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The therapeutic strategies against *Naegleria fowleri*

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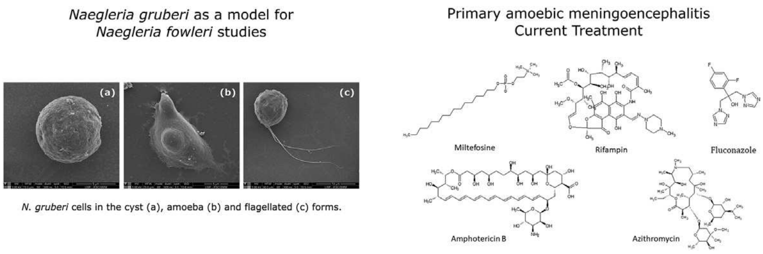
HIGHLIGHTS

- *N. fowleri* is an important human pathogen, causing a disease with high fatality rates.
- The fast development of the patients' clinical condition calls for a prompt diagnosis.
- Identifying sources of *N. fowleri* is critical for prevention and diagnosis.
- Existing therapy shows limitations regarding effectiveness and side effects.
- Studies point to new therapeutic alternatives that reveal promising treatment results.

GRAPHICAL ABSTRACT



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ABSTRACT

Naegleria fowleri is a pathogenic amoeboflagellate most prominently known for its role as the etiological agent of the Primary Amoebic Meningoencephalitis (PAM), a disease that afflicts the central nervous system and is fatal in more than 95% of the reported cases. Although being fatal and with potential risks for an increase in the occurrence of the pathogen in populated areas, the organism receives little public health attention. A great underestimation in the number of PAM cases reported is assumed, taking into account the difficulty in obtaining an accurate diagnosis. In this review, we summarize different techniques and methods used in the identification of the protozoan in clinical and environmental samples. Since it remains unclear whether the protozoan infection can be successfully treated with the currently available drugs, we proceed to discuss the current PAM therapeutic strategies and its effectiveness. Finally, novel compounds for potential treatments are discussed as well as research on vaccine development against PAM.

Contents

Abbreviations: DUWL, Dental unit waterlines; DWDS, Drinking water distribution system; Trp, Tritrpticin.

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1. Introduction

Free-living amoebae (FLA) include amphizoic protists capable of living in a variety of different habitats, not only in freshwater bodies, seawater and sewage systems, soil samples - which are actually expected habitats for amoebas to thrive in - but also in the air, dust samples, drinking water, dialysis units, eyewash solutions, contact lenses and dental treatment equipment (Trabelsi et al., 2012). Furthermore, some FLA species can be involved in both opportunistic as well as non-opportunistic infections in humans resulting in cerebral, skin or corneal infections. Pathogenic FLA belong to five genera, *Balamuthia, Acanthamoeba, Sappinia, Naegleria* and *Vermamoeba* (Abdul Majid et al., 2017; Teide et al., 2015). They share a life cycle that comprises a trophozoite (ameboid), feeding and replicating form, and a dormant cyst stage when faced with adverse environments. (Abdul Majid et al., 2017).

Particularly *Naegleria* is the unique genera of FLA that has both the amoeboid and cyst forms, besides a flagellate, motile, intermediate. (Fritz-Laylin and Cande, 2010; Khan et al., 2015). This ability to differentiate in a flagellate stage is one of the features that places *Naegleria* spp. as a member of the Vahlkampfiidae family, class Heterolobosea, together with Jakobozoa and Euglenozoa, the JEH clade, presently classified at the Excavata supergroup (Parfrey et al., 2006; Rodríguez-Ezpeleta et al., 2007). A previous research, using transcriptome approach, has analysed the enflagellation process of *Naegleria gruberi* showing that it takes about one hour to be completed and requires the transcription of a set of basal body and flagellar apparatus genes (Fritz-Laylin and Cande, 2010).

Similar to other FLA members, *Naegleria* is a free-living organism, feeding primarily on bacteria (De Jonckheere, 2011). To date over 40 different species of *Naegleria* have been identified (Abdul Majid et al., 2017). For the last 40 years attention has been focused on *Naegleria fowleri* (Carter, 1970) named in honour of its discoverers: the French zoologist Mathieu Naegler and the Australian doctor Malcolm Fowler. *N. fowleri* is the etiological agent of Primary Amoebic Meningoencephalitis (PAM), a devastating infection that targets the Central Nervous System (CNS) with high lethality rates (Grace et al., 2015).

N. fowleri is a thermophilic amoeboflagellate that have been isolated as a cyst resistant form, a trophozoite proliferative and feeding form and a motile flagellate form (reviewed by Grace et al., 2015; Baig et al., 2014; Heggie, 2010). All these stages have the ability to establish infection (Martinez and Visvesvara, 1997; Schuster and Visvesvara, 2004). Generally the trophozoite and flagellate forms are inhaled during swimming or diving, migrate through the neuroepithelium and have been found in affected tissue and the cerebrospinal fluid (De Jonckheere, 2002). The trophozoite attaches to human olfactory epithelium, move exuding pseudopodia and pass the cribriform plate to the brain through the olfactory cell axon (Visvesvara, 2010). The flagellate, a biflagellate form, once inside the nasal cavity transforms into a trophozoite taking several hours to complete differentiation (Marciano-Cabral, 1988), in contrast with *N. gruberi* fast differentiation process.

The spherical cysts, 8–12 μm in diameter, are naturally resistant to unfavourable environment, containing a single nucleus and a double-wall with pores through which the amoeba escape when conditions become favourable (Visvesvara, 2010). They are transported by dust and can occasionally enter the nasal mucosa, however they have not been encountered in the brain tissue (Martinez and Visvesvara, 1997). With a broad environment dispersion, the three *N. fowleri* stages have been found from fresh water and soil to airborne dust particles containing cysts (Martinez and Visvesvara, 1997). With the exception of Antarctica, its presence has been identified on all continents (De Jonckheere, 2011). Furthermore, due to its thermophilic nature, *N. fowleri* has been isolated from hot springs and thermally polluted rivers (Schuster and Visvesvara, 2004; Yoder et al., 2012).

