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Invited Review

New developments in Cryptosporidium research

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Abstract

*Cryptosporidium* is an enteric parasite that is considered the second greatest cause of diarrhoea and death in children after rotavirus. Currently, 27 species are recognized as valid and of these, *Cryptosporidium hominis* and *Cryptosporidium parvum* are responsible for the majority of infections in humans. Molecular and biological studies indicate that *Cryptosporidium* is more closely related to gregarine parasites rather than to coccidians. The identification of gregarine-like gamont stages and the ability of *Cryptosporidium* to complete its life cycle in the absence of host cells further confirm its relationship with gregarines. This opens new avenues into the investigation of pathogenesis, epidemiology, treatment and control of *Cryptosporidium*. Effective drug treatments and vaccines are not yet available due, in part, to the technical challenges of working on *Cryptosporidium* in the laboratory. Whole genome sequencing and metabolomics have expanded our understanding of the biochemical requirements of this organism and have identified new drug targets. To effectively combat this important pathogen, increased funding is essential.

Keywords: *Cryptosporidium*; Taxonomy; Cell culture; Vaccines; Genomics; Drug development
1. Introduction

_Cryptosporidium_ is an enteric protozoan parasite of medical and veterinary importance that infects a wide range of humans and animals worldwide. A recent epidemiological study investigating the cause and effect of diarrhoea in over 22,000 children (under 5 years of age), residing in four African and three Asian study sites, identified cryptosporidiosis as the second most common pathogen responsible for severe diarrhoea and was also associated with death in young children (12 - 23 months of age) (Kotloff et al., 2013). Globally, cryptosporidiosis is estimated to be responsible for 30 - 50% of the deaths in children under 5 years of age and is considered the second greatest cause of diarrhoea and death in children after rotavirus (Ochoa et al., 2004; Snelling et al., 2007; Striepen, 2013). Infection in this age group is also associated with developmental problems (Guerrant et al., 1999).

Cryptosporidiosis commonly results in watery diarrhea that may sometimes be profuse and prolonged (Current and Garcia, 1991; Chalmers and Davies, 2010; Bouzid et al., 2013). Other common symptoms include abdominal pain, nausea, vomiting and low-grade fever. Occasionally, non-specific symptoms such as myalgia, weakness, malaise, headache and anorexia occur (Current and Garcia, 1991). Immunocompetent individuals experience a transient self-limiting illness (up to 2 to 3 weeks). However, for immunocompromised patients such as HIV-infected individuals, symptoms may include chronic or protracted diarrhea and prior to the use of antiretroviral therapy cryptosporidiosis was associated with significant mortality (Manabe et al., 1998; Hunter and Nichols, 2002). Infections among HIV-infected individuals may also become extra-intestinal, spreading to other sites including the gall bladder, biliary tract, pancreas and pulmonary system (López-Vélez et al., 1995; Hunter and Nichols, 2002). Transmission of the parasite occurs via the fecal-oral route, either by ingestion of contaminated water or food, or by person-to-person or animal-to-human transmission (Xiao, 2010). Waterborne transmission is considered a major mode of transmission and _Cryptosporidium_ was the etiological agent in 60.3% (120) of the waterborne
protozoan parasitic outbreaks that have been reported worldwide between 2004 and 2010 (Balduresson and Karanis, 2011).

Current treatment options for cryptosporidiosis are limited and only one drug, nitazoxanide (NTZ), has been approved by the United States (US) Food and Drug Administration (FDA). This drug, however, exhibits only moderate clinical efficacy in children and immunocompetent people, and none in people with HIV (Abubakar et al., 2007; Amadi et al., 2009).

