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Free-living amoebae and other neglected protistan pathogens: Health emergency signals?

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Abstract

Protistan parasites have an undisputed global health impact. However, outside of a few key exceptions, e.g. the agent of malaria, most of these infectious agents are neglected as important health threats. The Symposium entitled "Free-living amoebae and neglected pathogenic protozoa: health emergency signals?" held at the European Congress of Protistology in Rome, July 2019, brought together researchers addressing scientific and clinical questions about some of these fascinating organisms. Topics presented included the molecular basis of pathogenicity in *Acanthamoeba*; genomics of *Naegleria fowleri*; and epidemiology of poorly diagnosed enteric protistan species, including *Giardia*, *Cryptosporidium*, *Blastocystis*, *Dientamoeba*. The Symposium aim was to excite the audience about the opportunities and challenges of research in these underexplored organisms and to underline the public health implications of currently under-appreciated protistan infections. The major take home message is that any knowledge that we gain about these organisms will allow us to better address them, in terms of monitoring and treatment, as sources of future health emergencies.

Keywords: Pathogenic protozoa; Neglected protozoa; Free-living amoebae; Enteric protozoa

Introduction

Protistan caused diseases are well-recognized sources for medical and public health concern. There have been substantial efforts in understanding and battling diseases such as Malaria, African Sleeping Sickness, and Chagas Disease and such efforts are paying dividends (Barry et al., 2016; Leggat et al., 2018). Nonetheless, beyond these high-profile examples, there are additional protistan pathogens and parasites about which we know a great deal less, but are very much worthy of consideration (Bertiaux and Bastin, 2020; Mungroo et al. 2019). The Symposium, "Free-living amoebae and neglected pathogenic protozoa: health emergency signals?" which took place at the ECOP meeting in Rome 2019 was intended to shine a light on some of these under-appreciated free-living amoebae and enteric protozoa which, in general terms, vary in their phylogenetic placement, the extent of basic scientific understanding regarding their pathogenicity, and the degree of their perceived threat.

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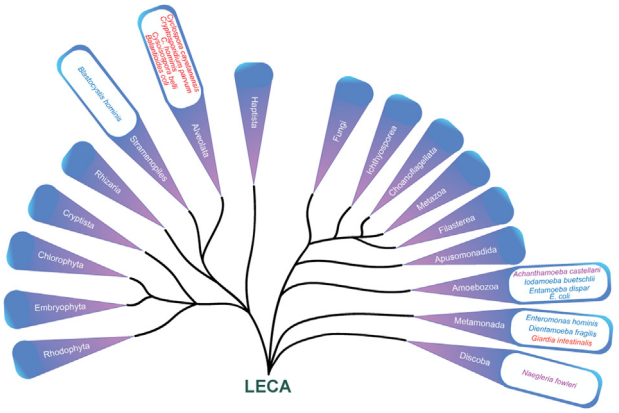

Fig. 1. Pathogen protists classification. Supergroup classification of the pathogen protists referred in the text, based on their parasitic nature in a scale from clear parasite (red), opportunistic pathogen (purple) and questionable parasitic relevance (blue). This tree redrawn after (More et al., 2020) with acknowledgement, and is based on the most recent Adl et al. classification (Adl et al., 2019).

The organisms mentioned in this review hail from across the breadth of eukaryotic diversity (Fig. 1). *Acanthamoeba castellanii*, *Entamoeba dispar, Entamoeba coli* and *Iodamoeba buetschlii* reside in the Amoebozoa (Supergroup Amorphea), *Naegleria fowleri* is a member of the Heterolobosea, within the supergroup Discoba, and *Dientamoeba fragils* is a member of the Metamonada, as are *Giardia intestinalis*, and *Enteromonas*. Finally, *Blastocystis hominis* belongs to the stramenopiles, while *Cyclospora cayetanensis*, *Cystoisospora belli, Cryptosporidium parvum, C. hominis, Balantioides coli* belong to the Alveolata all in the (T)SAR supergroup (Burki et al., 2019). Therefore, although these organisms are "neglected protistan parasites", there are so distantly related (Adl et al., 2019), that they are best considered separately with respect to their pathogenic mechanisms and perhaps most profitably considered in the light of their free-living or non-pathogenic relatives (Adl et al., 2019).

The same organisms also range in the degree to which they are understood at a cellular and clinical level. There is a deep and detailed cellular framework for *Acanthamoeba*, though open areas of investigation still remain particularly relating to inter-strain variability in pathogenicity, as highlighted below. At the same time, the basis of pathogenicity for *N. fowleri* remains unclear, particularly why this pathogen kills and all other *Naegleria* species are nearly harmless, and molecular cell biological knowledge for *Naegleria* is still in its infancy. Finally, there are some protistan organisms (e.g. *Blastocystis*) whose pathogenic status is still controversial, as evidence is equivocal as to whether they are normal members of the gut flora or disease-causing agents (Nieves-Ramírez et al., 2018; Stensvold and van der Giezen, 2018). Such self-resolving infections in immunocompetent patients often go unnoticed or misdiagnosed and their chronic course may induce lengthy suffering and possible complications in the patients (Partida-Rodríguez et al. 2017).

