitochondria, and rough endoplasmic reticulum. The kinetic apparatus consists of flagella and their associated kinetosomes, rhizoplast, and thin-walled fibrils. Neither centrioles nor kinetosomes are found in amoeboid stage, suggesting its synthesis *de novo* during transformation of into the flagellate form (152-154, 156). The rhizoplast is proteinaceous with subunit molecular weight of approximately 240 kDa (157). The flagellates express tubulin that is similar to other tubulins in molecular weight (55 kDa), amino

acid composition, electrophoretic mobility, and nucleotide composition (158, 159). There are 2 types of microfilament in the cytoplasm of *N. fowleri* (160). Thin, actin-like microfilaments, 5-7 nm in diameter in the non-motile amoebae. In actively motile amoebae, these microfilaments combine to form collateral bundles in close proximity to the plasma membrane. Thick, myosin-like microfilaments, 17-19 nm in diameter also occur in the amoebae cytoplasm (160). The purified actin share attributes with other actins, including molecular weight, strong binding to DEAE-cellulose, binding to DNase I, reversible polymerization to F-actin, binding of rabbit myosin subfragment 1 to give distinctive arrowheads, and activation of myosin Mg2+-ATPase (161, 162).

N. fowleri is shown to exhibit locomotion at 37°C (163). On an agar plate with *E. coli*, amoeba exhibits growth and movement at the rate of 1 to 3 mm per day at 23°C, 7 to 14 mm per day at 37°C, and 7 to 14 mm per day at 43°C (164), making it a useful model to study locomotion. The amoeba locomotion involves cytoplasmic streaming, pseudopod production, cell polarity and focal contact production that requires actin-based cytoskeleton in a chemotactic and chemokinetic-dependent manner towards live cells and extracts of bacterial species. *N. fowleri* responds to bacteria through chemokinesis, chemotaxis, and formation of food cups (165). For locomotion on a glass substrate, two types of contact are made: one, formed at a considerable distance from the substrate in deionized water, termed as “associated contact”, from which filopodia are produced, which form close contacts, termed as “focal contacts” (166). The associated contact is transient, in contrast to the focal contacts, which are stable. Focal contact sites are left behind on the glass surface (“footprints”) when the amoeba moves away. The locomotion is affected by the ionic strength of the medium and particularly the valency of the cation component (166). Similar to glass substrate, amoeba carries out locomotion at the water-

air interface, suggesting that the surface tension alone may deliver suitable properties for the adhesion and translocation of amoebae. The transitory swimming flagellate stage of *Naegleria* is capable of docking at the interface, making stable adhesions to it, and then reverting to the amoeboid phenotype. On the contrary, amoebae resident at the water-air interface can convert to swimming flagellates and escape into the bulk liquid phase (167).

The average cell dry mass is constant during log growth at 150 pg per amoeba, but decrease 30%, during stationary phase. During log growth, 80-85% of the cell dry mass is protein (120 pg per ameba). During log and stationary growth phases, carbohydrate content is approximately 15 pg per amoeba, and RNA is about 18 pg per amoeba. Total DNA content is 0.2 – 0.3 pg per amoeba during log growth, but it increases during transition from log phase to stationary phase and then gradually decreases to nearly initial levels. The peak in DNA content corresponds to an increase in the average number of nuclei per amoeba, following which the nuclear number decreases as the cells enter stationary growth phase. The RNA content is approximately 18 pg per amoeba (3, 168, 169). The trophozoites expresses high levels of surface glycoconjugates that contain alpha-D-mannose, alpha-D-glucose, and terminal alpha-L-fucose residues (170, 171). *N. fowleri* is shown to possess the thiol compound trypanothione, which was hitherto thought to occur in Kinetoplastida only. The trypanothione/trypanothione reductase system may also be useful "drug target" without targeting the human host (51). Similarly, Selenium (Se) is found in selenoproteins as the 21st amino acid (selenocysteine, Sec, or U), involved in growth, proliferation, cellular redox balance, and has been identified in *Naegleria*. Additionally, beta-glucosidase, beta-galactosidase (172), acid phosphatase and heme proteins (173), catalase, phospholipase A, and sphingomyelinase activity (3), pyrophosphate-dependent phosphofructokinase (PPi-PFK) (174), cytosolic heat shock protein 70 (Nf-cHSP70) (175), low-

molecular-mass thiol compounds (176), tet-like dioxygenase (177), sterol biosynthesis (178), malic enzyme, 3-hydroxybutyrate dehydrogenase, isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase, *L*-threonine dehydrogenase, glyceraldehyde-3- phosphate dehydrogenase, superoxide dismutase, hexokinase, phosphoglucomutase, uridine diphosphate-glucose pyrophosphorylase, 3-N-acetylglucosaminidase, aldolase, and glucose phosphate isomerase, proteases, lipases, phosphatases, esterases, calmodulins have been identified (179, 180).