2. Taxonomy

Cryptosporidium is an apicomplexan parasite and, until recently, belonged to the order Eucoccidiorida (which includes Toxoplasma, Cyclospora, Isospora and Sarcocystis) (Levine, 1984). Genomic and biochemical data indicate that Cryptosporidium differs from other apicomplexans in that it has lost the apicoplast organelle, as well as genomes for both the plastid and the mitochondrion (Zhu et al., 2000; Abrahamsen et al., 2004; Xu et al., 2004). Cryptosporidium also demonstrates several peculiarities that separate it from any other coccidian. These include (i) the location of Cryptosporidium within the host cell, where the endogenous developmental stages are confined to the apical surfaces of the host cell (intracellular, but extracytoplasmic); (ii) the attachment of the parasite to the host cell, where a multi-membranous attachment or feeder organelle is formed at the base of the parasitophorous vacuole (PV) to facilitate the uptake of nutrients from the host cell; (iii) the presence of two morpho-functional types of oocysts, thick-walled and thin-walled, with the latter responsible for the initiation of the auto-infective cycle in the infected host; (iv) the small size of the oocyst (5.0 x 4.5 µm for Cryptosporidium parvum) which lacks morphological structures such as sporocyst, micropyle and polar granules (Tzipori and Widmer, 2000; Petry, 2004); (v) the insensitivity to all anti-coccidial agents tested to date (Blagburn and Soave, 1997; Cabada and White, 2010); (vi) cross-reaction of an anti-cryptosporidial monoclonal antibody with gregarines (Bull et al., 1998), and (vii) the observation of the presence of novel gamont-like extracellular stages similar to those found in gregarine life cycles (Hijjawi et al.,
Molecular studies indicate that *Cryptosporidium* is more closely related to the primitive apicomplexan gregarine parasites rather than to coccidians (Carreno et al., 1999; Leander et al., 2003). Recent whole genome analysis comparing *Cryptosporidium* with the gregarine *Ascogregarina taiwanensis* supports this phylogenetic association (Templeton et al., 2010). *Ascogregarina* and *Cryptosporidium*, however, also possess features that unite them with the Coccidia, including an environmental oocyst stage, metabolic pathways such as the Type I fatty acid and polyketide synthetic enzymes, and a number of conserved extracellular protein domain architectures (Templeton et al., 2010). Future genomic studies of other gregarine parasites will hopefully provide a clearer understanding of the correct taxonomic placement of the genus *Cryptosporidium*. Further characterization of these novel gamont-like developmental stages, which are similar to those of gregarines, will also contribute to a greater understanding of the environmental ecology of *Cryptosporidium*, which is fundamental to its control (Barta and Thompson, 2006). A better understanding of the relationship between *Cryptosporidium* and gregarines will also open up new approaches into the investigation of pathogenesis, epidemiology, treatment and control of *Cryptosporidium*.

Delimiting species within the genus *Cryptosporidium* has also been controversial but currently 27 species are regarded as valid (Ryan et al., 2014, 2015). Three of these are avian *Cryptosporidium* spp.; *Cryptosporidium meleagridis*, *Cryptosporidium baileyi* and *Cryptosporidium galli*, 19 are species in mammals; *Cryptosporidium muris*, *C. parvum*, *Cryptosporidium wrairi*, *Cryptosporidium felis*, *Cryptosporidium andersoni*, *Cryptosporidium canis*, *Cryptosporidium hominis*, *Cryptosporidium suis*, *Cryptosporidium bovis*, *Cryptosporidium fayeri*, *Cryptosporidium macropodum*, *Cryptosporidium ryanae*, *Cryptosporidium xiaoii*, *Cryptosporidium ubiquitum*, *Cryptosporidium cuniculus*, *Cryptosporidium tyzzeri*, *Cryptosporidium viatorum*, *Cryptosporidium scrofarum* and *Cryptosporidium erinacei*; one (Cryptosporidium fragile) is a
species in amphibians; two (Cryptosporidium serpentis and Cryptosporidium varanii) are species in reptiles; and two (Cryptosporidium molnari and Cryptosporidium huwi) are species in fish (Ryan and Xiao, 2014; Ryan et al., 2014, 2015). There are also over 40 genotypes, with a high probability that many of these will eventually be given species status with increased biological and molecular characterisation.

