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Invited review

New developments in *Cryptosporidium* research

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1 **Invited Review**

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3 **New developments in *Cryptosporidium* research**

4

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6

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18

19 **Abstract**

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

32

33

34 **Keywords:** *Cryptosporidium*; Taxonomy; Cell culture; Vaccines; Genomics; Drug development

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36

37 1. Introduction

38 *Cryptosporidium* is an enteric protozoan parasite of medical and veterinary importance that
39 infects a wide range of humans and animals worldwide. A recent epidemiological study
40 investigating the cause and effect of diarrhoea in over 22,000 children (under 5 years of age),
41 residing in four African and three Asian study sites, identified cryptosporidiosis as the second most
42 common pathogen responsible for severe diarrhoea and was also associated with death in young
43 children (12 - 23 months of age) (Kotloff et al., 2013). Globally, cryptosporidiosis is estimated to be
44 responsible for 30 - 50% of the deaths in children under 5 years of age and is considered the second
45 greatest cause of diarrhoea and death in children after rotavirus (Ochoa et al., 2004; Snelling et al.,
46 2007; Striepen, 2013). Infection in this age group is also associated with developmental problems
47 (Guerrant et al., 1999).

48 Cryptosporidiosis commonly results in watery diarrhea that may sometimes be profuse and
49 prolonged (Current and Garcia, 1991; Chalmers and Davies, 2010; Bouzid et al., 2013). Other
50 common symptoms include abdominal pain, nausea, vomiting and low-grade fever. Occasionally,
51 non-specific symptoms such as myalgia, weakness, malaise, headache and anorexia occur (Current
52 and Garcia, 1991). Immunocompetent individuals experience a transient self-limiting illness (up to
53 2 to 3 weeks). However, for immunocompromised patients such as HIV-infected individuals,
54 symptoms may include chronic or protracted diarrhea and prior to the use of antiretroviral therapy
55 cryptosporidiosis was associated with significant mortality (Manabe et al., 1998; Hunter and
56 Nichols, 2002). Infections among HIV-infected individuals may also become extra-intestinal,
57 spreading to other sites including the gall bladder, biliary tract, pancreas and pulmonary system
58 (López-Vélez et al., 1995; Hunter and Nichols, 2002). Transmission of the parasite occurs via the
59 fecal-oral route, either by ingestion of contaminated water or food, or by person-to-person or
60 animal-to-human transmission (Xiao, 2010). Waterborne transmission is considered a major mode
61 of transmission and *Cryptosporidium* was the etiological agent in 60.3% (120) of the waterborne

62 protozoan parasitic outbreaks that have been reported worldwide between 2004 and 2010
63 (Baldursson and Karanis, 2011).

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

68

69 **2. Taxonomy**

70 *Cryptosporidium* is an apicomplexan parasite and, until recently, belonged to the order
71 Eucoccidiorida (which includes *Toxoplasma*, *Cyclospora*, *Isospora* and *Sarcocystis*) (Levine, 1984).
72 Genomic and biochemical data indicate that *Cryptosporidium* differs from other apicomplexans in
73 that it has lost the apicoplast organelle, as well as genomes for both the plastid and the
74 mitochondrion (Zhu et al., 2000; Abrahamsen et al., 2004; Xu et al., 2004). *Cryptosporidium* also
75 demonstrates several peculiarities that separate it from any other coccidian. These include (i) the
76 location of *Cryptosporidium* within the host cell, where the endogenous developmental stages are
77 confined to the apical surfaces of the host cell (intracellular, but extracytoplasmic); (ii) the
78 attachment of the parasite to the host cell, where a multi-membranous attachment or feeder
79 organelle is formed at the base of the parasitophorous vacuole (PV) to facilitate the uptake of
80 nutrients from the host cell; (iii) the presence of two morpho-functional types of oocysts, thick-
81 walled and thin-walled, with the latter responsible for the initiation of the auto-infective cycle in the
82 infected host; (iv) the small size of the oocyst (5.0 x 4.5 μm for *Cryptosporidium parvum*) which
83 lacks morphological structures such as sporocyst, micropyle and polar granules (Tzipori and
84 Widmer, 2000; Petry, 2004); (v) the insensitivity to all anti-coccidial agents tested to date
85 (Blagburn and Soave, 1997; Cabada and White, 2010); (vi) cross-reaction of an anti-cryptosporidial
86 monoclonal antibody with gregarines (Bull et al., 1998), and (vii) the observation of the presence of
87 novel gamont-like extracellular stages similar to those found in gregarine life cycles (Hijjawi et al.,