7.1. Genome of the genus *Naegleria*

The nuclear DNA content of *Naegleria* is approximately 0.2 – 0.3 pg per amoeba, whereas *Acanthamoeba* has 1 – 2 pg / amoeba, while *Amoeba proteus* is about 34 – 43 pg / amoeba (181, 182). DNA content is relatively constant during log to post-log phase transition (169). *N. fowleri* genome is diploid (66 MB), while the haploid genome size is 29.62 MB with GC content of 35.4% and 17,252 open reading frames (85, 183, 184). Of interest, the haploid genome size of *N. gruberi* is 40.96 MB with GC content of 33.1%, and composed of at least 12 chromosomes with sizes ranging from 0.7 to 6.5 MB. In addition, it contains a 14 kb extrachromosomal plasmid and a 50 kb mitochondrial genome (85, 185). In *N. gruberi*, mitochondrial DNA is estimated to be 14% of the total cell DNA (186), while *A. castellanii* contains 20 – 30% of the total cell DNA (181). The mitochondrial genome and a 60-kb segment of nuclear genome from *N. fowleri* is suggests the presence of potential pathogenicity factors, including ten novel *N. fowleri*-specific genes and a homolog of cathepsin B protease (185-187).

7.2. Classification

The International Society of Protistologists has classified Eukaryotes into six “Super Groups” namely, Amoebozoa, Opisthokonta, Rhizaria, Archaeplastida, Chromalveolata, and Excavata. Among free-living pathogens, genus *Acanthamoeba* and *Balamuthia* are included

under Super Group Amoebozoa: Acanthamoebidae; while genus *Sappinia* under Super Group Amoebozoa: Flabellinea: Thecamoebidae; and genus *Naegleria* under Super Group Excavata: Heterolobosia: Vahlkampfiidae (188). *N. fowleri* fit in the genus *Naegleria,* family Vahlkampfiidae in the class Heterolobosea. Like all other members of this class, it is a free-living protist that feeds typically on bacteria and contains both pathogenic and non-pathogenic species.

The rRNA genes have been used for classification. The rRNAgenes are transcribed together in the following order: small subunit (18S) rDNA, an internal transcribed spacer (ITS1), 5.8SrDNA, a second ITS (ITS2), and the large subunit (28S) rDNA (195). ITS sequence is rapidly evolving and has been used to classify over 40 species of *Naegleria* (185). *N. fowleri* remains the only species responsible for PAM. The rDNA of *N. gruberi* (28S, 18S, 5.8S) is encoded on an extrachromosomal circular nucleolar plasmid carrying all three rDNA genes. The 3,000 to 5,000 copies per cell of this 14-kilobase-pair circular plasmid convey all the 18S, 28S, and 5.8S rRNA genes. The presence of the ribosomal DNA of an organism completely on a circular extrachromosomal element is unusual (189). Comparison of the small-subunit ribosomal RNA gene with the rRNA sequences of other eukaryotes resulted in a phylogenetic tree that supports the proposed polyphyletic origin of *N. fowleri* and suggests a flagellate ancestry for *Naegleria* (190-192).

8. Cellular differentiation in *N. fowleri*

The ability of *N. fowleri* to transform into three distinct stages is remarakable. This property is stably inherited, and phenotypic changes occur without change in genotype (152). Amoebae encyst during late stationary phase (165). Encystation is categorised by an increase in cytoplasmic density (153, 154). The mitochondria are found closely concomitant with the