Regarding of PAM occurrences, 143 cases have been registered in the USA from 1962 to 2016 (Centers for Disease Control and Prevention (CDC), 2017), and about 440 reported worldwide (Abdul Majid et al., 2017; Coupat-Goutaland et al., 2016). The scarcity of cases seems to indicate a very rare type of infection. However, the number of reported cases appears to be increasing over the last years (Cope et al., 2016, 2015; Grace et al., 2015; Heggie, 2010; Linam et al., 2015; Stowe et al., 2017) and a precise diagnosis is an essential tool to obtain a veritable assessment of its distribution. Interestingly a small number of cases are reported in tropical areas as Africa and South America, probably resulting from a lack of interest in such an occasional disease, while millions of people are affected by other severe infections in those regions (De Jonckheere, 2011).

This leads to the underestimation in the number of cases around the globe, seriously aggravated by other factors such as the difficulty of an accurate diagnosis due to its short incubation period, leading to the patient death in 48 h after the appearance of the first symptoms (Chow and Glaser, 2014). Headache, fever, stiff neck, vomiting and mental confusion illustrate the onset of illness that frequently evolves to seizures, neurological impairment and cerebral haemorrhages, resulting in death (Heggie, 2010; Herwaldt, 2001; Siddiqui and Khan, 2014). When rarely diagnosed, the current therapy to treat PAM includes mainly Amphotericin B combined with several different drugs, as azoles, whose nonspecific treatment results in low survival efficacy (Heggie, 2010; Yoder et al., 2012). Not surprising, PAM high fatality rate has become a serious concern for public health agencies and officials, with an approximate mortality rate of 95%, and affecting mostly children in good health (De Jonckheere, 2011).

In this review we discuss the incidence of *Naegleria fowleri*, its environmental occurrence, the challenges in an accurate and fast diagnosis and how it has evolved. We proceed to present the most conflicting points of the current PAM therapy, related to drug efficacy and its side effects. In conclusion, new potential therapies are presented, including novel molecules with amoebicidal activity and possible vaccination strategies.

2. Environmental occurrence of *Naegleria fowleri*

Several studies have been conducted aiming at identifying *N. fowleri* trophozoites or cysts in the most varied environments, here classified under two categories of natural habitats and urban zones. Among natural habitats are grouped: rivers and freshwater lakes (Farra et al., 2017), ponds (Dobrowsky et al., 2016), hot springs and warm aquatic environments (Farra et al., 2017; Latifi et al., 2017). Under the urban zones category are grouped: recreational fountains (Morgan et al., 2016; Reyes-Batlle et al., 2017), pasteurized and unpasteurized water sources (Dobrowsky et al., 2016), domestic and hotel swimming pools (Farra et al., 2017), hospitals, pipe wall biofilms related to drinking water distribution system (DWDS) (Puzon et al., 2017), geothermal heated water (Streby et al., 2015), tap water used on nasal flooding (Cope et al., 2015; Streby et al., 2015), dental unit waterlines (DUWLs) (Leduc et al., 2012), contaminated drinking water (Morgan et al., 2016) and waterparks (Heggie and Küpper, 2017). Among these sites, those with higher water temperature, above 28 °C, are reported to harbour a greater number of *N. fowleri* (Heggie, 2010).

Furthermore, an occurrence that has been attracting attention is the association with bacteria and other eukaryotic organisms that live in symbioses with *Naegleria* spp. on biofilms of DWDS, known for their low chlorine levels (Miller et al., 2017). It has been shown that the higher the bacterial density the less viable is the coexistence with the amoebae (Morgan et al., 2016). The most common strategy to avoid amoebae associated with other bacteria in these distribution systems is the chlorination. The chlorine concentration usually applied, 0.5 mg/L, is not sufficient to eliminate *Naegleria* since they can differentiate into the resistant cyst form. A recent study outlines that a disinfection regimen consisting on a daily application of 1 mg/L of chlorine during 60 days is efficient against the amoebae (Miller et al., 2017).

This DWDS example reinforces the importance of amoeba genotyping to identify the appropriate elimination strategy, avoiding its re-emergence and decreasing PAM dissemination. Studies describing *N. fowleri* genetic diversity, linking different strains to their geographical occurrences, contribute to better comprehend the environmental reach of the strains (Al-Herrawy and Gad, 2015; Farra et al., 2017; Latifi et al., 2017; Tung et al., 2013). Among the main methods used to isolate and identify *Naegleria* in risk areas are morphological and molecular analyses (Bonilla-Lemus et al., 2014; Kang et al., 2015; Kao et al., 2014; Streby et al., 2015). The morphological approach is undertaken mainly as a complementary analysis by using wet sample mounts and visualizing them under an optical microscope, cultivating the organisms in non-nutrient agar (NNA) plates, conducting differentiation tests that induce enflagellation or encystment (Benterki et al., 2016; Latifi et al., 2017; Reyes-Batlle et al., 2017). Thermotolerance assays are usually carried out assessing their viability contributing to species identification (Abdul Majid et al., 2017).

The main identification approaches are those based in molecular techniques, using conventional and quantitative PCR (Polymerase Chain Reaction) associated with amplicon sequencing to confirm the morphological findings (Benterki et al., 2016; Dobrowsky et al., 2016; Liang et al., 2010; Régoudis and Pélandakis, 2016; Reyes-Batlle et al., 2017). Bioinformatics tools are essential for further data analysis. Table 1 summarizes the different primer sets used for *Naegleria* identification in case reports from the last 5 years, along with other relevant information. This molecular approach is of great importance since it allows not only for the recognition of known species, but also for the identification of new strains. Khwon and Park (2017) used rDNA ITS sequence of 45 *Naegleria* species and 18S rDNA sequence of 27 *Naegleria* species to describe three new species named *N. jejuensis*, *N. neojejuensis* and *N. koreanum*. Quantitative PCR analysis, in addition of been a detection tool, also allows for the quantification of amoebae cells using the number of gene copies and standard curves for calibration (Régoudis and Pélandakis, 2016). Furthermore, the melt curve analyses of the amplicons can be applied as primer specificity verification (Liang et al., 2010) as well as for strain genotyping (Benterki et al., 2016). Furthermore, Régoudis and Pélandakis (2016) used a single copy rDNA gene and applied both PCR techniques. The quantitative approach showed more reliable results when compared to the traditional PCR-based assay (Régoudis and Pélandakis, 2016).