In humans, nearly 20 Cryptosporidium spp. and genotypes have been reported, including C. hominis, C. parvum, C. meleagridis, C. felis, C. canis, C. cuniculus, C. ubiquitum, C. viatorum, C. muris, C. suis, C. fayeri, C. andersoni, C. bovis, C. scrofarum, C. tyzzeri, C. erinacei and Cryptosporidium horse, skunk and chipmunk I genotypes, with C. hominis and C. parvum most commonly reported (Xiao, 2010; Ryan et al., 2014). Other species, such as C. meleagridis, C. felis, C. canis, C. cuniculus, C. ubiquitum and C. viatorum are less common. The remaining Cryptosporidium spp. and genotypes have been found in only a few human cases (Xiao, 2010; Ryan et al., 2014). These Cryptosporidium spp. infect both immunocompetent and immunocompromised persons.

Molecular analysis using highly variable loci such as the 60 kDa glycoprotein (gp60) has revealed that C. hominis appears to be highly human-specific. Whilst some C. parvum subtypes such as the IIc subtype family are transmitted anthroponotically, other C. parvum subtypes are transmitted zoonotically (Xiao, 2010; Ryan et al., 2014).

3. Life cycle and cell culture

Cryptosporidium has a complex, monoxenous life cycle consisting of several developmental stages involving both sexual and asexual cycles (Fig. 1). The infective sporulated oocyst is excreted from the body of an infected host in the faeces. The oocysts possess a tough trilaminar wall, which is extremely resistant to chemical and mechanical disruption, and maintains the viability of the internal sporozoites under adverse environmental conditions (Fayer and Unger, 1986). This wall is
very rigid (Chatterjee et al., 2010) and atomic force microscopy shows that the oocyst wall resembles common plastic materials (Dumètre et al., 2013).

Once ingested, oocysts release sporozoites in the intestine, where infections are predominately localized to the jejunum and ileum. Cell invasion by the sporozoite is followed by intracellular development to a trophozoite stage which undergoes asexual proliferation to produce two different types of meronts. Merozoites released from type I meronts enter other intestinal epithelial cells and either develop into type II meronts or complete another cycle of type I meronts. Merozoites from type II meronts then multiply sexually to produce microgamonts and macrogamonts. The microgamonts fertilize the macrogamonts producing zygotes, which mature into oocysts (Hijjawi, 2010).

The presence of gamont-like extracellular stages in the life cycle of Cryptosporidium was first observed in a study by Hijjawi et al. (2002) and has since been reported by several investigators (Rosales et al., 2005; Karanis et al., 2008; Borowski et al., 2010; Koh et al., 2013, 2014; Huang et al., 2014). The origin of the extracellular stages is not known but it has been suggested that these stages might originate from sporozoites which failed to penetrate the host cells and developed extracellularly into motile trophozoite stages (Hijjawi et al., 2004; Rosales et al., 2005). Extracellular gamont-like stages have also been purified from mice infected with C. parvum (Hijjawi et al., 2004).

A major hurdle for research laboratories to facilitate biological, pathological, immunological and molecular and drug evaluation studies on Cryptosporidium has been the inability to continuously propagate Cryptosporidium in vitro. In addition, there are no methods allowing the indefinite storage of infectious material and isolates have to be continuously passaged through animals, usually calves or mice for C. parvum and piglets and gerbils for C. hominis (Tzipori and Widmer, 2008).