endoplasmic reticulum. Numerous vacuoles of low electron density arise in the vicinity of the nucleus and are scattered through the cytoplasm. The mature cyst wall is double wall, comprising of an inner thick component (200 – 450 nm) and an outer thin component (25 nm). The two layers are separated by a space filled with a network but joined at region of the cyst pore. The pores (about 600 nm) are sealed by a plug of electron-transparent material. During excystation, the amoeba cytoplasm becomes highly alveolar. The plug closing the pore in the wall, dissolves and the amoeba exits the cyst (153, 154). In the initial stages of encystation, the cisternae of the endoplasmic reticulum becomes densely filled with a fibrillar material (193). Vesicles with a similar content that appears to be derived from the cisternae is also observed in close contact with the plasma membrane. As encystation progresses, the fibrillar material becomes localized on the surface of the amoeba. Completely formed cysts possess 2 – 3 ostioles, each sealed by an operculum. When excystation is induced, small dense granules, which are in close contact with fibrillar material are observed in the cyst cytoplasm and in the peritrophic space. During excystation, the more compact component of the operculum moves to enable the pseudopod of the emerging trophozoite to penetrate the ostiole (194). Excystation of *N. fowleri* occurrs by rupture of the cyst wall (195). The optimum temperature for excystation is about 30°C and optimum pCO_2 in air is 5%. Inhibitors of carbonic anhydrase reduces excystation (196). The excystation is inhibited by actinomycin D, and DNA transcription is apparently obligatory. Once excystation initiates, it proceeds to completion in atmospheric CO_2. The cellular differentiation is affected by the presence of steroids (197). At high concentrations, progesterone and deoxycorticosterone prevent amoebae to change to the flagellate form. Flagella are made by filamentous extension from endoplasmic protrusion (198). A chromatin body of cytoplasmic origin is always located at the base of the protrusion or of the flagella, and is thought to be the

parabasal body. Reversion from the flagellate to the amoeba stage is achieved by absorption of the flagella, shedding of one or more flagella and absorption of the rest, or by casting-off a small part of the body to which the flagella are attached (198). Flagellates have an intersected flagellar apparatus, comprising of nucleus, rhizoplast and accessory filaments, basal bodies, and flagella (199). A basal body appears and assumes a position at the cell surface with its filaments perpendicular to the cell membrane. Axoneme filaments extend from the basal body filaments into a progressive evagination of the cell membrane which becomes the flagellum sheath. Continued elongation of the axoneme filaments leads to differentiation of a fully formed flagellum within 10 min after the appearance of basal bodies (199). Amoebae that are at the stationary phase of growth enflagellate more willingly than actively growing amoebae. Inhibitors of oxidative phosphorylation, protein synthesis, RNA synthesis, and DNA synthesis delay or block transformation, suggesting that RNA and protein synthesis are required (200). Flagellation is accompanied with a decrease in DNA synthesis. Flagellation formation begins 60 min after incubation in transformation medium. The nuclear DNA synthesis decreases, while mitochondrial DNA synthesis continues. The reduction in the nuclear DNA synthesis in differentiating cells is not due to inhibition of initiation of DNA replication, but rather to the termination of the DNA replicating process (181, 201).

9. Growth and life cycle

Naegleria feed on yeast, algae and both Gram-negative and Gram-positive bacteria (202). Food selectivity is observed with findings that filamentous cyanobacteria (e.g., *Anabaena*, *Cylindrospermum*, *Gloeotrichia*, and *Phormidium*) are consumed, while tight threads (*Oscilltoria*) and aggregates (*Aphanizomeno*n) are not ingested (203). Unicellular Chroococcaceae (e.g., *Synechococcus*, *Aphanocapsa*, and *Microcystis*) are excreted after

ingestion, indicating that food selection takes place inside food vacuoles. Ingestion depends on the satiation status of the amoebae, as starved amoebae feed at higher rates compared with satiated amoebae (203).

N. fowleri can be grown simply on the surface of non-nutrient agar overlaid with living or dead *Enterobacter aerogenes* or *E. coli* or other Gram-negative bacilli (202). Live bacteria support optimal growth compared with heat-killed bacteria. Under these conditions, the ameobae feed upon the bacteria, and as growth enters stationary phase and the food supply is used up, *N. fowleri* begin to encyst. Cysts, if kept from drying out, will remain viable for months, possibly years.