The final dimension to this comparison is our understanding of prevalence and how this prevalence may change as our environment does as well. All organisms discussed in this symposium review are water-related, with *Acanthamaeba* keratitis being not uncommon. Meningoencephalitis due to *N. fowleri* is relatively rare, but perhaps under-detected and thus under-estimated. As for the remainder of the organisms, general prevalence in industrialized countries is an open question and one with important implications for public health. Climate change effects, with the increase of water flow, due to heavy rainfall and consequent floods, determine the waste water reflux and increase water-related microbe (particularly of enteric origin) distribution and prevalence in the population (Angelici and Karanis, 2019; Boxall et al., 2009). Moreover, many zoonotic parasites are vulnerable to climate change and thus may influence their potential transmissibility (Polley and Thompson, 2009).

As part of this special issue of the European Journal of Protistology, this article encapsulates three separate mini-reviews on the topics presented by some of the presenters in the Symposium. It is a collaborative summary of the major ideas that emerged from this symposium and includes topics on these disparately related organisms, addressing questions of how they infect, how prevalent they really are and in some cases whether or not they even cause disease.

Acanthamoeba - A free-living protist and a facultative pathogen

Acanthamoeba is perhaps the best studied of the neglected protistan pathogens discussed in the Symposium. Here we focus on the current understanding of its pathogenicity.

Acanthamoebae are ubiquitous free-living amoebae that occur abundantly in water and soil worldwide, but also in man-made habitats such as swimming pools and air-conditioning systems. Their optimal growth temperature is at around 30 °C, but many strains grow well also at 37 °C, and as cysts, acanthamoebae can even survive under extreme conditions of pH, salinity, and temperature (Griffin, 1972; Khan, 2006). Generally, acanthamoebae do not need a host, but they can cause disease in a wide range of animals including humans upon accidental contact. In humans, they are the causative agents of a painful inflammation of the cornea, the so-called *Acanthamoeba* keratitis (AK), and of disseminating infections in immunocompromised individuals, potentially resulting in granulomatous amoebic encephalitis (GAE). The first cases of AK were reported in the early 1970ies and mainly affected persons with the history of a minor eye trauma (Jones et al., 1975; Nagington et al., 1974). Today, acanthamoebae are regarded as among the most important causative agents of keratitis in contact lens wearers. Due to the lack of awareness and delayed diagnosis and treatment, AK often shows a severe progression. Currently, there is no compound available exhibiting specific activity against

Acanthamoeba. While AK can mostly be treated successfully if started early, with a tight and lengthy regimen of topically applied disinfectants, there is no standard regimen for GAE and most cases have ended fatally (Lorenzo-Morales et al. 2015). In several recent cases, good outcomes have been achieved with repurposing of drugs, including miltefosine, various azoles and rifampicin. Novel approaches take advantage of high-throughput drug screening or nanotechnology (Elsheikha et al., 2020; Rice et al., 2020). The annual incidence of AK lies between 0.1-1 cases per 100,000 inhabitants, with a marked regional variation depending on contact lens wear habits and mode of water supply (Lorenzo-Morales et al., 2015, Walochni et al., 2015). For GAE, less than 500 cases have been estimated to have occurred worldwide since its discovery (Kalra et al., 2020; Khan, 2006; Visvesvara, 2013). As clinical *Acanthamoeba* isolates grow particularly well at temperatures between 34 °C (surface temperature of the human eye) and 37 °C (human body temperature) (Walochnik et al. 2000), it may be argued that climate change will have an impact on the epidemiology of *Acanthamoeba* infections. Certainly, temperature is a key driver for *Acanthamoeba* growth in various habitats.

Acanthamoeba spp. are facultative pathogens and the vast majority of humans never develop disease in spite of regular contact to *Acanthamoeba*. In the case of GAE, immunological deficiencies on the host side play a significant role: in the case of AK, microlesions in the cornea are important risk factors for the disease. However, *Acanthamoeba* strains also vary in their ability to cause disease and their virulence, respectively. The genus has been divided into currently 22 genotypes based on differences in their 18S rDNA, but a classification into virulent and non-virulent genotypes has not been possible (Fuerst et al., 2015; Gast et al., 1996; Stothard et al., 1998). Genotype T4 seems to be the most abundant one in most habitats and also the most common genotype in human infections (Booton et al., 2005; Fuerst and Booton, 2020), but by far not all T4 strains are pathogenic. So a key question is: what makes an *Acanthamoeba* strain pathogenic – and is this even a constant trait (Pumidonming et al., 2010)?

