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Research Article

Naegleria fowleri, the Causative Agent of Primary Amoebic Meningoencephalitis

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Abstract: Primary amoebic meningoencephalitis (PAM) is a fatal disease of central nervous system caused by free living amoebae, *Naegleria fowleri*. The disease is associated with exposure to contaminated water followed by brain tissue damage and death. Diagnosis of the disease is difficult due to similarity with bacterial meningoencephalitis. However, wet mount of cerebrospinal fluid (CSF), biochemical and various molecular techniques help in timely diagnosis of the disease. Treatment regimen includes high dose of amphotericin B alone or in combination with miconazole, sulfisoxazole and rifampin. However, several antibacterial and antifungal drugs have also shown potential antiamoebic activity. Exposure to *N. fowleri* can be prevented by chlorination of swimming pools and monitoring of recreational water bodies by the local authorities. This review provides an overview of the current knowledge about *Naegleria fowleri* as a disease causing pathogen along with recent diagnostic and treatment strategies to combat primary amoebic meningoencephalitis.

Keywords: Primary amoebic meningoencephalitis (PAM), *N. fowleri*, central nervous system

1. INTRODUCTION

Naegleria spp. belong to group of free living amoeba-flagellates present in diverse habitats worldwide [1]. More than 30 species of *Naegleria* have been identified so far and many of them are acknowledged on the basis of their small and large ribosomal subunit DNA and internal transcribed spacers (ITS). Among all, *Naegleria fowleri* is the most pathogenic to humans [2, 3]. It is the prime cause of primary amoebic meningoencephalitis (PAM). The ability of free living amoebae to cause disease was first taken into account by Fowler and Carter in 1965. Afterwards, the term PAM was introduced by Butt [4]. Primary amoebic meningoencephalitis mostly occurs in children or young adults who had exposure to water bodies. Mortality rate of the disease is 95% and death approaches within 72 hours of infection. Fewer than 100 cases of PAM have been reported on international level. PAM is difficult to diagnose because of its striking similarity with bacterial meningoencephalitis [5]. Timely diagnosis and effective treatment are important factors in saving lives of people infected with *N. fowleri.*

This review highlights the pathogenicity of *N. fowleri* and various diagnostic and treatment strategies used for management of the disease.

2. HABITAT

The favorable habitat for *Naegleria fowleri* is soil. Due to rains and water runoffs, it is also found in lakes, ponds and surface waters. It is thermo-tolerant and thermophilic, tolerating temperatures of 40°C-45°C. It has been isolated from various environments including coastal water, freshwater, ground water aquifers, river sheds, thermal spring recreations, sewage, heating and ventilation units, poorly chlorinated swimming pools, artificial lakes, warm water discharges of power plants, water treatment plants and cooling nuclear reactors [6-10]. It has also been isolated from tap swabs

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and neti pots from household. Leduc et al. [11] reported the isolation of *N. fowleri* from hospital environments. *N. fowleri* was isolated from water samples of dental units, suction lines and suction filters. It was also isolated from the dust samples of non-hospital environment having good records of cleanliness [12]. Different criteria affect the presence of *N. fowleri* in fresh waters. It include physical, chemical and biological parameters. Water sources containing cyanobacteria, eubacteria and coliforms have higher numbers of *N. fowleri* because these bacteria serve as food source for amoebae. Similarly, water containing higher concentrations of iron and magnesium has higher number of *N. fowleri* [10]. On the other hand, water characteristics such as conductivity, dissolved oxygen, temperature and pH does not affect the presence of *N. fowleri*. Number is also affected by predation by other protozoa and invertebrates, disturbance of water surface from boating and presence of bacterial or fungal toxins [8].

Naegleria spp. can also serve as hosts for facultative pathogens, such as *Legionella spp.* The coexistence of *Naegleria sp.* and *Legionella sp.* pose a public health threat [13].

3. EPIDEMIOLOGY

N. fowleri exposure is very common as revealed by the presence of antibodies against *N. fowleri* in general public. Disease is caused by inhalation of the pathogen by fresh water contact or contaminated dust. Cases of PAM have been taken in account from Virginia to Florida, California. The US Center for Disease Control and Prevention (CDC) announces 2-4 cases of waterborne diseases annually [4]. According to CDC, 133 cases of PAM caused by *N. fowleri* have been reported from 1964-2014 in U.S. [14].

