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Invited review

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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

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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

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protozoan parasitic outbreaks that have been reported worldwide between 2004 and 2010 (Baldursson 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.,

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2002; Rosales et al., 2005; Karanis et al., 2008; Borowski et al., 2010; Koh et al., 2013, 2014; Huang et al., 2014).

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 xiaoi, Cryptosporidium ubiquitum, Cryptosporidium cuniculus*, *Cryptosporidium tyzzeri, Cryptosporidium viatorum, Cryptosporidium scrofarum* and *Cryptosporidium erinacei*; one (*Cryptosporidium fragile*) is a

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114 species in amphibians; two (*Cryptosporidium serpentis* and *Cryptosporidium varanii*) are species in
115 reptiles; and two (*Cryptosporidium molnari* and *Cryptosporidium huwi*) are species in fish (Ryan
116 and Xiao, 2014; Ryan et al., 2014, 2015). There are also over 40 genotypes, with a high probability
117 that many of these will eventually be given species status with increased biological and molecular
118 characterisation.

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

128 Molecular analysis using highly variable loci such as the 60 kDa glycoprotein (*gp60*) has
129 revealed that *C. hominis* appears to be highly human-specific. Whilst some *C. parvum* subtypes
130 such as the IIc subtype family are transmitted anthroponotically, other *C. parvum* subtypes are
131 transmitted zoonotically (Xiao, 2010; Ryan et al., 2014).
132

133 **3. Life cycle and cell culture**

134 *Cryptosporidium* has a complex, monoxenous life cycle consisting of several developmental
135 stages involving both sexual and asexual cycles (Fig. 1). The infective sporulated oocyst is excreted
136 from the body of an infected host in the faeces. The oocysts possess a tough trilaminar wall, which
137 is extremely resistant to chemical and mechanical disruption, and maintains the viability of the
138 internal sporozoites under adverse environmental conditions (Fayer and Unger, 1986). This wall is

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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 undergos 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

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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% CO_2 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*

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191 *australiensis* and *Colletotrichum acutatum* (Woods and Upton, 2007). In the most recent study by
192 Koh et al. (2014), however, intense immunofluorescent labelling of the internal structures of
193 gamont-like stages using a *Cryptosporidium*-specific antibody counters this argument. The authors
194 suggested that the role of the gamont-like stage is to generate trophozoites and merozoites so that
195 more new oocysts can be produced without host encapsulation (Koh et al., 2014). The latter study
196 also demonstrated that *Cryptosporidium* has the ability to form a PV independent of a host (in both
197 biofilms and HCT-8 cell cultures) (Koh et al., 2014), which is consistent with the proposal by
198 Pohlenz et al. (1978) that *Cryptosporidium* does not require host encapsulation to form a PV.
199 Another study identified sporozoites, trophozoites and type I merozoites in cell-free cultures
200 by SEM and compared gene expression in cell culture and cell-free culture (Yang et al., 2015).
201 Findings from that study showed that gene expression patterns in cell culture and cell-free culture
202 were similar but in cell-free culture, gene expression was delayed in some genes and was lower
203 (Yang et al., 2015). A recent study conducted a genome-wide transcriptome analysis over a 72 h in
204 vitro culture of *C. parvum*-infected HCT-8 cells (Mauzy et al., 2012). Quantitative-PCR (qPCR) for
205 3,302 genes (87% of the protein coding genes) indicated that each gene has detectable transcription
206 in at least one time point assessed (Mauzy et al., 2012). Further studies which involve a wider range
207 of genes should be conducted to better understand the expression of *Cryptosporidium* genes in cell-
208 free culture.
209 Numerous studies have reported aggregations of trophozoites in cell-free culture (Hijjawi et
210 al., 2004, 2010; Boxell et al., 2008; Koh et al., 2014; Yang et al., 2015) and it has been suggested
211 that trophozoites may have fused together by a syzygy-like process (Hijjawi et al., 2004). More
212 recently, all life cycle stages from cell-free culture have been described using electron microscopy
213 (Aldeyarbi and Karanis, 2014, unpublished data). The authors also reported different
214 *Cryptosporidium* stages developing within the shells of the oocysts, the detection of gregarine-like
215 stages and syzygy and a PV (Aldeyarbi and Karanis, 2014, unpublished data). A cell-free in vitro
216 cultivation system for *Cryptosporidium* represents a significant advance that will be extremely

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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).