The infective and invasive form of *Acanthamoeba* is the trophozoite (Fig. 2A), which has a granuloplasma containing the organelles and a hyaloplasma producing sub-pseudopodia, the so-called acanthopodia. Trophozoites multiply rapidly under favourable conditions, most strains preferring temperatures of around 30 °C. However, many isolates can also grow at elevated temperatures, up to 45 °C. *Acanthamoeba* pathogenicity correlates to high growth rates and temperature tolerance (Griffin, 1972; Walochnik et al., 2000), but these characters have shown to be epigenetically regulated rather than genetically defined (Köhsler et al., 2008; Pumidonming et al., 2010). When there is a lack of nutrients or environmental conditions become unfavourable, the trophozoite starts to encyst. The cysts (Fig. 2B), although metabolically inactive, play an important role for the distribution of the amoebae on the one hand, and for their resistance against disinfection and treatment on the other hand. *Acanthamoeba* can also form cysts within host tissue and these cysts often lead to reinfections. The ability of *Acanthamoeba* to lyse other cells, including human cells, depends on cell-cell contact and is characterised by a contact-mediated cytolysis. Most studies on *Acanthamoeba* pathogenesis have focused on AK, but the general steps can be assumed to be comparable also in GAE. *Acanthamoeba* trophozoites, with the help of their acanthopodia, can very firmly attach to surfaces and their amoeboid locomotion enables them to pass through spaces as narrow as 2 μm (Bamforth, 1985). Both characters are important for the penetration of host tissue. Cell-cell contact is established primarily via a lectin-like adherence molecule, the mannose-binding protein (MBP), recognizing mannosylated glycoproteins in the membrane of other cells and allowing the amoebae to adhere to them (Garate et al., 2004, 2005; Yang et al., 1997). MBP is a 400 kDa cell surface receptor composed of multiple 130 kDa subunits. It consists of a large N-terminal extracellular domain, a transmembrane domain and a short C-terminal cytoplasmic domain (Garate et al., 2004). The cytoplasmic domain contains a number of phosphorylation sites and an NPLF motif, known for its ability to participate in cell-signalling events leading to cell spreading and shape change. Size, number and location of the carbohydrate recognition domains (CRDs) of the MBP of *Acanthamoeba* remain to be determined. However, apparently MBP contains at least one novel CRD (Panjwani, 2010). *Acanthamoeba* adhere to the surface of the cornea via the CRDs, which initiates signal transduction events via the cytoplasmic domain, eventually inducing cell lysis (see below). Besides MBP, several other molecules might be involved in cell-cell contact. For example, also the basement membrane components laminin and collagen IV and the adhesive glycoprotein fibronectin have been reported to function as binding sites (Gordon et al., 1993). Furthermore, 16 surface proteins, eight mannose glycoproteins and eight glycoproteins with N-acetyl glucosamine residues, have been detected in *Acanthamoeba* (Soto-Arredondo et al., 2014). Since human cornea epithelial cells express proteins with GlcNAc and Man residues on their surface (Panjwani et al., 1995), these proteins might represent potential receptor adhesins. Interestingly, *Acanthamoeba* seems to possess all ER glycosyltransferases involved in N-glycosylation as well as unusual fucosyl- and pentosyltransferases in the Golgi (Schiller et al., 2012). A 28.2 kDa laminin-binding protein (LBP) has been demonstrated to have definite binding specificity to mammalian laminin and higher expression levels in pathogenic strains (Hong et al., 2004), as has a 54 kDa LBP protein (Rocha-Azevedo et al., 2010).

The ability of acanthamoebae to lyse cells is mainly based on hydrolases and phospholipases, whereby the release of a 133-kDa serine protease termed mannose-induced protein (MIP)-133 seems to be a crucial step in the pathogenic cascade. Interestingly, MIP-133 is expressed in clinical but not in soil isolates (Hurt et al., 2003). MIP-133 activates matrix metalloproteinases (MMP), which have been shown to be expressed by corneal cells in response to pathogenic

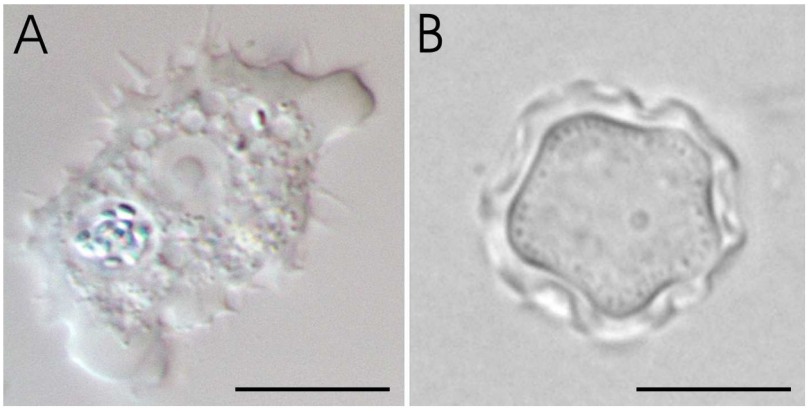

Fig. 2. *Acanthamoeba* cells. Trophozoite (A) and cyst (B) of *Acanthamoeba castellanii*. Scale bar: 10 μm.