Factors that affect the development and proliferation of Cryptosporidium in in vitro culture include the excystation protocol, age and strain of the parasite, stage and size of inoculum, host cell
type, maturity and culture conditions such as pH, medium supplements and atmosphere (Hijjawi, 2010; Karanis and Aldeyarbi, 2011). The majority of in vitro cultivation studies to date use human adenocarcinoma (HCT-8) cells, as this cell line supports superior development of the parasite in a conventional 5% CO₂ environment compared with other cell lines and atmospheres but still suffers from failure to propagate the parasite long-term, low yields of oocysts and/or lack of reproducibility (Hijjawi, 2010; Karanis and Aldeyarbi, 2011). Long-term culturing (up to 25 days) of Cryptosporidium in cell culture using pH modification, sub-culturing and gamma irradiation has been reported (Hijjawi et al., 2001). Reducing the volume of excystation medium and centrifugation of excysting oocysts onto the cell monolayer has also reportedly resulted in an approximately four-fold increase in sporozoite attachment and subsequent infection (King et al., 2011). Another study cultivated intact crypts from human intestinal fragments of intestinal layers with culture medium supplemented with growth factors and antiapoptotic molecules but only reported that Cryptosporidium development increased for >120 h (Castellanos-Gonzalez et al., 2013).

Nonetheless, high throughput screening of Cryptosporidium, using HCT-8 cultures, for viability and drug analysis has been achieved (King et al., 2011; Bessoff et al., 2013; Jefferies et al., 2015). Complete development of Cryptosporidium in a cell-free (axenic) in vitro cultivation system was first reported by Hijjawi et al. (2004). According to this report, new oocysts were present after 8 days post-culture inoculation (Hijjawi et al., 2004). Other researchers such as Girouard et al. (2006), who used similar but not identical serum-free cultivation systems, were unable to reproduce these results. However, multiplication of Cryptosporidium DNA from cell-free cultures has been reported by other researchers (Zhang et al., 2009; Hijjawi et al., 2010; Koh et al., 2013) and various Cryptosporidium developmental stages (sporozoites, trophozoites, large meronts, merozoites, microgamonts, gamont-like cells and extra-large gamont-like cells) have been identified from biofilms using various techniques including scanning electron microscopy (SEM) (Koh et al., 2013, 2014). A previous study had suggested that the presence of gamont-like stages in both cell-free and in-vitro cultures was due to contaminating debris or fungal infection resembling Bipolaris.
australiensis and Colletotrichum acutatum (Woods and Upton, 2007). In the most recent study by Koh et al. (2014), however, intense immunofluorescent labelling of the internal structures of gamont-like stages using a Cryptosporidium-specific antibody counters this argument. The authors suggested that the role of the gamont-like stage is to generate trophozoites and merozoites so that more new oocysts can be produced without host encapsulation (Koh et al., 2014). The latter study also demonstrated that Cryptosporidium has the ability to form a PV independent of a host (in both biofilms and HCT-8 cell cultures) (Koh et al., 2014), which is consistent with the proposal by Pohlenz et al. (1978) that Cryptosporidium does not require host encapsulation to form a PV.

Another study identified sporozoites, trophozoites and type I merozoites in cell-free cultures by SEM and compared gene expression in cell culture and cell-free culture (Yang et al., 2015). Findings from that study showed that gene expression patterns in cell culture and cell-free culture were similar but in cell-free culture, gene expression was delayed in some genes and was lower (Yang et al., 2015). A recent study conducted a genome-wide transcriptome analysis over a 72 h in vitro culture of C. parvum-infected HCT-8 cells (Mauzy et al., 2012). Quantitative-PCR (qPCR) for 3,302 genes (87% of the protein coding genes) indicated that each gene has detectable transcription in at least one time point assessed (Mauzy et al., 2012). Further studies which involve a wider range of genes should be conducted to better understand the expression of Cryptosporidium genes in cell-free culture.

Numerous studies have reported aggregations of trophozoites in cell-free culture (Hijjawi et al., 2004, 2010; Boxell et al., 2008; Koh et al., 2014; Yang et al., 2015) and it has been suggested that trophozoites may have fused together by a syzygy-like process (Hijjawi et al., 2004). More recently, all life cycle stages from cell-free culture have been described using electron microscopy (Aldeyarbi and Karanis, 2014, unpublished data). The authors also reported different Cryptosporidium stages developing within the shells of the oocysts, the detection of gregarine-like stages and syzygy and a PV (Aldeyarbi and Karanis, 2014, unpublished data). A cell-free in vitro cultivation system for Cryptosporidium represents a significant advance that will be extremely
useful in drug assessment and in research on developmental biology, avoiding the need for infectivity experiments with animal models or cell culture (Karanis and Aldeyarbi, 2011). It is hoped that with further advances in cell-free culturing, more researchers skilled in immunology, biochemistry and molecular biology will apply these skills to *Cryptosporidium*.