The recommended growth medium is Nelson's medium (0.4 mg of $MgSO_4$, 0.4 mg $CaCl_2$, 14.2 mg Na_2HPO_4, 13.6 mg KH_2PO_4, 12 mg NaCl, in 100 mL of distilled water and then addition of 0.17 g Liver infusion, 0.17 g glucose. This should be autoclaved for 25 min at 121°C, followed by the addition of sterile heat inactivated foetal calf serum (final concentration of 10% serum) just prior to use (3, 204, 205). *N. fowleri* reproduces during amoeboid form by binary fission and also gives rise to the cyst and flagellate stages. The approximate intervals of G1, S, G2, and M phases are 6% M phase (28 min), 38% G1 phase (180 min), 38% S phase (183 min), 19% G2 phase (90 min), with a total cell cycle time of 8 h. However, shorter estimates of S phase are obtained for monoxenic cultures (201). Throughout the stages of mitosis, the nucleolus is present. Throughout metaphase, numerous deeply stained DNA condensations following an elongated pattern are witnessed, conforming almost surely to tightly grouped chromosomes. The nucleus divides by cryptomitosis, a process in which the nuclear membrane does not disappear during mitosis, as demonstrated by ultrastructural observations. Centrioles are not found, and a

spindle of microtubules is witnessed running the length of the nucleus from pole to pole though, they do not come to a focal point (206).

9.1. Respiration in *N. fowleri*

N. fowleri contains many mitochondria and lives in aerobic aqueous environments and infects an oxygen-rich brain. Under agitated culture conditions, amoebae consume 30 ng of O per min per mg of cell protein during log growth. The lower oxygen consumption explains the presence of *N. fowleri* in warm waters where dissolved oxygen concentrations are substantially reduced. During stationary phase, the respiratory rate declines by 3-fold. Mitochondria rapidly oxidized glutamate, NADH, pyruvate, succinate, and other Krebs cycle intermediates but slowly oxidized lactate and glycerophosphate. The rates of substrate oxidation are ADP dependent and phosphorylative efficiencies (ADP:O ratios) are about 1.4 for NAD-linked substrates and 1.0 for succinate. The respiratory control ratios are 1.5 to 3 for 11 substrates and dependent on the addition of Pi, Mg^{2+} , and serum albumin to the reaction mixture. Cyanide, azide, malonate, and amytal inhibit oxidative phosphorylation of mitochondria, while rotenone inhibit both glutamate and succinate oxidation (207). The genome of *N. gruberi* showed its versatility, and although aerobic, its genome predicts anaerobic respiration (204, 208, 210). *N. gruberi* has a functional [FeFe]-hydrogenase, as determined by measuring hydrogen production (210). Hydrogenase enzyme is accredited to anaerobic organisms. Hydrogenase is localized in the cytosol, while no hydrogenase activity is linked with mitochondria.

9.2. Storage

Trophozoites can be stored in axenic culture medium and dimethylsulphoxide (DMSO) added to a final concentration of 5%. Cultures are placed directly at -20°C for 60 min, followed by a further 60 min at -70°C and then kept in liquid nitrogen. On rapid thawing at 37°C, revival

rates are about 8% for *N. fowleri* (211). Later studies showed tested a variety of conditions of cryopreservation (212). The average best conditions for freezing was 1 x 10^6 exponentially growing amoebae per mL of freezing medium, consisting of 12% DMSO, 20% heat-inactivated bovine calf serum, 4% glucose; 30 min equilibration at 23°C (room temperature), followed by 60 min at -20°C, with storage at -70°C (212). Under these conditions, *N. fowleri* viability after 1 month of freezing was 64%. After 12 months of freezing, viability was 47% and after 5 years, viability was 38%. The virulence did not decrease during 30 months of freezing (213). At 10 years of cryostorage, viability was 21% for *N. fowleri* (213, 214).

10. Ecology

N. fowleri is a free-living amoeba that is present in diverse environments including soil, water, and air. It has been discovered on all continents, except Antarctica (215, 216). Nonetheless, other species of *Naegleria* have been sequestered from the Antarctica. *N. fowleri* has been isolated from the air during the harmattan in Zaria, Nigeria that proved pathogenic to mice (217). *N. fowleri* is widespread in freshwater lakes (218). Populations in three of five lakes sampled routinely reached levels of one amoeba per 25 mL of water tested, during the hot summer months (218). When tested for seasonal distribution, population densities of free-living amoebae peaked in late summer (219). The occurrence of *N. fowleri* is often associated with elevated temperatures and/or industrial wastewater. *N. fowleri* showed 100% survival at pH ranging from 2.1 to 8.15 (220, 221). Artificial heating of water by power plant discharges facilitates proliferation (222), however *N. fowleri* was not detected outside the reach of the thermal pollution (223), suggesting that warm discharge water should not be used for sports and recreational purposes. Kasprzak *et al.*, (224) tested two complexes of lakes and canals supplying water for two electric power plants, their steam condensors and an adjoining river for the