In addition to PCR-based techniques, two new strategies to identify environmental occurrences have been proposed and showed promising results: microsatellites as neutral genetic markers (Coupat-Goutaland et al., 2016) and a metabolomic approach (Yu et al., 2017). The first is based on the fact that the *N. fowleri* specie has several different strains and can be isolated in the most diverse geographical sites. This issue was addressed by using microsatellites as strong population markers. Analyses of 47 *N. fowleri* strains using six microsatellites loci have described seven different genetic groups. Prior to these discoveries, only five were known (EA, WP, SP, CHO and CAT) adding NZ and RA. This new approach could help to better understand the genus population structure, contributing also with a better understanding of its evolutionary history (Coupat-Goutaland et al., 2016). The second strategy, metabolomics, aims to classify characteristic metabolites of each species that allow its rapid identification by using techniques such as ultra-performance liquid chromatography (UPLC) and mass spectrometry (MS). Among 550 metabolites studied, 4 have shown to be specie markers. Thereby, it can be used to examine water samples and on PAM diagnosis of CSF with the possibility to discriminate pathogenic from non-pathogenic *Naegleria* spp. (Yu et al., 2017).

3. Clinical diagnosis of PAM

Even considering PAM as a rare disease due to the scarcity of reported cases, the critical points are its high mortality rate (Coupat-Goutaland et al., 2016; Stubhaug et al., 2016) and the short incubation period (Chow and Glaser, 2014). Regarding its fast evolution, several PAM cases were only diagnosed *post-mortem*, through brain autopsies (Roy et al., 2014; Stubhaug et al., 2016). In this context, an accurate and fast diagnosis is likely to be the bottleneck for a better understanding of the global number of cases. Likewise, the PAM survival rate could be increased by allowing a fast and efficient medical intervention. This section presents the current methods used in PAM diagnosis.

The clinical diagnosis of PAM usually consists of three approaches that are time consuming and technically challenging. These are conducted together as often as possible and consist of: morphological analysis of cerebrospinal fluid (CSF) wet mount, molecular identification using the PCR, and differentiation tests with the organisms found on the CSF (e.g., enflagellation, encystment or thermotolerance tests) (Abdul Majid et al., 2017; Benterki et al., 2016; Streby et al., 2015; Stubhaug et al., 2016). In order to assess the clinical manifestations of the disease, the patients are primarily submitted to body temperature and blood pressure measurements, since any discrepancy from normal in these values can indicate an ongoing infection. The primary PAM symptoms are: headaches, fever, nausea, vomiting, exhaustion and lethargy (Heggie and Küpper, 2017; Linam et al., 2015; Stowe et al., 2017). This first phase of the infection is analogous to bacterial or viral meningitis. It has been shown that an in-depth CSF examination is required to distinguish a naegleriasis from a pneumococcal meningitis (Zahid et al., 2016). However, with the evolution of the

Table 1
Comprehensive primer set used for detecting Naegleria **upon environmental (E), clinical (C) and** in vitro **(V) contexts.** All primers are designed to search regions on ribosomal DNA of *Naegleria*, as the most recent papers have reported. Each target amplicon 18S_1 to 18S_3, ITS_1 to ITS_8 and 5.8S can be found in KT375442 strain, except ITS_4 found in M18732 strain both used as models to determine size and region of the amplicons.

Accession number/ PCR for	Target/Size (pb)	FW primer name/Sequence (5′ / 3′)	RV primer name/Sequence (5′ / 3′)	Context	Location	Reference
KT375442/*N. fowleri*	18S_1/153	NaeglF192/ GTGCTGAAACCTAGCTATTGTAACTCAGT	NaeglR344/ CACTAGAAAAAGCAAACCTGAAAGG	E	South Africa	(Dobrowsky et al., 2016)
				E	Georgia and U.S.	(Streby et al., 2015)
				C	Texas	(Barnett et al., 1996)
				C	California	(Johnson et al., 2016)
				C	Thailand	(Stubhaug et al., 2016)
				E	Louisiana (U.S.)	(Cope et al., 2015)
KT375442/*Naegleria*	18S_2/577	NF-ITS-F1/GACTTCATTCGTTCTTGTAGA	NF-ITSR1/CTCTTGCGAGGTCCAGAC	E	Louisiana (U.S.)	(Cope et al., 2015)
KT375442/*Naegleria*	18S_3/256	Nae3-For/CAAACACCGTTATGACAGGG	NII/AAATGATCCCTACGCAGGTT	E	Austria	(Scheikl et al., 2014)
KT375442/*N. fowleri*	18S_4/183	Nae3-For/CAAACACCGTTATGACAGGG	Nae3-R/CTGGTTTCCCTCACCTTACG	*V*	Republic of Korea	(Kang et al., 2015)
				E	Taiwan	(Kao et al., 2013)
				C	Taiwan	(Su et al., 2013)
				E	Taiwan	(Tung et al., 2013)
M18732/*Naegleria*	18S_4/183	Nae3-For/CAAACACCGTTATGACAGGG	51 R/CTGGTTTCCCTTACCTTGCG	E	Egypt	(Al-Herrawy and Gad, 2015)
KT375442/*N. fowleri*	ITSr_1/404	NGITSF/AACCTGCGTAGGGATCATTT	ITSRV/TTTCCTCCCCTTATTAATAT	E	Georgia and Florida	(Streby et al., 2015)
KT375442/*Naegleria*	ITSr_2/410	Veer.gs fw/GAACCTGCGTAGGGATCATTT	Veer.gs rv/TTTCTTTTCCTCCCCTTATTA	E	Northern Iran	(Latifi et al., 2017)
				V	Republic of Korea	(Kang et al., 2015)
				E	Taiwan	(Kao et al., 2014)
				E	Rural Western Australia	(Morgan et al., 2016)
				E	Rural Western Australia	(Miller et al., 2017)
KT375442/*Naegleria*	ITSr_3/405	Veer.gs fw/GAACCTGCGTAGGGATCATTT	ITSRV/TTTCCTCCCCTTATTAATAT	E	Central African Republic	(Farra et al., 2017)
KT375442/*N. fowleri*	ITSr_4/377	FWS/GTGAAAACCTTTTTTCCATTT	Veer.gs rv/TTTCTTTTCCTCCCCTTATTA	E	Western Australia	(Miller et al., 2017)
				E	Georgia and U.S.	(Streby et al., 2015)
				E	Rural Western Australia	(Morgan et al., 2016)
KT375442/*N. fowleri*	ITSr_5/311	Veer.ss fw/ TGAAAACCTTTTTTCCATTTACA	Veer.ss rv/ AAATAAAAGATTGACCATTTGAAA	E	Northern Iran	(Latifi et al., 2017)
				E	Taiwan	(Kao et al., 2014)
				E	Taiwan	(Tung et al., 2013)
				E	Egypt	(Al-Herrawy and Gad, 2015)
KT375442/*N. fowleri*	ITSr_6/311	FWS/GTGAAAACCTTTTTTCCATTT	Veer.ss rv/ AAATAAAAGATTGACCATTTGAAA	E	Rural Western Australia	(Morgan et al., 2016)
				E	Taiwan	(Kao et al., 2014)
KT375442/*N. fowleri*	ITSr_7/309	NFITSFW/TGAAAACCTTTTTTCCATTTACA	NFITSRV/ AATAAAAGATTGACCATTTGAAA	E	Central African Republic	(Farra et al., 2017)
				E	Southeast Asian	(Abdul Majid et al., 2017)
KT375442/*N. fowleri*	ITSr_8/125	JBVF/AGGTACTTACGTTAGAGTGCTAG	JBVR/ATGGGACAATCCGGTTTTCTCA	E	Georgia and U.S.	(Streby et al., 2015)
				E	Georgia	(Mull et al., 2013)
KT375442/*Naegleria*	5.8S/109	ITS-F/CAAAAAGCGATATGTAATGA	ITS-R2/ TTGATATAAAACTAGCACTCTAA	E	Taiwan	(Kao et al., 2014)