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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

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www.cryptodb.org). 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,

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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

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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.,

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2010; Gorla et al., 2012, 2013; Johnson et al., 2013). 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

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373 *Cryptosporidium*-positive and -negative human faecal samples (Ng et al., 2012). In that study,
374 higher levels of metabolites were generally detected in *Cryptosporidium*-positive patients,
375 suggesting that metabolic homeostasis and intestinal permeability were affected as a result of the
376 infection (Ng et al., 2012). Interestingly, a more controlled metabolomics analysis of faecal
377 metabolite profiles using experimentally infected mice reported that lower metabolite levels were
378 generally detected in faecal samples from *Cryptosporidium*-infected mice (Ng-Hublin et al., 2013).
379 Differences in metabolite profiles between different host types have been previously reported by
380 Saric et al. (2008). In that study, a comparison of faecal metabolite profiles from mice, rats and
381 humans showed that the levels of metabolites differed between the host species, presumably as a
382 result of different endogenous and exogenous perturbations, and differences in the gut microbiome
383 between species (Saric et al., 2008). Despite the differences in faecal metabolite profiles between
384 *Cryptosporidium*-infected humans and mice, metabolomic analysis in both studies was still able to
385 clearly differentiate between infected and uninfected hosts, as well as provide information on the
386 metabolic activity of the parasite during the infection based on faecal metabolite profiles.
387 Another study used metabolomic techniques coupled with statistical chemometric analysis
388 of viable and irradiated *Cryptosporidium* oocysts and identified a number of key metabolites
389 including aromatic and non-aromatic amino acids, carbohydrates, fatty acids and alcohol type
390 compounds that differentiated between the viable and non-viable oocysts (Beale et al., 2013).
391
392 **8. Perspectives**
393 Despite the evidence that *Cryptosporidium* is one of four pathogens responsible for the
394 majority of severe diarrhoea in infants and toddlers (Kotloff et al., 2013), *Cryptosporidium* research
395 lags behind the other three pathogens identified (rotavirus, Shigella and enterotoxigenic *Escherichia*
396 *coli*) (Striepen, 2013). Unlike those pathogens, no fully effective drug treatment or vaccine is
397 available for *Cryptosporidium*.

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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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413 **References**

414 Abrahamsen, M.S., Templeton, T.J., Enomoto, S., Abrahante, J.E., Zhu, G., Lancto, C.A., Deng, M.,
415 Liu, C., Widmer, G., Tzipori, S., Buck, G.A., Xu, P., Bankier, A.T., Dear, P.H., Konfortov,
416 B.A., Spriggs, H.F., Iyer, L., Anantharaman, V., Aravind, L., and Kapur, V., 2004. Complete
417 genome sequence of the apicomplexan, *Cryptosporidium parvum*. Science 304, 441 - 445.
418 Abubakar, I., Aliyu, S.H., Arumugam, C., Hunter, P.R., Usman, N.K., 2007. Prevention and
419 treatment of cryptosporidiosis in immunocompromised patients. Cochrane Database Syst. Rev.
420 1, CD004932.
421 Adams, C.P., Brantner, V.V., 2006. Estimating the cost of new drug development: is it really $802
422 million? Health Aff. (Millwood) 25, 420 - 428.
423 Amadi, B., Mwiya, M., Sianongo, S., Payne, L., Watuka, A., Katubulushi, M., Kelly, P., 2009.
424 High dose prolonged treatment with nitazoxanide is not effective for cryptosporidiosis in HIV
425 positive Zambian children: a randomised controlled trial. BMC Infect. Dis. 9, 195.
426 Andrews, K.T., Fisher, G., Skinner-Adams, T.S., 2014. Drug repurposing and human parasitic
427 protozoan diseases. Int. J. Parasitol. Drugs Drug Resist. 4, 95 - 111.
428 Baldursson, S., Karanis, P., 2011. Waterborne transmission of protozoan parasites: review of
429 worldwide outbreaks - an update 2004-2010. Water Res. 45, 6603 - 6614.
430 Barta, J.R., Thompson, R.C., 2006. What is *Cryptosporidium*? Reappraising its biology and
431 phylogenetic affinities. Trends Parasitol. 22, 463 - 468.
432 Beale, D.J., Marney, D., Marlow, D.R., Morrison, P.D., Dunn, M.S., Key, C., Palombo, E.A., 2013.
433 Metabolomic analysis of *Cryptosporidium parvum* oocysts in water: a proof of concept
434 demonstration. Environ. Pollut. 174, 201 - 203.
435 Bessoff, K., Sateriale, A., Lee, K.K., Huston, C.D., 2013. Drug repurposing screen reveals FDA-
436 approved inhibitors of human HMG-CoA reductase and isoprenoid synthesis that block
437 *Cryptosporidium parvum* growth. Antimicrob. Agents Chemother. 57, 1804 - 1814.