88 2002; Rosales et al., 2005; Karanis et al., 2008; Borowski et al., 2010; Koh et al., 2013, 2014;
89 Huang et al., 2014).

90 Molecular studies indicate that *Cryptosporidium* is more closely related to the primitive
91 apicomplexan gregarine parasites rather than to coccidians (Carreno et al., 1999; Leander et al.,
92 2003). Recent whole genome analysis comparing *Cryptosporidium* with the
93 gregarine *Ascogregarina taiwanensis* supports this phylogenetic association (Templeton et al.,
94 2010). *Ascogregarina* and *Cryptosporidium*, however, also possess features that unite them with the
95 Coccidia, including an environmental oocyst stage, metabolic pathways such as the Type I fatty
96 acid and polyketide synthetic enzymes, and a number of conserved extracellular protein domain
97 architectures (Templeton et al., 2010). Future genomic studies of other gregarine parasites will
98 hopefully provide a clearer understanding of the correct taxonomic placement of the genus
99 *Cryptosporidium*. Further characterization of these novel gamont-like developmental stages, which
100 are similar to those of gregarines, will also contribute to a greater understanding of the
101 environmental ecology of *Cryptosporidium*, which is fundamental to its control (Barta and
102 Thompson, 2006). A better understanding of the relationship between *Cryptosporidium* and
103 gregarines will also open up new approaches into the investigation of pathogenesis, epidemiology,
104 treatment and control of *Cryptosporidium*.

105 Delimiting species within the genus *Cryptosporidium* has also been controversial but
106 currently 27 species are regarded as valid (Ryan et al., 2014, 2015). Three of these are avian
107 *Cryptosporidium* spp.; *Cryptosporidium meleagridis*, *Cryptosporidium baileyi* and
108 *Cryptosporidium galli*, 19 are species in mammals; *Cryptosporidium muris*, *C. parvum*,
109 *Cryptosporidium wrairi*, *Cryptosporidium felis*, *Cryptosporidium andersoni*, *Cryptosporidium canis*,
110 *Cryptosporidium hominis*, *Cryptosporidium suis*, *Cryptosporidium bovis*, *Cryptosporidium fayeri*,
111 *Cryptosporidium macropodum*, *Cryptosporidium ryanae*, *Cryptosporidium xiaoi*, *Cryptosporidium*
112 *ubiquitum*, *Cryptosporidium cuniculus*, *Cryptosporidium tyzzeri*, *Cryptosporidium viatorum*,
113 *Cryptosporidium scrofarum* and *Cryptosporidium erinacei*; one (*Cryptosporidium fragile*) is a

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

139 very rigid (Chatterjee et al., 2010) and atomic force microscopy shows that the oocyst wall
140 resembles common plastic materials (Dumètre et al., 2013).

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

149 The presence of gamont-like extracellular stages in the life cycle of *Cryptosporidium* was
150 first observed in a study by Hijjawi et al. (2002) and has since been reported by several
151 investigators (Rosales et al., 2005; Karanis et al., 2008; Borowski et al., 2010; Koh et al., 2013,
152 2014; Huang et al., 2014). The origin of the extracellular stages is not known but it has been
153 suggested that these stages might originate from sporozoites which failed to penetrate the host cells
154 and developed extracellularly into motile trophozoite stages (Hijjawi et al., 2004; Rosales et al.,
155 2005). Extracellular gamont-like stages have also been purified from mice infected with *C. parvum*
156 (Hijjawi et al., 2004).