clinical condition of a PAM patient other complications may occur, such as bleeding and brain dysfunction (Benterki et al., 2016; Su et al., 2013).

With the identification of the primary encephalitis symptoms, a lumbar puncture is performed, collecting CSF to quantify glucose and protein levels, leukocyte count, fluid turbidity, among other parameters (Stowe et al., 2017; Su et al., 2013). Patients with positive results for PAM tend to present CSF with low glucose levels, high C-reactive protein concentration (around 260 mg/L) and leukocyte count (roughly 2100 cells/mm^3) (Stubhaug et al., 2016). The fresh CSF is also microscopically inspected for the presence of trophozoites. Once detected, further analyses must be performed investigating the quantity and granularity of the cytoplasmic vacuoles (Su et al., 2013), the amoebae length and width, the cysts diameter and number of pores (Khwon and Park, 2017). Moreover, by the CSF examination it is possible to verify the movement, size, pseudopod morphology and motility of the amoebas, contributing to species identification (Benterki et al., 2016).

Several staining methods have been suggested to aid in the amoebas identification (Abdul Majid et al., 2017; Heggie and Küpper, 2017). Table 2 summarizes the different histological techniques used for PAM diagnosis in case reports from the last 5 years. These morphological analyses are usually combined with immunochemical assays and radiological approaches, also indicated on Table 2. Imaging techniques such as contrast-enhanced computer tomography (CT) and magnetic resonance (MR) show a variety of CNS alterations as diffuse cerebral edema (Stowe et al., 2017), cortical sulci effacement and hydrocephalus herniation (Stubhaug et al., 2016). These conditions can evolve, worsening dramatically towards more advanced stages and possibly becoming necrotic areas, stenosis and aneurysms that lead to death (Stubhaug et al., 2016).

The aforementioned histopathological analyses are of extreme importance for a correct diagnosis. However, they allow the identification of amoebae only at the genus level (Abdul Majid et al., 2017). Therefore, molecular approaches, mainly PCR-based assays, have been developed. A growing improvement on the available molecular techniques over the past few decades such as sequencing, quantitative PCR, and the use of microsatellites gene markers, resulted in a more accurate identification of the target

Table 2

Clinical strategies used for PAM diagnosis (2013–2017). Regarding the staining information Ziehl-Neelsen is also known as acid-fast stain, H&E as hematoxylin and eosin stain, and PAS as Periodic acid–Schiff. The immunohistochemical (HI) methods groups IFF (indirect immunofluorescence staining), IHC (immune alkaline phosphatase staining), CD45 (pan-leukocyte marker) and CD68 (macrophage marker). The central nervous system analyses include cerebrospinal fluid (CSF) measurements, contrast enhanced computed tomography (CT), magnetic resonance (MR) and electroencephalogram (EEG).

Case Reports Gender (Year)	Staining Methods	IH	CSN Analysis	Culture assays	Molecular trials (PCR)	Reference
F (21)	–	–	CSF measurements	x	x	(Johnson et al., 2016)
M (11)	–	–		x	x	(Abrahams-Sandí et al., 2015)
M (42)	India ink preparation. Acid fast smear	–	CSF measurements, EEG	x	x	(Shariq et al., 2014)
M (6)	Ziehl Neelsen, India ink	–	CSF measurements	x		(Sood et al., 2014)
M (75)	Liu's stain, acidfast stain	–	CT, CSF measurements, MR	–	x	(Su et al., 2013)
M (4)	–	–	CT, EEG, MR, CSF measurements	–	x	(Cope et al., 2015; Stowe et al., 2017)
M(14)	–	IFF, IHC	CT, EEG	–	x	(Roy et al., 2014; Stowe et al., 2017)
F (71)	Mucicarmine stain, PAS, periodic acid staining	CD45, CD68	CSF measurements, CT	–	x	(Stubhaug et al., 2016)
F (12)	Giemsa, H&E, Wright	–	CSF measurements, CT	–	x	(Cope et al., 2016; Dunn et al., 2016; Heggie and Küpper, 2017; Linam et al., 2015)
M(8)	Wright	–	CSF measurements, CT	–	x	(Cope et al., 2016; Roy et al., 2014)
M (10)	H&E	IFF, IHC	CT	–	x	(Roy et al., 2014)
M (22)	–	–	–	–	x	
F (16)	–	–	–	–	x	
M (9)	Wright	–	–	–	x	

species. In most cases, total DNA is extracted from the cells found on the CSF and PCR is conducted using *Naegleria* specific primers (Fig. 1), frequently the 18S rDNA region. The internal transcribed sequence (ITS) regions (Benterki et al., 2016; Streby et al., 2015; Su et al., 2013) as well as the 5.8S and the 28S regions (Kao et al., 2014; Stubhaug et al., 2016) are targets also used for the identification, as illustrated in Fig. 1. The significance of using the rDNA as a PCR target is due to its inner variable regions allowing for individual genotype recognition (Pélandakis and Pernin, 2002; Tavares et al., 2006). Once the amplicons are obtained, sequencing is conducted, combined with bioinformatics tools to determine the species (Farra et al., 2017).