microorganisms (Fini et al., 1992). In human corneal cells, the interaction with MIP-133 leads to a significant increase of the expression of MMP-2 and MMP-3, potentially facilitating the invasion of trophozoites (Alizadeh et al., 2008). Also, MIP-133 can degrade human collagen types I and IV and is able to induce apoptosis (Hurt et al., 2003). Various other proteases seem to be important virulence factors, including especially several serine and metalloproteases (Alsam et al., 2005; Cirelli et al., 2020; Hadas & Mazur, 1993; Hasni et al., 2020). Upon contact to host cells, a 97 kDa serine protease is markedly upregulated as well as a cytotoxic 80 kDa metalloproteinase (Cao et al., 1998). Interestingly, protease activity profiles differ significantly between strains (Cirelli et al., 2020), and the secretome generally has been shown to depend on nutrient conditions (Goncalves et al., 2018). For some strains, protease activity has been shown to decline during long-term axenic culture but can be re-stored by cell-cell contact to human or animal cell lines (Köhsler et al., 2009). Besides protein-induced apoptosis, acanthamoebae may also induce apoptosis of host cells by the release of adenosine diphosphate (ADP) (Mattana et al., 2001). Moreover, also ecto-ATPases may play a role in *Acanthamoeba* pathogenesis and an association of ecto-ATPases and MBP has been suggested, since mannose increases ecto-ATPase activities in pathogenic strains (Sissons et al., 2004). Another important enzyme involved in the pathogenesis of AK is a 40 kDa *Acanthamoeba* plasminogen activator (aPA), a serine protease only expressed from pathogenic strains and facilitating the penetration of the trophozoites through the basement membrane by activating plasminogen (Alizadeh et al., 2007). Finally, acanthamoebae also express a pore-forming protein, the so-called acanthaporin, which has been shown to be cytotoxic for human neuronal cells and a variety of bacterial strains by permeabilizing their membranes (Michalek et al., 2013). Recently, it has been shown that *Acanthamoeba* can cause autophagy and necrosis in Schwann Cells (Castelan-Ramirez et al., 2020). Acanthamoebae generally show a neurotropism, which makes AK a particularly painful disease. A better understanding of the molecular armament of *Acanthamoeba* will not only help to better understand the diseases caused by *Acanthamoeba* but also facilitate the development of specific drugs (Elsheikha et al., 2020).

In conclusion, several factors contribute to *Acanthamoeba* pathogenicity, which is based on contact-mediated cytolysis. The contact is established via lectin-like amoebic adherence molecules and the ability to lyse cells is mainly based on hydrolases and phospholipases, whereby the release of a 133-kDa serine protease termed mannose-induced protein (MIP) 133 seems to play a major role. Importantly, some pathogenicity-related characters such as the expression of specific proteases indeed seem to differ between environmental and patient isolates, hinting at possible explanations for the differing pathogenicity between strains. However, protease activity gradually declines over long-term laboratory culture and thus seems to be epigenetically regulated.

Naegleria fowleri - Understanding an emerging pathogen in the molecular era

By contrast with the degree of detail known regarding *Acanthamoeba* pathogenesis at the molecular level, *Naegleria fowleri* is a disease-causing protist still begging exploration. It is the causative agent of a seemingly rare but highly fatal parasitic central nervous system infection of humans, Primary Amoebic Meningoencephalitis (PAM) (Carter, 1970). A thermophilic free living amoeba which is globally distributed in water and soil, *N. fowleri* has been isolated from natural water bodies, as well as pools, spas, domestic water supplies, and sewage (although not from seawater) (Schuster and Visvesvara, 2004; Trabelsi et al., 2012). The most common risk factor for infection is recreational water activities which result in exposure of the face and nasal cavity to water or mud, with inhalation or nasal exposure of infested water, as well as therapeutic sinus lavage or ritual ablution with infested water (Siddiqui and Khan, 2014). Accordingly, the majority of cases in temperate zones are reported in the summer months and cases tended to be in younger people and children. Given the ubiquity of this protozoan, exposure is much more common than disease,

supported by reported high prevalence of positive serology (Schuster and Visvesvara, 2004; Trabelsi et al., 2012), and the mechanisms of pathogenesis of severe disease as occurs in some individuals is unclear.