4. Vaccines

The immune status of the host plays a critical role in determining susceptibility to infection with this parasite as well as the outcome and severity of cryptosporidiosis. Therefore understanding host-parasite interactions and the essential elements of immunity to *Cryptosporidium* spp. are essential to the development of effective immunotherapies or vaccines (Mead, 2014). A complex sequence of events involving various components of the innate and adaptive host response has been shown to be important in the control of *Cryptosporidium* infection (Petry et al., 2010; McDonald et al., 2013). Yet the nature of these responses, particularly in humans, is not completely understood (Borad and Ward, 2010). However, as this parasite is largely localised to the intestinal tract, a vaccine that stimulates mucosal immune responses will likely be most beneficial (Mead, 2014). For example, commercially available mucosal vaccines against other enteric pathogens such as rotavirus, that are live and attenuated, have achieved considerable success in disease prevention and control in children in developed countries (Pasetti et al., 2011), but lower protection in children in developing countries (Vesikari, 2012). The use of an attenuated *Cryptosporidium* strain could therefore result in better immunological responses and protection from symptomatic disease and/or infection. Several lines of evidence support this. For example, dairy calves inoculated with gamma-irradiated *C. parvum* oocysts were protected against subsequent challenge (Jenkins et al., 2004). In pigs, *C. hominis*-specific immunity was sufficient to completely protect against challenge with the same species (Sheoran et al., 2012). In a second group of pigs, primary infection with *C. hominis* and subsequent infection with *C. parvum* resulted in a partial cross-protective immunity with milder symptoms and lower oocyst shedding than *C. parvum* only infected controls (Sheoran et al., 2012).
Studies in human volunteers have shown that re-challenge with the same *C. parvum* isolate, 1 year after recovery from cryptosporidiosis, did not prevent infection but did reduce its severity (Okhuysen et al., 1998). Indeed, it has been suggested that regular exposure to low doses of *Cryptosporidium* are beneficial, as *Cryptosporidium* infection rates were significantly higher for outbreaks associated with groundwater than surface water consumption (Craun et al., 1998; Hunter and Quigley, 1998). The authors argued that people who use surface water sources were regularly exposed to small numbers of oocysts and thus did not experience many outbreaks, unless there was a major breakdown in treatment (Craun et al., 1998; Hunter and Quigley, 1998). Development of vaccines containing *Cryptosporidium* parasites that have been rendered incapable of causing disease, through irradiation or genetic engineering, and identification of effective cryopreservation methods is likely the best approach for the development of potential vaccine strains (Striepen, 2012; Mead, 2014). However, it is important to remember that malnutrition and the associated reduction in immunity (Coutinho et al., 2008) may lower the effectiveness of any *Cryptosporidium* vaccine used on children in developing countries. This is particularly sobering in light of that fact that there are currently >842 million chronically malnourished persons worldwide (http://www.fao.org/docrep/018/i3458e/i3458e.pdf).