ACCEPTED MANUSCRIPT

438 Blagburn, B.L., Soave, R., 1997. Prophylaxis and chemotherapy: human and animal. In: Fayer, R.
439 (Ed.), *Cryptosporidium* and Cryptosporidiosis. CRC Press, Florida. pp.111 - 128.
440 Borad, A., Ward, H., 2010. Human immune responses in cryptosporidiosis. Future Microbiol. 5,
441 507 - 519.
442 Borowski, H., Thompson, R.C., Armstrong, T., Clode, P.L., 2010. Morphological characterization
443 of *Cryptosporidium parvum* life-cycle stages in an in vitro model system. Parasitology 137,
444 13 - 26.
445 Bouzid, M., Hunter, P.R., Chalmers, R.M., Tyler, K.M., 2013. *Cryptosporidium* pathogenicity and
446 virulence. Clin. Microbiol. Rev. 26, 115 - 134.
447 Boxell, A., Hijjawi, N., Monis, P., Ryan, U., 2008. Comparison of various staining methods for the
448 detection of *Cryptosporidium* in cell-free culture. Exp. Parasitol. 120, 67 - 72.
449 Bull, S., Chalmers, R., Sturdee, A.P., Curry, A., Kennaugh, J., 1998. Cross-reaction of an anti-
450 *Cryptosporidium* monoclonal antibody with sporocysts of *Monocystis* species. Vet. Parasitol.
451 77, 195 - 197.
452 Cabada, M.M., White, A.C., 2010. Treatment of cryptosporidiosis: do we know what we think we
453 know? Curr. Opin. Infect. Dis. 23, 494 - 499.
454 Carreno, R.A., Martin, D.S. and Barta, J.R., 1999. *Cryptosporidium* is more closely related to the
455 gregarines than to coccidia as shown by phylogenetic analysis of apicomplexan parasites
456 inferred using small-subunit ribosomal RNA gene sequences. Parasitol. Res. 85, 899 - 904.
457 Castellanos-Gonzalez, A., Cabada, M.M., Nichols, J., Gomez, G., White, A.C. Jr., 2013. Human
458 primary intestinal epithelial cells as an improved in vitro model for *Cryptosporidium parvum*
459 infection. Infect. Immun. 81, 1996 - 2001.
460 Chatterjee, A., Banerjee, S., Steffen, M., Moore, L.L., O'Connor, R.M., Ward, H.D., Robbins, P.W.,
461 Samuelson, J., 2010. Evidence for mucin-like glycoproteins that tether sporozoites of
462 *Cryptosporidium parvum* to the inner surface of the oocyst wall. Eukaryot. Cell 9, 84 - 96.
463 Chalmers, R.M., Davies, A.P., 2010. Clinical cryptosporidiosis. Exp. Parasitol. 124, 138 - 146.