Given that PAM is a rapidly developing, fatal meningoencephalitis with a very high mortality rate, underdiagnoses should be rare. However, the clinical presentation could be confused with rapidly fatal bacterial meningitis, so the possibility of missed cases exists. Patients present with fever, headache, photophobia, altered mental status, cognitive changes, and seizures. Because the organism migrates through the olfactory mucosa and the cribriform plate to access the CNS (Grace et al., 2015), local inflammation may result in smell and taste abnormalities, as well as a sensation of nasal blockage earlier in the course of disease. Disease progresses with intracranial hypertension, cerebral herniation, and death. Diagnostic CSF studies show classic meningitis findings with very low glucose, high protein, and very elevated white blood cells: therefore, it is suggested that severe meningoencephalitis cases should have diagnostic studies for PAM if routine microbiologic studies are negative (Matanock et al., 2018). Motile trophozoites can be seen on CSF wet mounts, preferably with a phase-contrast microscope, and Giemsa or trichrome stains may help in identifying trophozoite morphology (Pana et al., 2020). Neuroimaging findings are nonspecific, with the exception that lesions may tend to localize to the orbitofrontal and temporal lobes, base of the brain, cerebellum, and upper cord (Singh et al., 2006).

Based on published cases of survivors as well as extrapolation of treatments used for *Acanthamoeba* and *Balamuthia*, a variety of medications might be used including amphotericin B deoxycholate, rifampin, fluconazole (or posaconazole), miltefosine, and azithromycin (Cope et al., 2016; Linam et al., 2015). There have been very few documented survivors of robustly diagnosed PAM, generally with a history of early diagnosis and treatment with an amphotericin B based regimen (Gautam et al., 2012; Vargas-Zepeda et al., 2005; Yadav et al., 2013).

There are two main geographies of reported *N. fowleri* cases. In the Southern US, reported case numbers have remained fairly stable between 1960 and 2018 (Yoder et al., 2010; (Centers for Disease Control and Prevention, 2020). However, observed cases in the Indian Subcontinent particularly in Pakistan seem to be increasing markedly over the past 2 decades (Maciver et al., 2020) with clusters of cases thought to be related to ablution rituals, as well as lack of water disinfection with chlorine. It has been suggested *N. fowleri*'s tolerance for higher temperatures compared to related organisms may be promoting its expansion into an ecological niche increasingly defined by global warming. This possibility is consistent with the observed seasonality of PAM with higher incidence during hot seasons, as well as by its preferred geographic distribution in tropical rather than temperate zones (Maciver et al., 2020). This predilection for tropical geography, often in developing countries, also raises the possibility of under detection of cases. As noted, a fulminant PAM presentation may be clinically indistinguishable from severe bacterial meningitis, which is also common in younger people and particularly in many tropical and subtropical zones. Antemortem diagnostic studies and autopsy examinations are often not carried out in these locales and therefore, the perceived rareness of this devastating infection may be illusory. A concerted effort to raise awareness of this potential pathogen, and investigate the feasibility of surveillance is warranted.

With *N. fowleri* as a potentially increasingly important medical consideration in the future, understanding the basis of its pathogenic mechanism is more important than ever. Remarkably, while there are some other *Naegleria* species that can infect animals (e.g. *N. australiensis*), *N. fowleri* is the only species that readily infects humans and cause disease. This begs the question: "what factors make this species so deadly and its relatives benign?".

Various putative pathogenicity factors have been identified for *N. fowleri*. Based on traditional molecular parasitological studies, and consistent with the suggested pathogenic mechanism of tissue degradation and phagocytosis, proteases figure prominently on this list. As early as 1992 (Hu et al., 1992), cathepsins were identified as pathogenicity factors and have been fairly consistently implicated. Other proteases have also been suggested as involved, most prominently, the pore-forming protein, naegleriapore (Herbst et al., 2002; Leippe and Herbst 2004). The genomic era has provided an opportunity for a more broad-scale approach. In 2010 the genome of the non-pathogenic *N. gruberi* revealed a sophisticated encoded cellular complement and a surprisingly complete set of anaerobic metabolic enzymes (Fritz-Laylin et al., 2010). In 2013, a small-scale genomic analysis of mitochondrial genomes and a 60Kb contig of the nuclear genome hinted at contrasting levels of divergence between organellar and nuclear genomes (Herman et al., 2013). In 2014, the first genome of *N. fowleri* was sequenced and used as a guide for a comparative proteomic analysis of cultures grown in low versus high pathogenicity culture (Zysset-Burri et al., 2014). These highlighted proteins in gene ontology (GO) categories of cell membranes, vesicles, and cell projections. In 2018, the genome of the thermotolerant, but again, non-pathogenic, *N. lovaniensis*, as sequenced which revealed more similarity with that of the single sequenced strain of *N. fowleri* at the time than that of *N. gruberi* (Liechti et al., 2018). In 2019, a second strain of *N. fowleri* was reported, and re-emphasized the presence of a large number of proteins putatively associated with degradation, based on a signal P analysis of predicted proteins (Liechti et al., 2019).