presence of *N. fowleri*. Sixty-four strains of *N. fowleri* were isolated, from the steam condensor of the power station A and in waters polluted with warm water of this plant. *N. fowleri* strains occurred also in an adjoining river connected with the water system of the power plant. The results show the possible role of the steam condensor A as an incubator and regular source of pollution with pathogenic amoebae for its own system of cooling waters and even the adjoining river (224). During periods of thermal additions to cooling towers, amoebae concentrations amplified by as much as 2 orders of magnitude (225). A canal that was draining cooling water from a factory showed presence of *N. fowleri* (226). Amoebae have also been isolated from sewage samples (227). Based on these findings, it is widely acknowledged that polluted environments are the key sources of potentially pathogenic species of free-living amoebae. This was explained by flagellate-empty habitat hypothesis, i.e., human intervention and/or natural events eliminate usual competitors and the capability to transform to a motile flagellate confers an advantage to *N. fowleri* in recolonizing (228).

Swimming pool waters of Mexico city showd the prevalence at 16.77% of pools tested. *Naegleria* were recovered in their cyst forms. Indoor swimming pools require higher free-chloride residue (229). A high level of chlorine (5.31 mg per mL or more) would counter additional factors such as soil contamination. For example, Kadlec *et al.*, (230) located a reservoir of *N. fowleri* in the cracked wall of a swimming pool where repeated outbreaks of PAM were observed. *N. fowleri* is frequently found in or near geothermal baths (231). By installing a pipeline between the geothermal sources and the baths and by preventing flooding water from entering the baths after rainfall, it is possible to reduce the concentration of amoebae in geothermal baths (232). *N. fowleri* has been isolated from hot springs in Yellowstone and Grand Teton National Parks (233).

The widespread presence of amoebae in the environment is further demonstrated by prevalence of anti-*Naegleria* spp. antibodies in various wild mammals (234). In other studies, a total of 508 reptiles were captured at Canary Islands (Spain) and examined for free-living amoebas. Two hundred seventy-three clones of amoebas were isolated by culture of gut contents, 157 of them belonging to the genus *Acanthamoeba* and 12 to the genus *Naegleria* (235). *Naegleria* spp. have also been isolated from organs of freshwater fish (236, 237).

In addition, *Naegleria* spp. have also been isolated from the moist areas in physiotherapeutic departments of hospitals (238), dust samples in hospitals in Brazil (239), dental treatment units tested (240, 241). *N. fowleri* have been isolated from swabs taken from nose, mouth, and pharynx, suggesting that healthy patients are carriers of pathogenic protists (242, 243). Antibodies to *N. fowleri* have been detected in surveys of normal human sera and saliva with titres ranging from 1:5 to 1:20 and antibodies belonged to IgA, IgG, and IgM (3, 122, 124, 125, 244). Recently, Reveiller *et al.,* (124) developed ELISA for rapid identification of *N. fowleri* in environmental water samples. Flow cytometry has also proven efficient for detection of *N. fowleri* from river and surface water samples (245-247). PCR-based assays have been developed that can detect up to 10 trophozoites or cysts from environmental samples (248, 249). A multiplex PCR has also been developed to simultaneously detect *N. fowleri* and other *Naegleria* species in the environment (250, 251). Recently, Ahmad *et al.,* (252) developed a nested one-step PCR test, in combination with a direct DNA extraction from water or sediment material, for the quick and consistent detection of *N. fowleri* from the environment. Mull *et al.,* (253) used an immunomagnetic separation (IMS) procedure and real-time PCR TaqMan assay to recover and quantify *N. fowleri* in water and sediment samples. Kao *et al.,* (254) used PCR of 5.8S rRNA gene and ITS region to successfully identify *Naegleria* isolates and quantify the