The current state of the art in the diagnosis of naegleriasis is the combination of the morphological and molecular approaches just discussed. For instance, it was reported in 2013 a diagnostic of combined techniques which has been considereda successful pipeline in PAM diagnosis (Linam et al., 2015). This case describes a real-time PCR assay that testing positive for *N. fowleri* was of critical importance for the successful treatment, saving a young patient's life (Dunn et al., 2016; Heggie and Küpper, 2017). The analysis of the CSF showed a higher than normal white blood cells count (3675 cells/μL), glucose levels near 20 mg/dL and protein concentration around 370 mg/dL. In addition, microscope inspection of Giemsa-Wright stained CSF samples identified the presence of *Naegleria* trophozoites. Moreover the radiological images revealed blood in the brain frontal lobes and restricted diffusion in the

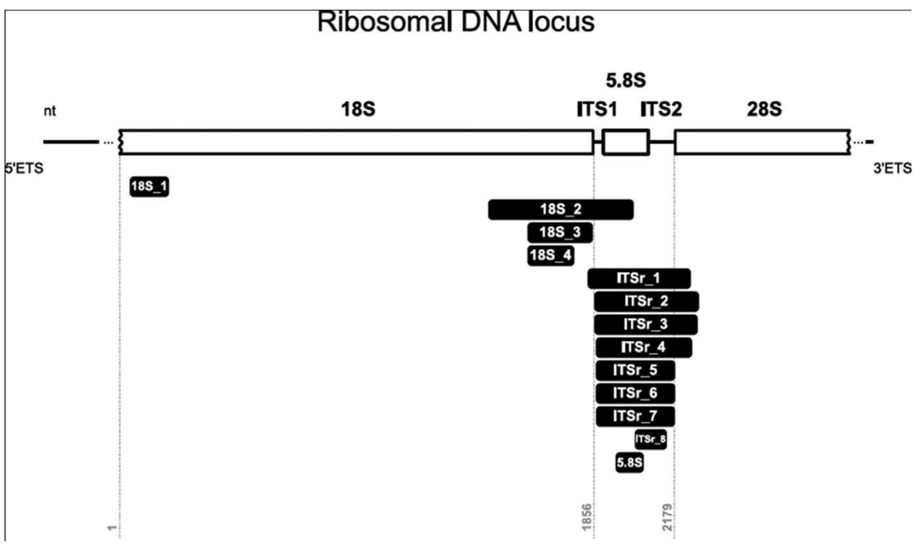

Fig. 1. Scheme of *Naegleria's* ribosomal DNA locus showing the set of primers used as a molecular approach to perform environmental and clinical investigations. The external transcribed spacers (5′ETS and 3′ETS) and internal transcribed spacers (ITS1 and ITS2) are also exhibited. The black boxes display the relative lengths of ampilicons and indicate the position chosen to perform the PCR. The forward and reverse set of primers used to amplify these amplicons are reported on Table 2.

cerebellum and multiple brain areas (Linam et al., 2015). The history of the patient, specially recent contacts with freshwater bodies, is relevant to further confirm a thorough diagnosis (Dunn et al., 2016). For instance, in the case reported by Linam et al. (2015), the patient was described as having swum in a water park right before the onset of the first symptoms (Linam et al., 2015). Other studies have also pointed out this correlation among PAM manifestations: recent use of water for recreational practices and the discovery of *N. fowleri* in the vicinities (Gyori, 2003; Morgan et al., 2016; Okuda et al., 2004; Yoder et al., 2010).

Simultaneously to the clinical aspects described, an improved environmental detection of *N. fowleri,* as described in the previous section, resulting in the characterization of its geographical dispersion, will render the implementation of prophylactic measures more effective.

4. The current therapy against PAM

Although some drugs commonly employed in PAM treatment show positive results (e.g., amphotericin B and miltefosine), it is actually unclear whether the disease can be successfully cured using a determined combination of them. Such combined therapy has been commonly applied in the last five years. Even when treated under similar therapeutic approaches, the fatality rate is still about 95% of the cases (Capewell et al., 2015). This section briefly describes the current treatment options for PAM (Grace et al., 2015; Pugh and Levy, 2016). Recently reported cases of PAM are summarized on Table 3, including their treatment strategy and its outcomes.

Amphotericin B (AmB) has been the most used drug for the treatment of PAM. All recent cases reporting treatment success have administered this compound either intravenously or intrathecally in combination with other drugs (Capewell et al., 2015). Best known for its antifungal activity, AmB is also indicated as an antiviral and antiprotozoal drug, with a minimal inhibitory concentration (MIC) of 0.075 μg/mL against *Naegleria* sp. (Carter, 1969). However, AmB administration is restricted by toxicity effects, generally accompanied by a series of severe side effects ranging from nausea and vomiting to acute kidneys damage (Sau et al., 2003). The conventional therapy consists on AmB deoxycholate administration, which increases the compound solubility.

Rifampin is an antibiotic commonly associated with AmB to potentiate the effects of the treatment. It has been proved to yield satisfactory results when used on bacterial and protozoan infections while at higher concentrations (Conti and Parenti, 1983; Vargas-Zepeda et al., 2005; Yoder et al., 2012). Therefore, it is widely present among the ensemble of drugs administered to PAM diagnosed patients (Capewell et al., 2015). Monotherapy with rifampin is normally discouraged due to the very rapid development of resistance during the treatment (Wehrli, 1983).

Another set of drugs employed on the treatment of PAM are the Azoles. They are prominently known as potent antifungals although their application is not restricted to it (Ghannoum and Rice, 1999). There is strong evidence of Azoles' activity against protozoa (De Macedo-Silva et al., 2013; Raether and Seidenath, 1984), and their use in this context is common (Kappagoda et al., 2011) with Miconazole, Fluconazole and Ketoconazole most frequently used in PAM treatment (Capewell et al., 2015).