Most recently, and as reported in this symposium, we have undertaken a large-scale comparative genomics analysis of three *N. fowleri* strains, as well as a transcriptomics analysis of high pathogenicity (mouse-passaged) vs low pathogenicity *N. fowleri*. Together these approaches highlighted some pathogenicity factors consistent with past results (e.g. proteases, naegleriapore), but also revealed metabolic and cellular systems not previously implicated.

With hundreds of factors unique to *N. fowleri* as compared with *N. gruberi*, dozens of differentially expressed genes, and extensively curated sets of genes associated with various cellular system, the analyses produced a rich set of putative pathogenicity factors for further investigation and the first comprehensive systems level model of *N. fowleri* pathogenesis (Herman et al., 2020, BioRxv doi: https://doi.org/10.1101/2020.01.16.908186).

Overall, genomic analysis is leading to a clearer understanding of the system-wide basis for *N. fowleri* pathogenesis, and converging on potential candidates for improved therapeutics. The development of genetic tools in *Naegleria* will be invaluable, allowing for testing of these candidates. While *N. fowleri* may be an increasingly relevant global pathogen, the prospect of understanding and combatting this pathogen is also very much on the rise.

Intestinal protozoa - unrecognized diseases requiring medical attention

Pathogenicity of *Acanthamoeba* and *Naegleria fowleri* may underestimated. Nevertheless, the molecular mechanisms that give rise to their pathogenicity are being studied, as are their infection prevalence, and both are still unquestionably disease-causing agents. On the contrary, there is a slate of intestinal protists the pathogenic nature and even prevalence of which is less clear.

Although highly prevalent in developing countries (https://www.who.int World Health Organization, 2017), intestinal parasitoses frequently afflict subjects in developed ones as well, as a consequence of both faecal contamination of aqueous environment (Angelici et al., 2018; Efstratiou et al., 2017; Plutzer and Karanis, 2016; Stensvold et al., 2020) and the globalization process or the immigration/adoption from endemic regions events. *Giardia intestinalis* and *Cryptosporidium parvum*/*C. hominis* are distributed worldwide and causative agents of acute gastroenteritis. These species cause waterborne infections frequent in many developed countries, including United States, Canada, New Zealand, Australia, England, Austria, Germany and others that have implemented governmental surveillance programs (Benedict et al., 2017; Fournet et al., 2013; McKerr et al., 2018; Fletcher et al., 2012). Thanks to this surveillance process these countries show a high prevalence for *Giardia* and *Cryptosporidium* infections with a certain number of outbreaks per year (Baldursson and Karanis, 2011; Centers for Disease Control and Prevention, 2017) https://www.cdc.gov/mmwr/index2017.html whereas in countries without such processes prevalence looks much lower as an effect of underestimation.

In developed countries, intestinal parasitoses caused by protozoa are often neglected because of many factors including the low specificity of symptoms and the poor training of physicians about these diseases. Moreover, only a few protozoa-caused diseases are submitted to European control strategies, resulting in others being underestimated, underrecognized and underreported (Calderaro et al., 2014).

There is so much more to discover about the role of the enteric protozoa in pathogenesis. Some intestinal protists are unquestionably pathogenic, such as those belonging to genera *Giardia*, *Entamoeba* and *Cryptosporidium*. Likewise, *Cyclospora cayetanensis*, *Cystoisospora belli*, and *Balantinoides coli*, are definitively known as pathogenic parasites and much information is available on their biology although their interaction mechanisms with hosts are not yet completely known (Barbosa et al., 2018; Legua and Seas, 2013; Giangaspero and Gasser, 2019).

Others are more enigmatic. *Blastocystis hominis* is the exemplary case, and one of the most contested. *Blastocystis hominis* is the most frequent protist in the human gut and often observed in high percentages of asymptomatic populations. It has therefore been proposed by some as non-pathogenic for the host (Stensvold and Clark, 2016). By contrast many authors disagree, reporting case studies of symptomatic patients (Kumarasamy et al., 2018) and experimental evidences of its pathogenicity (Yason et al., 2019; Wawrzyniak et al., 2013). Study approaches based on molecular biology revealed the existence of high genetic and genomic heterogeneity in this species and the presence of different pathogenic potential of the different genetic subtypes (Cakir et al., 2019; Gentekaki et al. 2017; Skotarczak, 2018; Yowang et al., 2018)

Similarly, despite multiple reports of clinical signs and symptoms in patients infected by *Dientamoeba fragilis,* the clinical significance of this protozoa as a human enteric pathogen, is still controversial (van Gestel et al., 2019). This is also despite the observations that symptoms resolved only after patients' treatment by antiprotozoan therapy and eradication of the infection, as also observed in our experience, on the symptomatic cases arrived to our attention, in a tertiary care University Hospital (Parma, Italy), (Calderaro et al., 2010b).