4. LIFE CYCLE & MORPHOLOGY

Life cycle of *N. fowleri* comprises of three distinct stages: the trophozoite, the flagellate and the cyst. Trophozoite stage is the active form ranging in size from 7-20 μm. They have elongated structure due to presence of rounded structures called lobopodia. The granular cytoplasm incorporates vacuoles, nucleus, rough endoplasmic reticulum and mitochondria. In axenic culture, the mitochondria of amoebae are round, oval and cylindrical in shape, the rough endoplasmic reticulum appears in the tubular or vesicular shape and vacuoles appear empty. Whereas, in infected mice brain tissue, mitochondria are dumbbell shape, rough endoplasmic reticulum is tubular and numerous food vacuoles with myelinated structures show excessive phagocytosis. Trophozoites depend on *Escherichia coli* for food. The flagellate stage is pear shaped and mobile. It is transient and returns back to trophozoite form when deprived of water. Cysts are dormant spherical structures 10 μm in diameter. They are double walled, refractile and uninucleate. They are the product of hostile environmental situations. The shape of nucleolus during movement is a distinct feature to differentiate between *Acanthamoeba* and *Naegleria* [10, 15]. Fig. 1 represents microscopic pictures of *N. fowleri* cyst, flagellate and trophozoite.

Various stains are used for studying different organelles or developmental stages of *N. fowleri*. Trichome-eosin and iron-hematoxylin stains

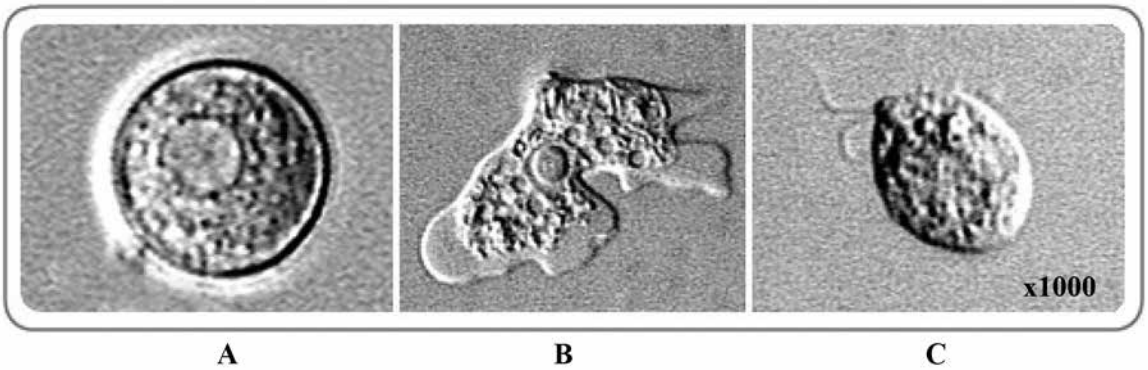

Fig. 1. *Naegleria fowleri* (A) Cyst (B) Trophozoite and (C) Flagellate. Magnification 1000X
Source: Adapted from [14].

produce contrast colors for the identification of all three stages: trophozoite, flagellate and cyst. Giesma's and Gramsstains only color trophozoite and flagellate stages. Modified Field's and modified acid-fast stains help to identify trophozoite stage only [3].

Mitosis in *Naegleria fowleri* can be studied by using 4', 6-diamidino-2-phenylindole (DAPI) and Feulgen stains which act as DNA markers and react with DNA in nuclei of amoebae. During metaphase, DNA is condensed and chromosomes are tightly grouped. Nuclear division takes place by crypto-mitosis. Nuclear membrane does not disappear, nucleolus is present throughout the mitotic process, centrioles are not present and spindle formation takes place [16].