Miltefosine is a breast cancer and anti-leishmanial drug that has shown good results when tested against FLA *in vitro* (Kim et al., 2008b; Schuster et al., 2006). In 2013 the drug was successfully administered to two patients diagnosed with PAM that have survived the infection (Cope et al., 2016; Linam et al., 2015). Even though it is an investigational drug, the CDC has expanded its access to clinicians for the treatment of FLA infections (Capewell et al., 2015; Centers for Disease Control and Prevention (CDC), 2013). The availability of Miltefosine and its apparent success has made the drug a potential option of the current therapeutic strategies against PAM. However, the latest case reports have not shown consistent results (Table 3).

Drug delivery is a key problem for the treatment of the central nervous system (CNS) amoebic infections. Reaching the site of infection at the effective concentrations is hampered by the blood-brain barrier. On the other hand, the transport to brain parenchyma through blood vessels presents a minor problem. Being able to successfully reach the brain parenchyma is a very important factor influencing the treatment efficacy. Therefore, the search for molecules that present these characteristics and with attractive amoebicidal effects can improve the therapeutic arsenal against *N. fowleri* (Schuster et al., 2006).

AmB, as previously stated, has been the most commonly used drug to treat PAM. However, alternatives such as lipid formulations (i.e., liposomal, lipid complex, colloidal suspension) (Botero et al., 2014) and nanoencapsulation (Diaz et al., 2015) have been proposed, showing promising results (Falci et al., 2015). A published study also proposes that a single dose of liposomal AmB could have the same efficacy as the conventional alternate day infusions of AmB deoxycholate for the treatment of visceral leishmaniasis, a

Table 3
Cases of PAM found in recent literature (2013–2017). IT – intrathecal; IV - intravenous.

Gender	Age	Outcome	Treatment	Location	Year of the Incident	Year of Publication	Reference
M	4	Death	Amphotericin B IV, fluconazole, rifampin, azithromycin, miltefosine	USA	2015	2017	(Stowe et al., 2017)
M	14	Death	Amphotericin B IV/IT, fluconazole, rifampin, azithromycin, miltefosine	USA	2015	2017	
M	12	Death	Amphotericin B IV, fluconazole, rifampin, azithromycin, miltefosine	USA	2013	2016	(Cope et al., 2016)
M	8	Survival	Amphotericin B IV, fluconazole, rifampin, azithromycin, miltefosine	USA	2013	2016	
F	21	Death	Dead before accurate diagnosis	USA	2015	2016	(Johnson et al., 2016)
F	12	Survival	Amphotericin B IV/IT, fluconazole, rifampin, azithromycin, miltefosine	USA	2013	2015	(Linam et al., 2015)
M	11	Death	Dead before accurate diagnosis	USA	2014	2015	(Abrahams-Sandí et al., 2015)
M	4	Death	Dead before accurate diagnosis	USA	2013	2015	(Cope et al., 2015)
M	42	Death	Amphotericin B IV/IT	Pakistan	Not reported	2014	(Shariq et al., 2014)
M	6	Survival	Amphotericin B, rifampin, fluconazole	India	Not reported	2014	(Sood et al., 2014)
M	10	Death	Rifampin, and vancomycin	USA	2009	2014	(Roy et al., 2014)
M	22	Death	Not reported	USA	2009	2014	
F	16	Death	Amphotericin B, azithromycin, fluconazole, rifampin	USA	2011	2014	
M	75	Death	Amphotericin B IV	Taiwan	Not reported	2013	(Su et al., 2013)

dose reduction which could drastically decrease the side effects of the treatment (Sundar et al., 2010). It is possible that a similar effect is observed for *N. fowleri.*

The Azoles are a set of drugs also commonly used as therapeutic agents against amoebic infections, but showing some drawbacks. For instance, the characteristic water insolubility of this group of molecules leads to drug processing in the liver and can induce drug interactions, or even toxicity, when taken in combination with other drugs. Voriconazole is a triazole generally used to treat invasive fungal infections. An *in vitro* study demonstrated its effects against different *N. fowleri* strains at concentrations as low as 1 µg/mL. Adding the drug to the culture medium at a concentration from 10 µg/mL up to 40 µg/mL, the amoebae still appeared viable after one week of incubation, but did not proliferate after transferring to a drug-free medium. Extensive lysis of amoebas was observed at concentrations higher than 1 µM. At those concentrations, Voriconazole showed no inhibitory effect on monolayers of monkey kidney cells, thus giving indications of reduced or no toxic effects on healthy mammalian cells. This characteristic, together with the molecule ability to penetrate the CSF and brain tissue, makes it a promising drug for further investigations towards developing new treatment for PAM (Schuster et al., 2006).

Chlorpromazine is primarily known as an antipsychotic compound while it is also used regularly in other contexts, such as an antiemetic agent. It can be further used for attenuating the replication of adenovirus as well as for the treatment of patients in shock. The drug showed promising amoebicidal properties in *N. fowleri*-infected mice compared to AmB treated animals. Additionally, marginal levels of liver and kidneys toxicity were found. The mechanism of action of Chlorpromazine may be connected to its lipophilic interactions with the plasma membrane of the amoebae or to the changes on proteins regulating the calcium metabolism. The fact that Chlorpromazine accumulates well in the CNS raises some interest regarding this drug as a potential useful agent on the treatment of PAM in humans (Kim et al., 2008a).

Antimicrobial peptides (AMPs) are recognized as an important component of the nonspecific host immune system against invading pathogens. The characteristics of AMPs include having small molecular size and cationic affinity, usually non-immunogenic and a short half-life. Their activities comprise membrane targeting, disrupting protein-protein interactions and the ability to penetrate tissues. Tiewcharoen et al. (2014) tested the effects of different AMPs against *N. fowleri* trophozoites and described that Tritrpticin (Trp) reduced the viability of the amoeba at concentrations as low as 100 µg/mL (Tiewcharoen et al., 2014). These findings are consistent with other reports in which Trp decreased the viability of *Trichomonas vaginalis* when tested at the same concentration (Infante et al., 2011). A combination of Chlorpromazine and AmB resulted in damage to *N. fowleri* trophozoites, causing bleb formation and disappearance of suckers and pseudopodia. Trp activity is comparable to this AmB-Chlorpromazine combination, with the advantage of not presenting human neuroblastoma SK-N-MC cells damage. These findings suggest Trp as an additional candidate drug against *N. fowleri* trophozoites (Tiewcharoen et al., 2014).