On the other hand, little is known regarding protozoa such as *Entamoeba dispar*, *Entamoeba coli*, *Enteromonas hominis* and *Iodamoeba buetschlii*, considered species that can live in commensalism with the human intestinal microbioma (Garcia, 2016). In the patients accepted at the University Hospital, in Parma, with intestinal parasitoses clinical suspicion, *Entamoeba dispar* and *Entamoeba coli* were frequently found, mainly in mixed infections with other pathogens and rarely as the only microbes diagnosed. On the other hand, *Enteromonas hominis* and *Iodamoeba buetschlii* have been rarely detected: in these latter cases a causative agent of the symptoms was not detected (A. Calderaro, unpublished data).

A contribution to the knowledge about these neglected protozoa can be achieved obtaining information about their circulation, in single infection or together with other protozoa such as *Giardia intestinalis*, and *Cryptosporidium parvum*/*C. hominis*. An accurate and reliable diagnosis of intestinal parasitoses is the starting point to understand the epidemi-

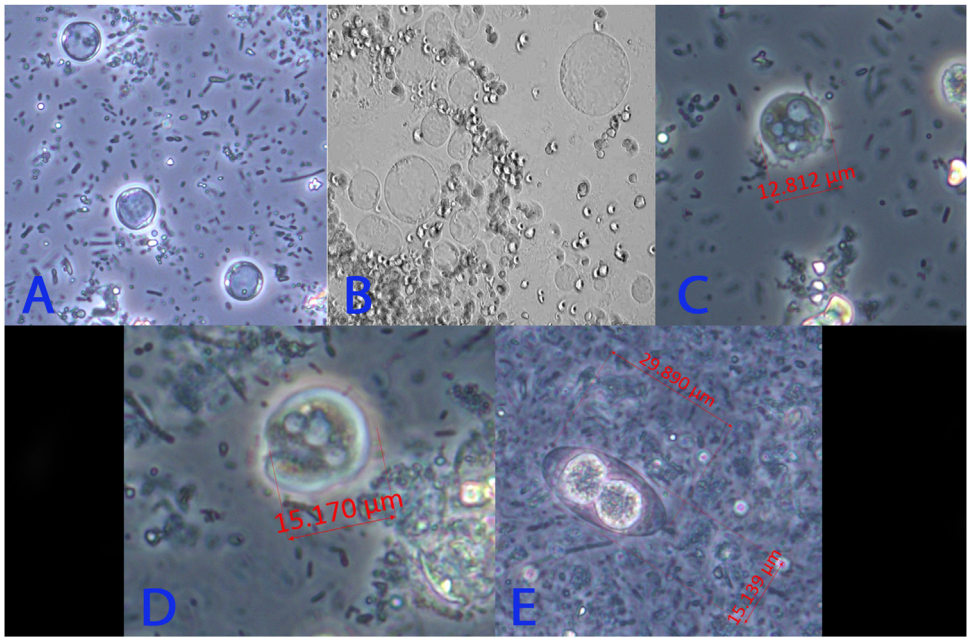

Fig. 3. Cells of different protistan pathogens. *Blastocystis hominis*cysts in an unstained wet mount from a stool sample. Microscopic examination by phase contrast microscopy (40x) (A); *B. hominis*trophozoites in Robinson's medium culture from a stool sample. Microscopic examination by phase contrast microscopy (50x) (B). *Dientamoeba fragilis* trophozoites in Robinson's medium culture from a stool sample (C, D). *Cystoisospora belli*oocysts in an unstained wet mount from a stool sample (E). Microscopic examination by phase contrast microscopy (40x).

ology of the parasitoses in a given area, achievable by the application of standard procedures. Macroscopic and microscopic examination of fresh/concentrated faeces, detection of *Giardia intestinalis* and *Cryptosporidium parvum/C. hominis* specific antigens, *in vitro* cultures to test the viability and infectivity of the isolated agents, are basic and essential methodology (Graczyk et al., 2003; Tan, 2008; Calderaro et al., 2010a, 2011). Fig. 3 shows the images captured by phase contrast microscopy of different stages of *B. hominis*, *D. fragilis* and *C. belli* in faecal samples analyzed in the Parma University Hospital's laboratories. Enteric protozoa are here examined by unstained wet mount directly from stool samples (*B. hominis* cysts and *C. belli* oocysts) or after Robinson's medium culture (*B. hominis* and *D. fragilis* trophozoites). Moreover, molecular techniques, as qualitative and real-time PCR amplifications, are applied to specific diagnosis of these enteric protozoa as crucial approaches to discriminate among cryptic species (i.e., *Entamoeba histolytica /E. dispar*) or to detect very frail organisms (*D. fragilis*) (Calderaro et al., 2006; Calderaro et al., 2010b; Intra et al., 2019; Laude et al., 2016).