5. PATHOGENICITY

N. fowleri is the causative agent of primary amoebic meningoencephalitis. The pathogen enters in the host by nasal passages which are exposed to *N. fowleri* flagellates during swimming or bathing in hot water springs or hot baths. Infection in humans can also be caused by inhalation of infectious cysts. After inhalation, ex-cystation process takes place and trophozoites invade brain through nasal mucosa and olfactory nerves. [10, 17]. Symptoms of PAM include severe headache, fever, nausea and vomiting [5]. The steps involved in disease progression after 8 hours of infection in mice include: amoebae in contact with mucous layer of olfactory epithelium, elimination of many amoebae by mucous shedding, the remaining amoebae reach nasal epithelium. After 24 hours, they enter the nasal epithelium and some invade through respiratory route. After 96 hours of infection, the inflammatory response become strong in olfactory bulb and brain. But this inflammatory response results in an increase in brain tissue damage and has no role in amoebae destruction [18]. At early stage of disease, *N. fowleri* does not involve lungs and blood vessels as the path to central nervous system (CNS). Later on, the amoebae enter the veins of CNS and bone marrow to spread the disease [19]. Target cells are destroyed by trogocytosis, which involves ingestion of target cells by forming food cups. The process is dependent on actin [20]. *N. fowleri* contain several pore forming peptides such as amoebapore A. These proteins can invade host cells and prevent the growth of phagocytosed bacteria by forming pores in their membranes [21]. Neuroimaging of patients infected with PAM showed multifocal parenchymal lesions, pseudotumoral lesions, meningeal exudates, hemorrhagic infarcts and necrosis in brain [22]. The suitable model for studying pathogenesis of *N. fowleri* is Human neuroblastoma (SK-N-MC) because these cells are most susceptible to *N. fowleri*. Upon infection, *N. fowleri* cause damage by phagocytosis or by producing amoebostomes. 100% destruction of human neuroblastoma cells occurs within 1 day [23-24]. Shibayama et al. [17] reported the pathogenic effect of *N. fowleri* on Madin-Darby canine kidney (MDCK) tight junction proteins which included claudin-1, occludin, ZO-1 and actin cytoskeleton. *N. fowleri* induced epithelial cell damage within just 3 hours infection. Also changes in junction proteins were observed except occluding. *N. fowleri* increased the epithelial membrane permeability which increased the trophozoites invasion.

In vitro cytotoxicity and pathogenicity of *N. fowleri* also depends on the culture medium. Burri *et al.* [25] reported that *N. fowleri* cultured in Nelson's medium were highly pathogenic in mice model. Whereas *N. fowleri* grown in PYNFH medium (in 1 litre of distilled water: 10 g proteose peptone, 10 g yeast extract, 1 g ribonucleic acid, 15 mg acid folic, 1 mg hemin, 0.2 g $Na_2HPO_4.2H_2O$, 36 g KH_2PO_4 and 100 ML fetal bovine serum) showed lower pathogenicity and slower growth. Addition of LH (Liver Hydrolase) in PYNFH medium increased the pathogenicity. *N. fowleri* cultured axenically in the presence of cholesterol, also show reduction in pathogenicity [26]. Also, long term maintenance of *N. fowleri* in axenic culture results in decrease in virulence and latent infections [27]. Similarly cryopreservation for 5-10 years at -70˚C decreases the viability of *N. fowleri* by 21% [28].

Genomic basis of *N. fowleri* pathogenicity is unclear. The genome is mostly compared to the close related nonpathogenic specie *N. gruberi.* By comparing the mitochondrial genome and fragment of nuclear genome (60 kb) of *N. fowleri* with that of *N. gruberi*, ten novel *N. fowleri* genes were found in nuclear genome which may be involved

in pathogenesis. Whereas, the mitochondrial genome of both species was highly similar. Also horizontal gene transfer over the evolutionary time period had incorporated a homolog of cathepsin B, which is involved in the pathogenicity of different eukaryotic pathogens [29]. Genome of *N. fowleri* contains a gene called heat shock 70 gene. After cloning and characterization, it was named as Nf-cHSP70. It is located in cytoplasm, pseudopodia and phagocytic food cups and play *important* role in pathogenicity. Knocking down of the gene with antisense oligomers resulted in 68.6% decrease in pathogenicity against Chinese hamster ovary (CHO) cells [30]. Another gene called Nf-actin1 gene located in pseudopodia also plays role in pathogenic activity of *N. fowleri* as it was involved in food cup formation [31, 20]. *In vitro* studies showed that *N. fowleri* trophozoites produce NO (Nitric oxide) isoforms which share epitopes with mammalian (NOS2) nitric oxide synthases. During infection in mice brain, the trophozoites reacted to the NOS2 antibodies which proved that NO may have role in pathogenesis [32]. *N. fowleri* also release different biological molecules called excretory-secretory proteins (ESPs) which are involved in pathophysiological and immunological events during infection. Important ESPs include 58 kDa of exendin-3 precursor, 40 kDa of secretory lipase, 24 kDa of cathepsin B-like proteases and cysteine protease, 21 kDa of cathepsin B, 18 kDa of peroxiredoxin, and 16 kDa of thrombin receptor [33].