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

163 Factors that affect the development and proliferation of *Cryptosporidium* in in vitro culture
164 include the excystation protocol, age and strain of the parasite, stage and size of inoculum, host cell

165 type, maturity and culture conditions such as pH, medium supplements and atmosphere (Hijjawi,
166 2010; Karanis and Aldeyarbi, 2011). The majority of in vitro cultivation studies to date use human
167 adenocarcinoma (HCT-8) cells, as this cell line supports superior development of the parasite in a
168 conventional 5% CO₂ environment compared with other cell lines and atmospheres but still suffers
169 from failure to propagate the parasite long-term, low yields of oocysts and/or lack of reproducibility
170 (Hijjawi, 2010; Karanis and Aldeyarbi, 2011). Long-term culturing (up to 25 days) of
171 *Cryptosporidium* in cell culture using pH modification, sub-culturing and gamma irradiation has
172 been reported (Hijjawi et al., 2001). Reducing the volume of excystation medium and centrifugation
173 of excysting oocysts onto the cell monolayer has also reportedly resulted in an approximately four-
174 fold increase in sporozoite attachment and subsequent infection (King et al., 2011). Another study
175 cultivated intact crypts from human intestinal fragments of intestinal layers with culture medium
176 supplemented with growth factors and antiapoptotic molecules but only reported that
177 *Cryptosporidium* development increased for >120 h (Castellanos-Gonzalez et al., 2013).
178 Nonetheless, high throughput screening of *Cryptosporidium*, using HCT-8 cultures, for viability
179 and drug analysis has been achieved (King et al., 2011; Bessoff et al., 2013; Jefferies et al., 2015).

180 Complete development of *Cryptosporidium* in a cell-free (axenic) in vitro cultivation system
181 was first reported by Hijjawi et al. (2004). According to this report, new oocysts were present after
182 8 days post-culture inoculation (Hijjawi et al., 2004). Other researchers such as Girouard et al.
183 (2006), who used similar but not identical serum-free cultivation systems, were unable to reproduce
184 these results. However, multiplication of *Cryptosporidium* DNA from cell-free cultures has been
185 reported by other researchers (Zhang et al., 2009; Hijjawi et al., 2010; Koh et al., 2013) and various
186 *Cryptosporidium* developmental stages (sporozoites, trophozoites, large meronts, merozoites,
187 microgamonts, gamont-like cells and extra-large gamont-like cells) have been identified from
188 biofilms using various techniques including scanning electron microscopy (SEM) (Koh et al., 2013,
189 2014). A previous study had suggested that the presence of gamont-like stages in both cell-free and
190 in-vitro cultures was due to contaminating debris or fungal infection resembling *Bipolaris*

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

217 useful in drug assessment and in research on developmental biology, avoiding the need for
218 infectivity experiments with animal models or cell culture (Karanis and Aldeyarbi, 2011). It is
219 hoped that with further advances in cell-free culturing, more researchers skilled in immunology,
220 biochemistry and molecular biology will apply these skills to *Cryptosporidium*.