Thanks also to the application of theses protocols, it is possible to observe an unexpectedly high prevalence of intestinal protozoan parasitoses in the local population of developed countries, a prevalence only partially attributable to recently arrived immigrants from developing countries where such endemic organisms are known to be more prevalent (Castelli and Sulis, 2017; Mohapatra et al., 2018). In a prevalence study performed in the University Hospital, in Parma, during the period 2006-2010, in the symptomatic population, protozoans detected included *B. hominis* (more than 60% of the detected agents), followed in prevalence by *D. fragilis* and *G. intestinalis* (about 8% of the detected agents for both) (Calderaro et al., 2014). During 2018, one case of infection by *C. belli* together with *B. hominis* in an HIV-positive patient with fever and diarrhoea and documented absence of other enteropathogenic agents (bacteria, parasites, viruses) was diagnosed (unpublished data). These results demonstrated how much these neglected protozoan infections are circulating in the developed population. They are undiagnosed and underestimated.

While in cases of mixed infection the causative agent of the observed abdominal symptoms may not be clear, in cases of patients presenting with abdominal symptoms and documented absence of enteropathogenic bacteria and/or viruses, the detected intestinal protozoan species may be considered the causative agent of the symptomatology. Could these intestinal protozoa, therefore, have clinical impact?

However, for most of the patients with intestinal parasitoses caused by protozoa, the detected agent was exactly among those for which the pathogenic role is still considered, by some authors, controversial, i.e. *B. hominis* and *D. fragilis*. So the question is again: could these neglected enteric protozoa have a clinical impact?

Among data collected from out-patients and in-patients in the laboratory of Parma's University Hospital (daily diagnostic activity in the last 3-year experience), 17% of the infections resolved after therapy, relative to both the para-

sites. More than 65% of cases could not be subjected to a follow up because to the symptoms resolution (A. Calderaro, unpublished data).

The data presented fit into the current knowledge about the circulation of protozoa in developed countries (El Safadi et al., 2016; Piubelli et al., 2019). The most prevalent protozoon was, as expected, *B. hominis*, underlining the need to deepen the studies related to protozoa, like this one, which are still the subject of debate. In general, the importance of considering protozoan infections even in non-endemic areas is reiterated, particularly in cases in which no enteropathogenic agents other than protozoa (bacteria, viruses) are present, and emphasizes the value of adopting adequate diagnostic methods.

Conclusions and perspectives

The goal of this Symposium, and this review, is to argue that neglected protistan pathogens should be neglected no more. The science underpinning their pathogenicity is a tractable and yet open opportunity for exploration. Their epidemiological importance is very likely under-appreciated and we may be doing so at our peril.

Substantial advances in understanding the mechanism of pathogenicity of *Acanthamoeba* show that molecular cell biology and biochemistry can give detailed knowledge of protistan pathogens. Genomic information is a powerful resource that can accelerate efforts towards this understanding and so the developments in genomics of *Naegleria fowleri* in advance of molecular genetic tools gives researchers an exciting headstart, just as such tools are on the horizon (Faktorova et al., 2020). Molecular understanding of pathogenic mechanisms and genomic resources of enteric parasites are at various stages of sophistication, with some such as *Giardia*, *Entamoeba*, and *Cryptosporidium* being highly advanced (Bouzid et al., 2013; Cunha et al., 2019; Fernández-López et al., 2019; Liu et al., 2018; Ryan and Hijjawi, 2015), others barely have been touched. For enthusiastic young scientists interested in these challenges, the opportunities have never been greater. Clinically, such knowledge is the first step in a path to diagnostics and treatment. Having better laboratory detection tools and making them ready for a wide use, should be helpful for general surveillance and critical for outbreak detection if needed.

Currently neglected protistan diseases also appear to be increasing medical problems, as highlighted by epidemiological evidence. *Acanthamoeba* and *Naegleria* are clearly pathogenic, but potentially underdiagnosed, at least for the latter. The remaining infections covered in the Symposium appear to be similarly more prevalent in industrial countries than currently suspected, even when in some cases, such as *Blastocystis* and *Dientamoeba,* their role in pathogenesis remains to be resolved. As the discipline gains a better understanding of the current epidemiology of these protistan agents and their potential as medical threats in the future, it may be necessary to develop programs for the mitigation of environmental changes and subsequent control of infections with environmental transmission.

Understanding and eventually mitigating infection by these currently neglected protistan pathogens will require a multidisciplinary approach, which was one reason that this Symposium included both basic science and clinical topics. Cross-discipline conversation will be crucial. By increasing the profile of neglected protistan pathogens, it is hoped that new investigators and teams will be engaged, new financial resources brought to bear and more attention paid by policy-makers. Whether in a direct infectious disease context, or a less urgent context of epidemiology and possibly shifting prevalence, the more that we know about potential threats, the better we will be prepared to face them.

Authors contributions

All authors contributed equally to this work.

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