Pathogenic study of *N. fowleri* upon incubation with Jurkat T cells showed that *N. fowleri* destroys host cells by lytic necrosis [34].

6. IMMUNE RESPONSES

N. fowleri, the causative agent of PAM, a disease of central nervous system (CNS), is also involved in the activation of various host innate immune responses. During early infection, the host secretes mucus due to activation of mucin producing gene (MUC5AC). Mucus entraps the trophozoites and provide first line of defense. It also reduces the cytotoxicity of host cells and disease progression. This process is followed by permeation of neutrophils into nasal cavity. The local inflammatory response is produced by activation of interleukins (IL-8 and IL-1 beta) via production of reactive oxygen species (ROS) which are responsible for signal transduction of these molecules. Most of the PAM infections are prevented due to these immune responses. However *in vitro* studies on Madin-Darby canine kidney (MDCK) and MUC5AC inducing cell line NCI-H292 showed that *Naegleria sp*. has a 37 kDa protein with mucolytic property. When the number of amoebae is sufficient, they may degrade mucins by proteolytic activity [35-36].

Inflammatory responses against PAM are also associated with astrocytes activation. The activated astrocytes activate interleukins (IL-beta and IL-6). These interleukins are dependent on extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 mitogen activated protein kinases (MAPKs) activation. The expression of interleukins causes the astrocytes to produce inflammatory response [37]. Antibodies also play an important role in defense against amoebic infections. IgA and IgM antibodies recognize *N. fowleri* (Nf) proteins and participate in resistance of disease. They provide defense by suppressing the attachment of trophozoites to the nasal mucosa [38-39].

While reviewing the cDNA expression library of *N. fowleri*, a gene called Nfa1, comprised of 357 bases was found. Nfa1 encoded Nfa1 protein which was 43% homologous to recombinant protein called myohemerythrin. It showed noticeable immunoreactivity in infected, immune anti-Nfa1 sera. In a SDS-PAGE study of *N. fowleri* lysate, two proteins (53 and 25 kDa) were found to be effective antigens for immunization. In another study, it was found that splenectomy has no effect on development of PAM in infected mice. Injection of monoclonal antibodies against *N. fowleri* in mice reduced the mortality rate. Similarly, transfer of splenocytes from immune mice to infected mice also reduced mortality rate. It was studied that zinc deficiency in mice minified the proliferation of splenocytes to concanavalin A and *N. fowleri* lysate with reduced IgG levels after immunization with *N. fowleri*. Natural killer (NK) cell activities in mice infected with *N. fowleri* were also studied. NK cells activity was noticeably increased on day 1 along with higher target binding abilities [15].

7. DIAGNOSIS

Progression of *N. fowleri* in central nervous system is fast and requires highly sensitive and rapid diagnostic tools for timely treatment [40].

The PAM is characterized by increased intracranial pressure and brain edema. Diagnosis is confirmed after isolation and identification of trophozoites from brain tissue or cerebrospinal fluid (CSF). CSF is analyzed by lumbar puncture technique which serves as basic diagnostic tool for PAM. Also the extent of necrosis and inflammation is determined by concentration of red blood cells (RBCs) in CSF. Normal CSF is yellowish white in color. The presence of red blood cells (2500/ mm^3) may indicate initial stage of disease. However, this number may increase up to (24,600/ mm^3) in advanced disease stage. Polymorphonuclear leukocytes (PMN) number in diseased state range from 300 to 26,000 cells/ mm^3. CSF pressure appears high (300-600 mm H_2O). Concentration of glucose may appear 10 mg/100 mL or lower and protein concentration may vary from 100 to 1000 mg/100 mL of CSF.

Wet-mount of CSF (Fig. 2) is observed using phase contrast microscopy. To identify trophozoites stained with immunofluorescent dyes UV light microscopy is used. Giesma's or Trichrome staining is used for visualization of trophozoites. Size of the trophozoites range from 7-15 µm and have single large nucleolus present. Several vacuoles are visible around nucleus and pseudopodia can be observed [40-41].

Naegleria fowleri utilize bacteria as food. Therefore, it is possible to isolate amoebae from clinical samples using non nutrient agar spread with bacteria. Vero monkey kidney cells (E6) and human lung fibroblasts (HLF) cells are also used as substrates for the amoebic growth. Growing amoebae destroy the confluent layer of cells in 2-3 days. Axenic cultures and chemically defined medium can also be used for growth [42].