ACCEPTED MANUSCRIPT

464 Chen, L., Pankiewicz, K.W., 2007. Recent development of IMP dehydrogenase inhibitors for the
465 treatment of cancer. Curr. Opin. Drug Discov. Devel. 10, 403 - 412.
466 Craun, G.F., Hubbs, S.A., Frost, F., Calderon, R.L., Via, S.H., 1998. Waterborne outbreaks of
467 cryptosporidiosis. J. Am. Water Works Assoc. 90, 81 - 91.
468 Coutinho, B.P., Oriá, R.B., Vieira, C.M., Sevilleja, J.E., Warren, C.A., Maciel, J.G., Thompson,
469 M.R., Pinkerton, R.C., Lima, A.A., Guerrant, R.L., 2008. *Cryptosporidium* infection causes
470 undernutrition and, conversely, weanling undernutrition intensifies infection. J. Parasitol. 94,
471 1225 - 1232.
472 Current, W., Garcia, L.S., 1991. Cryptosporidiosis. Clin. Microbiol. Rev. 4, 325 - 358.
473 Debnath, A., Ndao, M., Reed, S.L., 2013. Reprofiled drug targets ancient protozoans: drug
474 discovery for parasitic diarrheal diseases. Gut Microbes. 4, 66 - 71
475 Dumètre, A., Dubey, J.P., Ferguson, D.J., Bongrand, P., Azas, N., Puech, P.H., 2013. Mechanics of
476 the *Toxoplasma gondii* oocyst wall. Proc. Natl. Acad. Sci. U. S. A. 110, 11535 - 11540.
477 Fayer, R., Ungar, B.L., 1986. *Cryptosporidium* spp. and cryptosporidiosis. Microbiol. Rev. 50, 458
478 - 483.
479 Girouard, D., Gallant, J., Akiyoshi, D.E., Nunnari, J., Tzipori, S., 2006. Failure to propagate
480 *Cryptosporidium* spp. in cell-free culture. J. Parasitol. 92, 399 - 400.
481 Gorla, S.K., Kavitha, M., Zhang, M., Liu, X., Sharling, L., Gollapalli, D.R., Striepen, B., Hedstrom,
482 L., Cuny, G.D., 2012. Selective and potent urea inhibitors of *Cryptosporidium parvum* inosine
483 5'-monophosphate dehydrogenase. J. Med. Chem. 55, 7759 - 7771.
484 Gorla, S.K., Kavitha, M., Zhang, M., Chin, J.E., Liu, X., Striepen, B., Makowska-Grzyska, M., Kim,
485 Y., Joachimiak, A., Hedstrom, L., Cuny, G.D., 2013. Optimization of benzoxazole-based
486 inhibitors of *Cryptosporidium parvum* inosine 5'-monophosphate dehydrogenase. J. Med.
487 Chem. 56, 4028 - 4043.
488 Guerrant, D.I., Moore, S.R., Lima, A.A., Patrick, P.D., Schorling, J.B., Guerrant, R.L., 1999.
489 Association of early childhood diarrhea and cryptosporidiosis with impaired physical fitness

ACCEPTED MANUSCRIPT

and cognitive function four–seven years later in a poor urban community in northeast Brazil. Am. J. Trop. Med. Hyg. 61, 707 - 713.

Guo, F., Zhang, H., Fritzler, J.M., Rider, S.D. Jr, Xiang, L., McNair, N.N., Mead, J.R., Zhu, G., 2014. Amelioration of *Cryptosporidium parvum* infection in vitro and in vivo by targeting parasite fatty acyl-coenzyme A synthetases. J. Infect. Dis. 209, 1279 - 1287.

Hedstrom, L., 2009. IMP dehydrogenase: structure, mechanism, and inhibition. Chem. Rev. 109, 2903 - 2928.

Hijjawi, N.S., Meloni, B.P., Morgan, U.M., Thompson, R.C. 2001. Complete development and long-term maintenance of *Cryptosporidium parvum* human and cattle genotypes in cell culture. Int. J. Parasitol. 31, 1048 - 1055.

Hijjawi, N.S., Meloni, B.P., Ryan, U.M., Olson, M.E., Thompson, R.C.A. 2002. Successful *in vitro* cultivation of *Cryptosporidium andersoni*: evidence for the existence of novel extracellular stages in the life cycle and implications for the classification of *Cryptosporidium*. Int. J. Parasitol. 32, 1719 - 1726.

Hijjawi, N.S., Meloni, B.P., Ng'anzo, M., Ryan, U., Olson, M.E., Cox, P.T., Monis, P.T., Thompson, R.C.A., 2004. Complete development of *Cryptosporidium parvum* in host cell-free culture. Int. J. Parasitol. 34, 769 - 777.

Hijjawi, N., 2010. *Cryptosporidium*: New developments in cell culture. Exp. Parasitol. 124, 54 - 60.

Hijjawi, N., Estcourt, A., Yang, R., Monis, P., Ryan, U. 2010. Complete development and multiplication of *Cryptosporidium hominis* in cell-free culture. Vet. Parasitol. 169, 29 - 36.

Huang, L., Zhu, H., Zhang, S., Wang, R., Liu, L., Jian, F., Ning, C., Zhang, N., 2014. An *in vitro* model of infection of chicken embryos by *Cryptosporidium baileyi*. Exp. Parasitol. 147, 41 - 47.

Hunter, P.R., Quigley, C., 1998. Investigation of an outbreak of cryptosporidiosis associated with treated surface water finds limits to the value of case control studies. Commun. Dis. Public Health. 1, 234 - 238.