Naegleria spp. by TaqMan real-time quantitative PCR in reservoir water samples. Overall, these rapid, specific and sensitive assays are useful to facilitate studies of the physical, chemical, and biological factors associated with the presence and dynamics of *N. fowleri* in the environmental systems. Following detection of amoebae in the enviromental sample, chlorine was shown to be effective disinfectant against *N. fowleri* (255) in drinking water and swimming pools. Hypochlorite, at a concentration of 0.5 mg per L, killed 100% *Naegleria* after 1 h exposure (25°C, pH 7.3 – 7.4) (256). Later studies tested the efficacy of monochloramine against planktonic forms (trophozoites and cysts) and also biofilm-associated cells of *N. fowleri* as they are often associated with biofilms. The effective range was shown to vary from 4 to 17 mg Cl_2 per L at 25°C and pH 8.2 on both planktonic and biofilm associated cells (257). More recently, Miller et al., (258) showed that *N. fowleri* associated with drinking water distribution biofilm exhibit increased resistance to chlorine. The laboratory starin survived up to 0.6 mg per L chlorine. In comparison, parasite associated with an attached drinking water distribution biofilm survived more than 30 times (20 mg per L) the recommended concentration of chlorine for drinking water suggesting that *N. fowleri* can resist field drinking water distribution biofilm despite chlorination (258).

11. Host for other microbes

In addition to produce fatal infections, *N. fowleri* are recognized as environmental hosts. For the first time, Schuster (259) described intranuclear virus-like bodies in cultures of *Naegleria*. The particles were approximately 100 nm in diameter, and largely restricted to the nucleus. Passage of particles from the nucleoplasm into the cytoplasm was suggested by their association with tubular projections from the nuclear membrane, and particles were seen in the cytoplasm of the amoebae. It was suggested that the virus-like bodies resemble reovirus (260-

262). The subcellular infectious material in *Naegleria* was capable of infecting chick embryo cells and causing them to undergo cytopathic changes with the release of infectious material. The cytopathogenic agent was filterable and passageable and present in *N. gruberi* or *N. fowleri* (263, 264).

Phillips (64) reported the presence of intracellular diphtheroids. The organisms could be eliminated by antibiotic treatment but amoebae was shown to grow better in its presence suggesting a symbiotic relationship. Studies of the interaction of *Legionella pneumophila* with free-living amoebae showed that *Naegleria* could use *L. pneumophila* as a sole food source (265). Some *N. fowleri* cultures support the growth of *L. pneumophila*. Amoebae association with *L. pneumophila* show no increase in the pathogenic potential on intranasal inoculation of mice. Similarly, *L. pneumophila* propagate in infected *N. fowleri* cultures show no increase in virulence. Photomicrographs show intracellular vacuoles containing *L. pneumophila* in the process of binary fission (266, 267). Notably, non-*Legionella* bacteria (*E. coli*, *Aeromonas hydrophila*, *Flavobacterium breve*, and *Pseudomonas aeruginosa*) did not contest with *L. pneumophila* for uptake, proposing that the amoeba hosts took in *L. pneumophila* through a specific and presumably extremely efficient uptake mechanism. These results highlight *Naegleria* as a useful host for the replication and distribution of *L. pneumophila* (268). Bacterial attachment and uptake occurs through a receptor-mediated endocytosis, which requires *de novo* synthesis of host proteins. *L. pneumophila* show a high affinity to GalNAcbeta1-4Gal domain of the *N*-acetyl-D-galactosamine receptor of *Naegleria* (269). The knowledge of endosymbiont in *N. fowleri* is limited, however several lines of evidence suggest that endosymbionts play a key role in the biology of *N. gruberi* (270, 271). Using amoeba co-culture, Casson *et al.*, (272) isolated a *Naegleria* endosymbiont. Phenotypic, genetic, and phylogenetic analyses supported its

affiliation as *Protochlamydia naegleriophila* sp. nov. that may have a role in pneumonia (Fig. 6) (272). *V. cholerae* survive ingestion within amoebae and subsequent encystation (273). The high abundance in lakes, ponds and water circulation networks of these organisms indicate their potential symbiotic relationship, perhaps contributing to the survival and propagation of these pathogens to humans and other animals.

12. Conclusions and Future Studies

12.1. Rapid and non-invasive diagnosis

Given the rarity of the disease, lack of awareness, and similarities with bacterial meningitis, PAM is rarely thought of as the indicative cause. A high suspicion for PAM is considered following negative cultures for bacteria in the CSF and a history of swimming/nasal irrigation. Given the high intracranial pressure in PAM patients, CSF collection may complicate the clinical scenario leading to brain herniation (6, 274). Naturally, the emphasis is to lower the intracranial pressure. Amoebae enter the brain through the nasal passage, and then travel along the olfactory neuroepithelial route and infect inferior surface of the frontal lobe. Amoebae propogate in this area followed by dissemination in the CNS. It makes sense to collect nasal secretions from the primary site of infection for the identification of *N. fowleri* (275). In support, Singh *et al.,* (276) observed amoebae in the CSF and nasal secretions of a PAM-infected patient and confirmed *N. fowleri* through culture (276), demonstrating the usefulness of nasal secretions as a diagnostic tool. The wet preparation can be examined under the microscope or using PCR-based assays to detect *N. fowleri* for rapid, non-invasive identification of *N. fowleri* and this should be investigated in future research.