Isoenzyme analysis is a biochemical method used for specific identification of *N. fowleri* from clinical as well as environmental samples [40]. Enzyme linked immunosorbent assay (ELISA) also present as a specific diagnostic tool for *N. fowleri*. It utilizes a monoclonal antibody (5D12) which recognizes glycosylated epitope on proteins of *N.*

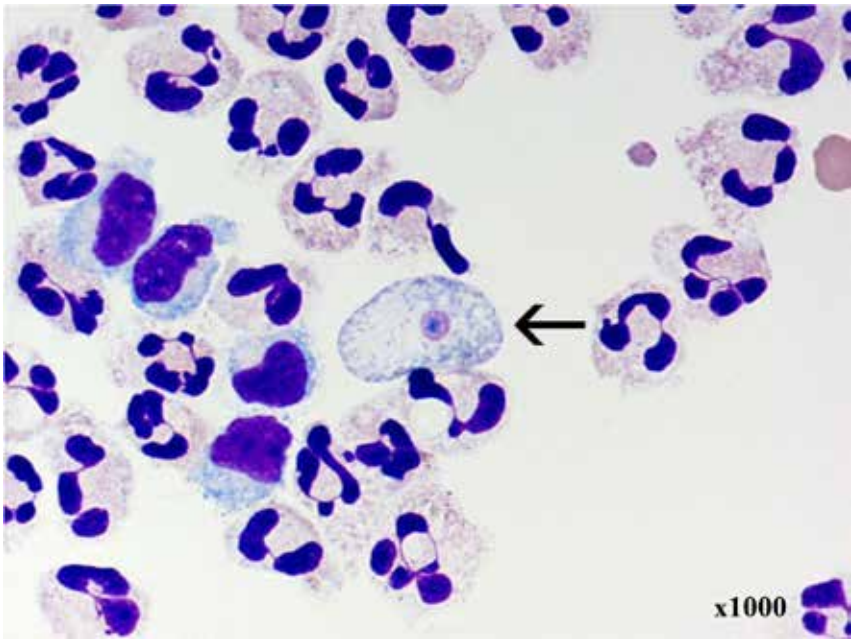

Fig. 2. A wet mount of fixed CSF showing trophozoite of *Naegleria fowleri* (arrow), lymphocytes and polymorphonuclear lymphocytes stained with Giesma-Wright amidst. Nucleus and nucleolus are visible inside the trophozoite. Magnification 1000X.

Source: Adapted from [57].

fowleri [43].

Several molecular techniques which include polymerase chain reaction (PCR) and real time PCR play an important role in detection of *N. fowleri* in clinical and environmental specimen [40]. Genetic variation in various strains of *N. fowleri* can be found by sequencing of 5.8S rRNA gene and internal transcribed spacers (ITS1 and ITS2) [44].

Multiplex PCR assay can be used to identify *N. fowleri* in CSF and brain tissue samples. Another sensitive and fast technique is real time PCR and melting curve analysis of 5.8S rRNA gene. It utilizes SYTO9 dye which intercalate DNA. This technique has been used for distinguishing various species of *N. fowleri* in environment [45].

8. TREATMENT

After diagnosis of PAM, treatment must be started urgently. Common treatment regimen include intrathecal and systemic administration of high dose amphotericin B alone or in combination with miconazole, sulfisoxazole and rifampin. However, administration of amphotericin B has some side effects as well. Various displeasing side effects in patients have been observed which include fever, shaking chills, vomiting, headache, hypotension, nausea, tachypnea, and dyspnea. Nephrotoxicity is also a serious side effect which can be severe.

Although amphotericin B showed promising results against *N. fowleriin-vitro* and *in-vivo*, but the success rate of the drug in PAM patientsremained low [40]. Due to the fact, several other antifungal, antimicrobial and antiparasitic drugs have been screened out for antiamoebic activity *in vitro* and *in vivo*. Examples of potent drugs include ketoconazole, clotrimazole, fluconazole, chlorpromazine and itraconazole [46-47].