221

222 4. Vaccines

223 The immune status of the host plays a critical role in determining susceptibility to infection
224 with this parasite as well as the outcome and severity of cryptosporidiosis. Therefore understanding
225 host-parasite interactions and the essential elements of immunity to *Cryptosporidium* spp. are
226 essential to the development of effective immunotherapies or vaccines (Mead, 2014). A complex
227 sequence of events involving various components of the innate and adaptive host response has been
228 shown to be important in the control of *Cryptosporidium* infection (Petry et al., 2010; McDonald et
229 al., 2013). Yet the nature of these responses, particularly in humans, is not completely understood
230 (Borad and Ward, 2010). However, as this parasite is largely localised to the intestinal tract, a
231 vaccine that stimulates mucosal immune responses will likely be most beneficial (Mead, 2014). For
232 example, commercially available mucosal vaccines against other enteric pathogens such as
233 rotavirus, that are live and attenuated, have achieved considerable success in disease prevention and
234 control in children in developed countries (Pasetti et al., 2011), but lower protection in children in
235 developing countries (Vesikari, 2012). The use of an attenuated *Cryptosporidium* strain could
236 therefore result in better immunological responses and protection from symptomatic disease and/or
237 infection. Several lines of evidence support this. For example, dairy calves inoculated with gamma-
238 irradiated *C. parvum* oocysts were protected against subsequent challenge (Jenkins et al., 2004). In
239 pigs, *C. hominis*-specific immunity was sufficient to completely protect against challenge with the
240 same species (Sheoran et al., 2012). In a second group of pigs, primary infection with *C. hominis*
241 and subsequent infection with *C. parvum* resulted in a partial cross-protective immunity with milder
242 symptoms and lower oocyst shedding than *C. parvum* only infected controls (Sheoran et al., 2012).

243 Studies in human volunteers have shown that re-challenge with the same *C. parvum* isolate, 1 year
244 after recovery from cryptosporidiosis, did not prevent infection but did reduce its severity
245 (Okhuysen et al., 1998). Indeed, it has been suggested that regular exposure to low doses of
246 *Cryptosporidium* are beneficial, as *Cryptosporidium* infection rates were significantly higher for
247 outbreaks associated with groundwater than surface water consumption (Craun et al., 1998; Hunter
248 and Quigley, 1998). The authors argued that people who use surface water sources were regularly
249 exposed to small numbers of oocysts and thus did not experience many outbreaks, unless there was
250 a major breakdown in treatment (Craun et al., 1998; Hunter and Quigley, 1998). Development of
251 vaccines containing *Cryptosporidium* parasites that have been rendered incapable of causing disease,
252 through irradiation or genetic engineering, and identification of effective cryopreservation methods
253 is likely the best approach for the development of potential vaccine strains (Striepen, 2012; Mead,
254 2014). However, it is important to remember that malnutrition and the associated reduction in
255 immunity (Coutinho et al., 2008) may lower the effectiveness of any *Cryptosporidium* vaccine used
256 on children in developing countries. This is particularly sobering in light of that fact that there are
257 currently >842 million chronically malnourished persons worldwide
258 (<http://www.fao.org/docrep/018/i3458e/i3458e.pdf>).

259

260 5. Genomics

261 The sequencing of the genomes of *C. parvum*, *C. hominis* and *C. muris* has been a major
262 advance in our understanding of the molecular biology of *Cryptosporidium* (Abrahamsen et al.,
263 2004; Xu et al., 2004; Widmer and Sullivan, 2012; Widmer et al., 2012). The genomes of *C.*
264 *parvum* and *C. hominis* display 95 - 97% DNA sequence identity and ~30% GC content, with no
265 large indels or rearrangements evident (Widmer and Sullivan, 2012). They are each 9.2 million
266 bases (Mb) in size and encode 4000 genes (Abrahamsen et al., 2004; Xu et al., 2004). The genome
267 of *C. parvum* is essentially fully assembled (13 scaffolds representing eight chromosomes; see
268 www.cryptodb.org), whereas the *C. hominis* genome still has some gaps (90 scaffolds; see

269 www.cryptodb.org). Approximately 75.3% of the *C. parvum* genome is annotated as coding
270 (Abrahamsen et al., 2004). *Cryptosporidium* (together with gregarines) has lost its apicoplast, and *C.*
271 *parvum* and *C. hominis* have a degenerate ‘mitosome’ instead of a mitochondrion, and have lost the
272 mitochondrial genome and nuclear genes for many mitochondrial proteins, including those required
273 for the tricarboxylic acid (TCA) cycle, oxidative phosphorylation, and fatty acid oxidation
274 (Abrahamsen et al., 2004; Xu et al., 2004; Templeton et al., 2010). Also absent are genes for de
275 novo biosynthesis of amino acids, nucleotides and sugars, as well as mechanisms for splicing RNA
276 and gene silencing (Abrahamsen et al., 2004; Xu et al., 2004). Loss of genes from multiple
277 metabolic pathways means *C. parvum* and *C. hominis* rely heavily on scavenging nutrients from the
278 host, salvage rather than de novo biosynthesis, and glycolysis or substrate-level phosphorylation for
279 energy production.