In a previous study (Kim et al., 2008b), seven different antibiotics were tested against *N. fowleri* (i.e., Roxithromycin, Hygromycin B, Zeocin, Clarithromycin, Erythromycin, Neomycin and Rokitamycin). Hygromycin B and Rokitamycin were the most effective *in vitro* drugs of this group, showing 100% of growth inhibition after 6 days. Roxithromycin also showed constant positive results along the same experimental time, maintaining a high rate of trophozoites growth inhibition. Other antibiotics such as Clarithromycin, Erythromycin, Neomycin and Zeocin did not have similar effects. During the *in vivo* tests, Rokitamycin exhibited a survival rate of 80% of the infected mice compared to 25% for Roxithromycin. Altogether, of the seven compounds tested, Rokitamycin has the greatest potential (Kim et al., 2008b).

Another study, Baig et al. (2014) tested compounds targeting vital biochemical pathways and receptors. Some of the compounds are currently used for treating disorders of the nervous system. Amlodipine reduced the viability of amoebas to about 19% compared to the control group. Other compounds revealed good results as well, with Haloperidiol and Apomorphine reducing the trophozoites viability to approximately 22% and 33%, respectively. Amiodarone and Loperamide exhibited less significant reductions in viability, both to values higher than 50%. Procyclidine, another tested compound, showed potential for amoebicidal activity while, similarly, Digoxin presented interesting lytic capabilities. However, future investigation is still necessary to validate their mechanism of action on *Naegleria*, both *in vivo* and *in vitro* (Baig et al., 2014).

Diamidines have shown interesting amoebicidal capabilities, including FLA, as well as antitrypanosomal, antileishmanial and antimalarial agents (Werbovetz, 2006). These molecules have the capability of crossing the blood-brain barrier, therefore being effective against CNS protozoan infections, representing an important class of compounds in the search for new therapeutic approaches. A recently published study (Rice et al., 2015) validated two new assays for high-throughput screening of molecules for new alternatives to treat PAM. Two compounds potentially suitable at the nanomolar range were identified among 150 amidino derivatives tested. From these, DB173 presented the best results. The authors concluded that the class of molecules derived from amidinos offer a very promising scenario in the development of new treatments for CSN infections (Rice et al., 2015).

The most commonly used methods for the discovery of new compounds with interesting amoebicidal effects are not generally cost and time effective when it comes to *Naegleria* infections. Aiming to find new alternatives to tackle this problem, a new high-throughput screening assay was proposed by Debnath and co-workers, directed towards accessing *Naegleria* viability (Debnath et al., 2012). Using *N. gruberi* as a model to comprehend *N. fowleri,* for safety reasons inhibitors to five kinases and an NK kappa B were identified as good hits during primary screens. Also, a recently identified compound belonging to the same antifungal class as AmB, namely Corifungin, was tested to determine its efficiency, yielding favourable results. Both *in vitro* and *in vivo* tests of Corifungin showed lower toxicity when compared to AmB, and a survival rate of 100% of *N. fowleri* infected mice compared to a 60% survival for mice treated with AmB (Debnath et al., 2012). A more recent study corroborated these results with *in vitro* testing of Corifungin against *Acanthamoeba castellanii*. Analysis of transmission electron microscopy showed several alterations on the cells when incubated with Corifungin, including swollen mitochondria, disordered nuclear chromatin and degeneration of cytoplasm architecture. It was also observed that the drug induced the cell encystment process while also lysing the cysts after long periods of incubation (Debnath et al., 2014).

Considering the results here described, several compounds with potential for treatment of PAM are been investigated, requiring validation and further confirmation. In summary, the current direction towards the reduction of PAM mortality rates comprises the development and availability of new treatments using these compounds, along with more rapid diagnostic techniques, and a careful environmental evaluation of incidence of *N. fowleri.*

5. Potential vaccination strategies against *N. fowleri*

Vaccination strategies are always an important part of the therapeutic arsenal against a pathogenic agent, and some studies here discussed have shown potential for the development of a vaccine against *N. fowleri*, which would represent a welcome achievement.

John et al. (1977) demonstrated that the immunization of mice with different preparations of *N. fowleri* protected the animal from lethal parasitemia doses. Mice immunized with living or formalinized *N. fowleri* or living *N. gruberi* presented a significant increase in their protection to successive challenges with *N. fowleri*. In general, intravenous inoculation conferred higher protection when compared to other administration routes; intact cells showed better results than cell fragments; and *N. gruberi* extracts seem a better immunogen than *N. fowleri* extracts (John et al., 1977). In a later study, Thong et al. (1983) noted that mice could be protected against *N. fowleri* after being immunized with the culture medium used for the amoebae growth, whose protection occurred in the nasal mucosa.

Other reports describe the use of the *Bacillus thuringiensis* Cry1Ac protoxin coadministered with amoebal lysate, increasing *N. fowleri* challenged mice survival compared to amoebal lysate alone. Mice humoral response was activated and IgG and IgA mucosal levels were increased, however, only IgG response persisted after a two months period (Saúl Rojas-Hernández et al., 2004). The interaction between trophozoites and IgA antibody in the nasal lumen was also observed. In comparison, in non immunized mice the trophozoites were able to invade the nasal mucosa (Jarillo-Luna et al., 2008).

A deeper investigation about the Cry1Ac as an adjuvant showed a 100% survival rate in STAT6+/+ mice co-administrating amoebic lysates and Cry1Ac with subsequent challenging with intranasal *N. fowleri* amoebae. On the other hand, STAT6−/− mice did not survive the same treatment, which suggest a Th2-biased immune response, related to the presence of the STAT6 protein. Other markers of Th2 response were observed, such as elevated levels of IgG1 and IL-4, while the STAT6−/− mice presented higher levels of IL-12, IFN-γ and Th1-associated IgG2a (Carrasco-Yepez et al., 2010).