ACCEPTED MANUSCRIPT

516 Hunter, P., Nichols, G., 2002. Epidemiology and clinical features of *Cryptosporidium* infection in
517 immunocompromised patients. Clin. Microbiol. Rev. 15, 145 - 154.
518 Jefferies, R., Yang, R., Who, C.K., Weldt, T., Milech, N., Estcourt, A., Armstrong, T., Hopkins, R.,
519 Watt, P., Reid, S., Armson, A., Ryan U.M., 2015. Target validation of the inosine
520 monophosphate dehydrogenase (IMPDH) gene in *Cryptosporidium* using Phylomer®
521 peptides. Exp. Parasitol. 148, 40 - 48.
522 Jenkins, M., Higgins, J., Kniel, K., Trout, J., Fayer, R., 2004. Protection of calves against
523 cryptosporiosis by oral inoculation with gamma-irradiated *Cryptosporidium parvum* oocysts.
524 J. Parasitol. 90, 1178 - 1180.
525 Johnson, C.R., Gorla, S.K., Kavitha, M., Zhang, M., Liu, X., Striepen, B., Mead, J.R., Cuny, G.D.,
526 Hedstrom, L., 2013. Phthalazinone inhibitors of inosine-5'-monophosphate dehydrogenase
527 from *Cryptosporidium parvum*. Bioorg. Med. Chem. Lett. 23, 1004 - 1007.
528 Karanis, P., Kimura, A., Nagasawa, H., Igarashi, I., Suzuki, N. 2008. Observations on
529 C*ryptosporidium* life cycle stages during excystation. J. Parasitol. 94, 298 - 300.
530 Karanis, P., Aldeyarbi, H.M., 2011. Evolution of *Cryptosporidium in vitro* culture. Int. J. Parasitol.
531 41, 1231 - 1242.
532 King, B.J., Keegan, A.R., Robinson, B.S., Monis, P.T., 2011. *Cryptosporidium* cell culture
533 infectivity assay design. Parasitology 138, 671 - 681.
534 Kirubakaran, S., Gorla, S.K., Sharling, L., Zhang, M., Liu, X., Ray, S.S., Macpherson, I.S., Striepen,
535 B., Hedstrom, L., Cuny, G.D., 2012. Structure-activity relationship study of selective
536 benzimidazole-based inhibitors of *Cryptosporidium parvum* IMPDH. Bioorg. Med. Chem.
537 Lett. 22, 1985 - 1998.
538 Koh, W., Clode, P.L., Monis, P., Thompson, R.C., 2013. Multiplication of the waterborne
539 pathogen *Cryptosporidium parvum* in an aquatic biofilm system. Parasit. Vectors. 6, 270.

ACCEPTED MANUSCRIPT

Koh, W., Thompson, R.C., Edwards, H., Monis, P., Clode, P.L., 2014. Extracellular excystation and development of *Cryptosporidium*: tracing the fate of oocysts within *Pseudomonas* aquatic biofilm systems. BMC Microbiol. 14, 281.

Kotloff, K.L., Nataro, J.P., Blackwelder, W.C., Nasrin, D., Farag, T.H., Panchalingam, S., Wu, Y., Sow, S.O., Sur, D., Breiman, R.F., Faruque, A.S., Zaidi, A.K., Saha, D., Alonso, P.L., Tamboura, B., Sanogo, D., Onwuchekwa, U., Manna, B., Ramamurthy, T., Kanungo, S., Ochieng, J.B., Omore, R., Oundo, J.O., Hossain, A., Das, S.K., Ahmed, S., Qureshi, S., Quadri, F., Adegbola, R.A., Antonio, M., Hossain, M.J., Akinsola, A., Mandomando, I., Nhampossa, T., Acacio, S., Biswas, K., O'Reilly, C.E., Mintz, E.D., Berkeley, L.Y., Muhsen, K., Sommerfelt, H., Robins-Browne, R.M., Levine, M.M., 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382, 209 – 222.

Leander, B.S., Clopton, R.E., Keeling, P.J. 2003. Phylogeny of gregarines (Apicomplexa) as inferred from small-subunit rDNA and beta-tubulin. Int. J. Syst. Evol. Microbiol. 53, 345 - 354.

Levine, N.D., 1984. Taxonomy and review of the coccidian genus *Cryptosporidium* (protozoa, apicomplexa). J. Protozool. 31, 94 - 98.

Li, W., Zhang, N., Liang, X., Li, J., Gong, P., Yu, X., Ma, G., Ryan, U.M., Zhang, X., 2009. Transient transfection of *Cryptosporidium parvum* using green fluorescent protein (GFP) as a marker. Mol. Biochem. Parasitol. 168, 143 - 148.

Li, W., Diao, Y., Gong, P., Suo, X., Li, J., Zhang, X., 2014. Transient reporter gene expression in oocysts and sporozoites of *Cryptosporidium parvum* controlled by endogenous promoters. Mol. Biochem. Parasitol. 194, 33 - 35.