11.2. Antiamoebic anesthetic agents

As recent studies showed anti-amoebic (amoebistatic and partial amoebicidal) effects of anesthetic agents, haloperidol and loperamide (277, 278), it is suggested that aforementioned agents should be added to complement therapeutic interventions by inhalation to exert anti-*N. fowleri* effects at the primary site of infection. The use a 'transcribrial route' (275) device for vaporized delivery of chemotherapeutic agents should be investigated in the future studies. Such a delivery method overcome challenges of the current intraventricular, intrathecal, intravenous methods that have added complications of side effects and/or difficulty in achieving MIC at the site of the infection, following intravenous injections, and/or drainage into the blood following CSF injections. Moreover the proposed route will avoid challenges of drug penetration through the highly selective blood-brain barrier and could be a useful method to apply drugs directly on the target site, without affecting other tissues due to systemic dissemination and diluting their effects needlessly. Further research is needed to determine the effectiveness of the proposed methods for effective management to help improve morbidity and mortality of *N. fowleri* infection.

11.3. Drug repurposing

Testing of drugs that are clinically-approved against various diseases with known mechanisms of action and targets/biochemical pathways offers useful research avenues, as the pharmacokinetics profiles, safety margins etc. have been already established. Recently, Baig *et al.,* (277, 278) have shown amoebicidal effects of some of the FDA-approved drugs such as digoxin and procyclidine. This approach can offer promising drug-leads for clinical practice. Based on the few drugs tested with promising anti-amoebic effects, there is a need for high throughput screening using available chemical libraries/FDA-approved to identify potential anti-

N. fowleri compounds. Once a number of potential compounds are identified *in vitro*, with known pharmacokinetics, and pharmacodynamics profiles, the findings can lead to *in vivo* testing at a faster pace. The drug discovery of effective anti-*N. fowleri* drugs over the last few decades has been disappointing, largely due to neglect by the funding agencies. The fact that PAM is a rare disease in developed countries and affects communities mostly in developing countries, where millions of people are dying due to other diseases, makes it logical to undertake drug repurposing approach in our search for effective anti-*N. fowleri* drugs.

11.4. Biomarkers

Absence of distinct biomarker(s) of PAM in the mucosal secretions, blood, CSF makes it difficult to study the impact of interventions against it. Biomarkers for PAM have been challenging to find, partly because of our inadequate understanding of its pathogenesis and pathophysiology. Thus there is a need to identify novel biomarkers for PAM. Using analytical tools such as mass-spectrometry, NMR etc., there is also a need to study and compare biochemical profiles of pathogenenic and non-pathogenic *Naegleria* as well as patients from different populations, gender, ethinicity to identify potential predisposing factors. The paucity of PAM studies during the last few decades is partly to blame for the lack of understanding of the pathogenesis and pathophysiology of PAM, and the subsequent lack of its good biomarkers. Major technological advances in studying pathophysiologic mechanisms in general have not been applied to the study of PAM so far. If we are to identify sensitive and specific biomarkers of PAM, we must first understand the pathophysiologic mechanisms that underlie this disease. The reasons for the lack of aforementioned studies are multifactorial. An important issue is that PAM is rare and/or occurs in resource-limited settings, which may not have the infrastructure to perform such experimentation or have other research priorities of health-related problems of

major significance. There, clearly, is a need to identify and test new promising candidate biomarkers of PAM in mucosal secretions, blood, and CSF, and then correlating them with the histopathologic features of affected patients, which will provide a mechanistic basis of PAM.

11.5. Drug targets

A complete understanding of the biology and pathogenesis of *N. fowleri* will identify molecules that could be of value to develop therapeutic interventions. Ondarza (279) suggested that exploration of several pathways can reveal drug targets, including, a) hydrolytic enzymes for host tissue invasion, b) glycolytic enzymes, that are differerentially expressed by the parasite, c) thiol-based redox metabolism pathways, d) oxidative stress pathway, e) trypanothione pathways, and f) encystation and excystation pathways.