Fluconazole showed promising results by reducing organ dysfunction and mortality by increasing antibacterial activity and introduction of neutrophils. It was capable of penetrating blood brain barrier effectively [40]. Another drug, Azithromycin has been tested *in vivo* for treatment of PAM in mice. It showed 100% results at a dose of 75 mg/kg/day for 5 days [48]. Intravenous administration of amphotericin B and fluconazole along with oral rifampicin has been reported to be successful mode of treatment against PAM patients [49].

Miltefosine and voriconazole, used as anticancer and antifungal drugs also showed effective antiamoebic activity against *Acanthamoeba spp, N. fowleri* and *Balamuthia mandrillaris in vitro* [50]. Several antibacterial drugs which included erythromycin, clarithromycin, rokitamycin, hygromycin, zoecin and roxithromycin were tested for their antiamoebic activity. Among the tested drugs, hygromycin B, roxithromycin and rokitamycin inhibited the growth of amoeba *in vitro*. Rokitamycin showed minimum inhibitory concentration of 6.25 µg/ML with survival rate of 80% over 1 month in experimental mice models [51].

Corifungin, a water-soluble polyene macrolide has shown better activity against *N. fowleri* as compared to amphotericin B. Mode of action of corifungin included disruption of plasma and cytoplasmic membranes of trophozoites, mitochondrial changes and complete lysis of amoebae. This macrolide also showed potent antiamoebic effects *in vivo*. Intraperitoneal dose of 9 mg/kg in mice resulted in 100% survival rate. However, amphotericin B was not much active at the same dose and survival rate was reduced. On the basis of these findings, U.S. FDA has given a status of orphan drug to corifungin for PAM treatment [52].

Recently, much research has been done to determine virulence factors of *N. fowleri* which can be targeted by therapeutic drugs to reduce pathogenicity [40]. One such target is Nfa1 gene, located on food cups and pseudopodia. Gene knockdown of Nfa 1 gene could result in loss of expression of pathogenicity factors in trophozoites [40, 53]. Nfa 1 DNA vaccines were administered via intranasal route in mice using retroviral and lentiviral vectors. DNA vaccination resulted in production of Nfa1 specific IgG antibodies (IgG2a and IgG1) in mice. These findings revealed that DNA vaccination can be an effective method for treatment of PAM infections [54]. In another study, it was reported that chlorpromazine showed more potency against *N. fowleri* trophozoites as

compared to amphotericin B and voriconazole. This drug inhibited expression of Nfa 1 gene which is an important virulence determinant of *N. fowleri* [55].

Diamidines also have shown potency against free living amoeba as they have the capability to cross the blood brain barrier effectively. In a study, mono and diamidino derivatives were investigated for antiamoebic activity. A compound (DB173) showed IC_{50} of 177 nM. This compound can be used as novel lead compound for the development of CNS infections associated with free living amoebae [56].

9. PREVENTION AND CONTROL

Thermophilic amoeba, *Naegleria fowleri* propagates in water when the temperature is above 30˚C. Due to global warming, it is anticipated that cases of PAM caused by *N. fowleri* will be reported first time in many countries. *N. fowleri* has been reported to have sensitivity towards chlorine (one part per million). Chlorination of swimming pools can be done to prevent amoebic proliferation. But the efficacy of chlorine is reduced in the presence of organic matter and sunlight. Another limiting factor is the fact that natural water bodies such as ponds, lakes and streams cannot be chlorinated due to which risk of *N. fowleri* proliferation is increased.

Local public health authorities should regularly monitor recreational waters for *N. fowleri* amoebae. Also, during hot summer months, appropriate warnings should be posted and water contact should be prohibited in suspected waters [41].

10. CONCLUSIONS

Primary amoebic meningoencephalitis (PAM) is a deadly disease of central nervous system caused by *Naegleria fowleri*. Pathogenicity mechanisms of *N. fowleri* are not well understood. However, it has been established that it produces pore forming proteins that lyse mammalian cells upon contact, produce phospholipases and proteases that play their role in degradation of mammalian tissue. Immune response against PAM include production of IgM antibody which along with IgA inhibits the adhesion of trophozoites to mucosal epithelium. Diagnosis of PAM is difficult due to its striking similarity with bacterial meningoencephalitis. However, after identification by wet mount of CSF, it is important to start the treatment immediately with amphotericin B alone or in combination with miconazole, sulfisoxazole and rifampin. Steps should be taken to eradicate *N. fowleri* proliferation in water bodies. Proper chlorination of swimming pools and monitoring of natural water bodies may help to reduce *N. fowleri* exposure.

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