5. Genomics

The sequencing of the genomes of *C. parvum*, *C. hominis* and *C. muris* has been a major advance in our understanding of the molecular biology of *Cryptosporidium* (Abrahamsen et al., 2004; Xu et al., 2004; Widmer and Sullivan, 2012; Widmer et al., 2012). The genomes of *C. parvum* and *C. hominis* display 95 - 97% DNA sequence identity and ~30% GC content, with no large indels or rearrangements evident (Widmer and Sullivan, 2012). They are each 9.2 million bases (Mb) in size and encode 4000 genes (Abrahamsen et al., 2004; Xu et al., 2004). The genome of *C. parvum* is essentially fully assembled (13 scaffolds representing eight chromosomes; see www.cryptodb.org), whereas the *C. hominis* genome still has some gaps (90 scaffolds; see
Approximately 75.3% of the *C. parvum* genome is annotated as coding (Abrahamsen et al., 2004). *Cryptosporidium* (together with gregarines) has lost its apicoplast, and *C. parvum* and *C. hominis* have a degenerate ‘mitosome’ instead of a mitochondrion, and have lost the mitochondrial genome and nuclear genes for many mitochondrial proteins, including those required for the tricarboxylic acid (TCA) cycle, oxidative phosphorylation, and fatty acid oxidation (Abrahamsen et al., 2004; Xu et al., 2004; Templeton et al., 2010). Also absent are genes for de novo biosynthesis of amino acids, nucleotides and sugars, as well as mechanisms for splicing RNA and gene silencing (Abrahamsen et al., 2004; Xu et al., 2004). Loss of genes from multiple metabolic pathways means *C. parvum* and *C. hominis* rely heavily on scavenging nutrients from the host, salvage rather than de novo biosynthesis, and glycolysis or substrate-level phosphorylation for energy production.

A recent comparison of the genome of the anthroponotic *C. parvum* isolate TU114 (gp60 IIC) and the reference genome originating from the zoonotic *C. parvum* isolate IOWA identified a small number of highly diverged genes (Widmer et al., 2012). Among these, transporters were significantly over-represented, which suggests that the ability to establish an infection in a particular host species may depend in part on transporters controlling the exchange of metabolites between the host cell and intracellular developmental stages of the parasite (Widmer et al., 2012). Further genome sequencing is required to confirm this. Interestingly, a three-way comparison of the newly sequenced anthroponotic *C. parvum* TU114 isolate, the reference zoonotic *C. parvum* (IOWA) and *C. hominis* identified at least three genes where the anthroponotic *C. parvum* sequence was more similar to *C. hominis* than to the zoonotic *C. parvum* reference. Because *C. hominis* and *C. parvum* IIC are human parasites, this raises the possibility that their evolution is driven by the adaptation of the parasite to different host species (Widmer et al., 2012).

A draft de novo assembly of the *C. muris* genome has been available from online public databases (e.g., CryptoDB, cryptodb.org) since 2008 (Widmer and Sullivan, 2012). The *C. muris* genome overall is similar in size, nucleotide composition and gene content to the other two species,
with a few notable exceptions, e.g., the nuclear genome encodes a complete set of TCA cycle enzymes, genes required for oxidative phosphorylation, and a functional ATP synthase (Mogi and Kita, 2010). Similar to *C. parvum* and *C. hominis*, *C. muris* lacks a cytochrome-based respiratory chain and shows no evidence of having a mitochondrial genome (Widmer and Sullivan, 2012).

However, the presence of mitochondrial structure and proteins that are absent from *C. parvum* and *C. hominis*, but present in *C. muris* and gregarines (Uni et al., 1987; Toso and Omoto, 2007; Mogi and Kita, 2010), supports the theory that the common ancestor of gregarines and *Cryptosporidium* had a larger complement of mitochondrial proteins than *C. parvum* and *C. hominis*, and that the loss of mitochondria in *C. parvum* and *C. hominis* occurred after they diverged from *C. muris* (Widmer and Sullivan, 2012).

Functional genomics in *Cryptosporidium* has been hampered by the lack of a transfection system due to the complex life cycle of the parasite and a lack of effective endogenous promoters. A transient expression system using GFP, has been developed based on the double-stranded RNA (dsRNA) *C. parvum* virus (CPV) harboured by *Cryptosporidium* (Li et al., 2009). More recently, a DNA-based transient transfection of yellow (YFP) or red (RFP) fluorescent protein in *C. parvum* oocysts and sporozoites controlled by the endogenous promoters of actin, alpha tubulin and myosin genes using the restricted enzyme-mediated integration technique has been described (Li et al., 2014). Further research is required to develop a stable transfection system, which will be helpful in determining the function and localization of novel *Cryptosporidium* proteins.