López-Vélez, R., Tarazona, R., Garcia Camacho, A., Gomez-Mampaso, E., Guerrero, A., Moreira, V., Villanueva, R., 1995. Intestinal and extraintestinal cryptosporidiosis in AIDS patients. Eur. J. Clin. Microbiol. Infect. Dis. 14, 677 - 681.

ACCEPTED MANUSCRIPT

McDonald, V., Korbel, D.S., Barakat, F.M., Choudhry, N., Petry, F., 2013. Innate immune responses against *Cryptosporidium parvum* infection. Parasite Immunol. 35, 55 - 64.

Manabe, Y.C., Clark, D.P., Moore, R.D., Lumadue, J.A., Dahlman, H.R., Belitsos, P.C., Chaisson, R.E., Sears, C.L., 1998. Cryptosporidiosis in patients with AIDS: correlates of disease and survival. Clin. Infect. Dis. 27, 536 - 542.

Mandapati, K., Gorla, S.K., House, A.L., McKenney, E.S., Zhang, M., Rao, S.N., Gollapalli, D.R., Mann, B.J., Goldberg, J.B., Cuny, G.D., Glomski, I.J., Hedstrom, L., 2014. Repurposing *Cryptosporidium* inosine 5'-monophosphate dehydrogenase inhibitors as potential antibacterial agents. ACS Med. Chem. Lett. 5, 846 - 850.

Maurya, S.K., Gollapalli, D.R., Kirubakaran, S., Zhang, M., Johnson, CR., Benjamin, N.N., Hedstrom, L., Cuny, G.D., 2009. Triazole inhibitors of *Cryptosporidium parvum* inosine 5′-monophosphate dehydrogenase. J. Med. Chem. 52, 4623 - 4630.

Mauzy, M.J., Enomoto, S., Lancto, C.A., Abrahamsen, M.S., Rutherford, M.S., 2012. The *Cryptosporidium parvum* transcriptome during in vitro development. PLoS One. 7, e31715.

Mead, J.R., 2014. Prospects for immunotherapy and vaccines against *Cryptosporidium*. Hum. Vaccin. Immunother. 10, 1505 - 1513.

Mogi, T., Kita, K., 2010. Diversity in mitochondrial metabolic pathways in parasitic protists *Plasmodium* and *Cryptosporidium*. Parasitol. Int. 59, 305 - 312.

Ng, J.S., Ryan, U., Trengove, R.D., Maker, G.L., 2012. Development of an untargeted metabolomics method for the analysis of human faecal samples using *Cryptosporidium*-infected samples. Mol. Biochem. Parasitol. 185, 145 - 150.

Ng Hublin, J.S., Ryan, U., Trengove, R., Maker, G., 2013. Metabolomic profiling of faecal extracts from *Cryptosporidium parvum* infection in experimental mouse models. PLoS One. 8, e77803.

Ochoa, T.J., Salazar-Lindo, E., Cleary, T.G., 2004. Management of children with infection-associated persistent diarrhea. Semin. Pediatr. Infect. Dis. 15, 229 - 236.

ACCEPTED MANUSCRIPT

591 Okhuysen, P.C., Chappell, C.L., Sterling, C.R., Jakubowski, W., DuPont, H.L., 1998. Susceptibility
592 and serologic response of healthy adults to reinfection with *Cryptosporidium parvum*. Infect.
593 Immun. 66, 441 - 443.
594 Pasetti, M.F., Simon, J.K., Sztein, M.B., Levine, M.M., 2011. Immunology of gut mucosal vaccines.
595 Immunol. Rev. 239, 125 - 148.
596 Petry, F., 2004. Structural analysis of *Cryptosporidium parvum*. Microscopy Microanal. 10, 586 -
597 601.
598 Petry, F., Jakobi, V., Tessema, T.S., 2010. Host immune response to *Cryptosporidium parvum*
599 infection. Exp. Parasitol. 126, 304 - 309.
600 Pohlenz, J., Bemrick, W.J., Moon, H.W., Cheville, N.F., 1978. Bovine cryptosporidiosis: A
601 transmission and scanning electron microscopic study of some stages in the life cycle and of
602 the host parasite relationship. Vet. Pathol. 15, 417 - 427.
603 Rosales, M.J., Cordón, G.P., Moreno, M.S., Sánchez, C.M., Mascaró, C., 2005. Extracellular like-
604 gregarine stages of *Cryptosporidium parvum*. Acta Trop. 95, 74 - 78.
605 Ryan, U.M. Xiao, L., 2014. Taxonomy and Molecular Taxonomy. In: Cacciò, S. M., Widmer, G.
606 (Eds.), *Cryptosporidium*: Parasite and Disease. Springer, New York, USA. pp. 1 - 22.
607 Ryan, U., Fayer, R., Xiao, L., 2014. *Cryptosporidium* species in humans and animals: current
608 understanding and research needs. Parasitol. 141, 1667 - 1685.
609 Ryan, U., Paparini, A., Tong, K., Yang, R., Gibson-Keuh, S., O'Hara, A., Lymbery, A., Xiao, L.,
610 2015. *Cryptosporidium huwi* n. sp. (Apicomplexa:Eimeriidae) from the guppy (*Poecilia*
611 *reticulata*). Exp. Parasitol. 150C, 31-35. doi: 10.1016/j.exppara.2015.01.009.
612 Saric, J., Wang, Y., Li, J., Coen, M., Utzinger, J., Marchesi, J.R., Keiser, J., Veselkov, K., Lindon,
613 J.C., Nicholson, J.K., Holmes, E., 2008. Species variation in the fecal metabolome gives
614 insight into differential gastrointestinal function. J. Proteome Res. 7, 352 - 360.