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

292 A draft de novo assembly of the *C. muris* genome has been available from online public
293 databases (e.g., CryptoDB, cryptodb.org) since 2008 (Widmer and Sullivan, 2012). The *C. muris*
294 genome overall is similar in size, nucleotide composition and gene content to the other two species,

295 with a few notable exceptions, e.g., the nuclear genome encodes a complete set of TCA cycle
296 enzymes, genes required for oxidative phosphorylation, and a functional ATP synthase (Mogi and
297 Kita, 2010). Similar to *C. parvum* and *C. hominis*, *C. muris* lacks a cytochrome-based respiratory
298 chain and shows no evidence of having a mitochondrial genome (Widmer and Sullivan, 2012).
299 However, the presence of mitochondrial structure and proteins that are absent from *C. parvum* and
300 *C. hominis*, but present in *C. muris* and gregarines (Uni et al., 1987; Toso and Omoto, 2007; Mogi
301 and Kita, 2010), supports the theory that the common ancestor of gregarines and *Cryptosporidium*
302 had a larger complement of mitochondrial proteins than *C. parvum* and *C. hominis*, and that the loss
303 of mitochondria in *C. parvum* and *C. hominis* occurred after they diverged from *C. muris* (Widmer
304 and Sullivan, 2012).

305 Functional genomics in *Cryptosporidium* has been hampered by the lack of a transfection
306 system due to the complex life cycle of the parasite and a lack of effective endogenous promoters.
307 A transient expression system using GFP, has been developed based on the double-stranded RNA
308 (dsRNA) *C. parvum* virus (CPV) harboured by *Cryptosporidium* (Li et al., 2009). More recently, a
309 DNA-based transient transfection of yellow (YFP) or red (RFP) fluorescent protein in *C. parvum*
310 oocysts and sporozoites controlled by the endogenous promoters of actin, alpha tubulin and myosin
311 genes using the restricted enzyme-mediated integration technique has been described (Li et al.,
312 2014). Further research is required to develop a stable transfection system, which will be helpful in
313 determining the function and localization of novel *Cryptosporidium* proteins.

314

315 **6. Drug discovery**

316 Progress in developing anti-cryptosporidial drugs has also been slow due to the limitations
317 of in vitro culture for *Cryptosporidium*, an inability to genetically manipulate the organism and the
318 unique metabolic features in this parasite, which has a highly streamlined metabolism and is unable
319 to synthesize nutrients de novo (Abrahamsen et al., 2004; Andrews et al., 2014; Guo et al., 2014).
320 As discussed, *Cryptosporidium* has completely lost the plastid-derived apicoplast present in many

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

330 Another enzyme pathway that has been extensively examined is the salvage of adenosine
331 from its host or environment as *Cryptosporidium* is unable to synthesize purine nucleotides de novo
332 (Striepen et al., 2004; Umejiego et al., 2004; Kirubakaran et al., 2012). *Cryptosporidium* does not
333 contain guanine salvage enzymes and consequently this pathway appears to be the only route to
334 source guanine nucleotides (Striepen et al., 2004; Kirubakaran et al., 2012). The inosine 5' -
335 monophosphate dehydrogenase (IMPDH) gene in *Cryptosporidium* appears to have been acquired
336 through lateral gene transfer from an ϵ -proteobacterium (Striepen et al., 2002, 2004) and recent
337 studies have shown that compounds optimised for inhibition of cryptosporidial IMPDH also have
338 antibacterial activity (Mandapati et al., 2014). Detailed kinetic analysis of this prokaryote-like
339 enzyme demonstrated that the *Cryptosporidium* IMPDH is very different from its human homologs
340 (Striepen et al., 2004; Umejiego et al., 2004). Furthermore, the “drugability” of IMPDH is well
341 established as inhibitors of human IMPDHs have been used clinically as immunosuppressants as
342 well as for the treatment of viral infections and cancer (Chen and Pankiewicz, 2007; Hedstrom,
343 2009). Thus, the exclusive reliance on the salvage pathway by *Cryptosporidium* and its high
344 metabolic demand for nucleotides due to the complicated lifecycle of this parasite make IMPDH a
345 potential drug target candidate. This hypothesis is supported by the recent discovery of several
346 *Cryptosporidium* IMPDH inhibitors (Umejiego et al., 2008; Maurya et al., 2009; Sharling et al.,