### 6. Drug discovery

Progress in developing anti-cryptosporidial drugs has also been slow due to the limitations of in vitro culture for *Cryptosporidium*, an inability to genetically manipulate the organism and the unique metabolic features in this parasite, which has a highly streamlined metabolism and is unable to synthesize nutrients de novo (Abrahamsen et al., 2004; Andrews et al., 2014; Guo et al., 2014).

As discussed, *Cryptosporidium* has completely lost the plastid-derived apicoplast present in many
other apicomplexans, and the remnant mitochondrion lacks the citrate cycle and cytochrome-based respiratory chain. Therefore, many classic drug targets are unavailable in Cryptosporidium and novel targets need to be identified for drug development (Guo et al., 2014). However, essential core metabolic pathways, including energy metabolism and lipid synthesis, are present in this parasite. Many enzymes within these pathways may serve as new drug targets because they are either absent in, or highly divergent from, humans and animals. For example, the C. parvum genome encodes three long chain fatty acyl-coenzyme A synthetases (LC-ACS) which are essential in fatty acid metabolism (Abrahamsen et al., 2004). A recent study reported good efficacy of the ACS inhibitor triacsin C against cryptosporidial infection in mice (Guo et al., 2014).

Another enzyme pathway that has been extensively examined is the salvage of adenosine from its host or environment as Cryptosporidium is unable to synthesize purine nucleotides de novo (Striepen et al., 2004; Umejiego et al., 2004; Kirubakaran et al., 2012). Cryptosporidium does not contain guanine salvage enzymes and consequently this pathway appears to be the only route to source guanine nucleotides (Striepen et al., 2004; Kirubakaran et al., 2012). The inosine 5’-monophosphate dehydrogenase (IMPDH) gene in Cryptosporidium appears to have been acquired through lateral gene transfer from an ε-proteobacterium (Striepen et al., 2002, 2004) and recent studies have shown that compounds optimised for inhibition of cryptosporidial IMPDH also have antibacterial activity (Mandapati et al., 2014). Detailed kinetic analysis of this prokaryote-like enzyme demonstrated that the Cryptosporidium IMPDH is very different from its human homologs (Striepen et al., 2004; Umejiego et al., 2004). Furthermore, the “drugability” of IMPDH is well established as inhibitors of human IMPDHs have been used clinically as immunosuppressants as well as for the treatment of viral infections and cancer (Chen and Pankiewicz, 2007; Hedstrom, 2009). Thus, the exclusive reliance on the salvage pathway by Cryptosporidium and its high metabolic demand for nucleotides due to the complicated lifecycle of this parasite make IMPDH a potential drug target candidate. This hypothesis is supported by the recent discovery of several Cryptosporidium IMPDH inhibitors (Umejiego et al., 2008; Maurya et al., 2009; Sharling et al.,
Another study used a yeast-two-hybrid system to identify “Phylomer®” peptides (constructed from the genomes of 25 phylogenetically diverse bacteria) that targeted the IMPDH of *C. parvum* and several interacting Phylomers® exhibited significant growth inhibition in vitro (Jefferies et al., 2015).

The prohibitive cost of de novo drug development, estimated to be between $500 million and $2 billion per compound successfully brought to market (Adams and Brantner, 2006), is another major limiting factor in the development of anti-cryptosporidial drugs and has resulted in drug repurposing. For example, drugs such as the human 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitor, itavastatin and Auranofin (Ridaura®) were initially approved for the treatment of rheumatoid arthritis and have been shown to be effective against *Cryptosporidium* in vitro (Bessoff et al., 2013; Debnath et al., 2013), which holds promise for further in vivo testing in animals and humans.