ACCEPTED MANUSCRIPT

615 Sharling, L., Liu, X., Gollapalli, D.R., Maurya, S.K., Hedstrom, L., Striepen, B., 2010. A screening
616 pipeline for antiparasitic agents targeting *Cryptosporidium* inosine monophosphate
617 dehydrogenase. PLoS Negl. Trop. Dis. 4, e794.
618 Sheoran, A., Wiffin, A., Widmer, G., Singh, P., Tzipori, S., 2012. Infection with *Cryptosporidium*
619 *hominis* provides incomplete protection of the host against *Cryptosporidium parvum*. J. Infect.
620 Dis. 205, 1019 - 1023.
621 Snelling, W.J., Xiao, L., Ortega-Pierres, G., Lowery, C.J., Moore, J.E., Rao, J.R., Smyth, S., Millar,
622 B.C., Rooney, P.J., Matsuda, M., Kenny, F., Xu, J., Dooley, J.S., 2007. Cryptosporidiosis in
623 developing countries. J. Infect. Dev. Ctries. 1, 242 - 256.
624 Striepen, B., White, M.W., Li, C., Guerini, M.N., Malik, S.B., Logsdon, J.M., Jr., Liu, C.,
625 Abrahamsen, M.S., 2002. Genetic complementation in apicomplexan parasites. Proc. Natl.
626 Acad. Sci. 99, 6304 - 6309.
627 Striepen, B., Pruijssers, A.J., Huang, J., Li, C., Gubbels, M.J., Umejiego, N.N., Hedstrom, L.,
628 Kissinger, J.C., 2004. Gene transfer in the evolution of parasite nucleotide biosynthesis. Proc.
629 Natl. Acad. Sci. 101, 3154 - 3159.
630 Striepen, B., 2013. Parasitic infections: Time to tackle cryptosporidiosis. Nature. 503, 189 - 191.
631 Templeton, T.J., Enomoto, S., Chen, W.J., Huang, C.G., Lancto, C.A., Abrahamsen, M.S., Zhu, G.,
632 2010. A genome-sequence survey for *Ascogregarina taiwanensis* supports evolutionary
633 affiliation but metabolic diversity between a Gregarine and *Cryptosporidium*. Mol. Biol. Evol.
634 27, 235 - 248.
635 Toso, M.A., Omoto, C.K., 2007. Ultrastructure of the *Gregarina niphandrodes* nucleus through
636 stages from unassociated trophozoites to gamonts in syzygy and the syzygy junction. J.
637 Parasitol. 93, 479 - 484.
638 Tzipori, S., Widmer, G., 2000. The biology of *Cryptosporidium*. Contrib. Microbiol. 6, 1 - 32.