In vitro experiments revealed that the inoculation of *N. fowleri* induces the activation of Neutrophil Extracellular Traps (NET), together with the release of other characteristic components, but the trophozoites were still able to evade the killing process. When the *N. fowleri* were opsonized with human IgG, the trophozoites were susceptible to neutrophil activity, suggesting a significant role of polymorphonuclear cells (PMNs), allowing NET formation in response to *N. fowleri* infection (Contis-Montes de Oca et al., 2016). Together, IgA and IgG up-regulation mentioned above with the PMN induction to liberate NET reduces trophozoite attachment to the olfactory mucosa (Rojas-Hernández et al., 2004).

By immunoscreening with sera obtained from both infected and immune mice, new antigenic molecules were identified (e.g., the nfa1 gene). Western blot experiments demonstrated that Nfa1 protein reacted strongly with infected and immune sera. Immunofluorescence experiments were able to identify the antigen to a pseudopodium-specific localization, suggesting a potential role in amoeba motility. Highly virulent amoebae presented faster movement in comparison to less virulent variants (Cho et al., 2003). Immunization with recombinant Nfa1, with or without adjuvant (i.e., Freund's complete), stimulated an immune response in mice exhibiting high levels of specific IgGs - including isotypes IgG2b, IgG2a and IgG3 - and IgA antibodies. The immunized mice were challenged with a lethal dose of *N. fowleri* trophozoites and a survival rate of 100% was observed after 9–10 days post infection (Lee et al., 2011).

In another study, inoculation of mice with recombinant Nfa1 protein and cholera toxin B subunit (CTB) or *Escherichia coli* heat-labile enterotoxin B subunit (LTB) activated the secretion of INF-γ (Th1 response), IL-4 (Th-2 response), IL-2 and IL-10 (T regulatory response) cytokines. Recombinant Nfa1 administration associated with CTB and LTB triggered Th1/Th2/Treg responses and the mice survival rates were 100% and 80%, respectively (Lee et al., 2015). Nfa1 was also used in a potential DNA vaccination strategy using a lentiviral vector (pCDH). Among the effects of this vaccine, increased IgG levels (IgG1 and IgG2a) and higher expression of IL-4 and IFN- γ were observed, suggesting a Th1/Th2 mixed response. The mice vaccinated with nfa1 DNA vaccine exhibited survival rates around 90% after a challenge with *N. fowleri* trophozoites with humoral and cellular immune responses activation (Kim et al., 2013).

Returning to some problems presented before as the diagnosis difficulties, i. e., the current drug arsenal with low efficacy and unreported cases mainly in tropical areas, this possible vaccination strategies calls attention. Assuming its large availability after validation, they could become an attractive method against increasing PAM deaths.

6. Conclusions

Potential risk factors for an increase in the number of PAM cases range from poor basic sanitation conditions, recreational activities on warm waters to nasal irrigations with contaminated water and other seemingly harmless factors. The current trend of global warming also pose a new factor in the rise of reported PAM cases as well as the development of drug resistance (Siddiqui and Khan, 2014). Solutions to these problems are as diverse as the factors leading to potential exposure to *N. fowleri* and include avoiding swimming in, or inhaling, warm contaminated water. Swimming should be forbidden whenever *N. fowleri* has been identified in the nearby environment, regardless its concentration (De Jonckheere, 2012) and the use of physical barriers to *N. fowleri* entry, such as nose clips, could be enforced. While taking baths, the water can be sterilized by boiling or filtering to be properly disinfected (Siddiqui and Khan, 2014). These recommendations, although seemingly simple to follow, are extremely difficult to be implemented at a large scale, especially in low-income areas, and sometimes are quite unpopular.

Prevention of infection with *N. fowleri* is dependent on two pillars: a rapid and accurate diagnosis for effective treatment, and the prevention of environmental contamination. Moreover, environmental surveys aid to chart the potential risk areas and pathogen sources. As described, *N. fowleri* occurrence has a worldwide distribution and it has been encountered from lakes, warm water and pools to hospitals and mineral water. The knowledge about population structure and its presence in public environmental allow the implementation of the elimination methods. An accurate and fast diagnosis method is of vital importance for treatment success. After identification of the initial symptoms, a combined investigation including histological analysis, neuroimaging showing necrotic process and immunoassays should be applied. Among these, histopathological studies are widely used and cost effective tools for the diagnosis of PAM. However, one of its drawbacks is the requirement of technical skill in the identification of the amoebae and is time consuming to yield conclusive results. The clinical diagnosis and environmental survey of *N. fowleri* have gained momentum with the popularization of molecular biology techniques, becoming affordable in research and clinical laboratories. A favourable horizon is envisioned as the development of this branch of science is still progressing as new techniques are developed and applied. The main molecular approach resulting in sensitive and precise identifications amplifies the rDNA conserved regions to genotype the species, with a preference for the 18S subunit. Therefore, investigating suspected cases of PAM by both histopathological and molecular approaches becomes a great strategy to diagnosis.

The fundamental PAM therapy in the last five years have employed amphotericin B combined with miltefosine. However, the survival outputs remain around 5% and the drug arsenal is commonly associated with a variety of side effects. It is undeniable that urgent improvement in the therapeutic arsenal against *N. fowleri* is necessary. Strategies include tests with drugs with known mechanisms of action that possess the ability to cross the blood–brain barrier. This initiative, also known as a "piggy-back" approach, decreases the cost and time required for development of new molecules and marketing approval of the drugs. In this article we presented several cases where these strategies were applied, such as for chlorpromazine, azoles and other classes of drugs, with promising results. Conversely, the search for new molecules is imperative on the long run and can contribute in the development of more effective drugs, with reduced side effects including drug resistance. High-throughput screening initiatives were able to detect new mono and diamidino derivatives with activity against *N. fowleri* and identified a promising drug, corifungin, as a candidate for PAM therapy.

Although not currently available, vaccination can represent an important strategy to cope with the potentially imminent surge in *N. fowleri* infections. Despite having many satisfactory different candidates to vaccination against *N. fowleri*, its practical application remains to be validated. Thereby more research around this topic is needed and if immunization is to be made available, it would surely represent an important contribution to areas where PAM might become a serious public health issue.

Conflict of interest

There is no conflict of interest regarding this paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.exppara.2018.02.010.

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