7. Metabolomics

Metabolomics, the study of intracellular and extracellular metabolites that are consumed and produced as a result of biological activity, is in its infancy in *Cryptosporidium* research but provides an avenue for biomarker discovery, drug targets and improved diagnostic techniques.

Genome sequencing and biochemical data has revealed that *Cryptosporidium* is highly reliant on its host/environment for nutrients as it is missing key metabolic pathways and lacks the ability for de novo synthesis of nucleosides, fatty acids and amino acids (Abrahamsen et al., 2004; Xu et al., 2004). An in silico genome-scale metabolic model of *C. hominis* identified 540 reactions performed by 213 enzymes (Vanee et al., 2010). Of these reactions, 514 were metabolic biochemical reactions involving intracellular metabolites and 26 were transport reactions representing the movement of metabolites across the cell membrane (Vanee et al., 2010).

A recent preliminary metabolomics study on *Cryptosporidium* developed a faecal metabolite extraction method for untargeted gas chromatography-mass spectrometry (GC-MS) analysis using
Cryptosporidium-positive and -negative human faecal samples (Ng et al., 2012). In that study, higher levels of metabolites were generally detected in Cryptosporidium-positive patients, suggesting that metabolic homeostasis and intestinal permeability were affected as a result of the infection (Ng et al., 2012). Interestingly, a more controlled metabolomics analysis of faecal metabolite profiles using experimentally infected mice reported that lower metabolite levels were generally detected in faecal samples from Cryptosporidium-infected mice (Ng-Hublin et al., 2013). Differences in metabolite profiles between different host types have been previously reported by Saric et al. (2008). In that study, a comparison of faecal metabolite profiles from mice, rats and humans showed that the levels of metabolites differed between the host species, presumably as a result of different endogenous and exogenous perturbations, and differences in the gut microbiome between species (Saric et al., 2008). Despite the differences in faecal metabolite profiles between Cryptosporidium-infected humans and mice, metabolomic analysis in both studies was still able to clearly differentiate between infected and uninfected hosts, as well as provide information on the metabolic activity of the parasite during the infection based on faecal metabolite profiles.

Another study used metabolomic techniques coupled with statistical chemometric analysis of viable and irradiated Cryptosporidium oocysts and identified a number of key metabolites including aromatic and non-aromatic amino acids, carbohydrates, fatty acids and alcohol type compounds that differentiated between the viable and non-viable oocysts (Beale et al., 2013).

8. Perspectives

Despite the evidence that Cryptosporidium is one of four pathogens responsible for the majority of severe diarrhoea in infants and toddlers (Kotloff et al., 2013), Cryptosporidium research lags behind the other three pathogens identified (rotavirus, Shigella and enterotoxigenic Escherichia coli) (Striepen, 2013). Unlike those pathogens, no fully effective drug treatment or vaccine is available for Cryptosporidium.
Increased funding for Cryptosporidium is essential to effectively combat this disease. The US National Institutes of Health (NIH) currently spends US$4.3 million each year on Cryptosporidium research, compared with approximately $300 million on more than 600 malaria projects (Striepen, 2013). In Australia, the National Health and Medical Research Council (NHMRC) has expended ~AUD $320,000 on Cryptosporidium research projects in the last 5 years compared with more than AUD $5 million on malaria research (www.nhmrc.gov.au/grants/research-funding-statistics-and-data). It is hoped that philanthropic organizations such as the Bill and Melinda Gates Foundation, USA, (www.gatesfoundation.org), which have focused previously on monitoring rather than intervention, will fund basic research on Cryptosporidium and that more research funding will become available from various governments.

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References


and cognitive function four–seven years later in a poor urban community in northeast Brazil.


Figure legend

**Fig. 1.** *Cryptosporidium* life cycle (reproduced with permission from Hijjawi et al. (2004)).
Highlights

- Relationship between Cryptosporidium and gregarine parasites
- Recent data supporting cell-free culture
- Vaccine prospects
- Recent developments in drug discovery and metabolomics