ACCEPTED MANUSCRIPT

639 Umejiego, N.N., Li, C., Riera, T., Hedstrom, L., and Striepen, B., 2004. *Cryptosporidium parvum*
640 IMP dehydrogenase: identification of functional, structural, and dynamic properties that can
641 be exploited for drug design. J. Biol. Chem. 279, 40320 - 40327.
642 Umejiego, N.N., Gollapalli, D., Sharling, L., Volftsun, A., Lu, J., 2008. Targeting a prokaryotic
643 protein in a eukaryotic pathogen: identification of lead compounds against cryptosporidiosis.
644 Chem. Biol. 15, 70 - 77.
645 Uni, S., Iseki, M., Maekawa, T., Moriya, K., Takada, S., 1987. Ultrastructure of *Cryptosporidium*
646 *muris* (strain RN 66) parasitizing the murine stomach. Parasitol Res. 74, 123 - 132.
647 Vanee, N., Roberts, S.B., Fong, S.S., Manque, P., Buck, G.A., 2010. A genome-scale metabolic
648 model of *Cryptosporidium hominis*. Chem. Biodivers. 7, 1026 - 1039.
649 Vesikari, T., 2012 Rotavirus vaccination: a concise review. Clin. Microbiol. Infect. 18, 57 - 63.
650 Widmer, G., Sullivan, S., 2012. Genomics and population biology of *Cryptosporidium* species.
651 Parasite Immunol. 34, 61 - 71.
652 Widmer, G., Lee, Y., Hunt, P., Martinelli, A., Tolkoff, M., Bodi, K., 2012. Comparative genome
653 analysis of two *Cryptosporidium parvum* isolates with different host range. Infect. Genet.
654 Evol. 12, 1213 - 1221.
655 Woods, K.M., Upton, S.J., 2007. *In vitro* development of *Cryptosporidium parvum* in serum-free
656 media. Lett. Appl. Microbiol. 44, 520 - 523.
657 Xiao, L. 2010. Molecular epidemiology of cryptosporidiosis: an update. Exp. Parasitol. 124, 80 - 89.
658 Xu, P., Widmer, G., Wang, Y., Ozaki, LS., Alves, J.M., Serrano, M.G., Puiu, D., Manque, P.,
659 Akiyoshi, D., Mackey, A.J., Pearson, W.R., Dear, P.H., Bankier, A.T., Peterson, D.L.,
660 Abrahamsen, M.S., Kapur, V., Tzipori, S., Buck, G.A., 2004. The genome of
661 *Cryptosporidium hominis*. Nature. 431, 1107 - 1112.
662 Yang, R., Elankumaran, Y., Hijjawi, N., Ryan, U., 2015. Validation of cell-free culture using
663 scanning electron microscopy (SEM) and gene expression studies. Exp. Parasitol. In press.

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664 Zhang, L., Sheoran, A.S., Widmer, G., 2009. *Cryptosporidium parvum* DNA replication in cell-free
665 culture. J. Parasitol. 95, 1239 - 1242.
666 Zhu, G., Keithly, J.S., Philippe, H., 2000. What is the phylogenetic position
667 of *Cryptosporidium*? Int. J. Syst. Evol. Microbiol. 50, 1673 - 1681.
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671 **Figure legend**

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

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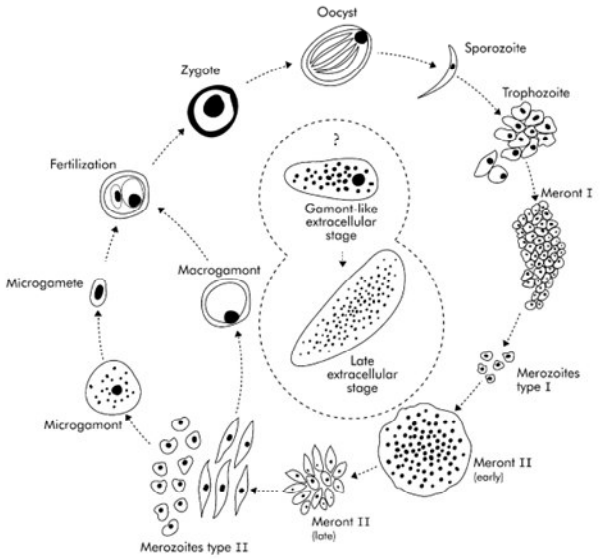

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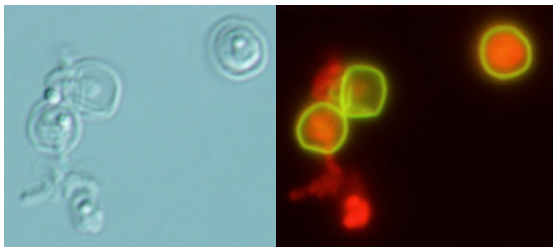

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680 **Highlights**

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682 • Relationship between *Cryptosporidium* and gregarine parasites
683 • Recent data supporting cell-free culture
684 • Vaccine prospects
685 • Recent developments in drug discovery and metabolomics

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