11.6. A model organism with the pathogenic potential

The biology of *N. fowleri* is fascinating. With its ability to switch phenotypes to an active trophozoite stage to grow vegetatively, or transform into a flagellate stage to travel long distance, or switch into a dormant cyst form to endure callous conditions, as well as the fact that it is a single-celled eukaryotic protist, it is an excellent organism for the study of biochemistry, cellular microbiology, molecular mechanisms of cell differentiation, physiology, cellular interactions with other microbes or human cells, phagocytosis, flagellar and amoebal motility, and evolutionary studies. A complete understanding of the molecular and cellular biology of *N. fowleri* to encyst, flagellate, excyst, transmit, evade host defences and produce disease will identify potential targets for the rational development of therapeutic interventions.

Future goals are to find means to identify individuals who are at risk and to provide them with rationally-designed strategies to protect against the infection. The preventative strategies may vary among different communities, from the use of chlorine or filters to decontaminate

water, or the use of nose clips or use of clean water for nasal cleansing/irrigation. It is known that binding of trophozoite to nasal mucosa is the first step in *N. fowleri* infection, and induction of mucosal immunity is another avenue to counter infection progression. Several virulence factors have been described, some of which are potential vaccine targets for the susceptible population. The availability of the genome information as well as *in vitro* and *in vivo* models will expedite the identification of novel drug targets further, through genomics, proteomics and bioinformatics. Given the rarity of the disease, chemotherapy will remain the primary choice to develop therapeutic interventions. Many of the drugs tested target the functional aspects of *N. fowleri*, however, being a eukaryote *N. fowleri* share functional homologies to mammalian cells. Consequently, many of the available drugs cannot be prescribed at effective concentrations due to their unwanted side effects. This is particularly relevant for *N. fowleri* infection, as drugs are given intravenously in most cases, and must get through the brain microvessels to target the intracerebral *N. fowleri*. In this process, drugs target all tissues, and can affect their physiology before reaching the target site, at effective concentration. Hence, there is a need to develop a targeted therapeutic approach, or identify drugs that can affect parasite viability without affecting host cells. Future research is needed to to identify drugs and/or chemotherapeutic approaches of potential value, together with improved understanding of drugs, targets, and their underlying mechanisms that will facilitate the development of more effective chemotherapies against this rare, but fatal infection. There is an urgent need for continued research in developing preventative strategies as well as understanding pathogenesis and designing effective anti-*N. fowleri* chemotherapeutic and immuno-therapeutic approaches against PAM and demonstrating their efficacy in the clinical practice. Furthermore, educational efforts are needed to increase

knowledge of this parasite, disease and associated risk factors, by the medical practitioners, public, health officials, and the local government.

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Figure legends

Figure 1. *Naegleria fowleri* (a) trophozoite form, (b) flagellate form, and (c) cyst form (courtesy: B. S. Robinson, Australian Water Quality Centre, South Australian Water Corporation). Bar is = 10 μm.

Figure 2. Pathological features of primary amoebic meningoencephalitis caused by *Naegleria fowleri*. (a) A cerebrospinal fluid smear stained with Giemsa stain. Note amoebae at arrows (× 1000). (b) A section through the cerebellum showing extensive inflammation. (c) An area of the cerebellum showing extensive destruction of the brain architecture with large numbers of amoebae in the perivascular area (× 1000). (d) A section, similar to the one shown in (c), but reacted with anti-*N. fowleri* serum. Note the brightly staining *N. fowleri* trophozoites (kindly provided by G. S. Visvesvara from FEMS Immunol Med Microbiol. 2007, 50, 1-26).

Figure 3. Thousands of people can be seen swimming in the canal that passes through the city of Lahore, Pakistan, without nearby facilities for defecation and urination.

Figure 4. Nasal cleansing/irrigation using neti pots can provide relief to patients with sinusitis by flushing out excess mucus and debris from the nose (a and b). Ablution involves nasal cleansing (c). The use of contaminated water together with forceful pushing up the nostrils even though it is not required as part of the ablution practice or swimming in contaminated water (d) or unchlorinated pools with amoebae can lead to parasite entry into the brain.

Figure 5. Factors associated with the pathogenesis of *Naegleria fowleri* infection.

Figure 6. *Naegleria lovaniensis* with *Protochlamydia naegleriophila*, strain KNic, as endoparasites (P). Bar is 1.0 µm (kindly provided by R. Michel and B. Hauröder, Central Institute of the Armed Forces Medical Service, Koblenz, Germany).