347 2010; Gorla et al., 2012, 2013; Johnson et al., 2013). Another study used a yeast-two-hybrid system
348 to identify “Phylomer®” peptides (constructed from the genomes of 25 phylogenetically diverse
349 bacteria) that targeted the IMPDH of *C. parvum* and several interacting Phylomers® exhibited
350 significant growth inhibition in vitro (Jefferies et al., 2015).

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

359

360 7. Metabolomics

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

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

371 A recent preliminary metabolomics study on *Cryptosporidium* developed a faecal metabolite
372 extraction method for untargeted gas chromatography-mass spectrometry (GC-MS) analysis using

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*.

398 Increased funding for *Cryptosporidium* is essential to effectively combat this disease. The
399 US National Institutes of Health (NIH) currently spends US\$4.3 million each year on
400 *Cryptosporidium* research, compared with approximately \$300 million on more than 600 malaria
401 projects (Striepen, 2013). In Australia, the National Health and Medical Research Council
402 (NHMRC) has expended ~AUD \$320,000 on *Cryptosporidium* research projects in the last 5 years
403 compared with more than AUD \$5 million on malaria research
404 (www.nhmrc.gov.au/grants/research-funding-statistics-and-data). It is hoped that philanthropic
405 organizations such as the Bill and Melinda Gates Foundation, USA, (www.gatesfoundation.org),
406 which have focused previously on monitoring rather than intervention, will fund basic research on
407 *Cryptosporidium* and that more research funding will become available from various governments.

408

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412

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671 **Figure legend**

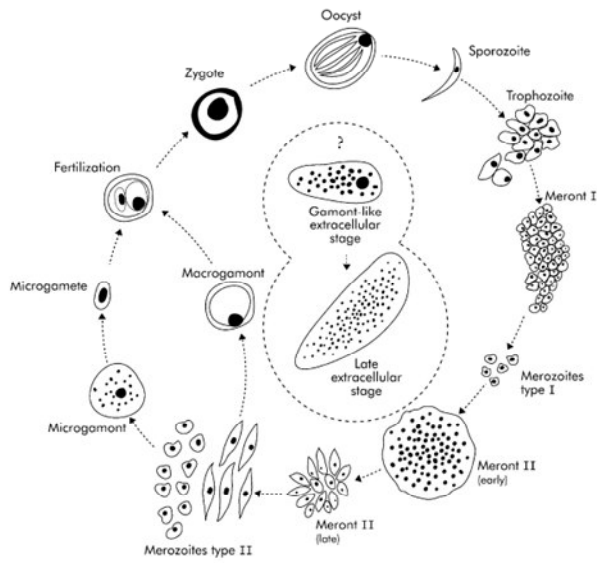
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673 **Fig. 1.** *Cryptosporidium* life cycle (reproduced with permission from Hijjawi et al. (2004)).

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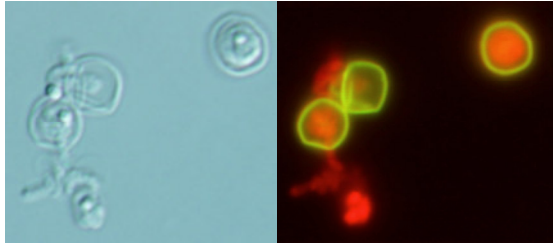


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680 **Highlights**

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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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